BASIC SCIENCE

Nanomedicine: Nanotechnology, Biology, and Medicine
37 (2021) 102415

Original Article

nanomedjournal.com

Therapeutic efficacy of cancer vaccine adjuvanted with nanoemulsion loaded with TLR7/8 agonist in lung cancer model

Jiae Koh, PhD[a,d,1], Sohyun Kim, BS[b,1], Sang Nam Lee, BS[b], Sun-Young Kim, PhD[b], Jung-Eun Kim, MS[b], Kyoung Young Lee, MS[d], Mi Soon Kim, BS[d], Jae Yeong Heo, MS[d], Young Mee Park, MS[d], Bo Mi Ku, PhD[d], Jong-Mu Sun, MD, PhD[c], Se-Hoon Lee, MD, Ph.D[a,c], Jin Seok Ahn, MD, PhD[c], Keunchil Park, MD, PhD[c], Siyoung Yang, PhD[e], Sang-Jun Ha, PhD[f], Yong Taik Lim, PhD[b,*], Myung-Ju Ahn, MD, PhD[a,c,**]

[a]*Department of Health Science and Technology, SAIHST, Sungkyunkwan University, Seoul, Republic of Korea*
[b]*SKKU Advanced Institute of Nanotechnology (SAINT), Department of Nano Engineering, School of Chemical Engineering, Sungkyunkwan University, Suwon, Gyeonggi-do, Republic of Korea*
[c]*Division of Hematology-Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University of Medicine, Seoul, Republic of Korea*
[d]*Research Institute for Future Medicine, Samsung Medical Center, Sungkyunkwan University of Medicine, Seoul, Republic of Korea*
[e]*Department of Pharmacology, Ajou University College of Medicine, Suwon, Republic of Korea*
[f]*Department of Biochemistry, College of Life Science & Biotechnology, Yonsei University, Seoul, Republic of Korea*

Revised 18 May 2021

Abstract

Although immune checkpoint inhibitors have significantly improved clinical outcomes in various malignant cancers, only a small proportion of patients reap benefits, likely due to the low number of T cells and high number of immunosuppressive cells in the tumor microenvironment (TME) of patients with advanced disease. We developed a cancer vaccine adjuvanted with nanoemulsion (NE) loaded with TLR7/8 agonist (R848) and analyzed its therapeutic effect alone or in combination with immune checkpoint inhibitors, on antitumor immune responses and the reprogramming of suppressive immune cells in the TME. NE (R848) demonstrated robust local and systemic antitumor immune responses in both subcutaneous and orthotopic mouse lung cancer models, inducing tumor-specific T cell activation and mitigating T cell exhaustion. Combination with anti-PD-1 antibodies showed synergistic effects with respect to therapeutic efficacy and survival rate. Thus, NE (R848)-based cancer vaccines could prevent tumor recurrence and prolong survival by activating antitumor immunity and reprogramming immunosuppression.

Key words: Immunotherapy; Adjuvant; Toll-like receptor agonist; Immunosuppression; Immune checkpoint inhibitor*Abbreviations:* APC, antigen-presenting cell; DC, dendritic cell; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; MDSC, myeloid-derived suppressor cell; NE, nanoemulsion; NE (R848), nanoemulsion loaded with a TLR7/8 agonist (R848); NK, natural killer; PD-1, programmed death-1; PD-L1, PD-ligand 1; TAM, tumor-associated macrophage; TEM, transmission electron microscopy; TGF, transforming growth factor; TL, tumor lysate; TLR, Toll-like receptor; TME, tumor microenvironment; TNF, tumor necrosis factor

Abbreviations: APC, antigen-presenting cell; DC, dendritic cell; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; MDSC, myeloid-derived suppressor cell; NE, nanoemulsion; NE (R848), nanoemulsion loaded with a TLR7/8 agonist (R848); NK, natural killer; PD-1, programmed death-1; PD-L1, PD-ligand 1; TAM, tumor-associated macrophage; TEM, transmission electron microscopy; TGF, transforming growth factor; TL, tumor lysate; TLR, Toll-like receptor; TME, tumor microenvironment; TNF, tumor necrosis factor

Funding: This work was supported by the National Research Foundation of Korea (NRF) grants funded by the Korean government (MSIT) (Grant Nos. 2017R1A5A1014560, 2018M3A9H4078701, 2020R1A2C3006888, 2017M3C9A6044633, and 2017H1A2A1044327).

Conflicts of interests: None declared.

* Correspondence to: Y. T. Lim, SKKU Advanced Institute of Nanotechnology (SAINT), Department of Nanoengineering, School of Chemical Engineering, Sungkyunkwan University (SKKU), Suwon, Gyeonggi-do, Republic of Korea.

** Correspondence to: M-J Ahn, Division of Hematology-Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University of Medicine, Department of Health Sciences and Technology, SAIHST, Sungkyunkwan University, Seoul, Republic of Korea.

E-mail addresses: yongtaik@skku.edu, (Y.T. Lim), silkahn@skku.edu. (M.-J. Ahn).

[1] These authors equally contributed to this work.

https://doi.org/10.1016/j.nano.2021.102415
1549-9634/

Please cite this article as: Koh J, et al, Therapeutic efficacy of cancer vaccine adjuvanted with nanoemulsion loaded with TLR7/8 agonist in lung cancer model. *Nanomedicine: NBM* 2021;37:102415, https://doi.org/10.1016/j.nano.2021.102415

Cancer immunotherapy, which encompasses cancer vaccines, immune checkpoint inhibitors, and adoptive cell therapy, is an effective therapeutic approach for treating various malignant tumors.[1,2] Cancer vaccines comprise peptide vaccines, vector-based antigen-specific vaccines, dendritic cell (DC) vaccines, and whole-cell vaccines, which deliver antigenic epitopes or entire antigens to patients and induce tumor-specific T cells that target cancer cells.[1,3] Thus, vaccine-based therapy may be one of the most efficient approaches to induce infiltration of T cells across a tumor.[4] However, several studies have shown that tumor and immune suppressive cells interfere with the infiltrating therapeutic immune cells such as antigen-presenting cells (APCs) and T cells, leading to diminished cancer vaccine efficacy. In the tumor microenvironment (TME) of solid tumors, suppressive immune cells such as myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) are abundant.[5,6] Moreover, because of the suppressive nature of TME, T cells express high levels of exhaustion markers, including programmed death (PD)-1, CD39, and CD73. Similarly, tumor-infiltrated $CD8^+$ T cells exhibit exhaustion markers such as high CD39 and CD73 expression, reducing tumor necrosis factor (TNF)-α and interleukin (IL)-2 expression and inhibiting interferon (IFN)-γ production.[7–9] One class of major suppressive immune cells in tumors and circulating blood is MDSCs, which are immature myeloid cells. MDSCs suppress T cell activity and proliferation by releasing molecules such as arginase I, and inducible nitric oxide synthase (iNOS), and anti-inflammatory cytokines, including transforming growth factor (TGF)-β and IL-10, and these cells differentiate into mature myeloid cells or TAMs.[10–12] MDSCs promote primary tumor progression and metastasis by aiding the spread and formation of tumor cells in other organs.[13,14] TAMs are highly plastic cells that can display on pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes.[15–17]

The PD-1 and PD-ligand 1 (PD-L1) pathways also play an important role in the TME, which hinders T cell activity.[18] Effector T cells in the tumor may become exhausted owing to the chronic inflammatory environment consisting of high PD-1, TIM3, or LAG-3.[19,20] With recent clinical successes, the application of immune checkpoint inhibitors of the PD-1/PD-L1 interaction has emerged as a new therapeutic approach, which has become an effective standard therapy for several advanced cancers, including lung cancer. However, studies have shown that only a small percentage of patients showed robust clinical benefits.[21–23] Particularly, the therapeutic efficacy of immune checkpoint inhibitors is very limited when few antigen-specific T cells and many immune suppressive cells are present. Conceivably, a combination of vaccine therapy that can boost the number of antigen-specific T cells with immune checkpoint inhibitors may synergistically induce antitumor immune responses by overcoming the suppressive TME.[24] Comparative studies have shown that a combination of cancer vaccines and immune checkpoint inhibitors is more effective than monotherapy for eliminating tumors.[25]

In this study, we evaluated the therapeutic efficacy of cancer vaccines adjuvanted with NE (R848) in subcutaneous and orthotopic mouse lung cancer models and analyzed both local and systemic antitumor effects when combined with anti-PD-1. We adopted R848 as a TLR7/8 agonist in nanoemulsion to induce robust cell-mediated immune responses and to reprogram immunosuppressive TMEs.[26] The antitumor effects and cell-mediated immune responses induced by a cancer vaccine adjuvanted with NE (R848) were studied by analyzing T cell proliferation and profiling suppressive immune cells, including MDSCs, TAMs, and Treg cells in tumors, lymph nodes, and the spleen.

Methods

Cell culture and animals

The ASB-XIV murine lung carcinoma cell line was purchased from Cell Lines Service (CLS, Eppelheim, Germany). ASB-XIV cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Gibco, Waltham, MA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) at 37 °C with 5% CO_2. BALB/c mice (7 weeks old, female) were purchased from Orient Bio, Inc. (Seongnam, Republic of Korea). All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committee (IACUC) of the Samsung Medical Center, Sungkyunkwan University School of Medicine. The animal experimental protocol was approved by the IACUC of the Samsung Medical Center, Sungkyunkwan University School of Medicine (SKKUIACUC2020-09-02-001).

Fabrication and characterization of NE (R848)

NE was manufactured by tip sonication of 5% squalene, 0.5% Tween 80, and 0.5% Span 80 in phosphate-buffered saline (PBS). NE (R848) was characterized by zeta potential and particle size analysis, UV–visible spectrophotometry, and transmission electron microscopy (TEM) (details described in the Supplementary Material).

In vitro splenocyte proliferation and activation assays

For the splenocyte proliferation assay, splenocytes (2×10^4) were incubated with the indicated concentrations of NE, free R848, and NE (R848) for 24 h, and analyzed using a WST-1 cell proliferation assay kit (Roche, Mannheim, Germany). For the splenocyte activation assay, splenocytes (1×10^6) were incubated with 50 ng/mL of NE, free R848, and NE (R848) for 24 h (details described in the Supplementary Material).

In vitro macrophage polarization assay

To assess the polarization of macrophages, M2 macrophages were incubated with NE, free R848, or NE (R848) for 24 h. The cell surface markers and cytokines secreted by M1 and M2 macrophages were evaluated (details described in the Supplementary Material).

In vitro MDSC polarization assay

To assess the polarization of MDSCs to APCs, purified MDSCs were incubated with 50 ng/mL of NE, free R848, or NE (R848) for 24 h. Cell surface markers and cytokines secreted by

tumoricidal APCs were measured (details described in the Supplementary Material).

Vaccination

For vaccination, 50 μL of PBS was used as a control, 10 μg ASB-XIV cancer cell lysate as tumor lysate (TL), NE (R848 25 μg) as NE (R848), and NE (R848 25 μg) + 10 μg ASB-XIV cancer cell lysate as NE (R848)/TL; 50 μL of PBS was used a control, NE (R848 25 μg) + 10 μg ASB-XIV cancer cell lysate as NE (R848)/TL, 100 μg anti-PD-1 (Clone: RMP1–14; BioXcell, Lebanon, NH, USA) alone, and NE (R848)/TL + anti-PD1 as combination were injected as previously described (details described in the Supplementary Material).[27]

Flow cytometry analysis

Single-cell suspensions from each organ were stained with the appropriate antibodies for 30 min and washed twice with PBS containing 1% fetal bovine serum (Supplementary Table 1; details described in the Supplementary Material).

Statistical analysis

All data presented in this study were obtained in triplicate for each experimental condition and are expressed as the mean ± standard deviation. Data were analyzed using an unpaired two-tailed Student's *t* test and one-way analysis of variance (ANOVA). Survival curves were obtained using the Kaplan–Meier method and comparisons were made using the log-rank (Mantel–Cox) test. All statistical analyses were performed using GraphPad Prism 5 (GraphPad, La Jolla, CA, USA). Statistical significance was set at $P < 0.05$.

Results

Multifunctional immunomodulatory effects of NE (R848) in vitro: T cell activations and polarization of immunosuppressive cells into immunostimulatory APCs

The multifunctional immunomodulatory effect of NE (R848)-based cancer vaccines stimulates immune responses and ameliorates immune suppression of the TME. NE denotes a squalene-based oil-in-water emulsion comprising 5% Span 85 and 5% Tween 80 surfactants to stabilize its structure. Briefly, NE (R848) was synthesized by dispersing R848 in squalene NE using oleic acid ($CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$), a fatty acid classified as monounsaturated omega 9 and a weak acid.[28] As R848 is a weak base because of the presence of a primary amine group in the quinoline moiety, it can be dissolved in oleic acid by a weak acid–base reaction and is well dispersed in squalene (Figure 1, *A*). The size, zeta potential, and polydispersity index of NE and NE (R848) measured by dynamic light scattering and an electrophoretic light-scattering photometer (Otsuka Electronics, Osaka, Japan) are shown in Table 1 (Figure 1, *B*). Based on a TEM image, NE (R848) consists of spherical nanoparticles with an average diameter of 178.5 ± 19.4 nm (Figure 1, *C*). The amount of R848 released from NE (R848) was measured *in vitro* and analyzed using a UV–visible spectrophotometer. The release of R848 from NE (R848) was sustained over time (approximately 70% of R848 was released from NE (R848) after 24 h) compared with that of free R848 (Figure 1, *D*). To confirm whether NE (R848) could reduce systemic toxicity, we measured the amount of IL-6 in serum from free R848 or NE (R848)-treated mice after a single injection. The systemic level of IL-6 in the NE (R848)-treated group was lower than that in the free R848-treated group. Therefore, the nanoemulsion-loaded imidazoquinoline TLR 7/8 agonist could be assumed to reduce systemic toxicity by the sustained release of free TLR 7/8 agonist (Supplementary Figure 1).

As the quality and the quantity of T cells are important for effective cancer immunotherapy, we sought to assess whether NE (R848) can directly influence the proliferation and activation of T cells. Splenocytes were incubated with the indicated concentrations of NE, free R848, and NE (R848) for 24 h. The splenocyte proliferation assay indicated that free R848 and NE (R848) treatment increased splenocyte proliferation in a concentration-dependent manner under 50 ng/mL (Figure 2, *A*). The expression level of CD69 and the secretion of IFN-γ from splenocytes were significantly higher in the NE (R848)-treated group than in the NE- or free R848-treated groups (Figure 2, *B*, *C*). These results suggest that NE (R848) induces the proliferation and activation of T cells more effectively than free R848.

TAMs and MDSCs are immunosuppressive myeloid cells in the TME that promote tumor growth; hence, their modulation is essential for effective cancer immunotherapy.[29–32] MDSCs promote tumor growth by suppressing T and NK cells and by inducing suppressive immune cells, such as Treg cells.[10] Based on a recent study, a TLR7/8 agonist differentiates TAMs and MDSCs into APCs; therefore, we evaluated whether NE (R848) could differentiate TAMs and MDSCs into APCs.[30–33] TAMs are categorized into two phenotypes modulated by signals in the TME; M1 macrophages can eradicate tumors, and M2 macrophages can promote tumor growth.[34,35] First, we assessed the polarization of M2 into M1 macrophages after the indicated treatments for 24 h. NE (R848)-treated macrophages demonstrated significant upregulation of CD40 expression and increased secretion of pro-inflammatory cytokines (TNF-α and IL-6), whereas the number of M2 macrophages expressing CD206 decreased (Figure 2, *D*, *E*). We also investigated the effects of NE (R848) on the polarization of MDSCs into APCs. When isolated MDSCs were incubated with NE, free R848, and NE (R848) for 24 h, the surface markers associated with DCs ($CD11c^+$), macrophages ($F4/80^+$), and pro-inflammatory cytokines (IL-12 and IL-6) were significantly enhanced in the NE (R848)-treated group (Figure 2, *F*, *G*). These results suggest that NE (R848) is effective in polarizing TAMs and MDSCs into APCs.

Intratumoral NE (R848) vaccination delays the growth of tumors and increases the overall survival of ASB-XIV tumor-bearing mice

To evaluate the efficacy of the NE (R848) cancer vaccine with or without tumor antigen in a lung cancer model, we subcutaneously injected 2×10^5 ASB-XIV cells into BALB/c mice. When the tumor volume reached 50 mm^3, we vaccinated

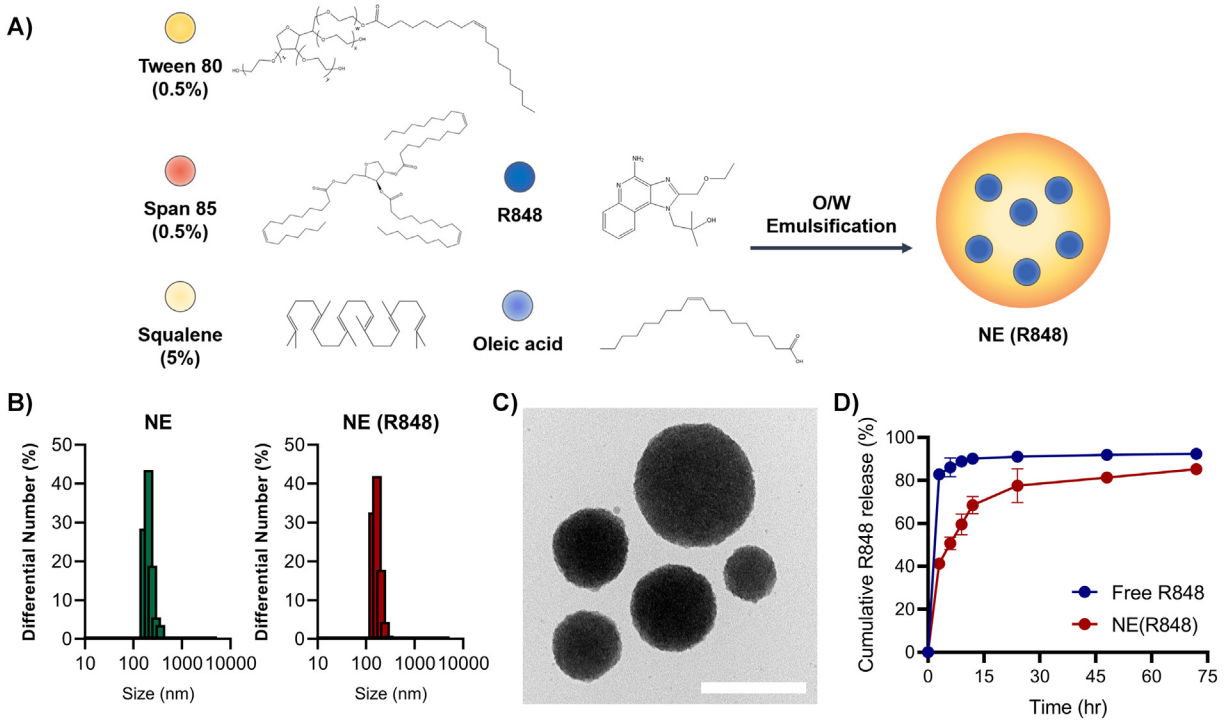

Figure 1. Physicochemical properties of nanoemulsion (NE) loaded with a TLR 7/8 agonist (R848) (NE [R848]). **(A)** Schematic diagram of NE (R848) synthesis. **(B)** Size distribution of NE and NE (R848). **(C)** Transmission electron microscopy (TEM) images of NE (R848). Scale bar = 200 nm. **(D)** *In vitro* cumulative release profile of R848 from NE (R848). R848 release from NE (R848) was measured by dialysis in PBS at each time point.

the mice intratumorally with TL, NE (R848), or NE (R848)/TL four times (on days 12, 15, 18, and 22). Notably, the NE (R848)/TL group showed significant inhibition of tumor growth compared with the TL and NE (R848) only group and exhibited complete tumor regression (Supplementary Figure 2). Furthermore, we assessed the tumor antigen-specific T cell response by isolating splenocytes and restimulating with ASB-XIV TL. The level of IFN-γ in the NE (R848)/TL-treated group was remarkably higher than that in the other groups (Supplementary Figure 3), confirming that NE (R848) vaccine with tumor antigen induced synergistic antitumor effects by triggering a tumor antigen-specific T cell response.

To evaluate the efficacy of the NE (R848) cancer vaccine in a mouse lung cancer model, we subcutaneously injected 2×10^5 ASB-XIV cells into BALB/c mice. When the tumor volume reached 50 mm^3, we vaccinated the mice intratumorally with NE (R848)/TL four times (on days 12, 15, 18, and 22) after tumor inoculation and injected 100 μg of anti-PD-1 mAb intraperitoneally (Figure 3, *A*). Compared with the control and anti-PD-1-treated groups, the NE (R848)/TL vaccine and combination groups showed significant tumor reduction on day 28 after tumor inoculation ($P_{ANOVA} = 0.0001$; Figure 3, *B*, *C*). When the tumor volumes were measured, a synergistic effect was exhibited by the combination therapy compared with that by the anti-PD-1 ($P = 0.0002$)- or NE (R848)/TL vaccine-treated ($P = 0.06$) groups. Furthermore, survival was significantly prolonged in the NE (R848)/TL-vaccinated and combination groups compared with that in the control or anti-PD-1-treated groups ($P = 0.001$; Figure 3, *D*). The combination group showed more prominent effects than the NE (R848)/TL vaccine alone-treated group ($P = 0.05$). Moreover, all mice in the control and anti-PD-1 groups showed tumor progression, whereas tumor-free survival was observed in four out of nine mice in both the NE (R848)/TL and combination groups (Figure 3, *E*). To further determine whether NE (R848) vaccination combined with anti-PD-1 induced a systemic immune memory response in a subcutaneous mouse lung cancer model, we reinjected 1×10^5 ASB-XIV lung cancer cells in mice ($n = 13$) that completely recovered from the primary tumor challenge. Thirty days after the initial tumor inoculation, the experimental group showed significant inhibition of tumor growth compared with the control group ($n = 13$; Figure 3, *F*). The combination group was mostly tumor-free (12 out of 13 mice) after the tumor rechallenge, whereas all mice in the control group showed continuous tumor growth (Figure 3,

Table 1
Physicochemical characteristics of NE and NE (R848).

	Size (nm)	Zeta potential (mV)	PDI
NE	211.3 ± 21.6	−13.33 ± 3.51	0.174
NE (R848)	178.5 ± 19.4	−17.67 ± 2.51	0.196

NE, nanoemulsion; NE (R848), nanoemulsion loaded with a TLR7/8 agonist (R848); PDI, polydispersity index.

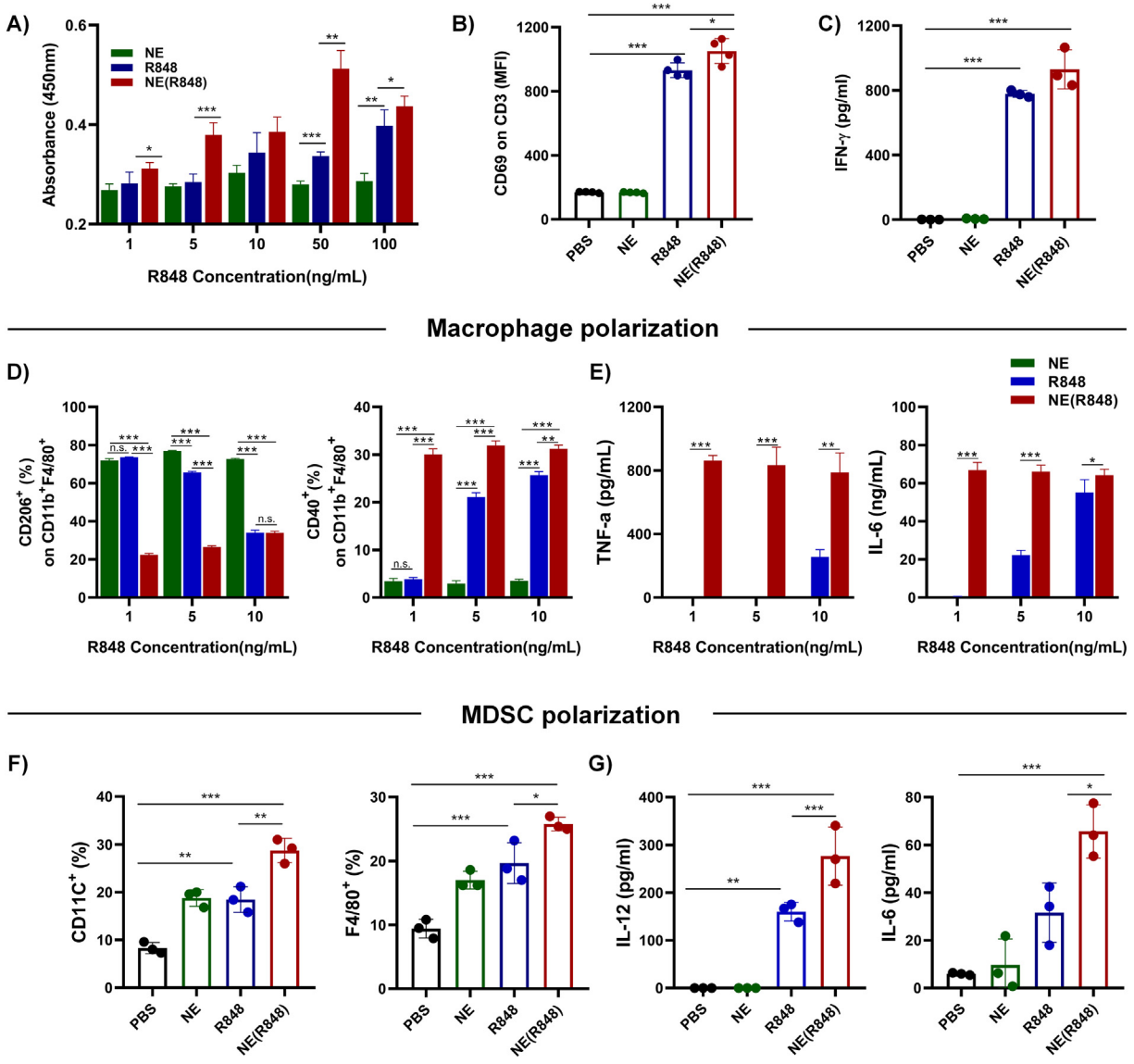

Figure 2. NE (R848) activates splenocytes and converts immunosuppressive TAMs and MDSCs into tumoricidal antigen-presenting cells (APCs). **(A)** *In vitro* splenocyte proliferation assay. **(B)** CD69 expression as a T cell activation marker on CD3 T cells. **(C)** IFN-γ secretion as T cell activation from splenocyte. **(D)** CD206 and CD40 expression on TAM. **(E)** Secretion of proinflammatory cytokines (TNF-α and IL-6) by TAM. **(F)** Expression of DC (CD11c) and macrophage-associated (F4/80) markers. **(G)** Secretion of proinflammatory cytokines (IL-12 and IL-6) by tumoricidal APCs. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

G). These results indicate that intratumoral NE (R848)/TL vaccination combined with anti-PD-1 generates a systemic memory immune response and triggers adaptive immunity.

Intratumoral NE (R848) vaccination increases the number of infiltrated immune cells and reduces that of suppressive immune cells in the TME

To investigate the underlying immunological mechanisms of the antitumor effects of NE (R848)-based cancer vaccines and to characterize the infiltration of immune cells, we harvested the tumor, spleen, and lymph nodes 1 day after the last vaccination. A representative flow cytometer gating strategy is shown in Supplementary Figure 4. We first profiled the immune cells in the lymph nodes and found that the frequency of $CD8^+$ T cells was higher in the NE (R848)/TL and combination groups than in the control and anti-PD-1 groups ($P_{ANOVA} = 0.05$; Figure 4, *A*, *B*). CD39 and CD73 are highly expressed in exhausted T cells, which define poor effector function and reduce the production of TNF-α and the expression level of coinhibitory receptors.[7–9] PD-1 expression is an established T cell exhaustion marker.[36,37] Therefore, we analyzed T cell exhaustion markers, including PD-1 and CD39, on $CD8^+$ T cells. We found that PD-1 expression level on $CD8^+$ T cells was lower in the combination group than in the control, anti-PD-1, and NE (R848)/TL groups, indicating that the combination group showed less exhausted T cell infiltration in the lymph nodes. Moreover, Treg cell frequency was lower in the combination group than in the only NE (R848)/

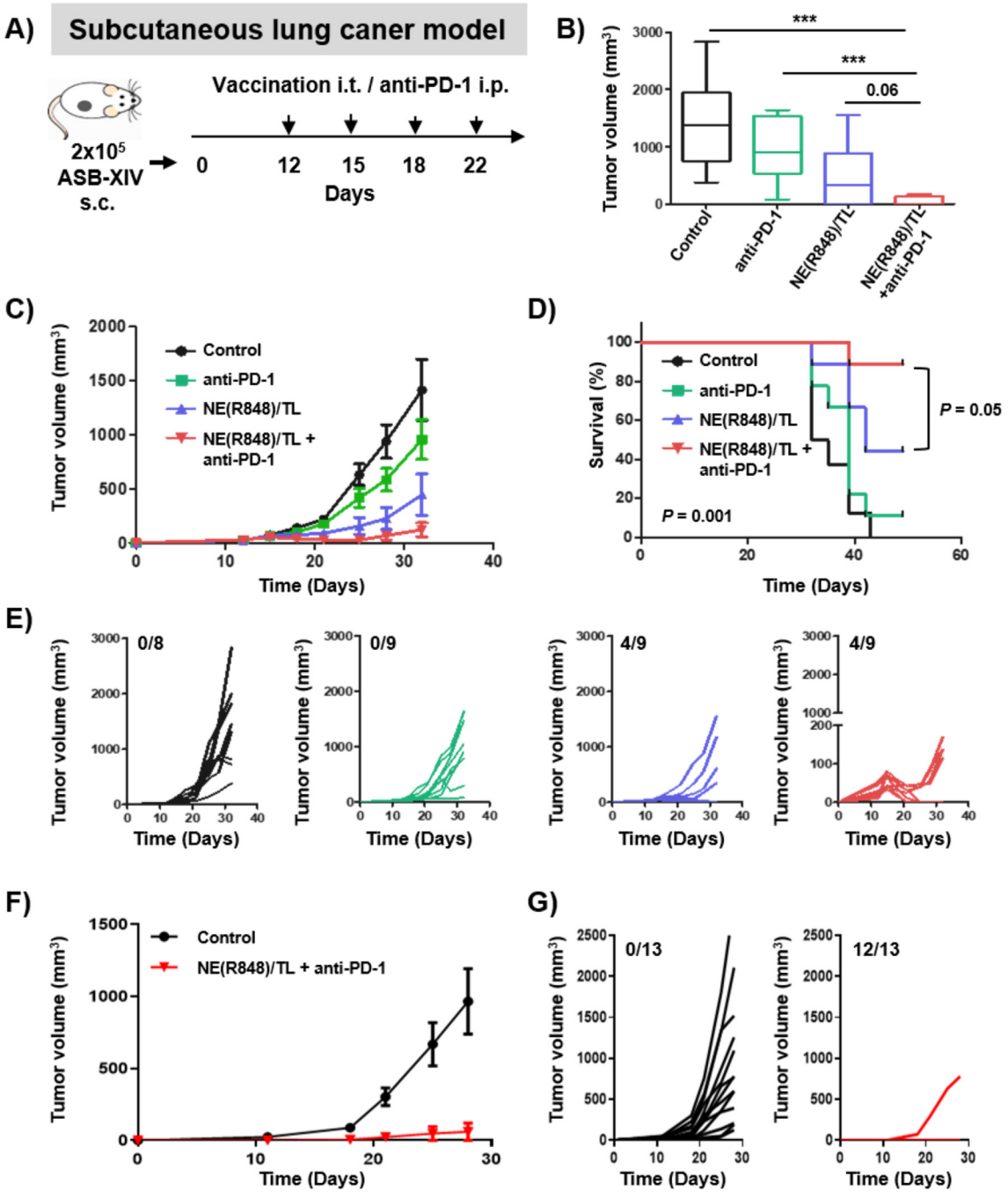

Figure 3. Efficacy of NE (R848) vaccine in a subcutaneous mouse lung cancer model. **(A)** Schematic representation of the NE (R848) vaccination protocol. **(B)** Tumor volumes (mm^3) measured on day 28 for control (n = 8), anti-PD-1 (n = 9), NE (R848)/TL (n = 9), and NE (R848)/TL + anti-PD-1 (n = 9) treated groups. **(C)** Tumor volumes (mm^3) measured every 2-3 days. **(D)** Overall survival of each group of mice by Kaplan–Meier curves. **(E)** Tumor volumes (mm^3) for each group of mice. **(F)** Tumor volume (mm^3) measured on day 28 after ASB-XIV tumor rechallenge for the control (n = 13) and NE (R848)/TL + anti-PD-1 treated groups (n = 13). **(G)** Individual growth curve of the reinoculated tumors in the tumor rechallenge study. *P < 0.05, **P < 0.01, ***P < 0.001.

TL-treated group (P_{ANOVA} = 0.04). We also evaluated suppressive immune cells in tumors, including Gr-1^+, Ly6G^+, and Ly6C^+ cells, which are the surface markers present in MDSCs, and CD206 expression on CD11b^+ and F4/80^+ cells, which are known as M2 macrophages. Ly6G^+ cell frequency was lower in the NE (R848)/TL and combination groups than in the control and anti-PD-1 groups (P_{ANOVA} = 0.0003). CD206 expression level in CD11b^+ and F4/80^+ cells was also lower in the NE (R848)/TL and combination groups (P_{ANOVA} = 0.002) than in the control and anti-PD-1 alone groups (Figure 4, *C*). Furthermore, the combination group showed a higher frequency of CD3^+ T cells than the NE (R848)/TL alone group (P = 0.04;

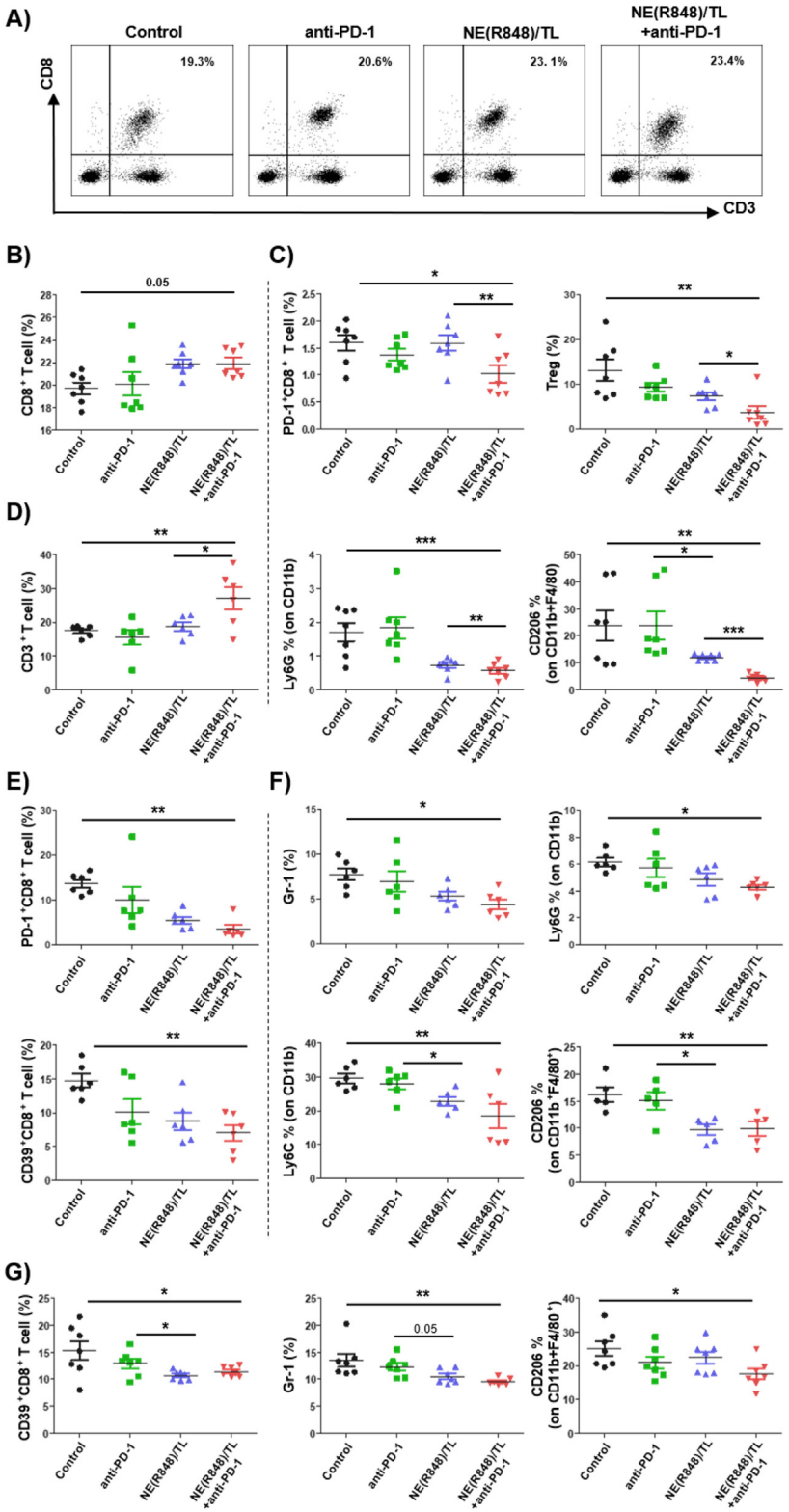

Figure 4. Immune cell profiling after NE (R848) vaccination. **(A)** Representative flow cytometry analysis images of $CD3^+CD8^+$ T cells for the control, anti-PD-1, NE (R848)/TL, and NE (R848)/TL + anti-PD-1 treated groups in lymph node ($n = 7$). **(B)** Frequency of $CD3^+CD8^+$ T cells in the lymph node. **(C)** $PD\text{-}1^+$ $CD8^+$ T cell population, Treg cells, $Ly6G^+CD11b^+$ cells as a marker for MDSC, and $CD206^+$ on $CD11b^+F4/80^+$ cells as TAM markers in the lymph node. **(D)** Tumor infiltrated $CD3^+$ T cells frequencies in % ($n = 6$). **(E)** Expression of the T cell exhaustion markers, PD-1 and CD39 on $CD8^+$ T cells. **(F)** Expression of $Gr\text{-}1^+$, $Ly6G^+$, and $Ly6C^+$ cells as MDSC markers, and CD206 expression on $CD11b^+F4/80^+$ cells as TAM markers at the tumor site. **(G)** Expression of the T cell exhaustion marker CD39 on $CD8^+$ T cells, $Gr\text{-}1^+$ cells as MDSC markers, and CD206 expression on $CD11b^+F4/80^+$ cells as M2 macrophage markers in the spleen. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Figure 4, *D*). Moreover, PD-1 ($P_{ANOVA} = 0.001$) and CD39 expression levels on $CD8^+$ T cells ($P_{ANOVA} = 0.004$) in tumors were relatively lower in the NE (R848)/TL and combination groups than in the control and anti-PD-1 groups (Figure 4, *E*). Consistent with our observations in the lymph node, MDSC frequency ($P_{Gr\text{-}1} = 0.02$, $P_{Ly6G} = 0.03$, and $P_{Ly6C} = 0.006$) and CD206 expression level in the $CD11b^+F4/80^+$ population of M2 macrophages ($P = 0.004$) in tumors were also low in the NE (R848)/TL alone and combination groups (Figure 4, *F*). Similar to the flow cytometry analysis, intratumoral $CD3^+$ T cells were higher, whereas $Gr\text{-}1^+$ cells were lower in the NE (R848)/TL and combination groups than in the control and anti-PD-1-treated groups, as confirmed by immunohistochemistry (Supplementary Figure 5). Furthermore, CD39 expression level on $CD8^+$ T cells in the spleen ($P_{ANOVA} = 0.01$) was low in the mice treated with NE (R848)/TL or the combination therapy. Gr-1 ($P = 0.005$) and CD206 ($P = 0.04$) expression levels on $CD11b^+F4/80^+$ cells in the spleen were also low in the NE (R848)/TL and combination groups (Figure 4, *G*). These results suggest that intratumorally injected NE (R848) induces a tumor-specific cytotoxic T cell response and reprograms the immunosuppressive TME. These effects were highly prominent when the NE (R848)-based cancer vaccine was combined with an immune checkpoint inhibitor.

Effects of initial tumor burden on the therapeutic efficacy of NE (R848) vaccination

To evaluate the efficacy of NE (R848) vaccination according to tumor burden, we vaccinated mice with different initial tumor sizes. First, we increased the initial inoculum of ASB-XIV cells to 5×10^5, which elicited more rapid tumor growth than the initial inoculation of 2×10^5 cells. When the tumor size reached 50 mm^3, we vaccinated each group of mice four times (on days 9, 12, 15, and 19; Figure 5, *A*). As expected, the NE (R848)/TL and combination groups showed significantly lower tumor volumes than the other groups, although the anti-PD-1 group also exhibited some antitumor effects compared with the control group on day 26 ($P_{ANOVA} < 0.0001$; Figure 5, *B*, *C*). Overall survival in the NE (R848)/TL and anti-PD-1 combination group was also greater than that in the NE (R848)/TL ($P = 0.07$) or anti-PD-1 group ($P = 0.02$). The median survival was significantly longer in the NE (R848)/TL and combination groups than in the control or anti-PD-1 treated groups (Figure 5, *D*). In the combination group, three out of seven mice showed complete remission. The NE (R848)/TL and anti-PD-1 treated groups had one tumor-free mouse out of seven, whereas all the control mice showed tumor progression (Figure 5, *E*). Thus, NE (R848) combined with anti-PD-1 immunotherapy exerted a potent antitumor effect even when the initial inoculation cell number was high and caused rapid tumor growth.

To further evaluate the effect of the NE (R848) vaccine combined with anti-PD-1 on more advanced tumors, we allowed the tumor to grow continuously before the initial vaccination. After inoculation of 5×10^5 ASB-XIV cells, when the tumor volume reached 150-200 mm^3, we started vaccination on days 19, 22, 26, and 30 after the tumor cell injection (Figure 5, *F*). Although most of the mice showed tumor progression, it was delayed in the combination group ($P_{ANOVA} = 0.0003$). The combination group showed significantly slower progression than the NE (R848)/TL-treated group ($P = 0.02$; Figure 5, *G*, *H*). Moreover, the overall survival time ($P = 0.006$) was prolonged in the combination group (Figure 5, *I*). In the combination therapy group, tumors were eliminated in one out of seven mice (Figure 5, *J*). Although most of the groups showed transient tumor progression but short overall survival when the initial volume of the tumor was greater than 150 mm^3, only the NE (R848)/TL vaccination and anti-PD-1 combination treatments prolonged overall survival and delayed tumor progression.

Subcutaneous NE (R848) vaccination elicits antitumor immunity and prevents tumor growth in an orthotopic lung cancer model

To test the systemic effect of the NE (R848) vaccine, we established an orthotopic lung cancer model in syngeneic BALB/c mice using ASB-XIV cells. We injected 5×10^4 ASB-XIV cells into the tail vein to develop tumor nodules in both lungs and vaccinated the mice subcutaneously close to the axillary lymph node with PBS, anti-PD-1, NE (R848)/TL, or a combination of NE (R848)/TL and anti-PD-1 four times (on days 1, 4, 7, and 11) after tumor inoculation (Figure 6, *A*). Compared with the control and anti-PD-1-treated groups, the NE (R848) and combination groups survived longer ($P = 0.0001$; Figure 6, *B*). Further, we examined nodule formation in both lungs in each group after vaccination on week 5 after tumor inoculation. The combination group showed fewer tumor nodules ($P = 0.01$) than the control group (Figure 6, *C*, *D*). MRI demonstrated a marked reduction in tumor size or complete elimination of tumors in the mice treated with NE (R848) alone or in combination with anti-PD-1 (Figure 6, *E*). To analyze the infiltrated immune cells in the lungs and lymph nodes, we isolated single cells from both organs. Representative flow cytometry analysis of $CD8^+$ T cells in the lymph nodes is shown in Figure 6, *F*. Consistent with that of the subcutaneous mouse model, the frequency of $CD8^+$ T cells was higher in the lymph nodes of the vaccinated groups than in those of the control and anti-PD-1 groups. The NE (R848)/TL and combination groups showed a synergistic effect with respect to NE (R848)/TL ($P < 0.0001$) or anti-PD-1 groups ($P = 0.009$; Figure 6, *G*). Moreover, CD39 expression level in $CD8^+$ T cells was significantly low in the NE (R848)/TL and combination groups ($P = 0.02$). CD206 expression level on $CD11b^+F4/80^+$ cells was also low in the NE (R848)/TL and combination groups ($P_{ANOVA} < 0.0001$; Figure 6, *G*). In the spleen, expression levels of CD39 and CD73 in $CD8^+$ T cells ($P_{ANOVA} < 0.0001$) and that of CD206 on $CD11b^+F4/80^+$ ($P_{ANOVA} = 0.001$) were low in the NE (R848)/TL and combination groups (Figure 6, *H*).

Discussion

In this study, we demonstrated both local and systemic antitumor effects of the NE (R848)-adjuvanted cancer vaccine alone and in combination with anti-PD-1 in subcutaneous and orthotopic mouse lung cancer models. Furthermore, we revealed the underlying mechanisms of antitumor activity of a cancer vaccine adjuvanted with NE (R848), which reprograms the TME by converting MDSCs into mature myeloid cells and M2 macrophages into M1 macrophages. These findings were

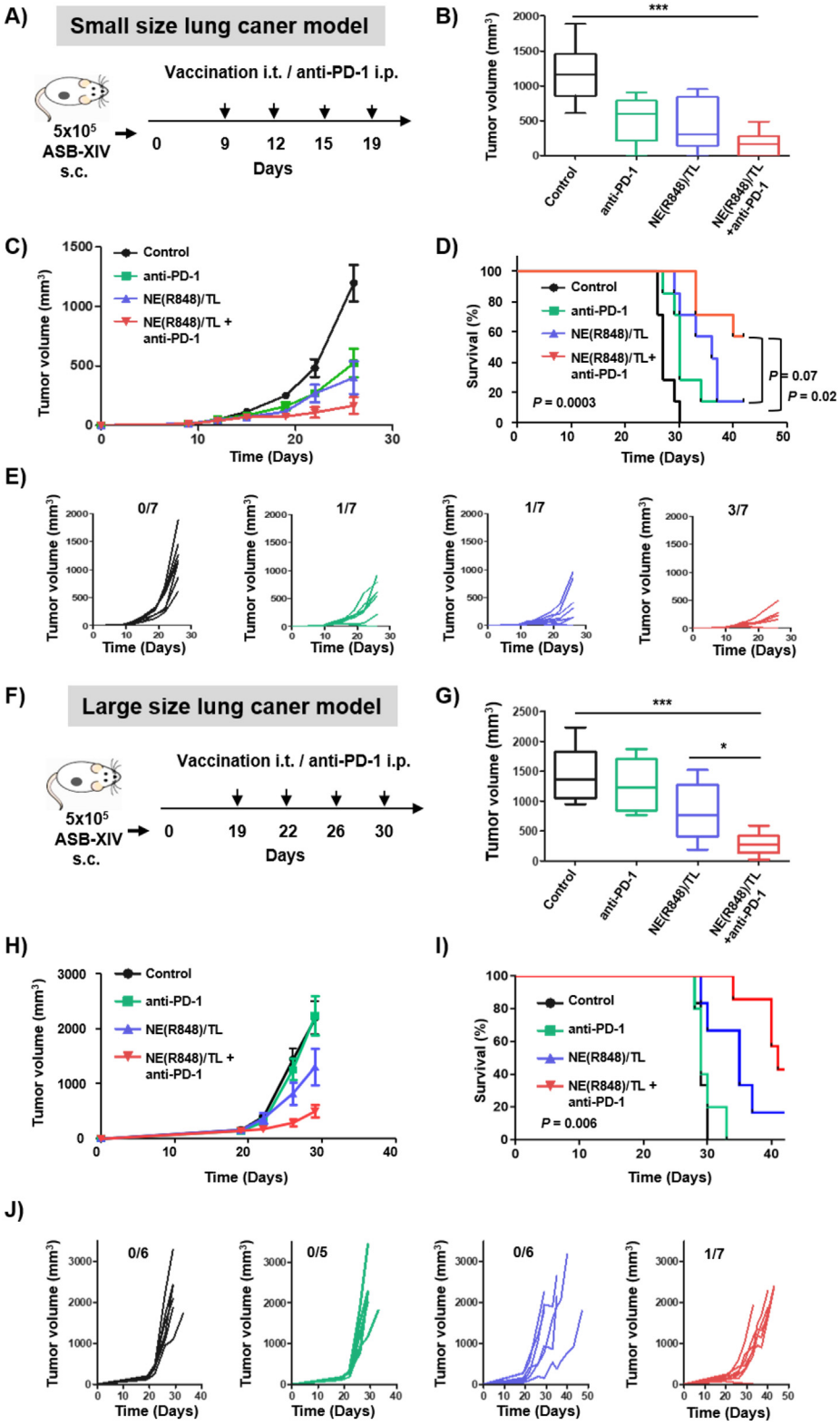

Figure 5. Efficacy of NE (R848) vaccine depending on initial tumor size. **(A)** Schematic representation of the NE (R848) vaccination protocol for small size lung cancer model. **(B)** Tumor volumes (mm^3) measured on day 26 for the control, anti-PD-1, NE (R848)/TL, and NE (R848)/TL + anti-PD-1 treated groups. **(C)** Tumor volumes (mm^3) measured every 2-3 days ($n = 7$). **(D)** Overall survival of each mouse group by Kaplan–Meier curves. **(E)** Tumor volumes (mm^3) for each group of mice. **(F)** Schematic representation of the NE (R848) vaccination protocol for large size lung cancer model. **(G)** Tumor volumes (mm^3) measured on day 28 for the control ($n = 6$), anti-PD-1 ($n = 5$), NE (R848)/TL ($n = 6$), and NE (R848)/TL + anti-PD-1 ($n = 7$) treated groups. **(H)** Tumor volumes (mm^3) measured every 2-3 days. **(I)** Overall survival of each mice group by Kaplan–Meier curves **(J)** Tumor volumes (mm^3) measured by each group of mice. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

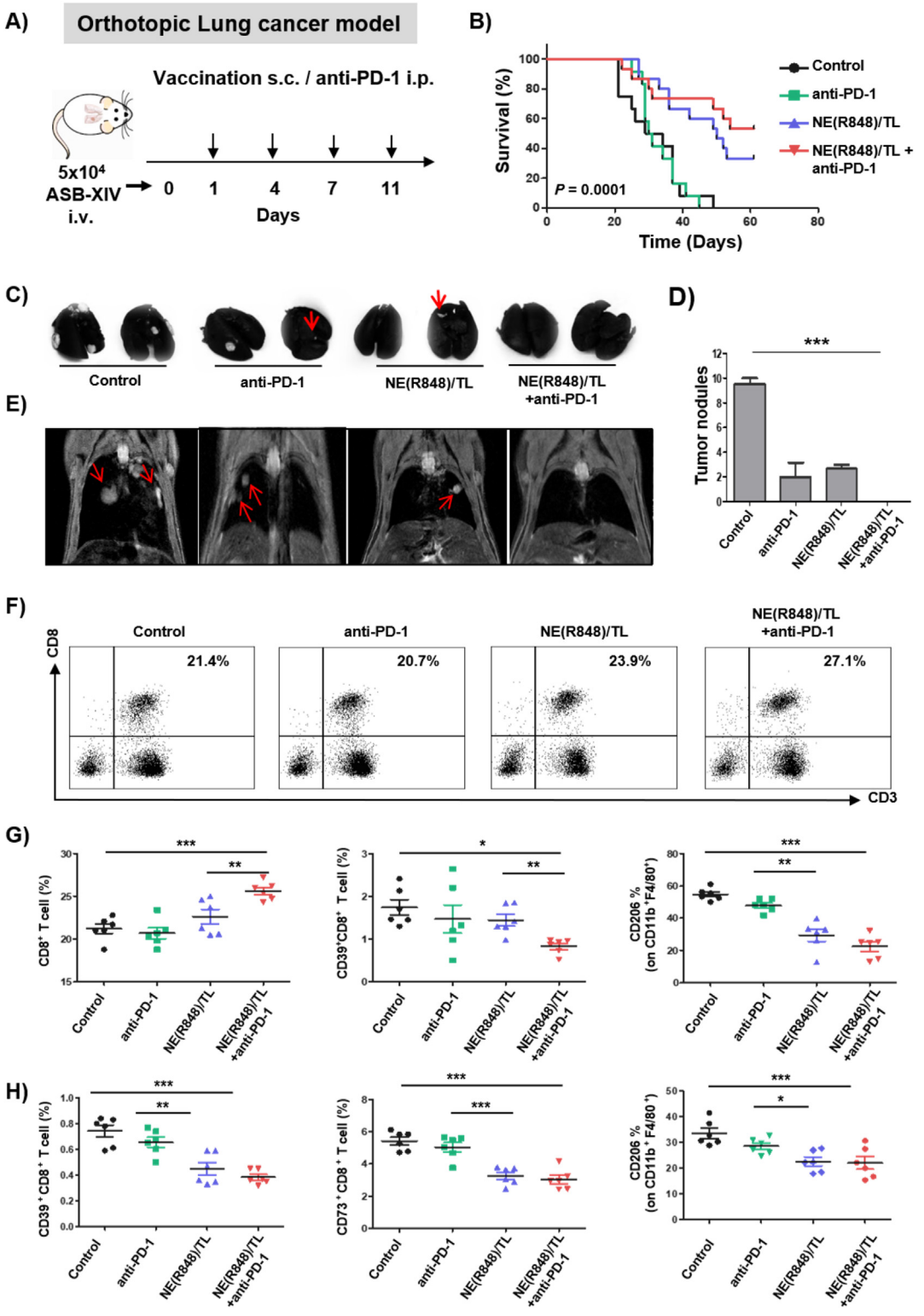

Figure 6. Efficacy of NE (R848) vaccine in an orthotopic mouse lung model. **(A)** Schematic representation of the NE (R848) vaccination protocol. **(B)** Overall survival of each mouse group by Kaplan–Meier curves (n = 14). **(C)** India ink staining of lung tumor from the mice treated with PBS, anti-PD-1, NE (R848)/TL, and NE (R848)/TL + anti-PD-1 (n = 3) on week 5 after tumor inoculation. **(D)** Quantification of lung tumor nodules from both lungs. **(E)** Representative of coronal MRI sections of lungs from each mouse group (n = 5) taken on week 5 of the initial tumor inoculation. **(F)** Representative flow cytometry analysis images of $CD3^+CD8^+$ T cells for the control, anti-PD-1, NE (R848)/TL, and NE (R848)/TL + anti-PD-1 treated groups in lymph nodes. **(G)** Frequency of $CD3^+CD8^+$ T cells in the lymph node, $CD39^+$ $CD8^+$ T cells, and the frequency of CD206 expression on $CD11b^+F4/80^+$ cells as TAM markers in the lymph node (n = 6). **(H)** Expression of T cell exhaustion markers CD39 or CD73 on $CD8^+$ T cells, and CD206 expression on $CD11b^+F4/80^+$ cells as TAM markers in the spleen. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

confirmed by the finding that the NE (R848)-treated mouse group had exhibited reduced infiltration of suppressive immune cells, including MDSCs, M2 macrophages, and Treg cells; $CD3^+$ and $CD8^+$ T cells were mainly recruited at the tumor site. We also found that T cell exhaustion markers such as PD-1, CD39, and CD73 expressed on $CD8^+$ T cells were low in the combination-treated groups. These findings indicated that the NE (R848)-adjuvanted cancer vaccine maintained T cells in an active state by reprogramming the TME, thus maintaining antitumor immunity. Although NE (R848) alone has antitumor activity, NE (R848)-adjuvanted cancer vaccine combined with anti-PD-1 further drives substantial antitumor activity, suggesting that combination strategies should be encouraged to maximize the anti-tumor effects. Notably, the NE (R848)-adjuvanted cancer vaccine alone or combined with anti-PD-1 induced significant tumor growth inhibition and extended survival with low tumor burden; however, these anti-tumor activities are not prominent in mice with high tumor burden, suggesting that cancer vaccines combined with anti-PD-1 are more effective at an early stage of cancer with low tumor burden.

Intratumoral injection of NE (R848) vaccine alone or in combination with anti-PD-1 induced a systemic memory response in a subcutaneous mouse lung cancer model. Notably, the antitumor activity of NE (R848)-adjuvanted vaccine alone or in combination with anti-PD-1 was also observed when the vaccine was administered *via* subcutaneous injection in an orthotopic mouse model. These results indicate that subcutaneous vaccination approach might have advantages in clinical applications because intratumoral injections are not a common injection route in lung cancer patients.

Although we demonstrated the promising therapeutic efficacy of NE (R848)-adjuvanted cancer vaccines by inducing host immune responses, there are some limitations. The NE (R848) vaccine alone and in combination with anti-PD-1 showed potential antitumor effects in the mouse lung model; however, some tumors were not completely eradicated in this experiment, suggesting that the dose and schedule of vaccination and anti-PD-1 should be further evaluated and optimized. Considering the aggressive growth rate of ASB-XIV cells in the orthotopic model generated *via* tail vein injection, further studies should be conducted to explore the ideal time for vaccination and anti-PD-1 therapy. Moreover, the efficacy of NE (R848) adjuvanted vaccine in preventing tumor relapse after lung cancer resection warrants further research. Nevertheless, a key advantage of the whole tumor cell lysate vaccines adopted in this research over other protein or peptide tumor antigen vaccines is that they can target multiple antigens.[4] Moreover, tumor lysate as a source of tumor-associated antigens bypasses the limitation of single antigen vaccines, thereby broadening the repertoire of antigen-specific T cell clones available for antitumor immunity regardless of HLA types.[38] A recent study of a meta-analysis showed that 1800 patients who were immunized with whole tumor cell lysate vaccines had a significantly higher response (8.1%) compared with patients who were immunized with defined tumor antigens (3.6%).[39] Also, prostate GVAX vaccine consisting of allogeneic prostate cancer cell lines LNCaP and PC-3 demonstrated encouraging results in 346 patients with hormone-refractor prostate cancer (HRPC) from several phase I/II studies, thus warranting the personalized whole tumor cell lysate vaccine approaches.[40,41] In conclusion, NE (R848) vaccination triggers innate and adaptive immune responses; the vaccine induces tumor-specific antitumor immunity and is highly efficacious when combined with anti-PD-1 immunotherapy, leading to robust anti-tumor activity and sustained overall survival in a mouse lung cancer model. Therefore, NE (R848) vaccination is expected to be a potential strategy for the clinical management of lung cancer.

CRediT Author Statement

Conceptualization and research design: YTL, M-JA, JK, SK, and S-YK; Conducting experiments: JK, SK, SNL, S-YK, J-EK, KYL, MSK, YMP, and JYH; Data acquisition: JK, SK, SNL, and KL; Data analysis: JK, SK, SNL, KYL, and BMK; Writing the manuscript: JK and SK; Providing expertise and feedback: J-MS, S-HL, JSA, KP, SY, and S-JH; Supervision and final manuscript confirmation: YTL and M-JA. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2021.102415.

References

1. Melief CJM, Van Hall T, Arens R, Ossendorp F, Van Der Burg SH. Therapeutic cancer vaccines. *J Clin Invest* 2015;**125**:3401-12.
2. Kirkwood JM, Butterfield LH, Tarhini AA, Zarour H. Immunotherapy of cancer in 2012. *CA Cancer J Clin* 2012;**62**:309-35.
3. Wang X, Fan S, Pan H, Chen W, Hua W. Cancer immunotherapy for metastasis: past, present and future. *Brief Funct Genomics* 2019;**18**:140-6.
4. Keenan BP, Jaffee EM. Whole cell vaccines—past progress and future strategies. *Semin Oncol* 2012;**39**:276-86.
5. Binnewies M, Roberts EW, Kersten K, Chan V, Fearon DF, Merad M, et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat Med* 2018;**24**:541-50.
6. Ohue Y, Nishikawa H. Regulatory T (Treg) cells in cancer: can Treg cells be a new therapeutic target? *Cancer Sci* 2019;**110**:2080-9.
7. Vignali DAA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* 2008;**8**:523-32.
8. Facciabene A, Motz GT, Coukos G. T-regulatory cells: key players in tumor immune escape and angiogenesis. *Cancer Res* 2012;**72**:2162-71.
9. Kim JH, Kim BS, Lee SK. Regulatory T cells in tumor microenvironment and approach for anticancer immunotherapy. *Immune Netw* 2020;**20**:1-17.
10. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009;**9**:162-74.
11. Kumar V, Patel S, Tcyganov E, Gabrilovich DI. The nature of myeloid-derived suppressor cells in the tumor microenvironment. *Trends Immunol* 2016;**37**:208-20.
12. Bizymi N, Bjelica S, Kittang AO, Mojsilovic S, Velegraki M, Pontikoglou C, et al. Myeloid-derived suppressor cells in hematologic diseases. *HemaSphere* 2019;**3**e168.

13. Trovato R, Canè S, Petrova V, Sartoris S, Ugel S, De Sanctis F. The engagement between MDSCs and metastases: partners in crime. *Front Oncol* 2020;**10**:1-16.
14. Condamine T, Ramachandran I, Youn JI, Gabrilovich DI. Regulation of tumor metastasis by myeloid-derived suppressor cells. *Annu Rev Med* 2015;**66**:97-110.
15. Gonzalez H, Hagerling C, Werb Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes Dev* 2018;**32**:1267-84.
16. Grenier JM, Yeung ST, Khanna KM. Combination immunotherapy: taking cancer vaccines to the next level. *Front Immunol* 2018;**8**:610.
17. van Dalen F, van Stevendaal M, Fennemann F, Verdoes M, Ilina O. Molecular repolarisation of tumour-associated macrophages. *Molecules* 2018;**24**:9.
18. Ostrand-Rosenberg S, Horn LA, Haile ST. The programmed death-1 immune-suppressive pathway: barrier to antitumor immunity. *J Immunol* 2014;**193**:3835-41.
19. Jiang Y, Li Y, Zhu B. T-cell exhaustion in the tumor microenvironment. *Cell Death Dis* 2015;**6**:e1792.
20. Maimela NR, Liu S, Zhang Y. Fates of CD8+ T cells in tumor microenvironment. *Comput Struct Biotechnol J* 2019;**17**:1-13.
21. Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, et al. Pembrolizumab for the treatment of non–small-cell lung cancer. *N Engl J Med* 2015;**372**:2018-28.
22. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus docetaxel in advanced nonsquamous non–small-cell lung cancer. *N Engl J Med* 2015;**373**:1627-39.
23. Rittmeyer A, Barlesi F, Waterkamp D, Park K, Ciardiello F, von Pawel J, et al. Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. *Lancet* 2017;**389**:255-65.
24. Zhao J, Chen Y, Ding ZY, Liu JY. Safety and efficacy of therapeutic cancer vaccines alone or in combination with immune checkpoint inhibitors in cancer treatment. *Front Pharmacol* 2019;**10**:1184.
25. Peng M, Mo Y, Wang Y, Wu P, Zhang Y, Xiong F, et al. Neoantigen vaccine: an emerging tumor immunotherapy. *Mol Cancer* 2019;**18**:1-14.
26. Del Giudice G, Rappuoli R, Didierlaurent AM. Correlates of adjuvanticity: a review on adjuvants in licensed vaccines. *Semin Immunol* 2018;**39**:14-21.
27. Kim SY, Kim S, Kim JE, Lee SN, Shin IW, Shin HS, et al. Lyophilizable and multifaceted toll-like receptor 7/8 agonist-loaded nanoemulsion for the reprogramming of tumor microenvironments and enhanced cancer immunotherapy. *ACS Nano* 2019;**13**:12671-86.
28. Thomas A. Fats and Fatty Oils. In: Ley C, Elvers B, Bellussi G, Bus J, Drauz K, Greim H, Hessel V, Kleemann A, Kutscher B, Meijer G, Moran S, Mukherjee J, Palkovits R, Pfaendner R, Qiao G, Roper M, Sundmacher K, Teles JH, Ulber R, Wagemann K, Wietelmann U, editors. *Ullmann's Encycl. Ind. Chem.* 7th ed. Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim; 2000.
29. Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. *Immunity* 2005;**23**:344-6.
30. Rodell CB, Arlauckas SP, Cuccarese MF, Garris CS, Li R, Ahmed MS, et al. TLR7/8-agonist-loaded nanoparticles promote the polarization of tumour-associated macrophages to enhance cancer immunotherapy. *Nat Biomed Eng* 2018;**2**:578-88.
31. Wang J, Shirota Y, Bayik D, Shirota H, Tross D, Gulley JL, et al. Effect of TLR agonists on the differentiation and function of human monocytic myeloid-derived suppressor cells. *J Immunol* 2015;**194**:4215-21.
32. Bayik D, Tross D, Klinman DM. Factors influencing the differentiation of human monocytic myeloid-derived suppressor cells into inflammatory macrophages. *Front Immunol* 2018;**9**:608.
33. Phuengkham H, Song C, Lim YT. A designer scaffold with immune nanoconverters for reverting immunosuppression and enhancing immune checkpoint blockade therapy. *Adv Mater* 2019;**31**:1903242.
34. Herd HL, Bartlett KT, Gustafson JA, McGill LD, Ghandehari H. Macrophage silica nanoparticle response is phenotypically dependent. *Biomaterials* 2015;**53**:574-82.
35. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol* 2017;**14**:399-416.
36. Antonioli L, Pacher P, Vizi ES, Haskó G. CD39 and CD73 in immunity and inflammation. *Trends Mol Med* 2013;**19**:355-67.
37. Canale FP, Ramello MC, Núñez N, Furlan CLA, Bossio SN, Serrán MG, et al. CD39 expression defines cell exhaustion in tumor-infiltrating CD8+ T cells. *Cancer Res* 2018;**78**:115-28.
38. Joshi VB, Geary SM, Gross BP, Wongrakpanich A, Norian LA, Salem AK. Tumor lysate-loaded biodegradable microparticles as cancer vaccines. *Expert Rev Vaccines* 2014;**13**:9-15.
39. Neller MA, López JA, Schmidt CW. Antigens for cancer immunotherapy. *Semin Immunol* 2008;**20**:286-95.
40. Chiang C, Coukos G, Kandalaft L. Whole tumor antigen vaccines: where are we? *Vaccine* 2015;**3**:344-72.
41. Lybaert L, Ah Ryu K, De Rycke R, Chon AC, De Wever O, Vermaelen KY, et al. Polyelectrolyte-enrobed cancer cells in view of personalized immune-therapy. *Adv Sci* 2017;**2**:1700050.