

Toll-like receptor agonist rMBP-NAP enhances antitumor cytokines production and CTL activity of peripheral blood mononuclear cells from patients with lung cancer

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Abstract. Toll-like receptor (TLR) agonists are known for their ability to inhibit tumor progression via enhancing antitumor cytokines production and cytotoxic T lymphocyte (CTL) activity. Recombinant *Helicobacter pylori* neutrophil-activating protein fused with maltose-binding protein (rMBP-NAP) has been reported as a novel TLR agonist for antitumor treatment in murine models. The present study aimed to determine the potential and efficacy of the rMBP-NAP for antitumor treatment prior to further clinical trials. The rMBP-NAP was expressed and purified for subsequent experiments. Peripheral blood mononuclear cells (PBMCs) from health donors and patients with lung cancer (LC) were incubated with PBS and 0.2 mg/ml rMBP-NAP. Antitumor cytokines production was assayed using ELISA and reverse transcription-quantitative polymerase chain reaction analysis. The cytolytic activity of PBMCs and the number of Interferon- γ (IFN- γ)-secreting cells were assayed using lactate dehydrogenase and Enzyme-linked ImmunoSpot assays, respectively. The results from the present study revealed that the expression of IFN- γ , interleukin (IL)-2, tumor necrosis factor- α and IL-12 of PBMCs from patients with LC and healthy donors were significantly increased following treatment with rMBP-NAP ($P < 0.05$). Additionally, rMBP-NAP

significantly upregulated the number of IFN- γ -secreting cells in PBMCs and prominently increased the cytotoxic activity of PBMCs ($P < 0.05$). Furthermore, the expression of TLR2 was significantly enhanced following rMBP-NAP stimulation ($P < 0.05$), which indicated that rMBP-NAP may serve an antitumor role via TLR2 signaling pathways. Overall, these results demonstrated that rMBP-NAP possesses the potential to be a novel immunomodulatory candidate drug and requires further evaluation in clinical trials.

Introduction

Toll-like receptor (TLR) alone or combined with other anti-tumor drugs have been used in cancer therapy due to the capability of TLR agonists to enhance the immune response of patients with cancer by improving the function of innate and adaptive immune cells (1,2). Currently, the US Food and Drug Administration (FDA) have licensed three TLRs agonists, including Bacille Calmette-Guérin (BCG), Monophosphoryl lipid A and Imiquimod, for use as prophylactics and/or therapeutic agents in cancer treatment (3-5). Although abundant natural and synthetic TLR agonists have been discovered and developed, a number of clinical research studies have reported that various TLR agonists that exhibited promising results in *in vivo* studies were not efficacious in humans (6,7). The ineffectiveness of TLR agonists in clinical studies may be ascribed to two primary reasons: i) The deviation of immune cell phenotypes and the differential expression of the TLR gene between human and animals; and ii) the immune system in patients with cancer is compromised (8,9). Therefore, comprehensive preclinical experiments are required to determine whether a novel TLR agonist may be an candidate antitumor drug.

Recombinant *Helicobacter pylori* neutrophil-activating protein fused with maltose-binding protein (rMBP-NAP), consisting *Helicobacter pylori* neutrophil activating protein fused with the *Escherichia coli* maltose binding protein, has been reported as a novel TLR agonist (10-12). In our previous research, local administration of rMBP-NAP inhibited tumor growth by activating TLR2 to trigger a significant Th1 type immune response in two murine models of hepatoma H22

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and sarcoma S180 (13). Furthermore, following rMBP-NAP treatment in a B16-F10-induced metastatic lung cancer model, the survival rates were significantly increased and the number of metastatic lung nodules were markedly reduced. During the treatment of the pulmonary metastasis, the release of antitumor cytokines, including interleukin (IL)-12, interferon- γ (IFN- γ), IL-27, chemokine (C-C motif) ligand (CCL)2, CCL20, vascular cell adhesion molecule (VCAM)-1 and intercellular cell adhesion molecule (ICAM)-1, were also significantly increased (14). These previous studies indicate that rMBP-NAP may be a potential novel antitumor immunomodulatory drug for cancer treatment.

The present study further assessed the stimulation of the immune response by rMBP-NAP treatment on PBMCs from patients with lung cancer and healthy donors. The results suggested that rMBP-NAP promoted antitumor cytokine production, including the production of INF- γ , IL-2, TNF- α and IL-12, and enhanced the cluster of differentiation (CD)4⁺ cytotoxic T lymphocyte (CTL) and CD8⁺ CTL cytotoxic effect. The present study further laid the groundwork for research into the efficacy of rMBP-NAP for clinical trials.

Materials and methods

Cell preparations. PBMCs from 12 health donors and 12 patients with lung cancer (LC) who had not previously received immunotherapy at the First Affiliated Hospital of Zhengzhou University (Zhengzhou, Henan, China) between April 2012 and October 2012 were isolated using Ficoll-Paque gradient centrifugation. Cells were centrifuged at 1,000 x g for 25 min at 24°C and cultured in Iscove's modified Dulbecco's medium supplemented with 10% inactivated fetal calf serum (both from Thermo Fisher Scientific, Inc., Waltham, MA, USA). Written informed consent was obtained from all participants and the present study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China).

Expression and purification of rMBP-NAP. The experimental procedures involved in the present study are all based on our previous published study (10). The rMBP-NAP was expressed by *E. coli* TB1 (*pMAL-c2x-napA*) at 37°C, isopropyl β -D-1-thiogalactopyranoside was added and induced for 3 h. The expression of rMBP-NAP was identified on 10% SDS-PAGE and was purified by amylose affinity chromatography. Endotoxin was removed with agarose bed columns containing immobilized polymyxin B (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). PBMCs from patients with LC and healthy donors were seed at a density of 2×10^6 cells/well and treated with rMBP-NAP (0.2 mg/ml) or PBS (as a control). The total RNA from the cultured PBMCs was extracted using TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using the PrimerScript 1st Strand cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan). The thermocycling conditions maintained were as follows: 37°C for 15 min; 85°C for 5 sec. RT-qPCR of the relative gene expression of TLR2 and IL-12p35 was

performed using a SYBR Premix Ex TaqTMII (Takara Bio, Inc.) on an ABI 7500 Fast instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). The genes were amplified with the following primers: TLR2 forward, 5'-CCTGGCCCTCTCTACAAACTT-3' and reverse, 5'-ACTGTGTATTTCGTGTGCTGGATA-3'; IL-12p35 forward, 5'-ATGGCCCTGTGCTTAGTAGT-3' and reverse, 5'-CGGTTCTTCAAGGGAAGATTTT-3'; and GAPDH forward, 5'-TGATGACATCAAGAGGTGG-3' and reverse, 5'-TTACTCCTTGGAGGCATGT-3'. The thermocycling profile was as follows: 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Relative mRNA expression levels were calculated for each gene following normalization with GAPDH using the $2^{-\Delta\Delta C_q}$ method (15).

ELISA. PBMCs from patients with LC and healthy donors were seeded at 2×10^6 cells/and treated with rMBP-NAP (0.2 mg/ml) or PBS (as a control) for 5 days. The cell culture supernatants were collected, and were used to detect the concentration of IFN- γ (cat no. 570209), IL-2 (cat no. 431804) and TNF- α (cat no. 570109) secreted using ELISA kits (all from BioLegend, Inc., San Diego, CA, USA). The absorbance was measured at 450 nm using a SpectraMax absorbance reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Enzyme-linked ImmunoSpot (ELISPOT). PBMCs from patients with LC and healthy donors were seed at 2×10^6 cells in each well and treated with rMBP-NAP (0.1 or 0.2 mg/ml) or PBS (as a control). The number of IFN- γ -secreting PBMCs were enumerated using Human IFN- γ ELISPOT kit (cat no. DKW22-1000-096, Dakewe Biotech Co., Ltd., Beijing, China). Spots were counted using a Bioreader 5000 (Bio-Sys, Karben, Germany).

Assay for cytotoxic activity. Cytotoxic activity of the CTLs was examined based on the measurement of lactate dehydrogenase (LDH) release using a LDH cytotoxicity assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. PBMCs (5×10^6 /ml) isolated from patients with LC and healthy donors were stimulated with rMBP-NAP (0.2 mg/ml) for 10 days, and used as the effector cells. EC-9706 and K562 were originally obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Iscove's modified Dulbecco's medium supplemented with 10% inactivated fetal calf serum (both from Thermo Fisher Scientific, Inc.) with 5% CO₂ at 37°C, which were used as target cells (16,17). EC-9706/K562 (5×10^3 cells/well) were co-cultured with various densities (6.25×10^4 , 1.25×10^5 and 2.5×10^5) of effector cells (PBMCs) at 37°C for 4 h. The following effector: Target cell ratios were used: 12.5:1, 25:1 and 50:1. The measurement of LDH activity was analyzed by using the LDH assay kit. 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)] x100.

Statistical analysis. All data are expressed as the mean \pm standard deviation. Differences between two groups were determined using an unpaired Student's t-test. One-way analysis of variance was used

for multiple comparisons followed by a Bonferroni Comparison post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed by using software SPSS 10.0 (SPSS, Inc., Chicago, IL, USA).

Results

Patient enrollment. PBMCs samples from 12 lung patients were collected in the present study (Table I). Of these patients, 75% (9/12) were male and 25% (3/12) were female, and the median age was 61 years (range, 40-86 years). In addition, 92% of patients (11/12) were diagnosed with advanced stage disease (stage III-IV). Positive metastasis was observed in eight cases (66.7%) and the middle stage of histological differentiation was detected in 10 cases (83.3%).

Antitumor cytokine secretion of PBMCs is increased following rMBP-NAP stimulation. The secretion of antitumor cytokines from PBMCs in patients with LC and healthy donors, which were stimulated by rMBP-NAP, were measured using ELISA (Fig. 1). IFN- γ , IL-2 and TNF- α expression were significantly increased following rMBP-NAP treatment compared with the PBS control group.

Number of IFN- γ -producing PBMCs is increased following rMBP-NAP stimulation. IFN- γ -producing cells serve an important role in antitumor immunotherapy (18). The number of IFN- γ -producing PBMCs in patients with LC and healthy donors, was measured using an ELISPOT assay (Fig. 2), and was demonstrated to be significantly increased following rMBP-NAP treatment compared with the PBS control group.

Cytolytic activity of PBMCs are increased following rMBP-NAP stimulation. The aforementioned results have demonstrated that antitumor cytokine secretion and the number of IFN- γ -producing cells increased in PBMCs following rMBP-NAP stimulation. Additionally, the cytolytic activity was evaluated using a LDH release assay. EC-9706 and K562 cells were used as target cells, and the effector/target ratio were 12.5:1, 25:1 and 50:1. As shown in Fig. 3, PBMCs from patients with LC and healthy donors stimulated with rMBP-NAP, exhibited noticeable cytolytic activity at the effector/target ratio of 25:1 and 50:1. Following treatment with rMBP-NAP, the killing rates of PBMCs from health donors at 50:1 targeting EC-9706 and K562 cells were 24.9 and 29.6%, respectively. The killing rates of PBMCs from patients with LC at 50:1 targeting EC-9706 and K562 cells were 26.3 and 31.9%, respectively.

rMBP-NAP stimulates IL-12 mRNA expression through the TLR2 signaling pathway in PBMCs. The PBMCs from health donors and patients with LC were stimulated with rMBP-NAP, and the mRNA expression level of TLR2 and IL-12 were examined by using RT-qPCR (Fig. 4). Following rMBP-NAP treatment, the levels of TLR2 and IL-12 mRNA were remarkably increased in PBMCs from health donors as well as patients with LC, compared with the PBS control groups. These results suggest that the upregulation of IL-12 mRNA expression by rMBP-NAP treatment was possibly mediated by the TLR2 signaling pathway in PBMCs.

Table I. Clinicopathological characteristics of patients with LC.

Characteristics	No. of patients with LC
Sex	
Female	3
Male	9
Age, years	
≥60	6
<60	6
TNM stage	
I	0
II	1
III-IV	11
Metastasis	
Positive	4
Negative	8
Histological differentiation	
Low	2
Middle	10
High	0
Total	12

LC, lung cancer; TNM, tumor node metastasis.

Discussion

A number of cytokines have important immunomodulatory effects, and are used in tumor immunotherapy, including INF- γ , IL-2 and TNF- α (19,20). The US FDA has approved the use of INF- γ and IL-2 as single agents for the clinical treatment of malignancies. Clinical trials of INF- γ and IL-2 therapies in solid malignancies, such as bladder carcinoma and ovarian cancer, have met with varying degrees of success (21,22). Several other cytokines, such as TNF- α , have been demonstrated to exhibit antitumor efficacy against cancer, for example sarcoma and breast cancer (23,24). ntTLR agonists have attracted increasing attention for its ability to promote the production of antitumor cytokines (e.g. IFN- γ , IL-2 and TNF- α) in PBMCs (25). For example, CpG-oligonucleotides (CpG-ODN), which has been reported as an effective TLR9 agonist for antitumor treatment, may induce proliferative responses in PBMCs from patients with LC, accompanied by elevated cytokine secretion, including that of IFN- α , IL-12 and TNF- α (26). BCG also induces Th1 cytokine production (IFN- γ , IL-12 and TNF- α) of human PBMCs through the TLR4 signaling pathway in bladder carcinoma (27). Following treatment with rMBP-NAP, the secretion of INF- γ from PBMCs, which serve important roles in antitumor treatment, was higher compared with that demonstrated by CpG-ODN in a previous study (26). The ability of rMBP-NAP, which induced antitumor cytokines production from the PBMCs, was lower compared with that of BCG. In our previous research, rMBP-NAP was observed to inhibit tumor growth by activating the TLR2 signaling pathway in murine models (14). Activated TLR2 induces

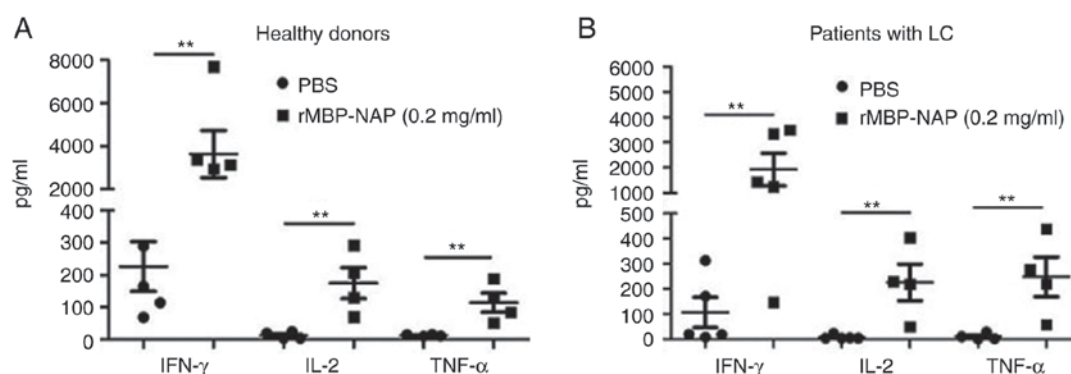


Figure 1. Expression levels of IFN- γ , IL-2 and TNF- α in PBMCs are upregulated following rMBP-NAP stimulation. PBMCs were stimulated with rMBP-NAP (0.2 mg/ml) for 5 days, and culture supernatants were collected for analysis of IFN- γ , IL-2 and TNF- α production in (A) healthy donors and (B) patients with LC. ** $P < 0.01$, rMBP-NAP treatment vs. the PBS control. PBMCs were provided by five patients with LC and four healthy donors. rMBP-NAP, Recombinant *Helicobacter pylori* neutrophil-activating protein fused with maltose-binding protein; PBMCs, peripheral blood mononuclear cells; LC, lung cancer; IFN- γ , Interferon- γ ; IL-2, interleukin-2; TNF- α , tumor necrosis factor- α .

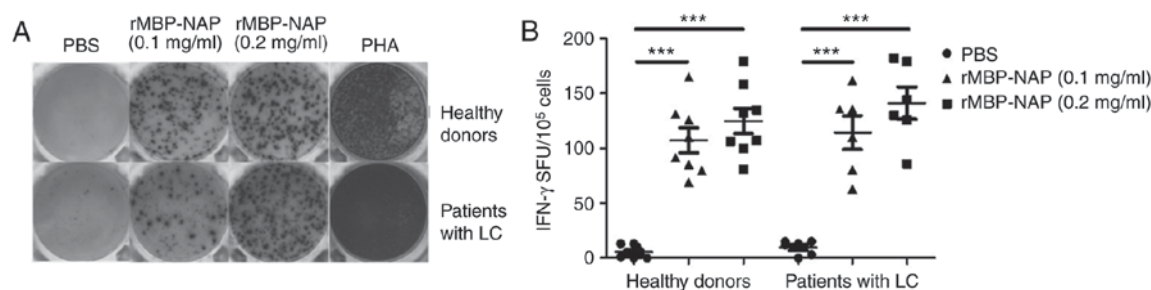


Figure 2. Number of IFN- γ -producing cells increases in PBMCs treated with rMBP-NAP. PBMCs from patients with LC and healthy donors were stimulated with 0.1 or 0.2 mg/ml rMBP-NAP for 5 days. (A) An ELISPOT assay was performed in 96-well microtiter plates pre-coated with antibodies against IFN- γ . (B) Spots were counted using an ELISPOT plate reader. *** $P < 0.001$, rMBP-NAP treatment vs. the PBS control. PBMCs were provided by six patients with LC and six healthy donors. ELISPOT, Enzyme-linked ImmunoSpot; rMBP-NAP, Recombinant *Helicobacter pylori* neutrophil-activating protein fused with maltose-binding protein; PBMCs, peripheral blood mononuclear cells; LC, lung cancer; PHA, phytohaemagglutinin; SFU, spot forming units.

the MyD88-dependent signaling pathway, which drives the activation of important pro-inflammatory transcription factors and subsequently promotes the production of Th1 cytokines (28). In the present study, the expression of TLR2 significantly increased following stimulation with rMBP-NAP, which indicated that the secretion of antitumor cytokines from PBMCs possibly depend on the TLR2 signaling pathway.

Cytotoxic lymphocytes, which serve an essential role in cancer immunotherapy, have been identified as CD4⁺ CTL and CD8⁺ CTL (29). The CD4⁺ CTLs produce multiple anti-tumor cytokines, including IFN- γ , and facilitate CD8⁺ T cell activation and differentiation (30). CD8⁺ CTLs can directly kill tumor cells by releasing cytotoxic molecules, such as perforin and granzymes, and also possess the capacity to secreting IFN- γ ; thus, modulating the antitumor immune response (31,32). Research has demonstrated the TLR agonists enhanced the ratio of IFN- γ -secreting cells, and the cytotoxicity of CD4⁺ and CD8⁺ CTLs in human PBMCs (33). It has been reported that the percentage of IFN- γ -secreting CD8⁺ CTLs in PBMCs from patients with LC, was increased 6-folds following CpG-ODN stimulation, which was compared with PBS (26). In the present study, the number of IFN- γ -secreting CTLs from PBMCs stimulated with rMBP-NAP increased 10 fold in comparison with the PBS control. Furthermore, the

cytolytic activity of PBMCs following rMBP-NAP treatment was evaluated. The cytolytic activity of PBMCs from patients with LC at 50:1 targeting EC-9706 and K562 cells was 3 fold higher compared with the PBS control, which is accordance with the 3 fold increase in the killing rate of PBMCs following BCG stimulation demonstrated in a previous study (34). These results suggest that rMBP-NAP effectively increased the number and the cytolytic ability of CD4⁺ and CD8⁺ CTLs similar to that as the classical TLR agonists BCG and CpG-ODN.

In a conclusion, the results of the present study demonstrated that rMBP-NAP induced the production of multiple antitumor cytokines and enhanced CTL responses in PBMCs from patients with LC. Further investigations on the antitumor effect of rMBP-NAP are required to strengthen the evidence for clinical trial research.

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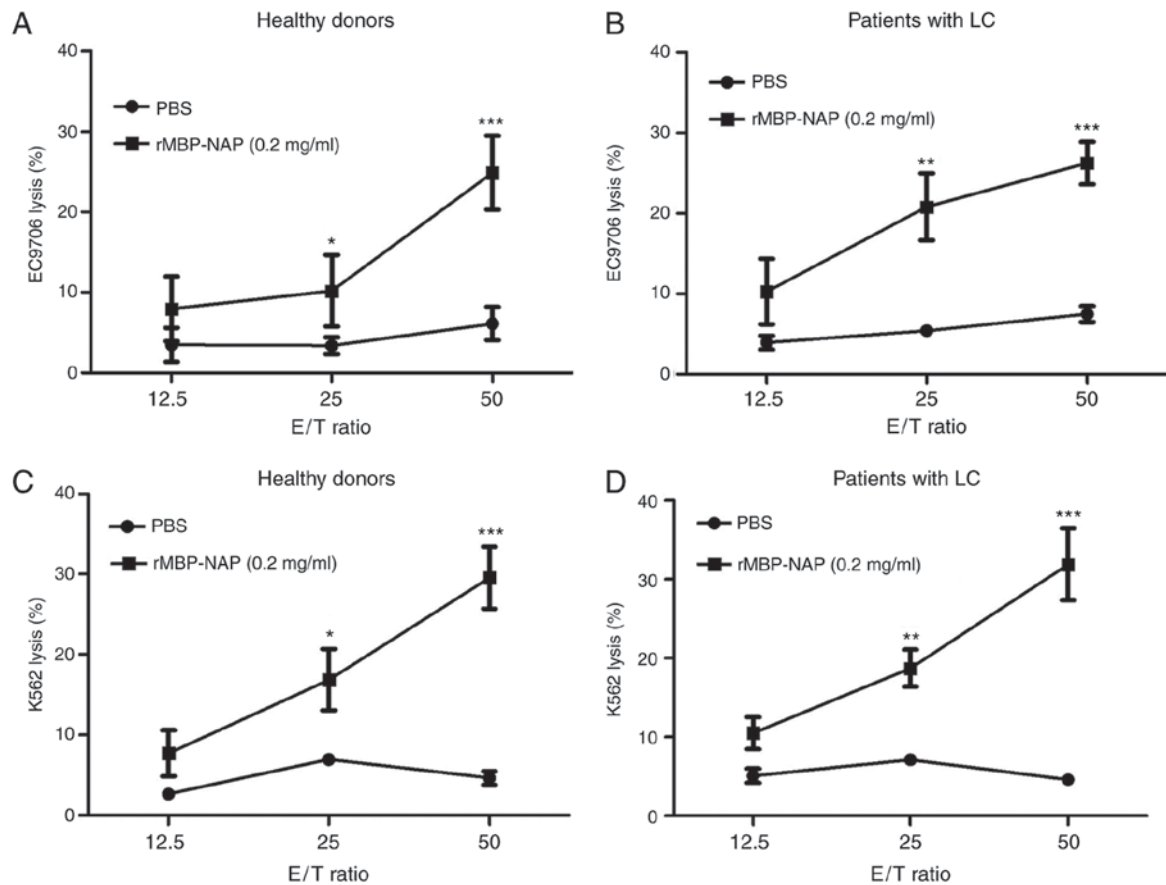


Figure 3. rMBP-NAP enhances the cytolytic activity of PBMCs. A lactate dehydrogenase release assay were performed to evaluate the cytolytic activity of PBMCs from health donor and patients with LC. PBMCs were stimulated with rMBP-NAP (0.2 mg/ml) for 10 days as effector cells. (A and B) EC-9706 and (C and D) K562 cells were used as target cells. The E/T ratios were 12.5:1, 25:1 and 50:1. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, rMBP-NAP treatment vs. the PBS control. PBMCs were provided by five patients with LC and five healthy donors. rMBP-NAP, Recombinant *Helicobacter pylori* neutrophil-activating protein fused with maltose-binding protein; PBMCs, peripheral blood mononuclear cells; LC, lung cancer; E/T, effector/target.

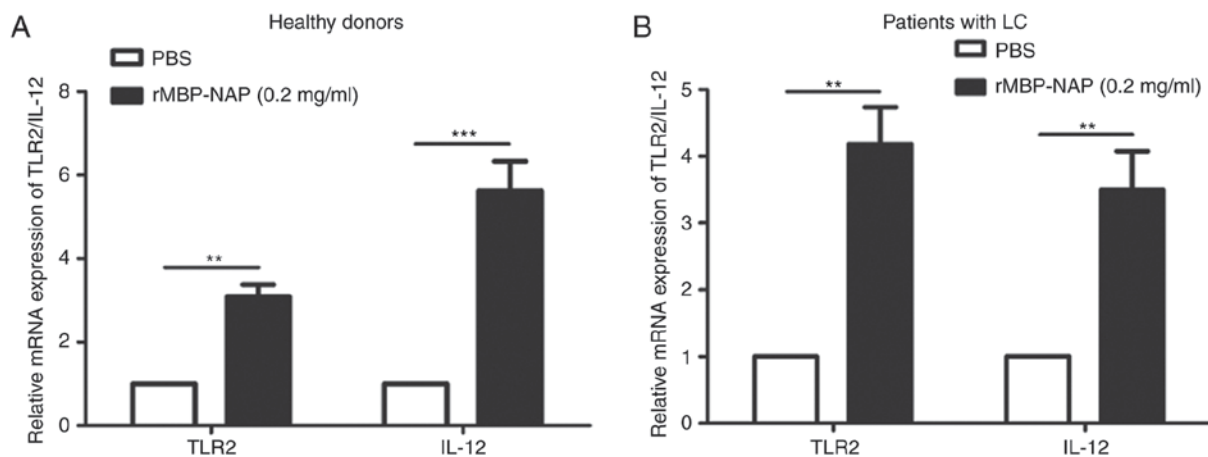


Figure 4. mRNA expression levels of TLR2 and IL-12 increase in PBMCs following rMBP-NAP stimulation. The mRNA relative expression of TLR2 for 16 h and IL-12 for 2 h stimulated with rMBP-NAP (0.2 mg/ml) in PBMCs from (A) healthy donors and (B) patients with LC was detected by reverse transcription-quantitative polymerase chain reaction. Data are expressed as the mean \pm standard deviation. ** $P < 0.01$ and *** $P < 0.001$, rMBP-NAP treatment vs. the PBS control. PBMCs were provided by four patients with LC and four healthy donors. TLR2, Toll-like receptor 2; rMBP-NAP, Recombinant *Helicobacter pylori* neutrophil-activating protein fused with maltose-binding protein; PBMCs, peripheral blood mononuclear cells; LC, lung cancer; IL-12, interleukin-12.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CD, LL, CZ, YZ, ZJ, TL and XG performed experiments, analyzed data, and reviewed the manuscript. XL and QK undertook project design and manuscript revisions.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of the First Affiliated Hospital of Zhengzhou University and study participants provided written informed consent.

Patient consent for publication

Written informed consent was obtained from each individual participant for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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