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Immune Activation Modulation via Magnetically Localized Bacteria Based Micro/Bio Robot (BBMBR)

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***Abstract*— Understanding tumor's microenvironment is one of the key factors in the cancer therapy. Especially, from the perspective of immunotherapy, immune desert or cold tumor is referred as significantly downregulated T cell infiltration due to lack of immune surveillance in the tumor microenvironment. There are many studies are dedicated to convert cold tumor to hot tumor for enhancing the efficacy of immunotherapy. In this study, we suggested selective immune activation system through the spatiotemporal control of the bacteria as an immune boosting agent. To this end, we have developed bacteria-based micro/bio robot system (BBMBR) by attaching bacteria with magnetic nanoparticles (MNP) so that the localization can be controlled through the magnetic field. The biomanufacturing results showed that BBMBR includes 6.6 ± 1.54 MNP attached and the presence ratio of bacteria-MNP out of total bacteria population reached 75.2 ± 3.37%. Spatial controllability experiments have shown that rotational and translation localization has been controlled as intended. The function of the immune modulation system through BBMBR was confirmed through experiments that magnetically driven BBMBR localization induced localized immune activation. M1-phenotype differentiation of macrophage cells were quantified CD80 staining, and overall immune response level was evaluated through IL-6 measurements. As the distance from the activation point increased, the population of M1 differentiated macrophages decreased, and when the movement of BBMBR was magnetically restricted, overall immune activation was found to be regulated downward. Proposed BBMBR and immune modulation framework could introduce a powerful new paradigm in cancer treatment by improving the localization controllability of immune-boosting agent and the spatial immune activation strategies.**

I. INTRODUCTION

Cancer is a disease found in many people and occurs in various parts of the body. Among the methods of treating cancer, immunotherapy treats cancer by causing an immune response through cells such as T cells and macrophages that cause immune activity. Depending on the distribution of T cells in the tumor microenvironment of cancer, they are referred to as cold tumor and hot tumor.[10] Among cold tumors, the fact that T cells are generated around the tumor but cannot penetrate is excluded, and non-existent tumors are called desert tumor.[4] Cancer cells have developed recognition and removal of the immune system, so cancerous tumors that inhibit and avoid the immune system are mainly classified as cold tumors [5]. Cold tumor has low expression of PD-L1 and MHCI on its own, making it difficult for T cell to penetrate.[9] In addition, cells such as tumor-associated macrophages (TAMs), T-regulatory cells (Tregs), and myeloid-derived suppressor cells (MDSCs) that inhibit immune activity are present, innate immune response is significantly downregulated.[9] There exist many studies regarding immune-boosting agents for improving the efficacy of immunotherapy in cold tumor by activating immune system for increasing the infiltration of T cells such as oncogenic pathway inhibitors, epigenetic modification inhibitors, transforming growth factor-β (TGF-β) inhibitors, and C-X-C chemokine receptor type 4 (CXCR4) inhibitors.[10]

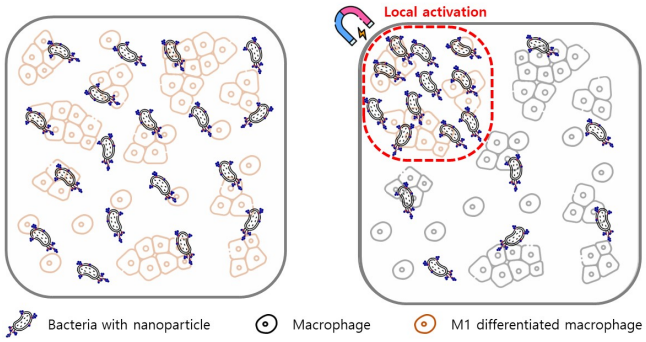

Fig. 1. Schematic diagrams of Immune Modulation through EMA system

Bacteria are promising immune-boosting agents that can initiate innate immune response for macrophages, dendritic cells, and neutrophils through exposing molecular patterns including lipopolysaccharides (LPS) and flagellin on the bacterial surface.[7] The activation mainly occurs based on the surface recognition of bacteria through toll-like receptors (TLR).[3] Macrophage is one of the antigen-presenting cells (APC), expressing major histocompatibility complexes (MHC) I and II, followed by T cells activation, which can lead boosting entire immunity.[8]

In this study, we utilized bacterial components, specifically pathogenic molecular pattern, as an immune system boosting agents in order to convert cold tumor into the hot tumor by promoting the immune activity. In particular, bacteria-based micro/bio robot system (BBMBR) was developed by attaching bacteria with magnetic nanoparticles so that the

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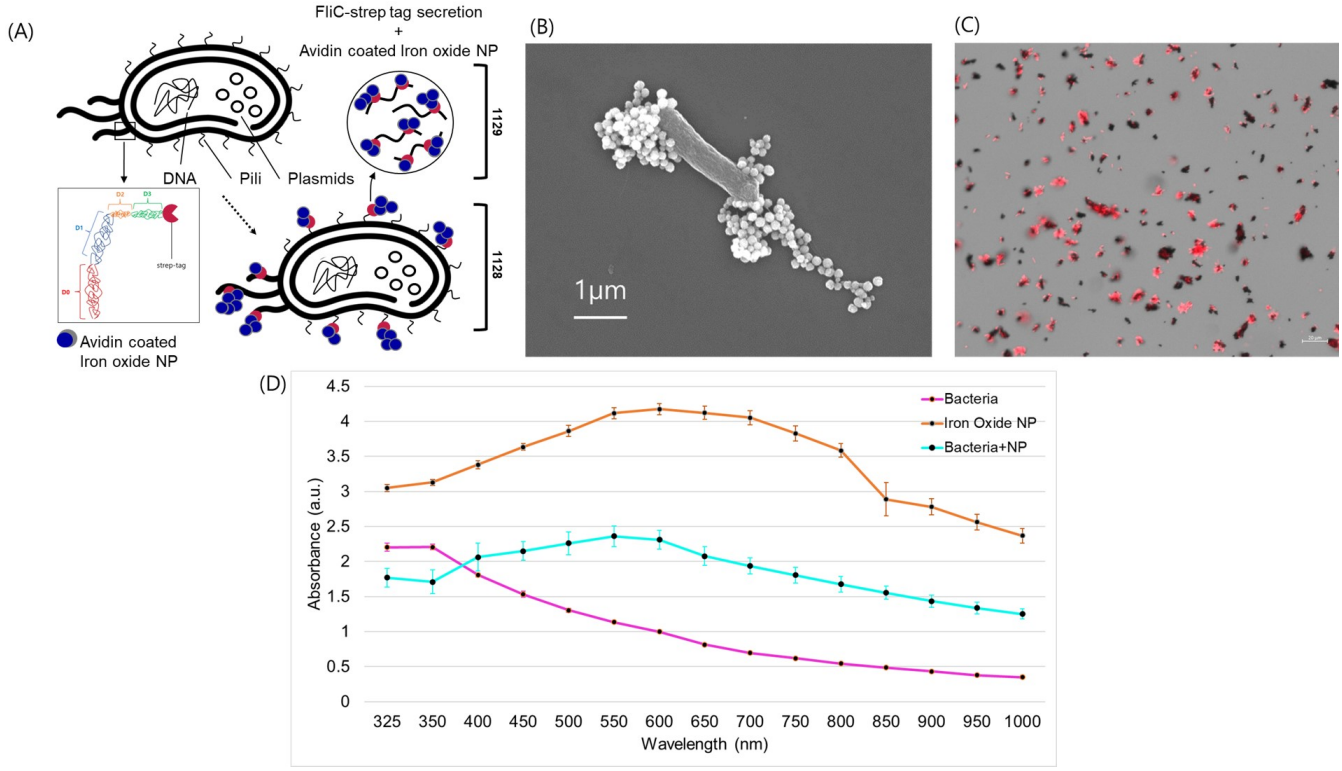

Fig. 2. Biomanufacturing BBMBR: (A) schematic of BBMBR synthesis, (B) scanning eletron microscope (SEM Regulus 8230) images of BBMBR, (C) Fluorescent micrograph image of BBMBR (Cy5: bacteria), and (D) Absorbance measurement

localization can be controlled through the magnetic field. In addition, the function of the immune modulation system through BBMBR was confirmed through experiments that magnetically driven BBMBR localization induced localized immune response as depicted in Fig. 1.

II. Materials and Methods

A. Biomanufacturing BBMBR

Plasmid FliC258-strep-tag-smuRFP-509 and FliC258-strep-tag-smuRFP-249 were transformed into Salmonella enterica Serovar Typhimurium aroA aroD for cellular surface functionalizing strain (1128) and secreting stain (1129), respectively. The aroA and aroD genes are responsible for the virulence of the bacteria, so we used an attenuated strain that had them removed.[2] The positive single colonies were inoculated in the Lysogeny broth (LB; 1% w/v of tryptone, 1% w/v of NaCl, 0.5% w/v of yeast extract, supplemented with 100ug/ml of ampicillin, and 15 ug/ml of chloramphenicol) and incubated in the shaker overnight at 37°C and 250 rpm. Confluent culture was diluted 100-fold in LB supplemented with the antibiotic and shaken at 37°C and 250 rpm until OD600 reaches 0.4. In order to initiate induction of strep tag, L-(+)-arabinose (Sigma-Aldrich, cat. A3256-100G) was added in the culture (0.2 g/ml) and incubated in the shaker at 37°C and 250 rpm overnight. For the culture of 1128, a 1.5ml aliquot of the overnight culture was washed three times using a centrifuge at 8000 rpm for 2 min at room temperature and suspended in PBS of pH 7.4 adjusting the concentration of the final culture at 1.0 of OD_{600}. For the culture of 1129, the overnight induced culture was transferred into the centrifugal devices (PALL, cat. MAP003C37) and concentrated in the centrifuge at 3400 rpm for 8.5 hours. The supernatant was separated from the concentrate in prior to biomanufacturing of BBMBR. In the supernatant, FliC, a flagellar filament structural protein, is present, and a strep tag is attached to the FliC. Magnetic nanoparticle (Fe_3O_4@Avidin) solution (1 mg/mL) in PBS at pH of 7.4 was separately combined with prepared strain (1128) at final concentration 1.0 of OD_{600} and supernatant for the strain (1129) and mixed for 30 min at 600 rpm in the vortex mixer in prior to the immune activation experiments.

B. In vitro BBMBR-mediated Immune Modulation Experiments

Murine macrophage RAW264.7 cells (KCLB 40071; Korean Cell Line Bank, Seoul, Korea) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% v/v penicillin/streptomycin (P/S) at T-25 flask in prior to the experiment. Macrophage cells were transferred in 35 mm petri dish (1.2×10^6 cells/mL) in DMEM with 10% FBS with 100 μg/mL of LPS (Sigma-Aldrich, L2630) at 37°C and 5% CO_2 overnight. 2 hours before the infection, the dish was washed with PBS and fresh media was added to the dish. Immune activation agents BBMBR-1128, BBMBR-1129, and PBS 300μl were separately added to the prepared cell dish and incubated at 5% CO_2 and 37°C for 2 hours. The

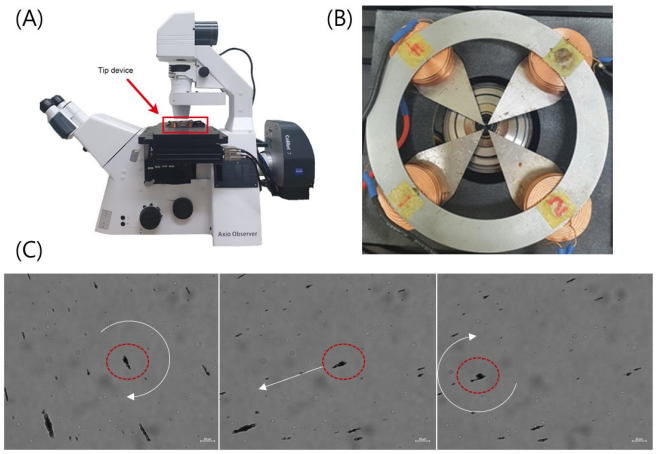

Fig. 3. Immune activation modulation experimental setup: (A) Customized electromagnetic actuation (EMA) on microscope, (B) EMA system consist of 4 tips for electromagnetic actuation, and (C) Magnetically induced actuation of BBMBR

movement of infecting agents were restricted during the co-incubation process by applying magnetic field at NW-tip in the magnetic field generating device as shown in Fig. 3(B).

C. Quantification of Macrophage Activation

The M1-phenotype differentiation of macrophages was quantified by CD80 staining, and IL-6, a substance that activates cytotoxic T cells and promotes the differentiation of B cells, was assessed with an ELISA kit (Invitrogen, cat.21435).[1] IL-6 cytokine concentrations (pg/ml) were calculated by obtained standard curves and 4PL logistics (Biotek, 800TS). Remaining cells attached on the dish was gently washed with PBS. 4% Paraformaldehyde (biosesang, cat. PC2031-100-00) was added to the dish for fixing for 20 min. After washing the cell with PBS, triton-x solution (0.15% w/v) (VWR LIFE SCIENCE, cat. 0694-1L) was treated for 30 min. After washing the cell with PBS, blocking buffer, BSA solution (3% w/v) was added and incubated at 37°C for 1 hour. Fluorescent conjugated CD80 antibody (Alexa Fluor, cat.104754) were diluted (0.1% v/v) in BSA solution (0.5% w/v), then added to cells and incubated at 37°C overnight before the imaging.

III. RESULTS

A. Bio manufacturing BBMBR

BBMBR was synthesized through two different bacterial induction as described as strain 1128 and 1129. The strain is designed to express strep tag, which shows high affinity to the avidin group [6], at the end terminal of FliC fragment, which exhibits immune activation molecular pattern. (Fig. 2(A)) The MNP attachment for BBMBR-1128 was evaluated through several methods including microscopic observation, scanning eletron microscope (SEM) image analysis, and absorbance pattern. Microscopic image observation showed fluorescent bacteria at 642/670 nm for excitation/emission were localized where the magnetic nanoparticles are located as shown in Fig. 2(C). Through SEM image analysis, we have quantified the average number of MNP loaded on a bacterium is 6.6 $\pm$ 1.54 and the presence ratio of bacteria-MNP out of total bacteria population reached 75.2 $\pm$ 3.37%. Lastly, absorbance for the BBMBR showed clear pattern shift at 350 nm which is also shown in the absorbance pattern of magnetic nanoparticles.

B. Actuation of BBMBR through Customized EMA system

In order to investigate the controllability of BBMBR localization, synthesized BBMBR was placed on the glass slide where the customized EMA system is placed on the microscope as shown in Fig. 3. The localization of BBMBR was observed through microscope where magnetic field is applied through clockwise actuation between the tips for the rotational movement and one side tip actuation for the translational movement. Upon actuating one side of tip for 15 seconds the average instantaneous speed of BBMBR reached to 20.54 $\pm$ 5.52 μm/s.

C. Spatial Immune Activation Modulation

In order to modulate the immune response of macrophage, BBMBR was guided towards northwest tip through EMA system so that the contact between immune cell and immune stimulant can be restricted as depicted in Fig. 4(A). ELISA results showed that IL-6 measurements for BBMBR-1128 and BBMBR-1129 were 6-fold to 15-fold higher than the control case indicating the immune system is activated through proposed BBMBR. Regardless of magnetic field application, supernatant based BBMBR (1129) showed 1.5-fold higher IL-6 level compared to bacteria based BBMBR (1128). It is because BBMBR-1129, a much smaller immune catalyst, is likely to respond easily and quickly in contact with immune cells, which results higher level of the immune activation. The EMA based spatial immunostimulant localization induced less IL-6 secretion. For the case of BBMBR-1128, the secretion level was decreased by 22% as EMA system restrict the movement. ($^*P<0.05$) BBMBR-1129 showed similar pattern with even larger suppression as 45%. ($^*P<0.05$) The difference in IL-6 secretion depending on whether a magnetic field is applied or not is 23% larger in the case of BBMBR-1129 because smaller size agents were more efficiently localized and thus more effectively limit the immune response.

The fluorescence intensity of CD80 expression was measured in the vicinity of the activation spot. The highest fluorescence intensity was observed within 20% of the activation spot and decreased with increasing distance. Beyond 80%, the fluorescence intensity of BBMBR-1129 was equivalent to that of PBS. A significant difference in fluorescence intensity was observed between BBMBR-1129 and BBMBR-1128 at the 60% section, with decreases of 60.6% and 35.6%, respectively, compared to the activation spot. The correlation between local IL-6 secretion and CD80 expression suggests that the fluorescence intensity ratio contributes to immune activity. Within 20% of the magnetic location, BBMBR-1129 accounted for 37.3% of the total immune response and BBMBR-1128 accounted for 27.7%. This difference

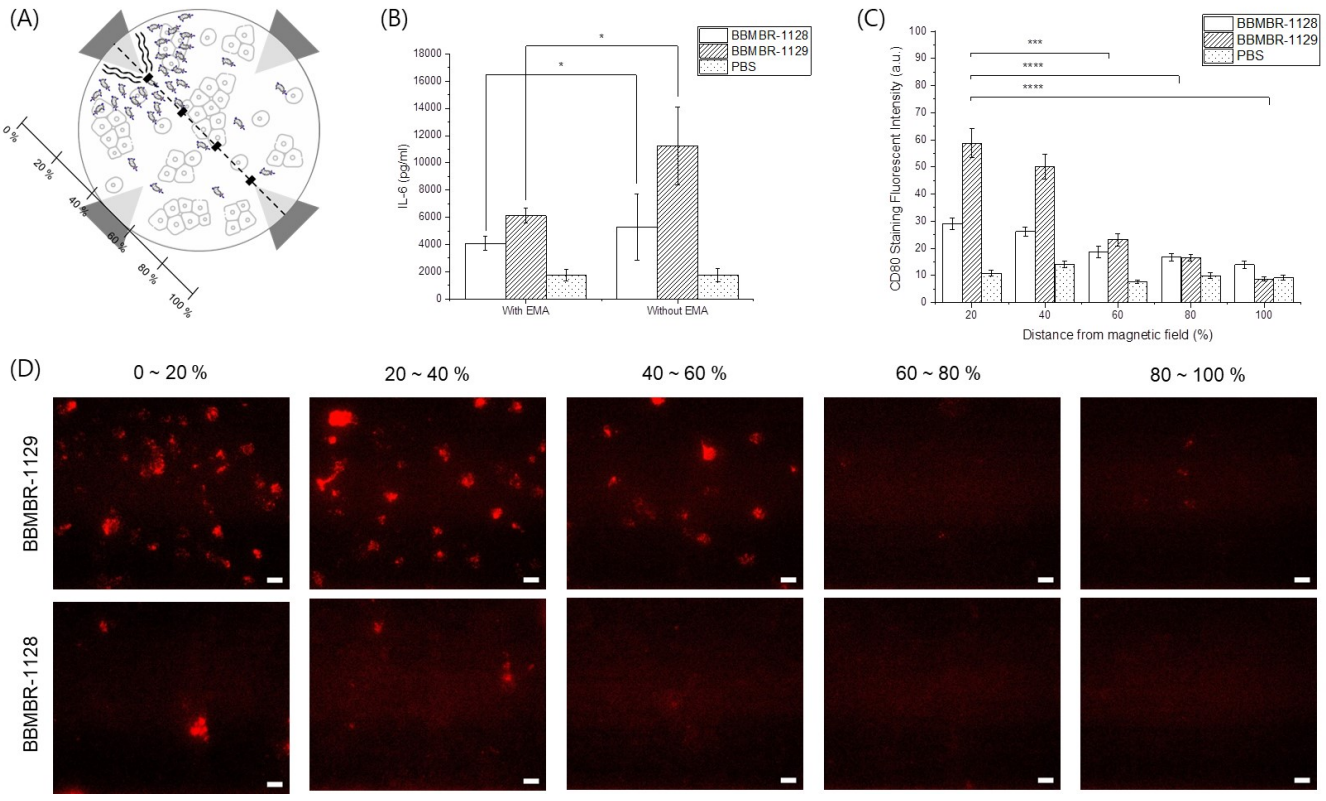

Fig. 4. Immune activation modulation results: (A) Schematic of immune response modulation through EMA system, (B) IL-6 measurements, (C) CD80 staining, and (D) Micrograph images of CD80 staining results (scale bar = 20 um)

is attributed to the high immune-stimulating efficiency of the small-sized immunostimulant and improved localization control through magnetic field application.

IV. CONCLUSIONS

In this study, we developed a bacteria-based micro/bio robot system (BBMBR) by attaching magnetic nanoparticles to the bacterial surface, which enabled us to control the location of BBMBR through magnetic field actuation. Biomanufacturing results indicated that BBMBR contained 6.6 ± 1.54 MNP attached, with the presence ratio of bacteria-MNP out of the total bacteria population reaching $75.2 \pm 3.37\%$. The immune modulation system's function through BBMBR was confirmed by experiments showing that M1-phenotype differentiation levels of macrophage cells significantly decreased when the magnetic field was applied to limit BBMBR's movement. Spatial immune response modulation was also confirmed, with the number of M1 differentiated macrophage cells decreasing as the distance from the activation point increased. The proposed BBMBR and immune modulation framework could introduce a powerful new paradigm in cancer treatment by improving the localization controllability of immune-boosting agents and spatial immune activation strategies.

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