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Carrageenan as an Adjuvant to Enhance Peptide-based Vaccine Potency

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Abstract

New innovative therapies are urgently required in order to combat the high mortality and morbidity associated with advanced cancers. Antigen-specific cancer immunotherapy using peptide-based vaccination has emerged as an attractive approach for the control of cancers due to its simplicity and easy preparation. However, such an approach requires the employment of suitable adjuvants. In the current study, we explored the employment of a sulfated polysaccharide compound from red algae, carrageenan (CGN) as an adjuvant for their ability to generate antigen-specific immune responses and antitumor effects in mice vaccinated with human papillomavirus type 16 (HPV-16) E7 peptide vaccine. We found that carrageenan can significantly enhance the E7-specific immune responses generated by E7 peptide vaccination via the TLR4 activation pathway. In addition, carrageenan could enhance the protective and therapeutic antitumor effects generated by E7 peptide vaccination against E7-expressing tumors. Furthermore, the observed enhancement was not restricted to E7 antigen but was also applicable to other antigenic systems. We also found that other structurally similar compounds to CGN, such as dextran, also generated similar immune enhancement. Thus, our data suggest that CGN and its structurally related compounds may serve as innovative adjuvants for enhancing peptide-based vaccine potency.

Keywords

carrageenan; peptide vaccination; E7; human papillomavirus (HPV)

Introduction

Advanced stage cancers are difficult to control using conventional therapies such as chemotherapy, radiation and/or surgery. Thus, the development of innovative therapies for advanced stage cancers is urgently needed. Antigen-specific immunotherapy has the potency to eradicate systemic tumors at multiple sites in the body, as well as the specificity to

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discriminate between malignant and normal cells (for review see [1,2]). Thus, antigen-specific immunotherapy, such as peptide-based vaccination serves as a potentially promising alternate approach for the control of advanced stage cancers, and may be employed in conjunction with conventional therapies.

Peptide-based vaccination has emerged as a potentially important strategy for the development of therapeutic vaccination as they are considered to be safe, easy to produce, and stable [3,4] (for review see [5,6]). One of the important factors in the designing of therapeutic peptide-based vaccines is the choice of target antigen. In the case of human papillomavirus (HPV)-associated cancers, the virus encoded oncogenic proteins such as E6 and E7 represent ideal target antigens for vaccine development since they are consistently expressed in a majority of cervical cancers and its precursor lesions and are essential for transformation [7]. Previous studies have shown that vaccination with the HPV-16 E7-derived 35 amino-acid long peptide E7 (aa43-77), which contains both a CTL epitope and a Th epitope, in combination with incomplete Freund's adjuvant or CpG as adjuvants resulted in potent E7-specific CD8⁺ T cell immune responses [8]. This study suggests that peptide-based vaccine potency may be enhanced by employment of adjuvants to enhance vaccine potency through DC activation. Thus, it is important to identify suitable adjuvants that can be used to enhance peptide-based vaccine potency.

The peptide vaccine potency can be enhanced by employment of “alert” signals such as toll-like receptor (TLR) ligands as adjuvants that stimulate dendritic cells to mature and differentiate into potent activators of antigen-specific T cells (for review, see [9]). It is clear that toll-like receptors (TLRs) play a crucial role in enhancing innate and adaptive immune responses (for reviews, see [10-13]). TLR ligands, such as TLR4 ligands represent an important class of vaccine adjuvants. Carrageenan (CGN), a TLR4 ligand [14], is a sulfated polysaccharide compound extracted from red algae [15]. Recent studies have shown that CGN is a potent inhibitor of papillomavirus infection [16]. It has a structure similar to heparan sulfate, which is an HPV cell-attachment factor, and has been shown to prevent the binding of HPV virions to cells. Thus, carrageenan may potentially be used as an adjuvant to enhance the potency of HPV peptide vaccination.

In the current study, we explored the adjuvant effect of CGN in enhancing the E7-specific CD8⁺ T cell immune responses and therapeutic antitumor effects against E7-expressing tumors generated by HPV-16 E7 peptide (aa 43-62) vaccination. We found that CGN can significantly enhance the E7-specific CD8⁺ T cell immune responses generated by E7 peptide vaccination via the TLR4 activation pathway. CGN was also found to enhance the protective and therapeutic antitumor effects generated by E7 peptide vaccination. The immune enhancement generated by CGN was comparable to that generated by other TLR4 ligands such as monophosphoryl lipid A (MPL-A). Furthermore, the observed enhancement was not restricted to E7 antigen but was also applicable to other antigenic systems. We also found that other structurally related compounds such as dextran also generated similar immune enhancement as CGN. Our data has significant implications for future clinical translation.

Materials and Methods

Mice

Female C57BL/6 mice, 5 to 8 weeks of age, were purchased from National Cancer Institute. TLR4 knockout mice were purchased from the Jackson Laboratory. All of the mice were used in compliance with institutional animal health care regulations, and all animal experimental procedures were approved by the Johns Hopkins Institutional Animal Care and Committee.

Cells and antibodies

TC-1 cells, which are an E7-expressing murine tumor model, were obtained by co-transformation of primary C57BL/6 mouse lung epithelial cells with HPV-16 E6 and E7 and an activated ras oncogene as previously described [17]. They were maintained in RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2nM glutamine, 1mM sodium pyruvate, 20mM HEPES, 50μM β-mercaptoethanol, 100IUml⁻¹ penicillin, 100μg ml⁻¹ streptomycin and 10 % fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA, USA).

Subcutaneous (s.c.) vaccination

Mice were injected *s.c.* in the left flank with 10 ug E7 peptide (aa 43-62) and/or 10 ug carrageenan (CGN, Sigma) in a total volume of 100 ul PBS, and were boosted 7 days later. Tyrosinase-related protein 2 (TRP2) (aa 181-188) and/or CGN, E7 peptide (aa 43-62) and/or Dextran (Sigma), E7 peptide (43-62) and/or MPL-A (monophosphoryl lipid A, InvivoGen) vaccinations were performed in the same fashion and dose.

Intracellular cytokine staining and flow cytometry analysis

For characterization of E7-specific CD8⁺ T cells, splenocytes were harvested 7 days after the second vaccination. 5×10⁶ splenocytes from each treatment group were incubated with 1 ug/ml E7 peptide and 1 ul/ml GolgiPlug (BD Cytotfix/Cytoperm Kit) for 16 hours. Cells were then harvested, stained for CD8 and IFN-γ using a previously described standard protocol [18]. Samples were analyzed on a FACSCalibur flow cytometer, using CellQuest software (Becton Dickinson, San Jose, CA) as described previously [18]. All of the analyses shown were carried out with gated lymphocyte populations.

TC-1 tumor challenge

TC-1 tumor cells were injected *s.c.* into mice in a volume of 100 ul. For prophylactic study, 5×10⁴ TC-1 tumor cells were injected 7 days after the second vaccination. For therapeutic study, mice were injected with 1×10⁴ TC-1 tumor cells, followed by 3 vaccinations with 4 days interval. The tumor growth was monitored each week by palpation, and the mice were euthanized when the tumor size reached 2 cm.

QUANTI-Blue assay

HEK-Blue-4 cells (InvivoGen) were cultured in DMEM medium supplemented with 10% FBS and 1× HEK-Blue Selection. Cells were seeded in 96 well plate, and incubated with CGN (1 ug/ml) in a total volume of 200 ul for 24 hours. For QUANTI-Blue assay, 160 ul of QUANTI-Blue (InvivoGen) was mixed with 40 ul of the induced HEK-Blue-4 cell supernatant, and incubated at 37°C for 2 hours, and then SEAP level was determined using a spectrometer at 625nm.

In vivo fluorescence imaging

Mice were injected with 1 ul Alexa Fluor750-streptavidin (Strep*, Invitrogen) with or without 10 ug CGN in a total volume of 100 ul by *s.c.* injection in the left flank. 2, 24, and 48 hours after the injection, fluorescence imaging was conducted on a cryogenically cooled IVIS system (Xenogen/Caliper Life Sciences), and an acquisition time of 1 min was used. The isolated lymph nodes were imaged by using the same method. The signal intensity was analyzed by Living Image software 2.5 (Xenogen/Caliper Life Sciences).

Statistical analysis

All data expressed as means±standard deviation are representative of at least two independent experiments. Comparisons between individual data points were made using a Student's *t*-test.

Results

Co-administration of carrageenan can significantly enhance the E7-specific CD8+ T cell immune responses generated by E7 peptide vaccination

In order to determine if CGN can enhance the E7-specific immune responses generated by E7 peptide vaccination, C57BL/6 mice (5 per group) were vaccinated with E7 peptide (aa 43-62) alone, CGN alone, or E7 peptide (aa 43-62) in combination with CGN twice by *s.c.* injection. One week after the last vaccination, the splenocytes were restimulated with E7 peptide (aa 49-57), and the E7-specific T cell immune responses were characterized using intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 1, we have demonstrated that mice vaccinated with E7 peptide in combination with CGN demonstrated significantly higher E7-specific CD8+ T cell immune responses compared to mice vaccinated with E7 alone or CGN alone (approx. 4-fold increase) ($p<0.05$). We also optimized the dose of CGN used in combination with E7 peptide vaccination to generate the highest E7-specific CD8+ T cell immune responses (See Supplementary Figure 1). We also characterized the E7-specific CD4+ T cell immune responses in naïve mice vaccinated with E7 peptide vaccine in combination with CGN treatment. However, we observed only background levels of E7-specific CD4+ T cells in the treated mice (data not shown). Thus, our data suggest that co administration of CGN with E7 peptide vaccination can significantly enhance E7-specific CD8+ T cell immune responses in vaccinated mice.

Co-administration of carrageenan can significantly enhance the protective and therapeutic antitumor effects generated by E7 peptide vaccination

In order to determine if co-administration of carrageenan could enhance the protective antitumor effects generated by E7 peptide vaccination, C57BL/6 mice (5 per group) were vaccinated twice with E7 peptide vaccine (aa 43-62) alone, CGN alone, or E7 peptide (aa 43-62) in combination with CGN, followed by subcutaneous injection of TC-1 tumor cells in the left flank. The tumor growth was monitored each week. As shown in Figure 2, mice immunized with E7 peptide in combination with CGN demonstrated significantly higher percentage of tumor-free mice and prolonged survival compared to mice immunized with E7 peptide alone or CGN alone ($p<0.05$).

We also characterized the therapeutic antitumor effects of co-administration of CGN with E7 peptide vaccination. C57BL/6 mice (5 per group) were injected with TC-1 tumor cells by subcutaneous injection in the left flank, followed 4 days later by vaccination with E7 peptide vaccine (aa 43-62) alone, CGN, or E7 peptide (aa 43-62) in combination with CGN three times with 4-day intervals and tumor growth was monitored each week. As shown in Figure 3, tumor-bearing mice treated with E7 peptide in combination with CGN demonstrated significantly higher percentage of tumor-free mice and prolonged survival compared to tumor-bearing mice treated with E7 peptide alone or CGN alone ($p<0.05$). Thus, taken together our data indicates that co-administration of carrageenan can significantly enhance the protective and therapeutic antitumor effects generated by E7 peptide vaccination.

CGN leads to the enhancement of immune responses generated by E7 peptide vaccination via TLR4 activation pathway

In order to determine if the observed enhancement in antigen-specific CD8+ T cell immune responses by CGN is dependant on toll-like receptor 4 mediated signaling, we performed a

TLR4 reporter assay. We used HEK-Blue-4 cells to study the activation of TLR4 by monitoring NF- κ B activation. HEK-Blue-4 cells were generated by cotransfection of TLR4, MD2 and CD14 and optimized secreted alkaline phosphatase (SEAP) reporter gene under the control of NF- κ B inducible promoter into HEK-293 cells. HEK-Blue-4 cells were treated with CGN for 24 hours, and the stimulation of TLR4 was detected by using QUANTI-Blue assay. As shown in Figure 4A, administration of CGN led to significantly higher level of SEAP TLR4 activation compared to the control, thus confirming that CGN is a TLR4 ligand ($p < 0.05$).

In order to compare the E7-specific CD8⁺ T cell immune responses generated by E7 peptide vaccination in combination with CGN in wild-type (WT) C57BL/6 mice with those generated in TLR4^{-/-} mice, C57BL/6 mice or TLR4^{-/-} mice were vaccinated twice with E7 peptide vaccine (aa 43-62), CGN, or E7 peptide (aa 43-62) in combination with CGN with a 1-week interval. 7 days after the second vaccination, the splenocytes were restimulated with E7 peptide, and the E7-specific T cell immune responses were characterized using intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 4B and C, we found that the E7-specific CD8⁺ T cell immune responses generated by E7 peptide vaccination in conjunction with CGN were significantly diminished in TLR4^{-/-} mice compared to WT C57BL/6 mice (approx. 4-fold reduction) ($p < 0.05$). Thus, our data indicate that CGN leads to the enhancement of E7-specific CD8⁺ T cell immune responses generated by E7 peptide vaccination via TLR4 activation pathway.

Co-administration of carrageenan can enhance antigen loading in the lymph nodes

In order to determine if CGN can lead to activation of DCs in the lymph nodes, we employed an *in vivo* fluorescence imaging assay using fluorescently labeled streptavidin in mice treated with or without CGN. C57BL/6 mice (5 per group) were injected with Alexa Fluor750 labeled streptavidin (Strep*) with or without CGN by subcutaneous injection in the left flank. At 2, 24, and 48 hours after the injection, the mice were imaged using fluorescence imaging. In addition, the lymph nodes from the treated mice were isolated and imaged using fluorescence imaging. As shown in Figure 5A and B, mice injected with Strep* in combination with CGN demonstrated significantly higher fluorescence intensity compared to the mice without CGN treatment ($p < 0.05$). This suggests that co-administration of antigen with CGN can lead to a prolonged release of antigen over time. Furthermore, the lymph nodes isolated from mice injected with Strep* in combination with CGN also demonstrated significantly higher fluorescence intensity compared to the lymph nodes isolated from mice without CGN treatment ($p < 0.05$) (Figure 5C and D). This data indicates that co-administration of antigen with CGN leads to an increased level of antigen in the lymph nodes. This suggests that the antigen may be taken up by DCs, which in turn may lead to activation and migration of DCs to the lymph nodes. Thus, taken together, our data suggest that co-administration of carrageenan may potentially enhance activation and migration of dendritic cells to the lymph nodes.

Co-administration of carrageenan can enhance the TRP2 antigen-specific immune responses generated by peptide vaccination

In order to determine if CGN can enhance the other antigen-specific immune responses generated by peptide vaccination, C57BL/6 mice were vaccinated with the melanoma-associated antigen, tyrosinase-related protein 2 (TRP2) peptide (aa 181-188) alone, CGN alone, or TRP2 peptide (aa 181-188) in combination with CGN twice by *s.c.* injection. One week after the last vaccination, the splenocytes were restimulated with TRP2 peptide (aa 181-188), and the TRP2-specific T cell immune responses were characterized using intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 6, we have demonstrated that mice vaccinated with TRP2 peptide in combination with CGN

demonstrated significantly higher TRP2-specific CD8⁺ T cell immune responses compared to mice vaccinated with TRP2 alone or CGN alone (5-fold increase) ($p < 0.05$). Thus, our data suggest that co administration of CGN with peptide vaccination can significantly enhance antigen-specific CD8⁺ T cell immune responses in vaccinated mice.

Co-administration of CGN with E7 peptide vaccination can generate comparable E7-specific immune responses compared to other TLR4 ligands such as MPL-A

Monophosphoryl lipid A (MPL-A) has been shown to be a toll-like receptor 4 ligand and has been used as an adjuvant to enhance antigen-specific immunity (For review see [19]). In order to compare the enhancement in E7-specific immune responses generated by E7 peptide vaccination between CGN and MPL-A, C57BL/6 mice were vaccinated with E7 peptide (aa 43-62) alone, CGN alone, MPL-A alone, E7 peptide (aa 43-62) in combination with CGN or E7 peptide in combination with MPL-A twice by *s.c.* injection. One week after the last vaccination, the splenocytes were restimulated with E7 peptide, and the E7-specific T cell immune responses were characterized using intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 7, we have demonstrated that mice vaccinated with E7 peptide in combination with CGN demonstrated comparable E7-specific CD8⁺ T cell immune responses to mice vaccinated with E7 peptide in combination with MPL-A. Thus, our data suggest that co administration of CGN with E7 peptide vaccination can significantly enhance E7-specific CD8⁺ T cell immune responses in vaccinated mice, comparable to other TLR4 ligands, such as MPL-A.

Co-administration of other structurally similar compounds such as dextran with E7 peptide vaccination can generate E7-specific immune responses comparable to CGN

We then wanted to determine if other compounds structurally similar to CGN, such as dextran, can enhance the E7-specific immune responses generated by E7 peptide vaccination. We confirmed that dextran is also a TLR4 ligand using the TLR4 activation assay. As shown in Figure 8A, we found that dextran leads to significant activation of TLR4 comparable to CGN. C57BL/6 mice were vaccinated with E7 peptide (aa 43-62) alone, CGN alone, dextran alone, E7 peptide (aa 43-62) in combination with CGN or E7 peptide in combination with dextran twice by *s.c.* injection. One week after the last vaccination, the splenocytes were restimulated with E7 peptide, and the E7-specific T cell immune responses were characterized using intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 8B and C, we have demonstrated that mice vaccinated with E7 peptide in combination with CGN demonstrated comparable E7-specific CD8⁺ T cell immune responses to mice vaccinated with E7 peptide in combination with dextran. Thus, our data suggest that co-administration of other structurally similar TLR4 ligands, such as dextran, with E7 peptide vaccination can enhance E7-specific CD8⁺ T cell immune responses to a level comparable to carrageenan in vaccinated mice.

Discussion

In the current study, we found that co-administration of carrageenan can significantly enhance the E7-specific immune responses generated by E7 peptide vaccination via the TLR4 activation pathway. Co-administration of carrageenan was also found to enhance the protective and therapeutic antitumor effects generated by E7 peptide vaccination. Furthermore, the observed enhancement was not restricted to E7 antigen but was also applicable to other antigenic systems. In addition, we found that the immune enhancement generated by carrageenan was comparable to that generated by other TLR4 ligands, such as monophosphoryl lipid A (MPL-A). We also found that other structurally similar compounds such as dextran also generated similar immune enhancement as carrageenan. Our study

opens up a new category of immune adjuvants that may potentially be used to enhance peptide/protein-based vaccine potency.

There is an urgent need to identify suitable adjuvants for vaccine development. Our study showed that other compounds that are structurally similar to carrageenan, such as dextran were also capable of inducing significant enhancement of antigen-specific immune responses (See Figure 8). The encouraging results from our study warrant further exploration of other compounds that share similar features with carrageenan and dextran as potential adjuvants to enhance the protein/peptide-based vaccine potency.

For clinical translation, it is essential to address issues regarding the safety of the compounds used as adjuvants. Carrageenan has been commercially used as a thickening and stabilizing agent in the food and cosmetic industry. The safety of carrageenan as an additive is being analyzed by the World Health Organization Expert Committee on Food Additives [20]. Although there have been concerns regarding the safety of this compound via oral intake [21], our study employs subcutaneous injection of carrageenan. Thus it will be of interest to determine if s.c. administration of carrageenan as an adjuvant would generate significant side effects in the future.

In the current study, we also observed that co-administration of carrageenan also led to prolonged release of antigen over time and led to an increased uptake of antigen in the lymph nodes. This indicates that the antigen was taken up by DCs, which in turn led to activation and migration of DCs to the lymph nodes, which suggests that co-administration of carrageenan can enhance activation of dendritic cells in the lymph nodes. This may potentially be a mechanism by which carrageenan can enhance the antigen-specific immune responses and antitumor effects in vaccinated mice. Further exploration is warranted to clearly identify the role of CGN in activating bone marrow-derived DCs in vitro and in vivo. In addition, it will be of interest to characterize if other effector cells such as CD4⁺ T cells and NK cells can be activated by CGN in future studies.

In summary, we have identified a novel class of compounds that may potentially be used as adjuvants in the enhancement of vaccine potency through toll-like receptor 4 activation pathway. Continued exploration of compounds similar to carrageenan with the highest potency and minimal side effects is warranted for the enhancement of protein/peptide vaccine potency for future clinical translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Characterization of the E7-specific CD8+ T cell immune responses generated by vaccination with E7 peptide in combination with CGN
C57BL/6 mice were vaccinated with E7 peptide (aa 43-62) alone, CGN alone, or E7 peptide (aa 43-62) in combination with 10 μ g of CGN twice by *s.c.* injection. 7 days after the second vaccination, the splenocytes were restimulated with E7 peptide (aa 49-57), and the E7 specific T cell immune responses were characterized using intracellular cytokine staining followed by flow cytometry analysis. **(A)** Representative flow cytometry data depicting the number of E7-specific CD8+ T cells. **(B)** Bar graph representing the number of E7-specific CD8+ T cells/3 $\times$ 10⁵ splenocytes (mean $\pm$ s.e.). Data shown are representative of two experiments performed.

Figure 2. Characterization of the protective antitumor effects generated by E7+CGN vaccination in vaccinated mice

C57BL/6 mice (5 per group) were vaccinated subcutaneously twice with E7 peptide vaccine (aa 43-62), CGN, or E7 peptide (aa 43-62) in combination with CGN at 1-week intervals, followed by *s.c.* injection of 5×10^4 /mouse of TC-1 tumor cells in the left flank one week after the last vaccination. The tumor growth was monitored each week, and the death of mouse was arbitrary defined when the tumor size reached 2 cm. **(A)** Line graph depicting the percentage of tumor free mice among vaccinated mice challenged with TC-1 tumors. **(B)** Kaplan Meier survival analysis of vaccinated mice challenged with TC-1 tumors.

Figure 3. Characterization of the therapeutic antitumor effects generated by E7+CGN vaccination in tumor-bearing mice

C57BL/6 mice (5 per group) were injected with 1×10^4 /mouse of TC-1 tumor cells by *s.c.* injection in the left flank. Four days later, tumor-bearing mice were vaccinated subcutaneously with E7 peptide vaccine (aa 43-62), CGN, or E7 peptide (aa 43-62) in combination with CGN three times with 4-day intervals. The tumor growth was monitored each week, and the death of mouse was arbitrary defined when the tumor size reached 2 cm. **(A)** Line graph depicting the percentage of tumor free mice among tumor-bearing mice treated with E7 peptide alone, CGN alone, or E7 in combination with CGN. **(B)** Kaplan Meier survival analysis of TC-1 tumor bearing mice treated with E7 peptide alone, CGN alone, or E7 in combination with CGN.

Figure 4. Characterization of the role of TLR4 signaling pathway in the adjuvant effect of CGN (A) TLR4 reporter assay depicting the TLR4 activation by CGN. HEK-Blue-4 cells were treated with CGN (1 ug/ml) for 24 hours, and the activation of TLR4 was detected by using QUANTI-Blue assay. **B & C.** C57BL/6 mice or TLR4^{-/-} mice were vaccinated twice with E7 peptide (aa 43-62) in combination with CGN with a 1-week interval. 7 days after the second vaccination, the splenocytes were restimulated with E7 peptide (aa 49-57), and the E7-specific T cell immune responses were characterized using intracellular cytokine staining followed by flow cytometry analysis. (B) Representative flow cytometry data depicting the E7-specific CD8⁺ T cell immune responses generated by E7+CGN vaccination in TLR4^{-/-} mice and in WT C57BL/6 mice. (C) Bar graph depicting the E7-specific CD8⁺ T cell immune responses generated by E7+CGN vaccination in TLR4^{-/-} mice and in WT C57BL/6 mice.

Figure 5. In vivo luminescence imaging of marker protein administered with or without CGN in mice

C57BL/6 mice were injected with 1 μ l Alexa Fluor750-streptavidin (Strep*) with or without 10 μ g CGN in a total volume of 100 μ l by *s.c.* injection in the left flank. 2, 24, and 48 hours after the injection, fluorescence imaging was conducted on a cryogenically cooled IVIS system and an acquisition time of 1 min was used. **(A)** Representative luminescence images depicting the fluorescence intensity of marker protein Strep* administered with or without CGN in mice over time. **(B)** Line graph depicting the fluorescence intensity of Strep* administered with or without CGN in mice over time. **C & D.** The lymph nodes from the treated mice were isolated and were imaged using IVIS system. **(C)** Representative luminescence images depicting the fluorescence intensity of the lymph nodes isolated from mice treated with Strep* with or without CGN. **(D)** Bar graph depicting the fluorescence intensity in the lymph nodes isolated from mice treated with Strep* with or without CGN.

Figure 6. Characterization of the TRP2-specific CD8+ T cell immune responses generated by vaccination with TRP2 peptide in combination with CGN

C57BL/6 mice were vaccinated subcutaneously with TRP2 peptide (aa 181-188) alone, CGN alone, or TRP2 peptide (aa 181-188) in combination with CGN twice at 1-week intervals. 7 days after the second vaccination, the splenocytes were restimulated with TRP2 peptide (aa 181-188), and the TRP2-specific T cell immune responses were characterized using intracellular cytokine staining followed by flow cytometry analysis. **(A)** Representative flow cytometry data depicting the number of TRP2-specific CD8+ T cells. **(B)** Bar graph representing the number of TRP2-specific CD8+ T cells/ 3×10^5 splenocytes (mean $\pm$ s.e.). Data shown are representative of two experiments performed.

Figure 7. Characterization of the E7-specific CD8+ T cell immune responses generated by vaccination with E7 peptide in combination with MPL-A or CGN

C57BL/6 mice were vaccinated subcutaneously with E7 peptide (aa 43-62) alone, MPL-A alone, CGN alone, E7 peptide (aa 43-62) in combination with MPL-A or E7 peptide (aa 43-62) in combination with CGN twice with 1-week interval. 7 days after the second vaccination, the splenocytes were restimulated with E7 peptide (aa 49-57), and the E7-specific T cell immune responses were characterized using intracellular cytokine staining followed by flow cytometry analysis. **(A)** Representative flow cytometry data depicting the number of E7-specific CD8+ T cells. **(B)** Bar graph representing the number of E7-specific CD8+ T cells/ 3×10^5 splenocytes (mean $\pm$ s.e.). Data shown are representative of two experiments performed.

Figure 8. Characterization of the E7-specific CD8+ T cell immune responses generated by vaccination with E7 peptide in combination with dextran or CGN
(A) TLR4 reporter assay depicting the TLR4 activation by CGN and dextran. **B & C.** C57BL/6 mice were vaccinated subcutaneously with E7 peptide (aa 43-62) alone, CGN alone, dextran alone, E7 peptide (aa 43-62) in combination with CGN or E7 in combination with dextran twice at 1-week intervals. 7 days after the second vaccination, the splenocytes were restimulated with E7 peptide (aa 49-57), and the E7-specific CD8+ T cell immune responses were characterized using intracellular cytokine staining followed by flow cytometry analysis. **(B)** Representative flow cytometry data depicting the number of E7-specific CD8+ T cells. **(C)** Bar graph representing the number of E7-specific CD8+ T cells/ 3×10^5 splenocytes (mean $\pm$ s.e.). Data shown are representative of two experiments performed.