BILINGUAL PUBLISHING CO.
Advances in Modern Oncology Research
doi: 10.30564/amor.v5i2.234

Article

Synthesis of Nano-grapheneoxide for Anti-tumor Photothermal Therapy and Immunogenic Death

Deep Fernandes[1] **Kamini Yadav**[2*]

1. Department of Community and Family Medicine, AIIMS, Bhubaneswar, Odisha, India
2. Department of Radiodiagnosis, AIIMS, Bhubaneswar, Odisha, India

***Correspondence Author:** Kamini Yadav, Department of Radiodiagnosis, AIIMS, Bhubaneswar, Odisha, India; Email: kyadv@yahoo.com

Abstract: Nano-grapheneoxide (NGO) is a good photothermal conversion agent with strong absorption and good photothermal conversion efficiency at 808nm. The study investigated the photothermal anti-tumor effect and immunogenic death mediated by NGO under the stimulation of near-infrared (NIR) light. Cell viability experiments confirmed that NGO has a good photothermal conversion effect to kill tumor cells effectively. In addition, NGO can stimulate macrophages to up-regulate the expression of interleukin-6 (IL-6) and tumor necrosis factor (TNF-α), thereby enhancing antigen presentation to trigger immunogenic death. The experiments of local primary tumors and metastatic tumors were simulated. The results showed that NGO-mediated photothermal therapy was effective in ablation of local tumors, and immunogenic death significantly reduced the growth rate of distant tumors, which suggested that photothermal therapy based on NGO may induce the anti-tumor immune response. The enhanced immune system would locally killing the tumor in situ, thus achieving the effect of inhibiting the growth of distant tumors.

Keywords: Nano-grapheneoxide; Photothermal therapy; Immunogenic death

Received: 10 February 2019; ***Accepted:*** 26 February 2019; ***Published Online:*** 1 April 2019

1. Introduction

Tumor, as a disease with high morbidity and mortality, is not satisfactory due to its complexity and transferability [1]. It is reported that more than 90% of patients with malignant tumors die from the recurrence and metastasis of tumors [2]. Traditional chemotherapy or radiotherapy would cause damage to normal tissues, severe biological toxicity limited the development of chemotherapy and radiotherapy. Meanwhile, multi-drug resistance of tumor cells also largely limits the efficacy of chemotherapy [3]. Therefore, the development of anti-tumor therapy in the future urgently need to explore a safer, effective and biosafety treatment.

In recent years, high hopes have been placed on stimuli-responsive treatment. Photothermal therapy for tumor depends on the sensitivity of tumor cells to heat, photothermal materials absorb NIR light and convert it to heat to kill the hyperthermia-sensitive tumor cells [4-5]. Meanwhile, there are minimal damages on the surrounding normal cells. Grapheneoxide (GO) has been widely used in biomedicine because of its special physicochemical and biological properties [6-7]. GO carries a variety of active groups, making it more dispersible in aqueous solutions. In addition, it is an highly biocompatible material with extremely low cytotoxicity and low cost [8-9]. Importantly, the study found that GO has a good photothermal conversion ability. It can quickly rise to more than 45 °C in a short

time by absorbing NIR light, resulting in excessive heat that can directly kill tumor cells [10-12].

Immunotherapy, as a promising tumor treatment strategy, has obvious cure rate and survival advantage [13]. Photothermal therapy can cause tumor cells to die and generate cell fragments, which can cause immune response. Cell fragments, as an antigen, can activate, proliferate and differentiate immune cells. Then the immune effector substances antibodies and sensitized lymphocytes were produced in the process, which was defined immunogenic death. In addition, immunogenic death combined with immune adjuvant can induce specific anti-tumor immune response and effectively inhibit distal metastatic tumor [14-15].

2. Methods

2.1 Synthesis of Materials

1 g of graphene was dispersed in 23 ml of 98% sulfuric acid for 8 hours at the temperature of <20°C, and adding $KMnO_4$ (3g) slowly. The solution was mixed at 35°C~40°C for 30min and at 65°C~80°C for 45min. Then 46 ml of water was added in the solution, and the mixture was heated at 100°C for 30 min. Then 140 mL distilled water and 10 mL of 30% H_2O_2 solution were added to stop the reaction. The mixture was centrifuged and filtered repeatedly using 5% HCl aqueous solution and distilled water. 10 mL of GO solution (1g/L) was added in 1.2 g NaOH and dissolved for 3h to adjust pH to 1, washing with water for 3 times. The solution was then aminated with six-branched glycol (2g/L) and crushed by ultrasound for 5min. 1 mmol of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, Sigma-Aldrich, USA) were added in the solution, and ultrasonically crushing for 1 h. Afterwards, the GO solution was centrifuged at a speed of 12000 r/min, using 2 times the phosphate buffer to remove the polymer and multilayer graphene. After centrifugation and cleaning for 8 times, it was filtered in a 100 kda MWCO centrifugal filter (microporous) to obtain the NGO treated with aminoated six-branched-chain ethylene glycol used in this research.

2.2 Characterization of Materials

A ultraviolet-visible-near-infrared light spectrophotometer UV-Vis-NIR spectrophotometer was used to detect the absorption spectrum of NGO at 600~1100nm. Atomic force microscope (AFM) and dynamic light scattering instrument (DLS) were used to detect the morphology and size characteristics of NGO.

2.3 Photothermal Conversion Ability

100μl of NGO solution with a concentration of 40mg/L or 80mg/L were added into a 1mL centrifuge tube respectively, 808 nm laser were used to irradiate NGO solution at a power of 0.5~2.5W/cm^2 for 5min, and using an infrared thermal imaging camera to record the temperature every 30 s.

2.4 Anti-tumor Effect in vitro

4T1 cells were cultured with 1640 medium containing 10% FBS in an incubator containing 5% CO_2 at 37 °C. NGO solutions with different concentrations were added to 4T1 cells respectively, and they were irradiated with NIR lasers of different powers for 5 minutes. After 24 hours of culture, the survival rates of tumor cells were detected. 10μl of MTS (Promega, USA) were added to each well to incubate for 2h, then detecting the absorbance (A) value of each well at 490nm with a microplate reader. The blank holes of the simple culture medium in parallel were used as zero with the experiment, recording the value of A. Repeat each experiment 3 times and take the average value, and the cell survival rate can be expressed as:

Survival rate = (experimental group A value - background A value) / (blank group A value - background A value) × 100%.

Apoptosis detection: The 4T1 cells with different treatment were subjected to FITC-AnnexinV/7AAD (Millipore, Germany) double staining, and then flow cytometry was used to analyze the killing effect of tumor cells.

2.5 Detection of Immune Response

Different concentrations of NGO were added to the macrophage RAW264.7 (2×10^5), and the expression levels of IL-6 and TNF-α (R&D, USA) in the cell supernatant were detected by ELISA after 24 hours. 4T1 cells (2×10^5) were divided into three groups for different treatments: blank control group, 808 nm laser (2W/cm^2, 5min) group, 808nm laser (2W/cm^2, 5min) combined with 40 mg/L of NGO group, and treated tumors cells were co-cultured with macrophages (2×10^5). After 24 hours, the concentrations of IL-6 and TNFα in the cell supernatant were detected by ELISA.

2.6 Anti-tumor Effect in vivo

4T1 breast cancer cells were simultaneously implanted on both sides of the back of female Balb/C mice, and treat the left tumor when the tumor volume grows to

10.30564/amor.v5i2.234

about 100 mm^3. The laser treatment group treated with 808 nm NIR laser (2W/cm2, 5min). The laser + NGO group was injected with NGO (1 mg/kg) locally and irradiated with 808 nm laser (2W/cm^2, 5min). After 6 hours, the temperatures of the tumor surface were recorded by infrared thermal imager during the treatment. After treatment, the tumor volume changes on the left and right sides were observed.

3. Results and Discussion

3.1 Characterization of Materials

NGO were two-dimensional and crystalline. In the sp2 hybrid orbital domain, monolayer atomic layers were arranged in a honeycomb lattice [16-17]. UV-Vis-NIR spectrophotometer was used to measure the absorption capacity of NGO in the NIR region. As shown in Figure 1, NGO had a significant absorption peak at 600~1100 nm. Further, AFM and DLS were used to observe the morphological characteristics and size of NGO. The image of AFM showed that the NGO had a layered dispersed structure (Figure 2a). DLS results showed that NGO had a peak at 40-60 nm and a low peak at 200-400 nm (Figure 2b). The results indicated that NGO have been successfully prepared and have good absorption in the NIR region with the potential of photothermal therapy.

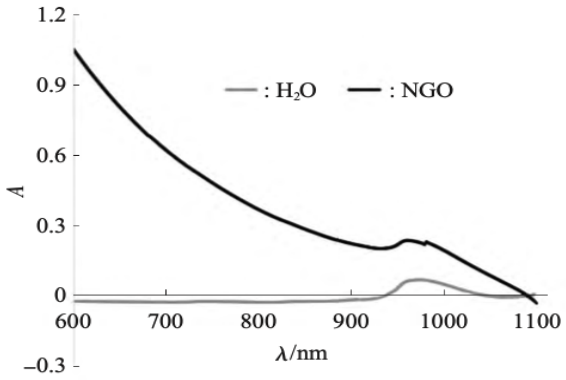

Figure 1. Absorption spectra of NGO

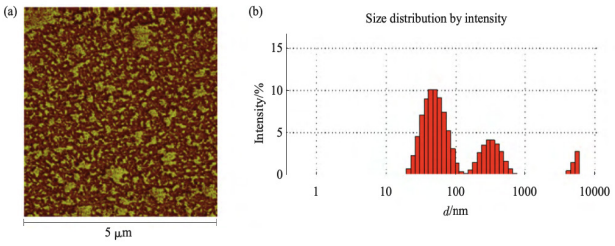

Figure 2. The morphological characteristics of NGO. (a) The images of NGO detected by AFM. (b) The size distribution of NGO detected by DLS

3.2 Photothermal Conversion Performance

The photothermal conversion performance of NGO was evaluated by the temperature change over time with an 808 nm laser irradiated by different concentration (2 W/cm^2). As shown in Figure 3a, at the same light intensity, the temperature of NGO solution was significantly higher than that of water, indicating that NGO had a good photothermal conversion capability. In addition, the NGO had a significant dependence on light intensity, and it could be heated to 60 °C under the 2.5 W/cm^2 in 60s (Figure 3b). As the concentration of NGO increased, the efficiency of the photothermal conversion became faster, reaching the critical temperature of tumor cell death within 40s at the concentration of 80mg/L.

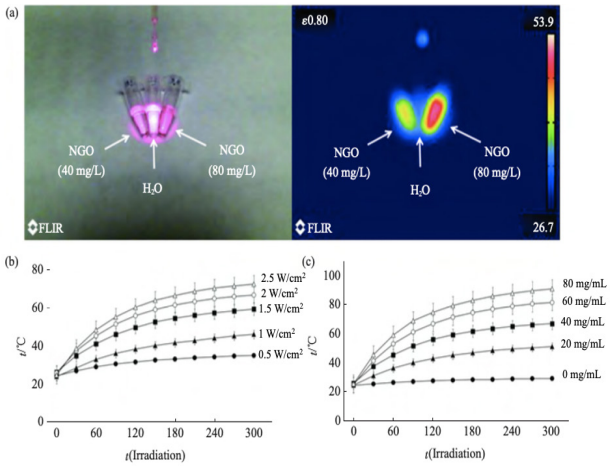

Figure 3. The photothermal conversion effect of NGO under laser irradiation. (a) A representative thermographic image of NGO solution (40 mg/mL and 80 mg/mL) and H_2O under laser irradiation (2 W/cm^2). (b) Temperature increase of NGO solution (40 mg/mL) under the laser irradiation with different doses

3.3 Anti-tumor Effect in vitro

The MTT experiment was used to detect the survival rate of 4T1 cells after the cells were incubated with NGO (40 mg/L) under the different power of laser, and the results were shown in Figure 4. With the increase of laser power, the killing effect of laser combined with NGO treatment on tumor cells has gradually increased. Under the power of 2W/cm^2, a greater killing effect can be achieved. However, the killing effect of laser treatment alone on tumor cells was not obvious, which indicates that the effect of laser irradiation at 808 nm alone on the activity of tumor cells was limited. Combined with NGO, the photothermal conversion can be effectively enhanced to achieve the anti-tumor effect.

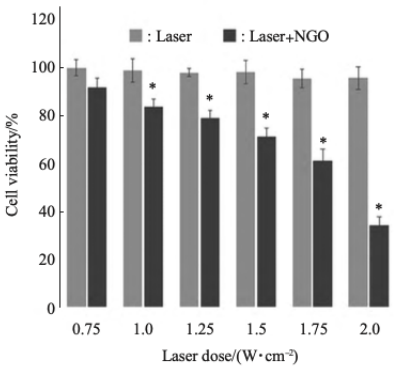

Figure 4. The anti-tumor effects in vitro of NGO (40 mg/mL) combined with the different doses of laser irradiation (*P<0.05)

Subsequently, the 4T1 cells were treated with 808 nm laser (2W/cm^2) combined with NGO treatment at two concentrations of 20mg/L and 40mg/L, and the killing effects of tumor cells were analyzed by flow cytometry. As shown in Figure 5, compared with the control group and the laser treatment group alone, the laser treatment combined with NGO was effective in anti-tumor effect. Compared with NGO with a concentration of 20mg/L, NGO with a concentration of 40mg/L were able to achieve better tumor cell killing effect.

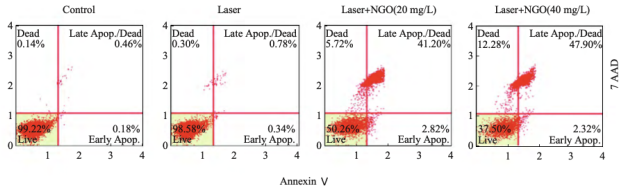

Figure 5. Cell death analysis under laser irradiation combined with NGO

3.4 Immunogenic Death Mediated by NGO

Macrophages are involved in innate and adaptive immune responses, such as phagocytes, antigen presenting cells and effector cells of delayed hypersensitivity[18]. The RAW264.7 is a kind of immortalized macrophages, which was used in the study. IL-6 and TNF-α are important cytokines for immune cells to control and affect the ability of cancer cells to proliferate, so they were used as common indicators for studying anti-tumor immune responses [19-25]. The macrophages RAW264.7 were co-incubated with the different concentrations of NGO, and immune-related cytokines were detected by ELISA kits. As shown in Figure 6a, TNF-α and IL-6 gradually increased with the increase of NGO concentration, which indicates that NGO has a concentration-dependent immune stimulation effect. Subsequently, tumor cells with different treatment (cells treated with saline: Control group, cells treated with laser: Laser group, cells treated with Laser+NGO: Laser+NGO group) were co-cultured with macrophages, and the results showed that the cytokine release level of the laser combined with NGO group was significantly higher than that of the blank control group and the laser treatment group alone (2W/cm^2, the concentration of NGO: 40mg/L), which showed that laser combined with NGO therapy effectively stimulated macrophage activation at the cellular level (Figure 6b). In vivo, laser combined with NGO therapy significantly increased tumor site temperature compared to the laser treatment group alone.

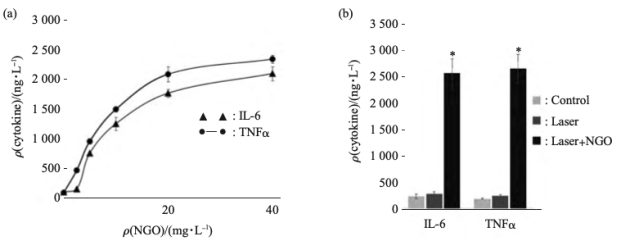

Figure 6. Cytokines secretion by macrophages stimulated by indicated treatments. Cytokines secretion by macrophages stimulated by NGO at different concentrations (a) and treated tumor cells with 40mg/mL of NGO (b) (*P<0.05)

3.5 Anti-tumor Effect in vivo

As shown in Figure 7, the temperature in tumor site were significantly increased in the mice treated with laser combined with NGO, which was higher than that of laser treatment group alone. The inhibition of NGO on tumor metastasis in mice was then tested. Tumors were planted on both sides of the back of the mice, and only left side tumors were treated (the NGO dose used in animal experiments was 1mg/kg). After that, tumor growth at the treated site and the untreated site on the right side were observed. As shown in Figure 8a, the tumor growth curve on the left side of the back showed that compared with the blank control group, the laser treatment group slightly inhibited tumor growth, while the laser combined with NGO treatment group significantly inhibited tumor growth. According to the analysis of tumor growth curve, after 3d, the tumors in the laser combined with NGO treatment group began to shrink, while the tumor volume in the control group and laser treatment group continued to grow. At 15d, the tumor volume in the laser combined with NGO treatment group was basically eliminated, while the tumor volume in the control group and laser treatment group reached more than 600mm^3 (Figure 8b). The results showed that laser combined with NGO can produce significant photothermal effect and kill tumor effectively.

10.30564/amor.v5i2.234

The tumor growth curve on the right side of the back showed that compared with the blank control group and the single laser treatment group, the laser combined with NGO treatment group significantly inhibited the growth of tumors. At 15d, the tumor volume of the laser combined with NGO treatment group was maintained at about 300 mm^3, while that of the control group and laser treatment group was up to about 500 mm^3. The laser combined with NGO did not directly affect the tumor on the right side of the back of mice, however, the growth of the tumor on the right side of the back was significantly inhibited. The results showed that the combination of laser and NGO photothermal therapy may stimulate the immune response of the body, thus inhibiting the growth of the same tumor in distant sites.

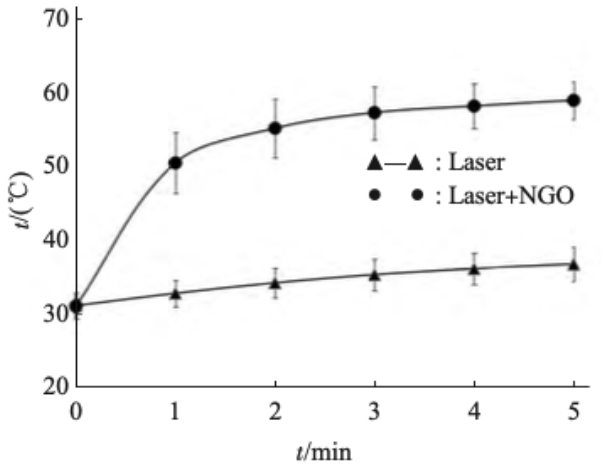

Figure 7. Temperature increase on the surface of tumor under laser irradiation with or without NGO (Laser: 2W/cm^2, 5min; NGO: 1mg/kg) (*P<0.05)

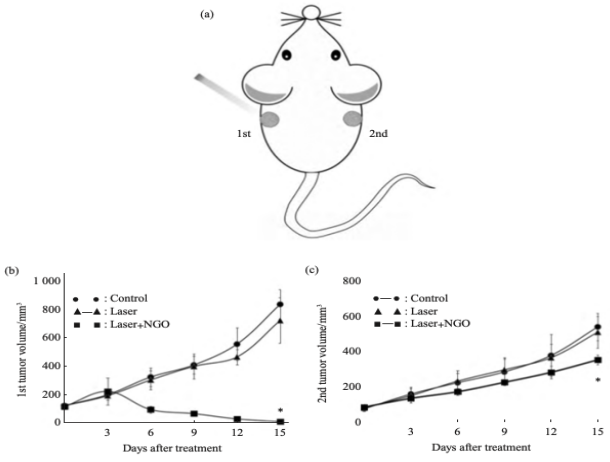

Figure 8. Volume of tumors under different treatments. (a) Diagram of the tumor-bearing animal model. (b) Volume of the 1st tumor treated under indicated 2nd tumor after the treatment on the 1st tumor (*p<0.05)

4. Conclusion

Metastatic malignancies have the characteristics of high refractory and high mortality in clinic. Surgical treatment is often not appropriate for patients with advanced tumors with distant metastasis. Traditional treatments such as radiotherapy and chemotherapy are limited in clinical application due to high side effects and patient intolerance. Therefore, looking for new, low-toxicity, high-efficiency, and new treatment strategies, which can simultaneously produce therapeutic effects on metastatic tumors, is of great significance for the treatment of advanced tumors. NGO has a good photothermal conversion ability, combined with laser treatment, can effectively produce overheating effects, so as to achieve the purpose of tumor killing. The results of photothermal experiments showed that in the NGO combined laser treatment strategy, both laser power and NGO concentration exist dose-dependent effect. In addition, with the increase of laser power and NGO concentration, the killing effect of tumor cells has also been significantly improved. And in cell experiments, NGO solution can effectively stimulate macrophages to produce IL-6 and TNF-α. Compared with the control group and the laser group, NGO combined with laser treatment can effectively promote the production of IL-6 and TNFα by macrophages， which suggested that NGO combined Laser treatment can effectively stimulate the production of anti-tumor immune responses. In vivo experiments have shown that compared to laser treatment group, NGO combined with laser treatment induced a more ideal photothermal response, producing higher thermal effects, which effectively damaged tumor tissue. In addition, in the NGO combined laser treatment group, although distal tumors did not receive any local treatment, but the growth rate was significantly lower than that of the control group and the laser treatment group. So, the combination of NGO and laser treatment can locally kill the tumor in situ, and at the same time may induce immunogenic death, thereby achieving the effect of inhibiting the growth rate of tumors in remote sites.

References

[1] Torre L A, Bray F, Siegel R L, et al. Global cancer statistics, 2012. CA Cancer J Clin, 2015, 65(2): 87-108.

[2] Fidler I J, Kripke M L. The challenge of targeting metastasis. Cancer Metastasis Rev., 2015, 34(4): 635-641.

[3] Chen W R, Adams R L, Bartels K E, et al. Chromophore-enhanced in vivo tumor cell destruction using an 808-nm diode laser. Cancer Lett, 1995, 94(2): 125-131.

[4] Chen W R, Adams R L, Higgins A K, et al. Photothermal

effects on murine mammary tumors using indocyanine green and an 808-nm diode laser: an in vivo efficacy study. Cancer Lett, 1996, 98 (2): 169-173.

[5] Geim A K, Novoselov K S. The rise of graphene. Nat Mater, 2007, 6(3): 183-191.

[6] Bitounis D, Ali-Boucetta H, Hong B H, et al. Prospects and challenges of graphene in biomedical applications. Adv Mater, 2013, 25(16): 2258-2268.

[7] Zhou X, Liang F. Application of graphene/graphene oxide in biomedicine and biotechnology. Curr Med Chem, 2014, 21 (7): 855-869.

[8] Yang K, Zhang S, Zhang G, et al. Graphene in mice: ultrahigh in vivo tumor uptake and efficient photothermal therapy. Nano Lett, 2010, 10(9): 3318-3323.

[1] Luo N, Weber J K, Wang S, et al. PEGylated graphene oxide elicits strong immunological responses despite surface passivation. Nat Commun, 2017, 24(8): 14537-14547.

[10] Takemoto M, Kuroda M, Urano M, et al. The effect of various chemotherapeutic agents given with mild hyperthermia on different types of tumours. Int J Hyperthermia, 2003, 19(2): 193-203.

[11] Wen Q L, He L J, Ren P R, et al. Comparing radiotherapy with or without intracavitary hyperthermia in the treatment of primary nasopharyngeal carcinoma: a retrospective analysis. Tumori, 2014, 100(1): 49-54.

[12] Hainfeld J F, Lin L, Slatkin D N, et al. Gold nanoparticle hyperthermia reduces radiotherapy dose. Nanomedicine, 2014, 10(8): 1609-1617.

[13] Zhou F, Li X, Naylor M F, et al. InCVAX-a novel strategy for treatment of late-stage, metastatic cancers through photoimmunotherapy induced tumor-specific immunity. Cancer Lett, 2015, 359(2): 169-17.

[14] Chen W R, Liu H, Ritchey J W, et al. Effect of different components of laser immunotherapy in treatment of metastatic tumors in rats. Cancer Res, 2002, 62(15): 4295-4299.

[15] Yeung H Y, Lo P C, Ng D K, et al. Anti-tumor immunity of BAM-SiPc-mediated vascular photodynamic therapy in a BALB/c mouse model. Cell Mol Immunol, 2017, 14(2): 223-234.

[16] Kalluru P, Vankayala R, Chiang C S, et al. Nano-graphene oxide- mediated In vivo fluorescence imaging and bimodal photodynamic and photothermal destruction of tumors. Biomaterials, 2016, 95(16): 1-10.

[17] Yang K, Gong H, Shi X, et al. In vivo biodistribution and toxicology of functionalized nano-graphene oxide in mice after oral and intraperitoneal administration. Biomaterials, 2013, 34 (11): 2787-2795.

[18] De Santis M, Locati M, Selmi C. The elegance of a macrophage. Cell Mol Immunol, 2017 [Epub ahead of print]

DOI: 10.1038/cmi. 2017.64

[19] Cheng X L, Ding F, Li H, et al. Activation of AMPA receptor promotes TNF-α release via the ROS-cSrc-NFκB signaling cascade in RAW264.7 macrophages. Biochem Biophys Res Commun, 2015, 461(2): 275-280.

[20] Lu C L, Zhu W, Wang D M, et al. Inhibitory effects of chemical compounds isolated from the rhizome of smilax glabra on nitric oxide and tumor necrosis factor-α production in lipopolysaccharide-induced RAW264.7 cell. Evid Based Complement Alternat Med, 2015, 2015: 602425.

[21] Tripsianis G, Papadopoulou E, Anagnostopoulos K, et al. Coexpression of IL-6 and TNF-α: prognostic significance on breast cancer outcome. Neoplasma, 2014, 61(2): 205-212.

[22] De Simone V, Franzè E, Ronchetti G, et al. Th17-type cytokines, IL-6 and TNF-α synergistically activate STAT3 and NF-κB to promote colorectal cancer cell growth. Oncogene, 2015, 34 (27): 3493-3503.

[23] Feito M J, Vila M, Matesanz M C, et al. In vitro evaluation of graphene oxide nanosheets on immune function. J Colloid Interface Sci, 2014, 432(15): 221-228.

[24] Chen X M, Song E W. The role of tumor microenvironment in cancer immunotherapy. Prog Biochem Biophys, 2017, 44 (8): 641- 648.

[25] Chen Y F, Hong J, Shen J J, et al. Progress on clinical immunotherapy of malignant tumor. Prog Biochem Biophys, 2017, 44(8): 709-716.

10.30564/amor.v5i2.234