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To demonstrate that Chitosan nanoparticles can be adapted as a platform for delivering secretory proteins of *M. tb.*, the mice were immunized with Group I-Chitosan nanoparticles (200µl/mice), Group II- CFP-10 *per se* (16µg/200µl/mice), Group III- CFP-21 *per se* (16µg/200µl/mice), Group IV- CFP-10 encapsulated chitosan nanoparticles (16µg/200µl/mice), Group V- CFP-21 encapsulated chitosan nanoparticles (16µg/200µl/mice) and Group VI- PBS (200µl/mice) via intraperitoneal routes thrice an interval of three weeks (Goonetilleke, et al., 2003).

CFU assay of lung from the pre-immunized Balb/c mice was performed 15 days post *M. tb.* challenge. Protection was expressed as decrease in the number of colonies. The fig. 7.1a clearly indicated a reduction in the number of colonies CFP-10 *per se*, i.e., 1.67x10^5. Although, CHNP CFP-10 showed a significant decrease in colony count (1.2x10^5) in comparison to PBS (5.8x10^5). In the group of mice immunized with CFP-21 *per se* the colony counts were 2.45x10^5 and in the group of mice immunized with CHNP CFP-21 the colony counts were 1.99x10^5 while in the control (PBS) group of mice the colonies count was 5.8x10^5. Maximum growth inhibition was observed by CHNP CFP21. CFP-21 *per se* too, inhibits bacterial growth. Interestingly, the colony count in the group of mice immunized with *void* chitosan nanoparticles was 6.75x10^5 which is comparable to control group of mice.

There was a significant decrease in the number of colony count in the group of mice immunized with CHNP CFP-10, which was 70% less than control (PBS) group of mice post 30 days of *M. tb.* infection (Fig 7.2a). Fig 7.2b indicated that the number of colonies in the group of mice immunized with CHNP CFP-21 (0.17x10^5) reduced significantly i.e were ~70% less than control (PBS) group of mice (0.7x10^5). *Void* chitosan nanoparticles apparently enhanced the bacterial growth in terms of the number of colonies (1.74x10^5) when compared to the control group.

The bacterial growth post 60 days of *M. tb.* infection in the group of mice immunized with *void* Chitosan nanoparticles exhibited 4.7 x10^5 colony counts. But mice immunized with CFP-10 *per se* the number of colonies observed was 0.6 x10^5.
95% growth inhibition was observed in the CHNP CFP-10 immunized mice (0.125 x10^5) when compared to the control (PBS) group (1.6x10^5) (Fig 7.3a).

Progressive reduction in colony count was observed. ~ 95% decrease in the number of colonies was observed with CHNP CFP-21 immunized mice. Maximum growth inhibition in terms of protection was indicated in CHNP CFP-21 immunized mice. Secretory proteins encapsulated in chitosan nanoparticles definitely appeared to be imparting protection over a longer period of time. Therefore, the efficacy of the secretory protein was not compromised by encapsulation.

Fig. 7.4a showed a time dependent comparative analysis of the effect of immunization of various groups on the ability to form colonies post *M. tb.* infection. Protection was induced in CHNP CFP-10 immunized mice as compared to CFP-10 *per se*. The bacterial growth in CHNP and control (PBS) were comparable. Increased colony count was observed on day 15 and 30 post *M. tb.* infection, in mice immunized by *void* chitosan nanoparticles, but by day 60 the number of colonies count decreased that maybe attributed to a heightened immune response. Remarkably, in the group of mice immunized with CHNP CFP-10 the number of colonies reduced significantly from day15 to day 60. CHNP CFP-21 showed maximum protection (Fig. 7.4) in CHNP CFP-21 immunized mice. CHNP and control (PBS) appeared to support similar bacterial growth which was 95% higher than the CHNP CFP 21 treated mice.

Bacterial CFU were enumerated post 30 days of *M. tb.* infection (Plate 1) that were incubated at 37°C in a 5% CO₂–95% air atmosphere. Reduced colony count was evident in the CHNP CFP-10 and CHNP CFP-21 immunized mice when compared to the *void* chitosan nanoparticles, PBS and CFPs *per se* immunized mice.
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**CFU Assay of CFP-10 15th Day**

Fig. 7.1a CFU assay of lung from the pre-immunized Balb/c mice with *Void* chitosan nanoparticles, CFP-10 *per se*, CHNP CFP-10 and control group (PBS). CFU assay was performed on 15 days post *M. tb.* challenge. Protection was expressed as decrease in the number of colonies. The above fig. clearly indicated a reduction in the number of colonies by CFP-10 *per se*, i.e., (1.67x10^5). CHNP CFP-10 showed a significant decrease in colonies count (1.2x10^5) in comparison to PBS (5.8 x10^5).

**CFU Assay of CFP-21 15th Day**

Fig. 7.1b CFU assay of lung from the pre-immunized Balb/c mice with *void* chitosan nanoparticles, CFP-21 *per se*, CHNP CFP-21 and control group (PBS). CFU assay was performed on 15 days post *M. tb.* challenge. In the group of mice immunized with CFP-21 *per se* the colonies count was 2.45 x10^5 and in the group of mice immunized with CHNP CFP-21 the colonies count was 1.99x10^5 while in the control (PBS) group of mice the colonies count was 5.8x10^5. It revealed that CHNP CFP-21 has more potential to inhibit the bacterial growth in comparison to CFP-21 *per se*. Interestingly, the colonies count in the group of mice immunized with *void* chitosan nanoparticles was 6.75x10^5 which is more than control group of mice.
Fig. 7.2a CFU assay of lung from the pre-immunized Balb/c mice with *void* chitosan nanoparticles, CFP-10 *per se*, CHNP CFP-10 and control group (PBS). CFU assay was performed on 30 days post *M. tb.* challenge. The colonies count in the group of mice immunized with *void* chitosan nanoparticles, CFP-10 *per se*, CHNP CFP-10 and PBS was 1.74x10^5, 0.35x10^5, 0.16x10^5 and 0.7x10^5 respectively. There was a significant decrease in the number of colonies count in the group of mice immunized with CHNP CFP-10, which was ~4 times less than control (PBS) group of mice.

Fig. 7.2b CFU assay of lung from the pre-immunized Balb/c mice with *Void* chitosan nanoparticles, CFP-21 *per se*, CHNP CFP-21 and control group. CFU assay was performed on 30 days post *M. tb.* challenge. The fig. indicated that there was significant decrease in the number of colonies in the group of mice immunized with CHNP CFP-21 (0.17 x10^5) which was ~ 3 times less than control (PBS) group of mice (0.7x10^5). Mice immunized with *void* chitosan nanoparticles the number of colonies (1.74 x10^5) was more than PBS. This revealed that chitosan nanoparticles were supporting the growth of *M. tb.*
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**Fig. 7.3a CFU assay was performed on 60 days post M. tb. challenge.** In the group of mice immunized with *void* Chitosan nanoparticles the number of colonies was \( 4.7 \times 10^5 \) in the group of mice immunized with CFP-10 *per se* the number of colonies was \( 0.6 \times 10^5 \), in the group of mice immunized with CHNP CFP-10 the number of colonies was \( 0.125 \times 10^5 \) while in the control (PBS) group of mice the number of colonies was \( 1.6 \times 10^5 \). Therefore, secretory proteins encapsulated in chitosan nanoparticles definitely appeared to be importing protection over a longer period of time.

**Fig. 7.3b CFU assay was performed on 60 days post M. tb. challenge.** The trend in the colony count observed was different at day 60. Progressive reduction in colony count was observed. The number of colonies in the group of mice immunized with CHNP CFP-21 was \( 0.8 \times 10^5 \) while in the PBS group of mice the number of colonies was \( 1.6 \times 10^5 \); this showed maximum protection in the group of mice immunized with CHNP CFP-21. This data indicated that CHNP CFP-21 has significantly more potential to inhibit the growth of *M. tb.* than other group of mice.
Fig. 7.4a Comparative analysis of CFU assay of lung on day 15, day 30 and day 60 from the pre-immunized Balb/c mice with void chitosan nanoparticles, CFP-10 per se, CHNP CFP-10 and control group (PBS). The fig. showed more protection induced in the group of mice immunized with CHNP CFP-10 than CFP-10 per se, CHNP and control (PBS). At day 15 and 30 void chitosan nanoparticles promoted the M. tb. growth whereas at day 60 the number of colonies count was less than control (PBS) group of mice. Remarkably, in the group of mice immunized with CHNP CFP-21 the number of colonies reduced significantly from day 15 to day 60.

Fig. 7.4b Comparative analysis of CFU assay of lung on day 15, day 30 and day 60 from the pre-immunized Balb/c mice with void chitosan nanoparticles, CFP-21 per se, CHNP CFP-21 and control group (PBS). The fig. showed more protection induced in the group of mice immunized with CHNP CFP-21 than CHNP and control (PBS). This fig. indicated that CFP-21 encapsulated chitosan nanoparticles have more potential to reduce the bacterial count than CFP-21 per se.
Table 7A: Log$^{10}$ CFU values in lung tissue from mice immunized intraperitoneally by three booster doses of the experimental groups at an interval of 15, 30, 60 days post M. tb. challenge.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 15</th>
<th>Day 30</th>
<th>Day 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (n=5)</td>
<td>5.8±1.1</td>
<td>0.7±0.07</td>
<td>1.6±0.13</td>
</tr>
<tr>
<td>Void CHNP (n=5)</td>
<td>6.75±1.06</td>
<td>1.74±0.02***</td>
<td>4.7±0.19**</td>
</tr>
<tr>
<td>CFP 10 per se (n=5)</td>
<td>1.67±0.21*</td>
<td>0.35±0.05**</td>
<td>0.6±0.15</td>
</tr>
<tr>
<td>CHNP CFP-10 (n=5)</td>
<td>1.2±0.2**</td>
<td>0.16±0.03***</td>
<td>0.125±0.03</td>
</tr>
<tr>
<td>CFP 21 per se (n=5)</td>
<td>2.45±0.59**</td>
<td>0.37±0.05**</td>
<td>0.7±0.12</td>
</tr>
<tr>
<td>CHNP CFP-21 (n=5)</td>
<td>1.99±0.9*</td>
<td>0.17±0.04***</td>
<td>0.08±0.02</td>
</tr>
</tbody>
</table>

CFU: Colony forming units
Values are presented as Means ± S.D.
*P < 0.1, **P<0.01, ***P < 0.001 as compared with controls (according to ANOVA)

Fig. 7.5 CFU assay on day 30 in the group of mice immunized with CFP-10 per se, CFP-21 per se, CHNP CFP-10, CHNP CFP-21 and control (PBS). The above figures showed less colony count was evident in the group of mice immunized with CHNP CFP-10 and CHNP CFP-21 in comparison to void chitosan nanoparticles and PBS.