Gold nanoparticles targeted to tumor-associated macrophages in combination with radiotherapy enhance antitumor effect of radiotherapy

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Gold nanoparticles targeted to tumor-associated macrophages in combination with radiotherapy enhance antitumor effect of radiotherapy

Directed by Professor Ki Chang Keum

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ABSTRACT

Gold nanoparticles targeted to tumor-associated macrophages in combination with radiotherapy enhance antitumor effect of radiotherapy

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Background and purpose: Tumor-associated macrophages (TAMs) exhibit the M2 phenotype and serve as critical tumor-promoting immune cells in the tumor microenvironment. As TAMs are an important target, we examined the effect of gold nanoparticles (GNPs) with radiotherapy (RT) on M2 TAMs in tumors.

Materials and methods: We synthesized CD163 antibody-conjugated GNPs (CD163-GNPs) that were specifically recognized by M2 TAMs. Bone marrow-derived macrophages and Raw 264.7 macrophages were polarized into M1 and M2 phenotypes. The effect of GNPs combined

with RT was evaluated in a CT26 xenograft mouse model. Immunostaining, flow cytometry, microscopic analyses, enzyme-linked immunosorbent assay, quantitative real-time polymerase chain reaction, and tumor growth delay assay were performed following irradiation combined with GNP treatment.

Results: We observed selective phagocytosis of CD163-GNPs by Raw 264.7 macrophages following M1/M2 polarization. Immunostaining analyses revealed higher numbers of CD163-GNPs taken up by M2 macrophages than M0 or M1 type. CD163-GNPs combined with RT significantly reduced tumor growth in the CT26 xenograft mouse model. Macrophages subjected to the combination treatment showed increased expression of M1 markers.

Conclusion: The depletion of M2 TAMs in tumors upon combination treatment with CD163-GNPs enhances the efficiency of RT.

Key words: Tumor-associated macrophage; Gold nanoparticle; Radiotherapy; Tumor microenvironment

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I. INTRODUCTION

The therapeutic effect of radiation is mainly mediated through DNA damage, resulting in cytocidal activity. Studies on cancer immunology have shown that immune activation by ionizing radiation is another important mechanism for mediating cytocidal effects[1]. Radiation enhances the recruitment of macrophages at the tumor site and affects tumor-associated macrophage (TAM) polarization [2, 3].

Macrophages have a critical role in the innate and adaptive immunity [4], and are involved in inflammatory process, antigen recognition, and homeostasis.

Tumor cells recruit macrophages by producing chemokines and cytokines[5]. Macrophages promote tumor cell invasion, migration, and proteolysis and facilitate their survival [6]. Macrophages are differentiated into type 1 and/or type 2 macrophages, and their relative abundance varies according to the tumor microenvironment [7]. The M2 TAMs are known to strongly promote tumor cell invasion and angiogenesis as compared with M1 type macrophages [8]. The high density of M2 TAMs is associated with poor prognosis [9].

Nanoparticles (NPs) in combination with radiotherapy (RT) are well known for enhancement of antitumor effects [10, 11]. Uptake of NPs by macrophages reduces their circulation time in the body and has been considered a barrier in nanomedicine applications in the past studies [12]. However, recent studies have considered macrophages that uptake NPs as a target for antitumor treatment[13, 14]. The modulation of macrophage polarization is important for the maintenance of host defense and may serve as a strategy for the treatment of associated diseases[15].

We hypothesized that M2 TAM-targeting gold nanoparticles (GNPs) in combination with RT may increase the population of M1 TAMs and reduce the number of M2 TAMs in the tumor microenvironment, resulting in the enhancement of antitumor effects. We investigated the effect of GNPs combined with RT on TAMs and evaluated the potential of GNPs to increase the antitumor effects of RT.

II. MATERIALS AND METHODS

1. Reagents

Gold (III) chloride trihydrate 99.9% (Sigma-Aldrich, St. Louis, Missouri, USA), sodium citrate tribasic dehydrate (Sigma-Aldrich, St. Louis, Missouri, USA), polyvinylpyrrolidone (PVP) K-15 (Junsei, Tokyo, Japan), ethyl alcohol 99.9% (Duksan, Ansan, Korea), ammonia water 30% (Duksan, Ansan, Korea), tetraethyl orthosilicate (TEOS) 99% (Sigma-Aldrich, St. Louis, Missouri, USA), 3-aminopropyl triethoxysilane (APTES) (Sigma-Aldrich, St. Louis, Missouri, USA), 5-Azido-2-nitrobenzoic acid N-hydroxysuccinimide ester (ANB-NOS) (Thermo Fisher scientific, Waltham, MA, USA), deionized (DI) water (Samchun, Seoul, Korea), CD163/M130 polyclonal antibody (Bioss antibodies, Boston, Massachusetts, USA), phosphate-buffered saline (PBS; pH7.4) (Gibco, Carlsbad, CA, USA), phosphate buffer (pH 8.5) (Bioworld, Dublin, Ohio, USA), and N,N-dimethyl formamide (DMF) 99.9% (Duksan, Ansan, Korea) were the chemicals purchased.

2. Synthesis of silica-coated gold nanoparticles (GNP@SiO_2)

Fifty nanometer GNPs were prepared by Turkevich/Frens reaction system using sodium citrate to reduce Au^{3+} and enlarge gold seeds [16]. To prevent aggregation of GNPs, 2 mL of GNP (170 μg/mL) solution and 0.42 mL of PVP solution in DI water (25.6 mg/mL) were mixed for 24 h under vigorous stirring. The PVP-treated GNP solution was washed once with DI water and dispersed in 4 mL of ethanol. The solution was treated with 6 μL of TEOS in 200 μL of ammonia solution or 2 μL of TEOS in 300 μL of ammonia solution to obtain 20 or 50 nm silica layer, respectively, under continuous stirring for 24 h at room

temperature. After being washed twice with ethanol, 20 and 50 nm GNP@SiO_2 were dispersed in 1 mL of ethanol.

3. Immobilization of antibody on GNP@SiO_2

A total of 1 mL of GNP@SiO_2 was reacted with 50 μL of APTES solution and gently mixed for 1 h at room temperature using a vertical rotating mixer, followed by 1 h curing at 90°C. After the removal of excess APTES by centrifugation (twice) with ethanol, the APTES-modified GNP@SiO_2 were dispersed in 1 mL of phosphate buffer (pH 8.5) and treated with 5 mM ANB-NOS in DMF using vertical rotating mixer for 2 h in the dark. The unreacted ANB-NOS was removed by centrifugation and the modified particles were dispersed in 0.5 mL of PBS (pH 7.4) and incubated with 0.5 mL of CD163 antibody solution (20 μg/mL) diluted in PBS. The solution was exposed to UV light (320 to 500 nm) for 180 s to conjugate the amide functional group of proteins with the terminal azide group of ANB-NOS-treated GNP@SiO_2. The antibody-conjugated GNP@SiO_2 were washed with PBS to remove excess antibodies by centrifugation (once) and stored in PBS (Figure 1A).

4. Cell culture conditions

Bone marrow-derived macrophages (BMDMs), Raw 264.7 macrophages, and a murine colorectal cancer cell line CT26 were used. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics and maintained at 37℃ in a 5% CO_2 incubator. The cells were subcultured every 3-4 days to maintain exponential growth.

5. Monocyte isolation and macrophage polarization

To confirm macrophage differentiation and polarization, fluorescence-activated cell sorting (FACS) and real-time quantitative polymerase chain reaction (RT-qPCR) were performed. BMDMs and Raw 264.7 cells were treated with indicated cytokines. For polarization into M2, cells were treated with 20 ng/mL of interleukin 4 (IL4) and IL13 for 72 h, followed by washing with fresh media. For polarization into M1, cells were incubated with 10 ng/mL of lipopolysaccharide (LPS) and 20 ng/mL of interferon (IFN)-γ for 72 h, followed by washing with fresh media. After incubation, the cells were stained for major histocompatibility complex II (MHC II)-allophycocyanin (APC) (Biolegend, San Diego, CA, USA), CD206-phycoerythrin (PE) (Biolegend, San Diego, CA, USA), and CD11b-fluorescein isothiocyanate (FITC) (BD biosciences, San Jose, CA, USA) for 30 min following polarization. Cells were evaluated by flow cytometry analyses using FACSVERSE flow cytometer (BD biosciences, San Jose, CA, USA). Total RNA was extracted using RNeasy plus mini kit (QIAGEN, Hilden, Germany) and reverse transcribed to cDNA as a template for PCR amplification. PCR was performed using Taq polymerase and 10 pmol/L of primers (Arg1(72)) under following conditions: initial denaturation, 40 cycles of denaturation, annealing, extension, and final elongation. PCR products were analyzed with StepOne^{TM} Software v2.3.

6. Immunofluorescence staining

Phagocytosis was studied by immunohistochemical analyses. Macrophages were cultured in a 24-well plate for 24 h. M1 and M2 cells were treated with CD163-conjugated GNP@SiO_2 (CD163-GNP) (100 μg/mL) for 24 h and incubated with fluorescent probes, followed by quantification using a fluorescence microscope (Zeiss, Germany). Fluorescence signal intensity

was compared using corrected total cell fluorescence (CTCF) between cell groups.

7. Xenograft tumor model and tumor growth delay (TGD) assay

We complied with the National Institutes of Health guide for the care and use of Laboratory animals. A CT26 mouse colon cancer model was developed to investigate the effect of GNP combined with RT on tumor growth. We subcutaneously injected 1×10^6 CT26 mouse colon cancer cells into the thigh of BALB/c mice. After the tumor volume reached 200 mm^3, 100 μL of CD163-GNPs (750 μg/mL) were injected into tumors. The tumors were irradiated with 10 Gy in a single fraction using X-Rad 320 irradiator (Precision X-Ray). The mice were placed at a distance of 69 cm from the radiation source and treated at a dose rate of 150 cGy/min with 300 kVp X-rays using 12.5 mA and an X-ray beam filter consisting of 2.0 mm aluminum. Tumor volume was calculated by the formula $0.5 \times ab^2$, where a is the long axis and b is the short axis of two orthogonal diameters.

8. CT26 colorectal cancer cell and Raw 264.7 cell co-culture

For polarization into M2, Raw 264.7 cells were treated with 20 ng/mL of IL4 and IL13 for 72 h, followed by washing with fresh media. A total of 4×10^4 M2 TAMs were plated in the lower chamber of a permeable transwell and 5×10^4 CT26 mouse colon cancer cells were plated in the upper chamber. Cells were treated with CD163-GNPs (5 μg/mL) for 6 h and washed with new media. The cells were irradiated with 5 Gy in a single fraction using X-Rad 320 irradiator (Precision X-Ray). After irradiation, co-cultures were maintained in RPMI-1640 medium supplemented with 10% FBS (Thermo Fisher scientific, Waltham, MA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin

(Thermo Fisher scientific, Waltham, MA, USA) for 24 and 48 h. The cells were treated with trypan blue and cell viability was evaluated using TC20TM Automated Cell Counter (BIO-RAD, Hercules, CA, USA). To confirm macrophage differentiation and polarization after co-culture, RT-qPCR was performed. Raw 264.7 cells previously polarized into M2 TAMs were treated with indicated cytokines (inducible nitric oxide synthase [iNOS], tumor necrosis factor alpha [TNFα], and arginase 1 [Arg1]). Total RNA was extracted using RNeasy plus mini kit (QIAGEN, Hilden, Germany) and reverse transcribed into cDNA as a template for PCR amplification. PCR was performed using Taq polymerase and 10 pmol/L of primers (Arg1(72)) using the following conditions: initial denaturation, 40 cycles of denaturation, annealing, extension, and final elongation. PCR products were analyzed with StepOne^{TM} Software v2.3.

9. Macrophage phenotype in tumor

To evaluate the phenotype of macrophages after treatment with the combination of CD163-GNPs and RT, FACS, immunohistochemical staining, and RT-qPCR were performed.

10. Statistical analysis

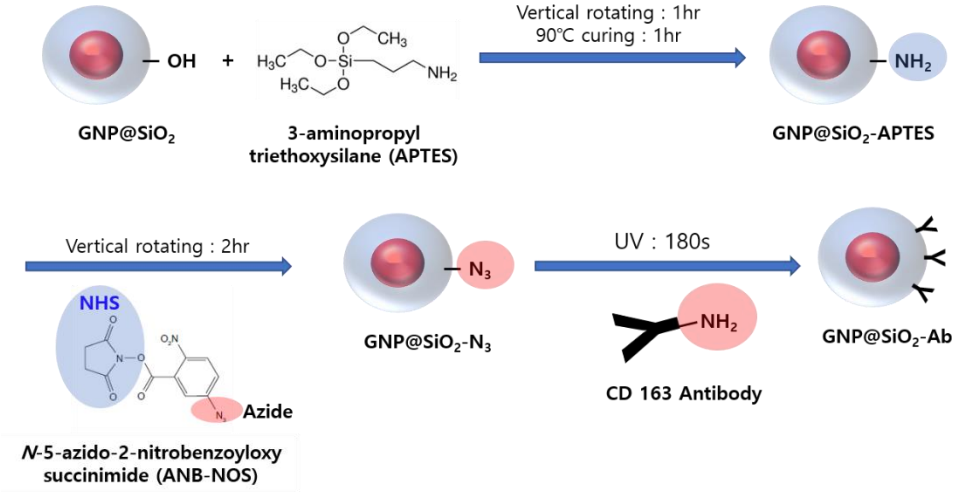
All graphs and statistical analyses were performed using the GraphPad *Prism* version 5.04 software (GraphPad Software, San Diego, CA, USA). Comparison of results was carried out using t-test and repeated-measures analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

III. RESULTS

1. GNP characterization

The formation of GNP@SiO_2 was confirmed with transmission electron microscopy (TEM) analysis as shown in Figure 1B. TEM image confirmed the core shell structure of NPs, wherein the thickness of the silica shell could be controlled with the addition of different amounts of ammonia and TEOS solutions. For targeting M2 TAMs, the antibody was immobilized on the silica surface via ANB-NOS, which is an amine-amine cross-linker that allows covalent attachment of proteins. Fourier transform infrared (FTIR) and UV-vis absorbance experiments were carried out to investigate the conjugation of antibody to GNP@SiO_2 (Figure 1C, 1D). FTIR spectra confirmed that only the antibody-conjugated GNP@SiO_2 samples showed two strong peaks at 1,542 and 1,575 cm^{-1} attributed to N-H stretch (amide II) and C=O stretch (amide I), respectively, that originated from the peptide bonds in the antibody protein (Figure 1C). The absorbance spectra in Figure 1D show that all particles had distinct, characteristic absorption peaks at approximately 540 nm, attributed to the surface plasmon absorbance of GNPs. Moreover, the absorbance of GNPs, GNP@SiO_2, and GNP@SiO_2-Ab showed a sequential red shift phenomenon owing to an increase in particle size, which also confirmed the apparent immobilization of antibody.

A



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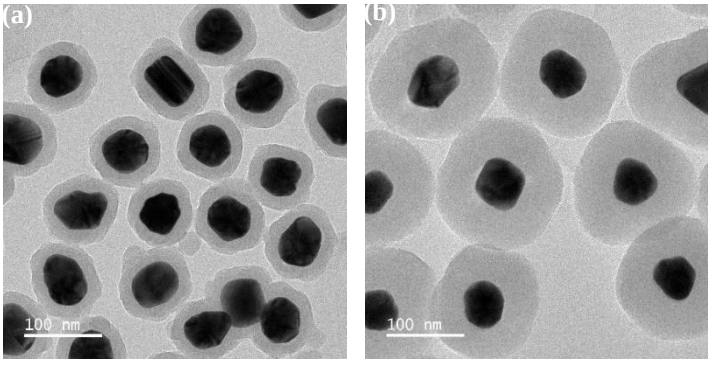


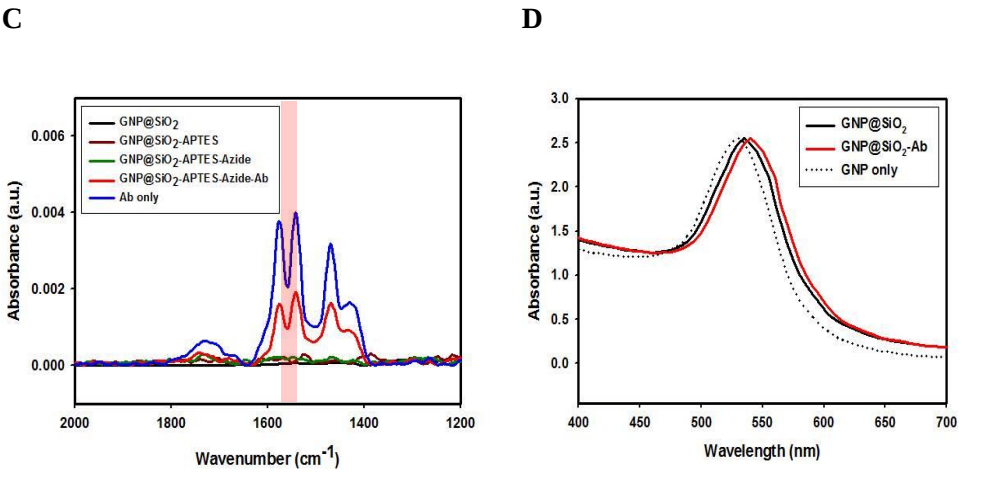


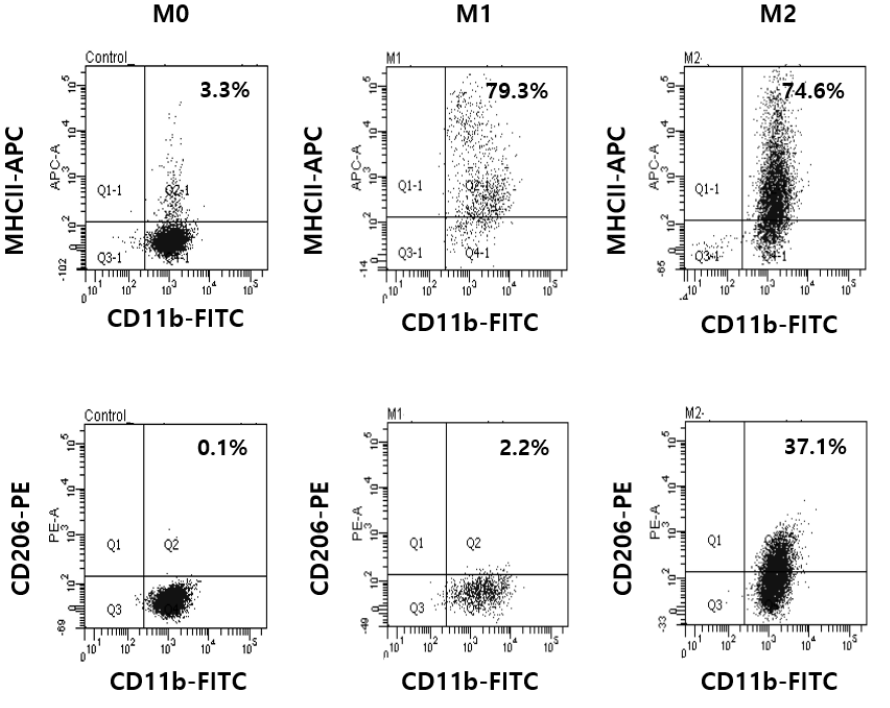
Figure 1. Gold nanoparticle (GNP)s

(A) Schematic illustration of the synthesis of antibody-conjugated silica-coated gold nanoparticle (GNP@SiO2) (B) Transmission electron microscopy image of GNP@SiO2 (a) 20㎚ of silica layer (b) 50㎚ of silica layer (C) Fourier transform infrared spectra at each steps of antibody immobilization of GNP@SiO2 (D) UV-vis absorption spectra of GNP, GNP@SiO2 and GNP@SiO2-Ab.

2. Polarization of macrophages

The polarization of macrophages was assessed with FACS and RT-qPCR. After treatment with IL4 and IL13, Raw 264.7 cells showed an increase in the expression of an M2 marker, CD206 (Figure 2A). Macrophages after treatment with LPS and IFN-γ showed an increase in the mRNA expression of iNOS and TNFα. Macrophages after treatment with IL4 and IL13 showed an increase in the mRNA expression of Arg1 (Figure 2B).

A



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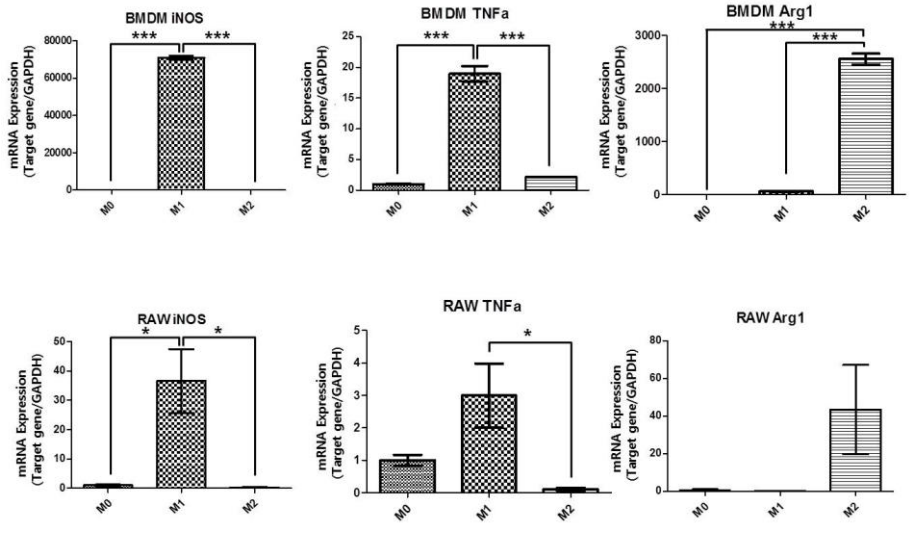

Figure 2. Polarization of macrophages (* = P-value <0.05, *** = P-value <0.0001)

(A) Fluorescence-activated cell sorting (FACS) results. Type 1 macrophage marker: MHCII, type 2 macrophage marker: CD206, pan marker: CD11b. (B) The mRNA expression of type 1 and type 2 macrophage markers in bone marrow derived monocyte (BMDM) and RAW 264.7 cells treated with cytokines.

3. GNP phagocytosis

We tested the M2-targeting capacity of CD163-GNPs using immunofluorescence and TEM image. Phagocytosis was determined after 24 h treatment with CD163-GNPs or GNPs (Figure 3). CD163-GNPs were stained with PE (red) (Figure 3A). Red fluorescence was observed more often in M2. The amount of phagocyted GNPs was compared using CTCF. The CTCF of M2 was significantly higher than that of M1 (Figure 3B). Phagocyted CD163-GNPs and GNPs within M2 were imaged using TEM (Figure 3C-3F). We evaluated the number of phagocyted GNPs within M2 using image J. CD163-GNPs were phagocyted more than GNPs in M2 (Figure 3E).

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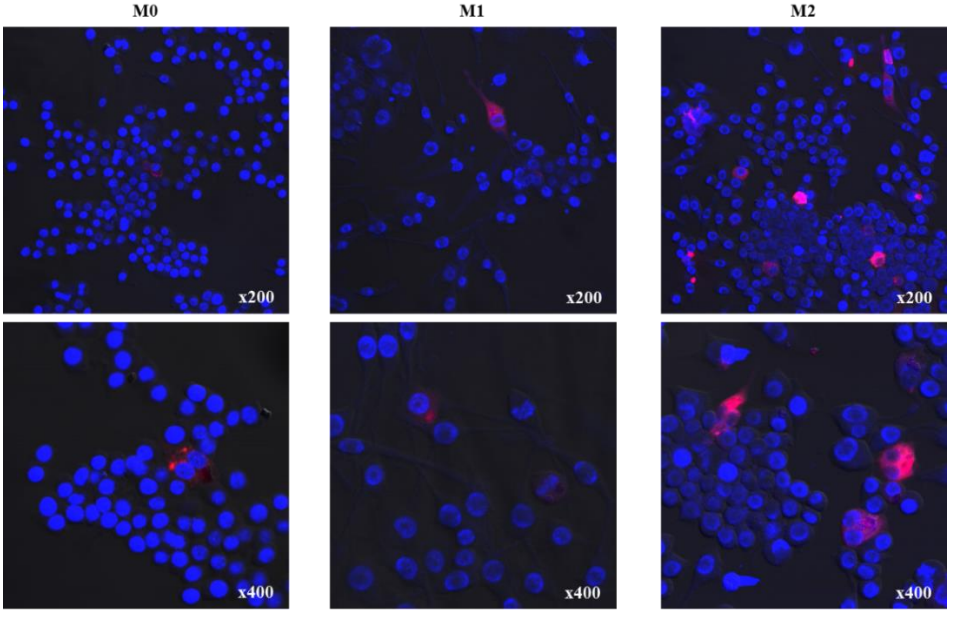

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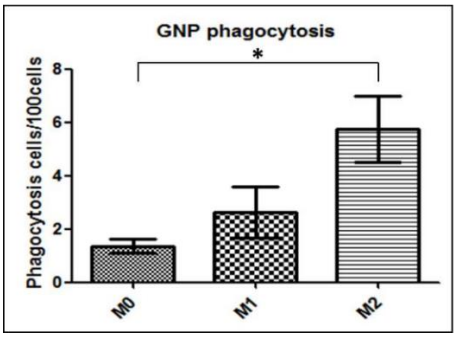

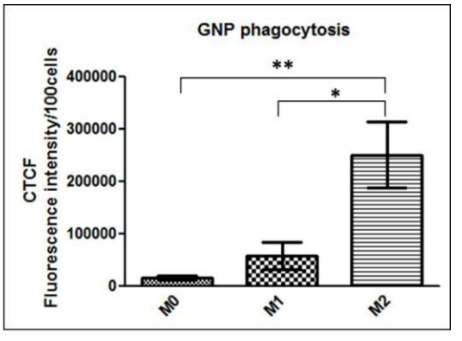

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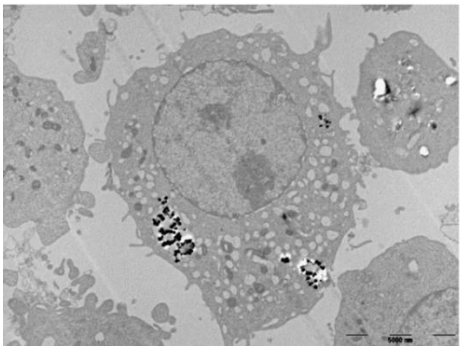

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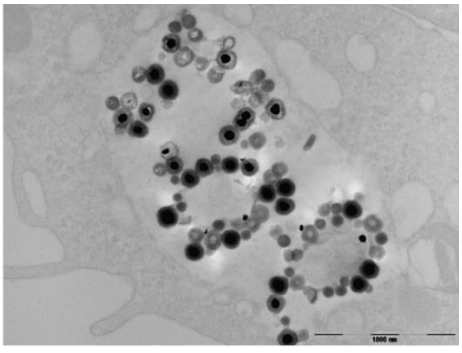

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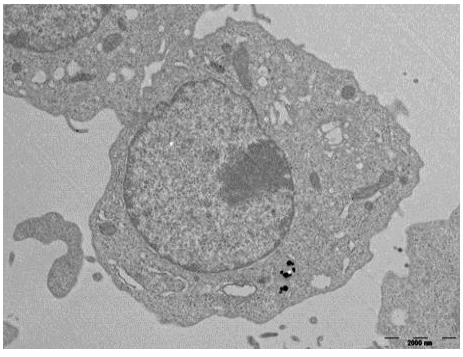

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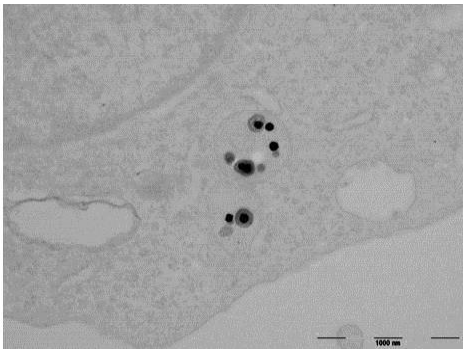

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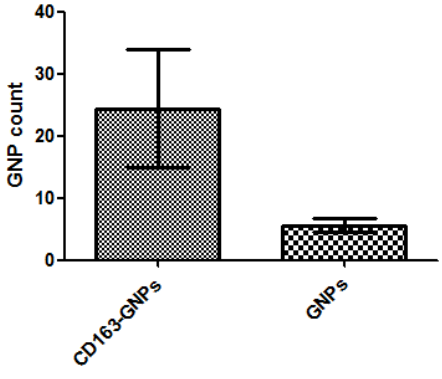

Figure 3. CD163-GNP@SiO_2 phagocytosis by macrophages (* = P-value <0.05, ** = P-value <0.005)

(A) Phagocyted CD163-GNP@SiO_2 in macrophages (red) (B) Corrected total cell fluorescence (CTCF) of macrophages, (C, D) Transmission electron microscopy images of phagocyted CD163-GNP@SiO_2 in type 2 macrophage (E, F) Transmission electron microscopy images of phagocyted GNP@SiO_2 in type 2 macrophage (C, E: 6000X magnification, scale bar = 5000nm, D, F: 40000X magnification, scale bar = 1000nm) (G) Number of phagocyted GNPs according to antibody conjugation.

4. TGD assay

To investigate the effect of the combination of GNPs and RT on TGD, tumors were treated with CD163-GNPs in the absence or presence of radiation. Tumor volume was compared in different treatment groups (Figure 4). CD163-GNPs alone showed no delay in the growth of tumor. In comparison with the control and GNP alone groups, the radiation group showed a decrease in the tumor volume 1 day after irradiation. Five days after irradiation, the tumor volume of RT alone group and the combination of CD163-GNP and RT group showed a difference. A week after irradiation, tumors from RT alone (10 Gy in a single fraction) group started to grow; however, tumors from the combination group continuously decreased.

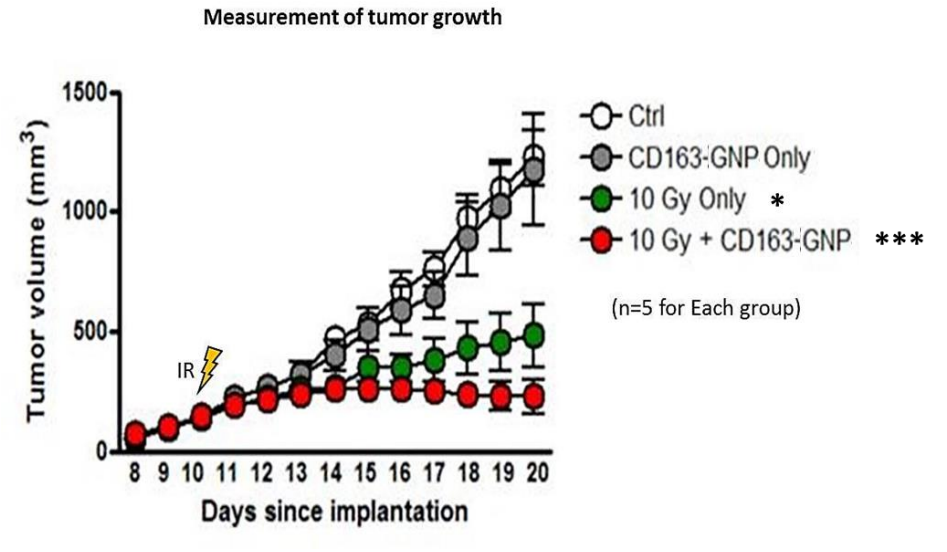

Figure 4. Tumor growth delay (* = P-value <0.05, *** = P-value <0.0001)
Empty circle: control, grey: tumor treated with CD163 antibody conjugated gold nanoparticles (CD163-GNP) only, green: tumor treated with 10 Gy in a single fraction only, red: tumor treated with CD163-GNP combined with 10 Gy radiation.

5. Polarization of macrophages in tumors after combination treatment

The polarization of macrophages in tumors was assessed with FACS, immunohistochemical staining, and qRT-PCR. After treatment of cells with the combination of CD163-GNPs and RT, the expression of MHCII (M1 marker) increased and that of CD206 (M2 marker) decreased (Figure 5A). Immunohistochemical staining showed an increase in M1 TAMs after combination treatment (Figure 5B, upper row). In comparison with control and GNP alone groups, the RT alone and combination treatment groups showed an increase in the yellow color signal (presenting macrophages). The combination

group had more yellow signal than the RT alone group. In contrast to M1 TAMs, M2 TAMs decreased after the combination treatment (Figure 5B, bottom row). In comparison with other groups, the combination treatment group showed lower amount of yellow signal. The results of RT-qPCR analysis were consistent with these observations (Figure 5C). We compared the mRNA expression and found that the combination treatment group showed an increase in *iNOS* and *TNF*α expression and a decrease in *Arg1* expression as compared with RT alone group.

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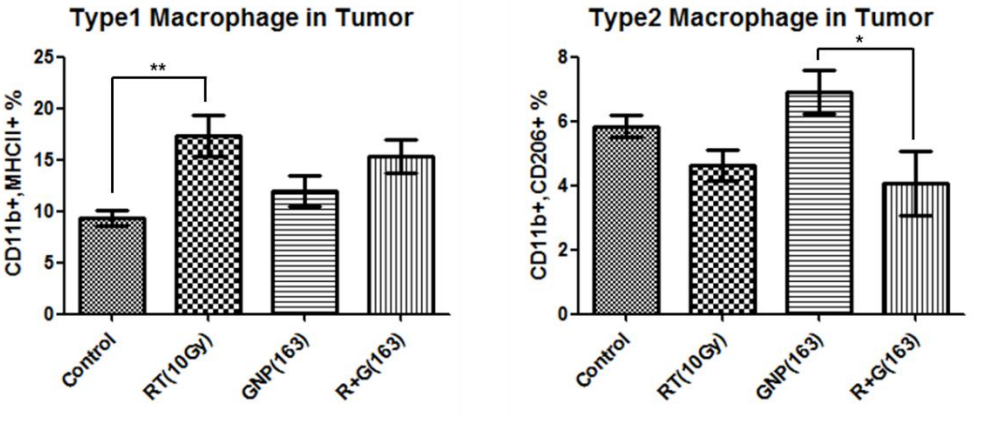

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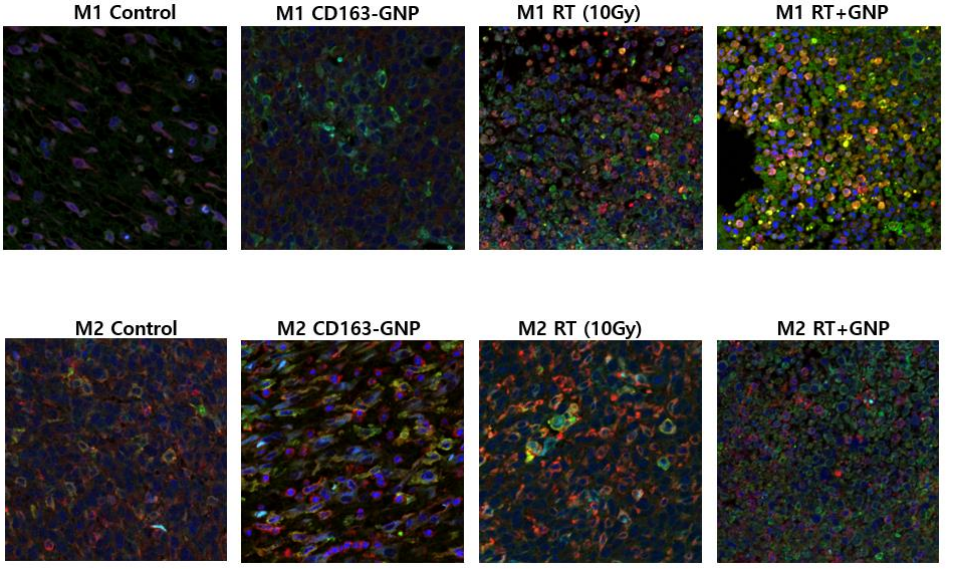

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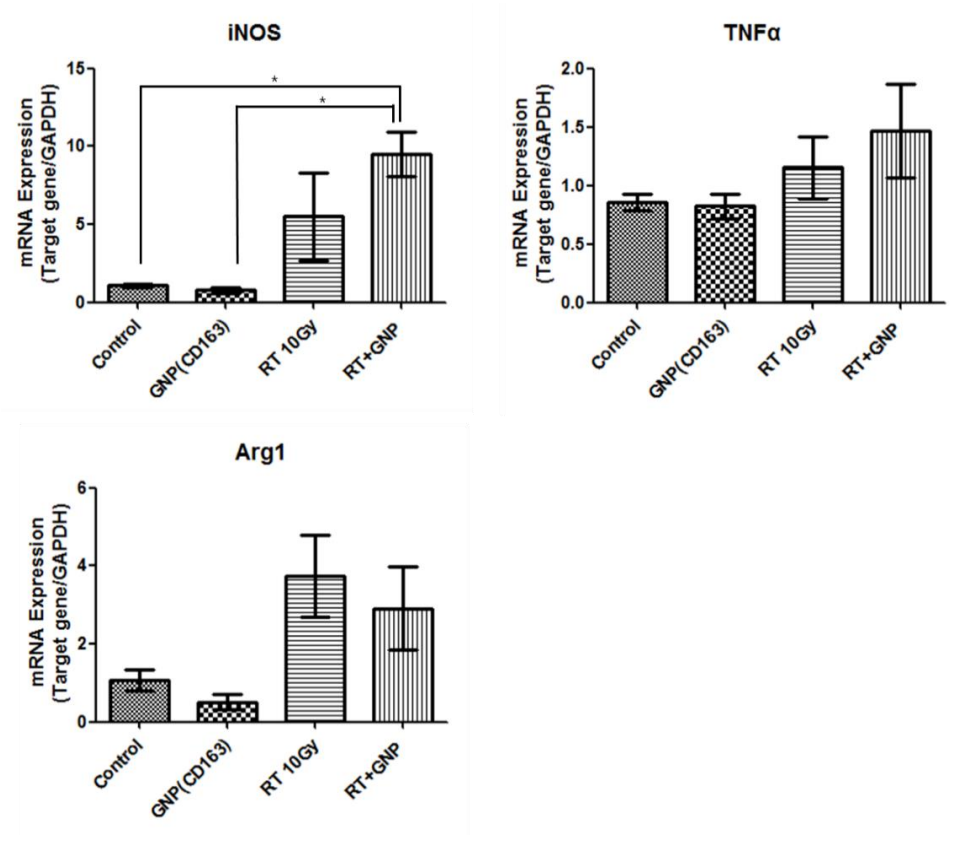


Figure 5. Polarization of macrophages in tumor after combined treatment

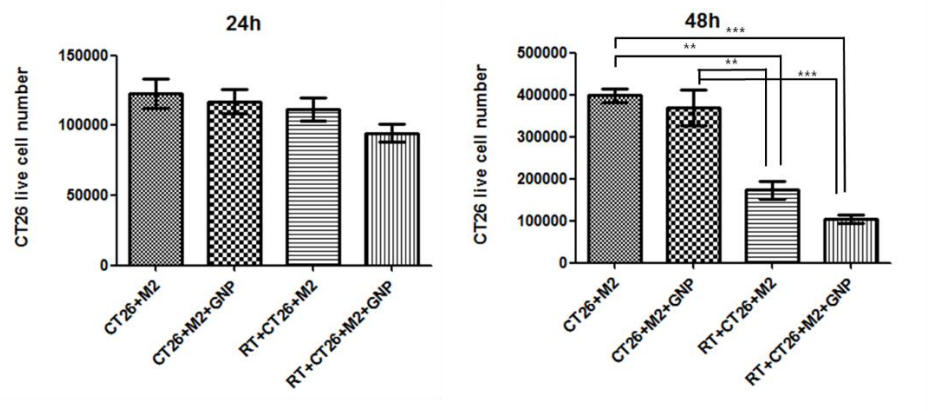
(* = P-value <0.05, ** = P-value <0.005)

(A) Fluorescence-activated cell sorting (FACS) results. Pan marker: CD11b, Type 1 macrophage marker: MHCII, type 2 macrophage marker: CD206 (B) Immunohistochemical staining. Upper row - red: MHCII, green: F4/80, yellow: type1 macrophage; Lower row – red: CD206, green: F4/80, yellow: type 2 macrophage (C) The mRNA expression of type 1 and type 2 macrophage markers in tumor after CD163-GNP combined with RT.

6. Effect of the combination of CD163-GNPs and RT on co-cultured cells

We co-cultured CT26 tumor cells with Raw 264.7 cells previously treated with IL4 and IL13. CT26 tumor cell viability was determined after 24 and 48 h treatment with CD163-GNPs in the absence or presence of radiation (Figure 6A). In comparison with the control group, the combination of CD163-GNP and RT reduced CT26 tumor cell viability. The combination therapy was more effective than radiation alone. The effect of the combination treatment was more obvious at 48 h. To exclude M1 TAMs, we treated macrophages with IL4 and IL13 and sorted only M2 TAMs. After co-culture of M2 TAMs and CT26 tumor cells, we performed RT-qPCR analysis for M2 TAMs to evaluate the change in mRNA expression. Although CD163-GNP alone showed a similar pattern, the combination of CD163-GNPs and RT resulted in an increase in the mRNA expression of *iNOS* and *TNF*α and a decrease in the mRNA expression of *Arg1* (Figure 6B).

A



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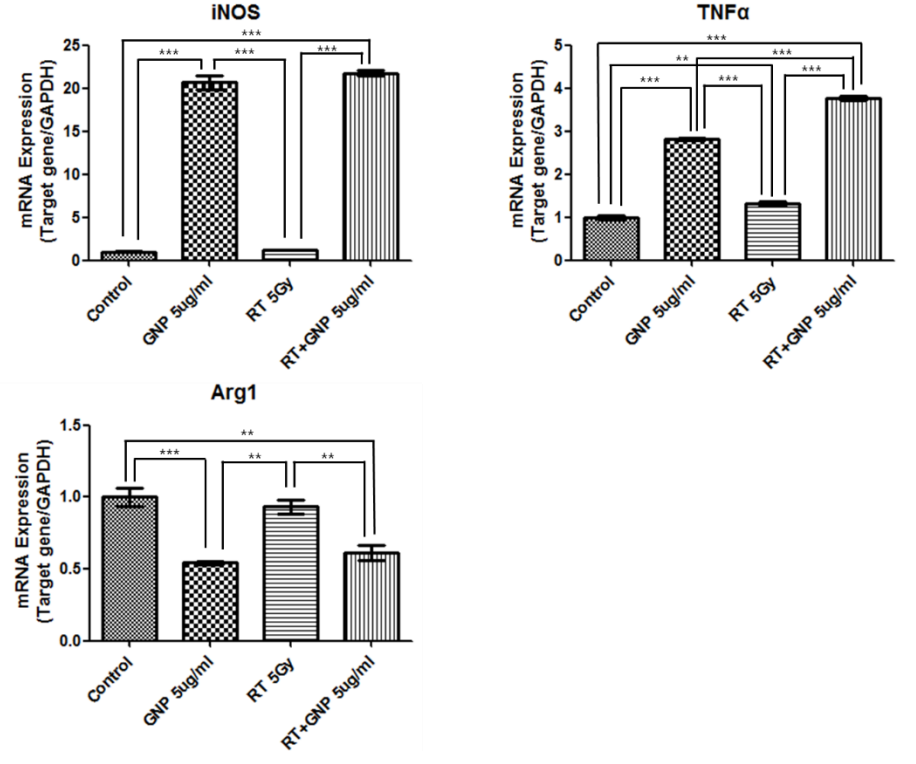

Figure 6. Effect of CD163-GNP combined with RT on co-cultured cells (** = P-value <0.005, *** = P-value <0.0001)

(A) Cell viability (B) The mRNA expression of type 1 and type 2 macrophage markers in M2 tumor-associated macrophages after CD163-GNP combined with RT.

IV. DISCUSSION

In the present study, we produced M2 TAM-targeting CD163 antibody conjugated GNPs. M2 TAMs phagocytosed more CD163-GNPs than M1 TAMs. We observed a delayed growth in tumors after treatment with the combination of GNPs and RT. Radiation alone also showed a significant growth-delaying effect on tumors. However, CD163-GNPs combined with RT exhibited sustained and effective TGD than radiation alone. In addition, the combination treatment decreased the number of M2 TAMs and increased the population of M1 TAMs.

In the tumor microenvironment, macrophages/monocytes interact with various types of cells [9]. These undergo differentiation into M1 or M2 TAMs via cytokines and co-exist in different ratios according to tumor types and individual tumors [7]. Many studies have reported that most TAMs in the tumor microenvironment are preferentially differentiated into M2 TAMs [17-19]. However, TAMs may change in response to the variations in the tumor microenvironment [20, 21]. M1 TAMs are the classically activated macrophages that increase the generation of reactive oxygen species and improve the ability of antigen-presenting cells [22]. On the other hand, M2 TAMs or alternatively activated macrophages reduce the antigen-presenting ability of T cells and promote angiogenesis and metastasis [23].

M2 TAMs have been regarded as potential therapeutic targets, and several studies targeting M2 TAMs are currently ongoing. Bao et al. reported the antitumor effects of a prostaglandin E4 (EP4) antagonist, E7046, through the modulation of TAMs and myeloid-derived suppressor cells (NCT02540291, American association of cancer research (AACR) 2015, abstract #275). Their recent study showed that the combination of E7064 and RT had synergistic effects on tumor control and rejection via an antitumor memory response [24].

These authors are conducting a multicenter phase 1b study of E7046 in combination with chemoradiotherapy for rectal cancer (NCT03152370). Pal et al. have also shown the antitumor activity of GNPs and silver NPs mediated through the modulation of M2 TAMs [25]. These authors have reported that metal NPs modulate the production of reactive oxygen species and reactive nitrogen species, resulting in the suppression of the antioxidant system of TAMs. Although these ongoing researches have focused on M2 TAMs, studies of RT targeting M2 TAMs are limited.

Yang and Zhang have categorized therapeutic strategies against TAMs into four groups [15]. Several studies have reported the induction of M2 macrophages into M1 phenotype, one of the important strategies for targeting M2 TAMs. The MD Anderson Cancer Center has shown antitumor activities of antisense oligonucleotide targeting signal transducer and activator of transcription factor 3 (STAT3), which is overactivated in M2 TAMs [26, 27]. These authors have reported that the antisense oligonucleotide decreased STAT3 expression and exerted antitumor effects in heavily treated patients with lymphoma and non-small cell lung cancer. The University of Porto has reported an IFN-γ delivery system using chitosan/poly(r-glutamic acid)multi-layered films, wherein IFN-γ modulated macrophages toward M1 phenotype and reduced the stimulation of cancer cell invasion [28].

In the present study, we did not reveal the actual mechanism underlying macrophage polarization with the combination of CD163-GNPs and RT. Studies have reported that Toll-like receptor 4 (TLR4) and IFN-γ receptors on macrophages are involved in the signaling pathways necessary for macrophage polarization toward the proinflammatory phenotype [29-31]. STAT1 and nuclear factor kappa B (NFκB) are mainly associated with macrophage polarization towards M1 TAMs [31]. Ionizing radiation induces NFκB activation and NFκB-dependent TNFα transactivation and secretion [32]. Pinto et al. revealed the activation of the NFκB signaling pathway mediated by ionizing radiation and

changes in macrophages towards proinflammatory phenotype after radiation [3]. TNF is an important regulator of TAM polarization. Franz et al. revealed the increase in M2 marker mRNAs in the absence of type 1 TNF receptor (TNFR) signaling using TNFR1 knockout mice [33]. These authors also found that TNF blocked IL13 expression from eosinophils, wherein IL13 is a key cytokine that activates M2 TAMs. In addition, the elevated TNFα level after radiation correlated with favorable treatment response [34].

The mRNA expression patterns observed after the treatment of co-cultured cells (M2 TAMs and CT26 tumor cells) with the combination therapy or CD163-GNP alone were similar. Although no statistical significance was observed, CD163-GNP treatment alone resulted in a decrease in the expression of *Arg1* mRNA in the tumor. Therefore, M2 TAMs targeting CD163-GNP alone may exert effects on polarization. However, RT in combination with CD163-GNPs induced more effective macrophage polarization. Several studies have addressed the effect of NPs on macrophage polarization. Fuchs et al. reported that polystyrene NPs functionalized with carboxyl and amino groups inhibited macrophage polarization towards M2 TAMs. Although this study did not address the actual mechanism of inhibition, the authors revealed the reduced expression of CD163, CD200R, and IL10 in M2 macrophages without any changes in the expression of CD86, nitric oxide synthase 2 (NOS2), and TNFα in the presence of polystyrene NPs [35]. Pal et al. reported that metal NPs maintained the levels of reactive oxygen and nitrogen species and activated proinflammatory signaling pathway [25]. Laskar et al. found that the levels of iron-related proteins are important to sustain the polarized state of macrophages and focused on the difference in intracellular iron levels between the macrophage subtypes. M1 macrophages had higher levels of ferritin and cathepsin L than M2 macrophages. Iron in super paramagnetic iron-oxide NPs upregulated the level of intracellular ferritin and cathepsin L in M2 macrophages, resulting in the induction of M1 phenotype [36]. Su et al. explained

that the phenotypic changes in macrophages are involved in cell to cell interactions mediated by the ligands on adjacent cells and the receptors on the surface of macrophages. These authors showed that glycocalyx-mimicking NPs interacted with the receptors on M2 macrophages and induced phenotypic change from M2 to M1 [37].

We hypothesized that macrophage repolarization is one of the most important mechanisms underlying the antitumor effects of the combination of CD163-GNPs and RT. Additional investigation of the actual mechanism underlying macrophage polarization is warranted in future studies.

In this study, we used antibody-conjugated, silica-coated GNPs that were administered via intratumoral injection. The development of more effective GNPs is desirable to enhance the effects of RT along with the development of methods to improve the *in vivo* biodistribution of GNPs.

V. CONCLUSION

In this study, we produced M2 TAM-targeting GNPs. The GNPs in combination with RT reduced the population of M2 TAMs in the tumor microenvironment, resulting in enhanced antitumor effects. Further studies are warranted to evaluate the mechanism underlying macrophage polarization in response to the combination treatment.

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APPENDICES

Abbreviation lists

TAM = tumor-associated macrophage, GNP = gold nanoparticle, RT = radiotherapy, NP = nanoparticle, PVP = polyvinylpyrrolidone, TEOS = tetraethyl orthosilicate, APTES = aminopropyl triethoxysilane, ANB-NOS = 5-Azido-2-nitrobenzoic acid N-hydroxysuccinimide ester, DI = deionized, PBS = phosphate-buffered saline, BMDM = bone marrow-derived macrophage, FBS = fetal bovine serum, FACS = fluorescence-activated cell sorting, RT-qPCR = real-tome quantitative polymerase chain reaction, IL = interleukin, LPS = lipopolysaccharide, IFN = interferon, MHC II = major histocompatibility complex II, APC = allophycocyanin, CTCF = corrected total cell fluorescence, TGD = tumor growth delay, iNOS = inducible nitric oxide synthase, TNFα = tumor necrosis factor alpha, Arg1 = arginase 1, TEM = transmission electron microscopy, FTIR = Fourier transform infrared, STAT = signal transducer and activator of transcription factor, NFκB = nuclear factor kappa B

ABSTRACT(IN KOREAN)

Gold nanoparticle과 방사선치료의 병용요법 시 종양 억제 효과의 증대

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배경: 종양관련대식세포와 악성종양의 높은 연관성이 밝혀지면서 종양관련대식세포는 진단/예후의 생체표지자와 치료 표적으로 활용되고 있다. 종양관련대식세포는 종양미세환경에 따라 M1 형질과 M2 형질이 서로 다른 비율로 존재한다. M2 형질 종양관련대식세포는 종양미세환경에서 종양의 성장과 전이를 유도하며, M2 형질의 수가 많을수록 예후가 나쁜 것으로 알려져 있다.

목적: 본 연구는 M2 형질 종양관련대식세포를 표적하는 gold nanoparticle과 방사선치료 병용요법이 종양관련대식세포에 미치는 영향을 확인하고, 나아가 병용요법이 기존 방사선치료의 종양억제효과를 증대 시킬 수 있는지 확인하고자 하였다.

방법: M2 형질 종양관련대식세포를 표적하기 위해 CD-163 항체 결합 gold nanoparticle을 제작하였다. gold nanoparticle과 방사선치료의 병용요법 시행 후 세포와 동물모델에서 대식세포의 분화를 확인하였고, 종양억제효과 비교를 위해 종양성장지연 분석을 시행하였다.

결과: 면역형광염색과 전자현미경을 이용하여 식작용된 gold nanoparticle을 확인하였고, M1 형질보다 M2 형질 종양관련대식세포 내부에서 유의하게 많은 gold nanoparticle이 관찰되었다. 종양성장지연분석 상, 병용치료 시행군에서 방사선치료 단독시행군보다 지속적이며, 높은 종양성장억제효과를 보였다. 동물모델에서 병용요법 후 면역화학염색을 시행했을 때, 종양내 M1 형질 종양관련대식세포 증가와 M2 형질의 감소를 보였고, mRNA를 확인했을 때에도 M2 형질의 표지자인 Arg1이 감소하고, M1 형질의 표지자인 iNOS, TNFα는 증가했다. 종양세포와 공생배양한 대식세포에서도 병용요법 후 유사한 mRNA 변화가 관찰되었다.

결론: 본 연구에서는 M2 형질 종양관련대식세포를 표적하는 gold nanoparticle을 제작하여 이를 방사선치료와 병용했을 때, M2 형질을 감소시키며, 기존 방사선치료의 종양억제효과를 증대 시킬 수 있었다.

핵심되는 말 : 종양관련대식세포; Gold nanoparticle; 방사선치료; 종양미세환경