



Immunotherapy With 5, 15-DPP Mediates Macrophage M1 Polarization and Modulates Subsequent *Mycobacterium tuberculosis* Infectivity in rBCG30 Immunized Mice

Faraz Ahmad^{1†}, Mohd. Saad Umar¹, Nazoora Khan¹, Fauzia Jamal¹, Pushpa Gupta², Swaleha Zubair³, Umesh Datta Gupta² and Mohammad Owais^{1*}

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*Correspondence:

Mohammad Owais
owais_lakhnawi@yahoo.com

†Present address:

Faraz Ahmad,
ICMR-National Institute of Pathology,
New Delhi, India

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¹ Molecular Immunology Lab, Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, India, ² Bio-Safety Level (BSL)-3 Animal Experimentation Facility, Indian Council of Medical Research (ICMR)-National Japanese Leprosy Mission for Asia (JALMA) Institute for Leprosy and Other Mycobacterial Diseases, Agra, India, ³ Department of Computer Science, Aligarh Muslim University, Aligarh, India

Tuberculosis (TB) is a significant and continuing problem worldwide, with a death toll of around 1.5 million human lives annually. BCG, the only vaccine against TB, offers a varied degree of protection among human subjects in different regions and races of the world. The majority of the population living near the tropics carries a varying degree of tolerance against BCG due to the widespread prevalence of non-tuberculous mycobacteria (NTM). Interestingly, ~90% of the *Mycobacterium tuberculosis* (*Mtb*) infected population restrain the bacilli on its own, which strengthens the notion of empowering the host immune system to advance the protective efficacy of existing mycobacterial vaccines. In general, *Mtb* modulates IL-10/STAT3 signaling to skew host mononuclear phagocytes toward an alternatively activated, anti-inflammatory state that helps it thrive against hostile immune advances. We hypothesized that modulating the IL-10/STAT3 driven anti-inflammatory effects in mononuclear cells may improve the prophylactic ability of TB vaccines. This study investigated the immunotherapeutic ability of a porphyrin based small molecule inhibitor of IL-10/STAT3 axis, 5, 15-diphenyl porphyrin (DPP), in improving anti-TB immunity offered by second generation recombinant BCG30 (rBCG30-ARMF-II[®]) vaccine in mice. The DPP therapy potentiated vaccine induced anti-TB immunity by down-modulating anti-inflammatory responses, while simultaneously up-regulating pro-inflammatory immune effector responses in the immunized host. The employed DPP based immunotherapy led to the predominant activation/proliferation of pro-inflammatory monocytes/macrophages/DCs, the concerted expansion of CD4+/CD8+ effector and central memory T cells, alongside balanced Th17 and Treg cell amplification, and conferred augmented resistance to aerosol *Mtb* challenge in rBCG30 immunized BALB/c mice.

Keywords: tuberculosis, vaccine, immunotherapeutics, IL-10 (interleukin 10), 5, 15-diphenylporphyrin

INTRODUCTION

As a strategy to counter immune onslaught, *Mycobacterium tuberculosis* (*Mtb*), the causative agent of human tuberculosis (TB), evokes anti-inflammatory responses in the host (1–6). It is tempting to speculate that the down-modulation of anti-inflammatory machinery with simultaneous mobilization of pro-inflammatory effectors, may serve as a deliberate host approach to control *Mtb* infection. Several recent studies have established that mononuclear phagocytes are crucial for imparting protection against *Mtb* infection (7–10). Interestingly, they also possess T cell-like memory capacity against re-infection (9, 11, 12). Along this line, the relatively modest potency of TB vaccines developed to date suggests that most of the T-cell targeting candidate TB vaccines do not contribute to any significant advancement in anti-TB prophylaxis programs. This and the above-specified observations, in turn, indicate the need to develop effective prophylactic strategies that can simultaneously activate both innate and adaptive arms of the immune system.

As a crucial component of first-line immune defense, mononuclear phagocytes, especially macrophages, encounter *Mtb* early during an invasion. It is well established that mononuclear phagocytes restrict the replication of invading *Mtb* long before the involvement and participation of specialized T cells (13). The macrophage subpopulation displaying pro-inflammatory classical or M1 phenotypes play a crucial role in the efficient clearance of invading *Mtb* (14). To withstand the immune onslaught, *Mtb* subverts inflicted macrophages to switch toward an anti-inflammatory M2 phenotype (6, 14–16). However, prolonged inflammation in chronic granulomatous infections (*cf. Schistosoma mansoni*) (17) is detrimental and triggers immunopathological consequences in the host. In general exuberant innate inflammation is associated with poor resolution of TB (18). Thus, a well-coordinated and temporally balanced expansion of both pro- and anti-inflammatory monocytes/macrophages is essential for achieving optimal protection against *Mtb*.

The role of IL-10 in exacerbating anti-mycobacterial immunity is well established (19–21) and has been reviewed elsewhere (22). The administration of IL-10 neutralizing monoclonal antibody during BCG vaccination ameliorated protection against subsequent *Mtb* challenge, in both- susceptible and resistant mice strains (23). Furthermore, immunization of IL-10 knockout mice with BCG resulted in elevated anti-mycobacterial immunity upon challenge with *Mtb* (24). Considering these facts, it is tempting to explore strategies that can simultaneously reinforce both the innate and adaptive arms of the immune system as a means to achieve desirable prophylaxis against *Mtb*. On this line, IL-10 mediated activation of JAK-STAT pathway and subsequent STAT3 activity is well established (5, 25, 26). STAT3 is a long established therapeutic target in cancer (27, 28). IL-10 driven STAT3 signaling in macrophages has been associated with the development of ocular angiogenesis and macular degeneration (26). Administration of the small molecule, 5,15-di phenyl porphyrin (DPP), a selective inhibitor of IL-10/STAT3 signaling, has been shown to reduce pathologic neovascularization and Age-related Macular Degeneration (AMD) *via* inhibition of alternative activation and

M2 polarization of macrophages (26). DPP is a small molecular weight organic compound containing a central porphyrin ring similar to phytochrome chlorophyll or heme, an integral component of hemoglobin. Due to its natural origin, it is a relatively safe and attractive STAT3 inhibitor over many other synthetic STAT3 antagonists. The role of the IL-10/STAT3 signaling axis has also been discussed recently in multiple reports, implicating this cascade in modulating monocytes/macrophages toward an anti-inflammatory, alternatively activated state conducive for *Mtb* outgrowth (3, 6, 29–33).

Therefore, targeting and transiently disrupting the IL-10/STAT3 axis mediated immune-suppressive effects could be effective. This study tested this approach to improve the anti-TB efficacy of the second-generation rBCG30-ARMF-II[®] vaccine. The vaccine is devoid of antibiotic resistance marker and expresses 2.6 fold more Ag85B than the original construct (34), which was shown to be safe and immunogenic in a phase I clinical trial (35). The rBCG30-ARMF-II[®] vaccine provided improved protection against *Mtb* aerosol challenge than classical BCG in guinea pigs (34). It also induced a strong, cross-protective immune response against *M. leprae* antigens in mice (36).

In the present study, we attempted to bolster rBCG30 mediated anti-TB immunity by modulating host IL-10/STAT3 signaling orchestrated anti-inflammatory effects. We administered DPP, a small molecule inhibitor of the IL-10/STAT3 signaling (26, 37), in mice at the post- rBCG30 vaccination stage (designated as Post Vaccination Immunotherapy or PVI). In an alternative approach, DPP was administered post challenge with *Mtb* in already vaccinated (with rBCG30) animals (herein called Post Infection Immunotherapy or PII). The treatment of mice with DPP resulted in a reduced expansion of pathogen permissive AAMs (Alternatively Activated Monocytes/Macrophages) along with the reciprocal predominance of pro-inflammatory CAMs (Classically Activated Monocytes/Macrophages), the cells that resist the establishment of successful *Mtb* infection. The results of the present study suggest that the modulation of the IL-10/STAT3 axis mediated anti-inflammatory effects can be a viable new anti-TB strategy, especially in vaccinated hosts.

RESULTS

Immunotherapy With DPP Skews Mononuclear Phagocytes Toward a Pro-Inflammatory CAM State

Ly6C⁺ mononuclear phagocytes are largely divided into two subpopulations-Ly6C^{hi} and Ly6C^{low}, which are further defined as pro-inflammatory M1 or CAMs/CADCs and anti-inflammatory M2 or AAMs/AADCs, respectively (38–42). There is a direct correlation between expression of Ly6C and the degree of the functional differentiation state of mononuclear phagocytes following infection with *Mtb* (42, 43), and other intracellular pathogens (17, 40). Considering this, we intended to restrict the *Mtb* induced polarization of monocytes/macrophages/DCs toward permissive AAMs/AADCs. We treated rBCG30-immunized animals with DPP, either before (PVI) or after (PII) challenge

with *Mtb*, to transiently block IL-10/STAT3 mediated anti-inflammatory signaling. Transient blockade of IL-10/STAT3 signaling was used as a means to allow augmented differentiation of mononuclear phagocytes into pathogen-clearing CAMs/CADCs. Interestingly, immunotherapy with DPP in rBCG30-immunized animals (PVI) resulted in significant up-regulation of CAM phenotype in monocytes/macrophages, while fewer monocytes/macrophages with AAM phenotype were present among both peritoneal exudates cells (PEC) population (**Figure S1**) and splenic mononuclear cells (**Figure S2**). DPP immunotherapy in rBCG30 vaccinated animals post-challenge with *Mtb* (PII) also resulted in significant augmentation of the CAMs among monocyte/macrophage population from peritoneal exudates (**Figures 1A, B**). Although in the spleen, only monocytes were significantly expanded into CAMs, while macrophages were predominantly AAMs, when compared with either controls or other experimental groups (**Figures 1C, D**).

The relative abundance of splenic CAMs/CADCs and AAMs/AADCs was assessed at 4- and 8-weeks PC 4- and 8-weeks PC as well. At 4 week PC, rBCG30 immunized animals (administered with either PVI or PII) displayed augmented expansion of both monocytes and DCs with pathogen restricting classical phenotype (CAMs/CADCs). While there was a concomitant reduction in the frequency of the pathogen permissive cells bearing alternative phenotype (AAMs/AADCs), as compared to either untreated, BCG- or rBCG30-immunized control groups. In contrast, macrophages from the PII, but not the PVI group, displayed a similar rise in the expansion of CAMs with a relatively reduced expansion of AAMs, when compared with the rBCG30 control animals (**Figure 2**). This increased expansion of CAMs among splenic macrophages from the PII group indicates the systemic induction of M1 polarized environment in infected macrophages, possibly as a direct effect of DPP therapy.

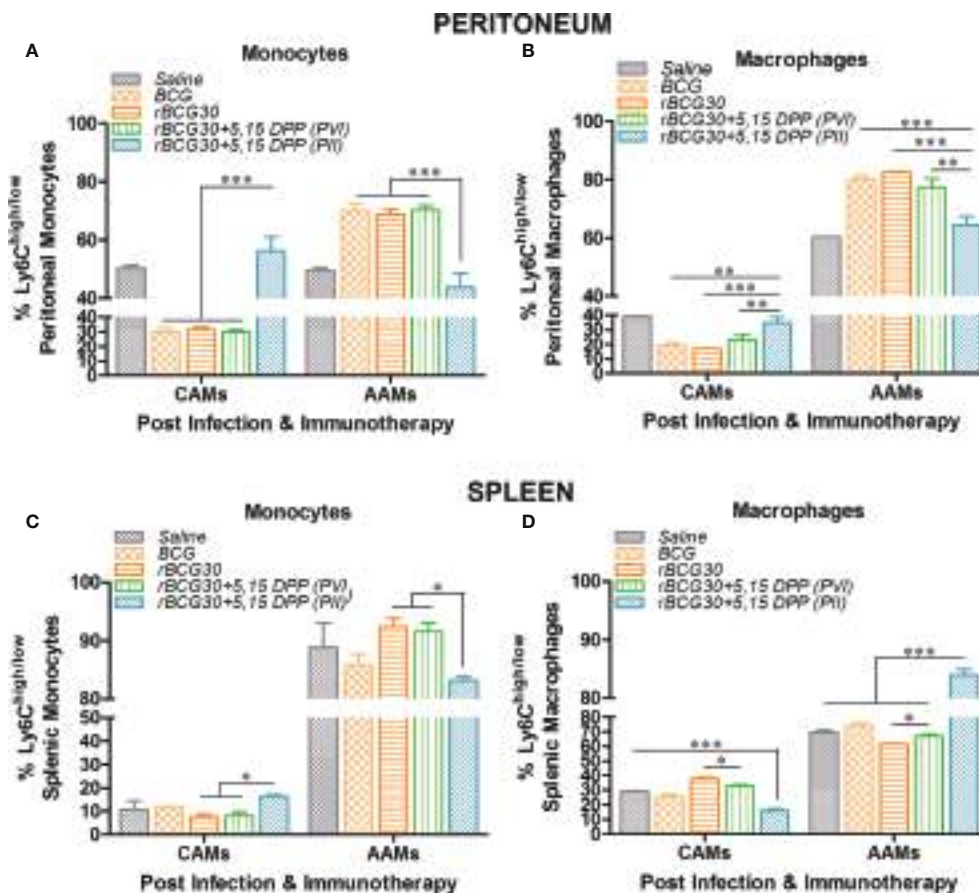


FIGURE 1 | The magnitude and phenotype of peritoneal and splenic monocyte/macrophage populations at 2 weeks post-challenge. Bar graphs in the figure represent percent numbers of Ly6C^{high} CAMs and Ly6C^{low} AAMs among (A, C) CD11b⁺F4/80⁺SSC^{low} monocytes, and (B, D) CD11b⁺F4/80⁺SSC^{low} macrophages. The cells were isolated from either peritoneum (upper panel) or spleen (lower panel) of the mice (n=3) belonging to various experimental groups the next day following completion of post-infection immunotherapy (PII) schedule (2 weeks PC with *Mtb*) and immunophenotyped employing flow cytometry. The results depicted in the figure are representative of two independent experiments and are presented as means \pm SEM of one of the two experiments with similar observations. The statistical significance of the difference between various groups was performed by employing two-way ANOVA followed by Bonferroni's multiple comparison post-test. The *p* values, <0.05(*), <0.01(**), <0.001(***) were considered as significant for analysis and interpretation of experimental data.

Some pathogens, such as *Mtb* (6) and *Brucella* (40) adapt intracellular parasitism and sought shelter in pathogen permissive macrophages of the host. Upon activation, the IL-10/STAT3 axis renders monocytes/macrophages toward an alternative state of activation during *Mtb* infection (6). This generally ensues in the establishment of chronic infection in the host. Taking this into account, we probed the effect of IL-10/STAT3 inhibiting immunotherapy in the modulation of the AAM population during the chronic phase of *Mtb* infection. At week 8 PC, the abundance of CAMs was still significantly higher in the rBCG30 +PPI group, as compared to the rBCG30+PVI group ($p<0.05$,

Figure 3). In addition, the administration of DPP (PII) was successful at restricting the expansion of pathogen permissive AAMs (macrophages) in the rBCG30+PII group even during the late phase of infection, as compared to either rBCG30+PVI or rBCG30 alone groups ($p<0.05$, **Figure 3D**).

DPP Therapy Induces Pro-Inflammatory Cytokines and Dampens the Production of Key Anti-Inflammatory Cytokines

Splenocytes from immunized animals were harvested on day 1 post completion of immunotherapy (either PVI or PII), and

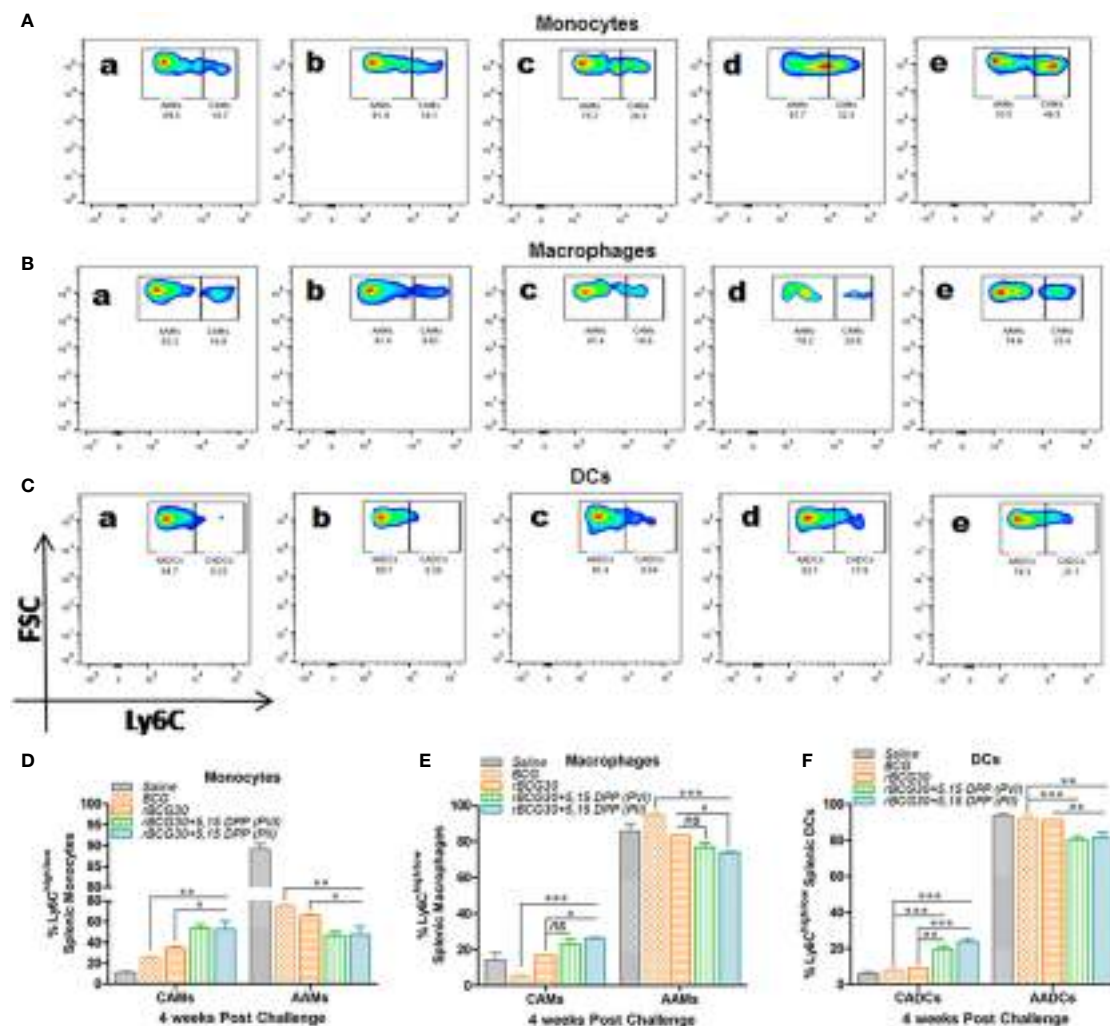


FIGURE 2 | Comparative abundance of the classical and alternative phenotype bearing splenic monocytes/macrophages/DCs at week 4 post-challenge. Representative FACS micrographs (A–C) and corresponding bar graphs (D–F) in the figure represent the percent frequency of Ly6C^{high} CAMs/CADCs and Ly6C^{low} AAMs/AADCs among (A, D) CD11b⁺F4/80⁺SSC^{low} monocytes, (B, E) CD11b⁺F4/80⁺SSC^{low} macrophages, and (C, F) CD11b⁺F4/80⁺SSC^{low} DCs isolated from the spleen of mice belonging to various groups and profiled using flow cytometry. The groups included in the study and depicted in the FACS micrographs were: (a) Saline, (b) BCG, (c) rBCG30, (d) rBCG30+5,15-DPP (PVI), and (e) rBCG30+5,15-DPP (PII). The results depicted in the figure are representative of two independent experiments and are presented as means \pm SEM of one of the two experiments with similar observations. The significance testing of differences between various groups was performed employing two-way ANOVA followed by Bonferroni's multiple comparison post-test. The p values, <0.05 (*), <0.01 (**), <0.001 (***) were considered as significant for analysis and interpretation of data. ns, non-significant.

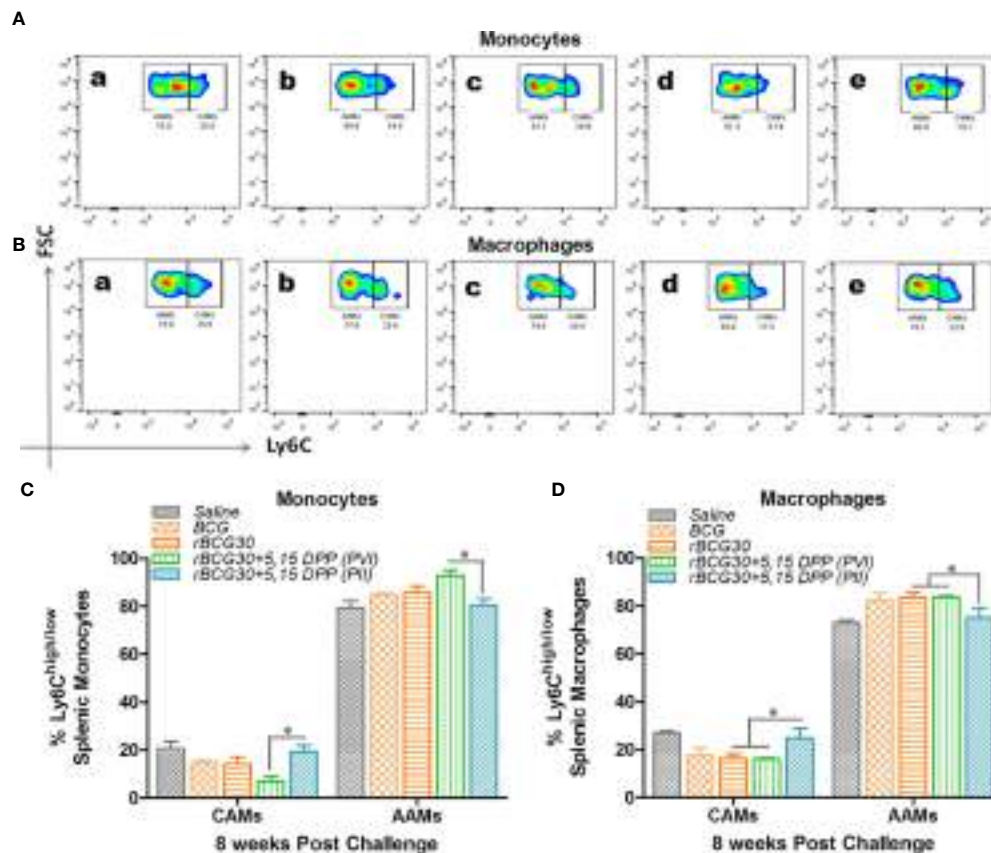


FIGURE 3 | Comparative abundance of classical (CAMs) and alternative (AAMs) splenic monocytes/macrophages at week 8 post-challenge. Representative FACS micrographs and bar graphs in the figure represents the percent frequency of Ly6C^{high} CAMs and Ly6C^{low} AAMs among (A, C) CD11b⁺F4/80⁺SSC^{low} monocytes and (B, D) CD11b⁺F4/80⁺SSC^{low} macrophages isolated from the spleen of mice (n=4-5) belonging to various groups and profiled using flow cytometry. The various groups included in the study and depicted in the FACS micrographs were: (a) Saline, (b) BCG, (c) rBCG30, (d) rBCG30+5, 15-DPP (PVI), and (e) rBCG30+5, 15-DPP (PII). The results depicted in the figure are representative of two independent experiments and are presented as means \pm SEM of one of the two experiments with similar observations. The significance testing of differences between various groups was performed employing Two-way ANOVA followed by Bonferroni's multiple comparison post-test. The *p* values, <0.05(*), <0.01(**), <0.001(***) were considered as significant for analysis and interpretation of the data.

cultured *ex vivo* as specified in the method section. Signature anti-inflammatory cytokines IL-10 and IL-4, and pro-inflammatory cytokines IFN- γ , IL-12, IL-1 β , and IL-6, were assessed in the culture supernatant employing sandwich ELISA. The level of Th1 specific pro-inflammatory cytokines, IFN- γ and IL-12, was found to be elevated in animals that received immunotherapy with DPP post vaccination with rBCG30 (PVI). On the other hand, the level of innate pro-inflammatory effectors, either IL-6 or IL-1 β , was not significantly up-regulated, as compared to controls (Figure 4A). However, the employed immunotherapy resulted in diminutive expression of IL-10 and IL-4 cytokines in the culture supernatant. The level of these two cytokines was found to be significantly reduced following DPP treatment in the PVI group (Figure 4B).

The splenocytes obtained from the group of animals vaccinated with rBCG30 and administered DPP for two weeks following *Mtb* challenge (PII scheme) were also assessed for their potential to produce both pro- (IFN- γ , IL-12, IL-1 β , and IL-6) as well as anti- (IL-10 and IL-4) inflammatory cytokines *ex vivo*. Splenic cells from

rBCG30 vaccinated animals (belonging to the PII group) displayed impressive production of Th1 cytokines, IFN- γ , and IL-12 ($p < 0.001$, Figure 4C). We observed a significant reduction in the level of anti-inflammatory cytokines IL-10 ($p < 0.05$) and IL-4 ($p < 0.001$) in the same group of animals, as compared to rBCG30-immunized controls (Figure 4D). Additionally, at 2 weeks PC, the level of either IL-1 β or IL-6 was un-influenced by post-infection immunotherapy, when compared to controls (Figure 4C).

DPP Therapy Up-Regulates Pro-Inflammatory Cytokines Until 8 Weeks Post-Challenge in the Immunized Animals

Splenocytes from various groups of immunized animals were assessed for their potential to produce signature pro- and anti-inflammatory cytokines at 4- and 8-weeks PC. The level of IL-12, the master cytokine of the Th1 cell-mediated immunity, was found to be significantly elevated in the rBCG30+PII group both at 4 ($p < 0.001$) and 8 ($p < 0.01$) weeks PC (Figure 5A). The expression of IFN- γ , another important effector cytokine of the Th1 type

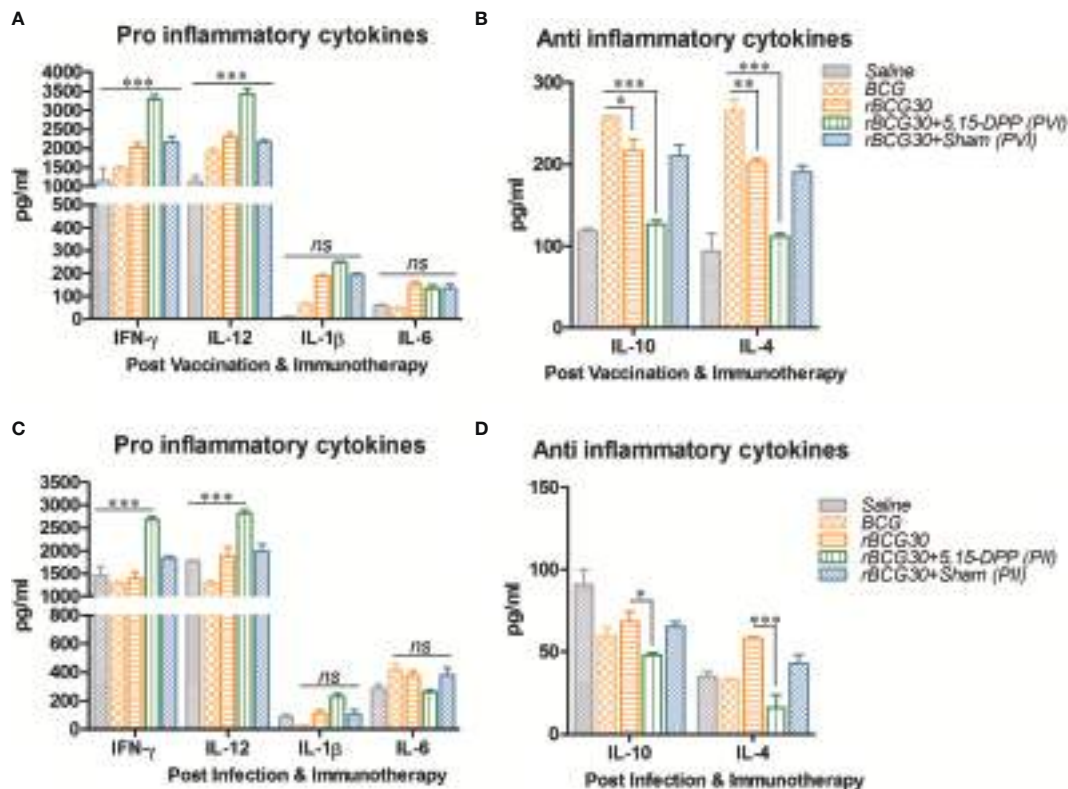


FIGURE 4 | Splenocytes cytokine expression at post vaccination and immunotherapy (PVI) as well as post infection and immunotherapy (PII). The level of signature innate cytokines (IL-1 β and IL-6); Th1 cytokines (IFN- γ and IL-12), and anti-inflammatory cytokines (IL-10 and IL-4), was estimated following (A, B) PVI and (C, D) PII, using sandwich ELISA in the splenocyte culture supernatants belonging to the various immunized groups. Cells were uniformly stimulated with native Ag85B antigen from *Mtb* (5 μ g/ml). Differential expression levels of cytokines in various immunized groups are presented as bar diagrams. The data were analyzed by employing Two-way ANOVA followed by Bonferroni's multiple comparison test and are shown as the means (\pm SEM) of one of the two experiments with similar observations performed in triplicate from the pooled cells of at least three animals per group, where p values; viz. $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered significant. ns, non-significant.

immunity, was also significantly up-regulated at week 8 PC in the PII group, as compared to rBCG30 alone ($p < 0.001$). There was profuse induction of IFN- γ in the PVI group as well when compared with the rBCG30 alone group ($p < 0.01$) (Figure 5D).

We then assessed another crucial pro-inflammatory cytokine IL-6 in various groups of immunized animals. The cytokine IL-6, besides being a crucial effector of innate immune response in TB (12), is also critical for amplification of Th17 cells responses in *Mtb* infected animals (44). Contrary to the 2 week PC time point (Figure 4C), the level of IL-6 was up-regulated significantly in rBCG30-immunized animals belonging to the PII group at both 4 ($p < 0.001$) and 8 ($p < 0.01$) weeks PC time points (Figure 5B). The level of IL-6 was almost identical in rBCG30+PVI, BCG and saline administered groups of animals at both 4 and 8 weeks PC time points.

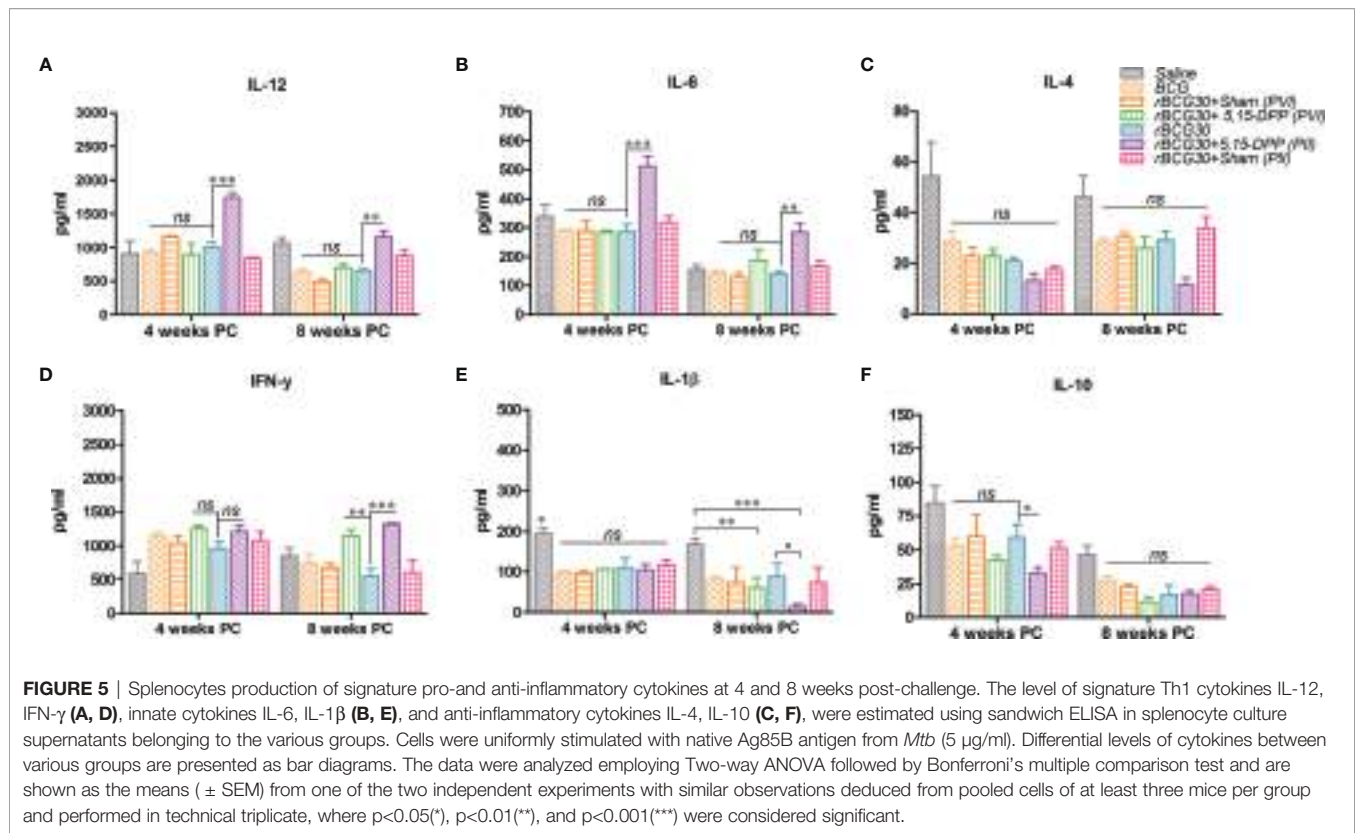
IL-1 β , an important innate cytokine, is primarily a product of either NLRP3 or AIM2 inflammasome assembly and is reported to play a crucial role in regulating the outcome of *Mtb* infection (18, 45). The level of IL-1 β was not significantly influenced by either of the immunotherapeutic strategies employed, until up to 4 weeks PC. Instead, the IL-1 β level was found to be increased in

the saline (*Mtb* infection control) group, as compared with all other experimental or control groups ($p < 0.05$) (Figure 5E). On the other hand, there was a considerable decrease in IL-1 β levels in both the rBCG30+PVI ($p < 0.01$) and rBCG30+PII ($p < 0.001$) groups at week 8 PC (Figure 5E).

The level of signature anti-inflammatory cytokines, IL-10 and IL-4, was probed at stipulated time points post *Mtb* challenge. At 2 weeks PC, the level of both IL-10 and IL-4 was significantly reduced in the rBCG30+PII group (Figure 4D). In contrast, at later time points (4 and 8 weeks PC), differences waned, especially the level of IL-4 (Figure 5C). However, the level of IL-10, the main regulator of anti-mycobacterial immunity, remained low at 4 weeks PC in animals vaccinated with rBCG30 and treated with DPP following *Mtb* challenge (PII) (Figure 5F).

Post-Infection Immunotherapy With DPP Strengthened the Expansion of CD4+ Multifunctional T Cells

Induction of functionally superior multifunctional T cells (MFTs) following immunization and/or infection is an important feature of T cell mediated immunity. Multifunctional T cells are known as



important anti-pathogen effectors that are crucial for immunity and protection against intracellular infectious agents including *Mtb* (46–50). The abundance of antigen specific CD4 $^{+}$ T cells induced in various immunized and immunotherapy groups were assessed *ex vivo* for simultaneous expression of Th1 effector cytokines (IFN- γ and TNF- α) at 2 as well as 4 weeks PC (Figure 6). Interestingly, rBCG30-immunized animals treated with DPP following *Mtb* challenge (PII scheme) produced significantly more CD4 $^{+}$ MFTs, as compared with rBCG30 alone ($p < 0.05$) or rBCG30+PVI group ($p < 0.01$) at 2 weeks PC. In contrast, PVI treated animals did not induce any better level of CD4 $^{+}$ MFTs either at 2 or 4 weeks PC, as compared to rBCG30 controls ($p > 0.05$) (Figures 6A, C). The frequency of CD4 $^{+}$ MFTs induced in saline, BCG, and rBCG30+PVI administered animals was almost identical at 2 weeks PC (Figure 6C). The MFTs response was further evaluated at 4 weeks PC; however, no statistically significant difference was found among experimental or control groups. Nevertheless, the level of induced CD4 $^{+}$ MFTs was still higher in PII treated animals compared to all other immunized groups (Figures 6B, D).

DPP Based Immune-Modulation Scheme Ensues in Development of Effector as Well Central T Cell Memory Following *Mtb* Challenge

Splenocytes isolated from various experimental and control animals were evaluated at 2 week PC for the presence of

central (CD44 $^{\text{high}}$ CD62L $^{\text{high}}$) as well as effector (CD44 $^{\text{high}}$ CD62L $^{\text{low}}$) memory phenotype on both CD4 $^{+}$ and CD8 $^{+}$ T cell population. As is evident from Figure 7, IL-10/STAT3 directed therapy PC with *Mtb* (PII) resulted in an early and significant expansion of effector memory T cells (Tem) in rBCG30 vaccinated mice. Animals in the rBCG30+PII group had a significantly higher percentage of CD4 $^{+}$ and CD8 $^{+}$ Tem cells than animals in either rBCG30 or rBCG30+PVI groups ($p < 0.001$). However, at 2 weeks PC, the frequency of CD4 $^{+}$ central memory T cells (CD4 $^{+}$ Tcm) was not significantly altered among the groups. Nevertheless, the frequency of CD4 $^{+}$ Tcm was still higher in the rBCG30+PII animals as compared to other control groups (Figure 7C). At 2 weeks PC, the level of CD8 $^{+}$ Tcm cells was negligible and inseparable among various groups (Figure 7D).

DPP Therapy Led to Sustained and Systemic Transition of T Cell Memory in rBCG30 Immunized Mice

The effect of DPP based immunotherapeutic strategy on the maintenance of T cell memory was further evaluated at 4 and 8 weeks PC. The splenic T cells from immunized animals were examined for expression of established T cell memory markers CD44 and CD62L. Interestingly, in contrast to 2 weeks PC time point, both CD4 $^{+}$ and CD8 $^{+}$ short lived Tem cells were found to be significantly down-regulated at 4 weeks PC in rBCG30 immunized animals administered PII ($p < 0.001$). The long lived

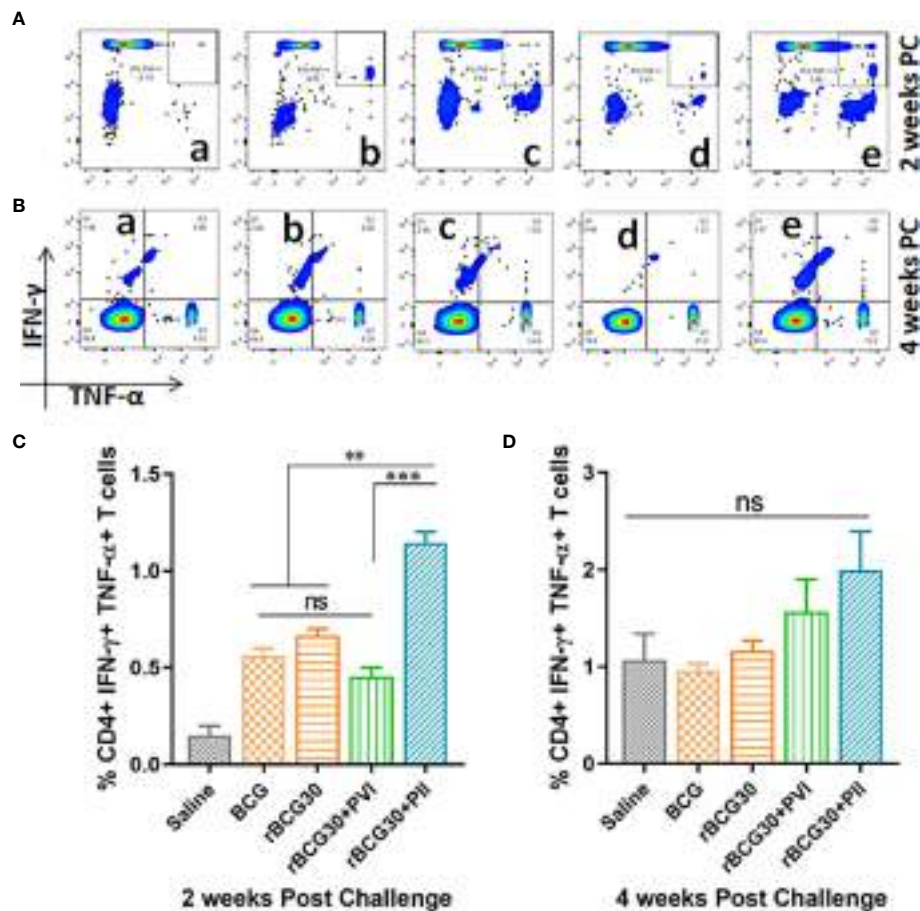


FIGURE 6 | Multifunctional CD4⁺ T cell response post-challenge with *Mtb*. Splenocytes isolated from various immunized and immunotherapy treated animals were profiled employing flow cytometry for simultaneous production of IFN- γ and TNF- α by CD3⁺CD4⁺ T cells at 2 as well as 4 weeks PC. The figure depicts representative FACS plots of CD4⁺ MFTs belonging to (a) Saline, (b) BCG, (c) rBCG30, (d) rBCG30+5,15-DPP (PVI), and (e) rBCG30+5,15-DPP (PII) groups, at (A) 2 and (B) 4 weeks PC, respectively, and corresponding quantitative assessment in the form of bar graphs (C, D). Cells were stimulated with native Ag85B antigen from *Mtb* (5 μ g/ml). The data were analyzed using One-way ANOVA followed by Tukey's multiple comparison test and are shown as the means (\pm SEM) from one of the two independent experiments with similar observations performed in at least three biological replicates, where $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered significant. ns, non-significant.

CD4⁺ Tcm cells were augmented in the same rBCG30+PII group of animals at 4 weeks PC ($p < 0.05$). The CD8⁺ Tcm cells remained undetectable at this time point (Figures 8A, B). Specifically, at 4 weeks PC, the frequency (cumulative) of CD4⁺ Tem cells was reduced to $\approx 40\%$ (Figure 8A) from $\approx 70\%$ at 2 weeks PC (Figure 7A). The level of CD8⁺ Tem cells at 4 weeks PC (Figure 8B) remained closely similar to its level at 2 weeks PC (Figure 10B). Interestingly, the level of CD4⁺ Tcm cells was increased in the rBCG30+PII group, as compared to either rBCG30 ($p < 0.05$) or rBCG30+PVI ($p < 0.01$) group (Figure 8A).

The decreasing trend of effector memory and augmentation of central memory T cells in the rBCG30+PII group was found to continue until 8 weeks PC with a significant drop in the cumulative level of short lived CD4⁺ Tem cells (from $\approx 44\%$ to $\approx 29\%$) (Figure 8). Conversely, the level of CD4⁺ Tcm cells in the rBCG30+PII group remained high even at week 8 PC, as

compared with either rBCG30 ($p < 0.05$) or BCG ($p < 0.01$) immunized groups (Figure 8C). Notably, the expansion of previously undetected CD8⁺ Tcm cells was spotted at week 8 PC. The CD8⁺ Tcm cells were induced significantly in rBCG30-immunized animals that received DPP therapy (PII) ($p < 0.001$; Figure 8D). To our understanding, the significant induction of CD8⁺ Tcm cells along with CD4⁺ Tcm cells during the late phase of infection embodies the final combined push to resist the pathogen's attempt to acquire dominance during the late phase of infection. Moreover, the failure of BCG is correlated with the predominant expansion of effector memory and weak (or no) central memory T cells. We observed predominance of Tem cells and few central memory cells early after infection. Nevertheless, in contrast to classical BCG or rBCG30 (control groups) based immunization, the level of both CD4⁺ and CD8⁺ Tcm cells at the later time point (week 8 PC) was high in the animals that received immunotherapy following aerosol *Mtb*

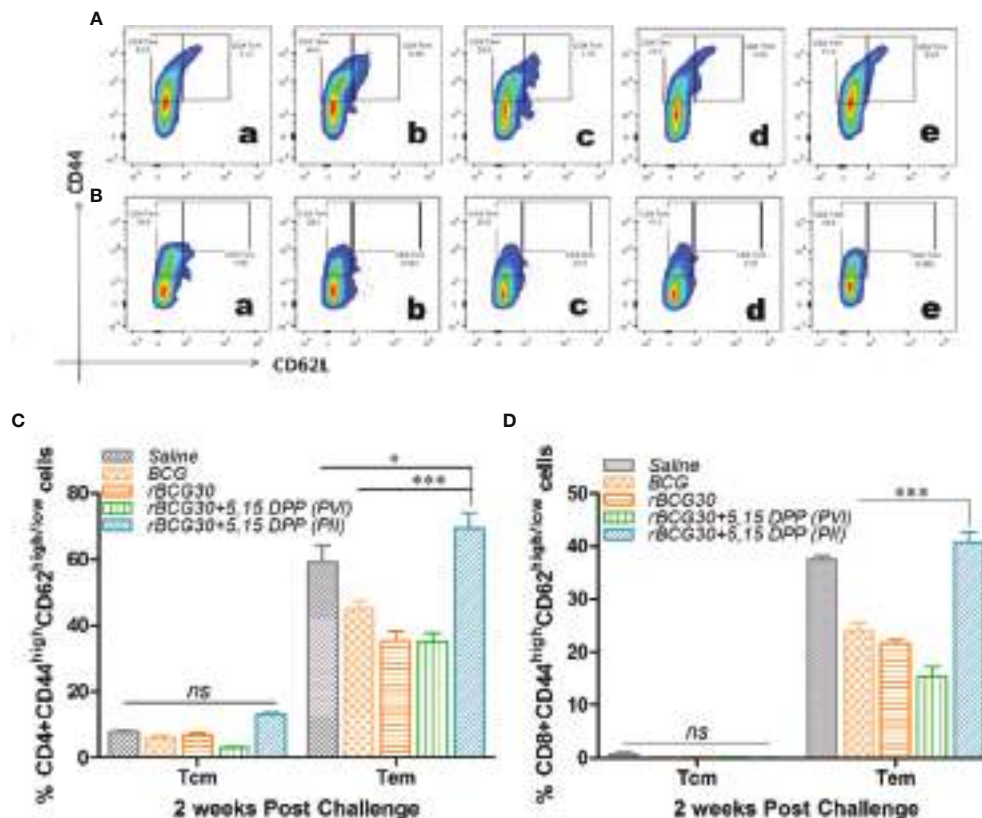


FIGURE 7 | Memory T cell response at week 2 post-challenge. Antigen-specific CD4⁺ and CD8⁺ T central and effector memory response was assessed in the splenocytes isolated from various groups at 2 weeks PC. Shown in the figure are representative FACS plots depicting cumulative frequencies of **(A)** CD4⁺ and **(B)** CD8⁺ memory T cells from (a) Saline, (b) BCG, (c) rBCG30, (d) rBCG30+5,15-DPP (PVI), and (e) rBCG30+5,15-DPP (PII) groups, respectively. Bar graphs in the figure are depicting comparative magnitude (in percent) of effector memory (CD44^{high}CD62L^{low}) (Tcm) and central memory (CD44^{high}CD62L^{high}) (Tem) among **(C)** CD4⁺ and **(D)** CD8⁺ T cells. The data were analyzed by employing Two-way ANOVA followed by Bonferroni's multiple comparison test and are shown as means (\pm SEM) from one of the two independent experiments with similar observations performed in at least three biological replicates, where $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered significant. ns, non-significant.

infection (PII) that presumably helped prevent the establishment of chronic disease (**Figures 8C, D**).

DPP Therapy Following *Mtb* Challenge Led to the Preferential Proliferation of Th17 Cells Over Immunosuppressive Tregs

To further establish the efficacy of DPP based immunomodulatory strategy targeting the immunosuppressive IL-10/STAT3 signaling axis; the comparative expression of CD4⁺ Th17 and Treg cells was assessed. The role of Th17 cells in providing protection from TB is much debated as they are considered dispensable for protection against *Mtb* (44). Nevertheless, the Th17 subset plays an adjunctive role in enhancing the diminished Th1 environment, consequent to the activity of anti-inflammatory mediators, including IL-10, during chronic *Mtb* infection (24). Immunosuppressive FoxP3⁺ T regulatory cells (Tregs) are known to inhibit effector antimycobacterial T cell responses and cause a delay in the onset of adaptive immunity (51–53). Thus, the magnitude of both Th17 and Treg cells was investigated. An attempt was made to establish the

causal relationship between the two subsets by transforming their relative frequencies as a relative ratio (Th17:Treg) (**Figure 9**). While Th17 cells are known to back up deficient Th1 responses (24), Tregs are supposed to brake-off the proliferation of both Th1 and Th17 cells during mycobacterial infection (51, 53). We anticipate that the immunotherapeutic strategy directed towards fine-tuning of Th17/Treg counterbalance, predominantly towards Th17, may prove beneficial to achieve optimal protective immunity against TB.

In the present study, we observed improved Th17 cells proliferation over Tregs in the animals vaccinated with rBCG30 and those that received immunotherapy following *Mtb* challenge (PII) (**Figure 9**). At 4 week PC, animals belonging to the rBCG30+PII group displayed a significantly high Th17 to Treg ratio ($p < 0.05$; **Figure 9E**), and the difference observed was even more at week 8 PC ($p < 0.001$; **Figure 9H**). The observed heightened proliferation of IL-17 producing Th17 cells and relatively low frequencies of FoxP3⁺ Tregs reflect the ability of employed immunotherapeutic regimen to induce pro-inflammatory Th17 lymphocytes, while simultaneously limiting the differentiation of immunosuppressive Tregs.

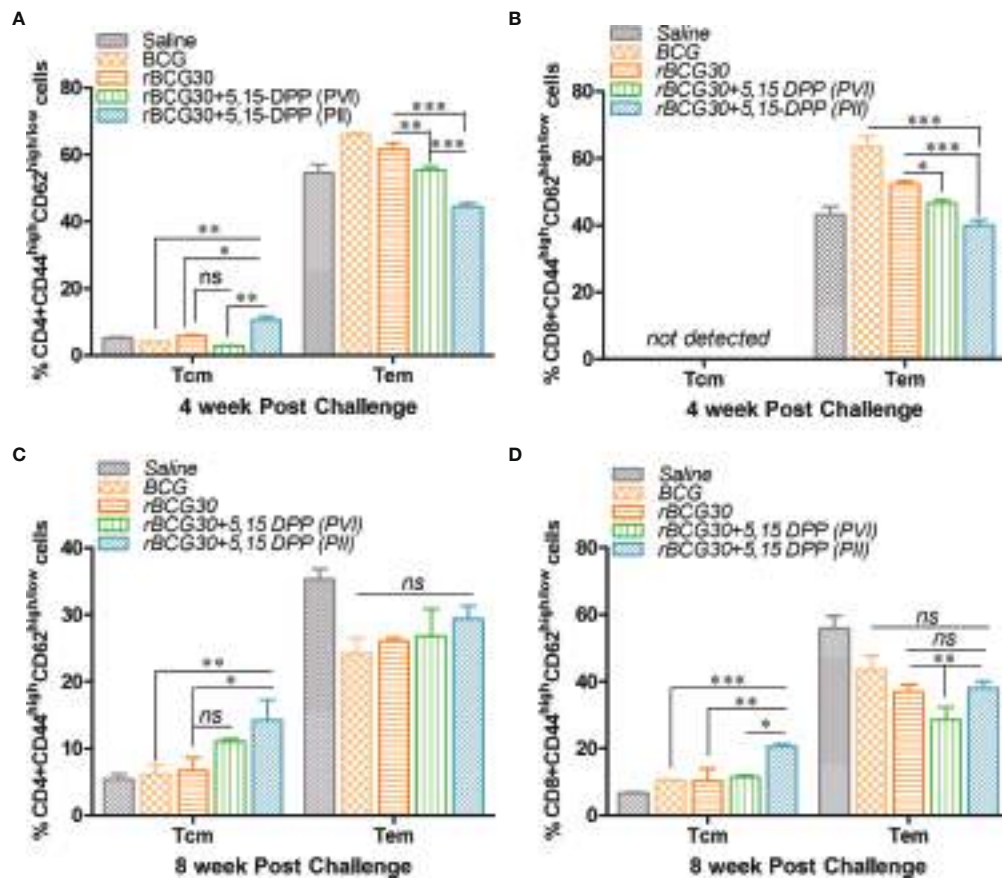


FIGURE 8 | Evaluation of T cells memory response at 4 and 8 weeks post-challenge. Antigen-specific CD4+ and CD8+ T central and effector memory response was assessed in splenocytes isolated from various groups of mice at both 4 and 8 weeks PC. The bar graphs in the figure depict the comparative magnitude (in percent) of T effector (Tem) (CD44^{high}CD62L^{low}) and T central (Tcm) (CD44^{high}CD62L^{high}) cells among (A, C) CD4+, and (B, D) CD8+ memory T cells at 4 (upper panel) as well as 8 (lower panel) weeks PC, respectively. The data were analyzed by employing two-way ANOVA followed by Bonferroni's multiple comparison test and are shown as means (± SEM) from one of the two independent experiments with similar observations performed in at least three biological replicates, where $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered significant. ns, non-significant.

The Compound DPP Lacks Intrinsic Anti-Mycobacterial Activity Against *Mtb* Strain H37Rv *In Vitro*

In addition to immunomodulatory activity, one can argue that the DPP may possess direct anti-mycobacterial activity as well. To rule out the possibility of intrinsic anti-mycobacterial activity of the immunomodulator DPP, we performed *in vitro* antimycobacterial susceptibility testing employing 96 well microtiter plate based alamar blue assay on avirulent *Mtb* strain H37Ra as well as MGIT assay employing standard virulent *Mtb* strain H37Rv (Figure S3). The antibacterial assay data employing the above specified assays suggested that DPP does not possess intrinsic anti-mycobacterial activity. The DPP exhibited a level of antibacterial activity comparable to that of DMSO in inhibiting *Mtb* H37Ra or Rv outgrowth *in vitro* at the tested concentrations. The *in vitro* data ruled out any intrinsic anti-mycobacterial activity at the tested concentration.

DPP Immunotherapy Following *Mtb* Infection Conferred Superior Protection in rBCG30 Vaccinated Mice

The *in vivo* efficacy of employed host-directed immunotherapy was assessed for its potential to augment the protective capacity of the rBCG30 vaccine against murine TB. The mice were immunized with rBCG30 and administered DPP based immunotherapy, either post vaccination or post-infection (with *Mtb*), were assessed for their ability to combat pulmonary *Mtb* infection. To evaluate the effect of immunotherapy on the control of mycobacterial infection *in vivo*, we examined mycobacterial loads in infected lungs and spleen from the various experimental and control groups of animals at 4 and 8 weeks PC.

Surprisingly, animals treated with DPP following aerosol challenge with *Mtb* (PII) displayed superiorly improved bacillary clearance in the lungs, when compared with either rBCG30 ($p < 0.001$) or rBCG30+PVI groups ($p < 0.01$). In spleen, the bacillary reduction in the rBCG30+PII group was significant in

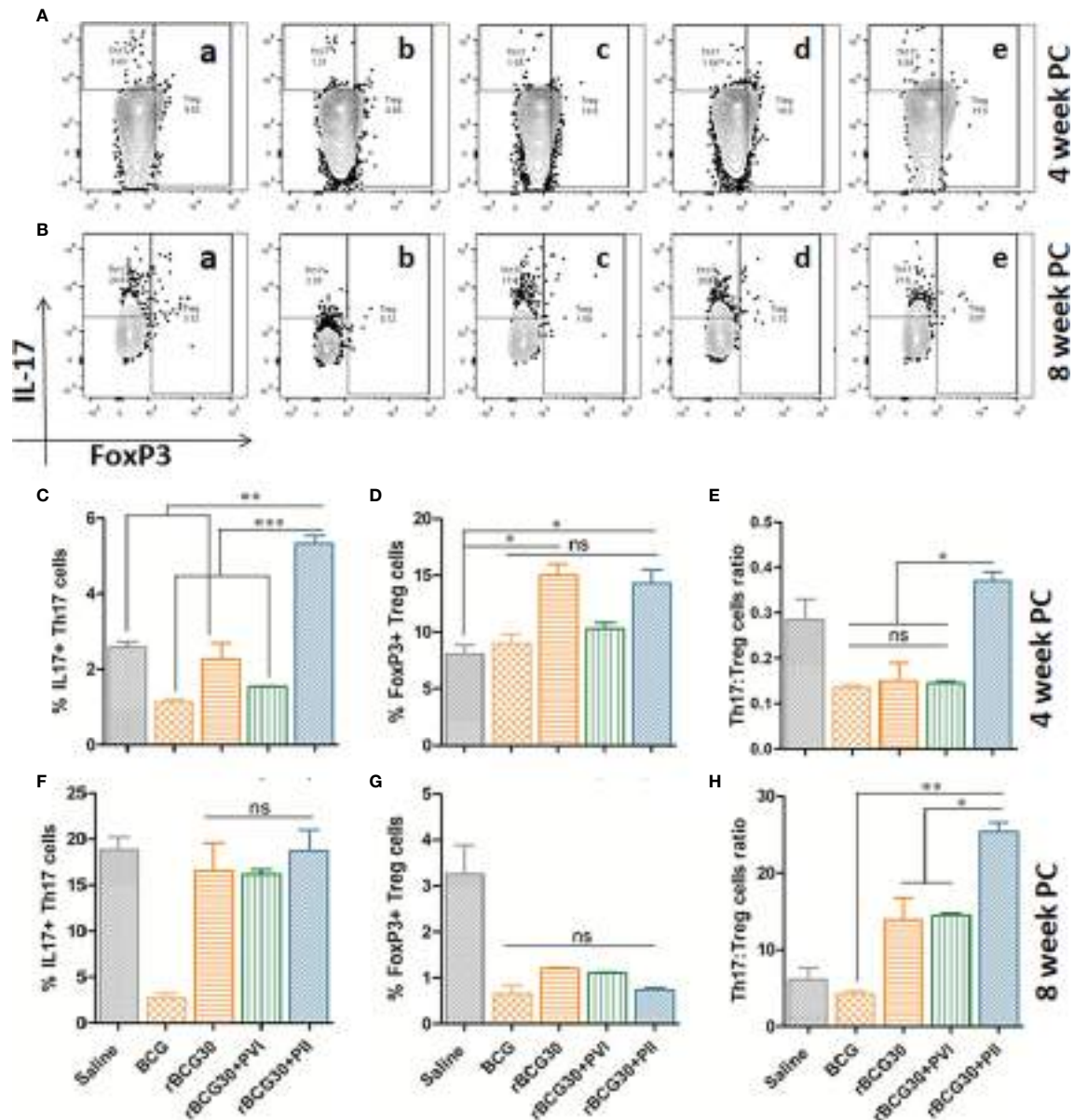


FIGURE 9 | Th17/Treg cells dynamics in response to DPP based immunotherapy. Representative FACS micrographs in upper panel depicting Th17/Treg frequency at (A) 4 and (B) 8 weeks PC with groups labels (a) Saline, (b) BCG, (c) rBCG30, (d) rBCG30+5,15-DPP (PVI), and (e) rBCG30+5,15-DPP (PII), respectively. Bar graphs in the figure are depicting quantitative magnitude (in percent) of Th17 cells at weeks (C) 4 and (F) 8, Treg cells at weeks (D) 4 and (G) 8, and the ratio of Th17 to Tregs at (E) 4 and (H) 8 weeks PC. The level of significance of the data was tested using Two-way ANOVA followed by Tukey's post-test and are shown as means (\pm SEM) from one of the two independent experiments with similar observations performed in at least three biological replicates, where $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered significant. ns, non-significant.

comparison with the rBCG30 group ($p < 0.05$), but not with the rBCG30+PVI group ($p > 0.05$). Moreover, in comparison with standard BCG control, organ bacillary reductions were prominent in PII treated animals ($p < 0.001$), as well as the PVI treated animals (lungs, $p < 0.001$; spleen, $p < 0.01$), at 4 week PC (Figures 10A, B).

Bacillary burdens in the lungs and spleen of infected animals were further evaluated at week 8 PC. Similar to at 4 weeks PC

time point, the CFU burden at week 8 PC was significantly suppressed in rBCG30-immunized animals that received PII with DPP. The adopted immunotherapeutic scheme was also able to reduce bacterial loads significantly at week 8 PC in rBCG30+PII treated animals, as compared to either classical BCG ($p < 0.001$) or rBCG30 vaccinated controls (lungs, $p < 0.001$; spleen, $p < 0.01$), and/or post-vaccination immunotherapy (PVI) administered animals ($p < 0.01$) (Figure 10C, D).

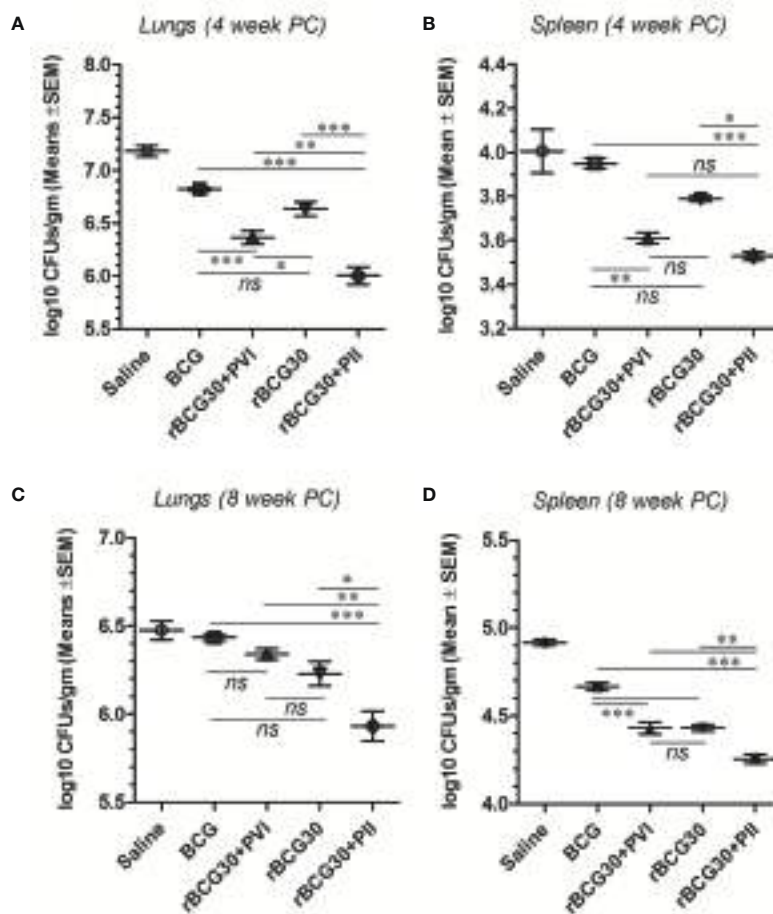


FIGURE 10 | Residual *Mtb* burdens in the lungs and spleen of the infected mice. *Mtb* bacillary load was assessed in rBCG30-immunized and DPP treated (both PVI and PII setups) mice at week 4 and 8 post aerosol *Mtb* challenge. CFU loads in the organ homogenates were enumerated and expressed as log₁₀ CFU/g of tissue. The scatter plots in the figure represent log₁₀ CFU/g ± SD (of at least five biological replicates per group) in lungs and spleen at (A, B) 4; as well as (C, D) 8 weeks post-challenge, respectively. CFU data were analyzed by employing one-way ANOVA followed by Tukey's post-test, where $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered significant. ns, non-significant.

Comparative mycobacterial loads in the lungs of various immunized and treated groups of animals were also evaluated by AFB staining of the lungs tissue sections. Formalin-fixed lungs tissue sections were subjected to Ziehl–Neelsen staining to assess the comparative tissue bacillary loads in various immunized and treated mice at 4 as well as 8 weeks PC. Mice administered DPP therapy following *Mtb* challenge displayed significantly fewer numbers of AFB+ bacilli in lungs tissue sections stained with Ziehl–Neelsen dye at either 4 or 8 weeks PC, as compared to either saline, BCG, or rBCG30 administered animals (Figure 11). Additionally, rBCG30 immunized and PVI treated animals didn't display improved bacillary clearance at either 4 (Figure 11D) or 8 (Figure 11I) weeks PC time points and the gross tissue mycobacterial loads were equivalent to that present in rBCG30 immunized control animals. Interestingly, while in all other groups bacilli were stained as multibacillary aggregates, in rBCG30+PII administered animals there were usually one or two bacilli at particular foci (paucibacillary), which suggest restrained

Mtb growth in the group. In concordance to CFU enumeration data, lung tissue from PII treated animals displayed the least AFB + burdens, as compared to rBCG30 alone or rBCG30+PVI treated mice at 4 as well as 8 weeks PC (Figure 11).

Overall, there was a maximum reduction in organ CFU burden in the animals vaccinated with rBCG30 and treated PC with DPP (PII). The *in vivo* protective efficacy data of the present study further underscore the protective potential of IL-10/STAT3 targeted DPP therapy (PII) in bolstering host resistance as well as the efficacy of rBCG30 vaccine against *Mtb* challenge in mice.

DISCUSSION

Host directed (immuno) therapeutic (HDT) strategies against *Mtb* have become a research focus in the eradication of TB disease (reviewed elsewhere) (54). Incidentally, most of the tested

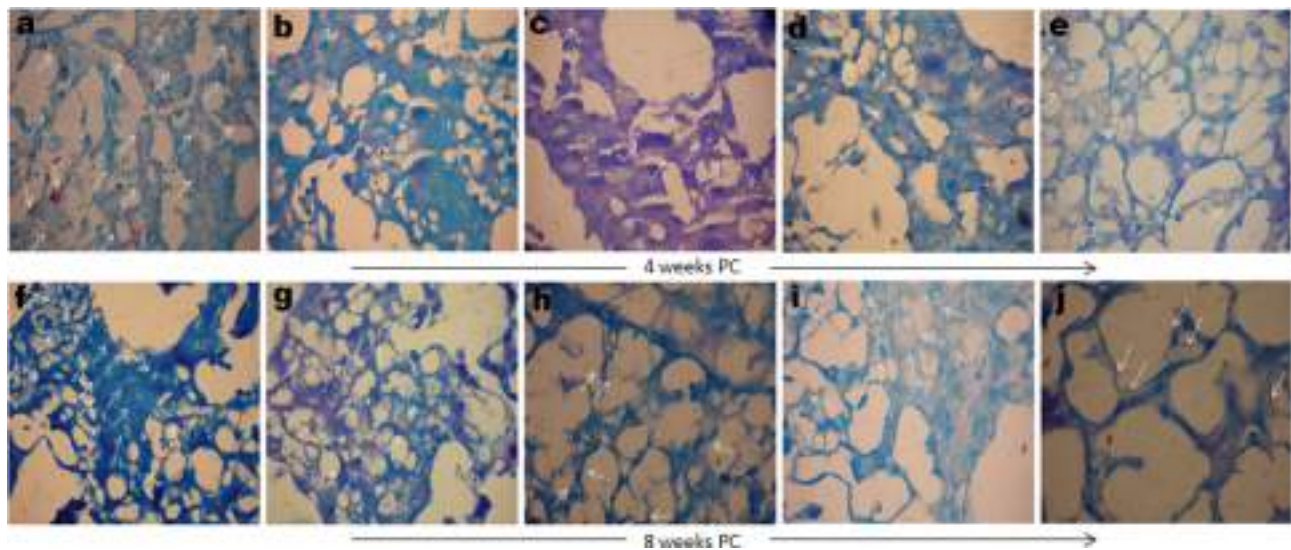


FIGURE 11 | Gross tissue mycobacterial loads in the infected lungs. Representative photomicrographs showing differential *Mtb* bacillary loads in the lungs tissue sections of immunized and DPP immunotherapy administered animals at 4 (upper panel) and 8 (lower panel) weeks PC. Arrows indicating Ziehl–Neelsen dye stained *Mtb* bacilli in various immunized and treated groups (Original magnification—1000x in oil immersion lamp). The various groups were as follows: **(A, F)** Saline **(B, G)** BCG **(C, H)** rBCG30, **(D, I)** rBCG30+PVI, and **(E, J)** rBCG30+PII, respectively.

schemes were proposed either as stand-alone or as an adjunct to standard anti-TB chemotherapy. Despite this, efforts aimed towards using host immune machinery to convalesce the prophylactic immunity conferred by BCG or its engineered recombinant version are relatively limited (23, 53, 55). The IL-10/STAT3 signaling axis has been implicated in generating pro-angiogenic/anti-inflammatory responses in the host (5, 25, 26). Multiple reports have shown that IL-10 persuades STAT3 to skew *Mtb* infected monocytes/macrophages towards pathogen permissive, anti-inflammatory AAM state (3, 6, 31–33). IL-10 has been considered to be a potent pro-mycobacterial cytokine that opts to weaken/disable anti-mycobacterial immune responses (21, 56). Apart from its active role in the direct inhibition of Th1 cell expansion (57), IL-10 delays the migration of DCs from *Mtb* infected lungs to draining lymph nodes (22). Rather, the cytokine IL-10 rejuvenates the chronic/latent *Mtb* infection (20). An optimum vaccination strategy to counter *Mtb* could thrive if it induces a protective pro-inflammatory response in the host while simultaneously restraining pathogen-triggered anti-inflammatory or regulatory pathways. Bearing this in mind, the present study explored DPP based immunotherapy as a host targeted intervention to enhance rBCG30 vaccine mediated immunity against experimental murine TB.

The DPP based therapeutic modulation of host immunity resulted in the significant expansion of M1 monocytes/macrophages (CAMs) along with concomitant reductions in the numbers of M2 monocytes/macrophages (AAMs) in the peritoneum as well as the spleen of the PVI (**Figures S1, S2**) or PII (**Figures 1A–C**) administered animals; except a feeble expansion of classical macrophages in the spleen of the PII

administered group (**Figure 1D**). The differential repertoire of peritoneal and splenic monocytes/macrophages in the PII group can be explained based on two propositions. First, intra-peritoneal administration of DPP therapy might have re-programmed the peritoneal monocytes/macrophages toward a classical state more promptly as compared to their splenic counterparts. Second, because aerogenic *Mtb* takes around two weeks to reach the splenic compartment from the aerosol infected lungs (22), the splenic resident cells in PII administered animals might not have been signaled to alter their steady state phenotype, as nearly 70–80% splenic monocytes/macrophages were found to be in the M2 state until then. By contrast, at 4 weeks PC, both classical monocytes and DCs were significantly expanded in the PVI group, macrophages from the same group of animals did not display any significant difference in numbers. Interestingly, animals belonging to the PII group showed increased numbers of monocytes, macrophages, and DCs with a classical phenotype at this point (**Figure 2**). This suggests that PII with DPP was still better at 4 weeks PC in maintaining a pro-inflammatory environment in macrophages, the primary cells *Mtb* thrives in, as opposed to PVI.

The phenotypic polarization status of the mononuclear phagocytes was further assessed at the week 8 PC time point. As the population of DCs was below the detection level; therefore, we confined our investigation to monocytes and macrophages only. It is noteworthy that the animals from the PVI group failed to maintain a pro-host innate immune response for long and instead displayed predominant differentiation of anti-inflammatory monocytes/macrophages (AAMs) at week 8 PC (**Figure 3**). The weak induction of macrophages bearing classical phenotypes during the late phase of *Mtb* infection can be

correlated with the failure of the PVI strategy to execute bacterial clearance from the host. The maintenance of increased CAMs numbers in the PII group can have a direct correlation with the sustained and controlled expansion of pro-inflammatory milieu that might, in turn, ensued in superior protection of the immunized animals (**Figure 3**). As assessed from the mononuclear phagocytes dynamics, it can be inferred that the post vaccination immunotherapy (PVI), executed before infection with *Mtb*, may be of minimal benefit to the host. It could also be attributed to the fact that avirulent BCG strains are fundamentally different from virulent *Mtb* in terms of pathogenesis (58–60).

For its unhindered intracellular survival, *Mtb* skews invading monocytes/macrophages/DCs toward the M2 state and inhibits the secretion of Th1 cytokines (22, 61). Notably, we found up-regulation of Th1 cytokines (IL-12, IFN- γ) and down-regulation of hallmark Th2 cytokines (IL-10, IL-4) in DPP treated animals following the conclusion of both PVI or PII immunotherapy schedules (**Figure 4**). The level of Th1 cytokine IL-12 was found up-regulated even until 8 weeks PC in the PII group of animals, but not in the PVI group (**Figure 5A**). However, a transient dip in the level of another Th1 cytokine IFN- γ was observed at 4 weeks PC in both PII and PVI group of animals, which was dramatically regained later at week 8 PC (**Figure 5D**). This transient decline in the IFN- γ level was coincident with the significantly augmented level of IL-6 (**Figure 5B**) and IL-17+ Th17 cells expansion (**Figure 9C**) in PII administered animals. The observed cytokine modulation was in concordance with the previous report, where mycobacteria inhibited Th1 response was backed up by IL-23 (and possibly IL-6) mediated Th17 response, followed by restoration of functional Th1 response (24). The levels of innate cytokines IL-1 β and IL-6 were not significantly affected in either PVI or PII treated animals until 2 weeks PC with *Mtb* (**Figure 4**). However, at 4 and 8 week PC, there was a significant increment in the level of IL-6 in PII administered animals (**Figure 5B**). The level of hallmark innate cytokine IL-1 β was not significantly influenced during the course of infection (**Figure 5E**). Interestingly, IL-1 β was least induced in rBCG30+PII administered animals at week 8 PC. The significantly reduced level of IL- β in the rBCG30+PII group may be considered as a correlate of the control of IL- β mediated exuberant inflammation during uncontrolled *Mtb* infection, as also reported previously (18). As expected, the DPP based immunotherapy transiently abrogated the expression of IL-10 until 4 weeks PC (**Figure 5F**), while IL-4 levels were indifferent in either of experimental and control groups at 4 or 8 weeks PC (**Figure 5C**), as opposed to significant inhibition observed at 2 weeks PC (**Figure 4D**). The observed transient reductions in the level of anti-inflammatory cytokines, especially IL-10, are in line with our efforts to fine-tune the anti-inflammatory signaling during the establishment of infection, instead of abrogating it permanently. The transient blockade of IL-10 signaling was desirable as it help avoid the inhibition of efficient immune priming (22). A complete absence of IL-10 increases the likelihood of developing pathological and auto-immune responses in the host. Consistently, these results implicate

IL-10 as a primary culprit that limits vaccine-induced protective anti-mycobacterial immunity by modulating mononuclear phagocytes toward an alternative/anti-inflammatory state of activation. Taken together, the cytokines expression data concord to the previous proposition that the design of host directed therapies against TB must attain an optimal balance between pro- and anti-inflammatory signaling/responses, so as to induce the protective immunity in a manner that inflict minimal collateral tissue pathology (62).

Protection against intracellular pathogens including *Mtb* cannot be achieved without optimum stimulation of the T cell repertoire. In this regard, the multifunctionality of induced T cells upon vaccination or challenge with an infection is an important correlate to assess protective immunity (47). The enhanced proliferation of CD4+ MFTs at 2 weeks PC in the PII group of the animals (**Figure 6**) plausibly accentuated the immune-stimulatory profile and protective potential of the rBCG30 vaccine in immunotherapy (PII) administered animals. The induction of CD4+ MFTs has been considered as an important correlate of a candidate vaccination scheme to impart protective immunity against experimental TB (49, 63).

T cell memory response is an obligatory component of vaccine design to mediate impeccable protection against *Mtb* (64). The DPP immunotherapy mediated strategic manipulation of the IL-10/STAT3 anti-inflammatory axis led to the concerted proliferation of CD4+ and CD8+ effector (CD44^{high}CD62^{low}) and central (CD44^{high}CD62^{high}) memory T cells in the vaccinated host. The memory T cell compartment was initially (2 weeks PC) dominated by both CD4+ and CD8+ effector memory T cells (Tem) in the PII group (**Figure 7**), which later declined and was replaced by CD4+ and CD8+ central memory T cells (Tcm) subsets at week 4 and 8 PC (**Figure 8**). The frequency of Tcm (either CD4 or CD8) was heightened at week 8 PC in PII treated animals, with a significantly high level noted of the CD8+ Tcm population (**Figure 8**). It is well established that antigen exposed 'epigenetically programmed' effector T cells generally result in a subsequent burst of long lived memory T cells (65). Intriguingly, the observed 'systemic transition of Tem population to Tcm', observed in the present study is of particular interest and is in line with current dogma, which argues for differentiation of long lived Tcm cells from the pool of antigen experienced, memory precursor Tem cells, in an ordered, epigenetically regulated 'On-Off-On' process (65–67). The T cell memory response data of the present study concord to this fact, as there was an early preponderance of Tem, and late differentiation of Tcm cells that probably were expanded from among contraction phase survived Tem cells. The greater proportion of effector T cells surviving the contraction phase and entering into a long-lived memory state is indeed an outcome of DPP immunotherapy. This eventually led to unusual induction of T cells with central memory phenotype during the late phase of infection that actually might have restricted the establishment of chronic *Mtb* infection. The observed decline in short lived Tem cell population, specifically at later time points, underscores the relevance of the proposed immunotherapy strategy in restricting surplus effector responses.

Notably, the classical BCG vaccine predominantly induces Tem cells and fails to evoke sufficient protective Tcm cells (68). Thus, the employed immunotherapy may also be explored concomitantly with chemotherapy for its potential to rejuvenate the host immune armory in BCG vaccinated subjects who present with clinical *Mtb* infection.

Fine-tuning between Th17 and Treg cells proliferation is another important aspect in effectively tackling *Mtb* infection (69, 70). In this regard, we measured the relative proliferation of Th17 and Treg cells in various immunized groups and employed the Th17/Treg ratio as an indicator to determine the protective capacity of the proposed immunotherapeutic strategy. We observed increased proliferation of Th17 cells with concomitant downregulation in the frequency of FoxP3+ Treg cells in the immunized animals under the PII scheme (Figure 9). While Th17 cells play an important role in protection against TB (23, 70), Tregs are known to dampen anti-TB immunity and contribute to the progression of the disease (51, 71). The increased Th17:Treg ratio, both at 4 and 8 weeks PC, further affirms the ability of DPP based immunotherapy to selectively inhibit the anti-inflammatory cues following *Mtb* infection in the vaccinated animals (Figure 9).

To assess the protective efficacy of the proposed immunotherapeutic strategy, an *in vivo* protection study was conducted in the mice. The prophylaxis data further affirm that the immunotherapy under the PII scheme resulted in improved protection against aerosol *Mtb* challenge. The immunotherapy (PII) was successful in reducing the bacillary loads in the lungs and spleen of infected mice up to weeks 8 PC, as compared with rBCG30-immunized and untreated control groups (Figure 10). Additionally, the results from histopathological studies (Figure 11) further establish the superior effectiveness of DPP therapy in reducing *Mtb* bacillary loads in the lungs of rBCG30 immunized animals. The mice immunized with rBCG30 and given DPP therapy following aerosol *Mtb* challenge (PII), significantly controlled lungs *Mtb* burdens, as compared to other control and treatment groups (Figure 11). The results corroborate the findings of the CFU counting assay and provide additional evidence of the superior efficacy of DPP mediated post-infection immunotherapy (PII) in rBCG30 immunized and *Mtb* challenged animals. We also evaluated whether DPP possesses any intrinsic anti-mycobacterial activity in an *in vitro* investigation conducted on the H₃₇Ra as well the H₃₇Rv strain of *Mtb* (Figure S3). The results from *in vitro* investigation ruled out any direct anti-mycobacterial activity of DPP and corroborate the central finding of this work that the DPP indeed targets the host and not the bacilli.

The augmented efficacy of the rBCG30 vaccine in the PII administered group of animals establishes the utility of host IL-10/STAT3 depletion (transient) as a viable means to improve TB prophylaxis. It is worth re-mentioning that the successful control of TB relies primarily on the host immune system as the majority of TB infected subjects (~90%) never develop active disease. Emerging evidence suggests that the smart manipulation of the host immune system during vaccination or infection can improve the level of host immunity provided by BCG (53, 55) and BCG based recombinant anti-TB vaccines.

The role of the IL-10/STAT3 signaling axis has been subjected to intense investigations lately and is suggested to have an active role in establishing pro-mycobacterial immune ambiance in the infected host (3, 6, 29, 31–33). SOCS3 and STAT3 are reported as two major regulators during TB infection (5). Mycobacteria possess the ability to impair the SOCS3 feedback loop that limits the IL-6 mediated pro-inflammatory environment. The ensuing IL-10 cytokine induces prolonged activation of STAT3 that in turn promotes an anti-inflammatory environment in the host (5). STAT3 had been associated with numerous mycobacterial survival strategies including blockade of phagolysosome maturation, dampening of reactive oxygen and nitrogen species production, inhibition of autophagy and apoptosis, feeble secretion of Th1 cytokines, reduced presentation of antigens, and eclipsing of co-stimulatory molecules on APCs (5). Few earlier reports have also demonstrated the potential of IL-10 inhibition during vaccination as an effective strategy to enhance anti-mycobacterial immunity (19, 23). Moreover, few recent reports emphasize the role of STAT3 in promoting *Mtb* infection in human subjects (33) and surrogate models (3, 6).

To the best of our knowledge, the present report is the first such attempt to use a small molecule, DPP, that inhibits IL-10 induced STAT3 dimerization and nuclear translocation by binding to either SH2 (Src homology 2) domain (37) or *via* interaction with cytosolic thioredoxin oxidoreductase (TrxR1) (72, 73). Enzyme TrxR1 has recently been described as a direct target of perceived STAT3 inhibitors (including DPP) (72, 73). The majority of available STAT3 inhibitors have been reported to directly bind with cytosolic oxidoreductase TrxR1 *in vitro* in a concentration dependent manner (73). The reduction of oxidized STAT3 by TrxR1 has been proposed to induce dimerization and nuclear translocation of STAT3 (72). It has been speculated that the majority of STAT3 inhibitors work indirectly rather than direct binding to STAT3 (72, 73). In the present study, we made use of the DPP as an immunomodulator/adjuvant, which also targets enzyme TrxR1 to down-modulate STAT3 exerted anti-inflammatory effects. Findings by Busker et al. (72, 73) and Mtwebana et al. (74) indicate that DPP may not directly bind to STAT3 and the observed effects might be STAT3 activation/phosphorylation independent, as also reported by Uehara and colleagues (37) and O'Farrell et al. (25). However, it is unlikely that they operate independently of the transcriptional activity of STAT3, which is extensively established to have a key role in generating an anti-inflammatory environment conducive for infection or cancer outgrowth.

Though this study does not provide direct evidence regarding STAT3 activation/inhibition, the presented evidence from cellular/immunological and protection experiments could be considered a direct correlate of DPP's ability to tame anti-inflammatory effectors, most likely *via* its action on STAT3. We aim to discern the precise role and mechanism of DPP in providing protection against TB in further studies, which will seek to address the critical mechanistic gaps of the present study.

The present study establishes the potential of intercepting host IL-10/STAT3 (and possibly TrxR1) signaling as a crucial

means to bolster innate immune defenses and subsequent adaptive immunity during *Mtb* infection. The proposed strategy is a proof-of-concept that highlights the exploration of IL-10/STAT3/TrxR1 directed immunotherapy as a promising approach to enhance vaccine immunity against TB. Moreover, the chemical nature of the immunomodulator DPP has great advantages in terms of cost-effectiveness, ease of production, stability, and delivery issues, as compared to antibodies or other bio-therapeutics based approaches employed in the earlier studies. The proposed immunotherapeutic approach can be exploited as a viable strategy in improving the protective profile of existing and forthcoming TB vaccines. In addition, it would also be equally interesting to evaluate if the DPP in conjunction with standard anti-TB chemotherapy may serve to enhance the chemotherapeutic index of standard anti-TB drug regimens.

MATERIALS AND METHODS

Reagents

All standard reagents used in the study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. The bacterial culture reagents, Middlebrook 7H9 broth; Middlebrook 7H11 agar, and Oleic Acid Albumin Dextrose and Catalase (OADC) supplement were procured from Difco Laboratories (Sparks, MD, USA). Plasticware was purchased from BD Biosciences (San Jose, CA, USA).

Animals

6–8 weeks old, BALB/c mice (n=111) of male sex were used in the study. Animals were acclimatized for a week before commencing the experiments. All animals were kept in a resting state with free access to food and water for 2 months from the day of vaccination, except the post vaccination immunotherapy (PVI) group that was administered DPP daily for one week after vaccination with rBCG30.

Immunization and Immunotherapy Schedule

Mice were immunized subcutaneously with either commercial BCG vaccine manufactured by Serum Institute of India or in-house propagated rBCG30-ARMF-II[®] Tice. Vaccines were equilibrated to deliver $1\text{--}1.5 \times 10^5$ CFUs in 100 μ l of normal saline and administered subcutaneously. As a placebo, normal saline was administered in one group of animals (saline control group). Animals immunized with rBCG30 were divided into two sub-groups. The first group received post vaccination immunotherapy (PVI) with DPP (Sigma-Aldrich) daily for one week commencing at day 1 post vaccination. The second group received post-infection immunotherapy (PII) on alternate days for two weeks starting at day 1 post aerosol *Mtb* challenge. To negate the effect of DMSO used as a solvent and vehicle to deliver DPP, DMSO sham control groups, rBCG30+sham (PVI) and rBCG30+sham (PII), were also included in the study. No specific effect was observed on the DMSO carrier that was used as a solvent to deliver DPP. All the treatment with DPP or vehicle (DMSO) was given intra-peritoneum. The dose used of DPP was

15 mg/kg of body weight as described previously (26), in injection volume of 200 μ l/mice/day.

Mycobacterium Bovis BCG, rBCG30, and *Mycobacterium Tuberculosis* H₃₇Rv Strains

rBCG30-ARMF-II[®] Tice was provided by Professor Marcus A. Horwitz (UCLA) and obtained under the limited, non-commercial use agreement between UCLA (USA) and AMU (India). The Bacille Calmette–Guerin (Russian strain) was obtained commercially (marketed as Tubervac[®] by Serum Institute of India) *Mtb* H₃₇Rv strain was kindly provided by The Director, National JALMA Institute for Leprosy and Other Mycobacterial Diseases (NJIL and OMD), Agra, India. *Mtb* was cultured in Middlebrook 7H9 broth containing 0.2% glycerol and 0.05% Tween-80 supplemented with 10% OADC at 37°C as a shaking culture. rBCG30 was cultured in Middlebrook 7H9 broth containing 0.02% Tween-80 supplemented with 10% OADC at 37°C with continuous shaking. The *Mtb* H₃₇Rv used in the study was passaged in mice on regular basis to ascertain its virulence. The viability was determined by culturing the bacteria on Middlebrook 7H11 medium supplemented with OADC and counting the number of colony forming units (CFUs).

Antigens for Ex Vivo Stimulation of Immune Cells

Purified native Ag85B (5 μ g/ml) was used to stimulate the cell cultures derived from various experimental and controls groups. Purified native Ag85B (NR-14857) was procured through BEI Resources (Manassas, VA, USA) under a TB research material procurement contract between NIAID (USA) and AMU (India). As per the manufacturer, the protein was purified from the culture filtrate proteins of *Mycobacterium tuberculosis* (strain H₃₇Rv).

Antibody Based Reagents and Assay Kits

The following fluorochrome-labeled anti-mouse antibodies were procured from e-Biosciences and BD Biosciences: anti CD3 (145-2C11), anti CD4 (GK 1.5), anti CD8 (53-6.7), anti CD44 (IM7), anti CD62L (MEL-14), anti CD11b (M1/70), anti F4/80 (T45-2342), anti Ly6C (AL-21), anti TNF- α (MP6-XT22), and anti IFN- γ (XMG1.2). Mouse Th17/Treg phenotyping kit (Cat. No. 560767) and IL-1 β , IL-4, IL-6, IFN- γ , IL-12, IL-10 BD OptEIA cytokine ELISA kits were procured from BD Biosciences (USA).

Ethics Statement

All animal experiments were approved by the Institutional Animal Ethics Committees (IAEC) of the ICMR-National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India. All animal experiments were performed according to the National Regulatory Guidelines issued by CPCSEA.

Establishment of Infection and Determination of Tissue Mycobacterial Loads

Two months post vaccination; all animals were challenged with virulent *Mtb* (H₃₇Rv) through aerosol route. A bacterial suspension corresponding to 5×10^7 bacteria/ml in 10 ml

normal saline was added to the nebulizer unit of the Aerosol Inhalation Exposure System (Glas-Col, USA). To determine the number of viable bacilli delivered to and surviving in mice lungs, 3 animals were euthanized within 16 h post-challenge (Day 1), and the entire lung homogenates were plated onto 7H11 agar plates to approximate the average implanted CFUs in the lungs of infected animals. On average, ≈ 110 viable bacilli were deposited into the lungs of each mouse. All animal challenge/infection studies were performed in the BSL-3 level containment facility at ICMR-National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India. Standard biosecurity and institutional safety procedures have been adhered to as per institutional SOPs and guidelines.

To evaluate the protective efficacy of the employed immunotherapeutic strategy, we determined the bacterial load in the lungs and spleen of experimental animals at 4 and 8 weeks post *Mtb* aerosol challenge. At stipulated time intervals, a minimum of 4 animals from each group were euthanized; their spleen and lungs were removed aseptically and homogenized in 7H9 media. Four different dilutions of prepared homogenate were plated onto 7H11 agar plates supplemented with OADC. Thiophene carboxylic acid hydrazide (TCH) at a concentration of 2 mg/ml was added to inhibit the growth of BCG or rBCG30 in immunized groups. All the plates were incubated for 3–4 weeks at 37°C in a CO₂ incubator with a constant supply of 5% CO₂. After incubation, colonies were counted to calculate the bacterial load. Bacterial loads were interpreted and expressed as mean log₁₀ CFU/g in the lungs and spleen of infected animals.

Isolation of Lymphocytes, Mononuclear Phagocytes, and Peritoneal Exudates Cells

Mice from various experimental groups ($n = 3-5$) were euthanized at specific time points, i.e. 2 weeks (next day after the conclusion of post-infection immunotherapy (PII), 4 weeks, and 8 weeks post-challenge (PC) to assess immunological parameters in the immunized animals. Single cell suspensions of the spleens were prepared according to previously reported procedures (75). Briefly, spleens from animals representing various groups were macerated using frosted glass slides and passed through a 70 μ m cell strainer to obtain single cell suspensions. The cell suspension was treated with ACK lysis buffer to lyse erythrocytes. Next, the cells were washed with Hanks Balanced Salt Solution (HBSS) three times and re-suspended in a complete RPMI 1640 medium. Peritoneal macrophages or Peritoneal Exudates Cells (PECs) were also isolated from PVI or PII treated animals the day after the completion of DPP therapy to assess their activation state specific phenotype. Isolated lymphocytes and mononuclear phagocytes from the spleen or peritoneum were characterized by assessing the presence of specific cell surface markers employing flow cytometry.

Cytokine Assay: Assessment of Antigen Induced Cytokine Profile

Both pro (IFN- γ , IL-12, IL-1 β , IL-6) and anti (IL-4, IL-10) inflammatory cytokines induced in *ex vivo* re-stimulated

splenocyte culture supernatants (from various experimental and control groups) were estimated using OptEIA sandwich ELISA kits (BD Biosciences). Briefly, 100 μ l of the purified capture antibodies were adsorbed overnight on polystyrene micro-titer plates (Maxisorp, Thermo Scientific) at 4°C in the kit recommended coating buffer. Plates were washed five times with PBST and blocked with 1% BSA. After washing, 100 μ l of the supernatant (isolated from cultured splenocytes after 24 h) was dispensed in each well. After incubation for the time stipulated, the plates were thoroughly washed and incubated with respective biotinylated anti-mouse detection antibody. Afterward, the plates were washed three times with PBST. Subsequently, 100 μ l of streptavidin-HRP conjugate was added to each well, and the plate was incubated for 30 min at room temperature (RT). The plates were again washed three times with PBST and finally a colored complex was developed with tetra methyl benzidine (TMB). The absorbance was read at 450 nm with a micro-titer ELISA plate reader (Bio-Rad).

Flow Cytometric Phenotyping of T Lymphocytes and Mononuclear Phagocytes

Ex vivo re-stimulated splenic lymphocytes/phagocytes or PECs (unstimulated) were harvested and stained for flow cytometric analysis following a protocol provided by BD Biosciences. Briefly, 1×10^6 splenocytes were washed twice with FACS staining buffer (PBS with 1% BSA and 0.1% sodium azide). Cells were incubated with Fc block (2.4G2) or with appropriate fluorochrome tagged monoclonal antibodies against CD3, CD4, CD8, CD44, CD62L, CD11b, F4/80, and Ly6C for 30 minutes at 4°C. After washing, cells were fixed with 4.0% paraformaldehyde (PFA). The flow cytometry data were acquired using FACS Aria-II platform with FACS Diva software (BD Biosciences) and a minimum of 10 000 events were recorded for each sample. Data were further analyzed with FlowJo software (Treestar Inc., USA). The cells of a definite phenotype [CD4⁺CD44^{high}CD62L^{low/high} (CD4 Tmem), CD8⁺CD44^{high}CD62L^{low/high} (CD8 Tmem), CD11b⁺F4/80⁺SSC^{low}Ly6C^{low/high} (Macrophages), CD11b⁺F4/80⁺SSS^{low}Ly6C^{low/high} (Monocytes), CD11b⁺F4/80⁺SSS^{low}Ly6C^{low/high} (DCs)] were deduced as percentage of the gated cell population, as determined by flow cytometry.

Intracellular Cytokine Staining for Multifunctional CD4⁺ and Th17/Treg Cells Detection

Splenocytes isolated and stimulated *ex vivo* for 24 h from various immunized mice were collected, washed with PBS, and stained for surface markers *viz.* CD3, CD4, and CD8 followed by fixation using Cytofix buffer (BD Biosciences). Thereafter, cells were permeabilized with Perm/Wash buffer (BD Biosciences), followed by intracellular staining to probe IFN- γ and TNF- α in the examined cells. GolgiStop solution (BD Biosciences) was added for the last 4 h of incubation before collecting the cells for staining and was removed subsequently through washing. Stained cells were subsequently acquired. The relative proportions of CD4⁺ Th17 and Treg cells populations were

determined with a Th17/Treg phenotyping kit following the manufacturer-supplied protocol (BD). The stained cells were subsequently acquired on the FACS Aria-II platform with FACS Diva software (BD) and later analyzed by FlowJo software. For intracellular staining, a minimum of 50,000 events were recorded.

Acid Fast Staining of Lungs Tissues

The experimental animals were sacrificed and their lungs were perfused fixed in 10% buffered formalin. Later, tissue blocks (of 3 mm × 5 mm dimensions) were processed for paraffin embedding, and subsequently, 10-mm thick sections were cut with a rotary microtome. Sections were subjected to Ziehl-Neelsen staining to identify and estimate the relative load of the acid-fast bacilli (AFB) in the stained tissue sections. Stained representative tissue sections were observed under a light microscope (Nikon). Observations were recorded and interpreted independently by an experienced histopathologist. Photomicrographs were taken from granulomatous regions of samples showing AFB+ staining.

Microplate Alamar Blue Assay to Evaluate the Anti-Mycobacterial Activity of DPP

The direct anti-mycobacterial activity of DPP was evaluated following a previously published protocol with slight modifications (76). The avirulent H37Ra strain of *Mtb* (as a surrogate to check anti-*Mtb* activity in alamar blue assay) was grown in 100 ml of Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol (Sigma), 10% OADC (Difco), and 0.05% Tween 80 (Sigma) on continuous shaking (180 rpm) at 37°C until the culture reached an optical density of 0.5 to 0.6 at 600 nm. The obtained bacterial suspension was washed with normal saline and suspended in 7H9 medium with OD adjusted to 0.01. The culture was then used for inoculation in 96 well microplate alamar blue assay in 100 µl 7H9 media (in duplicate). The compound DPP (dissolved in DMSO) was dispensed in each well at a two-fold diluting concentration (250 µg/ml to 1.95 µg/ml). The concentration of DMSO was maintained in all the wells and DMSO alone control was also included. Outer perimeter wells of the plate were filled with sterile water to prevent dehydration in experimental wells and the plate was sealed with parafilm. Medium alone was taken as a negative control and bacteria only as a positive control. Plates were incubated at 37°C for 5 days. On the fifth day post incubation in dark at 37°C, 50 µl of 0.3% alamar blue solution was added to all the wells, and the plate was further incubated for another 6 hrs. Post incubation, the absorbance of the colored complex was recorded at 570 nm (in absorbance mode) using a microplate spectrophotometric reader with wavelength correction at 600 nm (Eon, BioTek Instruments).

Evaluation of the Direct Anti-Mtb Activity of DPP Using BACTEC MGIT960 System

We also determined the direct anti-*Mtb* activity of DPP *in vitro* using BACTEC mycobacterial growth indicator tubes (MGIT)

960 system (Cat. No. 445870-BD Biosciences). The MGIT960 system utilizes fluorescence readout as an indicator of mycobacterial growth. The MGIT tubes contained a fluorescent compound embedded in silicone on the bottom of the tubes. The fluorescent compound was sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. As the mycobacterial cells grow, they utilize dissolved oxygen and thus allow the dequenching of the fluorescent probe. The machine had a provision to automatically indicate when there is sufficient *Mtb* growth or at 42 days (cut-off if no growth). Upon unloading the tube from the machine, a trail gets generated for each unloaded tube that contains arbitrary fluorescent units. This enabled us to better understand the approximate growth of the culture.

The MGIT960 grown culture of *Mtb* H37Rv (approx. 10^5 - 10^6 CFU/ml) was used to perform the experiment. The freshly grown culture was diluted 100 times (GC100) and 100 µl of the diluted culture was added to 7 ml BBL MGIT tubes (Cat. No. 245122-BD Biosciences) supplemented with OADC, but no antibiotics were added. The DPP was added (in duplicate) at 50 µM concentration (100 µl volume) in tubes inoculated with GC100 and allow to grow in the system. In a negative control tube, 100 µl DMSO alone was added. Positive control (only bacteria) tubes were also included. The graph of fluorescence readout (arbitrary units) was plotted for experimental and control tubes.

Statistical Analysis of Data

The data of various immunological studies (pertaining to various immunized and immunotherapy treated groups) was compared by employing either two-way or one-way ANOVA (as appropriate) followed by Bonferroni's or Tukey's multiple comparison post-test (as appropriate) using Graph Pad Prism software version 5.03. Data presented in various immunological assays are representative of at least 3 animals from each group and 2 similar experiments. The *p* values, <0.05(*), <0.01(**), <0.001(***) were considered as significant for analysis and interpretation of experimental data.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Ethics Committees (IAEC) of ICMR-National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India. All animal experiments were performed according to the National Regulatory Guidelines issued by CPCSEA.

AUTHOR CONTRIBUTIONS

FA conceived the idea of the manuscript, carried out all major experiments, analyse and interpret most of the data and wrote the first draft of the manuscript. MO provided overall supervision of the study, analyzed the data, edited the manuscript critically, and acquired/provided funding for the completion of the study. PG and UG helped executed animal challenge studies and contributed reagents and resources. MSU, NK, and FJ helped carry out experimental investigations. SZ provided software resources and data analysis services. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.706727/full#supplementary-material>

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Amyloid Form of Ovalbumin Evokes Native Antigen-specific Immune Response in the Host

PROSPECTIVE IMMUNO-PROPHYLACTIC POTENTIAL

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Saba Tufail^{†1}, Mohammad Owais^{‡2}, Shadab Kazmi[‡], Renu Balyan[§], Jasneet Kaur Khalsa[§], Syed Mohd. Faisal[‡], Mohd. Asif Sherwani[‡], Manzoor Ahmad Gattoo[¶], Mohd. Saad Umar[‡], and Swaleha Zubair^{||3}

From the [†]Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, U.P.-202002, the [§]National Institute of Immunology, New Delhi, the [¶]Department of Biochemistry, JNMC, Aligarh Muslim University, Aligarh, U.P.-202002, and the ^{||}Women's College, Aligarh Muslim University, Aligarh, U.P.-202002, India

Background: Amyloids have recently been found to be reversible and dynamic. They release the precursor peptide/protein in a slow and sustained manner.

Results: Antibodies produced in response to amyloid immunization could recognize native antigen.

Conclusion: OVA amyloids release proteins harboring native antigen epitopes.

Significance: The slow and sustained release of native antigen from amyloids makes them potential candidates for inducing a protective antibody response.

Amyloids are highly organized protein aggregates that arise from inappropriately folded versions of proteins or polypeptides under both physiological as well as simulated ambiances. Once thought to be irreversible assemblies, amyloids have begun to expose their more dynamic and reversible attributes depending upon the intrinsic properties of the precursor protein/peptide and experimental conditions such as temperature, pressure, structural modifications in proteins, or presence of chemicals in the reaction mixture. It has been repeatedly proposed that amyloids undergo transformation to the bioactive peptide/protein forms under specific conditions. In the present study, amyloids assembled from the model protein ovalbumin (OVA) were found to release the precursor protein in a slow and steady manner over an extended time period. Interestingly, the released OVA from amyloid depot was found to exhibit biophysical characteristics of native protein and reacted with native-OVA specific monoclonal as well as polyclonal antibodies. Moreover, antibodies generated upon immunization of OVA amyloid aggregates or fibrils were found to recognize the native form of OVA. The study suggests that amyloids may act as depots for the native form of the protein and therefore can be exploited as vaccine candidates, where slow antigen release over extended time periods is a pre-requisite for the development of desired immune response.

There is a general consensus that under destabilizing conditions, abnormally folded intermediates of several proteins have a strong tendency to self-aggregate into a polymeric amyloid

fibril (1, 2). Various proteins and peptides have been found to form amyloids in diverse conditions implying that amyloid formation is a generic feature of peptide and proteins (3–5). The amyloid fibrils harbor a core formed by cross- β -structures where β -strands are oriented perpendicularly to the main fibril axis (1, 6). The β -cores can bind to the amyloid binding dyes thioflavin T (ThT)⁴ and Congo Red (7, 8). Amyloid bodies have been associated with the pathogenesis of several neurodegenerative diseases such as Alzheimer, Parkinson, or Creutzfeldt-Jakob disease etc. (9–11). In downright contrast, a few naturally existing amyloids have been found to perform non-pathogenic rather beneficial functions that are crucial for the survival of the host, such as curli fibrils expressed by *Escherichia coli* assist in cell-cell contact (12), amyloid protein of chorion protects oocyte, and developing embryo of silkworm (13). Likewise, fungal prions including yeast and HETs prions in certain cases enhance survival of the host (14–16), whereas Pmel17 amyloid promotes skin pigmentation in humans (17). Also, peptides and protein hormones present in the pituitary secretory granules have been found to carry amyloid-like cross- β -sheet rich conformation (18). Amyloids have also been associated with transfer of genetic information or synaptic changes linked to memory (19–22). Moreover, some recent findings enumerate that artificially or *in vitro* synthesized amyloids can also perform beneficial biological activities *in vivo* (23, 24).

Amyloid fibrils classically viewed to be highly stable structures capable of withstanding perturbing environmental conditions have begun to be realized as more of dynamic entities that may revert back to their native form. Variations in temperature (25) and pressure (26, 27), contamination by chemical denaturants (28), and structural modifications in proteins (29, 30) have been found to destabilize amyloid fibrils. Employing hydrogen/deuterium exchange experiments, Carulla *et al.* (31) have inge-

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² To whom correspondence may be addressed: Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, U.P.-202002, India. E-mail: owais_lakhnawi@yahoo.com.

³ To whom correspondence may be addressed: Women's College, Aligarh Muslim University, Aligarh, U.P.-202002, India. E-mail: swalehazubair@yahoo.com.

⁴ The abbreviations used are: ThT, thioflavin T; DTH, delayed type hypersensitivity; PB, phosphate buffer; CD, circular dichroism; ELISA, enzyme linked immunosorbent assay; Th1, type 1 T helper; Th2, type 2 T helper.

niously demonstrated that monomeric Src homology domain 3 molecules incorporated into Src homology domain 3 amyloid fibrils continuously recycle between the fibril and the monomer state by a dissociation/re-association mechanism at the fibril ends. The recent findings by Kardos *et al.* (25) enumerated dissociation of β_2 -microglobulin fibrils to be a reversible and dynamic process reaching equilibrium between fibrils and monomers following time kinetics of the order of a few minutes. In an earlier study, β_2 -microglobulin fibrils were found to completely dissociate to monomeric β_2 -microglobulin upon treatment with dimethyl sulfoxide (28). Binger *et al.* (30) have shown that hydrogen peroxide-mediated oxidation of methionine residues in the preformed apoCII fibrils reverses their assembly and dissociates the monomer in a time-dependant manner. Also, approximately 2–4% of the monomers have been found to remain unpolymerized at the culmination of A β 1–40 fibril formation (32). The presence of the “unpolymerized free monomeric pool” provides further indication for the existence of an equilibrium between the monomers present in solution and those incorporated into fibrils. Moreover, in a few recent reports non-fibrillar as well as fibrillar aggregates generated from the same protein have been found to exhibit varied release kinetics depending upon the compactness and ordered nature of the aggregates (23, 24). Aggregates obtained at earlier incubation time periods have been found to attain rapid saturation in the release profile in comparison to fibril species formed at later stages. Release of monomers from some of the aggregates could reach saturation only after a period of over 15 or 30 days, which indicates that in such cases equilibrium between the fibril or other amyloid aggregates and the released monomers are attained after long time periods and therefore the aggregates could subsequently release the monomeric protein in a sustained manner over an extended time course. Although overwhelming information is available regarding conformation of amyloid fibrils and release of proteins from them, there remains a paucity of reports on the conformation of the released proteins and peptides.

In the present study, OVA (a non-inhibitory member of serpin protein family) was taken as a model protein for the synthesis of amyloid bodies. OVA was continuously agitated at varying pH conditions (pH 2.5, 7.0, and 10) and the aggregates obtained after various incubation periods were characterized by turbidity measurements, Rayleigh scattering studies, ThT, and Congo Red binding, CD spectral measurements etc. Finally, the formation of fibrils was confirmed by transmission electron microscopy. Furthermore, release of OVA from various aggregates was monitored over an extended time period followed by assessing the state of released product and its conformational integrity. The amyloid aggregates formed at either pH 2.5 or 7.0 were then studied for their immunological properties. The antibodies generated in response to the immunizations were investigated for their affinity with the native state of the antigen employing ELISA and dot blot analysis. We further evaluated Th1/Th2 cytokine bias, lymphocyte proliferation, delayed type hypersensitivity, and nitric oxide (NO) production induced by OVA aggregates in immunized animals to investigate their potential to evoke desired immune responses in the host.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—All the reagents used were of the highest purity available. OVA (A2512), thioflavin T, Congo Red, and fetal calf serum were purchased from Sigma. IgG2a (R35–95) isotype control was procured from eBiosciences (San Diego, CA). IgG1 and IgG2a isotypes (550487), and cytokines *viz.* IL-4, interferon- γ , and IL-12 cytokine estimation kits were procured from BD Biosciences, OptEIA (Franklin Lakes, NJ). RPMI 1640, antimycotic solution, and plasticwares were purchased from BD Biosciences (San Diego, CA). Anti-OVA monoclonal antibodies 2D11 and 3G2E1D9 were purchased from Santa Cruz Biotechnology, Inc.

Fibril Formation—OVA was dissolved in PBS (pH 7.0) (24) or glycine-HCl (pH 2.5) or glycine-NaOH (pH 10) buffers (carrying 0.01% azide) at a concentration of 1 mg/ml and incubated at room temperature under continuous agitation (at 90 rpm). Aliquots collected at various time points were pelleted down at $15,000 \times g$ for 15 min using Sigma 3K30 (Germany) microcentrifuge. Under these conditions, monomeric OVA does not sediment and thus remained in solution only. Fibril formation was established by ThT and Congo Red binding assays and transmission electron microscopy. There was no aggregate formation upon incubation of OVA at pH 10 (data not shown).

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.

Turbidity Measurements—For turbidity measurements, the incubated samples collected at various time points were monitored by UV absorbance at 350 nm using a PerkinElmer UV-visible spectrometer model λ 25 in a 1-cm path length cuvette at room temperature.

Congo Red Binding Studies—Congo Red solution (20 μ M) prepared in phosphate buffer (PB) (pH 7.4) (using a stock solution (1 mM) of Congo Red prepared in ethanol) was incubated with 100 μ g of aggregates for 30 min at room temperature. UV absorbance was measured in the spectral range at 300–700 nm using a PerkinElmer UV-visible spectrometer model λ 25. Congo Red solution (20 μ M) in phosphate buffer served as a control (Congo Red-only spectrum), and absorbance of 100 μ g of native OVA mixed with 20 μ M Congo Red solution was also measured.

ThT Binding Studies—OVA aggregates (100 μ g) obtained at various time points were incubated with 30 μ M ThT solution (30 μ l of 1 mM ThT stock solution) at room temperature and fluorescence was measured on Hitachi F-4500 fluorescence spectrophotometer after 30 min. The bound ThT amyloid was excited at 450 nm and spectra were recorded from 460 to 560 nm. The excitation and emission slit widths were fixed at 5 and 10 nm, respectively.

Interaction of various amyloid species obtained at various time points with ThT was also assessed by fluorescence microscopy. Incubated OVA aggregates obtained at various time points, *viz.* days 2, 4, 7, 10, and 15 were pelleted down at $15,000 \times g$ for 15 min. The pellets obtained were incubated with 20 μ M ThT for 30 min at room temperature and then trans-

ferred onto a glass slide to be analyzed under fluorescence microscope (Axio, HBU 50/AC; Zeiss, Gottingen, Germany).

CD Measurements—A JASCO spectropolarimeter (J-815) was used for circular dichroic measurements using quartz cell with 0.1-cm path length. The temperature was controlled at room temperature using a Peltier Thermostat with Multitech water circulator and the instrument was calibrated using D10-camphor sulfonic acid. A scan speed of 100 nm/min and response time of 2 s were used for spectra collection. Scans were performed for each sample in the range of 200–250 nm with final protein concentrations being 200 μ g/ml.

Transmission Electron Microscopy—For electron microscopy, 6 μ l of protein sample (100 μ g) was spread on a carbon-coated copper grid that was further negatively stained with 2% (w/v) uranyl acetate. The grid was examined under a JEOL transmission electron microscope operating at an accelerating voltage of 200 kV.

In Vitro Release Kinetics—OVA (1 mg/ml of PBS, pH 7.0, and glycine-HCl, pH 2.5) samples incubated for fibrillation were withdrawn at various time points. The amyloids formed were isolated by pelleting at $15,000 \times g$ for 15 min. The pellets obtained were washed three times with PB (pH 7.4) and re-suspended in PB (pH 7.4). The kinetics of OVA release into PB (pH 7.4) was monitored spectrophotometrically at 280 nm (24) as well as by determining intrinsic fluorescence measurements (32). The supernatant obtained after centrifugation was analyzed at various time points for approximately 15 days using PerkinElmer UV-visible spectrometer model λ 25 and Hitachi F-4500 fluorescence spectrophotometer, respectively.

Size Exclusion Chromatography—Five milliliters of the sample containing OVA released from various aggregates after 12 days (approximately 10 mg) was applied to a 83-cm long column with an internal diameter of 2.3 cm, filled with about 340 ml of preswollen Bio-Gel P10 (Bio-Rad), and equilibrated with 20 mM phosphate-HCl buffer (pH 3.0) containing 18 mM NaCl. The flow rate was adjusted to 35 ml/h and 1.0-ml fractions were collected. Absorbance of the collected fractions was measured at 280 nm using PerkinElmer UV-visible spectrometer model λ 25 and plotted against the elution volume.

Western Blot Analysis of the OVA Released from Various Aggregates—Released OVA from various aggregates after various incubation periods was resolved by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred onto PVDF membrane. After blocking in 5% nonfat dry milk prepared in phosphate-buffered saline (PBS) with Tween (PBST), the membrane was washed three times with PBST and incubated for 2 h at 37 °C with either anti-OVA polyclonal or 3G2E1D9 monoclonal antibody. After incubation and stipulated washing steps, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (1:5000) for 1 h at 37 °C. Finally, post washing, the bands onto the membrane were developed by enhanced chemiluminescence (ECL) using ECL kit (Bio-Rad).

Animals—Inbred female BALB/c mice (6–8 weeks old, 20 ± 2 g) were obtained from the Animal House Facility of the Institute. The BALB/c mice were housed in commercially available polypropylene cages and maintained under controlled temperature conditions on a 12-h light/dark cycle and had free access

to food and water *ad libitum*. OT-I and OT-II mice originally obtained from The Jackson Laboratory were a gift from Dr. Satyajit Rath (National Institute of Immunology, New Delhi, India). OT-I and OT-II mice were bred and maintained in the Animal House Facility of the National Institute of Immunology (New Delhi, India). All the animal experiments were performed according to the National Regulatory Guidelines issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Mode and Schedule of Immunization—Animals were immunized subcutaneously in the lower abdominal region aseptically with 50 μ g of OVA amyloid bodies (obtained from fibrillation reaction at various time points). A booster was given 3 weeks after the first immunization with 25 μ g of the corresponding form of protein fibril.

Collection of Sera—The blood was collected from mice after 5 days of last booster. Sera were separated from the clotted blood by centrifugation at $1500 \times g$ for 10 min at 4 °C. Finally, the supernatant was collected for ELISA analysis and purification of polyclonal antibodies.

Determination of Antigen-specific Total IgGs by ELISA—Antigen-specific total IgGs against the native as well as OVA aggregates were determined in the sera of mice immunized with various aggregates of OVA formed at various time points following the protocol as described elsewhere (33). Briefly, 100 μ l (2 μ g) of native OVA as well as OVA aggregates obtained at various time points were dissolved in carbonate/bicarbonate buffer (0.05 M, pH 9.6) and poured into 96-well microtiter plates that were further incubated overnight at 4 °C. The plates were then incubated with serially diluted sera at 37 °C for 2 h, after the usual washing and blocking steps. Furthermore, the plates were washed again and 100 μ l of (1:5000 dilution of stock) horseradish peroxidase-conjugated goat anti-mouse antibodies were added to each well and the plates were incubated at 37 °C for 1 h. After the usual plate washing, 100 μ l of substrate solution (6 mg *o*-phenylenediamine) in 12 ml of substrate buffer with 5 μ l of 30% H₂O₂ was added to the wells and the plates were finally incubated at 37 °C for 40 min. The reaction was terminated by the addition of 50 μ l of 7% H₂SO₄. The absorbance was read at 490 nm with a microtiter plate reader (Bio-Rad).

ELISA of OVA Released from Various Aggregates—Briefly, 96-well microtiter plates were incubated overnight with 100 μ l (2 μ g) of OVA released from various aggregates 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 anti-native OVA-specific polyclonal and 2D11 monoclonal antibodies at 37 °C for 2 h. After washing the plates, 100 μ l of (1:5000 dilution of stock) horseradish peroxidase-conjugated goat anti-mouse antibodies were added to each well and the plates were incubated at 37 °C for 1 h. Substrate solution (100 μ l) was added to the wells after the usual plate washing, which were finally incubated at 37 °C for 40 min. For terminating the reaction, 50 μ l of 7% H₂SO₄ was added to the wells. The absorbance was read at 490 nm with a microtiter plate reader (Bio-Rad).

Amyloid Immunization Induces Native Antigen-specific Antibodies

Determination of Antibody Isotype in Sera of Immunized Mice—Sera from mice immunized with day 4 and 7 aggregates formed at both pH 2.5 and 7.0 were analyzed for antibody isotypes using the protocol described elsewhere (34). Briefly, 2 μ g (100 μ l) of antigen in carbonate/bicarbonate buffer (0.05 M, pH 9.6) was added to 96-well microtiter plates that were incubated overnight 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, 100 μ l of (1:5000 dilution of stock) goat anti-mouse anti-IgG1, and IgG2a antibodies were added in each well and incubated for 1 h at 37 °C. Plates were again washed and 100 μ l of (1:5000 dilution of stock) horseradish peroxidase-conjugated rabbit anti-goat antibodies were added to each well and each plate was incubated at 37 °C for 1 h. After further washing of the plates, 100 μ l of substrate solution was added to the wells and the plates were finally incubated at 37 °C for 40 min. The reaction was stopped by the addition of 50 μ l of 1 M H₂SO₄. The absorbance was read at 490 nm with a microtiter ELISA plate reader (Bio-Rad).

Measurement of DTH Response—The animals were immunized with day 4 and 7 OVA aggregates generated at both pH 2.5 and 7.0 in their inguinal region and the delayed type hypersensitivity (DTH) response was assessed at various time points. The mice were footpad tested to determine their DTH reactions to native as well as various OVA aggregate forms. DTH reactions were elicited by the injection of various OVA aggregates (50 μ g) as well as native OVA 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 h post-injection of OVA 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 h): (left footpad at testing time – left footpad at 0 h).

Determination of NO Production—Mice were sacrificed 1 week post booster, and peritoneal macrophages were isolated. The peritoneal macrophages were grown in culture plates and pulsed with OVA (final concentration 10 μ g/well). After 24 h, 100 μ l of culture supernatant was collected from each well, mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% H₃PO₄), and further incubated for 10 min at 25 °C and absorbance was determined at 550 nm in an ELISA reader (Bio-Rad).

T Lymphocyte Isolation from Spleens of Immunized Mice—Mice immunized with native as well as various aggregated forms of OVA were sacrificed on post day 5 after the last booster. T lymphocytes were isolated from the spleens of sacrificed mice as described elsewhere (34). Briefly, spleens isolated from animals belonging to various immunized groups were macerated and suspension was treated with ACK lysis buffer (0.15 mol/liter of ammonium chloride, 10 mmol/liter of potassium/bicarbonate, and 88 mmol/liter of edetic acid) for lysis of the red blood cells. The cell suspension was centrifuged at 1500 \times g for 5 min, and the cell pellet was washed with Hanks' balanced salt solution 3 times and resuspended in RPMI 1640

medium containing 10% fetal calf serum and 0.1% antimycotic mixture.

T-cell Proliferation Assay for Lymphocytes Isolated from Immunized BALB/c Mice—Lymphocyte proliferation assay was performed as described elsewhere (34). Briefly, lymphocytes isolated from the spleens of mice belonging to various immunized groups were incubated in round bottomed 96-well plates (2 \times 10⁵ cells/well) in 200 μ l of RPMI 1640 medium with 10% fetal calf serum. Splenic cells isolated from groups of mice immunized with native or appropriate OVA aggregates were incubated with 20 μ g of the corresponding matching forms of OVA. After 72 h, the cultures were pulsed with 0.5 μ Ci of [³H]thymidine. After 16 h, the plates were harvested onto glass-fiber filter mats by the use of a Tomtec-Harvester-96 (Tomtec). The incorporated radioactivity was measured with liquid scintillation spectroscopy (Wallac-1450 Microbeta Trilux; PerkinElmer Life Sciences).

Lymphocyte Proliferation Assay for TCR-transgenic OT-I and OT-II Cells—OVA-specific T cell proliferation assay for TCR-transgenic OT-I and OT-II cells was performed by measuring [³H]thymidine incorporation, for determining antigen-specific response to different OVA aggregates. The assays were performed in Immunobiology Laboratory-2 at the National Institute of Immunology, New Delhi. Briefly, OT-I and OT-II splenocytes were isolated and cultured in 96-well culture plates at 1 \times 10⁵ splenocytes/well in 200 μ l of complete RPMI medium. The splenocytes were stimulated with titrating concentrations of OVA aggregates. The plates were incubated in a humid atmosphere of 5% CO₂ at 37 °C and pulsed after 60 h with 0.5 μ Ci/well for measurement of [³H]thymidine incorporation at the end of 72 h. Cells were harvested using a Filler-Mate cell harvester (PerkinElmer Life Sciences) and incorporation of [³H]thymidine was determined by a Top Count scintillation counter (PerkinElmer Life Sciences).

Cytokine Assay: 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 various aggregates of OVA were estimated using appropriate and specific biotinylated antibody pairs according to the manufacturer's protocols. Briefly, 96-well microtiter plates were coated with 50 μ l of the purified capture antibodies in carbonate/bicarbonate buffer (pH 9.5) at 4 °C. After the usual washing and blocking steps, 50 μ l of the supernatant (isolated from cultured splenocytes after 48 h) was poured in each well for determining the level of cytokine induced. Plates were washed and incubated with biotinylated polyclonal goat anti-mouse cytokine detection antibody. Furthermore, after washing the plates, 100 μ l of streptavidin-horseradish peroxidase conjugate was added to each well and plates were incubated for 30 min at room temperature. The plates were then washed and the colored complex was developed with tetramethylbenzidine. 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 the level of the given cytokine in the samples tested, and the concentration was expressed as pg/ml.

Dot Blot Assay—The native form of OVA (10 μ g), various aggregate forms, and OVA released from various aggregates were spotted onto PVDF strips (with dimension of \sim 10 by 4

mm), which were further allowed to dry at room temperature. The strips were rinsed briefly in PBS (pH 7.4) containing Tween 20 (PBST) and incubated overnight at 4 °C in 5% nonfat 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 various aggregates were incubated with antibodies obtained from the animals immunized with corresponding aggregates. The native OVA-coated strips were then incubated with various anti-amyloid OVA antibodies produced in mice. The strips spotted with released OVA from various aggregates were allowed to react with native OVA-specific polyclonal and 2D11 monoclonal antibodies. After the stipulated incubation, the strips were washed three times in PBST and further incubated for 1 h at 37 °C with horseradish peroxidase-conjugated goat anti-mouse antibody (1:5000). The strips were washed with PBST three times and finally the immunoreactive dots were developed on x-ray film using a ECL kit (Bio-Rad). A clearly defined greyish to black spot at the site where the antigen was spotted was considered a positive result. A nonspecific protein (Human Serum Albumin) was spotted onto membranes as control in all the dot blot assays performed.

Interaction of Macrophages with Amyloid Aggregates—Macrophages (1×10^6 cells) were isolated from the intraperitoneal cavity of thioglycollate-primed BALB/c mice following the protocol as described elsewhere (35). The recovered cells consisted of >99% macrophages as demonstrated by histochemistry and FACS analysis using labeled anti-F4/80 antibodies (data not shown). The macrophages were incubated with 100 μ g of FITC-tagged aggregates for 1–2 h 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).

Ovalbumin Molecular Modeling—A three-dimensional ribbon model of OVA was drawn with PyMOL by importing the crystal structure of OVA from the Protein Data Bank with code 1OVA. The selected sequences were highlighted with different colors.

Statistical Analysis—Data were analyzed and two groups were compared using Student's *t* test and one-way analysis of variance (Holm-Sidak method) was used to compare all groups to each other. *p* values < 0.05 were considered significant. SigmaPlot (versions 10 and 11; SigmaPlot software, San Jose, CA) software was used for data presentation.

RESULTS

Continuous Shaking over an Extended Time Period Induces Aggregation of Soluble OVA—Fluorescence intensity at 350 nm is a useful tool to detect aggregation in proteins (36). Taking this fact into consideration, the extent of light scattering was measured in OVA samples obtained after various incubation times (day 0 to 15) when OVA solution in PBS (pH 7.0) or glycine-HCl (pH 2.5) was shaken at 90 rpm. With an increase in incubation time, an enhancement in fluorescence intensity was observed in samples at both pH 2.5 as well as pH 7.0. The increase in fluorescence intensity for pH 2.5 samples was more prominent and significant when compared with samples incubated at pH 7.0. When compared with native OVA, fluorescence intensity was found to increase consistently with incuba-

tion times from 1 to 36 h suggesting aggregate formation. However, the magnitude of increment was less than 5-fold (data not shown). As shown in Fig. 1A, *a*, all the samples collected (at both pH 2.5 and 7.0) after various time periods exhibited a more than 7-fold increase in fluorescence intensity in comparison to native OVA in a time-dependent manner (up to day 15). The turbidity measurements determined at 350 nm for different forms of OVA obtained at various time periods also revealed a pattern in concordance with fluorescence intensity measurements thus offering further evidence in support of aggregate formation (Fig. 1A, *b*).

Extended Incubation with Shaking Leads to the Formation of β -Sheet-rich OVA Aggregates—The OVA samples obtained after various incubation periods were assessed for the presence of β -sheets employing Congo Red and ThT binding assays as well as CD spectroscopy. Congo Red, an amyloid binding dye, is used as a tool to monitor the formation of protein aggregates. The β -sheet conformation of amyloids is crucial for Congo Red binding. Congo Red exhibits a red shift in the absorbance spectrum upon binding to the amyloids (7). In the present study, Congo Red was incubated with aggregates obtained at various time points. The aggregates formed at longer incubation periods showed an increase in absorbance along with a slight red shift. Interestingly, a prominent red shift was observed in aggregates obtained upon incubation of protein for 15 days at pH 2.5 indicating conversion of all the secondary structures to β -sheet conformation. Protein aggregates formed at pH 7.0 did exhibit augmentation of absorbance at increasing incubations but the red shift in wavelength was lower compared with samples incubated at pH 2.5 (Fig. 1B).

ThT, another dye, exhibits enhanced fluorescence emissions upon binding to β -sheet-rich structures like amyloid aggregates of both fibrillar or non-fibrillar nature (8). The aggregates obtained at various time points were characterized employing the ThT binding assay. In general, the aggregates obtained at increasing incubation periods were found to bind ThT more explicitly (Fig. 1C). The aggregates obtained for pH 2.5 samples exhibited significantly enhanced bound fluorescence as compared with the corresponding aggregates obtained at pH 7.0. Interestingly, aggregate generated at pH 7.0 at the same incubation time period showed only 50% fluorescence of that observed for the pH 2.5 sample. The data suggest the formation of β -sheet-rich aggregates for both pH 2.5 and 7.0. However, the greater ThT binding exhibited by aggregated protein generated at pH 2.5 compared with pH 7.0 samples reflects that the fibrillar form for pH 2.5 aggregates to possibly appear earlier than the corresponding pH 7.0 aggregates (Fig. 1C). ThT binding with OVA aggregates was also observed using fluorescence microscopy. In concordance with ThT fluorescence spectral studies, fluorescence microscopy also revealed a similar pattern, *i.e.* aggregates obtained at longer incubations (both at acidic as well as neutral pH) exhibited increased fluorescence (Fig. 2A). ThT and Congo Red binding assays suggest that samples agitated for longer periods (either at pH 2.5 or at 7.0) harbor more β -rich structures than aggregates obtained at shorter incubations.

The far-UV CD spectrum of a protein is highly sensitive to its conformation. In fact, it is especially useful for offering

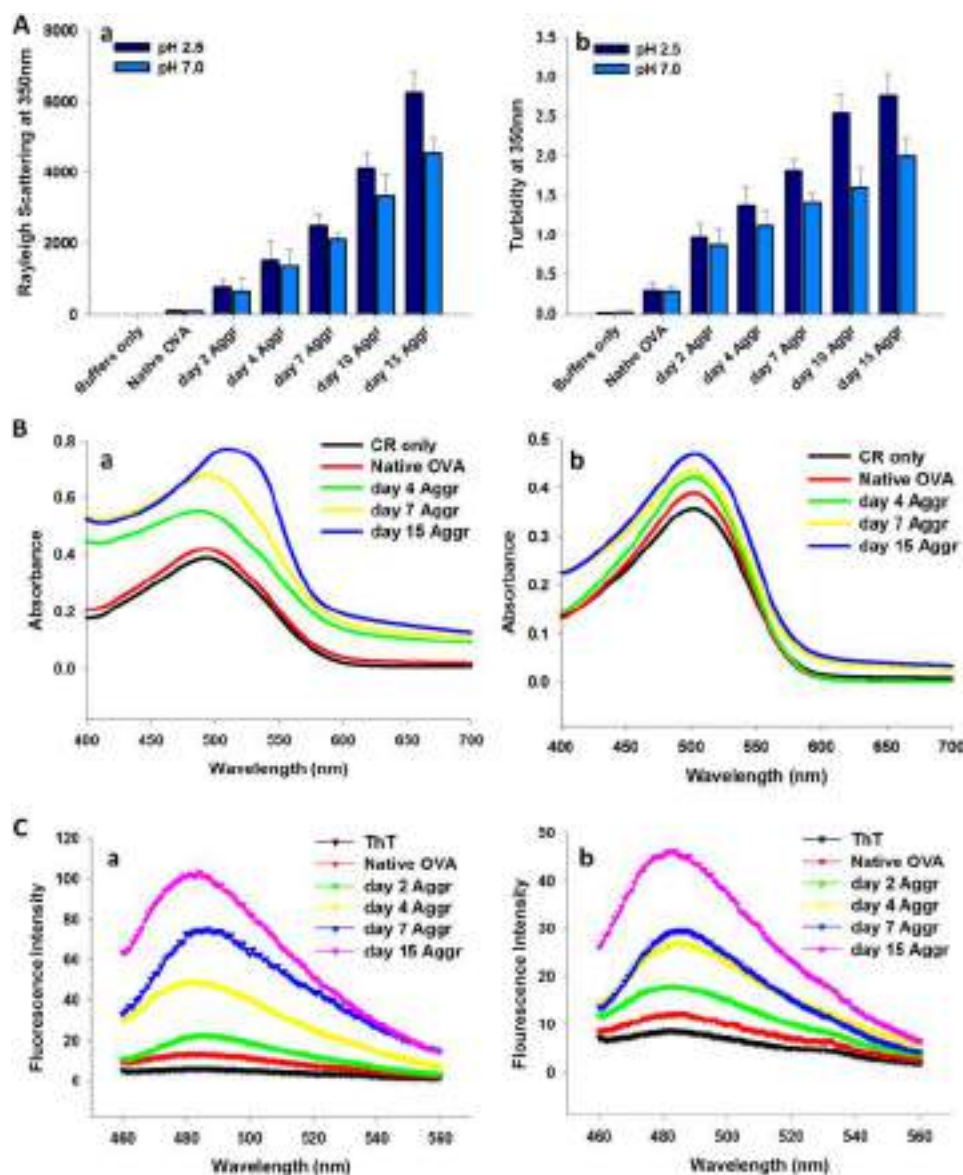


FIGURE 1. Continuous agitation for extended time periods induces aggregation of soluble OVA. A, Rayleigh scattering measurements at 350 nm for aggregates formed at various time points in both acidic and neutral ambiances (a). Turbidity measurements for OVA aggregates formed at various time points at pH 2.5 and 7.0 (b). Error bars exhibit standard error of three independent experiments. B, steady state absorption spectra of Congo Red bound to various aggregates obtained at pH 2.5 (a) and 7.0 (b). The aggregates were scanned in the 400–700 nm range. C, ThT binding assay of various OVA aggregates formed at pH 2.5 (a) and 7.0 (b). ThT emission spectra were obtained by excitation at 450 nm and emission in the range of 460–560 nm. Data are representative of at least three independent experiments with similar observations.

insight regarding the secondary structure of a protein. Fig. 2B highlights shaking induced conformational changes in the structure of OVA. Native OVA exhibited two minima, one at 208 nm and another at 222 nm, which is indicative of a α -helical structure. With an increase in shaking time, the negative peak at 208 nm began to disappear, whereas the peak at 222 nm started shifting toward the left to lie between 215 and 222 nm, which indicates loss of the α -helical structure with the appearance of β -sheets. The aggregates formed at both acidic as well as neutral pH conditions exhibited two negative peaks although the negative ellipticity was decreased in comparison to native OVA. A marked difference for pH 2.5 and 7.0 samples was observed upon further incubations. The aggregate obtained on day 4 (pH 2.5) exhibited a significant loss in negative ellipticity at 208 nm and the

minimum at 222 nm shifted so as to lie between 222 and 215 nm. On the other hand, the protein aggregate generated at the same time period upon incubation at pH 7.0 did not exhibit a significant change when compared with the pH 2.5 sample (Fig. 2B). Day 7 sample, formed upon incubation at pH 7.0, exhibited CD spectrum very similar to that obtained for the day 4 aggregate (pH 2.5) showing almost the same degree of loss in ellipticity at 208 and 222 nm peaks. Interestingly, day 7 aggregate obtained at pH 2.5 exhibited a CD spectrum with a different pattern than its pH 7.0 counterpart, which matched with the spectrum obtained for the day 10 aggregate formed at pH 7.0. These aggregates exhibited a much higher loss in negative ellipticity at 208 nm than the aggregates formed earlier and also showed a shift in the 222 nm peak toward 215 nm. Similar results were obtained for

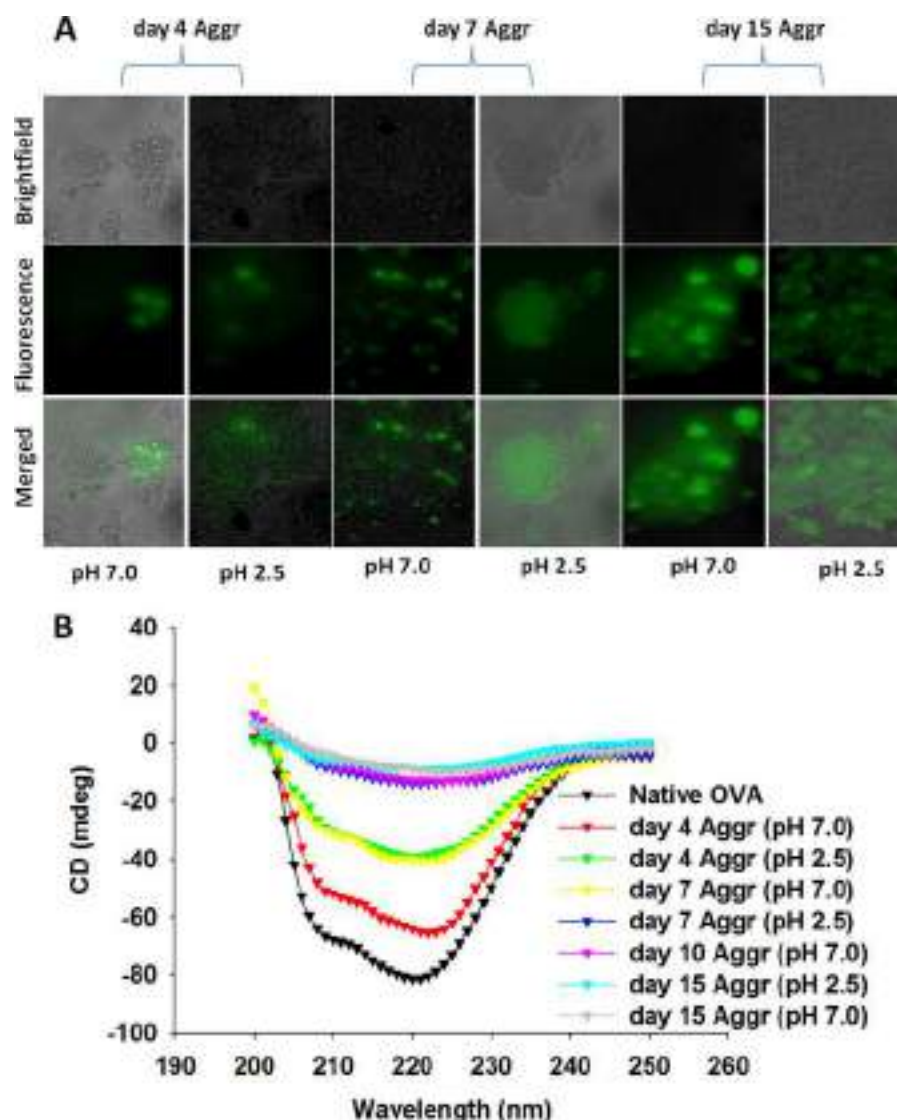


FIGURE 2. Extended shaking of soluble OVA leads to formation of β -sheet-rich aggregates. *A*, ThT binding to various OVA aggregates (100 μ g) as revealed by fluorescence microscopy. ThT (30 μ M) was incubated with OVA aggregates for 30 min; 5 μ l of the suspension was placed onto the glass slide and observed under fluorescence microscope. *B*, secondary structure determination of various OVA aggregates by far UV-CD. Native OVA and various aggregates (200 μ g/ml) were scanned in the range of 200–250 nm. Data are representative of at least three independent experiments with similar observations.

day 15 aggregates formed at both pH 2.5 and 7.0 with almost complete loss in 208 nm minima and a major shift toward 215 nm indicating the presence of only β -sheets, a structural feature of fibrillar amyloids (Fig. 2B).

Aggregates Formed at pH 7.0 Have Fibrillar whereas Aggregate Species Generated at pH 2.5 Have Suprafibrillar Morphology— The morphology of aggregates formed after various incubation periods at various pH conditions was elucidated employing electron microscopy. As shown in Fig. 3, aggregates obtained upon shaking at different pH conditions acquired varied morphologies after the same incubation periods. Fibrillar structures resembling typical amyloid fibrils appeared on day 10 postincubation for OVA incubated at pH 2.5 (Fig. 3A), whereas incubation at pH 7.0 ensued in amyloid fibril formation only after 15 days of incubation. In contrast, incubation of OVA at pH 2.5 revealed a branched suprafibrillar structure on day 15 postincubation (Fig. 3A).

The Sturdy Fibrillar Amyloids Release OVA in a Sustained Manner— The release of precursor OVA from its aggregates (formed at pH 2.5 and 7.0) was monitored over an extended time period (Fig. 3B). The protein aggregates (formed at pH 2.5 or 7.0) are found to release OVA in a time-dependent manner. The protein aggregates generated at pH 7.0 exhibited better release than the corresponding pH 2.5 aggregates. Release kinetics of various aggregates was monitored for a total of 15 days (Fig. 3B). Aggregates generated on day 2 of incubation (at pH 2.5 and 7.0) showed a burst release for the initial 3 to 4 days that leveled off subsequently. The aggregates formed at day 4 (for both pH 2.5 and 7.0) were found to release the precursor OVA in a steady manner that peaked at day 12 (for pH 2.5) and 10 (for pH 7.0) and plateaued at day 15. Aggregates generated upon 7 days of incubation exhibited varied release kinetics, the one formed at pH 2.5 could only feebly release OVA in contrast to the day 7 aggregate generated at pH 7.0, which exhibited a

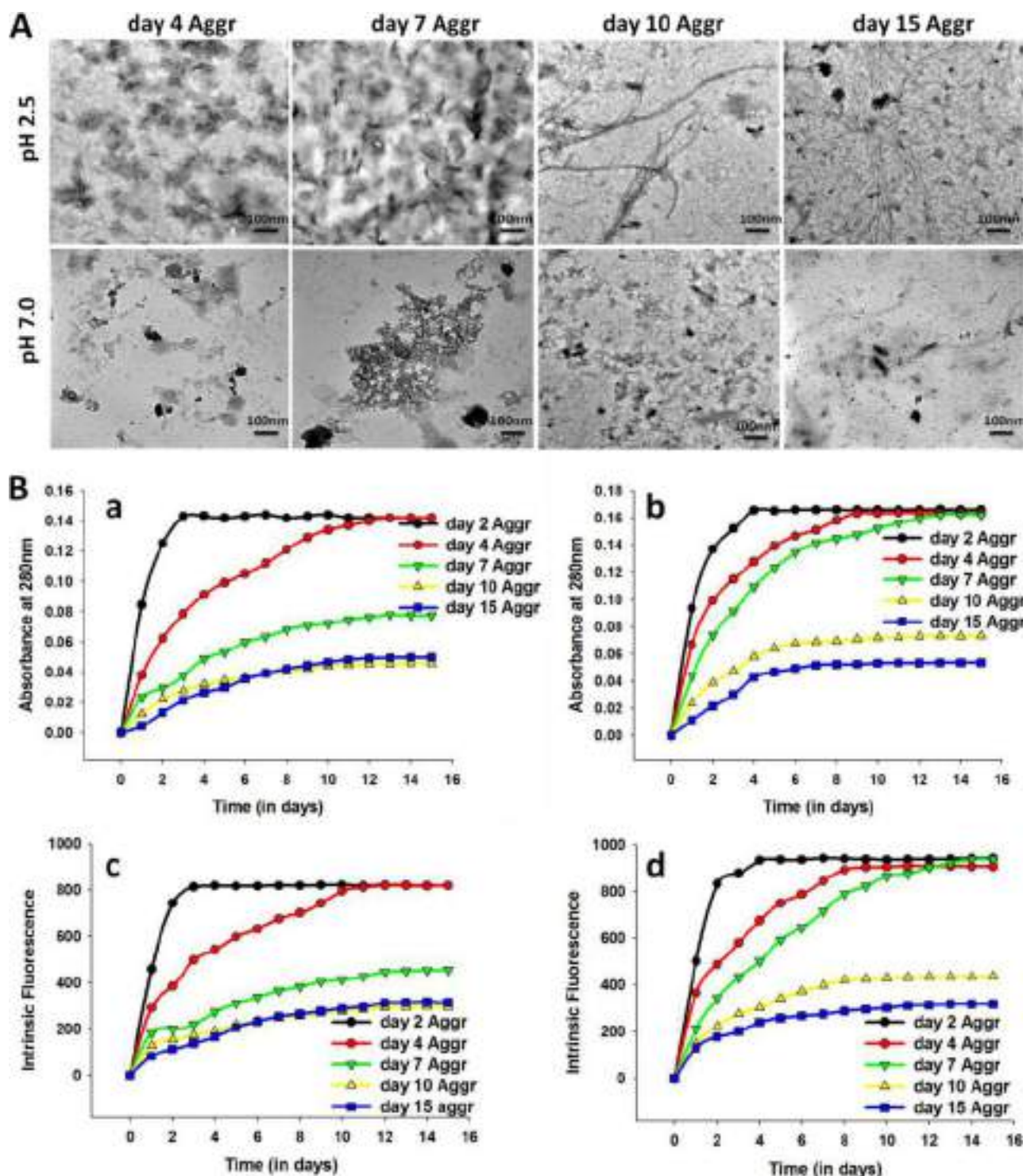


FIGURE 3. A, fibril maturation as revealed by transmission electron microscopy. Various aggregates (100 μ g) were coated onto the grid. Images shown are those selected after similar observation of at least two independent experiments performed for each sample. B, kinetics of OVA release from various OVA aggregates. *In vitro* release kinetics of OVA from various OVA aggregates obtained at pH 2.5 (a) and 7.0 (b) for a period of 15 days as measured by determining absorbance at 280 nm. Kinetics of OVA release from various OVA aggregates generated at acidic (c) and neutral (d) pH as monitored by measuring intrinsic fluorescence with excitation at 280 nm and emission in the range of 300–400 nm. Data are representative of three independent experiments with similar observations.

slow and sustained release that reached plateau after 13 days. The aggregates obtained upon 10 and 15 days of incubation at pH 2.5 or 7.0 were also observed to release OVA. Hence, both fibrillar as well as suprafibrillar forms of OVA were found to release the protein.

With an increase in the protein concentration in a given sample, its intrinsic fluorescence increases because of the increased number of Trp and Tyr residues. Therefore, we also monitored the intrinsic fluorescence of the protein released in the surrounding milieu (PB, pH 7.4). The intrinsic fluorescence profile

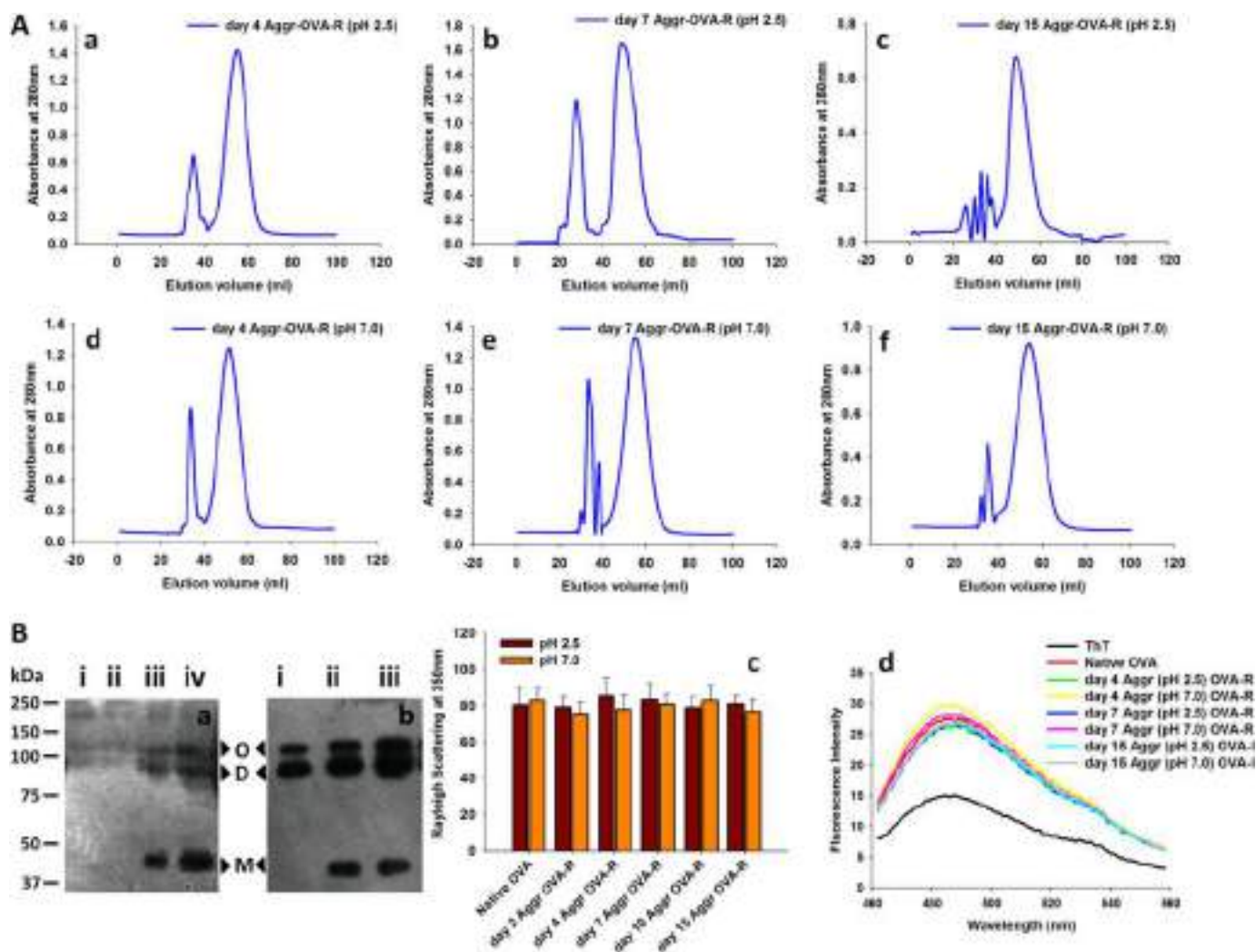


FIGURE 4. Besides oligomeric forms, aggregates release monomeric OVA that exhibit biophysical characteristics similar to that of native OVA. A, size exclusion chromatography of released OVA. Size exclusion profile of OVA released after 12 days of incubation from various aggregates exhibiting release of oligomers, dimers in addition to monomers. a–f, show SEC profile of OVA released after 12 days of incubation from day 4 aggregate formed at pH 2.5, day 7 aggregate formed at pH 2.5, day 15 aggregate formed at pH 2.5, day 4 aggregate formed at pH 7.0, day 7 aggregate formed at pH 7.0, and day 15 aggregate formed at pH 7.0, respectively. B, Western blot analysis and biophysical characteristics of released OVA. Probing with OVA specific polyclonal and 3G2E1D9 monoclonal antibodies reveals the presence of monomeric, dimeric, as well as oligomeric OVA released from OVA aggregates in surrounding milieu. a, immunoblot of OVA released from day 7 aggregate formed at pH 7, developed using anti-OVA polyclonal antibody; lanes i–iv exhibit OVA released after 4, 8, 12, and 15 days, respectively, from the day 7 aggregate. b, OVA released from day 7 aggregate formed at pH 7 probed using anti-OVA 3G2E1D9 monoclonal antibody; lanes i–iii show OVA released post 8, 12, and 15 days of incubation, respectively. M, D, and O denote monomers, dimers, and oligomers respectively. Rayleigh scattering studies (c) and ThT binding assay (d) of the OVA released from various aggregates. OVA-R represents released OVA from various aggregates. Error bars represent S.E. of three independent experiments. At least three independent experiments were carried out for each sample and data obtained with similar results are presented.

of the released OVA for various aggregates was in agreement with those observed for spectrophotometric results. Intrinsic fluorescence of the materials released from day 15 aggregates formed either at pH 2.5 or 7.0 attained a substantial value suggesting release of OVA (Fig. 3B, c and d).

Besides Monomer, the Aggregates Possibly Release Dimeric as Well as Oligomeric Forms of OVA—The elution profile of OVA released after 12 days of incubation from various aggregates is shown in Fig. 4A. In almost all the fractions, peaks are observed in the void volume apart from another fully resolved and distinct peak corresponding to the released monomeric form of protein. The peaks corresponding to the void volume may be dimeric or multimeric forms of released OVA. This indicates that the released fraction of OVA from various aggregates is a mixture of monomeric, dimeric, as well as multimeric OVA

(Fig. 4A). Western blot analysis employing OVA-specific polyclonal and 3G2E1D9 monoclonal antibodies further supported the possible presence of various forms (monomeric/dimeric/oligomeric) of OVA released from aggregates (Fig. 4B, a and b). Immunoblots stained with polyclonal anti-OVA antibody showed OVA release from day 7 aggregate formed at pH 7.0 after 4, 8, 12, and 15 days corresponding to lanes i, ii, iii, and iv, respectively (Fig. 4B, a). Surprisingly, post 4 and 8 days of release, no monomeric band was observed, although bands of higher molecular weight that might be corresponding to dimer or higher oligomeric forms can be seen in Fig. 4B, lanes i and ii. After 12 and 15 days of release, a distinct monomeric band in addition to higher molecular weight proteins can be observed in Fig. 4B, lanes iii and iv. Similarly, Western blot performed using anti-OVA 3G2E1D9 monoclonal antibody shows concordant

result with that obtained for polyclonal antibody (Fig. 4B, *b*). Lanes *i*, *ii*, and *iii* correspond to OVA released after 8, 12, and 15 days, respectively, from day 7 aggregate formed at pH 7. Monomeric as well as higher oligomeric forms of OVA are seen to be present in the released materials after 12 days of release.

The Released OVA Seems to Acquire a Native Configuration and Does Not Exhibit General Characteristics of Amyloid Aggregates—A fixed amount of released OVA from various aggregates (generated at pH 2.5 and 7.0) was analyzed for light scattering at 350 nm. As shown in Fig. 4B, *c*, the protein materials released from various aggregates exhibited light scattering very similar to that of native OVA. Moreover, the released OVA from various aggregates also showed binding to ThT more or less very similar to native OVA (Fig. 4B, *d*).

Antibodies Developed Upon Administration of Aggregated Forms of OVA Can Recognize Native Antigen—The immune response evoked in the host upon immunization with various aggregate forms of OVA was assessed on the basis of ELISA and dot blot assay. First, wells of ELISA plates were coated with various forms of OVA aggregates and were allowed to react with antibodies generated upon immunization with the corresponding aggregate forms. OVA fibrils formed either at pH 2.5 or 7.0 were found to react explicitly with the generated antibodies. Antibodies developed against aggregates formed either at pH 2.5 or 7.0 exhibited very similar reactivities. Dot blot assay results supported the ELISA findings that antibodies reactive to various OVA aggregate forms are formed upon their immunization (Fig. 5A).

Subsequently, the potential of the antibodies to recognize the native form of OVA was assessed. For ELISA, wells of microtiter plates were coated with native OVA and evaluated for its reactivity with antibodies developed against both native as well as various other forms of OVA aggregates. ELISA analysis revealed that antibodies generated against various forms of aggregates (formed both at pH 2.5 or 7.0) could recognize the native form of OVA (Fig. 5B, *a* and *b*). Aggregates generated on day 10 as well as day 15 (at both pH 2.5 and 7.0) were also successful in generation of anti-native OVA antibodies. Interestingly, day 4 aggregate for pH 2.5 as well as days 4 and 7 aggregates for pH 7.0 exhibited better antibody production than the native OVA itself. The specificity of the generated antibodies was further ascertained by dot blot assay. To perform dot blot, native OVA was coated on PVDF strips and its reactivity was checked for antibodies generated against various forms of OVA aggregates (both for pH 2.5 and 7.0 samples) formed at various time points. The antibodies developed upon immunization with both non-fibrillar as well as fibrillar OVA aggregates were found to recognize native OVA (Fig. 5B, *c*).

OVA Released by Various Forms of OVA Aggregates Interacts with Anti-native OVA Antibodies of Monoclonal as Well as Polyclonal Origin—The incubation of fibrils as well as other forms of OVA resulted in the release of precursor OVA into the surrounding milieu. We wondered whether various *in vitro* released species of OVA are reactive to native OVA-specific antibodies (of polyclonal as well as monoclonal origin); to ensure this we performed indirect ELISA and a dot blot assay. For ELISA, we coated the wells with OVA (2 μ g/well) released *in vitro* from each aggregate (both non-fibrillar or fibrillar) and

allowed it to interact with polyclonal anti-native OVA antibodies (which may recognize mature aggregates/fibrils as well). Proteins released from various aggregates generated either at pH 2.5 or 7.0 were found to react with anti-native OVA antibodies (Fig. 5C, *a* and *b*). Next, we performed a dot blot assay by coating the released OVA onto PVDF strips and checked their reactivity with antibodies raised against native OVA as done for ELISA. In concordance with ELISA data, the anti-native OVA antibodies recognized the OVA released from various aggregates (Fig. 5C, *c*).

To work on our hypothesis that the released OVA might be attaining a native conformation, we exploited the 2D11 antibody, which is an anti-OVA mouse monoclonal antibody showing high affinity for OVA in its native form, but is not able to recognize denatured OVA. ELISA as well as dot blot results enumerate the recognition of released OVA from various aggregates with 2D11 antibody (Fig. 5D, *a* and *b*). Native OVA as well as OVA released from various aggregates exhibit practically similar reactivities with 2D11 antibody. Similarly, dot blot data also reveals that 2D11 antibodies recognize both mono as well as oligomeric forms of released OVA. However, 2D11 antibodies fail to recognize the mature aggregates/fibril form of proteins (Fig. 5D, *c*).

Macrophages Phagocytose OVA Aggregates—The sustained release kinetics observed for various OVA aggregates persuaded us to analyze if they can be exploited as vaccine candidates. Because aggregates obtained at day 4 and 7 for pH 2.5 and 7.0, respectively, were found to release OVA in a slow and sustained manner. Therefore, we investigated their uptake by macrophages, which are professional antigen presenting cells. As shown in Fig. 6*a*, a punctuate fluorescence (co-localization with endo-lysosomal compartment) along with a diffuse pattern localized throughout the intracellular compartment of the macrophages is observed.

The OVA Aggregate-based Immunization Evokes Th1 Cytokines in the Host—Th1/Th2 bias was assessed in animals immunized with day 4 and 7 aggregates (formed at pH 7.0 and 2.5). The levels of Th1 cytokines, IL-12 and IFN- γ , were found to be significantly elevated in animals immunized with day 4 (formed at pH 7.0 and pH 2.5) and day 7 (formed at pH 7.0) aggregates in comparison to native OVA ($p < 0.01$) (Fig. 6, *b* and *c*). Although day 7 aggregates formed at pH 2.5 led to the generation of higher levels of Th1 cytokines, the increase was only moderate as compared with native OVA ($p < 0.05$). On the other hand, insignificant levels of Th2 cytokine (IL-4) were induced in the groups immunized with day 4 (generated at pH 7.0 and 2.5) and day 7 aggregates (formed at pH 7.0) (Fig. 6*d*). The day 7 aggregate (generated at pH 2.5) also exhibited generation of moderately lower levels of IL-4 as compared with native OVA, which showed elevated levels of IL-4 in comparison to control (Fig. 6*d*).

We also determined antibody isotype switching by evaluating the OVA aggregate-specific IgG1 and IgG2a response in the serum of immunized animals. Animals immunized with day 4 (formed at pH 2.5 and 7.0) and day 7 (generated at pH 7.0) aggregates showed a significantly higher IgG2a/IgG1 ratio as compared with native OVA ($p < 0.01$). On the other hand, day 7 aggregates formed at pH 2.5 showed only a moderate increase

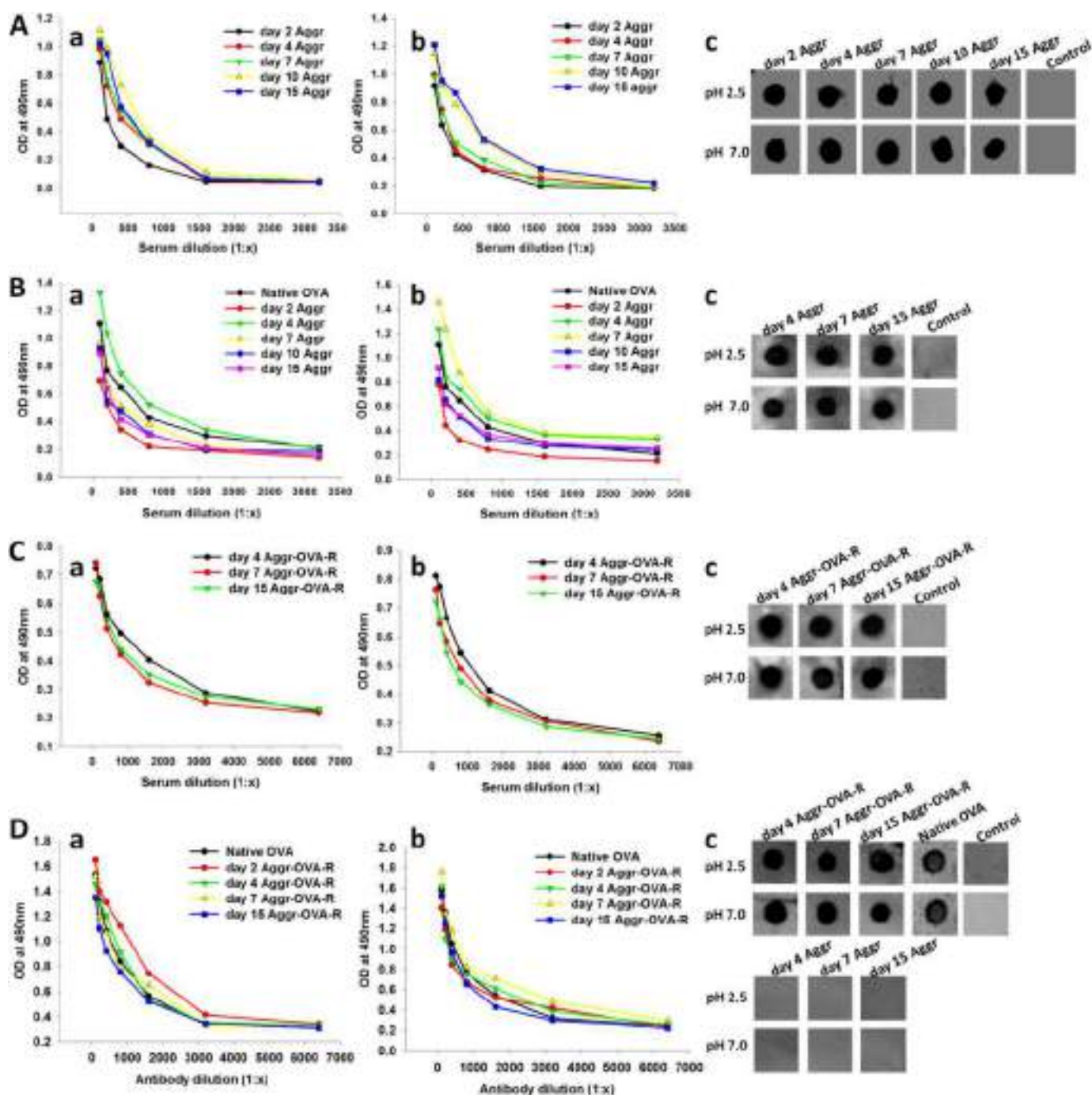
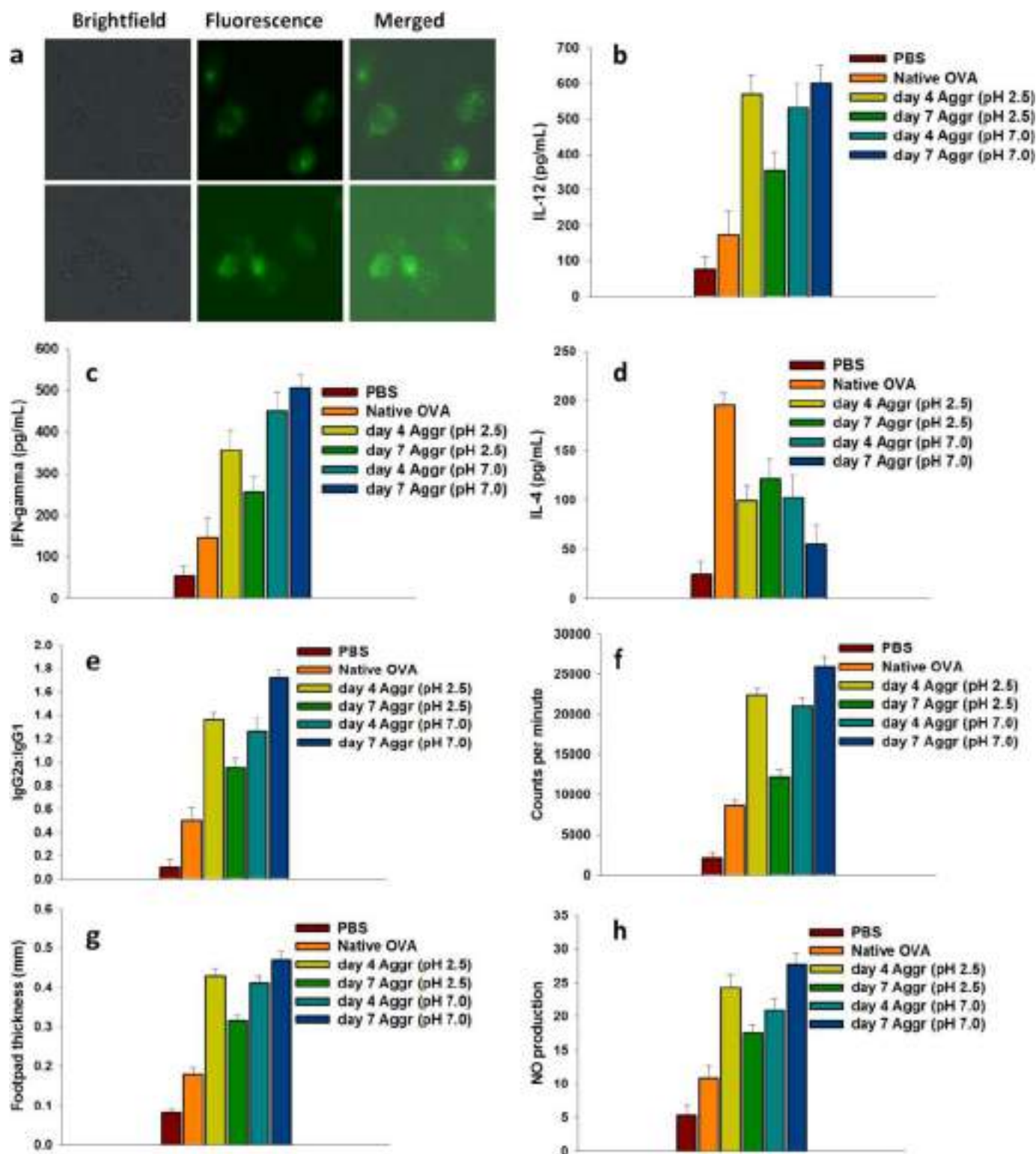


FIGURE 5. A, induction of aggregate-specific antibodies upon immunization with OVA aggregates. OVA aggregates formed at pH 2.5 (a) and 7.0 (b) were allowed to interact with aggregate-specific antibodies employing ELISA. In the next set of experiments, various amyloid aggregates were coated onto PVDF strips and investigated for binding with antibodies generated upon immunization with corresponding aggregates (c). B, OVA aggregate immunization also induces native OVA-specific antibodies. The ELISA plate was coated with native OVA antigen as described under "Experimental Procedures" and reacted with sera obtained from the animals immunized with various aggregates as revealed by dot blot assay (c). C, OVA released from various aggregates interacts with native OVA-specific antibodies. ELISA plate was coated with OVA released from aggregates formed at pH 2.5 (a) and 7.0 (b) and allowed to react with polyclonal antibodies produced in response to immunization with native OVA. For the dot blot assay, OVA released from various amyloid aggregates were coated onto PVDF strips and evaluated for reactivity with anti-native OVA polyclonal antibodies (c). D, OVA released from various amyloid aggregates reacts with 2D11 anti-OVA monoclonal antibody that recognizes only native but not denatured OVA. ELISA plate was coated with OVA released from aggregates formed at pH 2.5 (a) and 7.0 (b) and allowed to react with 2D11 anti-native OVA monoclonal antibodies. OVA released from various amyloid aggregates and amyloid aggregates themselves were coated onto PVDF strips and evaluated for reactivity with anti-native OVA 2D11 monoclonal antibody by dot blot assay (c). 2D11 antibody recognized OVA released from various aggregates as well as native OVA but failed to react with aggregates themselves. For control, a nonspecific protein (human serum albumin) was coated onto the membrane and allowed to react with the respective antibody. OVA-R denotes OVA released from various aggregates. Data are representative of at least three independent experiments carried out for each sample.

Amyloid Immunization Induces Native Antigen-specific Antibodies

in the IgG2a/IgG1 ratio in comparison to native OVA ($p < 0.05$) (Fig. 6e).

T-cell Proliferative Response in Lymphocytes Isolated from Immunized BALB Mice—Lymphocyte proliferation in response to immunization with a prospective candidate vaccine is used as a parameter to assess its vaccine potential. Lymphocytes isolated after 1 week post-booster from the spleens of mice immunized with native OVA as well as day 4 and 7 aggregates formed at both pH 2.5 and 7.0 exhibited proliferation upon their exposure to native OVA (Fig. 6f). The proliferative response of lymphocytes obtained from animals immunized with day 4 (formed at pH 2.5 and 7.0) and day 7 (formed at pH 7.0) aggregates was significantly higher when compared with native OVA ($p < 0.01$), however, day 7 aggregate formed at pH 2.5 showed a



moderately higher T cell proliferation in comparison to native OVA ($p < 0.05$). Graph plots (Fig. 6f) reveal the lymphocyte proliferative response at a fixed dose of OVA (20 μg) at the 1 week post booster time point.

OVA Aggregates Evoke Delayed Type Hypersensitivity in Immunized Animals—To evaluate the ability of various OVA aggregates to induce a cell-mediated immune response, we immunized mice subcutaneously in the lower abdominal region. Native OVA was used as immunogen to determine whether they need an adjuvant for evoking a desirable immune response. Seven days after immunization, mice were exposed to native OVA or PBS via the footpad and footpad swelling was measured at various time intervals. Immunization with day 4 (generated at pH 2.5 and 7.0) and day 7 aggregates formed at pH 7.0 resulted in the generation of a strong DTH response ($p < 0.01$), whereas the day 7 aggregate formed at pH 2.5 resulted in generation of a moderate DTH response ($p < 0.05$) as compared with native OVA (Fig. 6g).

Nitric Oxide Production—Formation of NO is used as an index of the activated macrophage population. One week post booster, generation of NO was evaluated in macrophages isolated from mice immunized with native OVA and day 4 as well as day 7 OVA aggregates. Maximum NO production was observed in macrophages isolated from mice given day 7 aggregate formed at pH 7.0 after 24 h of pulsation ($p < 0.01$), followed by day 4 aggregate generated at pH 2.5 and day 4 aggregate formed at pH 7.0. An augmented NO production in comparison to native OVA was also observed in day 7 aggregate formed at pH 2.5 ($p < 0.05$) but was not as significant as observed for other aggregates (Fig. 6h).

Lymphocyte Proliferative Response in Splenocytes Isolated from TCR-transgenic OT-I and OT-II Mice—To obtain a clear picture about the humoral and cellular immune responses evoked, we also analyzed lymphocyte proliferative response in splenocytes isolated from TCR-transgenic OT-I and OT-II mice that exhibited T cells with TCR specific for OVA Class I and OVA Class II peptides, respectively. Splenocytes isolated from TCR-transgenic OT-I and OT-II mice were treated with native OVA as well as day 4 and 7 OVA aggregates formed at pH 2.5 and 7.0, respectively. As shown in Fig. 7, the proliferative response of OT-I and OT-II splenocytes with day 4 (formed at pH 2.5) and day 7 (formed at pH 7.0) aggregates was significantly higher when compared with native OVA ($p < 0.05$). Day 7 OVA aggregate formed at pH 7.0 also showed a slightly higher

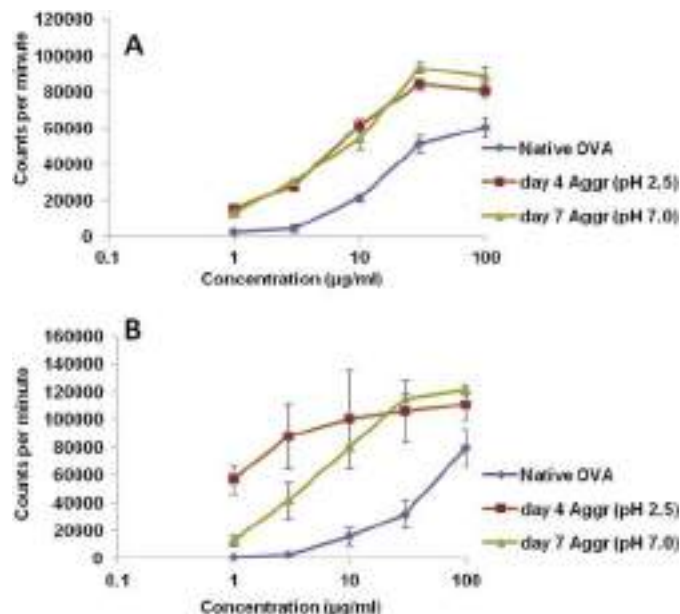


FIGURE 7. Lymphocyte proliferation response in TCR-transgenic OT-I and OT-II lymphocytes upon stimulation with titrating concentrations of various OVA aggregates. T cell proliferation response in TCR-transgenic OT-I (A) and TCR-transgenic OT-II (B) cells when co-cultured in the presence of increasing concentrations (1 to 100 $\mu\text{g/ml}$) of day 4 aggregate formed at pH 2.5 and day 7 aggregate formed at pH 7.0 in 96-well plates. After 60 h of culture, the plates were pulsed with 0.5 $\mu\text{Ci/well}$ of [^3H]thymidine. The radioactivity incorporation was determined at the end of the 72-h incubation in proliferating cells and represented in terms of counts/min values to denote the level of stimulation. Data are expressed as mean \pm S.E. of triplicate cultures. Statistical significance of the data was determined using Student's *t* test analysis with $p < 0.05$ level of significance. For OT-I as well as OT-II lymphocytes, day 4 aggregate (formed at pH 2.5) versus native OVA, $p < 0.05$; day 7 aggregate (formed at pH 7.0) versus native OVA, $p < 0.05$.

T cell proliferation in comparison to day 4 OVA aggregate formed at pH 2.5.

DISCUSSION

Amyloid fibrils belong to the group of self-assembled ordered nanostructures generated upon improper folding of polypeptides/proteins (2). Although pathogenesis of several human diseases is linked to amyloids, growing evidence indicates that amyloids might be actively participating in several biological events. A few recent reports have elaborated that *in vitro* synthesized amyloids when administered into animals elicit biological functions of native protein (23, 24). Maji *et al.* (23) found that long-acting gonadotrophin releasing hormone analogs are

FIGURE 6. OVA aggregates are taken up by professional antigen presenting cells and also evoke Th1 biased immune response in the immunized animals. a, green fluorescence shows the uptake of day 4 aggregate obtained at pH 2.5 (upper panel) and day 7 aggregate obtained at pH 7.0 (lower panel) by macrophages isolated from thioglycollate-primed BALB/c mice. Data are representative of at least three independent experiments carried out for each sample. OVA aggregates (day 4 and 7 aggregates formed both at pH 2.5 and 7.0) mediated Th1/Th2 bias was ascertained by determining the cytokine response in splenocyte culture supernatant belonging to various immunized groups following the protocol described under "Experimental Procedures," IL-12 (b), IFN- γ (c), and IL-4 (d). To further confirm the Th1/Th2 polarization upon immunization with day 4 and 7 aggregates (generated at pH 2.5 and 7.0), the sera of immunized animals were analyzed for the presence of IgG2a and IgG1 isotype (e). Moreover, OVA aggregates induce strong DTH response, lymphocyte proliferation, and enhanced NO production as revealed by f–h, respectively in BALB/c mice. The DTH response was evaluated by measuring footpad swelling in mice immunized with day 4 aggregate, day 7 aggregate, native OVA, and PBS. The lymphocyte proliferation assay was performed to measure lymphocyte proliferation of OVA-specific T lymphocytes. Lymphocytes isolated from various immunized groups were stimulated with a corresponding set of matching formulations of OVA. The cells were allowed to grow in the presence of [^3H]thymidine as described under "Experimental Procedures." The incorporation of labeled nucleotide during DNA synthesis of proliferating cells was measured and denoted as cpm. NO was determined in macrophages isolated from mice immunized with various aggregates. The data represent mean of three determinants \pm S.D. and are representative of at least three independent experiments with similar observations. Various immunized groups were compared to determine the statistical significance of the data using Student's *t* test analysis with $p < 0.05$ level of significance. Day 7 aggregate formed at pH 7.0 versus native OVA, $p < 0.01$; day 4 aggregate formed at pH 7.0 versus native OVA, $p < 0.01$; day 7 aggregate formed at pH 2.5 versus native OVA, $p < 0.05$; day 4 aggregate formed at pH 2.5 versus native OVA, $p < 0.01$.

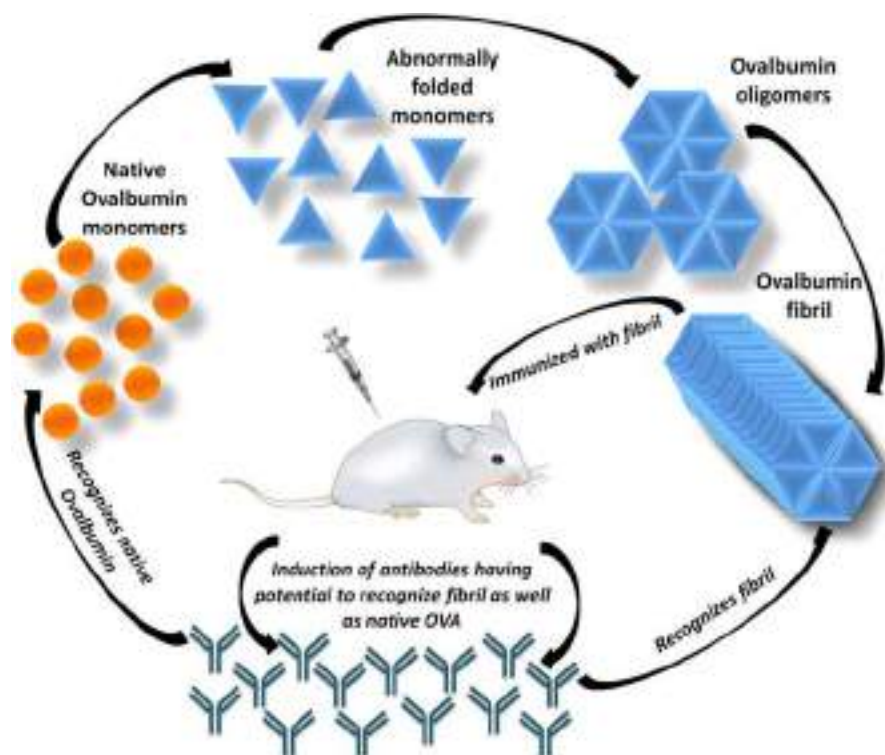


FIGURE 8. Schematic illustration depicting kinetics of fibril formation and potential of OVA aggregates to induce generation of antibodies that have ability to recognize both native as well as fibrillar OVA. The model depicts that formation of fibril from monomeric native OVA encompasses various intermediates such as abnormally folded monomers and oligomers. More specifically, it highlights that fibril immunization leads to generation of antibodies reactive to native as well as fibril OVA.

able to form amyloids *in vitro*, which can sustain the release of monomeric drug, the therapeutic molecule, both *in vitro* and *in vivo*. Similarly, a controlled release of insulin monomers from *in vitro* synthesized pre-fibrillar oligomers made from insulin has also been reported (24), demonstrating them to be a potential therapeutic agent of long-acting peptide drugs. The investigators propose that amyloid fibril or pre-fibril forms can act as depots and possibly release the bioactive form of the peptide/protein in a sustained manner. The bioactivity of the released peptide is realized from the fact that it binds to the specific receptors (*c.f.* gonadotrophin releasing hormone receptor and insulin receptor) for which obviously a perfectly fitting conformation is required to activate the downstream signaling cascade. In these reports, it has been proposed that the amyloid form of protein may release the bioactive peptide retaining the conformational properties of the native state of the peptide. In the present study, in-house synthesized amyloid fibrillar and non-fibrillar aggregates of OVA were injected into mice and the antibodies raised against them were evaluated for their specificity with native form of OVA.

Morphological alterations in amyloids formed from the same protein under differing incubation ambiances are among the most intriguing aspects of amyloid attributes (37). The pH of the surrounding medium has been observed to be one of the most influencing factors on the characteristic morphology and properties of the resulting amyloid fibrils. It has been found that by manipulating pH conditions, whereas keeping other extrinsic factors constant, fibrils of various shapes and sizes of the same protein can be generated (24). In the present study, buffers with three different pH, glycine-HCl (pH 2.5), glycine-

NaOH (pH 10), and PBS (pH 7.0), were used to induce OVA amyloid formation. Although pH 2.5 and 7.0 buffers could induce aggregation upon shaking, pH 10 buffer failed to induce aggregation (data not shown). Acidic ambience was found to induce better protein aggregation than neutral pH conditions (Fig. 1). Interestingly, the amyloid aggregates for OVA obtained at two different buffering conditions exhibited altered properties with respect to content of β -sheet-rich structures and also to the morphological characteristics of formed aggregates (Figs. 1–3).

Moreover, the data presented in Figs. 1–3, in general, clearly reveal that pH 2.5 induces early fibril formation as compared with pH 7.0. This is particularly well demonstrated in Fig. 3A where the day 15 aggregate formed at pH 2.5 shows hugely branched supra-fibrillar assembly in contrast to the pH 7.0 sample revealing two fibrillar threads separately lying over the surface. This might be explained on the premise that at pH 2.5, earlier formed fibrils would further adhered laterally upon longer incubation conditions that eventually led to the formation of suprastructures. The data also suggest that amyloid formation involves a hierarchical process where precursors formed at the initial stages act as a template for the growth of mature fibers that subsequently interact laterally to generate supra-fibrillar structures. A schematic representation of the hierarchical process of fibril formation is outlined in Fig. 8. The two pH conditions, *i.e.* neutral *versus* acidic, seem to induce $\alpha \rightarrow \beta$ transitions in the protein following the same sojourn, however, this transition is more prompt at acidic conditions when compared with neutral pH conditions. It seems that the variations in the electrostatic repulsions or attractions in response to differing

pH conditions may lead to formation of aggregates of varied morphologies. This implicitly suggests that the amyloid fibrils may have the potential to exhibit distinct forms depending on the incubation conditions.

Once it was ascertained that both acidic and neutral pH conditions induce the formation of amyloid aggregates and fibrils of OVA with differing compactness and morphologies, we set about to study their tendency to release OVA, the precursor protein. The day 4 and 7 aggregates formed either at pH 2.5 or 7.0 were found to release OVA over a period of 10–15 days in a sustained manner. Beyond this period, OVA release from the aggregates attained equilibrium. Interestingly, the fibrillar aggregates formed at days 10 and 15 could also release OVA although the release was not much pronounced when compared with aggregates obtained at the day 4 and 7 incubation periods (Fig. 3B). To analyze the state of OVA released from various aggregates, size exclusion chromatography and Western blot analyses of released OVA from various aggregates were performed. As seen in the size exclusion chromatography analysis (Fig. 4A), the released OVA from various aggregates seems to be in the monomeric, dimeric, as well as multimeric states although the monomeric OVA seems to be a dominant species in the mixture for all the samples. As can be seen in Western blot analysis performed using 3G2E1D9 monoclonal as well as polyclonal anti-OVA antibodies, the appearance of monomeric as well as oligomeric forms of the OVA can be detected by day 12 postincubation (Fig. 4B, *a* and *b*). Until day 8, bands corresponding to higher oligomeric forms of OVA can be seen but no monomeric band is observed. Immunoblot studies appear to indicate that release of OVA from various aggregated forms seem to follow a multistep kinetics. It is to be noted that the OVA dimers/oligomers that are released from the aggregates are SDS-insoluble, hence, did not run as a monomer in a reducing SDS gel, which is in concordance with several reports enumerating the formation of SDS-insoluble amyloid fibrils (38–41). This supports the view that the respective species are actually released from amyloid fibrils.

Furthermore, antigen antibody interactions revealed that antibodies generated upon immunization with various aggregates obtained at pH 2.5 or 7.0 could recognize native OVA in addition to aggregate forms (Fig. 5, *A* and *B*). A schematic illustration of the production of antibodies capable of recognizing fibrillar as well as native OVA upon fibril immunization is shown in Fig. 8. Induction of antibodies reactive to native OVA upon immunization with various aggregates suggests that either the fibrils themselves carry native antigen epitopes or the released OVA might be retaining the native epitopes, also both possibilities may happen simultaneously. Interestingly, day 4 aggregate formed at pH 2.5 and both day 4 as well as day 7 aggregates obtained at pH 7.0 exhibited elevated antibody production against native OVA than the sera obtained upon immunization with native antigen (Fig. 5B, *a* and *b*). The observed interaction of OVA with specific polyclonal antibody could be explained on the premise that aggregates may have acted as depots for the slow and sustained release of the antigen, which boosted immune cells over the time to generate antigen-specific antibodies. Moreover, OVA released under simulated conditions *in vitro* (from each aggregate obtained at pH 2.5 and

7.0) was tested for its reactivity with antibodies generated against the native form of OVA. The anti-native OVA polyclonal antibodies recognized the released OVA substantially, indicating possible retention of native epitopes in the released proteins (Fig. 5C). The observation was validated by employing 2D11 monoclonal anti-OVA antibody that specifically recognizes the native form of OVA but not the denatured form. Interestingly, anti-native OVA monoclonal 2D11 antibody recognizes native OVA as well as OVA released from various aggregates but not aggregates themselves (Fig. 5D). It indicates that the 2D11 antibody is specific to an epitope (conformational epitope) exposed in native OVA but inaccessible in aggregated forms. The reactivity of 2D11 antibody with native and released OVA but not to its aggregated form gives an indication that protein when released from aggregates may undergo a conformational change. The released precursor protein seemingly attains the conformation similar to that of native protein leading to the exposure of the epitopes accessible in native form, which, however, may be buried in the aggregated protein. The reactivity of two different OVA-specific monoclonal antibodies (3G2E1D9; capable of recognizing denatured OVA and 2D11; reactive to native OVA only not denatured form) used in the present study clearly demonstrates that OVA aggregates have the potential to release precursor proteins with intact linear as well as conformational epitopes. Biophysical characteristics such as light scattering and ThT binding of released OVA from various aggregates was also found to be very similar to that observed for native OVA (Fig. 4B, *c* and *d*). Moreover, the far UV CD spectra of the proteins released from various aggregates were also found to be similar to the spectrum obtained for native OVA (data not shown). Although these findings suggest that the released OVA possibly refolds to native protein and hence may harbor the conformational properties of the native form of the protein. However, it would be too premature to conclude that the proteins released from amyloids completely refold to the native protein conformation, as from one intact conformational epitope, we cannot declare conformational integrity of the overall structure.

The immune response data of the present study categorizes aggregate form of protein to act as a potential vaccine, whereby, slow release of antigen over extended time periods can elicit desirable immune responses without repeated booster requirements. Because the day 4 and 7 aggregates generated both at pH 2.5 and 7.0 exhibited slow and sustained release of OVA, we analyzed Th1/Th2 bias, lymphocyte proliferation, DTH response, and NO production in animals immunized with these aggregates. Day 4 and 7 aggregates (formed at pH 2.5 and 7.0) were found to induce significantly higher levels of Th1 cytokines as compared with native OVA (Fig. 6). Strong T cell proliferation and heightened DTH response also indicate a polarized bias for the cell-mediated response in animals immunized with OVA aggregates formed at pH 2.5 and 7.0, but not in those immunized with its native form. Moreover, a significantly higher amount of NO was produced by macrophages isolated from mice immunized with OVA aggregates (Fig. 6). Lymphocyte proliferative responses in OT-I and OT-II lymphocytes in response to stimulation with day 4 and 7 OVA aggregates obtained at pH 2.5 and 7.0, respectively (Fig. 7), are found in

concordance with the T cell proliferative responses obtained for lymphocytes isolated from immunized BALB/c mice. This indicates that OVA aggregate forms are successful in evoking both humoral as well as cell-mediated immune responses in the host. As shown in Fig. 6, *b-h*, the most desirable response among various aggregates was observed for day 7 aggregate (formed at pH 7.0) followed by day 4 aggregate (formed at pH 2.5), which was further trailed by day 4 (formed at pH 7.0) and day 7 (formed at pH 2.5) aggregates. The pattern observed appears to be correlated to the release kinetics of OVA from various aggregates because the day 7 aggregate (formed at pH 7.0) exhibited the best release kinetics when compared with other aggregates. It was followed by day 4 aggregate (formed at pH 2.5), which was found to exhibit better release kinetics than day 4 (formed at pH 7.0) and day 7 (formed at pH 2.5) aggregates. The results are consistent with a previous report where it has been demonstrated that the generation of desirable humoral and cellular immune responses is a function of kinetics of antigen delivery (42). Demento *et al.* (42) have shown that OVA despite being a weak immunogen in free form could elicit a strong humoral and cellular immune response when delivered in poly(lactic-co-glycolic acid) nanoparticles. They ruled out the adjuvant properties of encapsulating material, specifically poly(lactic-co-glycolic acid) (a biodegradable and biocompatible polymer). Rather, their findings enumerate that the slow and sustained release of OVA from poly(lactic-co-glycolic acid) nanoparticles led to better humoral and cellular immune responses in contrast to liposomes, which exhibited a burst release of OVA. Interestingly, they also found that sustained antigen release plays a crucial role in shaping a long-lasting effector T cell memory response, which led to effective clearance of *Listeria monocytogenes*, an intracellular pathogen (42).

Besides specific affinity between the TCR-peptide-MHC interface, the phenotype of T lymphocytes generated against a given antigen is generally regulated by its dose, chemical composition, and also by factors such as differential expression of co-stimulatory molecules, presence of adjuvants, cytokine milieu *etc.*, which play important roles in differential Th1/Th2 bias (43–45). Although the potential of OVA aggregates to induce a strong humoral response can have a direct correlation with a sustained release potential, however, a Th1 bias of various observed immunological responses remained intriguing. It seems that preferential uptake of OVA amyloids by macrophages (Fig. 6*a*) because of their particulate nature has some co-relationship with the Th1-biased response as in an earlier study, it has been reported that OVA linked to beads is presented via MHC class I molecules by macrophages approximately 10^4 times more efficiently than soluble OVA (46).

Fig. 9*A* highlights various sequential and conformational B-cell epitopes present in OVA. The various sequential epitopes have been predicted by COBEpro (47), an algorithm dedicated to predicting continuous B-cell epitopes. The conformational B-cell epitopes have been predicted using CBTOPE prediction algorithm (48).

The cell-mediated immunity exhibited by OVA amyloid aggregates can also be in part due to some of the primary sequence stretches that have a strong propensity to form amyloids and simultaneously bear T cell antigenic determinants.

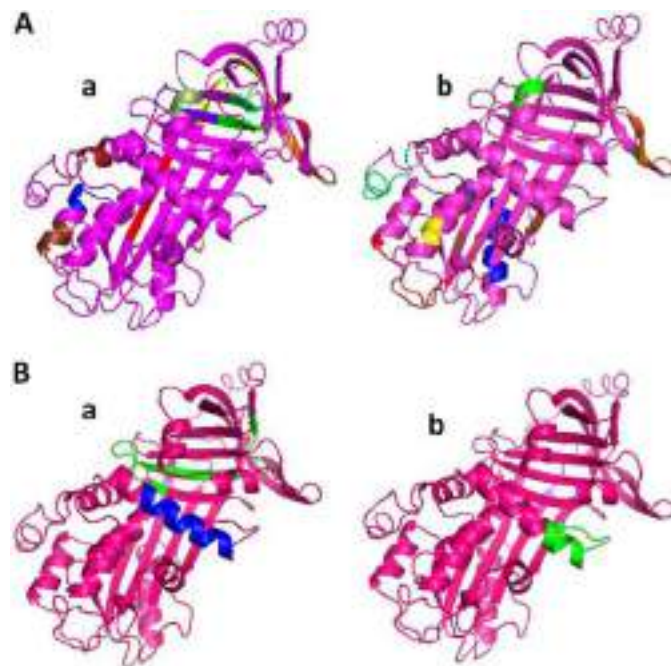


FIGURE 9. *A*, putative B-cell epitopes present in OVA. *a*, various sequential B-cell epitopes present in OVA as retrieved using COBEpro, an algorithm used for predicting continuous B-cell epitopes. Sequences with scores between 0.75 and 0.85 have been presented: ¹¹²PILPEY¹¹⁷ (red), ¹³⁵QTAAQ¹⁴⁰ (blue), ²⁴⁹VSGLEQ²⁵⁴ (green), ²¹⁴GLFRV²¹⁹ (orange), ¹⁹³TQAMPF¹⁹⁸ (yellow), ²³⁴FASGTM²³⁹ (lime green), ¹⁶⁶VDSQTA¹⁷² (brown), ⁹²KPNVDVYS⁹⁸ (firebrick), ²⁷²VMEERK²⁷⁷ (forest), ²⁴⁵LPDEVSG²⁵¹ (purple blue), ²²²MASEKM²²⁷ (olive), and ¹²⁶RGGLEPI¹³². *b*, conformational B-cell epitopes present in OVA as predicted by CBTOPE prediction algorithm: ⁷²QCG⁷⁴ (yellow), ⁸⁰HS⁸¹ (deep blue), ⁸⁶ILN⁸⁸ (lime green), ¹¹⁸LQ¹¹⁹ (raspberry), ¹²¹VKELYRGG¹²⁸ (red), ¹⁵⁴NGIIRNVLPQS¹⁶⁴ (blue), ²⁰³QESKPVMYQ²¹³ (orange), ²¹⁶LF²¹⁷ (forest), ²⁷³MEER²⁷⁶ (green), ²⁹⁶VL²⁹⁷ (chocolate), ³⁰⁸SS³⁰⁹ (olive), and ³²²KISQAVH³²⁸ (firebrick). *B*, three-dimensional ribbon model of OVA illustrating regions of β -aggregation propensity and T-cell antigenic determinants. *a*, β -aggregation propensity regions recognized for OVA: ³²IAIMSALAMVYL⁴³ (blue), ¹⁷²MVLVNAIVFK¹⁸¹ (red), and Phe³⁶⁴-Val³⁸³ (green). *b*, sequence found to be a T-cell epitope (green), ³⁹AMVYLGAKDSTR⁵⁰. The whole OVA sequence was taken into consideration during numbering.

Tanaka *et al.* (49) identified three high β -aggregation propensity regions in OVA using algorithms like TANGO, PASTA, and AGGRESAN; ³²IAIMSALAMVYL⁴³, ¹⁷²MVLVNAIVFK¹⁸¹, and Phe³⁶⁴-Val³⁸³. They further proved by *in vitro* studies that ³⁸LAMVYL⁴³ (a stretch of ³²IAIMSALAMVYL⁴³ sequence) has a very strong tendency to form amyloid fibrils. Interestingly, a sequence, ³⁹AMVYLGAKDSTR⁵⁰, has been attributed to be a T-cell epitope of OVA, which harbors ³⁹AMVYL⁵⁰. The high β -aggregation propensity regions and T-cell epitope of OVA are shown in Fig. 9*B*.

Our results support the hypothesis that amyloid immunization induces native protein-specific immune responses because of the release of precursor proteins from the amyloid depot, which may bear conformational properties of native protein. However, it cannot be ignored that native protein-specific antibodies may also be generated in response to various linear epitopes that remained intact in the fibrils during their formation from precursor proteins. In other words, antibodies specific for linear epitopes of the native protein may cross-react with the fibril form. Nevertheless, our purpose of exploiting amyloid as a vaccine candidate is fulfilled even if the host immune system favors the latter option. It has been reported for

several amyloidogenic proteins to induce the formation of antibodies capable of recognizing both the fibrils as well as the native protein. Antibody HMB50 against the amyloid-forming protein Pmel17 is an example, in that this antibody recognizes fibrils (51) and also efficiently immunoprecipitates newly synthesized native, not yet aggregated protein (52). Many fibril-reactive antibodies additionally recognize SDS-denatured protein. An example would be the Pmel17-specific antibody HMB45 (51). Moreover, aggregation of recombinant epotetin (EPO) has been shown to induce antibodies that bind to native EPO, leading to pure red cell aplasia in subjects (53, 54). It is to be noted that in all the above discussed reports, release of native protein from amyloids has not been studied; nevertheless, the generation of native protein-specific immune responses is well elucidated. Reckoning with these findings, even if we do not take into consideration the release of soluble native protein from the amyloid depot, the presence of intact fibril itself in the system may boost the immune system to elicit a desirable immune response. In fact, amyloids significantly withstand biological proteolytic degradation when compared with the native form of the protein (2, 23, 24), which ensues in extending the residence time of the antigen much longer in circulation. This "stability effect" could strongly enhance its potency as a vaccine even if we ignore release of any precursor protein from amyloid as it may still facilitate the generation of antibodies that may cross-recognize native protein.

CONCLUSIONS

Although the immunoprophylactic responses can always be manipulated by including desirable immunomodulators, the categorical immune response induced in the host upon administration of OVA aggregates suggests that the system can be used as vaccine without inclusion of external excipients. Taking immune activation features into consideration, the self-assembled amyloid bodies can be exploited as potential vaccine candidates against both extra as well as intracellular pathogens.

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Amyloid Form of Ovalbumin Evokes Native Antigen-specific Immune Response in the Host: PROSPECTIVE IMMUNO-PROPHYLACTIC POTENTIAL

Saba Tufail, Mohammad Owais, Shadab Kazmi, Renu Balyan, Jasneet Kaur Khalsa, Syed Mohd. Faisal, Mohd. Asif Sherwani, Manzoor Ahmad Gatoo, Mohd. Saad Umar and Swaleha Zubair

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Development, characterization and efficacy of niosomal diallyl disulfide in treatment of disseminated murine candidiasis

Maroof Alam, PhD^a, Swaleha Zubair, PhD^b, Mohammad Farazuddin, PhD^a,
Ejaj Ahmad, PhD^a, Arbab Khan, MSc^a, Qamar Zia, MSc^a,
Abida Malik, MD^c, Owais Mohammad, PhD^{a,*}

^aInterdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, India

^bWomen's College, Aligarh Muslim University, Aligarh, India

^cDepartment of Microbiology, Jawaharlal Nehru Medical College, Aligarh, India

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Abstract

In the current study, a novel niosome based formulation of diallyl disulfide (DADS) was evaluated for its potential to treat disseminated candidiasis in mouse model. Among various non-ionic surfactants tested, niosome formulation prepared using Span 80 was found to be most efficient in the entrapment of DADS. The DADS loaded niosomes had size dimensions in the range of 140 ± 30 nm with zeta potential of -30.67 ± 4.5 . Liver/kidney function tests as well as histopathologic studies suggested that niosome-based DADS formulations are safe at the dose investigated. When administered to *Candida albicans* infected animals, the DADS bearing niosomal formulation cleared the fungal burden and increased their survival much efficiently than its free form.

From the Clinical Editor: In this study, a novel niosomal formulation of the antifungal DADS was utilized in a murine candidiasis model, resulting in more efficient fungal clearance compared to the free formulation.

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Key words: Diallyl disulfide; Niosome; Antifungal efficacy; Candidiasis

Candida albicans, in a manner similar to other opportunistic fungal pathogens, causes serious infections, often systemic, among immuno-debilitant subjects such as AIDS patients, people undergoing cancer chemotherapy or those being treated with immune-suppressive drugs (eg, during organ transplantation).^{1–4} Incidentally, research and development of novel antifungal drugs did not receive remarkable attention because of the low prevalence of human infection in the pre-AIDS era. The emergence of *Candida* isolates less susceptible to widely used antifungal drugs such as azoles has further aggravated the situation.⁵ This necessitates administration of relatively high doses of presently available

antifungal drugs, which brings with them a specter of drug-associated toxicity in patients.⁶

Plant-based therapeutics having improved antimicrobial activity with less toxicity, are being increasingly accepted as alternatives to conventional antibiotic therapy.⁷ For example, garlic, a traditionally used anti-infective agent, has been widely prescribed in treatment of both fungal and bacterial infections. The wonder herb, garlic, owes its antimicrobial activity primarily to the compound allicin (allyl 2-propene thiosulfinate) formed after the crushing or cutting of garlic cloves.^{8–12} As allicin rapidly undergoes degradation that makes its clinical usage difficult, steam-distillation of mashed garlic pulp is carried out to convert allicin to garlic oil (composed of methyl and allyl sulfide derivatives of allicin), which relatively enables its usage in medicinal formulations.¹³ Allyl constituents of garlic oil have been evaluated with varying success for their anticancer, antimicrobial, and other pharmacologic activities.¹⁴ Among these, DADS, a member of allyl sulfides family (diallyl sulfide, diallyl disulfide, and diallyl trisulfide)^{15–17} has been reported to possess both anti-bacterial as well as anti-fungal activity.¹¹ An exhaustive study on role of these sulfides suggest that number of

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*Corresponding author: Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh-202002, India.

E-mail address: owais.lakhnawi@yahoo.com (O. Mohammad).

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disulfide bonds might have direct correlation with antimicrobial activity of a given allyl sulfide.^{17–19}

The work of Lemar et al.²⁰ on the antifungal mechanism of DADS against *C. albicans*, as model fungal pathogen, strongly suggests that increased oxidative stress through decreased GSH levels generally mediates the drastic death response induced in the organism. This study further suggests that impairment of mitochondrial respiration elicited by the action of DADS on ATP-synthase and a site(s) located among complexes II–IV in the electron transport chain is also likely to be involved in the killing of *C. albicans*.²⁰ Despite showing strong antimicrobial activity, hydrophobicity of DADS is considered to be a major hurdle in the development of its suitable dosage form. This is the reason why desirable pharmacologic properties of this wonderful molecule cannot be translated in clinical settings till now.

With a view to overcome the solubility issues of DADS, we developed a non-ionic surfactant based niosome formulation. Niosomes are bilayered structures, made from non-ionic surfactants and can be used as carrier of both hydrophobic as well hydrophilic drugs. They are biodegradable, biocompatible, and more importantly non-immunogenic with minimum toxicity.²¹ The niosome mediated delivery has potential to increase efficacy of the associated drug molecules.^{22–26} Interestingly, unlike liposomes that are made up of phospholipids, the constituents that are more vulnerable to heat and oxidation mediated degradation, niosomes show better stability with increased shelf life of entrapped drugs.^{26,27}

Incidentally, *C. albicans* adapts intracellular parasitism as a strategy to avoid antibody onslaught and resides in macrophages. We envisaged that being particulate in nature the drug bearing niosome would be avidly taken up by macrophages. This eventually will help in targeted delivery of niosome-loaded DADS to pathogen harboring macrophages. To test our hypothesis, a number of non-ionic surfactants of the “SPAN” series (sorbitan monoesters) were screened to prepare desirable DADS-bearing niosome formulations. The ideal formulation was evaluated for favorable entrapment efficiency of the active constituent, as well as other parameters such as acceptable release kinetics, size and zeta-potential. Finally, we examined the potency of DADS niosomal formulation in treatment of experimental systematic candidiasis in Balb/c mice.

Methods

Chemicals

DADS, cholesterol, sorbitan monoester non-ionic surfactants viz. Span 20 (sorbitan mono laurate), Span 40 (sorbitan mono palmitate), Span 60 (sorbitan mono stearate), Span 80 (sorbitan mono oleate), dicetyl phosphate (DCP), and amphotericin B were purchased from Sigma Aldrich (St. Louis, Missouri). Detection kits for liver and kidney function test parameters were purchased from Span Diagnostics (Gujarat, India). The rest of the chemicals were of analytical grade of purity and procured locally.

Strains

The test strain of *C. albicans* (ATCC 18804) was obtained from ATCC. Yeast-peptone-dextrose (YPD) agar/broth was used

for growing the *C. albicans* strain. In addition, for in vitro antifungal activity tests, clinical isolates of *C. albicans* (29 isolates), *C. glabrata* (19 isolates), and *C. krusei* (15 isolates) were obtained from the repository of Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, India.

Preparation of DADS-bearing niosomes

We used sonication method for preparation of Span-based niosomal formulations of DADS after published procedure as standardized in our laboratory.²⁸ The details of the preparation method have been incorporated in Supplementary Material (available online at <http://www.nanojournal.com>).

Determination of entrapment efficiency of niosome-intercalated DADS

The entrapment efficiency of DADS was determined by dissolving an aliquot of the in-house prepared niosomal DADS formulation in chloroform:methanol (1:9 v/v) mixture, followed by estimation of DADS content by high performance liquid chromatography (HPLC) method employing published procedure.²⁹ The details of the experimental protocol are given in the Supplementary section.

In vitro release kinetics of DADS from niosomal formulation

Release of DADS from various niosomal formulations was studied using a dialysis method as published earlier.^{30,31} Briefly, niosomal DADS formulation (containing 1 mg DADS) was dispersed in 1.0 mL of phosphate buffered saline (PBS) (pH 7.4) and placed in a dialysis bag. It was fully immersed under release medium (PBS, pH 7.4) taken in a glass beaker and kept for gentle stirring on a magnetic stirrer at room temperature (25°C). Aliquots of the medium were withdrawn for analysis of released drug at different time intervals and the volume of the suspension buffer was replenished by adding same volume of fresh medium.

Determination of particle size and zeta potential of DADS-bearing niosomes

The zeta potential gives an indication of the charge acquired by particulate system on its suspension in aqueous medium. In fact, it is a measure of surface charge on a given particle that regulates its half-life both in vitro as well as in vivo. Zeta potential of different formulations was measured by the instrument Zeta sizer Nano ZS (Malvern Instrument Limited, Worcestershire, United Kingdom). Briefly, lyophilized form of the formulation was taken in microfuge tube and the samples were suspended in 20 mM PBS, pH 7.4, and then introduced into the instrument following the guidelines of the manufacturer.

Niosome-mediated transfer of entrapped molecules to macrophages

The successful elimination of intracellular pathogen *C. albicans* requires effective delivery of drug pay load to infected macrophages. We determined uptake of niosome encapsulated fluorescent dye (calcein) by macrophages as a parameter to assess ability of newly developed niosomal formulation to home intercalated drug to *C. albicans* harboring

macrophages. The details of the niosome uptake study have been provided in Supplementary section.

Animals

For in vivo studies, female Balb/c mice of weight 20 ± 2 g were procured from the institute's animal house facility and were kept on standard pellet diet (Hindustan Lever Ltd.) and water ad libitum. Animals were checked daily for their mortality and morbidity before commencement of the study and only healthy animals were included in the experiments. The techniques used for bleeding, injection as well as for the killing of animals were strictly performed after mandates approved by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India).

Toxicity assessment of niosomal DADS formulation

Before introducing the novel niosomal formulation of DADS as potential antifungal therapy approach, we determined its intrinsic toxicity issues in both in vitro as well as in vivo system. Preliminarily, acute drug toxicity was assessed through in vitro erythrocyte lysis test, wherein hemoglobin released as a result of membrane leakage or disruption caused by exposure to the drug formulation, was measured.³² The details of the procedure have been included in the Supplementary Material section.

In vivo hepatic and renal toxicities for free drug as well as niosomal DADS formulations were assessed by administering $10 \times$ multidoses intra-peritoneally (ip) in Balb/C mice (total three doses, at alternate days). Biochemical profiles of serum creatinine, alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) were determined using commercial detection kits (Span Diagnostics, India). Blood was collected by retro-orbital puncture from the mice belonging to various experimental groups at day 1 (before administration of first drug dose) and a day after the last dose administration. The serum was separated from blood for determination of creatinine, ALP, and GGT contents according to specific protocols provided by the manufacturer.

The toxic effect of DADS (if there was any) on vital organs (viz, liver and kidney) of the experimental animals was also assessed by histopathologic studies. For histopathologic examination of liver and kidney, mice from each group were fixed by intracardiac perfusion of 10% buffered formalin. Tissue blocks of liver and kidney (1×0.2 cm) were processed for paraffin embedding. Tissue sections (10 μ m-thick) were stained with regular hematoxylin and eosin stains and examined under light microscope (Olympus-BX 40, Japan) at $\times 200$.

For in vivo toxicity studies the animals were divided in following groups, with eight mice in each group:

- Group I No drug (Control)
- Group II Free DADS (60 mg/kg bw)
- Group III Free DADS (120 mg/kg bw)
- Group IV Niosomal DADS (60 mg/kg bw)
- Group V Niosomal DADS (120 mg/kg bw)

Where bw stands for body weight of mouse.

In vitro antifungal testing and minimum inhibitory concentration (MIC) of DADS for *Candida* spp.

To determine antifungal susceptibility, the *C. albicans* strain used in the study was exposed to amphotericin B (final concentration range of 0.02 to 5 mg/L), a standard antifungal agent using broth microdilution method performed in 96-well round bottom microtitre plates, as per NCCLS recommendations.³² Further the in vitro antimicrobial activity of DADS against a total of 63 isolates of *Candida* spp. viz *C. albicans* (29 isolates), *C. glabrata* (19 isolates), and *C. krusei* (15 isolates) was determined simultaneously. These isolates were also tested for any resistance to amphotericin B. DADS was solubilized in 5% DMSO and various aliquots of drug solution were further diluted in RPMI 1640 medium (pH 7.0) containing a seeded final inoculum concentration of 2×10^3 cells/mL. DADS was tested over a final concentration range of 1 mg/L to 100 mg/L. The wells containing fungal inoculum with different concentrations of drugs and appropriate controls (drug-free as well as inoculum-free) were incubated at 37°C for 48 hours. Turbidity was measured spectrophotometrically at 530 nm. The MIC was defined as the lowest concentration of drug that produced 80% reduction in turbidity.

Preparation of inoculum

For infection purpose, *C. albicans* was cultured in 5% dextrose broth at 37°C for 36 hours. The cell suspension was centrifuged at $5000 \times g$ for 15 minutes at 4°C, followed by washing with sterile normal saline.

Induction of experimental candidiasis

For induction of experimental candidiasis, each mouse was challenged via the tail vein with 1×10^7 colony forming units (CFU) of *C. albicans* suspended in 200 μ L of sterile normal saline (150 mM, pH 7.4). Our pilot studies suggest that this inoculum size consistently produced disseminated infection within 48 hours of injection and ultimately ensuing in death of animals within 7 days postchallenge with infection (data not shown).

Assessment of antifungal efficacy

For antifungal efficacy, animals were treated with various formulations given by ip route. The efficacy of Span 80 niosomal DADS was assessed by monitoring the survival of the infected animals and determining the residual *C. albicans* burden in their vital organs viz. liver, spleen, and kidney. For survival and residual fungal burden studies two separate but similar experiments were set up simultaneously. In each experiment the infected animals were divided in six different groups as listed below. Each group had 10 animals.

- Group I Control (Only PBS)
- Group II Control (Sham niosome)
- Group III Free DADS (6 mg/kg bw)
- Group IV Free DADS (12 mg/kg bw)
- Group V Niosomal DADS (6 mg/kg bw)
- Group VI Niosomal DADS (12 mg/kg bw)

For survival studies, mortality of the animals was observed twice each day during 50 days of observation. Quantitative

Table 1
Niosome forming ability and %EE of SPAN-based niosomes with regard to DADS

Surfactant Percent mol	Cholesterol Percent mol	DCP Percent mol	%EE \pm SD			
			Span 20	Span 40	Span 60	Span 80
100	0	0	9.2 \pm 2.4	No vesicles	12.3 \pm 4.7	No vesicles
70	30	0	25.3 \pm 3.8	27.9 \pm 2.7	49.5 \pm 4.2	23.4 \pm 4.2
50	50	0	36.52 \pm 4.6	35.3 \pm 3.4	65.7 \pm 2.6	72.7 \pm 4.2
60	30	10	17.8 \pm 3.9	32.4 \pm 4.3	37.2 \pm 4.3	29.3 \pm 4.3
50	40	10	15.2 \pm 5.15	34.2 \pm 3.3	51.8 \pm 4.5	32.5 \pm 4.7
47.5	47.5	5	35.2 \pm 4.17	41.2 \pm 2.8	68.6 \pm 3.3	74.5 \pm 3.2

DADS, diallyl disulfide.

assessment of the fungal burden in various vital organs viz. liver, spleen, and kidney was performed following published procedure.³³ Antifungal treatment was commenced 24 hours postchallenge of animals with *C. albicans* infection. Treatment was repeated at day 3 and day 5 postinfection. The animals (three from each group) were killed on day 7, 12, and 15 postinfection, and vital organs viz. liver, spleen, and kidney were taken out aseptically. The organs were washed extensively with hypotonic buffer, homogenized, and serially diluted with normal saline. Various dilutions of each organ homogenate were dispersed on YPD agar plates containing gentamycin to avoid bacterial contamination. After incubation of 12–24 hours at 37°C, the colonies were counted and the fungal load was determined by multiplying with the dilution factor.

Tissue distribution studies

Tissue distribution of DADS in various organs was determined following published procedure.^{34,35} Both normal as well as infected mice were administered with free as well as niosomal DADS (12 mg/kg) via intravenous route. The details of the procedure are available in Supplementary Material section.

We also determined plasma level of DADS on administration of DADS bearing niosome in the experimental animals. The animals were injected with 10 \times amount of therapeutic dose via intraperitoneal route in both normal healthy as well as *C. albicans* infected mice. Blood was withdrawn by retro orbital puncture at different time intervals. Serum was separated by centrifugation at 795 \times g for 5 minutes. It was then precipitated with methanol and centrifuged at 8832 \times g for 10 minutes. Supernatant was taken out and analyzed for DADS content by HPLC method.

Statistical analysis

The results were analyzed with one-way analysis of variance (ANOVA) of mean values with the Student *t* test. *P* value of < 0.05 was considered statistically significant.

Results

Effect of composition on the formation and entrapment efficiency of DADS-containing niosome formulations

The DADS-loaded niosomes were prepared using various molar ratios of the Span surfactants, cholesterol, and DCP. The percent entrapment efficiency (% EE) for DADS is summarized in Table 1. Niosomes prepared using Span 20 and Span 40 had very

low % EE irrespective of the molar ratios of constituents used. The maximum entrapment was achieved by Span 80-based niosomes (74.5 \pm 3.2%) at molar ratio of 47.5:47.5::SPAN 80:cholesterol:DCP and 72.7 \pm 4.2% at 50:50:0::SPAN 80:cholesterol:DCP % molar ratios, respectively. This was followed by Span 60 niosomes (68.6 \pm 3.3% and 65.7 \pm 2.6% for molar ratios 50:50:0 and 47.5:47.5::SPAN 60:cholesterol:DCP, respectively). Exclusion of DCP during preparation of either Span 60 or Span 80-based niosomes resulted in formation of large aggregates within 24 hours of preparation. Based on earlier reports that Span 80 and Tween 80 niosomes are more stable than other detergents and our supporting data of entrapment efficiency, the Span 80-based niosomes (47.5:47.5:5 molar composition) were subsequently chosen for characterizations and assessment of efficacy studies against experimental murine candidiasis.

Particle size, zeta potential and shape of DADS-bearing Span 80 niosomes

The zeta-potential of DADS-loaded Span 80 niosomes was found to be -30.67 ± 0.45 mV. The size of the novel DADS-bearing niosome formulation was established using zeta average diameter analysis. It shows niosome to possess size range of 140 ± 30 nm.

Niosome-mediated transfer of entrapped molecules to macrophages

To know fate of calcein loaded niosomes after their uptake, they were incubated with mouse J774 A.1 cells and subsequently examined by fluorescence and phase contrast microscopic techniques. As evident from Figure 1, A and B, the incubation of niosomes with macrophages resulted in punctate fluorescence suggesting their uptake by active endocytosis of the target cells. Both normal and infected macrophages were able to internalize calcein-loaded niosome; however, uptake was more obvious in case of infected macrophages.

Release profile of niosomal DADS

Figure 2 shows the release pattern of DADS from Span 80 niosomes wherein approximately 20% of the intercalated drug was released in 24 hours and around total 33% of the drug was released in 72 hours. The release profile suggests that niosome-based formulation releases the drug in a sustained manner.

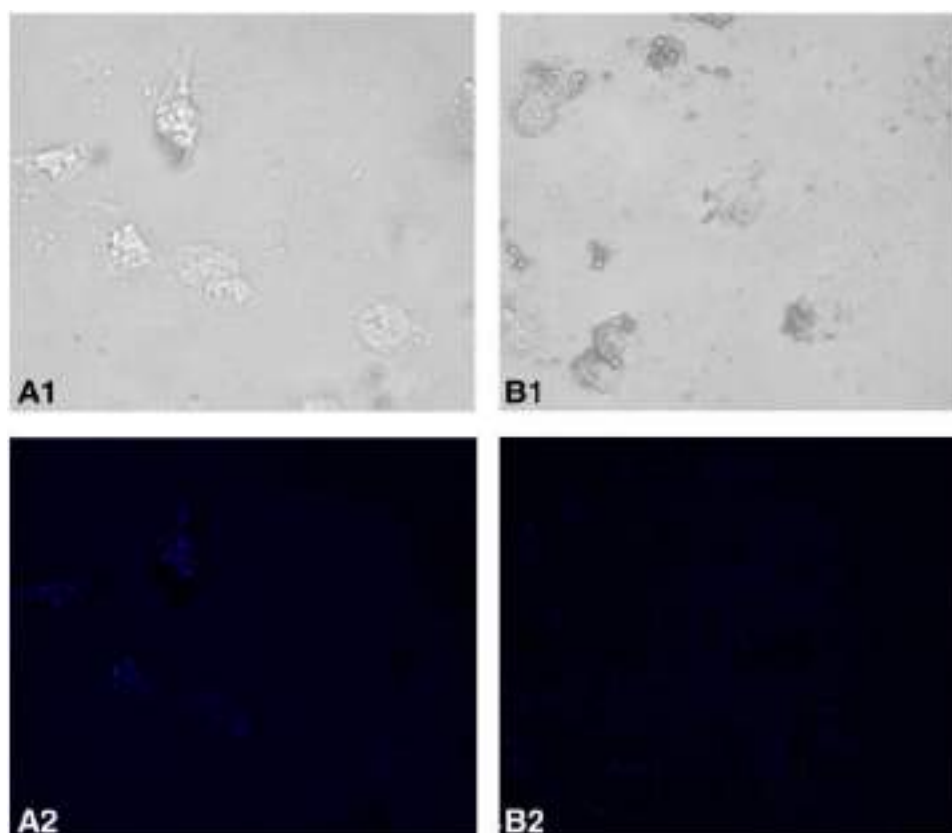


Figure 1. Fluorescence light micrographs of the macrophages J774 A.1 after their interaction with calcein (fluorescent probe) loaded niosome. Fluorescent and Phase contrast light micrographs of normal macrophages (A1 & A2) and *C. albicans* (B1 & B2) infected macrophages interacted with calcein loaded niosome for 60 min at 37°C as described in *Methods* section.

Toxicity tests

- (a) *In vitro hemolysis test.* The possibility of Span 80 niosomal DADS mediated lysis of erythrocytes was assessed using a dose that was almost 10 times greater to that of the therapeutic dose used in chemotherapy of the infected animals. Figure 3 displays extent of erythrocyte lysis induced by free as well as niosomal DADS with appropriate controls (Triton X-100, empty sham niosomes and DMSO). As niosome releases entrapped DADS molecules in regulated manner, it does not cause any lysis of RBCs, whereas free form of DADS had induced significant lysis.
- (b) *In vivo toxicity of DADS formulations.* Renal and hepatic toxicities of the in-house developed formulations were measured after the administration of a 10 times higher concentration of DADS when compared with that of the therapeutic dose used in *in vivo* efficacy experiments. As evident from the data, there was no apparent change in the RFT and LFT parameters of the animals receiving the DADS formulation when compared with the control (Table 2).

Histopathologic studies revealed that on treatment with Nio-DADS at 12 mg/kg dose, the liver appeared to possess normal hepatic lobular architecture and hepatocyte laminae

(Figure 4, B). Sinusoids were mildly dilated because of acute hyperaemia. Hepatocytes showed smooth contour and uniform staining without any sign of degeneration or necrosis and were identical with the healthy control animal, except for the dilated sinusoids (Figure 4, A and B).

Similarly, renal parenchyma of the kidney from drug treated animals (Figure 4, D) was quite comparable with the healthy animals (Figure 4, C). The kidney showed normal renal corpuscles in terms of glomerular mass, its cellularity, and urinary space as well as renal tubules where proximal convoluted tubule (PCT) and distal convoluted tubules (DCT) could be identified with their intact characteristic lining cells as well as interstitial space.

In vitro antifungal testing and MIC of DADS

The MIC of amphotericin B against *C. albicans* strain (ATCC 18804) was 0.125 µg/mL, which suggests that the strain is antifungal susceptible. Similarly all the 63 *Candida* isolates consistently showed amphotericin B MIC in the range of 0.15–3.68 µg/mL indicating absence of any resistance to standard antifungal drug (data not shown). DADS showed MIC of 6 ± 0.8 mg/L for the *C. albicans* strain (ATCC 18804). For the 63 clinical isolates of *Candida* spp. the DADS MIC was 7 ± 1.7 mg/L for *C. albicans* isolates, 10 ± 2.54 mg/L for the

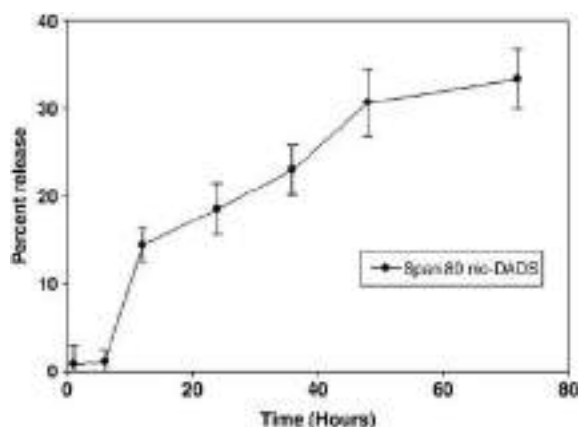


Figure 2. Release of DADS from DADS-based niosomes. Niosomal DADS suspension was incubated in sterile phosphate buffer saline (10 mM phosphate, 150 mM saline, pH 7.4). The amount of DADS released at different time points was spectrophotometrically analyzed at 254 nm as described earlier in *Methods* section. A standard curve of the drug was plotted at 254 nm by determining the area under curve corresponding to the known increasing amount (10–500 ng) of the drug. Release runs were continued for 72 h. The absorbance of the collected samples was measured at 254 nm. The calculated amount of the released drug was plotted against time. Data are means \pm SD of five independent experimental values.

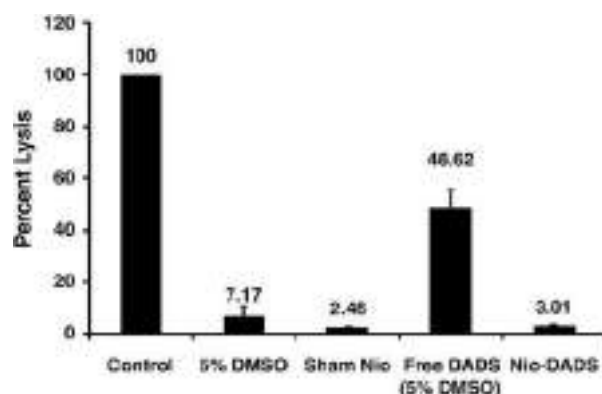


Figure 3. Erythrocyte lysis test for DADS-bearing niosomes. The extent of damage incurred to blood erythrocytes by DADS formulations was measured as percentage lysis of total erythrocytes used in the individual sample.

C. glabrata isolates, and 11.8 ± 3.62 mg/L for the *C. krusei* isolates (Supplementary Table S1).

Assessment of fungal load in vital organs of infected animals after treatment with niosomal DADS formulation

The severity of *C. albicans* infection was assessed by culturing the liver, spleen, and kidney homogenates, of animals belonging to various experimental groups onto YPD agar plates. As shown in Figure 5, A, there was significant decrease in the fungal load in liver, spleen, and kidney respectively from animals of Group VI treated with Span 80 niosomal DADS (12 mg/kg bw) when compared with animals of Group IV treated with 12 mg/kg bw free DADS treatment ($P < 0.04$). Untreated

Table 2

Concentrations of creatinine, alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT) in plasma of mice after drug therapy

Groups	Percent Creatinine \pm SD	ALP (in KA Unit)	GGT (IU/L serum)
Control	0.58 ± 0.09	0.00^a	1108.5 ± 113.84
Free DADS (120 mg/kg)	1.04 ± 0.17	3.78 ± 0.059	1265.0 ± 50.91
Niosomal DADS (120 mg/kg)	0.81 ± 0.29	0.51 ± 0.09	1064.05 ± 80.53

DADS, diallyl disulfide; SD, standard deviation.

^a Amount of ALP is calculated by subtracting the value of control from test. Hence, it has been shown as 0.

control animals of Group I did not survive beyond 5 days and were not available for killing for determining fungal load.

Survival studies

As shown in Figure 5, B, the animals treated with niosomal formulation of DADS (Nio-DADS) at dose of 12 mg/kg bw (Group VI) showed a survival rate of 80% as noted up to day 50 postinfection, whereas 50% animals survived in Group V (animals that were treated with niosomal-DADS at dose of 6 mg/kg bw). None of the animals survived beyond the 15th day postinfection in the Group IV that were treated with free form of DADS at dose of 12 mg/kg bw. The Nio-DADS formulation (12 mg/kg bw as well as 6 mg/kg bw) was more effective when compared with free DADS in improving survival rate of treated animals. The animals belonging to control groups (sham niosome and PBS treated) succumbed to death within 7–8 days postchallenge with infection.

Tissue distribution of DADS in vital organs

Figure 6, A, shows that on administration of free DADS, its concentration was increased in liver and spleen 1 hour postinjection but decreased significantly after 24 hours, whereas the drug concentration in kidney and lungs remained lower in this period. The DADS concentration in the animals receiving niosomized form was less in liver and spleen when compared with the free form of drug at 1 hour but increased by 24 hours postinjection indicating steady and gradual accumulation of drug to both organs. Figure 6, B, shows that DADS concentration was higher in all the organs when it was administered to the *C. albicans* infected animals. To determine serum concentration of DADS, animals were injected with free or niosomal DADS at the dose of 12 mg/kg bw. Although statistically nonsignificant, administration of free-DADS resulted in higher serum concentration, whereas niosomal DADS treated group had relatively low concentration at various time points (Figure 6, C). A time dependant increase in DADS concentration in the serum was observed in both the groups. On the other hand, increased plasma half-life of DADS was more noticeable in the case of the niosomal drug treatment of the infected animals when compared with the free drug indicating that DADS distribution in bio-phase is remarkably influenced by its incorporation in niosomes. Overall the significant altered distribution of the entrapped drug

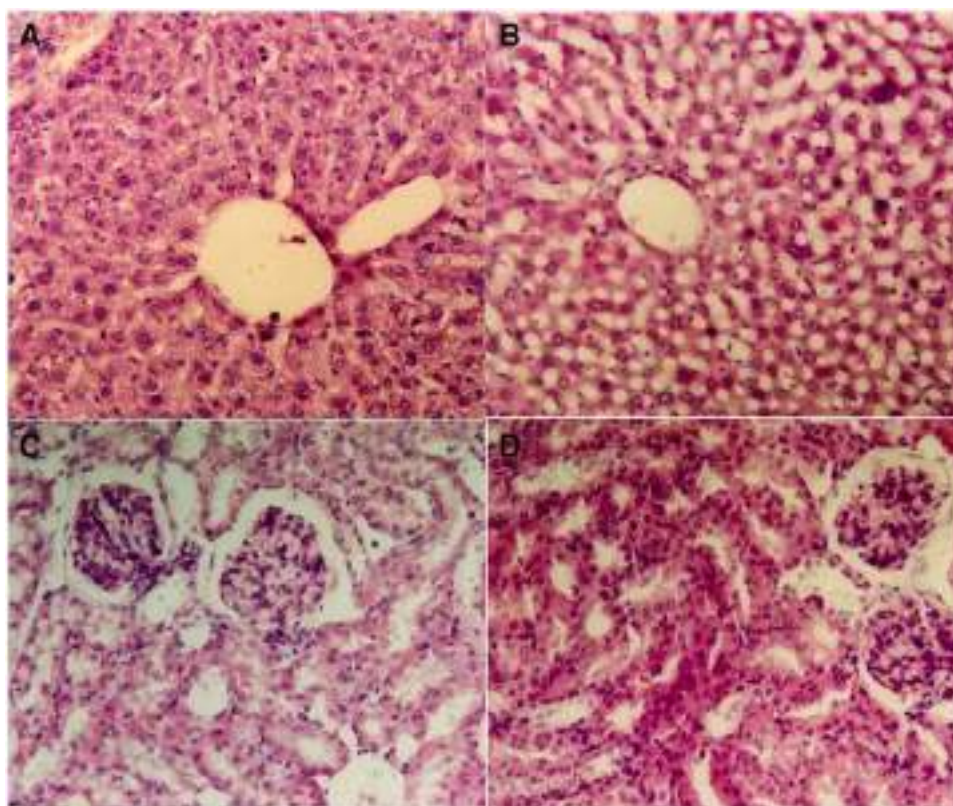


Figure 4. Histopathologic studies. Photomicrographs of mice liver samples from control (A) and niosomal DADS (12 mg/kg bw) treated group (B). In the treated group the lobular architecture, hepatic laminae and contour of hepatocytes are intact with usual presence of red blood cells in the sinusoids. There is no sign of hepatocyte necrosis. Photomicrographs of kidney samples from control (C) and niosomal DADS (12 mg/kg bw) treated group (D). Treated group shows intact renal corpuscle with normal glomerular cellularity, urinary space, and lining cells of renal tubules. $\times 200$, Stain H & E.

in healthy versus *C. albicans* infected animals suggest that the infection with *C. albicans* clearly influence the distribution of the drug in various organs of the host animal (Figure 6, C).

Discussion

In the current study, we have tried to establish antifungal potential of DADS, a plant based compound against systemic murine candidiasis in model animals. Keeping into consideration the fact that poor solubility of DADS hampers development of suitable dosage form that can otherwise facilitate its systemic distribution upon administration in the host, it was envisaged that incorporating DADS in the bilayer of niosomes, which are particulate vesicular entities can enable solubilization of the hydrophobic compound and modify its systemic distribution in the body.

To confirm our hypothesis, we developed niosome-based DADS formulation and evaluated its antifungal efficacy against experimentally induced disseminated *Candida* infection in mice. Span 80-based DADS niosomal formulation showed favorable drug entrapment efficiency, desirable surface charge, particle size and drug release kinetics (Figure 2). Recently it has been reported that higher fatty acid can also inhibit a range of microbes including *C. albicans*.^{36,37} The unsaturated fatty acids are found to be more potent antifungal agents than their saturated

counterpart, the reason why we opted for Span 80, an oleic acid ester of sorbitan over Span 60, which is an ester of stearic acid in spite of the fact that both Span 60 as well as Span 80 had comparable drug entrapment efficiency.³⁸ DCP was used to impart negative zeta potential to the prepared niosomes that may help in preventing or delaying aggregation when particles are suspended in aqueous medium. Besides, negative zeta potential may also increase plasma half-life of the niosome-based formulation. The surface properties and sustained drug kinetics from niosome-based formulation are desirable features that can have direct effect on efficacy of the drug.

To establish that in-house prepared niosomal formulation can deliver its contents to macrophages, the calcein (a water soluble fluorescent probe) bearing niosomes were incubated with macrophages in vitro. The interaction eventually resulted in punctuate fluorescence pattern referring to co-localization of niosome bound calcein in endo-lysosomal compartment of the macrophages, the site that *C. albicans* (yeast form) used as dwelling shelter inside the host (Figure 1, A and B).

Before proceeding for in vivo efficacy studies, we tested the niosomal DADS formulation for inherent toxicity issue if there was any. In this regard, toxicity of the formulation to living cells as revealed by in vitro erythrocyte lysis test (Figure 3) suggests that niosomal DADS induces negligible damage to red blood cells even at $10\times$ therapeutic dosage. Further, results of in vivo liver and renal function tests (Table 2) confirm that niosomal

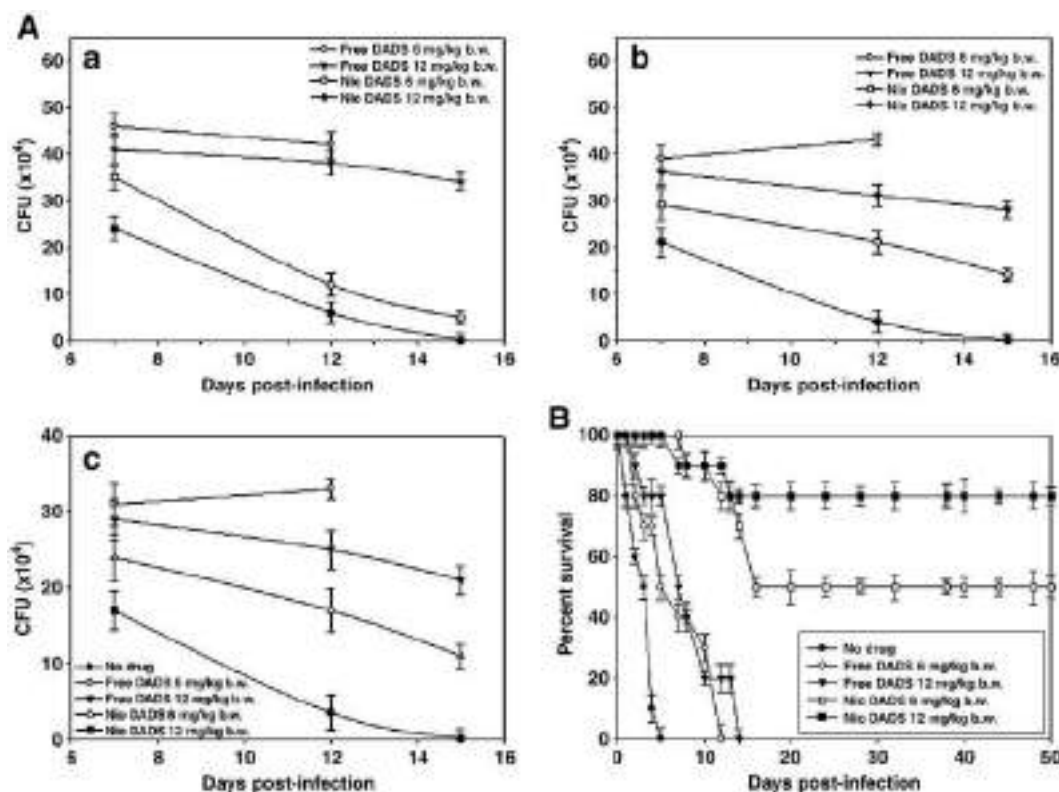


Figure 5. (A) Span 80 niosomal DADS mediated fungal load reduction in vital organs of *C. albicans* infected mice. Animals were challenged with 10^7 CFU of *C. albicans* and subsequently treated with various forms of DADS as described in *Methods*. Antifungal efficacy was determined as residual fungal load in the respective vital organ plotted against number of days postinfection. No animal survived in untreated control (No drug) beyond 5th day postinfection, therefore none was available for sacrifice. Fungal burden in **liver (a)** Nio-DADS (12 mg/kg bw) versus Free DADS (12 mg/kg bw), $P < 0.03$, **spleen (b)** Nio-DADS (12 mg/kg bw) versus Free DADS (12 mg/kg bw), $P < 0.05$, and **kidney (c)** of niosomal DADS treated mice Nio-DADS (12 mg/kg bw) versus Free DADS (12 mg/kg bw), $P < 0.05$. (B) Increase in survival of animals by Span 80 niosomal DADS. Animals were infected with 10^7 CFU and subsequently treated with various forms of DADS as described in *Methods*. The data is the mean of five sets of different experiments \pm SD. Nio-DADS (12 mg/kg) versus Free DADS (12 mg/kg), $P < 0.001$, Nio-DADS (6 mg/kg) versus Free DADS (6 mg/kg), $P < 0.001$.

DADS imparts mild toxicity to host liver and kidney when compared with its free form. In fact, non-ionic surfactant Span 80 used in preparation of niosome is a Food and Drug Administration (FDA)-approved food additive thus rules out any intrinsic toxicity constraint. The possible toxicity issues related with formulation were further excluded by histopathologic studies in which features of liver and kidney from DADS-treated animal (12 mg/kg bw) appeared very much similar to that of control healthy animal.

Our pilot studies suggested that in a manner similar to liposome-based formulation, DADS being hydrophobic in nature gets readily incorporated in the niosome bilayer. We established preferential distribution of DADS in niosome bilayer by sonication-resonance method that accounts for the presence of more than 95% of DADS in bilayer rather than aqueous core compartment (data not shown).

The efficacy studies suggest that, the Span 80-based formulation was dramatically effective in clearing fungal burden in liver, spleen and kidney of infected animals (Figure 5, A). In comparison, same dose of free form of DADS failed to eliminate fungal load in various vital organs of the experimental animals (Group III and Group IV). The in vivo survival study

(Figure 5, B) showed a significant reduction of mortality in animals treated with niosomal DADS. The higher efficacy of inhouse prepared DADS formulation was found to have direct correlation with specific distribution of the niosome intercalated DADS to infected organs mainly (Figure 1).

As observed in the tissue and plasma distribution studies (Figure 6), DADS accumulation in the vital organs and blood is influenced by its niosomization as well as presence of infection in the host animals. Incorporation of DADS in niosomes ensured its accretion in macrophage-rich organs that was more pronounced when the animals were infected with *C. albicans*. This is mostly because of the fact that subsequent to establishment of infection, body immune system recruits, and signals various immune cells to accumulate at the site of infection.^{39,40} The improved efficacy of niosome encapsulated DADS can also be correlated to the uptake of DADS-loaded particulate niosomes by the pathogen-harboring macrophages, whereas free form of DADS probably has inadequate access to macrophages and hence find it difficult to reach to the infection sites. In fact, macrophages seem to act as secondary depot for niosomal DADS and facilitate its distribution to the pathogen harboring organs of the host.

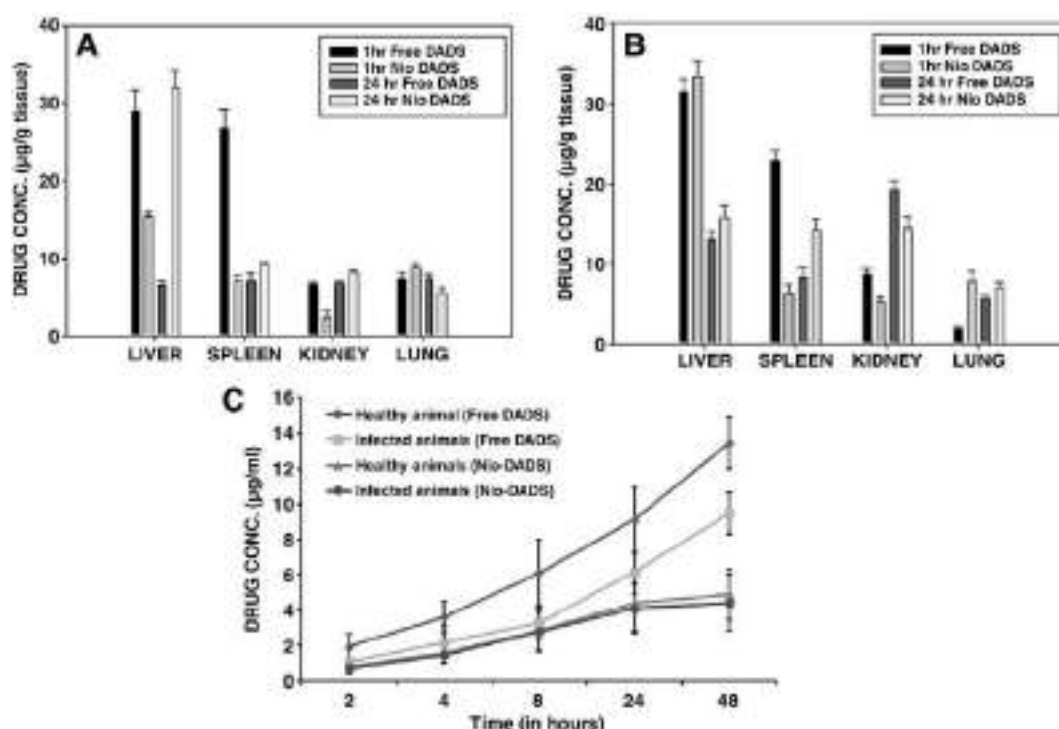


Figure 6. Tissue distribution study. DADS tissue distribution in liver, spleen, kidney, and lungs at 1 and 24 h posttreatment in un-infected (A) and infected (B) animals. DADS plasma concentration: DADS in free and niosomised form, was administered by ip route in healthy and infected animals and blood was withdrawn at different time intervals. Plasma DADS concentration in healthy and infected animals (C). Drug concentration in the organs and plasma was analyzed by HPLC as described in *Methods* section.

Recently, we have demonstrated efficacy of novel formulation of DAS,^{41,42} another member of allyl sulfide based essential oil present in garlic extract. However, with the emergence of azole resistant isolates, one cannot rule out possibility that MDR-ABC transporters⁴³ present in *C. albicans*; can extrude a variety of structurally unrelated compounds including DAS. The drug efflux will not allow attainment of effective concentration of DAS inside the cell. In the current study, we selected a far more potent analog of this family viz, DADS with the assumption that if retained inside the cell even at low concentration its presence may lead to successful elimination of pathogen from systemic circulation. In fact, earlier reports have shown that entrapment of drug in lipid-based delivery systems not only effectively targets multidrug-resistant cells but also remains unaffected by P-glycoprotein-mediated drug efflux.⁴⁴

The data of the current study offers an efficacious, negligibly toxic, and cost-effective alternative to the presently used allopathic drug formulations that have undesirable side effects when administered at pharmacologically effective dosage regimens. The promising findings of the current study corroborate our approach, whereby therapeutic efficacy of DADS can be enhanced by incorporation into niosomes. We hence infer that niosomal formulation of DADS possesses extra advantages over free form of DADS for the treatment of experimental candidiasis because of reduced toxicity and ability to facilitate drug accumulation at RES sites as well as prolonged and sustained release of the DADS over extended time period. Further, niosomal form of DADS are ought to offer great promise

in treatment of fungal infections in immuno-compromised persons who usually need prolonged administration of antimicrobial agents when compared to the healthy subjects.

More detailed study and optimizations to elucidate the mechanism of action of DADS, incorporation of target specific ligands (antibody, aptamer) on the surface of the DADS-loaded niosomes to enable active targeting to the site of infection thereby achieving better efficacy, and further amendments to increase shelf life of the formulation are needed to enable development of an effective nature-derived antifungal formulation that can provide an alternative to the presently available antifungal drugs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2012.07.004>.

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