**Oxidized carbon nanosphere-based subunit vaccine delivery system for tuberculosis**

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**Abstract**

**Background:** Tuberculosis (TB) is among the deadliest infectious disease worldwide. Current vaccine BCG showed limited protective efficacy against adult pulmonary tuberculosis. Novel TB vaccine is urgently needed. To develop a subunit TB vaccine, effective delivery system is a prerequisite.

**Methods:** Oxidized carbon nanosphere (OCN) was used as subunit TB vaccine delivery system. The ability to deliver recombinant *Mycobacterium tuberculosis* (Mtb)proteins, Ag85B and HspX, into bone marrow derived macrophages (BMDMs) was investigated. For immunization, OCN was mixed with the two antigens as well as adjuvant, monophosphoryl lipid A (MPL). Mice were subcutaneously immunized and serum and splenocytes were collected. Antibody titer and cytokine profiles from *in vitro* re-stimulated splenocytes were evaluated for immunogenicity. For monitoring cytotoxic T cell activation, re-stimulated splenocytes were stained for CD8+ T cell and intracellular granzyme B. Moreover, the protective efficacy was analyzed by aerosol Mtb challenge with virulent strain of Mtb and the bacterial burdens were measured.

**Results:** OCN is highly effective in delivery of Mtb proteins into BMDMs. Upon immunization, this vaccine formulas induced Th1 immune response characterized by cytokine profiles from re-stimulated splenocytes and specific antibody titer. More importantly, enhanced cytotoxic CD8+ T cells activation was observed. However, it did not reduce bacteria burden in lung and spleen from aerosol Mtb challenge.

**Conclusion:** OCN is highly effective in delivery of subunit protein vaccine and induces higher CD8+ T cell response. Collectively, this vaccine delivery system is suitable for application in settings where cell-mediated immune response is needed.

Keywords: carbon nanosphere, vaccine delivery, subunit vaccine, cytotoxic T lymphocyte; tuberculosis

**Introduction**

 *Mycobacterium tuberculosis* (Mtb) is an intracellular bacterial pathogen which is the causative agent of tuberculosis (TB). Currently, TB is still one of the major global health problems with higher mortality rate than HIV/AIDS (1). The live attenuated strain of *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) is the only TB vaccine available for human use since 1921. It provides protection against severe forms of TB in children but confers less protective efficacy against pulmonary TB in adults (2). Therefore, an effective vaccination strategy and novel TB vaccine are urgently needed to stop TB.

 Cell-mediated immune response (CMI), such as type I helper T cells (Th1) and cytotoxic T lymphocytes (CTL) are believed to play a major role to defense against Mtb (3, 4). Therefore, this type of immune responses became the main focus to develop TB vaccine. Antigen presenting cell (APCs) process and present antigens to T cells and the outcome of the responses are determined by various factors including route of antigen uptake, cytokine milieu. Conventionally, endogenous antigens (cytosolic antigen) are processed and loaded on to major histocompatibility complex (MHC) class I molecules then presented to CD8+ T cells (5). However, cross-presentation which is an alternative pathway also allow the exogenous antigens loading on to MHCI (5, 6). This mechanism is important for designing subunit vaccine to elicit CMI and CD8+ T cell response. Subunit vaccines increasingly become major type of vaccines due to high degree of safety and their production can be standardized (7). Therefore, proper adjuvants and novel delivery systems are increasingly needed with the aims to increase immunogenicity of peptides or recombinant antigens.

Recently, nanoparticle-based vaccine delivery system is wildly studied (8-10). Nanoparticles have many advantages for vaccine development. First, the size of nanoparticle is in the range of microorganisms and can be targeted and readily uptake by APCs. As a result, nanoparticles help to increase the way of antigen presentation to the immune cell (11). Second, encapsulated antigens can be protected from degradation rapidly by various enzymes in the environment before taken up by APCs and the antigen releasing can be controlled to prolong presentation by APCs (12-14). Third, as soluble antigens are less to generate cross-presentation, the combining of nanoparticles with soluble antigen can generate a particulate form that show the higher level of cross-presentation than the soluble antigens (15, 16). Because the properties of vaccine will determine the outcomes of immune responses, therefore using nanoparticle as a vaccine delivery system is highly attractive and show promising for TB vaccine development.

The types of nanoparticles including viral vector and liposome are applied in clinical trial TB subunit vaccines. For viral vectored vaccines, recombinant adenovirus, vaccinia virus and influenza virus are used for expressing Mtb antigen. In preclinical study, other types of nanoparticles such as poly (lactide-co glycolide) (PLGA), chitosan, poloxamer and pluronic-stabilized polypropylene sulfide are also reported (17).

Oxidized carbon nanosphere (OCN) is the spherical particle around 130 nm in diameter, water dispersible, stable, negatively charged and oxidized carbon nanoparticle. A starting material of OCN is graphite or graphene nanoplatelets (GNP) which are cheap (18). This nanoparticle shows promising properties including low toxicity, biocompatibility and good macromolecules carrier (18, 19) Additionally, OCN leaking from endosome in to cytoplasm by generating the transient pore at the lipid bilayer is also reported (19). Once the antigens are in the cytosol, the chance of antigen being loaded onto MHC I is expected that may lead to enhanced CD8+ T cells activation. Overall, the application of OCN as a delivery system for TB subunit vaccine is a promising system. To the best of our knowledge, carbon-based nanoparticle for TB vaccine development has never reported before.

 In this study, we prepared our prototype TB vaccine by mixing three components of (I) OCN, (II) two dominant Mtb antigens, Ag85B (a fibronectin-binding protein with mycolyltransferase activity, Rv1886c) and HspX (a heat shock protein, Rv2031c) and (III) monophosphoryl lipid A (MPL), Th1 potent adjuvant (20). Ag85B and HspX represent replicating state and dormant state protein (21, 22), respectively. The ability of OCN to deliver Mtb antigens in to macrophage cell was tested in *vitro*. Immunogenicity of the recombinant vaccine was evaluated by antibody titer and *in vitro* re-stimulation assay. Finally, the protective efficacy was tested by aerosol challenge with Mtb.

 **Methods**

**Reagents**

Dulbecco's Modified Eagle Medium (DMEM), sodium pyruvate, HEPES and penicillin-streptomycin were purchased from GE Healthcare Life Sciences (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Life Technologies (Waltham, MA, USA). Horse serum was purchased from Thermo Fisher Scientific. MPL was a vaccine grade and purchased from Invivogen (San Diego, CA, USA)**.** Recombinant proteins Ag85B and HspX were obtained from BEI Resources (**Manassas, VA, USA**).

**Animals**

BALB/c female mice (8 weeks old) were purchased from Nomura Siam International (Thailand). All experiment involving BALB/c mice experiments were approved by Chulalongkorn University Institutional Animal Care and Use committee (CU-IACUC) (No.1673005). For Mtb challenge, CB6F1/Crlfemale mice (6-8 weeks old) were used. All the experiments were performed following the ethical and legal requirements set by the UK Home Office guidelines referring to the welfare of experimental animals (23).

**BMDMs preparation**

 Bone marrow cells were isolated from tibias and femurs of BALB/c female mice by flushing and BMDMs were generated by incubating cells in BMDM media (DMEM supplemented with 10% (v/v) FBS, 1% (w/v) sodium pyruvate, 1% (w/v) HEPES, 100 U/ml penicillin, 0.25 mg/mL streptomycin, 20% L929 cell-conditioned media and 5% horse serum), followed by culture in this media for a week. Media were changed every 3 days (24).

**Formulation of OCN with recombinant antigens**

 OCN was prepared as described previously (18). OCN was autoclaved at 121°C and sonicated for 5 min before use in every experiment. OCN+Ag85B+HspX mixture was prepared by mixing OCN-Ag85B complex with OCN-HspX complex. Briefly, two µg of OCN was mixed with 2 µg of each protein separately in water with the total volume of each complex was 5 µl. The two mixtures were incubated overnight at 4°C before formulated into two protein mixture together. Five microliters of mixture were added in 500 µl DMEM completed media (DMEM supplemented with 10% (v/v) FBS, 1% (w/v) sodium pyruvate, 1% (w/v) HEPES, 100 U/ml penicillin, 0.25 mg/mL streptomycin) to obtain the final weight of 2 µg of OCN, 1 µg of Ag85B and 1 µg of HspX. The weight ratio of OCN to proteins was 1:1.

**Immunofluorescent staining**

 BMDMs cells were seeded at 1x105 cells/well on 8 well-chamber slides (Thermo Fisher Scientific, Waltham, MA, USA) and grown overnight in BMDM media at 37°C with 5%CO2. Media was changed to DMEM completed media and incubated further for overnight. Culture supernatant was removed and OCN formulation in DMEM completed media were added. Cultures were maintained at 37°C, 5% CO2 for 1 h. Cells were fixed, permeabilized and stained with standard immunofluorescent staining protocol. Ag85B staining was performed by using 1:200 dilutions of rabbit polyclonal anti-Ag85B (a kind gift from Prof. Watchara Kasinrerk, Faculty of Associated Medical Sciences, Chiang Mai University, Thailand) and detected with 1:500 dilutions of Alexa 555-labeled anti-rabbit IgG (Cell Signaling Technology, Danvers, MA, USA), while HspX was stained with 1:200 dilutions of monoclonal anti-HspX (BEI Resources, **Manassas, VA, USA**) and detected with 1:500 dilutions of Alexa 488-labeled anti-mouse IgG (Cell Signaling Technology) for 1 h each. Nuclei were stained with Hochest (Invitrogen, Waltham, MA, USA). Confocal microscopy was used for observing the intracellular uptake (Olympus FV10i, Tokyo, Japan).

**Vaccine preparation (HspX+Ag85B+OCN+MPL)**

One hundred microliters of vaccine per mouse were prepared by mixing OCN-Ag85B with OCN-HspX and MPL (Invivogen, San Diego, USA) together. Briefly, each mixture was prepared by mixing 10 µg of OCN with 10 µg of Ag85B or 10 µg of HspX separately (OCN: protein weight ratio was 1:1) in endotoxin free water at 4°C for overnight in 45 µl. After that, the two mixtures were mixed together and MPL (10µg) to reach the final volume of 100 µl. The final amount of OCN, Ag85B and HspX in 100 µl of vaccine were 20, 10 and 10 µg, respectively.

**Immunization strategy and challenge of mice with Mtb**

 For study the immunogenicity of the prototype TB vaccine, 8 weeks old BALB/c female mice (n=6 per group) were used. The day before the immunization, blood was collected from facial vein. One hundred microliters of formulas including saline, HspX+Ag85B+MPL and HspX+Ag85B+OCN+MPL were immunized 3 times subcutaneously 2 weeks interval (Fig. 2E). The animals were sacrificed at day 35 to collect blood and spleens.

 For protective efficacy, two immunization strategies were performed to test the protective efficacy of vaccine. First, mice were primed and boosted with the prototype vaccine. Second, mice were primed with BCG and boosted with prototype vaccine later. Six to eight weeks old CB6F1/Crlfemale mice (n=6 per group) were used. In the prime-boost strategy, mice were immunized subcutaneously with 100 µl of each formulas including PBS, HspX+Ag85B +MPL and HspX+Ag85B+OCN+MPL 3 times with 2 weeks interval. For standard control group, mice were received a single dose of BCG1331 (5x106 CFU/mouse). For boosting strategy, mice were primed with BCG 1331 and followed by a single boost of corresponding treatments on week 8. Six weeks after the last immunization, mice receiving 2 immunization strategies were subjected to an aerosol challenge with virulent H37Rv Mtb aiming for an infective dose level of 100 CFU/mouse. Body weight of mice were observed once a week. Lungs and spleens were harvested 6 weeks after challenge then processed and plated for *Mycobacterium* CFU counting.

**Splenocyte re-stimulation assay**

###  Splenocytes were collected by homogenization of spleen though 100 µm cell strainer with the syringe plunger. Red blood cell lysis buffer was used to lyse red blood cell. Splenocytes were seeded at 4x106 cells/well, total volume of 1 ml in 24 well plate. Specific antigen including Ag85B and HspX at the final concentration of 10 µg/ml were added then the plate was incubated at specific time point. For measurement of cytokine profiles by ELISA, incubation time for IL-2 detection was 48 h. while IL-5 and IFN-γ detection were 72 h. In the case of intracellular granzyme B staining, incubation time was 72 h and in the last 4 h, 1 µl of Golgi plug (Brefeldin A) (BD Biosciences, San Jose, CA USA), were added to the cultures.

**Specific Antibody Titer**

 Serum were collected to measure the level of specific IgG1 and IgG2a. Two micrograms per milliliter of Ag85B or HspX were coated on 96 well MaxiSorp plate (Nunc, Roskilde, Denmark) and incubated at 4°C overnight. Plates were washed and blocked with 10% FBS in PBS and serial dilutions of serum was added. After 1 h of incubation, plates were washed and sheep anti-mouse IgG-HRP (GE Healthcare, UK) or rabbit anti-mouse IgG1-HRP (Invitrogen, Camarillo, CA, USA) or rat anti-mouse IgG2a-HRP (Invitrogen, Camarillo, CA, USA) were added. After washing, 3,3 ,5,5’-tetramethylbenzidine ([Sigma Aldrich](https://www.google.co.th/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&cad=rja&uact=8&ved=0ahUKEwjfsZPc66_SAhUJjLwKHadjDfIQFggaMAA&url=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsigma%2Fc5275&usg=AFQjCNHCwJCPUUvO8IKgw-uW_52tzQCCnA), St. Louis, MO, USA) were added to develop the signal and 1N of H2SO4 was used to stop the reaction. The optical density (OD) at 450 nm was measured with microplate reader (Anthos 2010, Biochrom, UK).

**Intracellular cytokine staining**

 Cells were processed by using BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences, San Diego, CA, USA), according to manufacturer’s instructions. Briefly, cells were stained for cell surface markers, CD3 and CD8 with biotin-labeled anti-mouse CD3e (Biolegend, San Diego, CA, USA) and PE-labeled anti-mouse CD8a (Biolegend), respectively. PE/Cy7-labeled streptavidin (Biolegend) was used as secondary reagent for detection. After surface staining, cells were fixed, permeabilized and stained for granzyme B using FITC-labeled anti-human/mouse granzyme B (Biolegend). Isotype matched antibodies were used as a control. Data were collected using Flow cytometer (Beckman Coulter, USA) and analyzed by FlowJo software (Tree Star, USA).

**Measurement of cytokine production by ELISA**

 Cell culture supernatants were collected to measure level of IFN-γ, IL-2 and IL-5 using LEGEND MAXTM mouse IFN-γ, IL-2 and IL-5 ELISA kits (Biolegend) following the manufacturer's protocol. The absorbance was detected at OD450 nm using microplate reader.

**Statistical analysis**

All data were analyzed for statistical significance using GraphPad Prism (version 5.03) software (GraphPad, USA). All data were analyzed by one-way analysis of variance (ANOVA) for determining statistical significant differences between groups. Probability (*p*) values less than or equal to 0.05, 0.01 and 0.001 were considered as significance and labeled with one, two and three asterisks, respectively.

**Results**

**Enhancing Mtb proteins uptake in BMDMs by OCN**

To test the ability of OCN to deliver proteins into antigen presenting cells, recombinant Mtb proteins mixing with OCN at the weight ratio of 1:1 was tested in BMDMs. Immunofluorescence staining showed that two Mtb proteins, Ag85B and HspXwere observed inside BMDMs when combine with OCN at 1 hr of incubation (Fig. 1). Whereas no or low fluorescence signal was detected when Ag85B and HspX were used alone. Interestingly, most cells showed single antigen staining pattern suggesting that each antigen was separately uptake by each cell. Therefore, OCN effectively promoted protein antigen uptake.

**Immunogenicity of OCN with HspX, Ag85B and MPL**

To study the influence of OCN on the outcome of immune response, mice were immunized and serum and splenocytes were collected as depicted in Fig. 2A. For serological response, specific antibody against Ag85B and HspX in serum were measured. Because IgG2a and IgG1 titer corresponds to Th2 and Th1 immune responses, the titer of these two isotypes were compared. Overall adding MPL to the vaccine formulas induced higher IgG1 titer than antigens and OCN alone and this is more evident in HspX specific antibody titer (Fig. 2C). More importantly, the results showed that IgG1 titer in mice receiving HspX+Ag85B+OCN+MPL was higher than the other 2 groups that received only PBS or HspX+Ag85B+MPL, while there was no significance difference in the IgG2a titer between HspX+Ag85B+OCN+MPL and HspX+Ag85B+MPL groups (Fig. 2C, D). This is true for both antigens. Additionally, the ratio of IgG2a to IgG1 also demonstrated that there are no statistical significant differences between all groups in both Ag85B and HspX stimulation (Fig. 2E).

For the cytokine profiles, splenocytes from immunized mice were re-stimulated with HspX and Ag85B separately *in vitro*. High IL-5 production is associated with Th2-mediated immune response, while IFN-γ production is represent to Th1-mediated immune response. Under re-stimulation with Ag85B, IL-5 production in the HspX+Ag85B+OCN+MPL group was significantly lower than HspX+Ag85B+MPL group, but the difference between them was not observed in HspX re-stimulation (Fig. 2 F). For IFN-γ production, the group receiving HspX+Ag85B+OCN+MPL showed significantly higher level of IFN-γ than that receiving HspX+Ag85B+MPL in both Ag85B and HspX re-stimulation (Fig. 2G). The results of IL-2 production in both Ag85B and HspX re-stimulation also in the same trend with IFN-γ production (Fig. 2H). Taken together, OCN enhanced Th1 cytokine production while decreasing Th2 cytokine production in re-stimulation assay.

**OCN enhanced cytotoxic CD8+ T cell activation to Mtb protein**

 To study the effect of OCN on cytotoxic CD8+ T cell activation, splenocytes were re-stimulated with Ag85B or HspX and stained for CD3, CD8 and granzyme B (Fig. 3A, B). In HspX re-stimulated condition, the percentages of CD8+ T cells that producing granzyme B in HspX+Ag85B+OCN+MPL immunized mice were significantly higher than the other groups. With the re-stimulation of Ag85B, the percentage of CD8+ granzyme B+ cells in the group of HspX+Ag85B+OCN+MPL and HspX+Ag85B+MPL were similar and higher than the control saline group (Fig 3C). This result indicated that OCN augmented the frequencies of granzyme B producing cytotoxic CD8+T cells to HspX.

**OCN combined with HspX, Ag85B and MPL were not sufficient to protect mice against Mtb challenge**

 To evaluate the protective efficacy induced by subunit TB proteins combining with OCN and MPL with or without priming with BCG, immunized mice were respiratory challenged with virulent Mtb H37Rv strain six weeks after the last immunization. Changes in body weight was measured during the experimental time line and there were no significant differences in the body weight change among all groups and both immunization strategies showed the similar results (Fig. 4A and Fig.5A). Six weeks after challenge, bacterial burden in lung and spleen were evaluated. In the two experiments, BCG was used as a positive control standard. The numbers of bacteria in lung and spleen of BCG immunized mice was significantly lower when compared with PBS control group. Unfortunately, bacterial CFU in both organs of other tested groups were not decreased when compared with PBS group (Fig. 4B, C). In the BCG priming regimen, adding Ag85B+HspX+OCN+MPL did not reduce the bacterial burden further than those in the BCG alone (Fig. 5 C, D). These results indicated that the prototype TB vaccine combining OCN with two recombinant proteins was not sufficient to induce the protective immunity against Mtb infection in both prime-boost and boosting strategies.

**Discussion**

In this study, we evaluated a novel nanoparticle from oxidized carbon OCN as a delivery system for subunit TB vaccine**.** Because cell-mediated immune responses and Th1 responses are considered the major immune response against Mtb infection, designing a better delivery system that targets and enhances this type of immune response is desirable for TB vaccine candidate (4, 10, 25).

 APCs are cells that process and present antigens via MHC class I and II to T cells. Targeting antigen to these cells and engineering the route of antigen processing can drive appropriate immune responses. Consistent with the previous reports, OCN was efficiently taken up by macrophages. Previous report proposed that OCN can generate transient pore on lipid membrane of endosome, then leaks into cytosol and localize in the cytosol (19). Using this rationale, we expected that the antigens delivered by OCN would be processed and presented to CD8+ T cells as well as CD4+ T cells. In fact, the frequency of granzyme B producing CD8+ T cells is higher when OCN was combined with recombinant antigens, indicating that OCN possibly drives antigen presentation to CD8+ T cells. This mechanism was also reported when PLGA was used as a delivery system (15).

 Currently, one of the most advance TB vaccine is based on engineered BCG to expressed Ag85B and *Listeria monocytogenes* listeriolysin to facilitate endosomal escape of BCG vaccine (26, 27). The proposed protective mechanisms of this vaccine are the effective CD8+ T cell priming through antigen cross presentation by DC (26-28). Therefore, it is highly possible to target CD8 T cells and acquired protective immunity against Mtb.

The reason that we used MPL in our vaccine system is to increase Th1 immune response. MPL, a Toll-like receptor (TLR4) agonist is one of the few licensed adjuvants for human. It is an attenuated version of lipopolysaccharide (LPS) with less toxicity and maintaining its immunostimulatory activity (29). Signaling via TLR4 involves myeloid differentiation primary response 88 (MYD88)-dependent and TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathways, leading to pro-inflammatory cytokines and type I interferon production, respectively (29, 30). MPL generally drives the immune responses toward a Th1 type (29). Our cytokine profiles from specific antigen re-stimulated splenocytes indicated that MPL induces Th1 immune response.

Surprisingly, when OCN was added to the vaccine formula, the level of Th1 signature cytokine, IFN-γ was increased, while the level Th2 cytokine, IL-5 was significantly reduced. This result suggested that OCN can drive the immune response towards Th1. However, the cytokine profiles detected here did not corelate with specific antibody titer. The results of antibody titers from serum indicated that the response was polarized towards a Th2 immune response. The discrepancies between antibody isotypes and cytokine profiles are currently unknown.

# Even if the immunogenicity elicited by the novel vaccine formula seemed to be good, but the protective efficacy of this prototype vaccine was not achieved. The vaccine could not reduce the bacterial burden in lung and spleen of Mtb challenge mice in both the priming and boosting strategies. One of the reasons we did not observe protective response may lie in the recombinant antigens used in this study. We did not fuse the two antigens, but rather used them as separated antigens. Previously, it was shown that Ag85B-ESAT6 fusion protein and DDA/MPL or DDA/TDB as adjuvants can induce the protection against Mtb challenge in guinea pigs and mice (31, 32). Non-fusion proteins Ag85B and ESAT6 individually or combination, on the other hand, did not confer protection (31). The multi-stage subunit vaccine that consist of ESAT6-Ag85B-MPT64(190-198)-Mtb8.4-HspX combining with DDA/PolyI:C showed strong immunogenicity and long lasting protective efficacy against Mtb (33). H56, a clinical trial Mtb protein consisting of Ag85A, ESAT-6, and Rv2660c (dormant state protein), conferred highly protective efficacy in both before and after exposure to Mtb (34).

**Conclusion**

In summary, the present study reveals that OCN was a good subunit protein carrier for delivering protein into APC such as macrophages. Moreover, OCN mixed with two state proteins of Mtb, Ag85B and HspX, and MPL as adjuvant increased cytotoxic CD8+ T cells activation. The responses were polarized to Th1 immune response characterized by cytokine profiles. Unfortunately, the immune responses elicited by this vaccine were not sufficient for generating protection against Mtb.

**Acknowledgments**

 This work was supported by the Thai Government Annual Budget Newton Fund-Institutional Links (UK) and PS is supported by the 100th Anniversary Chulalongkorn University fund.

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Figure Legends

**Figure 1.** OCN increases Mtbantigen protein uptake by macrophages.

Complex of OCN:HspX/Ag85B (weight ratio 1:1) were tested in 1x105 cells of BMDM cell. After 1 hr of incubation at 37 Cᵒ, cells were fixed and subjected to an immunofluorescent staining. Ag85B and HspX were stained with rabbit polyclonal anti-Ag85B and monoclonal anti-HspX as a primary antibody, respectively. For detection, anti-rabbit IgG-Alexa 555 (Red) and anti-mouse IgG-Alexa 488 (Green) were used as a secondary antibody. Nuclei were stained with Hochest (blue). Cells were observed by confocal microscope.

**Figure 2.** OCN influences immune responses when combined with HspX, Ag85B and MPL.

(A) BALB/c (n=6) mice were immunized with saline, HspX+Ag85B+MPL or HspX+Ag85B+OCN+MPL via subcutaneous administration three times at 2 weeks intervals. One week after the last immunization, splenocytes and blood were harvested. (B-D) For serological responses to Ag85B or HspX, sera were collected for measuring specific total IgG titer, IgG1 and IgG2a. (E) The ratio of IgG2a to IgG1 was shown. (F) Splenocytes were stimulated with Ag85B (10µg/ml) or HspX (10 µg/ml) separately. (G-H) Culture supernatant were collected and used to measure IL-5 and IFN-γ at 72 h, and IL-2 at 48 h by ELISA. Data represents the means ± SEM. The significance of differences between groups was determined by one-way analysis of variance (ANOVA). \**p* < 0.05*, \*\*P*< 0.01 and *\*\*\*P*<0.001.

**Figure 3.** OCN augments the frequencies of cytotoxic CD8+ T cells activation.

(A-B) BALB/c (n=6) mice were immunized as indicted in Figure 2. One week after the last immunization, splenocytes were harvested and stimulated with Ag85B (10µg/ml) and HspX (10 µg/ml) separately *in vitro* for 72 h in the presence of brefeldin A for the last 4 h. Cells were collected for intracellular granzymeB staining. Representative flow cytometry results were shown. (C) The percentage of Ag85B and HspX specific CD8+ T cells producing granzymeB were summarized. Data represents the means ± SEM. The significance of differences between groups was determined by one-way analysis of variance (ANOVA). *\*\*\*P*<0.001*.*

**Figure 4.** Protective efficacy of OCN in combination with HspX, Ag85B and MPL in prime-boost strategy.

CB6F1/Crlfemale mice were immunized with PBS, HspX+Ag85B+MPL and HspX+Ag85B+OCN+MPL subcutaneously for 3 times at 2 weeks interval, except for BCG1331 (5x106 CFU/mouse) that was immunized as a single dose at the last immunized time. Six weeks later, mice were aerosol challenged with H37Rv Mtb (100 CFU/mouse). (A) The changes in body weight were monitored. (B-C) The protective efficacy was determined and demonstrated as the number of *Mycobacterium* CFU per lungs and spleens at 6 weeks after challenge. Error bar showed mean ± SD. Each line corresponds to a single experimental group. The significance of differences between groups was determined by one-way analysis of variance (ANOVA). *\*\*\*P*<0.001*.*

**Figure 5.** Protective efficacy of OCN in combination with HspX, Ag85B and MPL as a booster following BCG priming.

CB6F1/Crlfemale mice were primed with BCG1331 (5x106 CFU/mouse) on week 0 plus single dose of boost with corresponding vaccine candidates on week 8. The control group received only one dose of PBS on week 0. Six weeks later, mice were aerosol challenged with H37Rv Mtb (100 CFU/mouse). (A) The changes in body weight were monitored. (B-C) The protective efficacy was determined and demonstrated as the number of *Mycobacterium* CFU per Lungs and spleens at 6 weeks after challenge. Error bar showed mean ± SD. Each line corresponds to a single experimental group. The significance of differences between groups was determined by one-way analysis of variance (ANOVA). *\*\*\*P*<0.001*.*