

USE OF CRASSOCEPHALUM RABENS EXTRACT IN INDUCING HUMORAL IMMUNE RESPONSE

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and benefit of U.S. Provisional Patent Application Nos. 63/485,175 and 63/485,192, both filed on February 15, 2023. The entirety of the aforementioned applications is incorporated herein by reference.

FIELD OF THE DISCLOSURE

[0002] The present disclosure relates to use of *Crassocephalum rabens*, and more particularly to use of *Crassocephalum rabens* in clinical applications.

BACKGROUND OF THE INVENTION

[0003] A humoral response is typically desired to protect against viral or bacterial invaders. Humoral immune response is typified by high levels of antibody production by B cells. More and more research shows a strong positive correlation between B cells and cancer-specific survival and response to therapy. Anti-tumor auto-antibody (AA)-secreting tumor-infiltrating B cells (TIL-B) have been reported to improve clinical outcomes [Garaud, S., et al., JCI Insight, 2019. 5(18): e129641; Wang, S.S., et al., Cell Mol Immunol, 2019. 16(1): p. 6-18]. A higher proportion of follicular B cells in tertiary lymphoid structures and tumor-infiltrating plasma cells are correlated with better long-term survival in cancer patients [Wang, S.S., et al., Cell Mol Immunol, 2019. 16(1): p. 6-18]. AA development has a protective role in antitumor immunity in cancer patients under treatment.

[0004] Vaccination can trigger appropriate immunity against pathogen infection and provide long-term protection. Regardless of the type of vaccine, adjuvants need to be added to produce a stronger immune response to poorly immunogenic antigens (for example, synthetic peptides, subunit antigens, and DNA). Although various adjuvants have been developed, only few adjuvants are allowed to be used clinically due to their toxicity and side effects.

[0005] Considering efficiency and safety, a large volume of research reports show that plant-derived ingredients may have the potential to be developed as adjuvants and used in vaccine formulations. So far, the most promising plant-derived molecules for adjuvant development are saponins (Qiao N, Liu Q, Meng H, Zhao D., *International Immunopharmacology*. 2014;18(2):333-339), shikonin (Chen HM, Wang PH, Aravindaram K, et al., *Journal of*

Biomedical Science. 2012;19(1):42), polysaccharides (Sun B, Yu S, Zhao D, Guo S, Wang X, Zhao K., *Vaccine*. 2018;36(35):5226-5234), and lectins (Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. *Immunity*. 2010;33(4):492-503). Some medicinal herbs directly stimulate B cells to enhance humoral immunity, for example, fractions or components from *Echinacea angustifolia* (Razin MAF, Osman A, Ali MA, Bahgat MM, Maghraby AS., *Acta Microbiologica et Immunologica Hungarica*. 2017;64(3):313-330) and *Cuscuta cephalanthi* seed (Wang Z, Fang JN, Ge DL, Li XY., *Acta Pharmacologica Sinica*. 2000;21(12):1136-1140) enhanced B cell proliferation. These herbal extracts or components induced a higher titer of antibodies against the pathogenic antigens. Some herbs also have indirectly stimulatory activity on B cells.

[0006] Thus, there is a need for the development of therapy or adjuvant for generating strong humoral responses against a variety of antigens.

SUMMARY OF THE INVENTION

[0007] The present disclosure provides use of a *Crassocephalum rabens* plant or extract in inducing humoral immune response. Also, the present disclosure provides a *Crassocephalum rabens* plant or extract for use in a method for treating or preventing a humoral immune response-mediated disease.

[0008] The present disclosure provides a method for inducing humoral immune response in a subject in need, comprising administering to said subject an effective amount of *Crassocephalum rabens* plant or extract and optionally a pharmaceutically acceptable carrier or excipient.

[0009] In one embodiment, the method is for preventing or treating a humoral immune response-mediated disease. In some further embodiments, the disease is a cancer, infectious disease or a neurodegenerative disease. Examples of the neurodegenerative disease include, but are not limited to, Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS). Examples of a cancer include, but are not limited to, melanoma, adenocarcinoma, thymoma, lymphoma (e.g., non-Hodgkin's lymphoma, Hodgkin's lymphoma), sarcoma, lung cancer, liver cancer, colon cancer, leukaemia, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer and brain cancer. Examples of an infectious disease include, but are not limited to, bacterial infections (e.g. periodontitis, pneumonia, gastritis and other bacterial infections), immunodeficiency (e.g. HIV), papilloma (e.g. HPV), herpes (e.g. HSV), encephalitis,

influenza (e.g. human influenza virus A), SARS-CoV-2 virus and viral infections such as the common cold.

[0010] In some embodiments of the disclosure, the method is for attracting B cells.

[0011] In some embodiments of the disclosure, the method is for enhancing antibody production and/or enhancing antibody class switching. In some further embodiments of the disclosure, the antibody class switching comprises switching from IgM to IgG1, IgG2 or IgG3.

[0012] In some embodiments of the disclosure, the method is for inducing antibody-dependent cell cytotoxicity or antibody-dependent cell phagocytosis.

[0013] In some embodiments of the disclosure, the method is for increasing serum IFN- γ and IL-21.

[0014] In some embodiments of the disclosure, the method is for inducing B cell proliferation or B cell differentiation. In some further embodiments of the disclosure, the method is for inducing B cell proliferation or B cell differentiation through IL-21R/STAT3/Blimp-1 pathway.

[0015] In some embodiments of the disclosure, the humoral immune response is induced against a surface-bound cancer antigen; or an antigen associated with a neurodegenerative disease. In some further embodiments of the disclosure, the cancer is drug-resistant colorectal cancer.

[0016] In some embodiments of the disclosure, the *Crassocephalum rabens* is *Crassocephalum rabens* (Benth.) S. Moore.

[0017] In some embodiments of the disclosure, the *Crassocephalum rabens* plant is pieces of dried or fresh *Crassocephalum rabens*.

[0018] In some embodiments of the disclosure, the *Crassocephalum rabens* extract is alcohol extract of *Crassocephalum rabens*.

[0019] In some embodiments of the disclosure, the *Crassocephalum rabens* extract is manufactured by extracting *Crassocephalum rabens* with an alcohol solution to obtain alcohol extract; fractioning the alcohol extract to obtain a phytogalactolipid-enriched fraction.

[0020] In some embodiments of the disclosure, the fractioning step is performed with reverse phase medium pressure liquid chromatography (RP-MPLC). In some further embodiments of the disclosure, reverse phase high performance liquid chromatography (RP-HPLC) is further performed after RP-MPLC.

[0021] The present disclosure also provides a method for inducing humoral immune response in a subject in need, comprising administering to said subject an effective amount of galactolipid compounds from *Crassocephalum rabens* or a pharmaceutically acceptable derivative thereof and optionally a pharmaceutically acceptable carrier or excipient.

[0022] In one embodiment of the disclosure, the galactolipid compound is 1,2-di-*O*- α -linolenoyl-3-*O*- β -galactopyranosyl-*sn*-glycerol (dLGG) or 1,2-di-(α -linolenoyl)-3-[(α -D-galactosyl-(1-6)- β -D-galactosyl)-*sn*-glycerol (CRDG).

[0023] The present disclosure provides an adjuvant composition comprising an effective amount of *Crassocephalum rabens* plant or extract.

[0024] The present disclosure also provides an immunogenic composition comprising an antigen component and the adjuvant composition as described herein.

[0025] In some embodiments of the disclosure, the antigen component is a peptide or a protein.

[0026] The present disclosure also provides a method for enhancing immune response in a subject in need, comprising administering to said subject the adjuvant composition or the immunogenic composition as described herein.

[0027] In some embodiments of the disclosure, the adjuvant composition or the immunogenic composition is administered through an oral route.

[0028] In some embodiments of the disclosure, the method is for enhancing antibody production.

[0029] In some embodiments of the disclosure, the method is for enhancing antibody class switching. In some further embodiments of the disclosure, the method is for increasing the proportion of IgG2a, IgG2b or IgG3 in total IgG antibodies.

[0030] In some embodiments of the disclosure, the method is for triggering IL-21 and/or B cell activating factor (BAFF) release.

[0031] In some embodiments of the disclosure, the method is for triggering B cell differentiation.

[0032] In some embodiments of the disclosure, the method is for enhancing cell proliferation, uptake ability, and/or antigen presentation of phagocytes.

[0033] In some embodiments of the disclosure, the method is for increasing Th1 and Th2-related immune responses.

[0034] The present disclosure is described in detail in the following sections. Other characteristics, purposes and advantages of the present disclosure can be found in the detailed description and claims.

BRIEF DESCRIPTION OF DRAWINGS

[0035] **FIGs. 1A to 1E show tumor growth inhibition of CRA in a CRC tumor allograft mouse model.** FIG. 1A: The inhibitory effects of CRA on tumor growth. FIG. 1B: CRA inhibited tumor volumes in a dose-dependent manner at day 33. CT26.CL25 tumor-bearing mice were orally administrated with 25, 50, or 100 mg/kg of CRA every day. Tumor volumes were recorded weekly (n=5-10). *: $P < 0.05$, compared to the control group. After CRA treatment, (FIG. 1C) the proportion of splenic CD19+ B cells, and (FIG. 1D) different B cell subsets in splenocytes were determined (n=5). *: $P < 0.05$, compared to the control group. FIG. 1E: The expression of TIL-B cells (B220, red) in the tumor by IHC staining.

[0036] **FIGs. 2A to 2C show titers and recognition of CRA-induced anti-tumor antibodies.** FIG. 2A: Titers of specific anti-CT26.CL25 cell antibodies for control or CRA antisera on days 0 and 33. FIG. 2B: Effects of CRA on the class-switch of specific anti-CT26 cell antibodies. *: $P < 0.05$, compared to the control group. The titers of these antisera (1,000× dilution) were measured for IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA. FIG. 2C: Tumor specificity of CRA antisera. Normal sera, control antisera, or CRA antisera bound to CT26.CL25 (black bar) or MG-CAP-A1 cells (white bar) were indirectly detected with FITC-conjugated goat anti-mouse IgG antibody.

[0037] **FIGs. 3A to 3D show cytotoxicity and antitumor activity of CRA-induced antisera collected from CT26.CL25 tumor-bearing mice.** FIG. 3A: Cytotoxicity of CRA antisera. CT26.CL25 cells were treated with 2 μ L antisera and incubated for 48 h. Cell viability was determined by MTT assay. To make sCRA antisera, the anti-CT26.CL25 antibodies from CRA antisera were pre-subtracted by CT26.CL25 cell binding. The data are reported as the proliferation index. N: normal sera; Ctrl: control antisera; sCRA antisera: CRA antisera with the subtraction of anti-CT26.CL25 antibodies. FIG. 3B: CRA antisera-mediated ADCC. CFSE labeled CT26.CL25 cells serving as target cells were incubated with mouse splenic NK cells (effector cells) in an E:T ratio of 4:1 with 2 μ L normal sera or antisera for 4 h. FIG. 3C: CRA antisera-mediated ADCP. RAW264.7 macrophages treated with unopsonized or opsonized CT26.CL25 cells (incubated with 2 μ L antisera) for 4 h. The percentage of phagocytosis for normal sera and antisera is shown. FIG. 3D: CRA antisera induced CDC activity. CDC activity of CRA antisera used horse complements. Cell lysis was determined after the addition of antisera for 4 h. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$, compared to the control antisera group.

[0038] **FIG 4 shows the effects of CRA on IFN- γ and IL-21 release ex vivo.** Following different treatments, splenocytes harvested from CT26.CL25 tumor-bearing mice were treated with CT26 cell lysate. Then, IFN- γ and IL-21 release in supernatants were analyzed. All values were expressed as the mean $\pm$ SEM (n=6). *: $P < 0.05$; **: $P < 0.01$, compared to the control group.

[0039] **FIGs. 5A to 5H show effects of CRA on B cell differentiation in splenocytes *in vitro*.**

FIG. 5A: Cell proliferation of CD19⁺ B cells. LPS was used as a positive control for B cell proliferation. FIG. 5B: Cell population of plasmablasts (CD19⁺CD138⁺) and plasma cells (CD19⁻ or low CD138⁺) in CRA-treated mouse splenocytes were analyzed by flow cytometry. FIG. 5C: The proportion of plasmablasts (CD19⁺CD138⁺) and plasma cells (CD19⁻ or low CD138⁺). Mouse splenocytes were treated with serial dilution of CRA for 72 h. FIG. 5D: CRA increased IL-21 and IFN- γ expression in splenocytes. *: $P < 0.05$; ***: $P < 0.001$, compared to the control group (n=7). FIG. 5E: Cell proliferation of CD19⁺ B cells with anti-CD40 antibody (1 μ g/mL), mouse IL-4 (100 U/mL) stimulation. FIG. 5F: Cell population of plasmablasts (CD19⁺CD138⁺) and plasma cells (CD19⁻ or low CD138⁺) in CRA and/or anti-CD40/IL-4-treated mouse splenocytes were analyzed by flow cytometry. FIG. 5G: The proportion of plasmablasts (CD19⁺CD138⁺) and plasma cells (CD19⁻ or low CD138⁺) in anti-CD40/IL-4 stimulated splenocytes. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$, compared to the control group. FIG. 5H: CRA increased IL-21 and IFN- γ expression in anti-CD40/IL-4 stimulated splenocytes.

[0040] **FIGs. 6A to 6B show that CRA induced B cell differentiation through upregulating STAT3/Prdm1 signaling.** CRA increased the expression of STAT3 and Prdm1 in (FIG. 6A) unstimulated and (FIG. 6B) anti-CD40/IL4-stimulated splenocytes. Unstimulated or anti-CD40/IL4-stimulated splenocytes were treated with doses of CRA for 72 h, the expression of STAT3 and Prdm1 was determined by RT-PCR and western blot. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$, compared to the control group.

[0041] **FIGs. 7A to 7C show effects of CRA on B cell differentiation in splenic B cells *in vitro*.**

FIG. 7A: CRA did not affect IL-21 and IFN- γ expression in splenic B cells. ns: no significant difference, compared to the control group (n=6). M: cell culture medium. FIG. 7B: Cell population of plasmablasts (CD19⁺CD138⁺) and plasma cells (CD19⁻ or low CD138⁺) in CRA-treated mouse splenic B cells were analyzed by flow cytometry. FIG. 7C: The proportion of plasmablasts (CD19⁺CD138⁺) and plasma cells (CD19⁻ or low CD138⁺) in the mouse splenic B cells after treating with serial dilution of CRA for 72 h.

[0042] **FIGs. 8A to 8C show Effects of CRA on B cell differentiation in anti-CD40/IL-4-treated splenic B cells *in vitro*.**

FIG. 8A: CRA did not affect IL-21 and IFN- γ expression in anti-CD40/IL-4 stimulated splenic B cells. NC: splenic B cells without anti-CD40/IL-4 and CRA treatment. ns: no significant difference, compared to the control group (n=6). FIG. 8B: Cell population of plasmablasts (CD19⁺CD138⁺) and plasma cells (CD19⁻ or low CD138⁺) in CRA and/or anti-CD40/IL-4-treated splenic B cells were analyzed by flow cytometry. Splenic B cells were pre-stimulated with 1 μ g/mL of anti-CD40 antibody and 100 U/mL of mouse IL-4. FIG. 8C: The proportion of

plasmablast (CD19⁺CD138⁺) and plasma cells (CD19^{- or low}CD138⁺) in anti-CD40/IL-4 stimulated splenic B cells. *: $P < 0.05$; **: $P < 0.01$, compared to the control group.

[0043] **FIG. 9 shows Effects of neutralizing anti-IL21 antibodies on CRA-induced B cell differentiation.** Splenocytes were treated with CRA and 50 μg of anti-IL21 antibodies for 30 h. The proportion of plasma cells was analyzed by flow cytometry. *: $P < 0.05$, compared to the control group. ns: no significant difference.

[0044] **FIGs. 10A to 10E show CRA promoted IL-21 release from T cells by upregulating STAT3/BCL6/cMaf pathway.** FIG. 10A: CRA induced IL-21 release from T cells. After treating with CRA, the concentration of IL-21 in cell culture media was determined by ELISA. The effects of CRA on (FIG. 10B) STAT3, (FIG. 10C) BCL6, and (FIG. 10D) cMaf expression in splenic T cells. Splenic T cells were treated with different doses of CRA for 72 h. The expression of STAT3, BCL6, and cMaf was determined by qPCR. FIG. 10E: Schematic representation of the proposed mechanism of action of CRA.

[0045] **FIGs. 11A to 11F show CRA-API effectively induced B cell differentiation.** FIG. 11A: Cell population of plasma cells in CRA, CRDG, or CRG-treated mouse splenocytes. FIG. 11B: Cell population of plasma cells in CRA, CRDG, or CRG-treated mouse splenocytes with anti-CD40 antibody/IL-4 stimulation. FIG. 11C: Effects of CRDG or CRG on IL-21 release from splenic T cells. The effects of CRDG or CRG on (FIG. 11D) STAT3, (FIG. 11E) cMaf, and (FIG. 11F) BCL6 expression in splenic T cells. Splenic T cells were treated with different doses of CRDG or CRG for 72 h. The expression of STAT3, BCL6, and cMaf was determined by qPCR. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$, compared to the control group.

[0046] **FIGs. 12A to 12D show that CRA enhanced antibody production in BALB/c mice pulsed antigen.** FIG. 12A: Total titers of anti-AaHSP60 antibody were determined weekly after immunization and boost. Female BLAB/c mice were immunized and boosted with 100 μg of rAaHSP60 mixed with the 10 mg/kg CRA or vehicle by intraperitoneal (i.p.) injection. The values of anti-AaHSP60 antibody titers were collected at 1: 25,600 dilution. FIG. 12B: EC50 values of anti-AaHSP60 antibody at day35 were calculated. The x-axis depicts serial dilutions (log10) of the specific antibody binds to the rAaHsp60. Fifty percent binding (EC50) of CRA or vehicle (c)-induced anti-AaHsp60 antibodies was obtained. FIG. 12C: The antibody isotypes (IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA) were determined at day 35. FIG. 12D: B cell lineage were determined after 35-days immunization. All values were expressed as mean $\pm$ SD (n=5). *: $P < 0.05$, compared to the control group.

[0047] **FIG. 13 shows effects of CRA on expression of IL-21 and IFN- γ in the sera of immunized mice.** Female BLAB/c mice were immunized and boosted with 100 μg of rAaHSP60

mixed with the 10 mg/kg CRA or vehicle by intraperitoneal (i.p.) injection. The concentration of IL-21, IFN- γ , and TGF- β in the sera was determined by multiplex cytokine assay. All values were expressed as the mean $\pm$ SEM (n=6). *: $P < 0.05$, compared to the control group.

[0048] **FIGs. 14A to 14F shows effect of CRA on cell proliferation and cytokine releases *in vitro*.** **FIG. 14A: CRA induced cell proliferation in mouse splenocytes.** Mouse splenocytes were treated with different concentrations of CRA (1.5, 3, and 6 $\mu\text{g/ml}$) for 48h. The proliferation of the splenocytes was analyzed by CFSE. Effect of CRA on (FIG. 14B) IL-21 and (FIG. 14C) IFN- γ releases in mouse splenocytes. All values were expressed as the mean $\pm$ SEM (n=6). *: $P < 0.05$, compared to the control group. **FIG. 14D: CRA induced proliferation in AaHSP60-pulsed splenocytes.** Mouse splenocytes were treated with different concentrations of CRA (1.5, 3, and 6 $\mu\text{g/ml}$) and 1 $\mu\text{g/ml}$ of rAaHSP60 for 48h. Effect of CRA on (FIG. 14E) IL-21 and (FIG. 14F) IFN- γ release in AaHSP60-pulsed mouse splenocytes. #: $P < 0.05$, compared to the AaHSP60 group.

[0049] **FIGs. 15A to 15B show effect of CRA on B cell activation in mouse splenocytes.** **FIG. 15A: Effect of CRA on B cell proliferation.** The CFSE-labeled splenocytes were treated with different concentrations (1.5, 3, and 6 $\mu\text{g/ml}$) of CRA with or without 1 $\mu\text{g/ml}$ of rAaHSP60 for 72h. The proliferation of the CFSE-labeled CD19+ B cells was monitored by flow cytometry. **FIG. 15B: CD86 expression and MHCII expression in CRA-treated splenocytes with or without AaHSP60.** LPS was used as positive control. The fold of fluorescence mean was evaluated using the following equation: the mean fluorescence intensity of the sample divided by the mean fluorescence intensity of the control group. All values were expressed as the mean $\pm$ SEM (n=6). *: $P < 0.05$, compared to the control group.

[0050] **FIG. 16 shows effect of CRA on B cell differentiation in mouse splenocytes.** **Mouse splenocytes were treated with CRA with or without rAaHSP60 for 72h.** The proportion of plasma cells (CD19⁺CD138^{or low}) was analyzed by flow cytometry. *: $P < 0.05$, compared to the control group. #: $P < 0.05$, compared to the AaHSP60 group.

[0051] **FIGs. 17A to 17D show effects of CRA on activation and antigen presentation in macrophage.** **FIG. 17A: Effect of CRA on macrophage proliferation.** *, $P < 0.05$; **, $P < 0.01$, compared to the control group. **FIG. 17B: Antigen uptake ability of macrophage after treating with CRA.** **MHC class II (FIG. 17D) and CD86 (FIG. 17C) expression of macrophages after CRA treatment.** The fold of fluorescence mean was evaluated using the following equation: the mean fluorescence intensity of the sample divided by the mean fluorescence intensity of the control group. All values were expressed as the mean $\pm$ SEM (n=6). *: $P < 0.05$, compared to the control group. #: $P < 0.05$, compared to the AaHSP60 group.

[0052] **FIGs. 18A to 18B show that CRA induced BAFF released from mouse splenocyte.** FIG.

18A: The mRNA expression of BAFF in mouse splenocytes treated with CRA was detected by RT-PCR. FIG. 18B: BAFF released from mouse splenocytes treated with CRA was detected by ELISA. Mouse splenocytes were treated with different concentrations (3, 6, 12, and 24 µg/ml) of CRA for 24h. All values were expressed as the mean ± SEM (n=6). *; $P < 0.05$, compared to the control group.

[0053] **FIG. 19 shows that phytolectolipids CRA induced specific antibody production in**

BALB/c mice by oral administration. Female BLAB/c mice were weekly immunized and boosted with 100 µg of rAaHSP60 mixed with the 10 mg/kg CRA or vehicle control by oral administration. The titers of IgA-typed anti-AaHSP60 antibodies in saliva were determined at 1: 3,200 dilution.

DESCRIPTION OF THE INVENTION

[0054] The present disclosure can be more readily understood by reference to the following detailed description of various embodiments of the disclosure, the examples, and the chemical drawings and tables with their relevant descriptions. It is to be understood that unless otherwise specifically indicated by the claims, the disclosure is not limited to specific preparation methods, carriers or formulations, or to particular modes of formulating the extract of the disclosure into products or compositions intended for topical, oral or parenteral administration, because as one of ordinary skill in the relevant arts is well aware, such things can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0055] As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meaning:

[0056] It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, unless otherwise required by context, singular terms shall include the plural and plural terms shall include the singular.

[0057] As used herein, the term "or" is used to mean "and/or" unless explicitly indicated to refer to alternatives only, or the alternatives are mutually exclusive.

[0058] As used herein, the terms "subject" and "patient" are used interchangeably herein and will be understood to refer to a warm-blooded animal, particularly a mammal. Non-limiting examples of animals within the scope and meaning of this term include guinea pigs, dogs, cats, rats, mice, horses, goats, cattle, sheep, zoo animals, non-human primates, and

humans.

[0059] The term "effective amount" of an active ingredient as provided herein means a sufficient amount of the ingredient to provide the desired regulation of a desired function. As will be pointed out below, the exact amount required will vary from subject to subject, depending on the disease state, physical conditions, age, sex, species and weight of the subject, the specific identity and formulation of the composition, etc. Dosage regimens may be adjusted to induce the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. Thus, it is not possible to specify an exact "effective amount." However, an appropriate effective amount can be determined by one of ordinary skill in the art using only routine experimentation.

[0060] "Humoral immune response" as referred to herein relates to antibody production and the accessory processes that accompany it, such as for example T-helper 1 and 2 (Th1 and Th2) cell activation and cytokine production, isotype switching, affinity maturation and memory cell activation. It also refers to the effector functions of an antibody, such as for example toxin neutralization, classical complement activation, and promotion of phagocytosis and pathogen elimination. The humoral immune response is aided by CD4⁺Th1 and CD4⁺Th2 cells and therefore the activation or generation of this cell type is also indicative of a humoral immune response as referred to herein.

[0061] The phrases "adjuvant composition(s)" refer to a composition that when administered to a subject is capable of inducing an immune response in the subject. When administered in combination with an antigen, the "adjuvant compositions" are capable of eliciting an antigen-specific immune response. Adjuvants are generally used to accomplish two objectives: the slow the release of antigens from the injection site, and the stimulation of the immune system.

[0062] An "immune response" to an antigen or composition is the development in a subject of a humoral and/or a cellular immune response to molecules present in the antigen or composition of interest. A "humoral immune response" refers to an immune response mediated primarily by antibody molecules, while a "cellular immune response" is one mediated primarily by T-lymphocytes and/or other white blood cells.

[0063] The term "antigen" refers to any substance that can be recognized by the immune system (e.g., bound by an antibody or processed so as to elicit a cellular immune response by, e.g. T cells) under appropriate conditions. An antigen contains one or more epitopes. A B-cell epitope includes at least about 3-5 amino acids, for example, 4 or more amino acids. A hapten or a polysaccharide may also serve as a B cell epitope. A T-cell epitope, such as a cytotoxic T-

cell (CTL) epitope, may include at least about 7-9 amino acids, for example, 8 or more amino acids. A helper T-cell epitope may include at least about 12-20 amino acids. The term "antigen" denotes both subunit antigens (i.e., antigens which are separate from the whole organism with which the antigen is associated in nature), as well as, killed, attenuated or inactivated bacteria, viruses, fungi, parasites or other microbes, prions, allergens or any other disease causing agents. An antigen may be a modified protein that includes modifications, such as deletions, additions and substitutions (generally conservative in nature) to the native protein sequence. The term antigen also denotes nucleic acids (DNA or RNA) encoding a protein or peptide antigen.

[0064] The terms "polypeptide" and "protein" refer to a polymer of amino acid residues and are not limited to a minimum length of the product. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include postexpression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

[0065] The term "carrier" or "excipient" as used herein refers to any substance, not itself a therapeutic agent, used as a carrier and/or diluent and/or adjuvant, or vehicle for delivery of a therapeutic agent to a subject or added to a formulation to improve its handling or storage properties or to permit or facilitate formation of a dose unit of the composition into a discrete article such as a capsule or tablet suitable for oral administration. Suitable carriers or excipients are well known to persons of ordinary skill in the art of manufacturing pharmaceutical formulations or food products. Carriers or excipients can include, by way of illustration and not limitation, buffers, diluents, disintegrants, binding agents, adhesives, wetting agents, polymers, lubricants, glidants, substances added to mask or counteract a disagreeable taste or odor, flavors, dyes, fragrances, and substances added to improve the appearance of the composition. Acceptable carriers or excipients include citrate buffer, phosphate buffer, acetate buffer, bicarbonate buffer, stearic acid, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulfuric acids, magnesium carbonate, talc, gelatin, acacia gum, sodium alginate, pectin, dextrin, mannitol, sorbitol,

lactose, sucrose, starches, gelatin, cellulosic materials (such as cellulose esters of alkanolic acids and cellulose alkyl esters), low melting wax cocoa butter, amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins (for example, serum albumin), ethylenediamine tetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), sodium chloride or other salts, liposomes, mannitol, sorbitol, glycerol or powder, polymers (such as polyvinyl-pyrrolidone, polyvinyl alcohol, and polyethylene glycols), and other pharmaceutically acceptable materials. The carrier should not destroy the pharmacological activity of the therapeutic agent and should be non-toxic when administered in doses sufficient to deliver a therapeutic amount of the agent.

[0066] The term "a pharmaceutically acceptable derivative" or "pharmaceutically acceptable derivatives" as used herein denotes a compound that is modified from the compound of the disclosure but has properties and efficacies that are the same as or better than those of the compound of the disclosure. Preferably, the pharmaceutically acceptable derivative is a pharmaceutically acceptable salt, solvate, hydrate, or prodrug of the compound of the disclosure.

[0067] The compounds of the disclosure can also exist as solvates and hydrates. Thus, these compounds may crystallize with, for example, waters of hydration, or one, a number of, or any fraction of molecules of the mother liquor solvent. The solvates and hydrates of such compounds are included within the scope of this disclosure.

[0068] The present disclosure provides a method for inducing humoral immune response in a subject in need, comprising administering to said subject an effective amount of *Crassocephalum rabens* plant or extract and optionally a pharmaceutically acceptable carrier or excipient.

[0069] The *Crassocephalum rabens* plant or extract of the present disclosure, by inducing humoral immune response, may be capable of protecting a subject from a disease, disorder or ailment associated with an antigen capable of inducing a humoral immune response. Without limitation, this includes for example, cancers involving a membrane surface-bound cancer antigen which is recognized by an antibody, diseases where it is desirable to sequester antigen in circulation, like amyloid protein (e.g. Alzheimer's disease); neutralizing toxins with an antibody; neutralizing viruses or bacteria with an antibody; or neutralizing allergens (e.g. pollen) for the treatment of allergies. In another embodiment, the antigen may be or comprise a B cell epitope capable of inducing a humoral immune response. For example, the antigen may be or comprise a B cell epitope.

[0070] A humoral immune response can also be useful for combating cancer. B cell mediated

responses may target cancer cells through other mechanisms which may in some instances cooperate with a cytotoxic CD8 T cell for maximum benefit. Examples of mechanisms of B cell mediated (e.g. humoral immune response mediated) anti-tumor responses include, without limitation: 1) Antibodies produced by B cells that bind to surface antigens found on tumor cells or other cells that influence tumorigenesis. Such antibodies can, for example, induce killing of target cells through antibody-dependent cell-mediated cytotoxicity (ADCC) or complement fixation, potentially resulting in the release of additional antigens that can be recognized by the immune system; 2) Antibodies that bind to receptors on tumor cells to block their stimulation and in effect neutralize their effects; 3) Antibodies that bind to factors released by or associated with tumor or tumor-associated cells to modulate a signaling or cellular pathway that supports cancer; and 4) Antibodies that bind to intracellular targets and mediate anti-tumor activity through a currently unknown mechanism.

[0071] In another embodiment, the antigen may be an antigen associated with a disease where it is desirable to sequester the antigen in circulation, such as for example an amyloid protein (e.g. Alzheimer's disease). Thus, a composition of the invention may be suitable for use in the treatment and/or prevention of a neurodegenerative disease in a subject in need thereof, wherein the neurodegenerative disease is associated with the expression of an antigen. The subject may have a neurodegenerative disease or may be at risk of developing a neurodegenerative disease. Neurodegenerative diseases that may be treated and/or prevented by the use or administration of a composition of the invention include, without limitation, Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS).

[0072] As used herein, the terms "cancer", "cancer cells", "tumor" and "tumor cells", (used interchangeably) refer to cells that exhibit abnormal growth, characterized by a significant loss of control of cell proliferation or cells that have been immortalized. The term "cancer" or "tumor" includes metastatic as well as non-metastatic cancer or tumors. A cancer may be diagnosed using criteria generally accepted in the art, including the presence of a malignant tumor.

[0073] In some embodiments of the disclosure, a phytogalactolipid-enriched fraction (CRA) from *Crassocephalum rabens* (Benth.) S. Moore is shown to induce humoral immune responses, for example, against cancers. In some embodiments of the disclosure, CRA treatment attracted an abundance of B cells into a target site, such as the tumor. CRA enhances the anti-tumor antibodies in both sera and tumor resulted in anti-tumor antibodies undergoing a class-switch from IgM to IgG1, IgG2, and IgG3. CRA-induced antisera specifically

recognized surface antigens on the plasma membrane of cancer cells. CRA antisera not only shows cytotoxicity but also induces antibody-dependent cell cytotoxicity (ADCC) and phagocytosis (ADCP). In some embodiments, serum IFN- γ and IL-21 significantly increase after CRA treatment. CRA directly regulates STAT3 and cMaf, and let T cells secrete IL-21, in turn, inducing B cells proliferation and differentiation through the IL-21R/STAT3/Blimp-1 pathway. Together, CRA shows potent bioefficacy in regulating T cells to stimulate B cell activation, enabling a subject to be treated to trigger anti-tumor antibodies to impede cancer progression.

[0074] *Crassocephalum rabens* (Benth.) S. Moore from the family Asteraceae also known as *Crassocephalum crepidioides* (Benth.) S. Moore, is a common vegetable and a popular folk medicine whose ethanolic extract has been shown to be a safe supplement [Hsu, P.K., et al., Toxicol Rep, 2022. **9**: p. 58-63]. *C. rabens* has attracted attention due to its prominent anti-inflammatory and anticancer activity [Hou, C.C., et al., Cancer Res, 2007. **67**(14): p. 6907-15; Apaya, M.K., et al., Cancers (Basel), 2020. **12**(1): p. 199; Yang, C.C., et al., Int J Cancer, 2018. **143**(12): p. 3248-3261]. The glyceroglycolipid 1,2-di-*O*- α -linolenoyl-3-*O*- β -galactopyranosyl-sn-glycerol (dLGG) is a major component of *C. rabens* with chemopreventive activity in cancer or sepsis by inhibiting inflammatory mediators, such as TNF- α , IL-6, or bioactive lipid mediator oxylipins [Hou, C.C., et al., Cancer Res, 2007. **67**(14): p. 6907-15]. dLGG repressed the metastatic ability of melanoma cells by deregulating epithelial-mesenchymal transition (EMT), attenuating tight junction permeability of pulmonary vasculature and circulating oxylipin dynamics [Yang, C.C., et al., Int J Cancer, 2018. **143**(12): p. 3248-3261]. dLGG used alone or in combination with doxorubicin effectively attenuates triple-negative breast cancer (TNBC) recurrence and lung metastasis through inhibiting the fatty acid binding protein (FABP)/epoxy-eicosatrienoic acid (EET)-mediated signaling axes [Apaya, M.K., et al., Cancers (Basel), 2020. **12**(1): p. 199].

[0075] As used herein, the *Crassocephalum rabens* plant may be the whole plant or one or more parts thereof, including but not limited to, seeds, flowers, leaves, stems and roots. In an embodiment of the present disclosure, the *Crassocephalum rabens* plant is the whole plant. In another embodiment of the present disclosure, the *Crassocephalum rabens* plant is seeds, flowers, leaves, or any combination thereof.

[0076] The *Crassocephalum rabens* plant may be collected at various stages.

[0077] In one embodiment of the disclosure, the *Crassocephalum rabens* plant is a mixture obtained by removing some substances from the *Crassocephalum rabens*. In a preferred embodiment of the present disclosure, the *Crassocephalum rabens* plant is prepared by drying and crushing the *Crassocephalum rabens*, and is pieces of dried or fresh *Crassocephalum*

rabens.

[0078] In a preferred embodiment of the present disclosure, the *Crassocephalum rabens* extract is prepared by removing solid contents of the *Crassocephalum rabens* and the *Crassocephalum rabens* extract is liquid of *Crassocephalum rabens*.

[0079] In some embodiments of the disclosure, the *Crassocephalum rabens* extract is alcohol extract of *Crassocephalum rabens*. In some embodiments, the alcohol is C1 to C4 alcohol. The term "C1 to C4 alcohol" as used herein refers to linear or branched, substituted or unsubstituted, mono- or poly- functional, and saturated or unsaturated alcohol; preferably unsubstituted, mono-functional and saturated alcohol. In one embodiment of the disclosure, the C1 to C4 alcohol is selected from the group consisting of methanol, ethanol, n-propanol, isopropanol, n-butanol, iso-butanol, sec-butanol, tert-butanol. In some embodiments, the C1 to C4 alcohol is methanol or ethanol. The C1 to C4 alcohol can be used solely or in combinations.

[0080] In some embodiments of the disclosure, the *Crassocephalum rabens* extract is manufactured by extracting *Crassocephalum rabens* with an alcohol solution to obtain alcohol extract; fractioning the alcohol extract to obtain a phytogalactolipid-enriched fraction.

[0081] In some embodiments of the disclosure, the fractioning step is performed with reverse phase medium pressure liquid chromatography (RP-MPLC). In some further embodiments of the disclosure, reverse phase high performance liquid chromatography (RP-HPLC) is further performed after RP-MPLC.

[0082] The present disclosure also provides a method for inducing humoral immune response in a subject in need, comprising administering to said subject an effective amount of galactolipid compounds from *Crassocephalum rabens* or a pharmaceutically acceptable derivative thereof and optionally a pharmaceutically acceptable carrier or excipient.

[0083] In some embodiments, the active ingredients of comprises 1,2-di-O- α -linolenoyl-3-O-(6-O- α -galactopyranosyl- β -galactopyranosyl)-sn-glycerol (designated CRDG) and dLGG (designated CRG) in CRA.

[0084] The galactolipid compounds of the disclosure can be further converted into a pharmaceutically acceptable derivative, such as a pharmaceutically acceptable salt, solvate or prodrug, by any known methods.

[0085] The *Crassocephalum rabens* extract is preferably contained in an extraction composition.

[0086] The extraction composition according to the disclosure is preferably a pharmaceutical composition or food composition.

[0087] The pharmaceutical composition according to the disclosure is preferably administered topically or systemically by any method known in the art, including, but not limited to, intramuscular, intradermal, intravenous, subcutaneous, intraperitoneal, intranasal, oral, mucosal or external routes. The appropriate route, formulation and administration schedule can be determined by those skilled in the art. In the present disclosure, the pharmaceutical composition can be formulated in various ways, according to the corresponding route of administration, such as a liquid solution, a suspension, an emulsion, a syrup, a tablet, a pill, a capsule, a sustained release formulation, a powder, a granule, an ampoule, an injection, an infusion, a kit, an ointment, a lotion, a liniment, a cream, or a combination thereof. If necessary, it may be sterilized or mixed with any pharmaceutically acceptable carrier or excipient, many of which are known to one of ordinary skill in the art.

[0088] The extract composition can be added to a conventional food composition (i.e., the edible food or drink or precursors thereof) in the manufacturing process of the food composition. Almost all food compositions can be supplemented with the extract composition of the disclosure. The food compositions that can be supplemented with the extract composition of the disclosure include, but are not limited to, candies, baked goods, ice creams, dairy products, sweet and flavor snacks, snack bars, meal replacement products, fast foods, soups, pastas, noodles, canned foods, frozen foods, dried foods, refrigerated foods, oils and fats, baby foods, or soft foods painted on breads, or mixtures thereof.

[0089] The present disclosure provides use of *Crassocephalum rabens* plant or extract as an adjuvant for enhancing humoral immune response.

[0090] The present disclosure also provides an adjuvant composition comprising an effective amount of galactolipid compounds from *Crassocephalum rabens*.

[0091] In some embodiments of the disclosure, a galactolipid-enriched extract of *Crassocephalum rabens* (designated CRA) functions as adjuvant against pathogen infection. CRA dramatically enhances the titers of specific antibody against an antigen and increased the IgG2a, IgG2b and IgG3 proportion of total Ig. In some embodiments, CRA triggers IL-21 and B cell activating factor (BAFF) release resulting in B cell differentiation. CRA also enhanced cell proliferation, uptake ability, and antigen presentation phagocytes. Taken together, CRA possesses immunomodulatory activity and adjuvant effect that enhances mouse immunity against pathogenic antigens through specifically increasing both Th1 and Th2-related immune responses. Mechanistic study showed that CRA induced B cell differentiation through promoting IL-21 and BAFF, and functioned to produce higher titer and affinity of specific antibodies.

[0092] Certain embodiments as provided herein include vaccine compositions and immunological adjuvant compositions, including pharmaceutical compositions, that contain, in addition to *Crassocephalum rabens* plant or extract, at least one co-adjuvant, which refers to a component of such compositions that has adjuvant activity but that is other than *Crassocephalum rabens* plant or extract. A co-adjuvant having such adjuvant activity includes a composition that, when administered to a subject such as a human (e.g., a human patient), a non-human primate, a mammal or another higher eukaryotic organism having a recognized immune system, is capable of altering (i.e., increasing or decreasing in a statistically significant manner, and in certain preferred embodiments, enhancing or increasing) the potency and/or longevity of an immune response. In certain embodiments disclosed herein, *Crassocephalum rabens* plant or extract and a desired antigen, and optionally one or more co-adjuvants, may so alter, e.g., elicit or enhance, an immune response that is directed against the desired antigen which may be administered at the same time as *Crassocephalum rabens* plant or extract or may be separated in time and/or space (e.g., at a different anatomic site) in its administration, but certain invention embodiments are not intended to be so limited and thus also contemplate administration of *Crassocephalum rabens* plant or extract in a composition that does not include a specified antigen but which may also include one or more of a TLR agonist, a co-adjuvant, an imidazoquinoline immune response modifier, and a double stem loop immune modifier (dSLIM).

[0093] The present disclosure also provides an immunogenic composition comprising an antigen component and the adjuvant composition as described herein.

[0094] In some embodiments of the disclosure, the antigen component is a peptide or a protein.

[0095] When combined with an antigen such as in a vaccine or immunogenic composition, the adjuvant compositions of the present disclosure provide improved presentation of the antigen portion of the vaccine to the immune system of the recipient of the vaccine, when compared to previous vaccines or immunogenic compositions comprising adjuvants not provided in the present disclosure. Such improved presentation is in comparison to the same antigen when combined with an adjuvant composition that is not part of this disclosure. Preferably, the improved presentation permits the use of smaller or lower amounts of antigen to achieve the same level of immune system reaction. The level of immune system reaction can be measured by the strength of the response, as measured by markers of immune response, or can be measured by the duration of immunity, or combinations of these two indicators of immune system reaction. Even more preferably, the improved antigen

presentation permits the use of 95% of the amount of antigen, more preferably 90%, still more preferably 85%, 80%, 75%, 60%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.09%, 0.08%, 0.07%, 0.06%, 0.05%, 0.04%, 0.03%, 0.02%, 0.01%, 0.009, 0.008%, 0.007%, 0.006%, 0.005%, as well as ranges formed by any two members of this group, to achieve the same level of immune system reaction as the same antigen in combination with a different adjuvant when administered to an animal of the same species.

[0096] The present disclosure also provides a method for enhancing immune response in a subject in need, comprising administering to said subject the adjuvant composition or the immunogenic composition as described herein.

[0097] The method of vaccinating of the present disclosure preferably includes administration of the composition comprising the adjuvant of the present disclosure and an antigen, where administration is needleless or injected. For purposes of embodiments of the present disclosure, the administration method is preferably orally, intramuscularly, subcutaneously or transdermal administration, although other administration methods may be employed. In one embodiment, the administration method is selected from the group consisting of topical, intramuscular, nasal, oral, transdermal, mucosal, needleless administration methods and subcutaneous. Needleless administration methods include, but are not limited to, vaccine guns, transdermal patches, aerosols, mucosal administration methods, skin adhesion methods, dry particle projectiles, wet projectiles, gold/inert particle guns, and pneumatic guns.

[0098] The following Examples are given for the purpose of illustration only and are not intended to limit the scope of the present disclosure.

[0099] **Material and methods**

[0100] **Preparation of the bioactive fraction CRA from *C. rabens* plant and purification of two major chemical constituents, i.e., CRDG and CRG in CRA**

[0101] The fresh or dried whole plant materials of *C. rabens* were extracted with alcohols, e.g., 50 to 99.5% ethanol or methanol to yield the total crude extract of *C. rabens*, which was further fractionated with reverse phase medium pressure liquid chromatography (RP-MPLC) to obtain a phytogalactolipid-enriched fraction, designated CRA. The mobile phase of MPLC was composed of primary alcohol (MeOH, EtOH, etc.) and distilled water; the fraction CRA was eluted by a high percentage of alcohols, e.g., 70 to 100%. The CRA fraction was subjected to preparative reverse phase high performance liquid chromatography (RP-HPLC) to collect pure CRDG and CRG. The mobile phase of HPLC was composed of methanol or acetonitrile and

distilled water with a ratio of >90% organic solvent: <10% distilled water to obtain CRDG and CRG. The purity and structural elucidation of CRDG and CRG were examined using analytical HPLC, high-resolution mass spectrometry and NMR spectroscopy [Hou, C.C., et al., Cancer Res, 2007. **67**(14): p. 6907-15].

[0102] **Cells**

[0103] Mouse colorectal cancer CT26.CL25 cells were purchased from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). CT26.CL25 cells were maintained in RPMI-1640 culture medium (Invitrogen, Carlsbad, CA) supplemented with 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.1 mM non-essential amino acids, 10% fetal bovine serum, and 1% penicillin-streptomycin (Invitrogen). Mouse metastatic castration-resistant prostate cancer MG-Cap A1 cells were kindly provided by Dr. Pei-Wen Hsiao of the Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan, and maintained in DMEM supplemented with 10% fetal bovine serum, 10 µg/mL puromycin, 5 µg/mL blasticidin, and 1% penicillin-streptomycin.

[0104] **Animals**

[0105] Female BALB/c mice aged 5 weeks were applied. All animals were subjected to health monitoring and maintained on a 12:12-h light:dark cycle at a controlled temperature ($22 \pm 2^\circ\text{C}$) and humidity ($55 \pm 10\%$) in a specific pathogen-free animal facility. Animals were acclimatized with free access to a rodent standard diet (LabDiet 5010, St. Louis, MO) and water. All procedures were approved by the Institutional Animal Care and Use Committee of Academia Sinica (approved protocol no. 20-12-1592) and followed the guidelines for the Use of Laboratory Animals (National Academy Press, Washington, DC).

[0106] **The therapeutic effects of CRA treatment *in vivo***

[0107] BALB/c mice were inoculated subcutaneously with 1×10^6 CT26.CL25 cells in 100 µL of PBS. When the average tumor mass reached 100 mm³, the tumor-bearing mice were randomly divided into four groups. Animals were treated with 25, 50, and 100 mg/kg CRA via daily oral administration for 33 days. The tumor volumes were measured using a caliper every 2 days, and the volumes were calculated using the following formula: volume (mm³) = length × width × height. Before sacrifice, the sera of the mice were collected for experimental use.

[0108] **Phenotyping of splenic and tumor-infiltrating B cell subsets**

[0109] The splenic and tumor-infiltrating lymphocytes of CRA-treated or untreated mice were harvested. B cell subsets, such as naïve (CD19⁺CD23⁺), activated (CD19⁺CD38⁺), memory B (CD19⁺CD27⁺) and plasma cells (CD19⁻ or low CD138⁺) were probed with specific monoclonal antibodies (BioLegend, San Diego, CA) and analyzed by flow cytometry (BD Biosciences). The

indices of different types of B cell proportion were calculated as follows: (the number of splenocytes × the percentage of each B cell population in treated splenocytes)/(the number of splenocytes × the percentage of each B cell population in untreated splenocytes).

[0110] **Immunohistochemical staining**

[0111] Tumors isolated from CRA-treated mice were fixed with 4% paraformaldehyde at 4°C overnight. After washing with PBS, the tumor samples were placed in 30% sucrose at 4°C overnight. Then, the tumor samples were embedded in OCT, and 10 µm sections were made and processed for immunohistochemical staining. The sections were probed with Alexa Fluor 594-conjugated rat monoclonal anti-B220 (1:50 dilution; Biolegend) and PE-conjugated rat monoclonal anti-CD3 (1:50 dilution; Biolegend) antibodies. The sections were also probed with goat polyclonal anti-IL-21 (1:20 dilution; Thermo Fisher) at 4°C overnight, and the detection antibodies were recognized using a Alexa Fluor 488-conjugated anti-goat IgG antibody (1:100 dilution; Thermo Fisher). The immune complexes in the sections were visualized and photographed at 200× magnification using a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany).

[0112] **Titers and Ig classes of antitumor autoantibodies.**

[0113] Blood was collected from CT26.CL25-bearing mice treated with PBS or 25, 50 or 100 mg/kg CRA, and the titers of CT26.CL25-specific antibodies in the sera were measured by the following method. The wells of 96-well culture plates were seeded with CT26.CL25 (10⁶ cells/well). On the following day, the cells were fixed with 4% paraformaldehyde, washed, and blocked with 300 µl of 2% skim milk in PBST (PBS buffer with 0.05% tween-20) for 1 h. One hundred microliters of 1000-fold diluted sera in PBS containing 0.5% skim milk were loaded into each well and incubated at room temperature for 2 h. After washing 3 times, 100 µl HRP-conjugated anti-mouse Ig antibody (1:5000 dilution; Sigma-Aldrich, St. Louis, MO) were added to each well and incubated for 1 h. After washing 3 times, 100 µL of NeA-Blue (Clinical Science Products, Mansfield, MA) was added to each well, incubated for 20 minutes and stopped using 100 µL 1 N HCl. The optical density was measured at 450 nm using an ELISA reader (Tecan, Mannedorf, Switzerland). The isotypes of specific anti-CT26.CL25 cell antibodies in anti-sera (1:1600 dilution) were determined by using HRP-conjugated specific anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3 or IgA antibodies (Acris, Herford, Germany).

[0114] **Immunofluorescence analysis of antisera binding to tumor antigens on the cell surface**

[0115] CT26.CL25 or MG-Cap-A1 cells were incubated with 2 µL of normal sera, PBS antisera (control antisera) or CRA antisera for 1 h and detected with secondary antibodies conjugated

to FITC (Molecular Probes, Eugene, USA). Antisera binding to tumor antigens on the surface of CT26.CL25 and MG-Cap-A1 cells were analyzed by flow cytometry (BD Biosciences).

[0116] ***In vitro* ADCC, ADPC and CDC assay**

[0117] In *in vitro* ADCC assay, a total of 5×10^3 CFSE-labeled CT26.CL25 target cells were pre-incubated with 2 μ L of antisera. Mouse NK cells purified from mouse splenocytes were added at an effector with a target (E: T) cell ratio of 4:1. Assay plates were incubated at 37°C for 4 h. Cytolysis was determined by an ELISA Reader (BioTek, Winooski, VT, USA).

[0118] For the ADPC assay, a total of 1×10^5 labeled CFSE-labeled CT26.CL25 cells were incubated with 2 μ L of antisera for 30 minutes and then added to 3×10^5 macrophages. Four hours later, macrophages were probed with Alexa Fluor 647 conjugated rat monoclonal F4/80 antibodies (1:100 dilution; Biolegend). Phagocytosis was determined by counting double-labeled cells by flow cytometry (BD Biosciences).

[0119] For the CDC assay, a CT26.CL25 cell suspension containing 1×10^4 cells in 100 μ L of serum-free medium was mixed with 2 μ L of antisera and incubated on ice for 30 min. Two hundred microliters of Gibco horse serum (Thermo Fisher) without heating was added and incubated at 37°C for 2 h. Following washing with PBS, cell viability was determined by MTT assay.

[0120] **Cytokine assay**

[0121] Mouse splenocytes isolated from CRA treated mice were seeded at 3×10^5 cells/300 μ L in each well of a 96-well microplate and then cultured for 72 h. Cell culture supernatants were collected and analyzed by a cytokine multiplex assay (Inflammation Core Facility, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan).

[0122] For analysis of IL-21 and IFN- γ concentration, splenocytes or splenic T cells were seeded at 3×10^5 cells in a 96-well culture plate and then treated with various doses of CRA, CRG, or CRDG for 72 and 24 h, respectively. The levels of IL-21 in the culture media were determined by the ELISA kit (R&D System, Minneapolis, MN) according to the manufacturer's instructions.

[0123] **B cell proliferation and differentiation *in vitro***

[0124] Splenocytes (1×10^5 cells/well) were labeled with CFSE for 37°C for 15 min. Cells were then washed with PBS and suspended in fresh medium. CFSE labeled cells were treated with different concentrations of CRA for 72 h. B cell proliferation was analyzed by anti-CD19 antibody staining in CFSE-labeled cells. LPS (1 μ g/mL) was used as a positive control. Another experiment of *in vitro* proliferation, splenocytes (1×10^5 cells/well) were stimulated with 1 μ g/mL of anti-CD40 antibody and 100 U/mL of IL-4, and treated with CRA for 72 h. B cell

proliferation was determined as described above. For B cell differentiation, after treating with CRA for 72 h, the proportions of plasmablasts (CD19⁺CD138⁺) and plasma cells (CD19⁺ or low CD138⁺) were probed with specific monoclonal antibodies (BioLegend) and analyzed by flow cytometry (BD Biosciences).

[0125] **Quantitative polymerase chain reaction (qPCR)**

[0126] Splenocytes or splenic T cells were treated with CRA, CRG, or CRDG at indicated concentrations, and then the mRNA expression of Stat3, IL-21, Bcl6, and cMaf was measured by qPCR. Briefly, total cellular RNA was extracted with Trizol reagent (Invitrogen) and reverse-transcribed into cDNA using the Superscript RT-kit (Invitrogen). Primers of Stat3, Prdm-1, Bcl6, cMaf, and β -actin were designed using Primer-BLAST on NCBI website. The primers for Stat3 were: forward primer 5'-AGGAGTCTAACACGGCAGCCT-3' (SEQ ID NO: 1) and reverse primer 5'-GTGGTACACCTCAGTCTCGAAG-3' (SEQ ID NO: 2); the primers for IL-21 were: forward primer 5'-TAGACGCTCAGGAATGCAGG-3' (SEQ ID NO: 3) and reverse primer 5'-GTCTGTGCAGGAACCAAA-3' (SEQ ID NO: 4); the primers for Bcl6 were: forward primer 5'-CAGAGATGTGCTCCATACTGC-3' (SEQ ID NO: 5) and reverse primer 5'-CTCTCAGAGAAACGGCAGTCA-3' (SEQ ID NO: 6); the primers for cMaf were: forward primer 5'-AGCAGTTGGTGACCATGTCG-3' (SEQ ID NO: 7) and reverse primer 5'-TGGAGATCTCTGCTTGAGG-3' (SEQ ID NO: 8). All PCR reagents used to amplify the cDNA were purchased from Promega (Madison, WI, USA). β -Actin was used as an endogenous control. The qPCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA) and detected by ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Relative fold expression was calculated by the $2^{-\Delta\Delta CT}$ method using the following formulae: ΔCT (sample) = $CT_{\text{target gene}} - CT_{\text{reference gene}}$

[0127] **Statistical Analysis**

[0128] The results were expressed as mean $\pm$ SD and analyzed using the SAS statistical software package (SAS Institute, Cary, NC). The ANOVA test was used when comparing multiple samples. Differences with a P value of less than 0.05 were considered statistically significant.

[0129] **Animal immunization**

[0130] Female BLAB/c mice were immunized with 100 μ g of recombinant AaHSP60 mixed in 10 mg/kg of CRA by *intraperitoneal (i.p.) injection*. Subsequent immunizations of mice were also *i.p.* injected with a mixture of AaHSP60 and CRA every week and last for 35 days. The blood was collected from mice with pre- and post-immunization. Their titers of antisera were determined by ELISA.

[0131] **Titers and Ig classes of anti- AaHSP60 antibody**

[0132] Blood was collected weekly from AaHSP60/CRA-immunized mice. The titers of anti-AaHSP60 antibodies in sera were measured as described hereafter. One hundred ng of AaHSP60 was coated in each well of the 96-well plates at room temperature overnight. Subsequently, the wells were blocked with 300 μ L of 2% skim milk PBST (PBS buffer with 0.05% tween-20) for 1 h. One hundred microliters of serially diluted sera in PBS containing 0.5% skim milk were loaded into each well and incubated at room temperature for 2 h. After washing 3 times, 100 μ L HRP-conjugated anti-mouse Ig antibody (1:10000 dilution; Sigma-Aldrich, St. Louis, MO, USA) were added to each well and incubated for 1 h. After washing 3 times, 100 μ L of NeA-Blue (Clinical Science Products, Inc. Mansfield, MA, USA) was added to each well, incubated for 20 minutes and stopped using 100 μ L 1 N HCl. The optical density was measured at 450 nm using an ELISA reader (Tecan, Mannedorf, Switzerland). The isotypes of specific anti-AaHSP60 antibodies in sera (1:2000 dilution) were determined by using HRP-conjugated specific anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3 or IgA antibodies (Acris, Herford, Germany).

[0133] **The changes of B cell lineage in the AaHSP60/CRA-immunization mice**

[0134] After AaHSP60-immunization, mouse splenocytes were obtained as the previous description. The splenocytes were probed by fluorochrome conjugated anti-CD19, anti-CD-23, anti-CD-38, anti-CD27 and anti-CD138 antibodies and measured using flow cytometry (BD Biosciences). The types of B cell were defined as follows: naïve B cells (CD19⁺CD23⁺), activated B cells (CD19⁺CD38⁺), memory B cells (CD19⁺CD27⁺) and plasma cells (CD19^{low/-}CD138⁺).

[0135] **Cytokine profiles in sera from AaHSP60/CRA-immunized mice**

[0136] The sera isolated from the blood of AaHSP60/CRA-immunized mice were analyzed by cytokine multiplex assay following the Bio-Plex Pro Mouse Cytokine Standard Group I 23-Plex (Bio-Rad, Hercules, CA, USA) and Milliplex mouse IL-21, IL-33, and TGF β (Inflammation Core Facility, Academia Sinica, Taipei, Taiwan). IL-21, IFN- γ and TGF β in mouse serum or cultural media of CRA-treated cells were analyzed by ELISA assay kits (R&D System, Minneapolis, MN) according to the manufacturer's protocol.

[0137] **Cells and cell culture**

[0138] Splenocytes were isolated from BALB/c mice according to the following procedures. Briefly, BALB/c mice were sacrificed and their spleens were collected. The spleen was placed into the cell strainer and homogenized the spleen through the cell strainer into the petri dish. Erythrocytes in the homogenized spleen were lysed by ACK lysis buffer at room temperature for 5 min following the PBS wash. After the spin-down of cells, they were resuspended and cultured with RPMI1640 growth medium (Gibco/Invitrogen) supplemented with heat-

inactivated 10% fetal bovine serum (Gibco/Invitrogen) and 1% penicillin/streptomycin (Gibco/Invitrogen).

[0139] RAW264.7 mouse macrophages were purchased from BCRC (Hsinchu, Taiwan, ROC). RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco/Invitrogen) supplemented with heat-inactivated 10% fetal bovine serum (Gibco/Invitrogen) and 1% penicillin/streptomycin (Gibco/Invitrogen) in 5% CO₂ at 37°C.

[0140] **Cell proliferation assay**

[0141] Splenocytes (1×10⁵ cells/well) were labeled with CFSE for 37°C for 15 min, and then cells were washed with PBS and then suspended in fresh medium. CFSE labeled cells were treated with 1 µg/ml of AaHSP60 and different concentration of CRA for 72h. Splenocyte proliferation was determined by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). B cell proliferation was analyzed by anti-CD19 antibody staining in CFSE-labeled cells.

[0142] Macrophage RAW264.7 cells were seeded at 1×10⁴ cells/well overnight in a 96-well microculture plate. After treatment with serial concentrations of CRA in DMSO (final concentration of DMSO is 0.1%) for 48 h, macrophage cell proliferation was measured by MTT. The cell viability ratio (%) was calculated using the following equation: % viability = absorbance of test sample/absorbance of control × 100%.

[0143] **Surface markers expression**

[0144] After treating with AaHSP60 and CRA for 48h, the treated cells were probed with FITC-conjugated anti-mouse CD86, and anti-mouse MHC II monoclonal antibodies, and the surface fluorescence of the cells was measured using flow cytometry (Becton Dickinson). The data were expressed as the fold of fluorescent mean.

[0145] **Phagocytosis assay**

[0146] RAW 264.7 cells (3×10⁵ cells/well) were treated with 1 µg/ml of AaHSP60 and indicated concentrations of CRA for 48 h. Phagocytic activity of RAW264.7 cells were determined by the Phagocytosis Assay Kit (Cayman, Ann Arbor, MI) according to the product manual. Briefly, cells were incubated with the latex beads-rabbit IgG-FITC (1:400) at 37°C for 1 h. Then, 50 µL of trypan blue quenching solution (1: 50 dilute in Cell-Based Assay Buffer) was added to quench the surface fluorescence. Phagocytosis was analyzed by flow cytometer (Becton Dickinson).

[0147] **Analysis of mRNA expression of mBAFF by RT-PCR**

[0148] Splenocytes (1×10⁶ cells/well) were treated with CRA for 16 h. Then, total cellular RNA was extracted with TRIzol (Invitrogen Life Technologies, Carlsbad, CA) and reverse-transcribed into cDNA using the Superscript™-III kit (Invitrogen). PCR analysis was performed on aliquots

of the cDNA preparations to detect gene expression. The cDNA of mBAFF was then amplified by PCR. The primers for mouse BAFF were: forward primer 5'-TGGTGAGGCAAACAGGCTAT-3' (SEQ ID NO: 9) and reverse primer 5'-AGAAGGTGTCGTCTCCGT TG-3' (SEQ ID NO: 10) All PCR reagents used to amplify the cDNA were purchased from Promega (Madison, WI, USA). GAPDH cDNA in the samples was used to normalize the loading amounts in each reaction. Finally, PCR products were resolved by electrophoresis on 2% agarose gels, stained with ethidium bromide and photographed using the Uni-photo band tool (EZ lab, Taipei, Taiwan).

[0149] **Analysis of protein expression of BAFF by ELISA**

[0150] Splenocytes (1×10^6 cells/well) were treated with CRA for 24 h. The levels of BAFF in the culture media were determined by the ELISA kit (R&D System, Minneapolis, MN) according to the manufacture instructions.

[0151] **Statistical analyses**

[0152] The results are presented as the mean $\pm$ SEM. The statistical significance was evaluated using Student's *t*-test for testing the difference between two groups or one-way ANOVA followed by Newman-Keuls difference post hoc analysis for testing the difference among groups. $P < 0.05$ was considered significant.

[0153] **Example 1 CRA inhibited tumor growth and activated systemic and tumor-infiltrating B cells**

[0154] To examine whether CRA triggered immunoregulatory activity for the suppression of tumor growth, the effects of oral injection with 25, 50 or 100 mg/kg of CRA into CT26.CL25 tumors-bearing BALB/c mice were monitored. After treatment for 21 days, 50 and 100 mg/kg of CRA significantly inhibited the growth of CT26.CL25 tumors in BALB/c mice (FIG. 1A). At the end of treatment (day 33), much smaller tumor masses were observed in the CRA_50 (40.9%) and CRA_100 (44.3%) groups than the control (100%) group (FIG. 1B). Control and treatment mice exhibited similar body weights during the experiment (FIG. 1C).

[0155] To determine whether B cell activation was involved in the antitumor activity of CRA, the proportion of splenic CD19 positive B cells and their functional activity were analyzed. There was no significant difference between the control and CRA treatment mice in terms of the levels of splenic CD19 positive B cells (FIG. 1D). The different B cell subsets were further examined. CRA decreased the proportion of splenic naïve B cells, but increased the proportions of splenic activated, memory, and plasma cells (FIG. 1E). These results revealed that CRA did not change the levels of total B cells but CRA changed the proportion of B cell subsets. CRA increased the functional activity and promoted differentiation in systemic B cells. Using an immunohistochemistry (IHC) assay, it was shown that CRA treatments led to the

infiltration of B cells into the tumor area (FIG. 1F). These results suggested that CRA-induced B cell activation and differentiation may play an adjuvant role in CRA-induced tumor inhibition.

[0156] **Example 2 CRA increased the production and activity of anti-tumor autoantibodies**

[0157] Characterization of the anti-sera revealed that the titers of specific anti-CT26.CL25 antibodies in CRA antisera were higher than in control antisera. Even a low dose (25 mg/kg) of CRA still increased the titers of anti-CT26CL25 antibodies (FIG. 2A). The major classes of increased antibodies in CRA antisera were IgG1, IgG2a, IgG2b, and IgG3 (FIG. 2B). Like normal mouse sera, control antisera only somewhat recognized the CT26CL25 cells; however, the CRA antisera displayed a stronger binding affinity for the CT26CL25 cells (FIG. 2C). CRA antisera did not recognize the MG-Cap A1 cells (FIG. 2C), suggesting CRA-induced antibodies in antisera have tumor specificity.

[0158] **Example 3 CRA-activated humoral immune response suppresses tumor growth**

[0159] To determine whether CRA-triggered humoral immunity is involved in the antitumor activity, cytotoxicity assay was performed on CRA antisera or control antisera. CRA antisera significantly inhibited cell proliferation in CT26.CL25 cells, whereas sCRA antisera (pre-subtract anti-CT26.CL25 cell antibodies) had no effect (FIG. 3A). CRA antisera triggered major ADCC and ADCP and minor CDC response to kill tumor cells (FIGs. 3B, 3C and 3D). When the anti-CT26.CL25 cell antibodies were pre-subtracted, these three antibody-mediated cell death responses declined. These results indicated that CRA-activated humoral immunity contributes to the therapeutic efficacy of CRA treatment in cancer therapy.

[0160] **Example 4 Role of cytokine expression in CRA-induced B cell activation**

[0161] Cytokine expression examination of splenocytes isolated from CRA-treated mice showed that levels of IFN- γ and IL-21 were significantly increased (FIG. 4). These results suggest that IL-21 may be an important cytokine in modulation of B cell activation and differentiation.

[0162] **Example 5 CRA promoted B cell differentiation *in vitro***

[0163] To explore the effect of CRA on B cell activation, cell proliferation and differentiation following CRA treatment were measured in mouse splenocytes. CRA did not induce B cell proliferation in mouse splenocytes (FIG. 5A), but increased the proportion of plasma (CD19^{low}/-CD138⁺) cells (FIGs. 5B and 5C), indicating that CRA mainly promotes B cell differentiation rather than proliferation. CRA increased the IL-21 and IFN- γ release in splenocytes (FIG. 5D). In another set of *in vitro* studies, splenocytes were stimulated with anti-CD40 antibody and IL-4 and then treated with CRA. The results also showed CRA did not affect

cell proliferation of splenic B cells (FIG. 5E) but accelerated B cell differentiation in anti-CD40 antibody and IL-4 stimulated splenocytes (FIGs. 5F and 5G). In the culture media, the levels of IL-21 and IFN- γ also increased in anti-CD40 antibody and IL-4 to stimulate splenocytes after CRA treatment (FIG. 5H). Subsequently, CRA treatment increased the gene and protein expression of STAT3 and Blimp which are downstream of the IL-21/IL-21 receptor signaling in B cells (FIGs. 6A and 6B).

[0164] To understand whether CRA can directly differentiate B cells or requires IL-21 mediation, splenic B cells were purified and then treated with CRA. In the absence of IL-21, CRA did not trigger the differentiation response of splenic B cells with or without anti-CD40 antibody and IL-4 stimulation (FIGs. 7 and 8). Furthermore, CRA-induced differentiation declined when IL-21 released from CRA-treated splenocytes was neutralized by anti-IL-21 antibody (FIG. 9). These results suggest that CRA-induced B cell activation requires the presence of IL-21, which can trigger IL-21/IL-21R signaling leading to B cell differentiation.

[0165] **Example 6 Role of T cells in CRA-induced B cell activation**

[0166] The immune cells that produce both IL-21 and IFN- γ should be the key reactive cells for CRA-induced B cell activation. It has been reported that T cells produce IL-21 through STAT3/cMaf/BCL6 signaling. To investigate whether CRA activates T cells to release IL-21, the expression of STAT3, cMaf, and BCL6 in splenic T cells were determined. CRA not only increased IL-21 release (FIG. 10A) but also raised the expression of STAT3 and cMaf in a dose-dependent manner, whereas CRA increased the expression of BCL6 only at the highest dose (FIGs. 10B, 10C, and 10D). Thus, CRA induced T cells to produce IL-21 through the STAT3/cMaf/BCL6 pathway. IL-21 released from CRA-activated T cells allows B cell differentiation and produces high titers and affinity of antitumor autoantibodies to inhibit tumor growth (FIG. 10E).

[0167] **Example 7 Effects of active components of CRA on B cell differentiation**

[0168] CRA is composed of two major compounds, CRDG and CRG. To investigate which compound is the active ingredient of CRA for B cell differentiation, changes in the proportion of plasma cells in CRDG or CRG-treated splenocytes were determined. In this study, the effect of CRA on B cell differentiation was derived from these two compounds, CRDG and CRG (FIGs. 11A and 11B). Both CRDG and CRG induced splenic T cells to release IL-21 through the STAT3/cMaf/BCL6 pathway (FIGs. 11C, 11D, 11E and 11F), suggesting that CRDG and CRG are the active compounds of CRA-promoted B cell differentiation.

[0169] **Discussion**

[0170] This study revealed that a phytogalactolipid enriched fraction CRA fractionated from

C. rabens (Benth.) *S. Moore* can provide anti-tumor activity through activating a humoral response (FIG. 1) to generate anti-tumor autoantibodies (AAs) with a high titer, affinity, and cytotoxic activities (FIGs. 2 and 3) which were able to abolish CRC tumor growth in BLAB/c mice (FIG. 1). A proposed mechanism for CRA is shown in FIG. 8E. CRA increased STAT3, cMaf, and BCL6 activity in T cells to induce the release of IL-21 (FIGs. 8A-D), then it could engage the IL-21 receptors on the B cells (FIGs. 6A-D) to activate STAT3/BLIMP-1 signaling to promote B cell maturation and differentiation. Blockade of CRA-induced IL-21 by neutralizing monoclonal anti-IL-21 antibody significantly inhibited B cell differentiation (FIG. 6E). These results indicate that IL-21, triggered by CRA, is a critical cytokine for mediating B cell differentiation and antibody production. IL-21 is essential for B cell differentiation to plasma cells, promoting functional germinal centers and immunoglobulin production [Spolski, R. and W.J. Leonard, *Nat Rev Drug Discov*, 2014. **13**(5): p. 379-95]. The predominant mechanism underlying IL-21-induced B-cell differentiation is STAT3-mediated induction of BLIMP-1, which is a transcriptional repressor for the generation of plasma cells and establishment of long-lived antibody response [Avery, D.T., et al., *J Exp Med*, 2010. **207**(1): p. 155-71]. It had been reported that IL-21 can have both positive and negative effects on B cells in vitro. IL-21 increased the proliferation of murine splenic B cells that had been stimulated by anti-IgM and anti-CD40 [Konforte, D., N. Simard, and C.J. Paige, *J Immunol*, 2009. **182**(4): p. 1781-7]. Conversely, B cell proliferation induced by anti-IgM and IL-4 was inhibited by IL-21 [Konforte, D., N. Simard, and C.J. Paige, *J Immunol*, 2009. **182**(4): p. 1781-7]. In this study, CRA treatment did not affect proliferation in unstimulated splenocytes (FIG. 5A). In anti-CD40/IL4-stimulated splenocytes, B cell proliferation seemed to be inhibited by CRA treatment (FIG. 5E). These results are similar to IL-21 addition to splenic B cells stimulated by anti-IgM and IL-4 in vitro [Konforte, D., N. Simard, and C.J. Paige, *J Immunol*, 2009. **182**(4): p. 1781-7]. However, the B cell differentiation is still promoted in both unstimulated and anti-CD40/IL4-stimulated (FIGs. 5B, 5C, 5F, and 5G). IL-21 signaling primes CD40-stimulated human naïve B cells to enhance their differentiation into plasmablasts via STAT3 [Berglund, L.J., et al., *Blood*, 2013. **122**(24): p. 3940-50]. Therefore, the role of CRA-induced IL-21 contributes to the promotion of B cell differentiation.

[0171] CRA enhanced titers of anti-tumor autoantibodies (FIG. 2A) and the increased antibody isotypes are IgG1, IgG2a, IgG2b, and IgG3 (FIG. 2B). Cytokines secreted by activated helper T cells determine Ig class-switching. CRA treatment-induced IL-21 and IFN- γ release (FIGs. 4A-E). IL-21 modulates B cells to produce IgG1 and IgG3 antibodies whereas IFN- γ promotes IgG2a antibody production [Pène, J., et al., *J Immunol*, 2004. **172**(9): p. 5154-7;

Bossie, A. and E.S. Vitetta, *Cell Immunol*, 1991. **135**(1): p. 95-104]. Somatic hypermutation (SHM) and class switch recombination (CSR) generate antibody diversity. Stimulation with anti-CD40 mAb and recombinant IL-21 induces activation-induced cytidine deaminase (AID) expression and causes CSR to produce IgG1 and IgG3 antibodies through induction of $\gamma 1$ and $\gamma 3$ germline transcripts and $S\gamma/S\mu$ switch circular DNA [Konforte, D., N. Simard, and C.J. Paige, *J Immunol*, 2009. **182**(4): p. 1781-7; Pène, J., et al., *J Immunol*, 2004. **172**(9): p. 5154-7]. IFN- γ produced by Th1 cells enhances the secretion of IgG2a. Our results suggested that CRA-induced IL-21 and IFN- γ not only induced antibody class switching but also activated both Th1 and Th2 responses.

[0172] IL-21 is produced by T cell populations, with the highest production by T follicular helper (Tfh) cells and Th17 cells, and lower levels produced by natural killer T (NKT) cells and CD8+ T cells [Spolski, R. and W.J. Leonard, *Nat Rev Drug Discov*, 2014. **13**(5): p. 379-95]. In this study, CRA predominantly activated STAT3/c-Maf signaling and slightly activated Bcl6 signaling to produce IL-21. STAT3 expression in T cells was required for IL-21 production by multiple T helper subsets. In Tfh and Th17 cells, STAT3 activated by IL-6 and/or IL-21 is required for IL-21 production upon T cell receptor stimulation [Wei, L., et al., *J Biol Chem*, 2007. **282**(48): p. 34605-10; Dienz, O., et al., *J Exp Med*, 2009. **206**(1): p. 69-78]. STAT3 can directly bind the IL21 promoter for IL-21 production. In addition, c-Maf is required for IL-21 production through transactivation of the IL-21 promoter in Tfh and Th17 cells. In c-Maf^{-/-} mice, IL-21 expression is deficient suggesting that expression of IL-21 and c-Maf are mutually dependent [Bauquet, A.T., et al., *Nat Immunol*, 2009. **10**(2): p. 167-75]. Bcl6 increases IL-21 production by inhibiting Krüppel-like factor 2 (Klf2), which suppresses IL-21 expression. [Choi, J. and S. Crotty, *Trends Immunol*, 2021. **42**(4): p. 336-349]. Such a repressor-of-repressors mechanism of Bcl6 serves as IL-21 production in Tfh [Choi, J. and S. Crotty, *Trends Immunol*, 2021. **42**(4): p. 336-349]. These findings suggest that CRA is a STAT3 activator to increase c-Maf expression or directly activate c-Maf to induce IL-21 production. Furthermore, IL-21 also contributes to the growth and survival of T lymphocytes by both autocrine and paracrine of IL-21 [Liu, S.M. and C. King, *J Immunol*, 2013. **191**(7): p. 3501-6]. Generally, IL-21 also affects the expression of transcription factors, such as STAT3, c-Maf and Bcl-6, which are central to Tfh development [Liu, S.M. and C. King, *J Immunol*, 2013. **191**(7): p. 3501-6]. Therefore, our data indicated that CRA triggered IL-21 release through activating STAT3, c-Maf, and Bcl6 signaling, in turn IL-21 promoted the development of Th subsets.

[0173] Cancer patients have been proven to generate autoantibodies in their sera that recognize their own tumor antigens. These autoantibodies do not provide therapeutic benefit

for tumor elimination. In some cases, they accelerate tumor growth. In this invention, CRA improved the quality of autoantibodies by inducing a switch of the Ig class from IgM to IgG, thereby enhancing their binding affinity (FIG. 2B). In the human body, IgG has a longer half-life in the blood. IgG efficiently increases the killing by NK cells and phagocytosis by macrophages. Thus, the CRA-induced class-switch may be the reason that the CRA anti-sera causes more efficient inhibition of tumor growth than control anti-sera. CRA might enhance the process of somatic hypermutation in B cells that causes the autoantibodies in CRA anti-sera to have higher antigen binding affinities than those in control anti-sera, which resulted in the antitumor autoantibodies being able to stably bind on the surface antigens to provide cytotoxicity (FIG. 3A) or trigger more efficient ADCC and ADCP (FIGs. 3B and 3C).

[0174] **Conclusion**

[0175] This invention discovered the new pharmacological function of CRA on dominant humoral responses, involved T-cell-dependent induction of IL-21 through the IL-21R/STAT3/Blimp-1 pathway. CRA triggers a systemic and tumoral response to produce higher titer and better quality of antitumor autoantibodies than the untreated mice resulting in elimination of tumors in animals.

[0176] **Example 8 The adjuvant effect of CRA for the production of antibody *in vivo***

[0177] To evaluate the efficiency of CRA as an effective adjuvant *in vivo*, we utilized AaHsp60 as an antigen to immunize mice. After completion of the immunization protocol, the titers of anti-AaHsp60 antibodies in the sera were measured. FIG. 12A shows that CRA significantly induced titers of specific anti-AaHsp60 antibodies after 21-day treatment. The EC₅₀ range of CRA-induced anti-AaHsp60 antibody is 1:37301 to 1: 64028 dilution, when the dilution range of vehicle-induced anti-AaHsp60 antibody is 1:12595 to 1: 64028 (FIG. 12B). The isotypes of increased specific anti-AaHsp60 antibodies are IgM, IgG2a, IgG2b, and IgG3 (FIG. 12C). Furthermore, CRA enhanced proportion of plasma cells in splenocytes isolated from AaHsp60-immunized mice (FIG. 12D). These results showed that CRA enhanced the production and binding affinity of the specific antibodies. CRA-induced antibody class-switching was a reason for enhancing antibody binding affinity. CRA-induced B cell differentiation led to more antibody production.

[0178] Antibody class-switching and B cell differentiation are dependent on the types of cytokines that are produced following stimulation with antigens. To prove that the CRA induces which immune pathways, we examined the different types of cytokines that were induced by antigen stimulation following immunization. Table 1 and FIG. 13 showed that CRA significantly increased the serum levels of IL-21, IFN- γ , and TGF β in the AaHSP60-immunized

mice, whereas CRA did not affect Th2 cytokines (IL-4, IL-5, and IL-10) which are related to B-cell differentiation and antibody production. Cytokine results indicated that CRA-induced B cell proliferation through upregulating IL-21 expression. The antigen immunization with CRA also resulted in an increase in the number of IFN- γ -expressing T cells that produced IgG2a and IgG3 antibodies as well as TGF β -expressing T cells that induced IgG2b antibodies.

[0179] **Table 1.** Cytokines production in the sera from the CRA-treated mice.

Cytokines	Control	CRA
IL-1 β	1.56 $\pm$ 1.17	1.56 $\pm$ 10.2
IL-2	3.25 $\pm$ 3.48	2.56 $\pm$ 2.51
IL-3	0.51 $\pm$ 1.14	0.14 $\pm$ 0.31
IL-4	0.45 $\pm$ 0.35	0.41 $\pm$ 0.30
IL-5	4.08 $\pm$ 3.88	2.25 $\pm$ 1.65
IL-6	1.91 $\pm$ 1.94	1.66 $\pm$ 1.78
IL-9	30.28 $\pm$ 32.13	38.54 $\pm$ 47.05
IL-10	5.91 $\pm$ 6.41	11.9 $\pm$ 18.01
IL-12 (p40)	452.3 $\pm$ 262.4	447.2 $\pm$ 186.9
IL-12 (p70)	30.71 $\pm$ 41.04	13.79 $\pm$ 29.16
IL-13	47.11 $\pm$ 19.38	47.28 $\pm$ 39.90
IL-17	57.87 $\pm$ 51.11	24.27 $\pm$ 12.76
IL-21	82.43 $\pm$ 68.08	146.23 $\pm$ 83.69 *
Eotaxin	1314.8 $\pm$ 595.3	1324.9 $\pm$ 107.0
G-CSF	71.15 $\pm$ 15.49	37.39 $\pm$ 41.14
GM-CSF	22.18 $\pm$ 12.17	10.32 $\pm$ 9.99 *
IFN-γ	14.29 $\pm$ 8.37	37.66 $\pm$ 9.88 *
KC	27.31 $\pm$ 18.5	23.39 $\pm$ 10.12
MCP-1	76.08 $\pm$ 45.07	75.32 $\pm$ 41.26
MIP-1 α	2.16 $\pm$ 1.12	1.90 $\pm$ 0.95
MIP-1 β	62.92 $\pm$ 31.51	54.17 $\pm$ 50.19

RANTES	30.92 ± 18.77	20.36 ± 11.58
TNF- α	56.14 ± 59.87	41.51 ± 51.31

* $P < 0.05$ compared to the control group.

[0180] **Example 9 Effects of CRA on cell proliferation and cytokines production in splenocytes**

[0181] To evaluate the adjuvant effect of CRA, cell proliferation and cytokine release in splenocyte were analyzed. CRA efficiently induced cell proliferation in mouse splenocytes with or without AaHsp60 stimulation (FIGs. 14A and 14D). The releases of IL-21 and IFN- γ in CRA-treated splenocytes significantly increased (FIGs. 14B, 14C, 14E and 14F). Together, we demonstrated that CRA possesses potent immunomodulatory activity and adjuvant effect.

[0182] **Example 10 Effects of CRA on B cell activation and differentiation *in vitro***

[0183] To evaluate the effect of CRA on B cell activation and differentiation, cell proliferation and surface marker expression of CRA-treated B cells were analyzed. CRA unexpectedly did not influence the B cell proliferation in mouse splenocytes with or without AaHsp60 (FIG. 15A). CD86 and MHCII expression are associated with B cell activation and the ability of antigen presentation (acts as an APC). CRA also had no effects on CD86 and MHCII expression in CD19+ B cells (FIG. 15B). After CRA treatment, the proportion of plasma (CD19⁺CD138^{or low}) cells significantly increased (FIG. 16). These results indicate that CRA effectively induces B cell differentiation, even though CRA does not affect B cell proliferation and the ability to act as APC. Therefore, the adjuvant effect of CRA for the antibody production results from CRA-induced B cell differentiation.

[0184] **Example 11 Effects of CRA on antigen presentation in macrophages**

[0185] To evaluate the adjuvant effect of CRA on the efficiency of antigen presentation, cell proliferation, phagocytic activity, and surface marker expression of macrophages that is one of the antigen-presenting cells were analyzed. CRA induced 20-30% cell proliferation in macrophages (FIG. 17A). CRA did not affect the ability of antigen uptake in macrophages without antigen stimulation. However, CRA/AaHSP60 facilitated antigen uptake in macrophages by phagocytosis (FIG. 17B). CRA at 12 $\mu\text{g/ml}$ increased the uptake ability compared to vehicle control in AaHSP60-treated macrophages (FIG. 17B). Increased CD86 and MHC II expression are involved in APC maturation which leads to efficient antigen presentation. The effects of CRA on CD86 and MHCII expression involved in antigen presentation was determined. Although CRA did not affect CD86 expression (FIG. 17C), CRA effectively enhanced MHC II expression on the cell surface in macrophages with or without AaHSP60 stimulation (FIG. 17D). Interestingly, we found that CRA effectively induced the

mRNA expression and protein production of BAFF in macrophages (FIG. 18). These results demonstrated that CRA not only activated macrophages to be an APC, but also produced BAFF to help B cell differentiation.

[0186] **Example 12 Effects of CRA on antigen production through oral administration**

[0187] Female BLAB/c mice were immunized by oral administration of vehicle/rAaHSP60 (100 µg rAaHSP60) or CRA/rAaHSP60 (10 mg/kg CRA mixed with 100 µg rAaHSP60), respectively, and then one dose of CRA/rAaHSP60 was administered weekly for 5 weeks to enhance immunity. Saliva was analyzed for IgA-typed anti-AaHSP60 antibodies at a dilution of 1:3,200. As shown in FIG. 19, CRA achieves satisfactory effect on inducing antigen production through oral administration.

[0188] **Discussion**

[0189] We demonstrated a plant galactolipid-based extract, CRA, which has adjuvant effects for the enhancement of the immunogenicity of antigens. CRA not only enhanced the specific antibody production but also induced class-switch (FIG. 12A and 12C). CRA induced-IL21 and BAFF release allowed B cell differentiation (FIGs. 12D, 13 and 18). CRA induced Th1 responses including increases the levels of IgG2a (FIG. 12), IFN-γ (FIG. 13), as well as Th2 response including increases the levels of IgG2b (FIG. 12C), IL-21 (FIG. 13). Unexpectedly, the levels of typical Th2 cytokines, such as IL-4 and IL-10 in mouse serum did not change by CRA treatment. Therefore, IL-21 was suggested to play a critical role in CRA-induced B cell differentiation. IL-21 is produced by follicular Th (T_{fh}) cells in the primary and subsequent responses to T cell-dependent antigen to initiate and maintain long-lived humoral immunity (Bryant VL, Ma CS, Avery DT, et al., *Journal of Immunology*. 2007;179(12):8180-8190). IL21 elicits CD4+ T cell-mediated help for B cell differentiation to Ab-secreting plasmablasts (Bryant VL, Ma CS, Avery DT, et al. *Journal of Immunology*. 2007;179(12):8180-8190). IL-21 supports the generation and differentiation of Th2 cells by autocrine regulation. IL-21 belongs to Th2 cytokines which theoretically inhibit Th1 differentiation and GM-CSF production (Kastirr I, Maglie S, Paroni M, et al., *Journal of Immunology*. 2014;193(7):3322-3331), but IL-21 in synergy with IL-12, IL-15, or IL-18 enhances IFN-γ production in NK cells and T cells and augment the subsequent Th1-polarized immune response (Strengell M, Sareneva T, Foster D, Julkunen I, Matikainen S., *Journal of Immunology*. 2002;169(7):3600-3605). The levels of IgG2a antibody and IFN-γ increased in CRA treated mice increased (FIG. 1C), indicating that Th1 cells can be activated by CRA treatment. IL-21 signaling has been proposed to promote Th17 differentiation (Huber M, Brüstle A, Reinhard K, et al., *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105(52):20846-20851; Korn T, Bettelli E, Gao W, et al., *Nature*.

2007;448(7152):484-487) but our results showed CRA did not affect IL-17 production (Table 1), indicating Th17 was unchanged by CRA treatment in mice.

[0190] CRA induced Ig class switch from IgM to IgG2a, IgG2b and IgG3 (FIG. 12C). CRA enhanced the process of somatic hypermutation in B cells, resulting in antibodies with higher antigen binding affinities. The mechanisms of the CRA-induced Ig class switch and somatic hypermutation may be involved in the IL-21, IFN- γ and TGF β release. In addition to being a cytokine for the activation of B cells and T cells, IL-21 is also a switching factor for the IgG1 and IgG3 antibodies production. CD19+ B cells stimulated with recombinant IL-21 and anti-CD40 mAb resulted in the production of S γ /S μ switch circular DNA by increasing γ 1 and γ 3 germline transcripts and cytidine deaminase activation (Pène J, Gauchat JF, Lécart S, et al., *Journal of Immunology*. 2004;172(9):5154-5157). Whereas IFN- γ promotes of IgG2a antibodies production, TGF β modulated B cells to produce IgG2b and IgA antibodies (Bossie A, Vitetta ES., *Cellular Immunology*. 1991;135(1):95-104; Snapper CM, Waegell W, Beernink H, Dasch JR., *Journal of Immunology*. 1993;151(9):4625-4636). This disclosure observed that the increase in BAFF levels is also a modulator of CRA-induced Ig class switch. BAFF upregulates the expression of the transcription factor Pax5/BSAP, which sequentially increases the transcription of activation-induced cytidine deaminase (AID), an RNA editing enzyme responsible for IgH class switch recombination and somatic hypermutation (Xu Z, Pone EJ, Al-Qahtani A, Park SR, Zan H, Casali P., *Critical reviews in immunology*. 2007;27(4):367-397; Stavnezer J, Schrader CE., *Journal of Immunology*. 2014;193(11):5370-5378; Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T., *Cell*. 2000;102(5):553-563). Furthermore, in the murine immune system, IgG2a and IgG2b efficiently sensitize NK cells for cell killing (Clémenceau B, Vivien R, Pellat C, Foss M, Thibault G, Vié H., *MAbs*. 2013;5(4):587-594). With respect to IgG2a and IgG2b, the most potent IgG subclasses can bind to Fc γ Rs (IgG2a binds to Fc γ RI, III, and IV; IgG2b binds to Fc γ RIII and IV) to trigger NK cell-mediated ADCC (Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV., *Immunity*. 2005;23(1):41-51). IgG2a and IgG2b as well as IgG1 easily diffuse into extravascular sites to access antigens and have greater efficacy for activating the complement system. In some cases, IgG2a and IgG2b were found to be more effective than IgG1 in triggering ADCC and complement-mediated neutralization (Clémenceau B, Vivien R, Pellat C, Foss M, Thibault G, Vié H., *MAbs*. 2013;5(4):587-594; Ishizaka ST, Piacente P, Silva J, Mishkin EM., *The Journal of Infectious Diseases*. 1995;172(4):1108-1111). Mouse IgG3 binds to Fc γ RI on monocytes/macrophages and trigger *antibody*-dependent cellular phagocytosis (ADCP). Human IgG3 is the most effective subclass in terms of their activating effector functions because of their high binding

activity to C1q and FcγRs (Stapleton NM, Andersen JT, Stemerding AM, et al., *Nature Communications*. 2011;2:599). Thus, the CRA-induced class-switch should have the potential for future application inactivating antibodies against pathogens.

[0191] It has been reported that BAFF is also a potent cytokine with stimulatory effects on B and T cells (Smulski CR, Eibel H., *Frontiers in Immunology*. 2018;9:2285). The increase in systemic BAFF levels is able to augment a pathogen-specific immune response upon immunization with heat-killed *Pseudomonas aeruginosa* (Tertilt C, Joh J, Krause A, et al., *Infection and Immunity*. 2009;77(7):3044-3055). BAFF was used as an immune stimulator because of its trimerized property to enhance immune responses (Gupta S, Clark ES, Termini JM, et al., *Journal of virology*. 2015;89(8):4158-4169). The transient overexpression of BAFF in vivo enhances antigen-specific humoral immunity, which is considered a potential vaccine adjuvant design. CRA induced BAFF release and promoted anti-AaHSP60 antibody production, suggesting that the immune-stimulatory effects of CRA may be exploited as an adjuvant for pathogenic vaccines.

[0192] **Conclusion**

[0193] CRA revealed an adjuvant effect that enhances mouse immunity against pathogenic antigens and specifically increases both Th1 and Th2-related immune responses. CRA promotes B cell differentiation, and functions to produce higher titer and higher affinity antibodies against pathogenic antigens. CRA was proven to be a promising adjuvant for pathogenic vaccines.

[0194] While the present disclosure has been described and illustrated with reference to specific embodiments thereof, these descriptions and illustrations are not limiting. It should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the present disclosure as defined by the appended claims. The illustrations may not be necessarily drawn to scale. There may be distinctions between the artistic renditions in the present disclosure and the actual apparatus due to manufacturing processes and tolerances. There may be other embodiments of the present disclosure which are not specifically illustrated. The specification and drawings are to be regarded as illustrative rather than restrictive. Modifications may be made to adapt a particular situation, material, composition of matter, method, or process to the objective, spirit and scope of the present disclosure. All such modifications are intended to be within the scope of the claims appended hereto. While the methods disclosed herein have been described with reference to particular operations performed in a particular order, it will be understood that these operations may be combined, sub-divided, or re-ordered to

form an equivalent method without departing from the teachings of the present disclosure. Accordingly, unless specifically indicated herein, the order and grouping of the operations are not limitations of the present disclosure.

Claims

1. A method for inducing humoral immune response in a subject in need, comprising administering to said subject an effective amount of *Crassocephalum rabens* plant or extract or galactolipid compounds from *Crassocephalum rabens* or a pharmaceutically acceptable derivative thereof and optionally a pharmaceutically acceptable carrier or excipient.
2. The method according to claim 1, wherein the method is for attracting B cells, enhancing antibody production, enhancing antibody class switching, inducing antibody-dependent cell cytotoxicity, inducing antibody-dependent cell phagocytosis, increasing serum IFN- γ and IL-21, inducing B cell proliferation, or inducing B cell differentiation.
3. The method according to claim 2, wherein the antibody class switching comprises switching from IgM to IgG1, IgG2 or IgG3.
4. The method according to claim 2, wherein the method is for inducing B cell proliferation or inducing B cell differentiation through IL-21R/STAT3/Blimp-1 pathway.
5. The method according to claim 1, wherein the humoral immune response is induced against a surface-bound cancer antigen; or an antigen associated with a neurodegenerative disease.
6. The method according to claim 5, wherein the cancer is drug-resistant colorectal cancer.
7. The method according to claim 1, wherein the *Crassocephalum rabens* is *Crassocephalum rabens* (Benth.) S. Moore.
8. The method according to claim 1, wherein the *Crassocephalum rabens* plant is pieces of dried or fresh *Crassocephalum rabens*.
9. The method according to claim 1, wherein the *Crassocephalum rabens* extract is alcohol extract of *Crassocephalum rabens*.
10. The method according to claim 1, wherein the *Crassocephalum rabens* extract is manufactured by extracting *Crassocephalum rabens* with an alcohol solution to obtain alcohol extract; fractioning the alcohol extract to obtain a phytogalactolipid-enriched fraction.
11. The method according to claim 10, wherein the fractioning step is performed with reverse phase medium pressure liquid chromatography (RP-MPLC).
12. The method according to claim 11, wherein reverse phase high performance liquid chromatography (RP-HPLC) is further performed after RP-MPLC.
13. The method according to claim 1, wherein the galactolipid compound is 1,2-di-*O*- α -linolenoyl-3-*O*- β -galactopyranosyl-sn-glycerol (dLGG) or 1,2-di-(α -linolenoyl)-3-[α -D-galactosyl-(1-6)- β -D-galactosyl]-sn-glycerol (CRDG).

14. An adjuvant composition comprising an effective amount of *Crassocephalum rabens* plant or extract or galactolipid compounds from *Crassocephalum rabens* or a pharmaceutically acceptable derivative thereof.
15. The adjuvant composition according to claim 14, wherein the *Crassocephalum rabens* is *Crassocephalum rabens* (Benth.) S. Moore.
16. The adjuvant composition according to claim 14, wherein the *Crassocephalum rabens* plant is pieces of dried or fresh *Crassocephalum rabens*.
17. The adjuvant composition according to claim 14, wherein the *Crassocephalum rabens* extract is alcohol extract of *Crassocephalum rabens*.
18. The adjuvant composition according to claim 14, wherein the *Crassocephalum rabens* extract is manufactured by extracting *Crassocephalum rabens* with an alcohol solution to obtain alcohol extract; fractioning the alcohol extract to obtain a phytogalactolipid-enriched fraction.
19. The adjuvant composition according to claim 18, wherein the fractioning is performed with reverse phase medium pressure liquid chromatography (RP-MPLC).
20. The adjuvant composition according to claim 19, wherein reverse phase high performance liquid chromatography (RP-HPLC) is further performed after RP-MPLC.
21. The adjuvant composition according to claim 14, wherein the galactolipid compound is 1,2-di-*O*- α -linolenoyl-3-*O*- β -galactopyranosyl-*sn*-glycerol (dLGG) or 1,2-di-(α -linolenoyl)-3-[(α -D-galactosyl-(1-6)- β -D-galactosyl)-*sn*-glycerol (CRDG).
22. An immunogenic composition comprising an antigen component and the adjuvant composition according to claim 14.
23. The immunogenic composition according to claim 22, wherein the antigen component is a peptide or a protein.
24. A method for enhancing immune response in a subject in need, comprising administering to said subject the adjuvant composition according to any of claims 14 to 21 or the immunogenic composition according to claim 22 or 23.
25. The method according to claim 24, wherein the adjuvant composition or the immunogenic composition is administered through an oral route.
26. The method according to claim 24, wherein the method is for enhancing antibody production, enhancing antibody class switching, increasing the proportion of IgG2a, IgG2b or IgG3 in total IgG antibodies, triggering IL-21 and/or B cell activating factor (BAFF) release, triggering B cell differentiation, enhancing cell proliferation, uptake ability, and/or antigen presentation of phagocytes, or increasing Th1 and Th2-related immune responses.