Non-cell *Corynebacterium parvum* generated by nanotechnology: A promising immunomodulator with less side effects

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Abstract

Corynebacterium parvum (CP), a kind of immunomodulator, has been well documented in immunotherapy to tumor. However, severe side effects, such as intrahepatic granulomas and scleromes in injected areas, restrict its clinical application. To minimize side effects of CP, a non-cell *Corynebacterium parvum* product (NCPP) was prepared by disposing CP with Nanotechnology. In present study, we compared effect of NCPP with that of CP and found: (1) NCPP with non-formaldehyde residue was easy to be absorbed without swelling and sclerome in local injected areas; (2) NCPP caused no obvious liver injury in murine and macaques; (3) NCPP maintained powerful anti-tumor activity, increased splenic index, elevated macrophage number, phagocytosis and production of hydrogen peroxide (H_2O_2), and nitric oxide (NO); (4) Importantly, unparallel CP, NCPP could stimulate macrophages to produce low level of tumor necrosis factor-α(TNF-α) but high level of interferon-γ (IFN-γ), an inhibitor to fibrosis. Our study has led to the view that NCPP will evolve into a new valuable immunomodulator for clinical application.
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1. Introduction

Corynebacterium parvum (CP), a gram-positive, anaerobic bacillus, which can be isolated from the bone marrow of healthy individuals, is not obviously pathogenic to the human body. Similar to the Bacillus Calmette–Guerin vaccine (BCG), which has been used as an immune adjuvant, CP is also a powerful nonspecific immune stimulator [1–6]. It has been observed that CP can elevate immune functions via the activation of macrophages [7], as well as the regulation of T cell immune responses [8]. In addition, CP is also able to promote haematopoietic stem cells to differentiate into monocytes, which in turn activates body immune system to exert its anti-cancer immune responses [9]. Although the precise mechanisms underlying CP-elicited macrophage activation still remain obscure [10], nowadays, CP is considered as

one of the best agents able to activate macrophages. Importantly, available data suggest that the administration of heat-killed CP in mice can induce multiple immunomodulatory effects and especially, the inhibition of tumor progression [11–13]. Unfortunately, recent investigations have demonstrated that CP-treated mice could develop splenic intumescences and intrahepatic granulomas [14,15]. Such severe side effects of CP, no doubt, have extremely restricted its clinical applications. In this regard, strategies that minimize the adverse effects of CP while preserving its immunomodulatory functions have generated considerable interest. In fact, evidence has shown that cellular extraction from CP is also able to stimulate macrophage activation, prevent microbe infection and inhibit the lethal effect of sarcoma 180 [16], indicating that reconstructing CP while retaining its biological function is feasible. This has led us to prepare a neotype of an immunomodulator originated from CP using advanced nanotechnology.

Nanotechnology has gradually been introduced into multiple fields [17,18]. For instance, nanometer medicaments, the nano-size granules, always have the unique properties of being easily absorbed while preserving low concentrations and immunogenicity. In addition, the diversification of particle size will also exert effects on serum lifetime and the pattern of deposition in the primed objects. All these specialties prove that nanotechnology is a promising strategy for drug discovery and clinical application [19,20].

To date, no information has been available on the transformation of CP using Nanotechnology. Therefore, the present study was designed initially to dispose of CP, using Nanotechnology to prepare a neotype of immunomodulator, referred to as the Non-cell *Corynebacterium parvum* Product (NCPP), and then, to investigate the general pharmacological traits of NCPP, with a specific focus on the effect of NCPP on mononuclear phagocytes.

2. Materials and methods

2.1. Animals

Male C57BL/6 and BALB/c mice (6–8 weeks, 20–22 g) were obtained from the Shanghai Laboratory Animal Center, Chinese Science Academy (Shanghai, China). Macaques (3–4 years, 3–5 kg) and rabbits (2–3 years, 3–5 kg) were purchased from the Laboratory Animal Center, Chinese Military and Medical Academy of Sciences (Beijing, China). All animals were bred under specific pathogen-free conditions. We give assurance of humane practice in animal maintenance and experimentation and all animal care and experimental treatments were done with the approval of the Shandong University Ethics Committee.

2.2. Cell lines and reagents

The B16 melanoma cell line was purchased from the Chinese Military and Medical Academy of Sciences (Beijing, China). CP (77-1 strain) was obtained from the National Institute for the Control of Pharmaceutical & Biological products(Beijing, China). PE-conjugated anti-F4/80 and PE-conjugated anti-IgG antibodies were purchased from eBioscience (San Diego, USA).

2.3. Preparation of NCPP

The cultured CPs were collected, sterilized and then cracked into small particles in an extra-high voltage jet crushing machine (Nanonizer, Japan). These particles were precipitated by centrifugation and resuspended in 0.9% sodium chloride solution, named NCPP, and stored at 4 °C for use. The size of NCPP, as well as CP, was detected under the transmission electron microscope (Phillips Co. Germany).

2.4. Treatment

B16 melanoma cells were cultured in RPMI1640 medium (Gibico) supplemented with 10% fetal bovine serum (SiJi Qing, Hangzhou, China). For establishment of a tumor-bearing murine model, tumor cells ($1 \times 10^6/0.1$ ml) were inoculated subcutaneously in the left flank of each mouse. When the maximal diameter and the minimal diameter of tumors reached about 5×5 mm, mice were injected intraperitoneally with NCPP or CP (0.5 mg/mouse) or phosphate-buffered saline (PBS) as a control. Groups were described as B16/NCPP, B16/CP, B16/PBS and PBS, respectively. In the splenic activation test, mice were sacrificed at day 14 after mice were injected intraperitoneally with NCPP or CP (0.5 mg/mouse) and splenic index was calculated by the following formula: Splenic index = [splenic weight (mg) of control group/body weight (g) of control group]/ [splenic weight (mg) of treated groups/body weight (g) of treated groups]. In the cutaneous absorbability experiment, NCPP or CP (0.5 mg/rabbit) was cutaneously injected into a rabbit's back (4 rabbits/group), and local swelling and sclerome were evaluated at 24 h, 48 h and 72 h after injection.

2.5. Evaluation for histopathology

Briefly, NCPP or CP (3 mg/macaque) was injected intramuscularly into macaques twice a week for 90 days and macaques were sacrificed within 24 h after the last injection. Similarly, NCPP or CP (0.5 mg/mouse) was injected intraperitoneally into BALB/c mice and 10 days latter, all treated mice were sacrificed to detect liver histopathological alteration. In succession, liver tissues of macaques and mice were fixed in 4% formaldehyde(40% formalin 100 ml, Na_2HPO_4 6.5 g, NaH_2PO_4 4.0 g, DDW 900 ml) for 24 h and embedded in paraffin. The sections were prepared, deparaffinized and stained with hematoxylineosin and eosin. In the end, pathologic changes were observed under a light

Observation of CP under electron microscope (×8000)

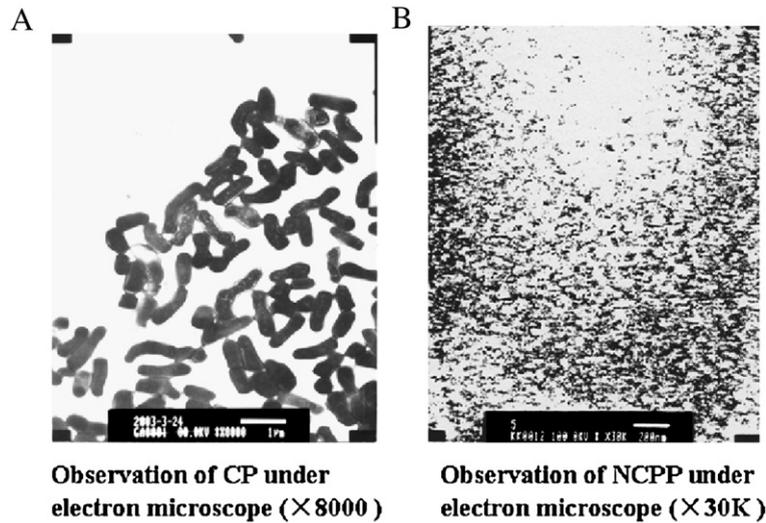**Observation of NCPP under electron microscope (×30K)**

Fig. 1. Observation under electron microscope. The CP (A) and NCPP (B) which was obtained by disposing *Corynebacterium parvum* in extra-high voltage jet crushing machine were observed under the transmission electron microscope.

microscope and grades were evaluated according to the following standard: 0 grade: normal liver tissue; 1 grade: slight inflammatory cell infiltration and spotty necroses; 2 grade: inflammatory cell infiltration and local necroses; 3 grade: comprehensive inflammatory cell infiltration in portal area and hepatic lobule accompanying comprehensive hepatic necroses.

2.6. The isolation and culture of macrophage

The celiac fluid containing macrophages was collected from mice at day 10 after the injection of NCPP, CP or PBS, and then cultured in dishes for 2 h. The attached macrophages were collected to evaluate the purity of celiac macrophage population by flow cytometry and then cultured the remanent cells in 96 well plates (5×10^5/well) in the presence of lipopolysaccharides (LPS, 10 ug/ml) at 37 °C for 48 h. Finally,

the supernatant was collected and used for the detection of cytokines, NO and H_2O_2.

2.7. Determination of cytokines, NO and H_2O_2

IFN-γ, TNF-α were detected by Enzyme-linked immuno-sorbent assay kits (BD Biosciences, USA) according to the instructions of the Cytokine ELISA Protocol. The level of NO and H_2O_2 was analyzed by a commercially available Kit (Beyotime Institute of Biotechnology, China) and measured the absorbance at 540 nm using a microplate reader (Biorad-Benchmark, USA).

2.8. Assay for phagocytic activity of macrophages

NCPP and CP were injected intraperitoneally into BALB/c mice. After 10 days, 1 ml of chicken red blood cell solution
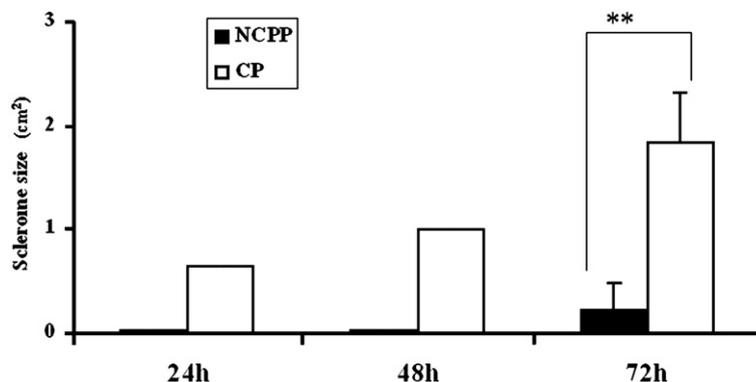

Fig. 2. The local inflammatory reaction in areas of NCPP or CP injection. NCPP or CP (0.5 mg/rabbit) was injected cutaneously into rabbit's back. Local swelling and sclerome were evaluated at 24 h, 48 h and 72 h after treatment. **$p < 0.01$, compared with CP groups ($n = 4$).

Fig. 3. The change of liver histopathology in macaques or mice after NCPP or CP treatment. A. liver histopathology of macaques and BALB/c mice: NCPP or CP was injected intramuscularly into macaques ($n=6$) twice a week for 90 days and histopathology of liver was determined at 24 h after the last injection, compared with PBS control. a: PBS control (0.1 ml/macaque); b: CP treatment (3 mg/macaque in 0.1 ml); c: NCPP treatment (3 mg/macaque in 0.1 ml); 0.1 ml of PBS (d) or 0.5 mg CP(e) or NCPP(f) in 0.1 ml of PBS was injected intraperitoneally into mice ($n=12$) and 10 days later, liver histopathology was evaluated under the microscopy by staining with H-E. B. Pathologic grades were evaluated according to liver histopathologic alteration: a: macaques; b: BALB/c mice.

was given intraperitoneally. Thirty minutes later, the mice were sacrificed. The celiac fluid containing macrophages was collected, centrifuged and washed twice with PBS. After staining with Wright's solution, phagocytosis of macrophages was observed under a light microscope, and the phagocytic rate and phagocytic index were calculated.

2.9. Flow cytometry

1×10^6 cells from bone marrow were washed twice with pyrogen-free PBS and then incubated with PE-conjugated anti-F4/80 antibody or PE-conjugated anti-IgG antibody in the dark at 4 °C for 30 min. After washing twice with PBS, cells were

analyzed by a Cytomics™ FC 500 instrument (Beckman Coulter, USA).

2.10. Statistical analysis

GraphPad Prism 4 Demo Software and Student's t-test were used to analyze the statistical difference between groups. The p value < 0.05 was considered to be a significant difference.

3. Results

3.1. Preparation of NCPP by nanotechnology

To decrease the side effects of CP, we prepared a non-cell *Corynebacterium parvum* Product (NCPP) by disposing *C. parvum* in an extra-high voltage jet crushing machine (Nanonizer, Japan). The sizes of NCPP and CP were observed under a transmission electron microscope. As shown in Fig. 1, CP (A) had an anomalistic stick configuration with 1–3 µm in length, whereas NCPP (B) was composed of small, uniform nano-scale sized particles. In addition, no formaldehyde was applied during the process of NCPP.

3.2. NCPP caused no obvious scleromes in the local injected areas

One of the side effects of CP is that it can cause scleromes at the site of injection. To determine the local reaction, NCPP and CP were cutaneously injected into a rabbit's back, and local reactions were evaluated at 24 h, 48 h and 72 h after injection. Results showed that NCPP administration caused no obvious scleromes at the administered sites, compared with CP (see Fig. 2).

3.3. NCPP possessed lower liver injury than CP

It has been known that CP may induce intrahepatic granulomas in the treated mice [11]. In the present study, we examined the influence of NCPP on liver histopathology and serum transaminase activity in both macaque and murine models. In macaque models, NCPP or CP was injected intramuscularly into the macaque twice a week for 90 days and the histopathology of the liver was determined at 24 h after the last injection. Results showed that CP treatment induced comprehensive fatty degeneration and inflammatory cell infiltration, whereas injection of NCPP did not induce significant pathological damage (see Fig. 3A). The parallel histopathology alteration has also been identified in the BALB/c mice at day 10 after NCPP or CP injection. As shown in Fig. 3A, there were many necrotic hepatocytes containing acidophilic bodies accompanying massive infiltrated inflammatory cells in the CP groups. However, there were only spotted inflammatory cells without necrotic hepatocytes in the NCPP group. Evaluation of the pathologic grade showed that pathologic lesions in the NCPP treatment group were significantly lower than that of the CP group both in BALB/c mice ($p = 0.0157$) and in macaques ($p = 0.0209$) (see Fig. 3B).

In addition, we examined ALT/AST alteration in the serum of BALB/c treated mice. Results showed that ALT/AST serum levels had no obvious increase after injection of NCPP for 24 h, 48 h and 72 h (data not shown).

3.4. NCPP maintained strong anti-tumor and splenic activation effects of CP

To identify the immunoregulatory function of NCPP, we investigated anti-tumor effects of NCPP in the tumor-bearing mice. As shown in Fig. 4A, the tumor growth in B16/NCPP and B16/CP mice went slower than that of the B16/PBS mice from day 11 after NCPP or CP treatment ($p < 0.01$), whereas there was no significant difference between the NCPP and CP groups. The splenic index assay showed that both NCPP and CP treatments had a significant splenic activation effect
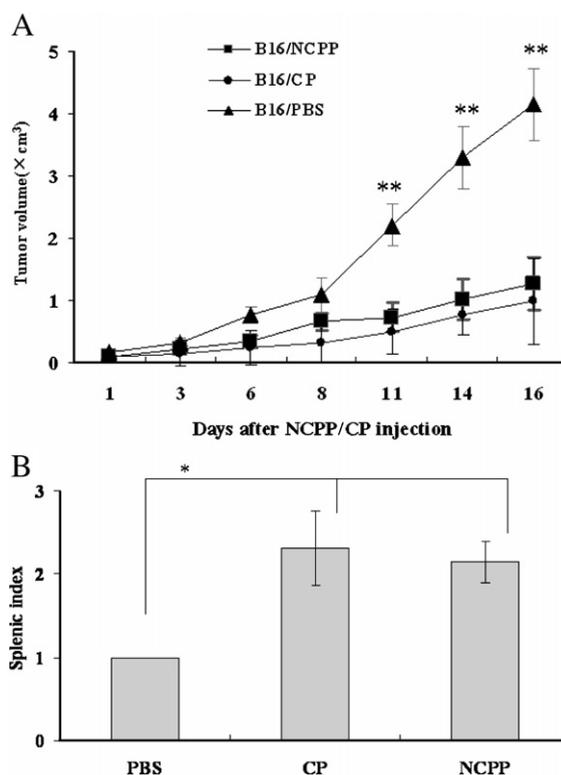

Fig. 4. Anti-tumor and splenic activation effect of NCPP. (A) B16 melanoma cells (1×10^6 /ml) were inoculated subcutaneously in the left flank of each mouse. When the maximal diameter and the minimal diameter of tumors arrived to about 5×5 mm, mice ($n = 12$) were injected intraperitoneally with NCPP or CP (0.5 mg/mouse) or PBS. The tumor volumes were measured three times a week. ** $p < 0.01$, compared with B16/PBS group. (B) Mice ($n = 12$) were sacrificed at day 14 after treatment and splenic index was calculated by the following formula: Splenic index=[splenic weight (mg) of control group/body weight (g) of control group]/[splenic weight (mg) of treated groups/ body weight (g) of treated groups]. * $p < 0.05$, compared with PBS control.
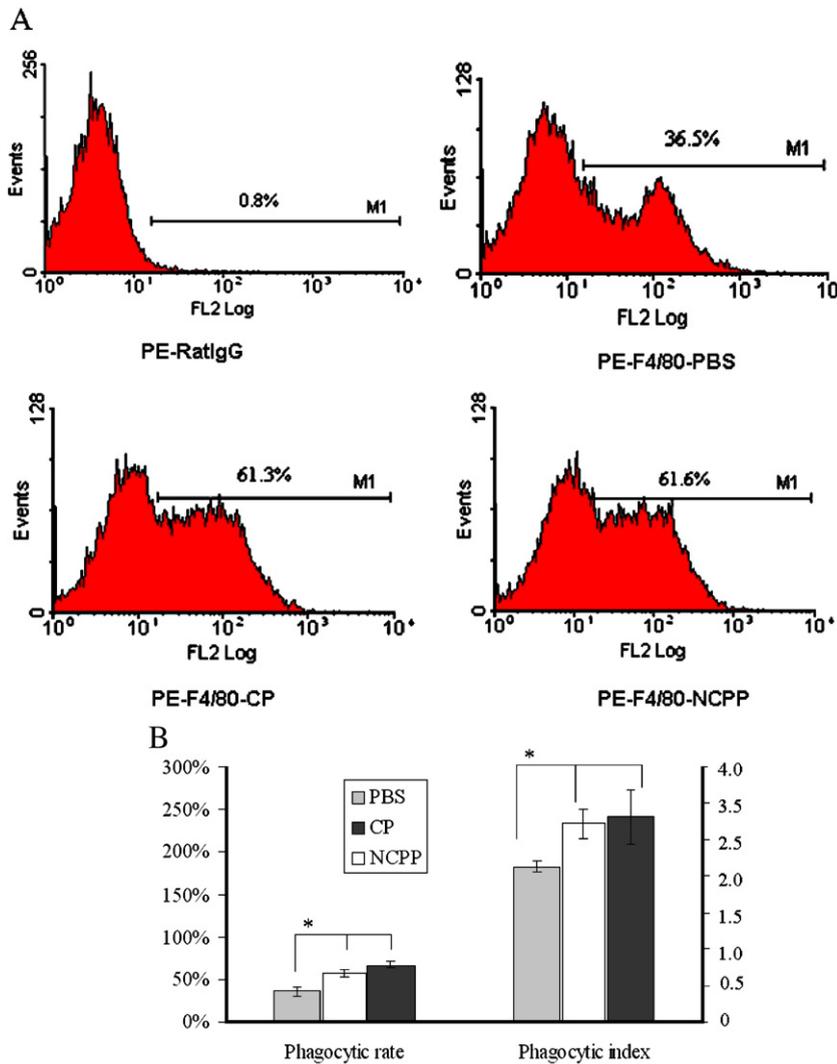
Fig. 5. Macrophage numbers and their phagocytic capacity. The cell suspension was prepared from bone marrow cells of mice treated by NCPP or CP for 10 days and stained by anti-F4/80 antibody and anti-IgG antibody. (A) The number of macrophages was detected by Flow Cytometry. (B) In phagocytic experiments of macrophages, celiac macrophages were prepared at day 10 after NCPP or CP (0.5 mg/mouse) treatment. Phagocytic rate and phagocytic index were evaluated according to the experimental instruction. $*p < 0.05$ ($n = 12$).

(Fig. 4B). This result demonstrated that NCPP maintained strong anti-tumor and splenic activation effects of CP.

3.5. NCPP could augment both macrophage number and their phagocytic capacity

It has been reported that CP can enhance immune functions via the activation of macrophages. To study effect of NCPP on macrophages, we examined macrophage number, phagocytic rate and phagocytic index at day 10 after NCPP or CP treatment. Results showed that both CP and NCPP could significantly increase the number of macrophages in the bone marrow, compared with the control group ($p < 0.05$) (see Fig. 5A). The ratio of F4/80$^+$ macrophages in bone marrow cells increased from $30.05 \pm 9.12\%$ in the control group to $55.85 \pm 7.71\%$ in the

CP group or to $59.45 \pm 3.04\%$ in the NCPP group. At the same time, the purity of celiac macrophage population as measured by flow cytometry was 92.7% F4/80+ with 1.2% CD3+ and 2% DX5+ indicating that we successfully obtained the purified macrophages. Moreover, NCPP as well as CP could enhance the phagocytic rate and phagocytic index of celiac macrophages, compared with the control (Fig. 5B, $p < 0.05$).

3.6. NCPP can enhance H_2O_2 and NO production of macrophages

There is growing evidence that activated macrophages may produce an enhanced level of H_2O_2 and NO. In the following test, we tried to determine the effect of NCPP on H_2O_2 and NO production of macrophages at 10 days after NCPP or CP

treatment. As shown in Fig. 6, both CP and NCPP could stimulate macrophages to produce higher levels of H_2O_2 and NO (CP vs control $P < 0.05$; NCPP vs control, $P < 0.01$). Notably, the level of NO in the NCPP treatment group was higher than in the CP treatment group ($p < 0.05$).

3.7. NCPP was able to stimulate macrophages to produce a higher level of IFN-γ but a lower level of TNF-α, compared with CP

To elucidate the influence of NCPP on the cytokine production of macrophages, macrophages were collected from celiac fluid of mice treated with NCPP or CP for 10 days and cultured in the presence of LPS for another 72 h. The level of IFN-γ and TNF-α in the supernatant was detected by the ELISA method. As shown in Fig. 7, macrophages from the control group mice secreted low levels of IFN-γ and TNF-α, whereas macrophages from NCPP or CP-treated mice could release enhanced IFN-γ and TNF-α. However, macrophages from NCPP-treated mice secreted lower levels of TNF-α and higher levels of IFN-γ compared with those from CP-treated mice ($p < 0.05$), suggesting that NCPP possessed some new characteristics that CP did not.

4. Discussion

It has been shown that CP, a strong immunological stimulant, can inhibit growth of primary and metastatic tumors and especially, the progression of cancer pleural fluid [21–25]. However, the major obstacle for further application of CP is attributed to its severe side effects. Previous works has demonstrated that the biological functions of diverse kinds of agents will be influenced once their physical configurations are transformed [26]. Thus, it is of great interest to determine whether the
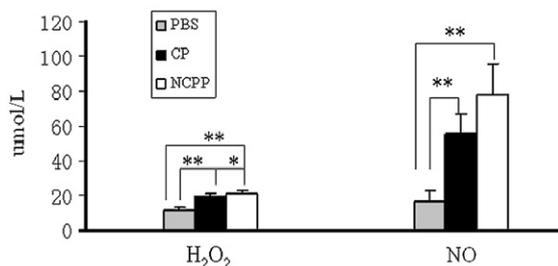

Fig. 6. The effect of NCPP on H2O2 and NO production of macrophages. To determine effect of NCPP on H_2O_2 and NO production of macrophages, the celiac fluid containing macrophages was collected from mice at day 10 after the injection of NCPP or CP or PBS, and then cultured in dishes for 2 hours. The attached macrophages were collected and cultured in 96-well plates (5×10^5 cells/well) in presence of LPS (10 ug/ml) at 37 °C for 48 h. Finally, the supernatant was collected and used for detection of NO and H_2O_2. *$p < 0.05$, **$p < 0.01$ ($n = 12$).
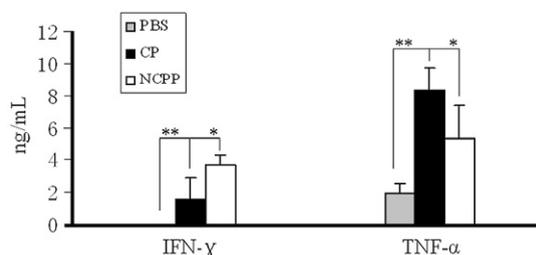

Fig. 7. The effect of NCPP on IFN-γ and TNF-α production of macrophages. Briefly, the celiac fluid was collected from mice at day 10 after the injection of NCPP or CP or PBS, and then cultured in dishes for 2 h. The attached macrophages were collected and cultured in 96-well plates (5×10^5 cells/well) in presence of LPS (10 ug/ml) at 37 °C for 48 h. Finally, the supernatant was collected and used for detection of IFN-γ or TNF-α levels with ELISA assay. *$p < 0.05$, **$p < 0.01$ ($n = 12$).

transformed CP still retains its original ability while reducing its adverse effects. In the present study, utilizing a nanometer technique, we, for the first time, tried to transform CP for the preparation of NCPP. Our results showed that NCPP did bear better general characteristics than CP (Fig. 1), which possessed small, uniform particles and contained low levels of pyrogen, suggesting that we successfully prepared a neotype of an immunomodulator. Surely, this provided the feasibility for the following experiments.

We next investigated whether NCPP possessed the biological functions pertinent to CP. In this work, we found that NCPP and CP could both significantly inhibit growth of B16 melanoma in the tumor-bearing mice compared with B16/PBS group. Moreover, other reports have shown that an increased splenic index was relevant to the anti-tumor effect of CP [27,28]. Our study demonstrated that both NCPP and CP administration were able to have significant splenic activation effects (Fig. 4). These data indicate that NCPP still maintains the immune functions similar to CP. A recent report has revealed that CP is able to modulate the proliferation and differentiation activities of macrophages [7]. In this study, we presented data that both NCPP and CP can not only enhance the numbers of macrophages, but also augment the phagocytic rate and phagocytic capacity of celiac macrophages in the treated mice compared with the PBS control groups (Fig. 5); thereby confirming that NCPP retains the functions of CP in the activation and regulation of macrophage. It has been shown that macrophages may continuously secrete obvious levels of NO once activated [29,30]. We showed that H_2O_2 and NO levels were significantly increased in response to NCPP, indicating that NCPP is another powerful stimulator of H_2O_2 and NO release in macrophages

(Fig. 6). All of the above data in this study clearly confirms that NCPP still preserves the fundamental immune functions possessed by CP.

Now that a novel immunomodulator had been obtained, conceivably, a major concern has arisen about its safety. In this investigation, NCPP injection could not induce obvious swelling and sclerome in the treated areas (Fig. 2), suggesting that NCPP is easy to be absorbed and causes minor local inflammatory reactions. Furthermore, the present assay for histopathology in NCPP-treated macaques and mice has shown that NCPP did not induce obviously pathological injury in the livers (Fig. 3), indicating that the toxicity of NCPP has been reduced compared with CP. In addition, we also identified the similar pathological alterations in the macaque's spleen, lung, kidney and local muscles for injection (data not shown). Our data demonstrates that, in addition to retaining the fundamental biological functions, NCPP has minimal side effects.

It has been well established that macrophages could deteriorate liver fibrosis and primary biliary cirrhosis through secreting many soluble cytokines including TGF-β, TNF-α, and IFN-γ [31,32]. Interestingly, TNF-α and IFN-γ may act with reverse roles in liver fibrosis, although they both can be secreted by activated macrophages. It has long been observed that IFN-γ is mainly produced by NK cells and T cells. But accumulated evidence has shown that IFN-γ can also be secreted by activated macrophages [33,34].

In this work, we presented that both NCPP and CP could significantly enhance IFN-γ and TNF-α secretion of macrophages (Fig. 7). Of note, a significant difference exists regarding the role of NCPP and CP in stimulating IFN-γ and TNF-α secretion, i.e., NCPP could activate macrophages to secrete a higher level of IFN-γ than CP ($p < 0.05$), whereas CP could induce higher TNF-α secretion than NCPP ($p < 0.05$), suggesting that NCPP may not induce fibrosis through the distinct immunoregulatory mechanism in stimulating specific cytokine secretion. Recent observations indicated that IFN-γ was a powerful anti-fibrogenic cytokine that might play central roles in the down-regulation of liver fibrosis, whereas TNF-α might aggravate this disease by influencing the balance of IFN-γ [35,36]. The role of NCPP from this study is, at least in part, in agreement with these conclusions.

In summary, we successfully reconstructed a neotype of immunomodulator, NCPP, through the transformation of CP utilizing nanometer technique. We confirmed that NCPP not only retains the fundamental biological functions of CP, but also possesses lower side effects, implying that NCPP may be a better immunoregulator

than CP. Further studies are required to elucidate the mechanisms of how NCPP affects macrophages and other immunocytes in order to fulfill its widespread clinical applications in the future.

Acknowledgments

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