**Recombinant protein rMBP-NAP restricts tumor progression
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Recombinant protein rMBP-NAP restricts tumor progression by triggering antitumor immunity in mouse metastatic lung cancer

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Abstract

Recombinant *Helicobacter pylori* neutrophil-activating protein fused with Maltose-binding protein (rMBP-NAP), a potential TLR2 ligand, was reported to possess immunomodulatory effects on in-situ tumor in our previous study. In present work, we attempt to elucidate the effect of rMBP-NAP at the local immune modulation in B16-F10-induced metastatic lung cancer. Our results demonstrated that growth of B16-F10 melanoma metastases in the lung was significantly arrested after rMBP-NAP treatment, along with marked reduction in metastatic lung nodules and significant increase in survival. The treatment induced both local and systemic immune responses, which associated with higher influx of CD4⁺/CD8⁺ T cells and drove toward Th1-like and cytotoxic immune environment. Moreover, rMBP-NAP also showed significant anti-angiogenic activity by reducing vascularization in lung tumor sections. In conclusion, rMBP-NAP could induce antitumor immunity through activating Th1 cells and producing pro-inflammatory cytokines, which are responsible for the effective cytotoxic immune response against cancer progression. Our findings indicate that rMBP-NAP might be a novel antitumor therapeutic strategy.

Keywords: rMBP-NAP; Metastatic lung cancer; Toll-like receptor; Antitumor immunotherapy; Immunomodulator

Introduction

Metastatic lung cancer is one of the principal causes of death. It is a low immunogenic cancer, resistant to the surveillance of the immune system (Pinto et al. 2011; Igney and Krammer 2002). Despite advances in treatment, the prognosis remains poor 5 years survival benefit. Although, in the past 20 years, the Food and Drug Administration has approved the systemic administration of interferon (IFN)- α for patients in stage II or III with high risk of metastatic disease, the stimulating interest is still focus on development of new therapeutic strategies (de Melo et al. 2015). The terminology of “cancer immunoediting” has been used to describe the process of shaping the immunogenicity of tumors, which relies upon the activation of the adaptive immune system to recognize and eliminate “transformed” cells (Prendergast 2008; Forte et al. 2012). However, the tumor microenvironment is characterized by a process of chronic inflammation, which can facilitate either tumor establishment or progression (Colotta et al. 2009). Therefore, the activation of the immune system could represent a means to induce tumor regression (Igney and Krammer 2002; Prendergast 2008).

Over a century ago, Dr. William Coley injected microorganisms that we now know include a number of Toll-like receptor (TLR) agonists into tumors and observed systemic antitumor responses (Mellman et al. 2011; Takeda et al. 2003; Liew et al. 2005). Following activation by ligands, TLRs can enhance the uptake of pathogens by phagocytic cells and also mediate leukocyte recruitment to pathologic focus through secreting multiple cytokines and chemokines (Mogensen 2009; Hajishengallis and Lambris 2011). On the other hand, TLRs also express on adaptive immune cells and modulate the correlation between subsets of T cells and innate immune cells (Caron et al. 2005; Zhang et al. 2011). Based on the broad immune

activation effect, TLR agonists become attractive candidates for adjuvants of cancer immunotherapy.

Increasing evidence suggests that TLRs are important regulators of tumor biology, however, little is known about their function in metastatic lung cancer. In order to understand the connection between TLRs and metastatic lung cancer progression, several TLR agonists are currently being tested as immunomodulator for anticancer therapies (Pinto et al. 2011; Forte et al. 2012). The TLR2 ligand, *Helicobacter pylori* neutrophil-activating protein (Hp-NAP), has been shown to possess a potential role as biological response modifier. Hp-NAP is a key factor driving Th1 inflammation in *H. pylori* infection. Furthermore, recombinant Hp-NAP itself could serve as a potential drug candidate in immunotherapy of cancer (Codolo et al. 2012; Iankov et al. 2012; Ramachandran et al. 2013). Our previous study demonstrated that the recombinant Hp-NAP fused with the maltose-binding protein of *Escherichia coli* (rMBP-NAP), possesses a significant immunomodulating effects on in-situ tumor, rather than direct killing of tumor cells (Wang et al. 2015).

The present study is focused on the effect of rMBP-NAP that acts as an immunomodulator in the experimental mouse model of B16F10 melanoma lung metastasis. We show that rMBP-NAP can induce both local and systemic immune responses, which associated with higher influx of CD3⁺CD4⁺T cells, CD3⁺CD8⁺T cells and drove toward a Th1-like and cytotoxic immune environment to tumor, with higher secreted interferon (IFN)- γ and interleukin (IL)-27 cytokines in lung tissue and spleen. Moreover, rMBP-NAP also showed significant anti-angiogenic activity by reducing vascularization in tumor section of lung in B16F10-bearing mouse. In conclusion, our study proves rMBP-NAP therapeutic efficacy via

the activation and maintenance of T cells immunity, the production of Th1-like cytokines and induction of anti-angiogenesis effect, which in turn facilitates efficient anticancer activity in a mouse model of metastatic lung cancer.

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Material and methods

Animals and cell lines

Animals were handled by specialized personnel in Henan Academy of Medical and Pharmaceutical Science (Certificate No. SCXK (Yu) 2011–0010, Henan, China). All procedures are specified in the project approved by the University committee for animal experiments. C57BL/6J mice, aged 6–8 weeks (initial weight 18 ± 2 g), were purchased from Vital River Laboratories Animal Technology Co., Ltd. (Certificate No. SCXK (Jing) 2012–0001, Beijing, China). The B16-F10 mouse melanoma cell line was maintained in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C in humidified 5% CO₂.

Mice treatment

Mice were injected (i.v.) with 2×10^5 B16-F10 cells (day 1), and after 24 h rMBP-NAP (0.5, 2.5, or 5 mg/kg; 6 mice each group) was administered by the i.p. route for 7 times once every two days. Mice were administrated saline or cisplatin (2 mg/kg; Sigma-Aldrich, Rome, Italy) as control group (n=6) and positive control group (n=6). Mice were euthanized 2 weeks after melanoma cells injection, the lungs were excised, and the pulmonary metastatic tumors were counted in a blind manner under a dissecting microscope.

For survival analysis, animals (n=5) were treated with 10 consecutive doses of the protein prior to tumor inoculation and were injected intravenously with 2×10^5 B16-F10 cells after rMBP-NAP treatment. We registered the day of death for each mouse and these data were used to Kaplan-Meier survival analysis.

Preparation of fusion protein rMBP-NAP

The involved experiment procedures were all based on our previous published works (Kang et al. 2005). Briefly, neutrophil-activating protein gene of *H. pylori* (Hp-napA) was subcloned from the recombinant plasmid pNEB-napA (restored in our laboratory), digested with *EcoR* I and *Sal* I (Takara, Japan) and inserted into the pMAL-c2x (New England Biolabs, Inc, USA) vector which prepared with the same enzymes to make pMAL-c2x-napA. The sequence confirmed plasmids were transformed into the *E. coli* strain TB1 (Invitrogen). The transformed cells were grown in LB medium containing 50 µg/ml kanamycin in 100 ml batches at 37 °C. IPTG was added to the medium to a final concentration of 0.3 mM when the OD₆₀₀ of the culture reached 0.5. The expression of the fusion proteins was allowed for 3 h at 37 °C. The cells were harvested and frozen at -20 °C overnight, sonicated in ice-water bath, centrifuged at 9000 rpm for 30 min at 4 °C. Soluble rMBP-NAP in the supernatant was purified by amylose affinity chromatography. Endotoxin was removed with agarose bed columns containing immobilized polymyxin B (Thermo scientific, USA).

Polymerase chain reaction (PCR)

Total RNA was extracted with Trizol® (Invitrogen, USA) according to the manufacturer's instructions. Homogenize tissue samples in Trizol (1 ml per 50 mg of tissue) in appropriate homogenizer. Add 0.2 ml of chloroform per ml of Trizol used. Centrifuge the resulting mixture at 12,000 rpm for 15 minutes at 2-8 °C. Transfer the aqueous phase to a fresh tube and add 0.5 ml of 2-propanol. Centrifuge and remove the supernatant, then wash the RNA pellet by adding a minimum of 1 ml of 75% ethanol and centrifuge at 12000 rpm for 5 minutes at 2-8 °C. Briefly dry the RNA pellet by air-drying. Total RNA was dissolved in RNase-free water and prepared for qRT-PCR analysis.

Quantitative real time PCR was performed using the SYBR Premix Ex Taq™ II (Takara, Japan). Data was acquired using a LightCycler 1.5 System (ligheCycler, German). Cycle threshold values were normalized to amplification of β -actin. Relative quantification of gene expression levels was calculated using the $2^{-\Delta\Delta C_t}$ methods. A list of the qRT-PCR primers used in the present study was given in suppl. Table S1.

Isolation of splenocytes, lymphocytes and PBMC

Spleens from individual mice were removed and disrupted by scratching in a sterile manner, Cells suspended in PBS were centrifuged at 1000 rpm for 5 minutes and red blood cell osmotic lysis was carried out after incubation with 10 ml of QB Lysing™ Solution Buffer (Quantobio, China). Centrifuging and removing the supernatant, the cell pellet was resuspended in 2 ml of RPMI-1640 medium and the viable splenocytes were counted by Trypan blue exclusion.

Lungs were removed, minced and incubated at 37°C for 45 min in PBS containing 1 mg/ml collagenase D and 0.2 mg/ml DNase I (Sigma-Aldrich, USA). After enzyme treatment, lung tissue was gently passed through a cell strainer (70 μ m, BD falcon, USA) and then washed twice in PBS. Cells were resuspended in 35% Percoll (GE Healthcare, USA) in PBS, layered over 70% Percoll, and spun at 2,500 rpm for 20 min. Lymphocytes collected from the interface were used for subsequent flow cytometry analysis.

Peripheral blood was collected by orbital sinus bleeding in a tube containing citrate sodium buffer. Anti-coagulated blood was layered onto Histopaque-1083 (Sigma-Aldrich, USA) and peripheral blood mononuclear cells (PBMC) were purified by gradient centrifugation according to the manufacturer's recommendations.

IFN- γ ELISPOT assay

IFN- γ -secreting splenocytes were enumerated using enzyme-linked immunospot (ELISPOT) (Lycke and Coico 1996). Briefly, the splenocytes (1×10^7 cells/ml) obtained from rMBP-NAP and PBS administrated tumor-bearing mice, were added to Immunospot plates (Dakewe Biotech, China), which precoated with an anti-IFN- γ monoclonal antibody (mAb). Then, rMBP-NAP or PBS was added respectively. The positive control well was added PHA (Sigma-Aldrich, USA). The plates were incubated overnight at 37°C under humidified air with 5% CO₂. The wells were washed with deionized water and wash buffer and then incubated with biotin-conjugated anti-IFN- γ mAb at 37°C for 1h. After washes, streptavidin-alkaline phosphatase was added and the plates were incubated for 1h at room temperature. The plates were again washed and added activator solution (Dakewe Biotech, China) to develop spots, then washed with distilled water to stop the reaction. Air dry completely at room temperature. Experiments were performed in triplicate. The number of spots in each well was counted using the BioReader 4000 Pro-X.

Cytotoxicity assay

The B16-F10 cells (0.5×10^4) in DMEM were used as target cells and incubated with PBMC, obtained from rMBP-NAP and PBS treated mice, for 4 h at 37 °C in a humidified atmosphere containing 5% CO₂. The cytotoxicity was determined by LDH release assay using a commercially available kit (Promega, USA). The percentage of lysis was calculated by the formula: % Specific lysis = (Experimental lysis – Spontaneous lysis / Maximum lysis – Spontaneous lysis) $\times$ 100%.

Flow cytometry

Freshly isolated splenocytes (1×10^6 cells/ml) were incubated for 30 min at 4 °C with FITC conjugated anti-CD3, PE conjugated anti-CD4 or PerCP conjugated anti-CD8 (eBioscience, USA). Antibody labeling was performed via a standard procedure. Flow cytometry analysis was carried out by using BD FACSCalibur cell analyzer (BD Biosciences, USA) and the data was analyzed with the FlowJo software.

Immunohistochemistry staining

Immunohistochemical studies were performed on 5-mm-thick sections prepared from formalin-fixed and paraffin-embedded tissue using standard staining protocols. Deparaffinization and antigen retrieval were carried out as a standard procedure. Microvessel density (MVD) represented by CD34 staining was measured according to the methods (Weidner et al. 1991). The resultant sections were first examined at low magnifications (4×) to identify the vascular-rich area in the tumor. Within this area, the CD34-positive microvessels were counted in a single high-power (20×) field. Any CD34 stained single or cluster of cells was considered a single countable microvessel.

Statistics

Results are expressed as means $\pm$ SEM. Changes observed in treated groups compared with controls were analyzed using GraphPad Prism 5.01 followed by unpaired two-tailed Student's *t* test, and/or two-way ANOVA where appropriate. The *p* values < 0.05 was considered to be statistically significant.

Results

rMBP-NAP decreases lung metastatic tumor growth and prolongs survival of mice

The immunotherapeutic effect of the association of rMBP-NAP was evaluated in the metastatic B16F10 melanoma model. The challenge with viable B16F10 tumor cells occurred on day 1, C57Bl/6 mice were treated with seven intraperitoneal inoculations of rMBP-NAP at one day intervals after the tumor bearing. 24 hours after the last treatment, mice were killed for lung nodules quantification and/or immunological assays (Fig. 1A).

Administration of rMBP-NAP promoted a marked reduction in the number of lung nodules in B16F10 injected mice (Fig. 1B). Lung metastasis frequencies significantly were reduced by 43.9%, 71.9%, and 56.9% in rMBP-NAP treated mice at dosage of 0.5mg/kg, 2.5mg/kg, 5mg/kg, respectively, and the number of lung foci in cisplatin-treated group was reduced to 41.6%, compared with that of PBS-treated mice (Fig. 1C). According to the results above the dose of 2.5 mg/kg of rMBP-NAP was chosen in the following assays. The weight of lung tissues was markedly reduced in response to rMBP-NAP vs PBS control, while no significant change in body weight (Fig. 1D and 1E). For survival analysis, mice inoculated with rMBP-NAP showed a prolonged survival in comparison to animals treated with PBS (Fig. 1F). This observation indicates that rMBP-NAP treatment reduces development of metastatic nodules in the lungs.

rMBP-NAP induces anti-tumor immune response against B16-F10 tumor cells in the spleen.

As we known, a Th2 cytokine profile is associated with down-regulation of the immune response and progression of melanoma. The polarization of Th1 cells is thought to contribute

to cellular immune response and increase CD8⁺ cytotoxic T lymphocytes. The cytokine profiles in spleen were measured by ELISPOT and qRT-PCR assay. IFN- γ -secreting cells were obviously increased after treated with rMBP-NAP (Fig. 2A). As shown in Fig. 2B, the expression of Th1 representative cytokines, IL-12 and IL-27, were effectively elevated, the typical Th2 cytokine IL-4 expression was dramatically reduced in the rMBP-NAP treated mice, which indicated a Th1-dominated milieu. To understand whether the immunomodulatory role of rMBP-NAP can elicit systemic T cytotoxic (Tc) 1 immune response, the cytotoxicity of splenic cells and PBMC towards B16-F10 cells was assessed after rMBP-NAP treatment. The results showed when an effector/target ratio (E/T ratio) of 10:1 was used, the cytotoxic efficacy of PBMC cells and splenic lymphocytes from rMBP-NAP treated mice were remarkably augmented compared with control mice (Fig. 2C).

rMBP-NAP can exhibit the immune regulatory effect in the tumor microenvironment

It is becoming clear that tumors can actively subvert the immune system through a variety of immune suppressive mechanisms within the tumor microenvironment. To verify the cytokine profiles induced by rMBP-NAP in the tumor microenvironment, we performed a quantification of cytokines in mice lung homogenates. To this end, lung tissues from rMBP-NAP treated or untreated mice were excised. As shown in Fig. 3A and 3B, the expression of Th1-like cytokine profiles such as IFN- γ and IL27 were significantly increased. In other hand, the mRNA expression of proinflammatory cytokines (IL-6, TGF- β) and chemokines (CCL2, CCL20) were remarkably decreased (shown in Fig. 3C). To verify the role of T lymphocytes in the protective antitumor effect induced by rMBP-NAP, we examined the types of infiltrating cells within the lung tissue and found that CD3⁺CD8⁺T cells were

significantly augmented after treatment (Fig.3D). In addition, the adhesion molecules ICAM-1, and VCAM-1 which is important regulator to the recruitment of effector T lymphocytes, were significantly enhanced compared with control (Fig.3E). These data indicated that rMBP-NAP has potential activity to regulate the tumor immunosuppressive microenvironment and enhance the adaptive cell immunity.

rMBP-NAP decreases VEGF levels in the lungs of tumor-bearing mice

To explore the involvement of suppression of angiogenesis in the antitumor activity induced by rMBP-NAP, we evaluated the inhibitory effect of rMBP-NAP on tumor-induced neovascularization by immunohistochemical staining of lung tissue section with anti-CD34 antibody. As shown in Fig. 4A, the microvessel which stained by anti-CD34 is much less in rMBP-NAP treated mice compared with control. Furthermore, qRT-PCR analysis also showed the production of antiangiogenic cytokines, VEGF-A and VEGF-D, were decreased in the tumor sections with rMBP-NAP treatment (Fig. 4B and 4C).

Discussion

Immunotherapy is emerging as a powerful and active tumor-specific approach against cancer via triggering the immune system. TLR agonists used as single agents can effectively eradicate tumors due to their potent stimulation of innate and adaptive immunity.

TLRs are fundamental elements of the immune system, which facilitate our understanding of the innate and adaptive immune pathways. TLR signaling could lead to tumor cell death, resulting in tumor regression or arrest via inducing Th1-like and cytotoxic immunity (Wang et al. 2006). Given that cancerous cells evade the immune system, the activation of TLRs could represent an efficient approach against cancer. Therefore, our currently study assessed the beneficial antitumor and anti-metastasis activity of rMBP-NAP on experimental pulmonary metastasis in mice.

The treatment with rMBP-NAP rendered protection against metastatic B16F10 melanoma by reducing the number of lung nodules and increasing the survival of treated mice. Nevala et al. demonstrated Th2 cytokine dominant systemic environment in patients with metastatic melanoma (Nevala et al. 2009). In current study, we showed that rMBP-NAP could stimulate IFN- γ production in the spleen, promote the mRNA expression of IL12 and IL27 and reduce the expression of IL4, which profoundly stimulated systemic antitumor Th1 response by inducing Th1/Th2 balance drift toward Th1. Moreover, we demonstrated that the cytotoxic efficacy of PBMC cells and splenic lymphocytes from rMBP-NAP treated mice were augmented, compared with PBS control mice. Our findings strongly prompted that the tumoricidal activity induced by rMBP-NAP was due to its capacity to elicit systemic Th1 and T cytotoxic (Tc) 1 immune response.

As we learn more about the processes that contribute to antitumor immune response, there is increased interest in altering the microenvironment directly in a manner that enhances cell-mediated immunity. Most recent investigation showed Th1 cells have an important role in cellular immune responses by secreting IFN- γ and capable of enhancing activity of CD8⁺ CTLs, traditionally been considered the most efficient CD4⁺ T cell subset to generate antitumor immunity (Knutson and Disis 2005). Indeed, upon manipulation of the melanoma microenvironment with rMBP-NAP, we observed a profound influx of both CD3⁺CD8⁺ and CD3⁺CD4⁺ effector T cells into the lung interstitium and increased levels of numerous Th1-like cytokines and inflammatory mediators (e.g., IFN- γ , IL12, IL27) in association with augmented amounts of Th1 cells and immunestimulative function, indicating an immunomodulatory effect of rMBP-NAP in melanoma lesions.

Tumor microenvironment is characterized by chronic inflammation represented by infiltrating leukocytes and soluble mediators, which lead to a local and systemic immunosuppression associated with cancer progression (Mantovani et al. 2008). The intraperitoneal administration of rMBP-NAP in mice promoted infiltration of lymphocyte in the lungs and reduced the local production of several proinflammatory cytokines (IL-6, TGF- β), indicating an anti-inflammatory effect in local area. Results from mRNA expression of chemokines consistently revealed that only rMBP-NAP treatment substantially decreased the expression of CCL2 and CCL20 in tumor tissues as compared with control mice. Chemokines CCL2 and CCL20 were also reported has capable to recruit MDSC and Regulatory T cells (Treg) to tumor microenvironment, which central to form immunosuppression (Borsig et al. 2014; Liu et al. 2015). These data together indicated that rMBP-NAP treatment induced different antitumor

immune responses in the tumor tissues. Intraperitoneal administration induced lymphocyte infiltration and regulated the expression of inflammatory mediators, improving the clearance of tumor of the lungs and increasing the survival of treated.

Since Folkman first demonstrated the importance of angiogenesis in tumor initiation, development and metastasis (Folkman 1990), it was thought of one of promising targets for tumor therapy (Kerbel 2008; Streit and Detmar 2003). So much attention had been paid to angiogenesis is required for a variety of physiological and pathological processes. At present, we stained pulmonary sections for the vascular marker CD34. A clear decrease was observed in overall vascularization in rMBP-NAP treated mice, along with the reduced expression of antiangiogenic cytokines, VEGF-A and VEGF-D. In parallel, the high levels of IFN- γ /IL27 could exert a strong antiangiogenic activity leading to a dramatic reduction of tumor vascularization (Yin et al. 2014; Murugaiyan and Saha 2013). Interestingly, we had currently shown that IL-27 was detected high increased level both in spleen and pulmonary tissue from rMBP-NAP treated-mice, suggesting that rMBP-NAP was capable of exerting an anti-angiogenic activity due to its capacity of creating an IFN- γ /IL27-enriched milieu by acting on innate immune cells, which plays an important role in its antitumor and antimetastatic activities. Although in vivo studies in humans will be necessary to assess the therapeutic efficacy of rMBP-NAP, our present results provide the basis to innovative therapies for relieving of metastatic lung cancer. Clearly, further studies are required for the validation of the possibility that rMBP-NAP, other than inducing Th1 responses, may promote antigen-specific immunity or act as tumor vaccine adjuvant against tumor represents an interesting issue that deserves further investigation.

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Conflict of Interest

The authors of this manuscript do not have any conflict of interest to disclose.

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Figure Captions

Figure 1. Treatment with rMBP-NAP protects mice against B16F10-induced metastatic lung cancer. (A) Treatment protocol. (B) Lung nodules quantification: animals from four independent experiments are represented individually. (C) The inhibition rate (IR) in response to rMBP-NAP at different doses and cisplatin 2mg/kg was analyzed according to the equation: $IR = (1 - \text{tumor nodules of rMBP-NAP/cisplatin-treated group} / \text{tumor nodules of PBS-treated group}) \times 100\%$. (D) Representative lung images and the mean lung weights in the tumor-bearing mice with or without rMBP-NAP treatment. (E) The body weights of each group from the first day (day 1) to the last day (day 14). (F) Survival analysis, $n=5$. Values were presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2. rMBP-NAP significantly elicited systemic Th1 and cytotoxic immune response. (A) The spots pictures and statistical analysis of IFN- γ ELISPOT assay in each group, $n=8$. (B) mRNA expression of IFN- γ , IL12, IL27 and IL4 in spleen. (C) The cytotoxicity of splenocytes and PBMC towards B16F10 cells was assessed using different E:T ratio. ** $p < 0.01$ in comparison with the PBS group. Values were presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. rMBP-NAP triggered T cell immune response in metastatic pulmonary tumor microenvironment. (A) and (B) The expression of Th1 like cytokines IFN- γ and IL-27. (C) qRT-PCR analysis of mRNA expression of inflammatory cytokines and chemokines in lung tumor tissues. (D) Flow cytometric analysis of the percentage of Th1 (CD3⁺CD4⁺ T cells) and Tc1 (CD3⁺CD8⁺ T cells) cells in single-cell suspensions of lung tissue in PBS or rMBP-NAP treated tumor-bearing mice. (E) The expression of adhesion molecules VCAM1 and ICAM1

in lung tissue. Values were presented as mean $\pm$ SEM. * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 4. rMBP-NAP inhibited melanoma metastasis tumor angiogenesis in lungs. (A) CD34 expression in lung tumor sections by immunohistochemistry; photos were taken using the inverted microscope (200 $\times$, scale bar 50 μ m). (B) quantification of microvessel density (MVD), n=8. (C) VEGF-A and VEGF-D expression in transcriptional level by qRT-PCR. Values were presented as mean $\pm$ SEM. . * p < 0.05, *** p < 0.001, difference versus control.

Draft

(A)

(B)

(C)

Table S1. Primers for qRT-PCR

Gene	Forward Primer (5)	Reverse Primer (3)	Product Size
IFN- γ	TCAAGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG	92 bp
IL-12	AGACATCACACGGGACCAAAC	CCAGGCAACTCTCGTTCTTGT	77 bp
IL-27p28	TCGATTGCCAGGAGTGAACC	CGAAGTGGTAGCGAGGAAG	127 bp
IL-27EBI3	GAGGGTCCGGCTTGATGATT	CACGGTGCCCTACATGCTAA	101bp
IL4	CATGGGAAAACCTCCATGCTT	ATGAATCCAGGCATCGAAAA	88 bp
IL-6	CTCTGCAAGAGACTTCCATCCAGT	GAAGTAGGGAAGGCCGTGG	71 bp
IL-10	CCAGAGCCACATGCTCCTAGA	AGCTGGTCCTTTGTTTGAAAGAA	75 bp
TNF- α	AGGGTCTGGGCCATAGAACT	CCACCACGCTCTTATGTCTAC	103 bp
TGF- β	GCTAATGGTGGACCGCAACAAC	GCACTGCTTCCCGAATGTCTG	101 bp
CCL2	GTTGGCTCAGCCAGATGCA	AGCCTACTCATTGGGATCATCTTG	80 bp
CCL20	CTTGCTTTGGCATGGGTACT	GAGGCAACAGTCGTAGTTGCT	71 bp
ICAM-1	GAAGGTGGTTCTTCTGAGCG	AGGCATGGCACACGTATGTA	370 bp
VCAM-1	AAGAGAACCCAGGTGGAGGT	GGGGCAACGTTGACATAAAG	352 bp
VEGF-A	AGCAGAAGTCCCATGAAGTGA	ATGTCCACCAGGGTCTCAAT	82 bp
VEGF-D	TCACGCTCAGCATCCCATC	ACTTCTACGCATGTCTCTCTAGG	125 bp
β -actin	GTGGCATCCATGAAACTACAT	GGCATAGAGGTCTTTACGG	71 bp