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MF59 formulated with CpG ODN as a potent adjuvant of recombinant HSP65-MUC1 for inducing anti-MUC1⁺ tumor immunity in mice

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ABSTRACT

MF59 is an oil-in-water emulsion adjuvant approved for influenza vaccines for human use in Europe. Due to its Th2 inducing properties, MF59 is seldom tested for cancer vaccines. In this study, MF59 formulated with a C-type CpG oligodeoxynucleotide (YW002) was tested for its Th1 adjuvant activity to induce immune responses to HSP65-MUC1, a recombinant fusion protein incorporating a mycobacterial heat shock protein (HSP65) and mucin 1, cell surface associated (MUC1) derived peptide. Combination of YW002 with MF59 (MF59-YW002) could confer a potent Th1 biasing property to the adjuvant, which enhanced the immunogenicity of HSP65-MUC1 to induce significantly higher levels of specific IgG2c, increased IFN- γ mRNA expression in splenocytes and the generation of antigen-specific cytotoxic T lymphocytes in mice. When prophylactically applied, MF59-YW002 adjuvant containing HSP65-MUC1 inhibited the growth of MUC1⁺ B16 melanoma and prolonged the survival of tumor-bearing mice. In contrast, adjuvant containing MF59 with HSP65-MUC1 in the absence of YW002, promoted the growth of MUC1⁺ B16 melanoma in mice. These results suggest that MF59 plus CpG oligodeoxynucleotide might be developed as an efficient adjuvant for tumor vaccines against melanoma, and possibly other tumors.

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1. Introduction

Ideally, a potent cancer vaccine should induce a strong T helper (Th) 1 cell mediated immune response and the generation of antigen-specific cytotoxic T lymphocytes (CTLs) [1]. However, as “self molecules”, tumor antigens in cancer vaccines are poorly immunogenic and therefore require the use of adjuvant to enhance their ability to elicit strong cellular immune responses. In general, an adjuvant serves to enhance the magnitude, breadth, quality and longevity of specific immune responses to antigens, and is composed of a vehicle and immune-stimulant [2]. The vehicle, such as mineral salts, emulsions and liposomes, functions to control the release and delivery of antigens. In the last decade, pathogen-associated molecular patterns (PAMPs) have been identified as innate immune stimulators, capable of activating antigen presenting cells, especially dendritic cells (DC) to process and present antigens, produce cytokines and chemokines, and up-regulate co-stimulatory molecules more efficiently. This has allowed the combination of PAMPs or their mimics with vehicle compounds such as emulsions to develop efficient adjuvants.

MF59, an oil-in-water nanoemulsion, has been licensed in Europe for use in the elderly as an adjuvant for seasonal influenza vaccines, and currently is under evaluation as an H1N1 pandemic vaccine [3–5]. MF59 is prepared with 4.3% w/w of squalene as an oil phase. Squalene, a biodegradable oil, is extracted from shark liver and occurs in higher organisms including humans as an intermediate in the cholesterol and vitamin D synthesis pathway [6]. In addition to its emulsion properties, MF59 enhances antigen uptake by activating DCs and facilitates Th2-biased immune responses to antigen [7]. Currently, MF59 is being evaluated in preclinical studies and clinical trials as an adjuvant for microbial antigens from hepatitis B virus (HBV) [8], hepatitis C virus (HCV) [9], cytomegalovirus (CMV) [10], human papillomavirus (HPV) [11], SARS coronavirus [12], serogroup B meningococcus [13], human immunodeficiency viruses (HIV) [14] and herpes simplex virus [15]. In all of these studies, MF59 is used to facilitate a Th2 biased antibody response to the microbial antigens.

To confer MF59 with Th1 immune response-promoting properties, PAMPs or their mimics, such as Toll like receptor (TLR) ligands have been added. In mice, MF59 combined with E6020, a chemically synthesized TLR4 agonist, induced a mixed Th1/Th2 immune response against hemagglutinin (HA) from H1N1 influenza virus, composed of IFN- γ and IL-5 producing T cells, and immunoglobulin (Ig) G1/IgG2a antibodies [16]. When MF59 was combined with a TLR9 agonist, CpG containing oligodeoxynucleotide (CpG ODN) to immunize mice, a more potent Th1 cellular immune response represented by

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higher IgG2a titers and the induction of a strongly enhanced IFN- γ response was observed in splenocytes [17].

Recently, CpG ODNs have been investigated as adjuvants for tumor vaccines due to their ability to induce and enhance both innate and adaptive immune responses, and consequently are being evaluated in clinical trials [18–20]. Based on functional characteristics, CpG ODNs are divided into A, B and C subtypes. A-type CpG ODNs induce plasmacytoid dendritic cells (pDC) to secrete large amounts of interferon- α (IFN- α). B-type CpG ODNs stimulate the proliferation and antibody production of B cells. C-type CpG ODNs share properties of A-type and B-type CpG ODNs [21]. In clinical trials, CpG ODNs, especially B-type CpG ODNs, have been evaluated for their efficacy as adjuvants in cancer vaccines [22]. In melanoma patients, CpG 7909, a B-type CpG ODN, formulated with incomplete Freund's adjuvant and melanoma antigen A, induced a vigorous cellular immune response that generated high numbers of melanoma-specific cytotoxic T lymphocytes (CTLs) [23]. CpG 7909 mixed with synthetic NY-ESO-1 peptide and incomplete Freund's adjuvant led to the early induction of specific integrated CD4⁺ Th cells and the development of NY-ESO-1 antigen-specific CD8⁺ immune responses in nine patients with NY-ESO-1 expressing tumors. Six of the nine patients survived for an average of 39 months, significantly longer than their predicted survival of 4 months [24,25]. In addition, vaccination with peptide epitopes LY6K-177 and TTK-567 derived from novel cancer-testis antigens in combination with CpG 7909 successfully elicited antigen-specific CD8⁺ T cell responses and enhanced innate immunity of patients with advanced esophageal squamous cell carcinoma [26].

In the current study, we investigated the use of PAMP mimics to confer a Th1 biasing capability to MF59 as an efficient adjuvant for tumor antigens. Using MF59 formulated with a C-type CpG ODN (YW002) [27] (MF59-YW002) enhanced the immunogenicity of HSP65-MUC1 [28], a recombinant fusion protein of mycobacterial heat shock protein (HSP65) and human MUC1 VNTR peptides (MUC-1) when prophylactically applied. The induction of vigorous Th1 cellular immune responses inhibited the growth of MUC1⁺ B16 melanoma in mice and prolonged the survival rate. Thus, the combination of MF59 and PAMP mimics may be useful for vaccination against tumor antigens in the clinic.

2. Materials and methods

2.1. Mice and cell lines

Female C57BL/6 mice (6–8 weeks) were purchased from Beijing Weitonglihua Laboratory Animal Co., Ltd. Mice were maintained in microisolator cages under pathogen-free conditions, and animal care conformed to the Guide for the Care and Use of Laboratory Animals (National Research Council).

Murine B16 melanoma cells of C57BL/6 origin were transfected with the pcDNA3-GFP-MUC1 VNTR plasmid encoding for human MUC1 VNTR peptide fused to GFP. The cell line was selected in complete medium containing G418 (500 mg/l) (Sigma-Aldrich, St Louis, MO) for MUC1⁺ cell clones, and the stable monoclonal transfected cells were verified by fluorescence microscopy. Complete medium was prepared with RPMI-1640 supplemented with 10% fetal bovine serum (FBS, v/v) and antibiotics (100 IU/ml penicillin, 100 IU/ml streptomycin), and the cell line was cultured at 37 °C in a 5% CO₂ humidified incubator.

2.2. Reagents and vaccine formulations

HSP65-MUC1 (MW = 68.2 kDa), was constructed in our lab, containing BCG HSP65 and two copies of the MUC1 VNTR core peptide (GSTAPPAGHGVTSAPDTRPAPGSTAPPAGHGVTSAPDNRPAL), expressed in *E. coli* and purified as previously described [28]. The recombinant protein was verified by western blotting analysis using anti-HSP65-

specific monoclonal antibody (mAb) (prepared in our lab) and mouse anti-human MUC1-specific mAb (BD Biosciences, FL, NJ).

MF59 (4.3% squalene, 0.5% Tween-80, 0.5% Span-85) was prepared in 10 mM sodium citrate buffer, as described [29]. Nuclease-resistant phosphorothioate-modified ODNs were synthesized by Takara Co. (Dalian, China). The CpG ODN used in this study was YW002 (C type, 5'-tcgcgaacgttcgcccgttcgaacggc-3'). Lowercase letters represent phosphorothioate linkage. The CpG ODN was diluted in PBS and tested for endotoxin using the Limulus amoebocyte lysate assay (Associates of Cape Cod, Inc., East Falmouth, MA). All reagents used were pyrogen-free.

Vaccines were prepared by mixing MF59 with HSP65-MUC1 and/or CpG ODN at a 1:1 (v/v) ratio. Soluble HSP65-MUC1 and/or CpG ODN at a 10 μ g/dose were added to the MF59 emulsion prior to immunization.

2.3. Animal experiments

Tumor-suppressing experiments were carried out in mice using the following two protocols for either prophylactic or therapeutic vaccination. In the prophylactic protocol, mice were immunized with HSP65-MUC1 alone or in combination with MF59 or MF59-CpG ODN, or with CpG ODN alone sub-cutaneously (s.c.) in the inguinal lymph node area on days -24, -17, -10 and -3. For the therapeutic protocol, mice were immunized on days 1, 8, 15 and 22. For tumor cell inoculation, each mouse was injected s.c. with 1.2×10^5 MUC1⁺ B16 cells into the back near the hind leg on day 0. Tumor size was measured every two days with calipers and tumors greater than 3 mm in diameter with progressive growth were recorded as positive. Survival of mice was monitored for approximately 70 days.

For immunogenicity studies, mice were immunized with HSP65-MUC1 alone or mixed with MF59 or MF59-CpG ODN s.c. in the inguinal lymph node area (200 μ l per mouse) two or four times at 7-day intervals. After sacrificing the mice, sera were collected for antibody analysis, and splenocytes were isolated for detection of IFN- γ and IL-4 mRNA expression and CD8⁺ T cell activation.

2.4. RNA isolation and real-time RT-PCR

The day after the fourth immunization, splenocytes were prepared for the isolation of total RNA using the TRIzol reagent (Invitrogen, Carlsbad, CA). The concentrations and contents of RNA were analyzed using a Unic UV2800 ultraviolet spectrophotometer. Ratios of A260/A280 (1.8–2.0) indicated that the RNA samples were highly purified and not degraded. Reverse transcription reactions were carried out using TIANScript M-MLV (Tiangen Biotech, Beijing, China), according to the manufacturer's instructions. The IFN- γ and IL-4 mRNA was quantified by real-time PCR using an ABI PRISM 7300 Fast Real-Time PCR System. All primers were synthesized by Sangon Biotech (Shanghai, China). The real-time PCR reactions were performed in a total volume of 20 μ l using a SYBR® Premix Ex Taq™ II quantitative augmentation reaction system (Takara, Dalian, China). The levels of IFN- γ and IL-4 mRNA in splenocytes were calculated after normalizing cycle thresholds against the housekeeping gene GAPDH and are presented as the fold change value ($2^{-\Delta\Delta\text{comparative threshold}}$) relative to control splenocytes of mice.

2.5. Determination of antigen-specific antibody subclasses by ELISA

Serum HSP65-MUC1-specific IgG1 and IgG2c in mice were detected on day 7 after the fourth immunization. ELISA plates were coated overnight at 4 °C with 100 μ l of 10 μ g/well HSP65-MUC1 in PBS (1 mmol/l KH₂PO₄, 10 mmol/l Na₂HPO₄, 137 mmol/l NaCl, 2.7 mmol/l KCl, pH 7.4). After washing three times with washing buffer (PBST, 1 mol/l PBS, 0.05% Tween-20), 200 μ l of blocking buffer was added (PBS, 5% skim milk) followed by incubating at 37 °C for 2 h. The sera were diluted

1:200 with sample buffer (PBS, 1% BSA, 0.05% Tween-20), added to wells in duplicate and incubated at 37 °C for 1 h. Afterwards, plates were washed followed by the addition of 100 μ l per well of goat anti-mouse IgG1/HRP and IgG2c/HRP (Sigma Chemical Co., St Louis, MO) at 1:2500 dilution and incubated at 37 °C for 1 h. After washing, the substrate solution of o-phenylenediamine dihydrochloride (OPD) (Amresco, Solon, OH) was added and incubated at room temperature for 5 min in dark for color development which was stopped with 50 μ l 1.25 mol/l sulfuric acid per well. The optical density (OD) values were measured at 492 nm on an automated ELISA plate reader. The results were expressed as the average OD₄₉₂ value \pm standard deviations (SD).

2.6. Mouse splenocyte cytotoxicity assay

C57BL/6 mice were injected s.c. with PBS, HSP65-MUC1, MF59/HSP65-MUC1 and MF59-YW002/HSP65-MUC1 at the both sides of the inguinal lymph node area on days 1 and 8. On day 18, splenocytes from 3 mice in each group were prepared and cultured with 20 μ g/ml HSP65-MUC1 for 5 days in a humidified atmosphere of 5% CO₂ at 37 °C. On day 3, mouse IL-2 (20U/ml) was added to the culture. The HSP65-MUC1 recalled splenocytes were collected as effector cells, and co-cultured with MUC1⁺ B16 melanoma cells, as target cells (1 \times 10⁴ per well), in a 96-well plate at effector/target (E: T) ratios 5:1, 15:1 and 45:1 at 37 °C for 6 h. After aspirating the supernatant containing the suspended splenocytes, the remaining adherent MUC1⁺ B16 cells were collected, stained with trypan blue and counted under light microscope by a double-blindness method [30]. Each counting was repeated 4 times. Under microscope, the live B16 melanoma cells are unstained and distinguishable from the residual splenocytes by distinctive larger size. The cytotoxicity of the splenocytes was calculated by the formula: Cytotoxicity (%) = [1 – viable cell number in experiment groups/viable cell number in medium control] \times 100%, where the viable cell number = total number of live B16 melanoma cells in four big squares/4 \times dilution factor \times 10⁴. The live cell number in four big squares should be between 200 and 500, and concentration of cells should be higher than 10⁴/ml.

2.7. Flow cytometry analysis

Mouse splenocytes from the immunized mice were prepared and cultured with 20 μ g/ml HSP65-MUC1 for 12 h, and then stained with FITC-labeled anti-CD8 mAb and PE-labeled anti-CD69 mAb to analyze the lymphocyte activation. Briefly, 1 \times 10⁶ splenocytes were stained with fluorescence conjugated monoclonal antibodies for 40 min on ice in the dark, washed with FACS buffer (1 \times PBS, containing 2 mmol/l EDTA and 20 ml/l FBS) and analyzed by flow cytometry.

2.8. Statistical analysis

Tumor growth curves were plotted based on tumor size until the first mouse died. One-way analysis of variance (ANOVA) was used to analyze the experimental data. A two-sided Student's *t*-test was adopted to compare the mean values of individual treatments when the primary outcome was statistically significant. Survival was estimated by the Kaplan–Meier method and evaluated with a log-rank test. A *p* value of <0.05 was considered statistically significant. All statistical analyses were performed with the SPSS 16.0 software.

3. Results

3.1. Immunization of mice with adjuvant containing MF59-CpG ODN and HSP65-MUC1 is prophylactic against anti-MUC1⁺ melanoma

To assess whether MF59 plus CpG ODN (MF59-CpG ODN) could be a potent adjuvant for cancer vaccines, we selected the recombinant fusion protein HSP65-MUC1 as an antigen for tumor vaccine against

MUC1-transfected melanoma cells (MUC1⁺ melanoma), which we established by transfecting a fusion gene encoding green fluorescence protein (GFP) and MUC1 VNTR peptide into murine B16 melanoma cells, as target tumor cells [28]. After preparation and identification by SDS-PAGE (Fig. 1A) and western blotting (Fig. 1B), HSP65-MUC1 alone, MF59/HSP65-MUC1, or combination of MF59 with a C-type CpG ODN (YW002) termed MF59-YW002/HSP65-MUC1 was subcutaneously (s.c.) immunized into C57BL/6 mice (*n* = 10) on both sides of the inguinal lymph node area four times in a 7 day interval (Fig. 2A). On day 3 after the last immunization, MUC1⁺ B16 melanoma cells (1.2 \times 10⁵ per mouse) were inoculated into mice (Fig. 1C). The tumor volumes of mice were measured every other day and survival of the mice was calculated. The results showed that MF59-YW002 could significantly boost the immunogenicity of HSP65-MUC1 to induce anti-MUC1⁺ melanoma immunity (*p* = 0.007 compared with the PBS group). Vaccination of HSP65-MUC1 alone displayed a very limited role in inhibiting tumor growth. Using either MF59 or YW002 alone as an adjuvant for HSP65-MUC1 failed to inhibit tumor growth. Surprisingly, when MF59 was used as the sole adjuvant with HSP65-MUC1 it promoted significant growth of MUC1⁺ B16 melanoma (*p* = 0.038) (Fig. 2B). Importantly, mice vaccinated with MF59-YW002/HSP65-MUC1 had a significantly prolonged survival rate, and on day 75 post-tumor challenge, 50% of vaccinated mice were alive. In contrast, only 11.1%, 20% and 20% of the mice

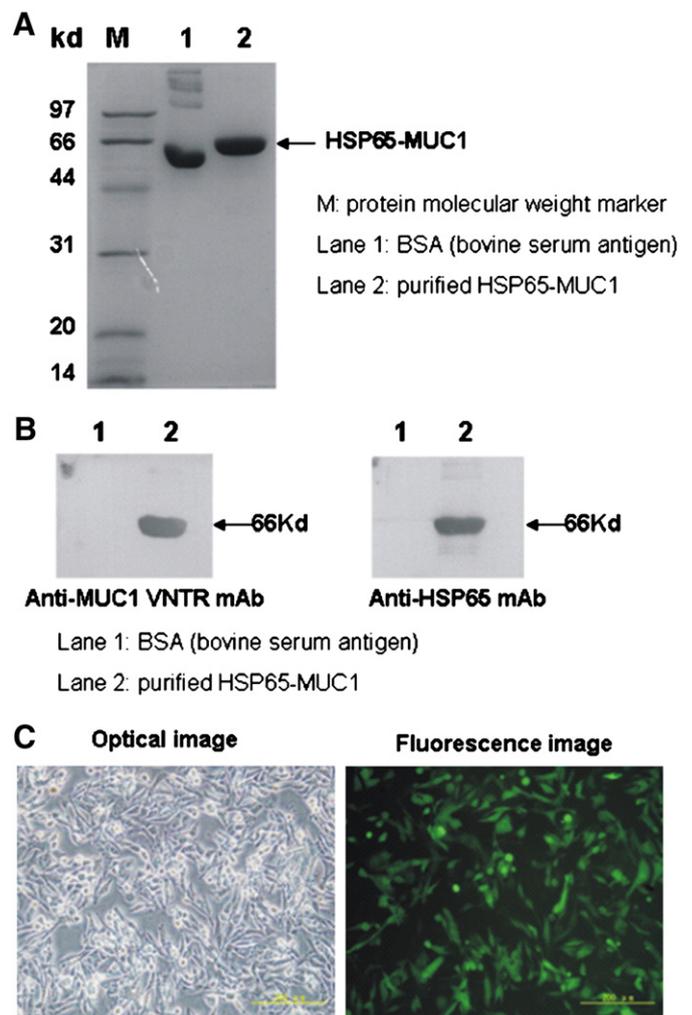


Fig. 1. Identification of HSP65-MUC1 and MUC1⁺ B16 melanoma cells. (A) SDS-PAGE analysis of purified HSP65-MUC1. (B) Western blots of HSP65-MUC1 using anti-MUC1 VNTR mAb or anti-HSP65 mAb. (C) MUC1⁺ melanoma B16 cells.

survived in the PBS, HSP65-MUC1 alone or YW002/HSP65-MUC1 groups, respectively. In the group vaccinated with MF59/HSP65-MUC1, all mice died at an early stage (Fig. 2C), consistent with the fast tumor growth. Tumor incidence was the highest (100%) in this group, compared with the PBS, HSP65-MUC1 alone or YW002/HSP65-MUC1 groups, where tumor incidence was 88.9%, 60% or 70%, respectively. In comparison, vaccination with MF59-YW002/HSP65-MUC1 significantly decreased tumor incidence to 30% (Fig. 2D). Taken together, MF59-CpG ODN may be a promising adjuvant to promote immunogenicity of HSP65-MUC1 to prophylactically inhibit MUC1⁺ tumors.

Next, we tested whether vaccination with MF59-YW002/HSP65-MUC1 could induce a therapeutic effect against anti-MUC1⁺ melanoma. Mice were inoculated with MUC1⁺ B16 melanoma cells (1.2×10^5 per mouse) followed by immunization with HSP65-MUC1 alone, or MF59-YW002/HSP65-MUC1 four times. The first immunization was performed on day 1 post-tumor challenge followed by three other immunizations in a 7 day interval (Fig. 3A). Tumor volumes of mice were measured every other day and survival of mice was calculated. Immunization with MF59-YW002 failed to enhance immunogenicity of HSP65-MUC1 to induce tumor inhibition in the therapeutic model (Fig. 3B). The survival rate of mice immunized with MF59-YW002/HSP65-MUC1 was

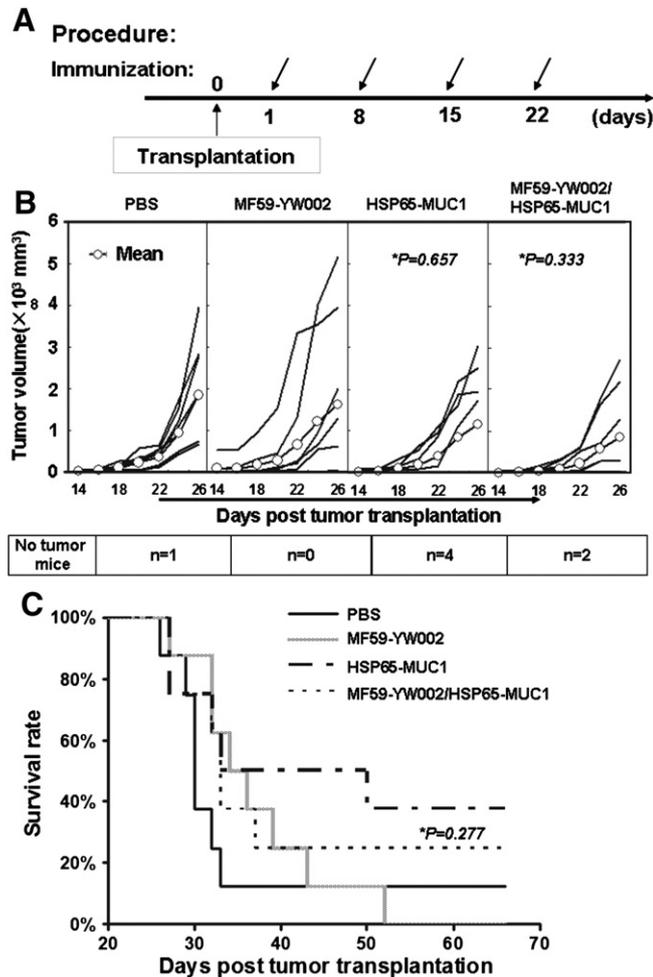


Fig. 3. Therapeutic anti-melanoma effect induced by immunization of MF59-YW002/HSP65-MUC1 in mice. C57BL/6 mice ($n=8$) were inoculated 1.2×10^5 MUC1⁺ B16 melanoma cells on day 0 and injected sub-cutaneously (s.c.) with HSP65-MUC1 alone, or combined with MF59-YW002/HSP65-MUC1, or MF59-YW002 on days 1, 8, 15 and 23. Tumor volume was measured every 2 days, and the survival of mice was calculated. PBS-injected mice were used as a negative control. * $p < 0.05$ compared with the PBS group. (A) Experimental procedure. (B) Tumor growth curves. Each line represents tumor growth kinetics in each mouse. (C) Survival of mice.

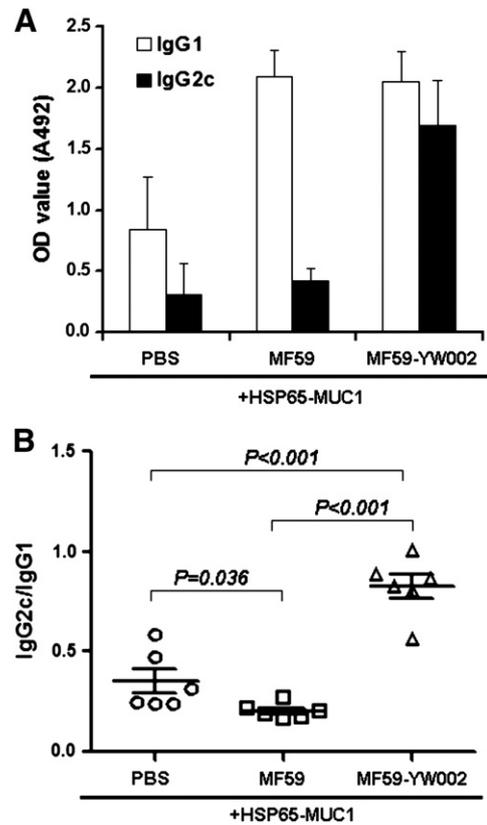


Fig. 4. HSP65-MUC1 specific antibody levels in sera of immunized mice. HSP65-MUC1 specific IgG1 and IgG2c levels in sera of immunized mice were determined by ELISA on day 7 after the last immunization. (A) Serum IgG1 and IgG2c levels. (B) IgG2c/IgG1 ratio.

only 22.2% compared with 38% in mice immunized with HSP65-MUC1 alone (Fig. 3C), indicating that MF59-CpG ODN may be not a suitable adjuvant for therapeutic application of HSP65-MUC1 to induce anti-tumor immunity.

3.2. MF59-CpG ODN enhances the immunogenicity of HSP65-MUC1 to induce a cellular immune response in mice

To explore how MF59-CpG ODN could enhance the anti-tumor effect of HSP65-MUC1 in mice, we measured the sera of mice immunized with HSP65-MUC1, MF59/HSP65-MUC1 or MF59-YW002/HSP65-MUC1, for HSP65-MUC1-specific antibodies. Mice immunized with MF59-YW002/HSP65-MUC1 and MF59/HSP65-MUC1 developed high levels of serum anti-HSP65-MUC1 IgG1 antibody compared with the HSP65-MUC1 group without adjuvant. However, serum IgG2c levels were significantly higher in mice immunized with MF59-YW002/HSP65-MUC1 compared with HSP65-MUC1 alone or MF59/HSP65-MUC1 (Fig. 4A). The ratio of IgG2c/IgG1 was higher in mice immunized with MF59-YW002/HSP65-MUC1, whereas MF59/HSP65-MUC1 induced a Th2 response as a lower HSP65-MUC1-specific IgG2c/IgG1 ratio was observed compared with that initiated by HSP65-MUC1 alone (Fig. 4B).

Next, spleens of mice were isolated on day 1 after the last immunization with HSP65-MUC1 alone, MF59/HSP65-MUC1 or MF59-YW002/HSP65-MUC1. The expression of IFN- γ and IL-4 mRNA in splenocytes was detected using real-time RT-PCR. Immunization with MF59-YW002/HSP65-MUC1 produced high levels of IFN- γ mRNA compared with HSP65-MUC1 alone or the MF59/HSP65-MUC1 group (Fig. 5). Comparatively, MF59/HSP65-MUC1 induced a higher level of IL-4 mRNA than HSP65-MUC1 alone ($p < 0.05$), while

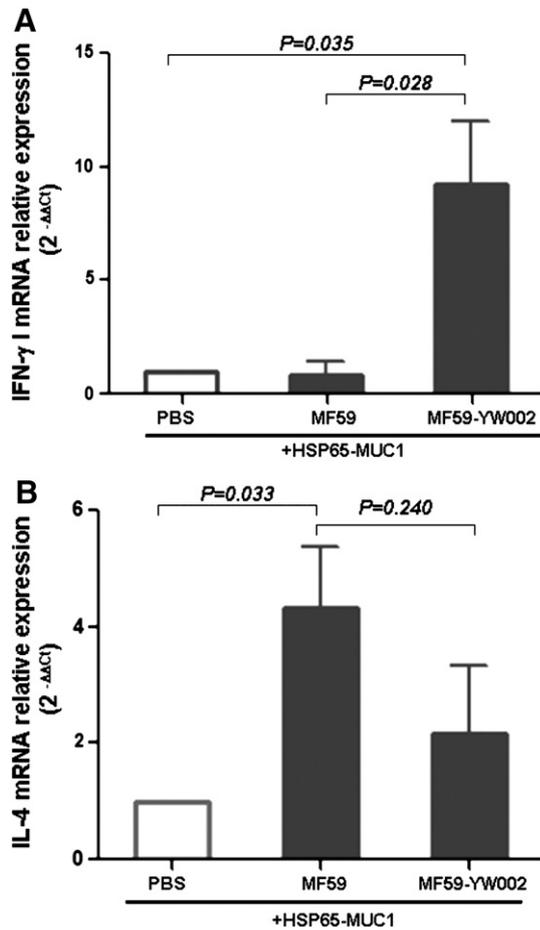


Fig. 5. Relative expression of IFN- γ and IL-4 mRNA in splenocytes from immunized mice. The IFN- γ and IL-4 mRNA expression in splenocytes of immunized mice was determined on day 1 after the last immunization. All results, normalized to the IFN- γ and IL-4 mRNA expression in splenocytes of HSP65-MUC1-immunized mice, were expressed as $2^{-\Delta\Delta Ct}$ ($\Delta Ct = Ct_{\text{IFN-}\gamma/\text{IL-4}} - Ct_{\text{GAPDH}}$, $\Delta\Delta Ct = \Delta Ct_{\text{purpose}} - \Delta Ct_{\text{HSP65-MUC1}}$).

MF59-YW002/HSP65-MUC1 tended to inhibit the up-regulation induced by MF59/HSP65-MUC1, although statistically insignificant (Fig. 5B). These results implied that YW002 could confer MF59/HSP65-MUC1 the property of inducing a Th1-biased immune response in mice.

Subsequently, spleens of mice immunized with HSP65-MUC1, MF59/HSP65-MUC1 or MF59-YW002/HSP65-MUC1 were isolated on day 18 after the last immunization. Splenocytes were pulsed with HSP65-MUC1 for 12 h and then stained with FITC-labeled anti-CD8 mAb and PE-labeled anti-CD69 mAb to analyze CD8⁺ T cell activation. When MF59-YW002 was used as an adjuvant, HSP65-MUC1 strongly induced the proliferation of CD8⁺ T cells as indicated by the presence of CD69⁺/CD8⁺ double positive cells (Fig. 6A). The ratio of CD69⁺/CD8⁺ T cells in splenocytes from mice immunized with MF59-YW002/HSP65-MUC1 was significantly higher ($3.80 \pm 0.85\%$) compared with mice injected with PBS ($2.13 \pm 0.28\%$, $p = 0.031$), HSP65-MUC1 ($2.26 \pm 0.29\%$, $p = 0.040$) and MF59/HSP65-MUC1 ($2.43 \pm 0.10\%$, $p = 0.049$) (Fig. 6B). The results suggest that MF59-CpG ODN as an adjuvant with HSP65-MUC1 may contribute to the production of tumor-specific immune responses.

To investigate whether MF59-CpG ODN enhanced the immunogenicity of HSP65-MUC1 to induce tumor-specific immune responses, splenocytes from immunized mice were cultured with HSP65-MUC1 for 5 days and then used as effector cells in a CTL killing assay. As shown in Fig. 6C, splenocytes from mice immunized with MF59-

YW002/HSP65-MUC1 lysed MUC1⁺ B16 melanoma cells tested at different effector:target (E:T) ratios. The highest cytotoxicity (44.1%) was observed at an E:T ratio of 45:1. Splenocytes from mice immunized with HSP65-MUC1 alone or MF59/HSP65-MUC1 could also lyse target cells compared to the PBS control group although at a lower cytotoxicity than the MF59-YW002/HSP65-MUC1 group. This data indicates that MF59-CpG ODN may be a promising adjuvant for the development of tumor vaccines.

3.3. MF59-CpG ODN enhances immunogenicity of HSP65-MUC1 to induce immunological memory to inhibit MUC1⁺ tumors

To investigate whether MF59 formulated with CpG YW002 could promote HSP65-MUC1 to generate immune memory to MUC1⁺ melanoma, five mice immunized with MF59-YW002/HSP65-MUC1 and challenged with MUC1⁺ B16 melanoma cells were re-challenged with 1.2×10^5 MUC1⁺ B16 melanoma cells per mouse on day 75 after the first challenge. Naïve mice ($n = 5$) inoculated with MUC1⁺ B16 melanoma cells were used as controls. The survival of the mice was calculated. Four naïve mice succumbed to melanoma on day 39, whereas only one mouse died in the re-challenged group. The average survival was significantly longer in re-challenged mice than naïve mice ($p = 0.032$) (Fig. 7). This data suggested that MF59-CpG ODN could assist HSP65-MUC1 to generate MUC1⁺ B16 melanoma-specific immunologic memory in mice.

4. Discussion

In this study, we demonstrate that an adjuvant containing MF59 plus CpG ODN (MF59-CpG ODN) and HSP65-MUC1 could prophylactically induce an anti-MUC1⁺ tumor immune response that inhibited MUC1-expressing melanoma growth and prolonged the survival of tumor bearing mice. Comparatively, adjuvant containing MF59 alone with HSP-MUC1 failed to induce protective immune responses in mice transplanted with MUC1-expressing melanoma cells. As reported, MF59 has been used as an adjuvant for microbial antigens, such as inactivated influenza virus or herpes simplex virus [31,32], to induce neutralizing antibodies against the viruses, with elevated serum Th2 cytokine levels including interleukin (IL)-4 and IL-5 [32]. The inability of MF59/HSP65-MUC1 to induce efficient immunity against MUC1⁺ tumors could be attributed to its Th2 immune response-biasing properties. Consistent with this, we showed that MF59/HSP65-MUC1 induced significantly elevated levels of HSP65-MUC1-specific IgG1, resulting in a lower IgG2c/IgG1 ratio. It has been shown that MF59 induces Th2 cells to secrete Th2 cytokines (IL-4, IL-5 and IL-10) that can induce isotype switching in B cells to produce IgG1 antibodies [31,32].

In clinical trials, MF59 facilitated H5N1 subunit vaccines to induce high titers of neutralizing antibodies and specific CD4⁺ T cells with a Th1 effector/memory phenotype (IL-2⁺ IFN- γ ⁻/IL-2⁺ IFN- γ ⁺) [33]. However, it is not considered a suitable adjuvant for tumor antigens to induce protective Th1 cellular immune responses against tumor cells. Furthermore, the unsuitability of MF59 for protective immune responses against cancer is demonstrated by the data from this study where immunization with MF59/HSP65-MUC1 promoted the growth of MUC1⁺ B16 melanoma. This phenomenon may arise from tumor growth promotion caused by Th2 immune responses and antibody mediated tumor invasion. Hypothetically, strong Th2 responses are often associated with a lack of Th1 cell-mediated immunity. *In vivo* studies showed that IL-4 and IL-10 could inhibit cell-mediated immunity synergistically [34]. Thus, the presence of Th2 cytokines would be unfavorable for beneficial Th1-mediated tumor responses. Indeed, Lewis lung carcinoma cells grew more aggressively in IL-10 transgenic mice where an inefficient APC capacity to induce MHC all-reactivity and impaired CTL responses were observed [35]. Additionally, endogenous IL-4 could up-regulate anti-apoptotic gene expression in

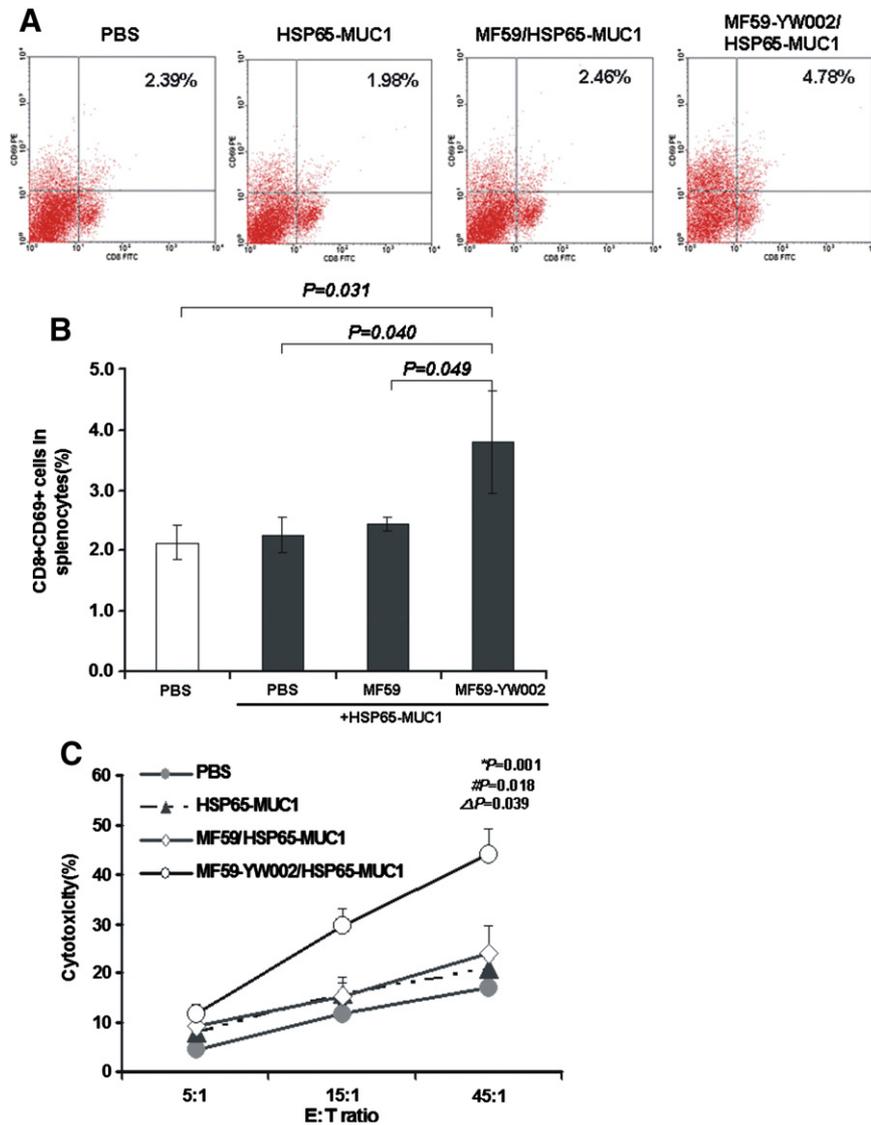


Fig. 6. Induction of MUC1-specific CD69⁺CD8⁺ T cells and cytotoxic T lymphocytes in immunized mice. C57BL/6 mice ($n = 3$) were immunized with HSP65-MUC1 alone, MF59/HSP65-MUC1 or MF59-YW002/HSP65-MUC1 on days 1 and 8. On day 18, mice were sacrificed for isolating splenocytes. After stimulation with HSP65-MUC1 for 12 h, the splenocytes were stained with FITC-labeled anti-CD8 mAb and PE-labeled anti-CD69 mAb and analyzed by flow cytometry. (A) Fluorescence analysis of CD8⁺CD69⁺ T cells in splenocytes. (B) Average ratio of CD8⁺CD69⁺ T cells in splenocytes from three mice. (C) Splenocyte cytotoxicity assay. Splenocytes were stimulated with HSP65-MUC1 for 5 days, and then co-cultured with the MUC1⁺ B16 melanoma cells at an effector/target (E:T) ratio of 5:1, 15:1, and 45:1, for 6 h. Statistical significance ($p < 0.05$) compared with other groups was represented as follows: * vs. PBS; # vs. HSP65-MUC1; Δ vs. MF59/HSP65-MUC1.

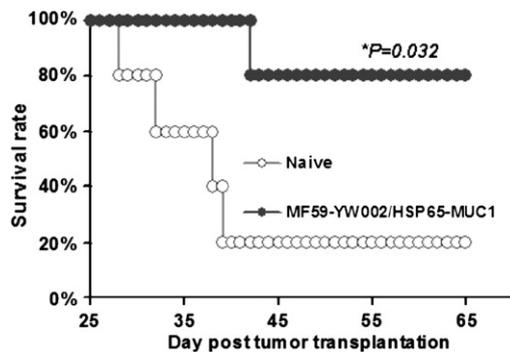


Fig. 7. Immunological memory is induced by immunization with MF59-YW002/HSP65-MUC1 in mice. Mice ($n = 5$) from the group receiving prophylactic immunization with MF59-YW002/HSP65-MUC1 followed by transfer of MUC1⁺ B16 melanoma cells were rechallenged sub-cutaneously (s.c.) with 1.2×10^5 MUC1⁺ B16 melanoma cells. Naïve mice ($n = 5$) were inoculated s.c. with 1.2×10^5 MUC1⁺ B16 melanoma cells as controls. Survival of mice was recorded after re-challenge. * $p < 0.05$ compared with naïve mice.

fibrosarcoma cells, therefore reducing apoptosis of tumor cells [36]. Thus, in a Th2 cytokine milieu, tumor antigen-induced specific antibodies could promote tumor growth. This may be supported by our observation that MF59/HSP65-MUC1 promoted B16 melanoma growth, possibly due to the specific antibodies induced by HSP65-MUC1 under Th2-biased settings created by MF59. This may be partly confirmed by the evidence that antisera or purified IgG derived from recombinant GRP78 (a tumor antigen expressed on melanoma cells)-immunized mice promoted the proliferation of murine melanoma cells *in vitro*, and that the antibody could cause accelerated B16F1 melanoma growth in GRP78-immunized mice [37]. In addition, the formation of antibody and tumor antigen complexes may induce granulocyte and macrophage-mediated tumor invasion and metastasis by promoting extracellular matrix degradation and angiogenesis [38].

Currently, B-type CpG ODNs are commonly used as adjuvants for tumor antigens in preclinical studies and clinical trials. In the present study, YW002, a C-type CpG ODN, when formulated with MF59, could enhance HSP65-MUC1-specific Th1-biased immune responses in mice, although the mechanism involved is unclear. However, the

combination of YW002 and MF59 might aid cross-presentation of exogenous HSP65-MUC1 by B cells to generate MUC1-specific CTLs. As reported, upon CpG ODN stimulation, naïve B cells could acquire the ability to present soluble antigens through MHC class I pathway to generate CD8⁺ T cells [39,40]. As shown in the present study, HSP65-MUC1 combined with MF59-YW002 induced MUC1⁺-specific cytotoxic lymphocytes. Second, the combination of YW002 and MF59 may induce HSP65-MUC1-specific Th1-biased immune responses against MUC1⁺ B16 melanoma by inducing high levels of IFN- α / β from pDCs. In addition, CpG ODN induced IFN- α could stimulate DCs to produce IL-12 and CD4⁺ T cells to produce IFN- γ , consequently promoting the differentiation of Th1 cells [41,42]. Similarly, our data demonstrated that MF59-YW002/HSP65-MUC1 induced high levels of antigen-specific IgG2c in mice and up-regulated the expression of IFN- γ mRNA in murine splenocytes *in vitro*. Noticeably, YW002 requires MF59 to induce a Th1-type adjuvant activity for HSP65-MUC1 as HSP65-MUC1 combined with YW002 alone was insufficient to inhibit MUC1⁺ B16 melanoma growth in mice. This may be explained by the promotion of DC maturation and antigen uptake by MF59, enhancing DC migration to lymph nodes [43], and providing a potent synergy with YW002 to induce cell-mediated immune responses more effectively. Overall, C-type CpG ODN might be more suitable to work with MF59 on inducing Th1-biased immune response to HSP65-MUC1. The assumption could be supported by our unpublished data that YW002, when formulated with MF59, facilitated HSP65-MUC1 to induce higher level of specific IgG2c and IFN- γ mRNA in mice than 2216, an A-type CpG ODN [44] or 2006, a B-type CpG ODN [45].

Taken together, this study demonstrated that MF59 formulated with a C-type CpG ODN (YW002) could enhance the immunogenicity of HSP65-MUC1 to induce a Th1-biased immune response and specific anti-MUC1⁺ B16 melanoma immunity prophylactically. This formulation could be applied to prepare tumor antigen-based vaccines for cancer patients, particularly for postoperative cancer patients using a prophylactic strategy to prevent tumor recurrence and eliminate residual tumor cells.

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