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ORIGINAL ARTICLE

TLR agonist rHP-NAP as an Adjuvant of Dendritic Cell-Based Vaccine to Enhance Anti-Melanoma Response

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ABSTRACT

Background: Melanoma is a common and malignant cutaneous tumor, which is responsible for a large proportion of skin cancer deaths. Dendritic cell (DC)-based vaccines have achieved positive results in the treatment of melanoma because of their ability to induce cytotoxic response to facilitate tumor elimination. **Objective:** To improve the efficacy of dendritic cell-based vaccines by the adjuvant activity of Helicobacter pylori neutrophil activating protein (HP-NAP), which is a virulence factor of *Helicobacter pylori*, has been proved as a TLR agonist with effective immunomodulatory activity. **Methods:** The recombinant HP-NAP (rHP-NAP) was expressed by using prokaryotic expression system. Dendritic cells (DCs) were cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. After treating with rHP-NAP, the maturation of DCs and dendritic cell-based vaccine were assayed by using flow cytometry and qRT-PCR. The activation and proliferation of T cells were measured by FCM, ELISA and MTT methods. The tumor specific cytotoxic response to resistant B16F10 was detected by using lactate dehydrogenase-release assay and qRT-PCR. **Results:** The recombinant HP-NAP (rHP-NAP), prepared from *E. coli* prokaryotic expression system, was able to significantly promote the maturation of dendritic cell-based vaccine loaded with tumor cell lysate (TCL) of B16F10 (DC-B16F10-TCL). Furthermore, it effectively induced the activation and proliferation of T cells and tumor specific cytotoxic response to resistant B16F10 melanoma tumor cells. **Conclusion:** These results suggested that rHP-NAP possesses the potential for use as an adjuvant of dendritic cell-based vaccine in anti-melanoma treatment.

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INTRODUCTION

Melanoma is one of the most malignant skin cancers for which treatment many methods have been explored with no satisfactory results effect (1). At present, the cancer immunotherapy has attracted much attention and achieved remarkable results in the treatment of melanoma (2,3). Dendritic cell (DC)-based vaccine therapy (dendritic cells loaded with tumor associated antigens) is regarded as a classical cancer immunotherapy approach because it is capable of activating the immune system and enhancing tumor specific cytotoxic response to kill tumor cells (4,5). Dendritic cell-based vaccines have been tested in different clinical phases with certain positive effects obtained in the therapy of melanoma. Dendritic cells (DCs) loaded with melanoma antigens gp100 were able to effectively prolong the overall survival (OS) without obvious treatment-related toxicities (6); autologous tumor lysate and dendritic cells were fused to create dendritoma vaccines which could improve the OS of stage IV melanoma patients in phase I/IIa clinical trial (7); CLBS20 which is a novel dendritic cell-based vaccine consists of autologous DCs loaded with antigens from irradiated autologous tumor cells of patient. This vaccine showed promising results in the early phase trials of advanced melanoma (8). In order to achieve better treatment outcomes, a variety of adjuvant have been used to enhance the efficacy of dendritic cell-based vaccines (9,10). Toll-like receptor (TLR) agonists, able to induce the maturation of dendritic cells, have attracted considerable attention and have been used to improve the efficacy of dendritic cell-based vaccine. Poly (I:C), a classic commercial TLR3 agonist, is a synthetic double-stranded RNA polymer also extensively studied as an immunomodulator for enhancing the immune response (11). Furthermore, poly I:C has been widely employed as an adjuvant of dendritic cell-based vaccine. It enhanced the activity of dendritic cells loaded with oxidized ovarian cancer cells and generated effector cells to kill autologous tumour (12). Dendritic cell exosome (Dexo)-based vaccine treated with Poly (I:C) could induce specific anti-tumor immune response to reduce tumor growth and improve the survival rate in both mouse melanoma models and mouse cervical cancer (13,14). Another classical TLR9 agonist CpG motif-containing oligodeoxynucleotides (CpG ODNs) which was safe and well-tolerated when administered as adjuvant to humans and in some cases increased vaccine-induced immune responses (15). Dendritic cell-based vaccine stimulated with CpG improved the survival of tumor-bearing mice, reduced the development of lung metastases, and generated immunological memory (16). CpG ODNs, as a part of dendritic cell-based vaccine STDENVANT, significantly improved survival and promoted tumor regression in a mouse model of glioma. In addition, combinatorial administration of anti-PD-L1 antibody and STDENVANT conferred a greater survival advantage (17). The role of some new TLR agonists is gradually being explored regarding the improvement in the efficacy of dendritic cell-based vaccines. Angelan, an acidic polysaccharide isolated from Angelica gigasNakai, demonstrated as a natural TLR4 ligand, increased the antitumor activity of dendritic cell-based vaccine in B16F10 syngeneic tumor model (18). Glycyrrhizauralensis water extract (GUWE) promoted dendritic cells maturation via TLR4 signaling pathway. Dendritic cells pulsed with human papilloma virus (HPV)-16E6/E7 peptides treated with GUWE significantly inhibited the cervical tumor growth (19).

Helicobacter pylori neutrophil-activating protein (HP-NAP) is a virulence factor of *Helicobacter pylori* and has been confirmed as a novel TLR agonist (20). HP-NAP has been reported to have effective immunomodulatory activity able to stimulate neutrophils

and monocytes releasing IL-12 and IL-23 in order to induce the innate immune response and regulate the adaptive immune response (21). In recent years, HP-NAP has been proven to possess the potential for enhancing immune response in anti-tumor treatment. In a model of bladder cancer, HP-NAP treatment was able to enhance the accumulation of both $CD4^+$ and $CD8^+$ IFN-γ-secreting cells within tumor and regional lymph nodes, probably because HP-NAP created IL-12-high milieu (22). Notably, HP-NAP, as an immunoactivator, can assist oncolytic viruses to inhibit tumor growth. In the mouse neuroendocrine tumor model, intra-tumoral administration of oncolytic adenovirus armed with secretory HP-NAP improved the median survival and increased the IL-12/23p40 cytokine secretion (23). In mouse pleural effusion xenograft model, intrapleural administration of oncolytic measles virus strains engineered to express secretory HP-NAP forms increased proinflammatory cytokine concentrations and doubled the median survival (24). These results suggest that HP-NAP has the potential to be an effective adjuvant to activate immune response. In the present study, a HP-NAP prokaryotic expression vector was constructed and recombinant HP-NAP (rHP-NAP) was successfully obtained. The rHP-NAP could significantly promote the maturation of dendritic cell-based vaccine DC-B16F10-TCL. This effectively induced the activation and proliferation of T cells as well as tumor specific cytotoxic response against melanoma cells. Our study suggested that rHP-NAP can be used as a novel adjuvant which can enhance the efficacy of dendritic cell-based vaccine in the treatment of melanoma.

MATERIALS AND METHODS

Animals. Female C57BL/6 mice (6–8 weeks old) were purchased from Vital River Laboratories Animal Technology Co., Ltd. (Certificate No. SCXK (Jing) 2012–0001, Beijing, China). Mice were housed in specific pathogen-free conditions (24 ± 1°C, 55 ± 5% relative humidity) and had free access to food and water under a 12 h light/dark cycle. All animals were acclimatized for environment at least one week prior to the experiments. Our study was approved by the Ethics Committee of Zhengzhou University.

Reagents and Antibodies. Recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4) were purchased from PeproTech (USA). FITC-dextran (40 kDa) and mitomycinC (MMC) were obtained from Sigma-Aldrich (USA). LPS was purchased from Invitrogen (USA), and nylon wool fiber was obtained from Polysciences (USA). The following FITC-, APC- or PE- conjugated monoclonal antibodies (Abs) were purchased from eBiosciences (USA): CD80, CD86, CD69, CCR7, and CD11c. PrimeScriptTM RT reagent Kit and SYBR Premix Ex TaqTMII were obtained from Takara (JPN). Cytokine ELISA Kit for IFN-γ was provided by Biolegend (USA). The CytoTox 96® Non-Radioactive Cytotoxicity Assay was obtained from Promega (USA). EtEraser™ Endotoxin Removal Kit was purchased from Xiamen Bioendo Technology Co., Ltd. (China).

Preparation of rHP-NAP. rHP-NAP was expressed in *E.coli* BL21 (pET-24b-napA) by induction of IPTG. The thallus was collected by equilibration buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 nM imidazole, 10 mM Tris base) and crushed by ultrasonic wave. The ultrasonic crushing program was 20 min of work on for 5 s, work off for 10 s. The supernatant passed through 0.45 μm filter to remove large cell fragments. Next, rHP-

NAP was purified by affinity chromatography with ProteinIso® Ni-NTA resin according to the instructions. The column was balanced with equilibration buffer, loaded with supernatant, and washed to remove miscellaneous protein. Finally, rHP-NAP was collected with equilibration buffer containing 500 mM imidazole and dialyzed in PBS. Endotoxin was removed with EtEraser™ Endotoxin Removal Kit and stored at -80°C.

Generation of Mouse Bone Marrow-Derived Dendritic Cells (BMDCs). The experimental procedures involved in the generation of mouse bone marrow-derived dendritic cells were all based on Lutz's previous published study (25). Briefly, cells from bone marrow flushed from femurs and tibiae of mice were cultured in 6-well culture plates at 1×10^6 cells/ml in complete RPMI culture medium supplemented with 10% FBS in the presence of IL-4 (10 ng/ml) and GM-CSF (20 ng/ml). On the third and fifth days, half of the medium containing the corresponding concentration of cytokines was exchanged. The immature BMDCs were obtained on the seventh day and stained to confirm the surface expression of CD11c by flow cytometry. The purity of dendritic cells was more than 90%, hence ready for use in the subsequent experiments.

T Cells Isolation. Nylon wool enrichment of T lymphocytes was based on the previous description (26). Briefly, the mouse spleen was made into single cell suspension under sterile conditions, and red blood cells were further removed. The single cell suspension flowing through sterile brushed nylon wool column and then incubated for 45 min at 37°C, 5% CO2 .This was followed by the elution of the non adherent T cells with warm RPMI 1640.

Flow Cytometry. For DCs maturation assay, (1×10^6 cells/ml) were incubated for 24 h with different concentrations of rHP-NAP (0.01 μM, 0.1 μM, 1 μM) in order to detect the expression of CD86 using anti-mouse CD86 antibody. PBS was used as negative control and LPS (1 μg/ml) as positive control. For DC-B16F10-TCL maturation assay, DCs (1×10^6 cells/ml) were treated with PBS+TCL (20 μg/ml), rHP-NAP (1 μM) + TCL (20 μg/ml), and LPS (1 μg/ml) + TCL (20 μg/ml) for 24 h to detect the expression of CD80 and CCR7 using anti-mouse CD80 and CCR7 antibody. For FITC-dextran uptake assay, DCs (1×10^6 cells/ml) were treated with PBS+TCL (20 μg/ml), rHP-NAP (1 μM) + TCL (20 μg/ml), and LPS (1 μg/ml) + TCL (20 μg/ml) for 24 h; the antigen phagocytosis was then detected using 1mg/ml FITC-dextran at 37°C for 30 min.

For T cells activation assay, DCs (1×10^6 cells/ml) were incubated for 24 h with PBS+TCL (20 μg/ml), rHP-NAP (1 μM) + TCL (20 μg/ml), and LPS (1 μg/ml)+ TCL (20 μg/ml) at 37°C; they were then co-cultured with T cells at a ratio of 1:10 (DC:T) in a U-bottom 96-well plate for 24 h to detect the expression of CD69 using anti-mouse CD69 antibody. Next, they were analyzed on a BD flow cytometer, and further analysis of the data was performed by use of FlowJo software.

T Cells Proliferation. DCs (1×10^6 cells/ml) were incubated with PBS+TCL (20 μg/ml), rHP-NAP (1 μM) + TCL (20 μg/ml), and LPS (1 μg/ml) + TCL (20 μg/ml) for 24 h at 37°C and then pre-treated with mitomycin C for 30 min at 37°C; they were ultimately co-cultured with T cells at a ratio of 1:10 (DC:T) in a U-bottom 96-well plate to detect the proliferation of T cells by MTT assay after 48h.

qRT-PCR. For DCs cytokine expression assay, DCs (1×10^6 cells/ml) were incubated for 2 h with different concentrations of rHP-NAP (0.01 μM, 0.1 μM, 1 μM) so as to detect the relative expression of IL-12p35 and IL-1β.

For DC-B16F10-TCL cytokine expression assay, DCs (1×10^6 cells/ml) were treated with PBS+TCL (20 μg/ml), rHP-NAP (1 μM) + TCL (20 μg/ml), and LPS (1 μg/ml)+

TCL (20 μg/ml) for 2 h in order to detect the relative expression of IL-12p35, IL-23p19, and IL-1β. For Granzyme B and Perforin expression assay of T cells, DCs (1×10^6 cells/ml) were treated with PBS+TCL (20 μg/ml), rHP-NAP (1 μM) + TCL (20 μg/ml), and LPS (1 μg/ml)+ TCL (20 μg/ml) for 24 h; subsequently, they were cultured with T cells at a ratio of 1:10 in 96-well culture plates for 24 h to detect the relative expression of granzyme B and perforine.

The total RNA was extracted using TRIzol reagent (Life Technologies, Thermo Fisher Scientific, Inc.). And 1 μg RNA was reverse transcribed into cDNA using the PrimerScript1st Strand cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan). The maintained thermocycling conditions were as follows: 37˚C for 15 min and 85˚C for 5 sec. Quantitative real-time PCR (qRT-PCR) was performed to examine the gene expressional changes. The primer sequences were utilized as follows: IL-12p35, Forward: AGAGGTGGACTGGACTCCCGA, Reverse: TTTGGTGCTTCACACTTCAG; IL-23p19, Forward: CTCTCGGAATCTCTGCATGC, Reverse: ACCATCTTCACACTGGATACG; IL-1β, Forward: GCCTGAACCCTGCATACCGT, Reverse: GCCAATAGCCCTCCCTGTCT; granzyme B, Forward: CTCTCGACCCTACATGG, Reverse: GCCCCCAAAGTGACATTTATT; perforine, Forward: GCACAAGTTCGTGCCAGG, Reverse: CGTCTCTCATTAGGGAGTTTTT; β-actin, Forward: GTGGCATCCATGAAACTACAT, Reverse: GGCATAGAGGTCTTTACGG. cDNA was amplified through the flowing PCR condition: 95°C for 10 min, 40 cycles of 95°C for 5 s, and60°C for 30 s. Relative mRNA expression levels were calculated for each gene following normalization with β-actin based on the $2^{-\Delta\Delta Cq}$ method.

***Ex vivo* Cytotoxic Assay.** Mice were intraperitoneally injected with 200 μg of B16F10 tumor cell lysate on days 0 and 5. On the 10th day, mice were sacrificed and T cells were enriched by nylon wool (27,28). DCs (1×10^6 cells/ml) were incubated for 24 h with PBS+TCL (20 μg/ml), rHP-NAP (1 μM) + TCL (20 μg/ml), and LPS (1 μg/ml) + TCL (20 μg/ml) at 37°C; after that, they were cultured with antigen-specific T cells at a ratio of 1:10 in 96-well culture plates for 72 h and then added with melanoma tumor cells (B16F10) for 6 h. The dissociation of B16F10 tumor cells was detected based on the release of lactic dehydrogenase (LDH). LDH activity was measured through the use of LDH assay kit (BioLegend, Inc., San Diego, CA, USA). The ratio of cell toxicity (% specific lysis) was calculated as follows: [(experimental release - effector spontaneous release - target spontaneous release) ⁄ (target maximum release - target spontaneous release)] × 100.

Measurement of IFN-γ by ELISA. DCs (1×10^6 cells/ml) were incubated for 24 h with PBS+TCL (20 μg/ml), rHP-NAP (1 μM) + TCL (20 μg/ml), and LPS (1 μg/ml) + TCL (20 μg/ml) at 37 °C and then co-cultured with T cells at a ratio of 1:10 (DC:T) in a U-bottom 96-well plate for 72 h (29,30). The supernatants were collected and evaluated using IFN-γ ELISA Kit (cat no. 570209), (BioLegend, Inc., San Diego, CA, USA).

Statistical Analysis. All the results were based on the average of three parallel experiments. Statistical tests with GraphPad Prism 5 were carried out and all values were expressed as mean ± SEM. Statistical significance of the differences between the two groups was determined on the basis of Student's t-test. One-way analysis of variance (ANOVA) was used for multiple comparisons followed by Bonferroni comparison post-hoc test. A value of $P<0.05$ was considered as statistically significant.

RESULTS

Purification of rHP-NAP.

We constructed a recombinant prokaryotic expression vector inserted with HP-NAP gene and further purified to obtain the rHP-NAP using prokaryotic expression system (Figure S1 and S2). As shown in Figure 1A, the molecular weight of rHP-NAP was 17 kDa. The rHP-NAP three-dimensional structure was predicted using Phyre 2 software. The results showed that rHP-NAP had four-helix bundled subunits (17 kDa) with a hollow central part as shown in Figure 1B.

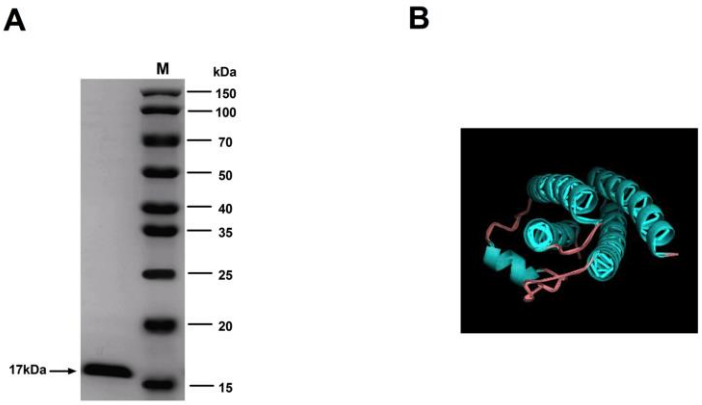

Figure 1. Purification of rHP-NAP. (A) rHP-NAP was purified and analyzed by SDS-PAGE. Lane1: rHP-NAP; Lane 2: protein marker; (B) The three-dimensional structure of the rHP-NAP predicted byPhyre 2 software.

rHP-NAP induce the maturation of BMDCs.

In order to evaluate the effect of rHP-NAP on the maturation of BMDCs, they were treated with different concentrations of rHP-NAP. As shown in Figure 2, following treatment with rHP-NAP, the expression of costimulatory molecular CD86 significantly increased in BMDCs. LPS was used as a positive control which also significantly upregulated costimulatory molecular CD86 expression. Furthermore, rHP-NAP enhanced the mRNA expression of IL-12p35 and IL-1β in a dose-dependent manner.

rHP-NAPpromotethe maturation of DC-B16F10-TCL.

Dendritic cells were loaded with tumor cell lysate of B16F10 and then stimulated with rHP-NAP. As observed in Figure 3A, B, D, and E, the expression of costimulatory molecular CD80 and chemokine receptor CCR7 of DC-B16F10-TCL were significantly upregulated after treatment with rHP-NAP. Also, LPS stimulating DC-B16F10-TCL dramatically increased the expression of CD80 and CCR7. Consistently, the antigen phagocytosis of DC-B16F10-TCL was attenuated by rHP-NAP and LPS (Figure 3C and F). Moreover, DC-B16F10-TCL treated with rHP-NAP significantly increased the expression of IL-12, IL-23 and IL-1β (Figure 3G, H and I).

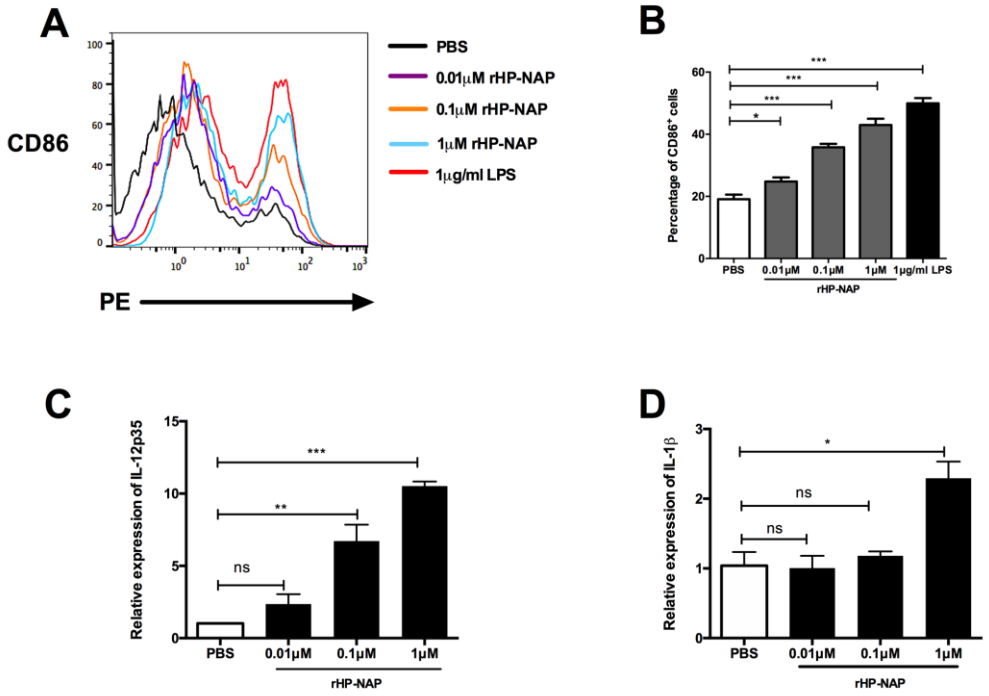

Figure 2. rHP-NAP promote the maturation of BMDCs. (A) The expression of CD86 on BMDCs after stimulated with rHP-NAP for 24 h. (B) Statistical summary of the expression of CD86. (C, D). The expression of cytokines in mRNA level after stimulated with rHP-NAP for 2 h. * P<0.05, ** P<0.01, ***P<0.001, and ns indicated no statistical significance, rHP-NAP or LPS treatment vs. the PBS control.

DC-B16F10-TCL treated with rHP-NAP induces the activation and proliferation of T cells.

The maturation of dendritic cell-based vaccine could efficiently trigger naïve T cells activation and proliferation. As shown in Figure 4A, B and C, the expression of early activation marker CD69 and the secretion of cytokine IFN-γ of T cells were significantly enhanced after being co-cultured with DC-B16F10-TCL treated by rHP-NAP and LPS. Moreover, DC-B16F10-TCL treated with rHP-NAP was more efficient in promoting the proliferation of T cells compared with the PBS (Figure 4D).

DC-B16F10-TCL treated with rHP-NAP induces cytotoxic response.

Cytotoxic T cells kill tumor cells through releasing granzyme B and perforine. The effect of DC-B16F10-TCL on inducing tumor specific cytotoxic response of T cells was estimated. As shown in Figure 5A, DC-B16F10-TCL treated with rHP-NAP and LPS increased the killing efficiency of T cells intargetingB16F10 tumor cells by three times compared with the control group. And in Fig.5B and C, the results showed that DC-B16F10-TCL treated with rHP-NAP enhanced the ability of cytotoxic T cells to secrete granzyme B and perforine.

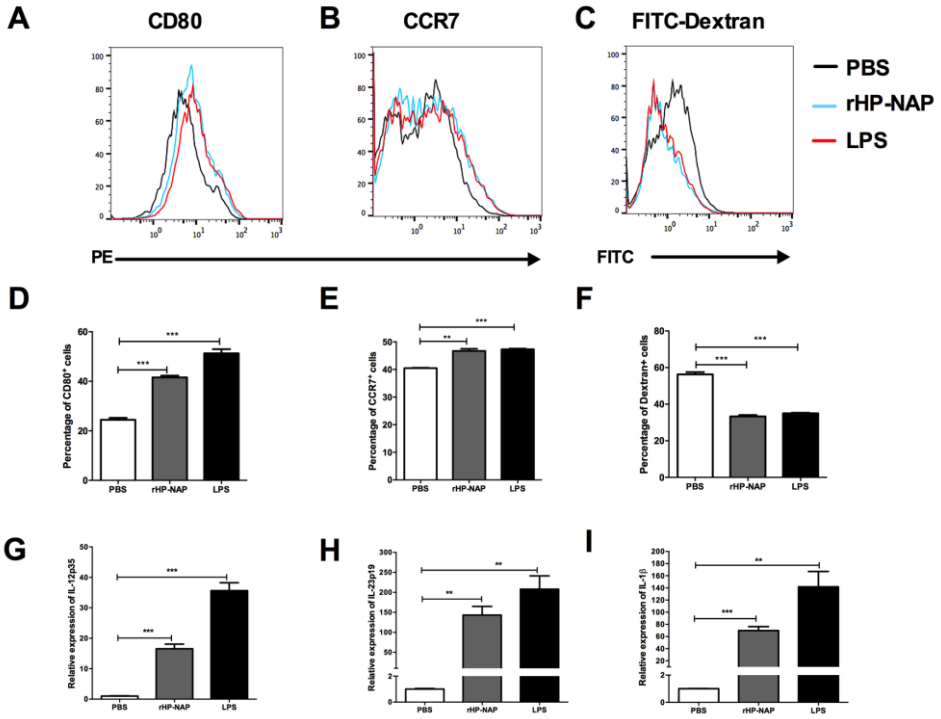

Figure 3. rHP-NAP promote the maturation of DC-B16F10-TCL. (A-C) The expression of CD80 and CCR7 as well as the antigen phagocytosis of DC-B16F10-TCL after stimulated with rHP-NAP for 24 h. (D-F) Statistical summary of the expression of CD80 and CCR7 as well as the antigen phagocytosis. (G-I). The relative expression of IL-12p35, IL-23p19 and IL-1β of DC-B16F10-TCL after stimulated with rHP-NAP for 2 h. **P<0.01, ***P<0.001, rHP-NAP or LPS treatment vs. the PBS control.

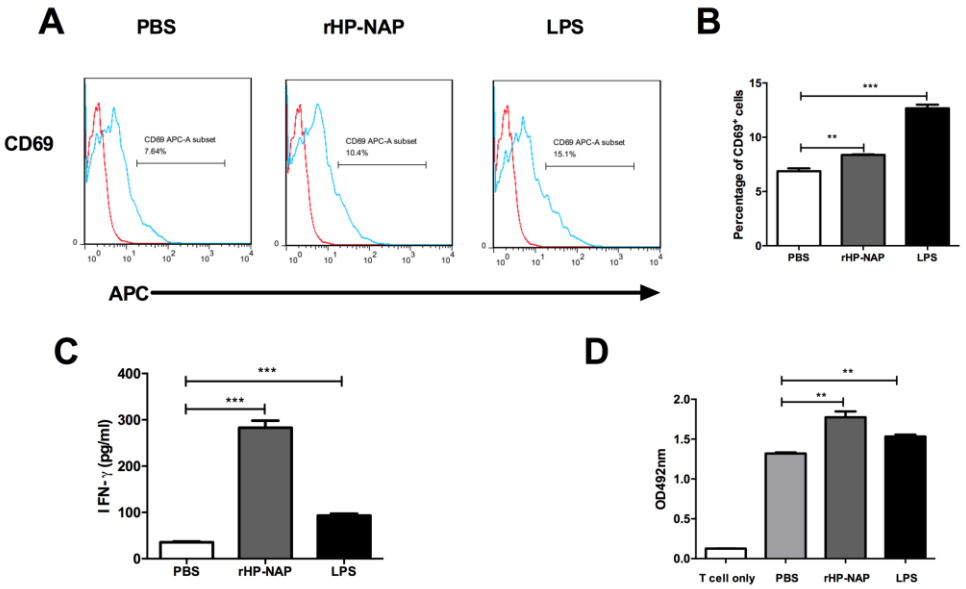

Figure 4. DC-B16F10-TCL treated with rHP-NAP induces the activation and proliferation of T cells. (A) The expression of CD69 on the surface of T cells after co-cultured with DC-B16F10-TCL for 24 h. (B) Statistical summary of the expression of CD69. (C) The cytokine IFN-γ secretion of T cells was detected after co-cultured with DC-B16F10-TCL for 72 h. (D) T cells proliferation was detected after co-cultured with DC-B16F10-TCL for 48 h. **P<0.01, ***P<0.001, rHP-NAP or LPS treatment vs. the PBS control.

DISCUSSION

TLR agonists have the potential to be an adjuvant of dendritic cell-based vaccine because it can effectively promote the maturation of dendritic cells. The maturation of dendritic cell-based vaccines enhances the expression of co-stimulatory molecules (CD80, CD86) and attenuates antigen endocytosis to promote antigen presentation and improve antigen processing ability. Furthermore, dendritic cell-based vaccines upregulate the chemokine receptors CCR7 which can enhance DCs migration in the draining lymph nodes and secretion of multiple cytokines in order to activate an anti-tumor immune response (31,10).

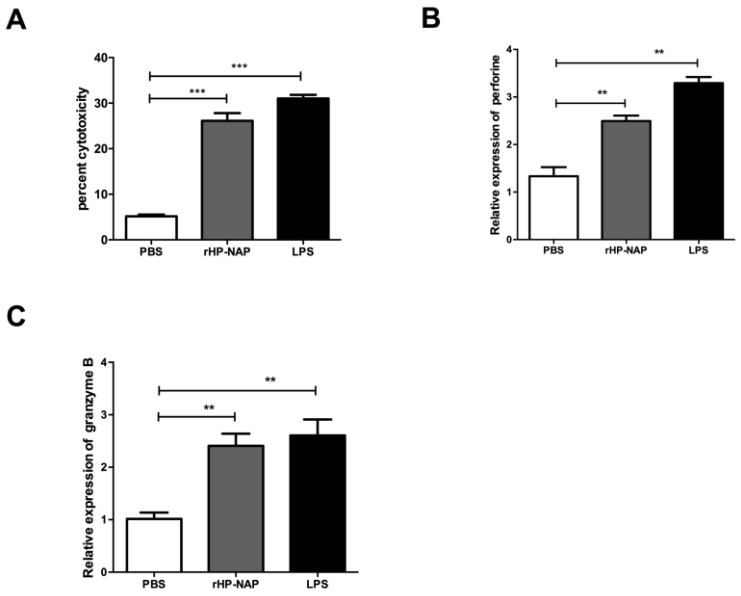

Figure 5. DC-B16F10-TCL treated with rHP-NAP inducecytotoxic response. (A) The cytotoxic responses of T cells to B16F10 was detected after co-cultured with DC-B16F10-TCL for 72 h. (B,C) The synthesis of granzyme B and perforine of T cells were detected after co-cultured with DC-B16F10-TCL for 24 h. **P<0.01, ***P<0.001, rHP-NAP or LPS treatment vs. the PBS control.

It has been reported that TLR9 agonist CpG treating antigen-pulsed DCs is able to increase the expression of CD86, MHC-II and promote the secretion of cytokines IL-12 and IFN-γ (16). Moreover, dendritic cell-based vaccine treated with the combination of TLR4 endogenous ligand HSP70407-426 and TLR4 exogenous ligand OK-432was able to can significantly increase CD80, CD86, CD40, and MHC molecules expression and enhance the production of Th1-type cytokines (32). Recent research has reported that Ampligen®-R848-IFN-containing cocktail is able to upregulate the expression of costimulatory molecules (CD80, CD86, CD83) and chemokine receptor CCR7 and markedly stimulate the IL-12p70 secretion of dendritic cell-based vaccine (33). The exosomes were obtained from BMDCs (Dexo) treated with poly (I:C) was positive for costimulatory molecules CD40 and CD80 as well as MHC II which was necessary for dendritic cell-based vaccine (14). In this research, the rHP-NAP was obtained by

prokaryotic expression system. Further assessed was the effect of TLR agonist rHP-NAP on the maturation of DC-B16F10-TCL. Our results showed that DC-B16F10-TCL treated with rHP-NAP was able to significantly upregulate the expression of costimulatory molecule CD80 and chemokine receptor CCR7, attenuate the phagocytosis ability, and enhance the secretion of proinflammation cytokines such as IL-12, IL-23, and IL-1β. TLR agonists promoted the migration of dendritic cells loaded with tumor antigens to lymphoid organs where they could stimulate the activation and proliferation of naïve T cells. Then, the activated T cells secreted IFN-γ, granzyme B and perforine to kill the tumor cells (34,35). In a recent study, DCs loaded with breast tumor cell lysates stimulated with CpG significantly promoted the proliferation of T cells [16].In addition,HSP70407-426 and OK-432-treating dendritic cell-based vaccine could induce significantly high response levels of lymphocytes proliferation and $CD8^+$ cytotoxic T-lymphocyte (CTL) against mouse hepatocellular carcinoma (HCC) (32). The Ampligen®-R848-IFN-containing cocktail improved the effect of dendritic cell-based vaccine on eliciting a cytotoxic T-lymphocyte response to autologous breast cancer cells *in vitro* (33). Peptide-pulsed DCs in combination with poly-ICLC was safe and induced a measurable tumor specific T cell population in patients with advanced pancreatic cancer (36). In the current research, it was observed that DC-B16F10-TCL treated with rHP-NAP was able to significantly promote the activation and proliferation of T cells. In addition, the cytotoxicity of T cells towards B16F10 was significantly enhanced with the increase in the expression of granzyme B and perforine. In conclusion, using the prokaryotic expression system, we successfully obtained rHP-NAP which can effectively induce the maturation of DC-B16F10-TCL, able to enhance the activation, proliferation and cytotoxic response of B16F10-specific T cells. Our results showed that rHP-NAP has the potential for use as an adjuvant of anti-melanoma dendritic cell-based vaccine.

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