The Same Combined Adjuvant NE+Rg1 Shows Different Immune Effects on Different Pathogen Antigens

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Research article

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Abstract

Background

New recombinant protein vaccines with high purity, clear ingredients and good safety are gradually replacing attenuated and inactivated vaccines in clinical practice. However, one of the main issues with use of these new vaccines is the need for adjuvants to enhance their immune effect. Aluminium salts (hydroxide and phosphate) were the first approved adjuvants used in human vaccines, but these salts have some limitations, such as their induction of primarily humoural immunity with weaker induction of cell-mediated immunity and the failure to clear intracellular viral infections. Therefore, there is a growing need for novel adjuvants. Combined adjuvants or adjuvant systems are increasingly being used to meet the need for adjuvant development for vaccines. Different pathogen antigens also need specific adjuvants to enhance the immune response and protection.

Methods

The present study evaluated the synergistic immunological effect of a combined nano emulsion (NE) with Ginsenoside Rg1, i.e. NE+Rg1, for the hepatitis B surface antigen (HBsAg) or H1N1 haemagglutinin (HA) of an inactivated influenza vaccine (split virion) in mice and the vaccine stability and safety. Non-parametric tests (Mann-Whitney test) and one-way analysis of variance (ANOVA) were used for statistical analyses. Tests were considered significant when $P < 0.05$.

Results

The combined adjuvant NE+Rg1 showed high stability, a mean diameter of 168.1 nm, and a zeta potential of -22.8 mV. When combined with HBsAg, it produced a similar or higher anti-HB and IgG1/IgG2a titre and elevated IL-2 and IFN-γ expression in spot forming cells (SFCs) compared with NE alone and higher IFN-γ and IL-2 expression in $CD8^+$ T cells than aluminium hydroxide. However, when combined with HA, HA+NE+Rg1 resulted in a comparable haemagglutination inhibition (HAI) titre as HA+NE compared to that in mice immunized with HA alone or HA+Rg1 and an even lower protection rate than HA+NE after PR/8/34 virus strain challenge in mice.

Conclusions

This research demonstrated that the same combined adjuvant NE+Rg1 had different immune effects on different antigens and suggests that the research and development of adjuvants must consider specific pathogens and should be studied on a case-by-case basis.

Background

The importance of adjuvants to vaccines such as recombinant proteins is self-evident. In clinical settings, adjuvants are often incorporated within vaccine formulations through physical or chemical association with antigens[1]. The development trend of adjuvants clearly shows that single components, such as

aluminium adjuvants, cytokine or chemokine adjuvants and TLR agonists, often fail to meet the needs of various refractory infectious diseases, and combined adjuvants show great promise in the development of adjuvants in the future. For example, $AS01_B$ is one type of combined adjuvant that is composed of a liposome, MPL and QS21, and it was used in a recombinant herpes zoster vaccine, Shingrix. It reduced the risk of herpes zoster infection by 97.2% in an elderly population aged 50 years and older and by 96.6%-97.9% for all age groups [2]. However, the efficacy of the marketed live attenuated herpes zoster vaccine Zostavax was only 69.8% in the population aged 50-59 years, and it was even lower in the elderly population aged over 60 years [3]. These results fully show the tremendous potential of combined adjuvants and their great effect on vaccines.

Panax ginseng is an edible plant with medicinal and effects, such as antitumour, anti-fatigue, immune boosting and anti-aging [4,5]. Ginsenoside is an important component of Panax ginseng, and it is a type of immunomodulator that has a wide range of pharmacological activities and significant effects on cardiovascular, central nervous system, endocrine and immune systems[6,7]. Ginsenosides are divided into three types according to the chemical structure of their aglycone: 1) ginsenediol saponins, including Ra1, Ra2, Ra3, Rb1, Rb2, Rb3, Rc and Rd; 2) ginsentriol saponins, including Re, Rf, Rg1, Rg2, Rh1 and notoginsenoside; and 3) ginsenoside of oleanolic acid as a glycoside [8]. Rg1 is the main component of ginseng, and it belongs to the protopanaxatriol type (PPT), which has antitumour and steroid hormone effects and improves the non-specific immune function of mice via an unclear mechanism of action. The structure of Rg1 is shown in Figure 1[9].

MF59 is an oil-on-water emulsion developed by Chiron that contains squalene, polysorbate 80, and sorbitan trioleate [10]. Compared to the unadjuvanted influenza vaccine, the MF59 adjuvanted vaccine produced a stronger and longer antibody response to the homotypic and heterotypic influenza strains in infants and the elderly. The first MF59™ adjuvant seasonal influenza vaccine was approved in Europe in 1997 for people over 65 years of age, and it is now available in nearly 30 countries [11]. Clinical evaluation of the safety and immunogenicity of Fluad®, an influenza vaccine that contains the MF59 adjuvant, in more than 2,600 volunteers of different ages (primarily people over 65 years old) showed that the MF59 adjuvant trivalent influenza vaccine was well tolerated, and its immunogenicity was better than the traditional vaccine [12]. Similar to that of the aluminium hydroxide adjuvant, the mechanism of MF59 is not fully understood. However, some studies suggested that MF59 and other adjuvants induce proinflammatory cytokines and chemokines, which are related to the recruitment, activation and maturation of antigen-presenting cells at the injection site [13].

The present study evaluated the physical and chemical characteristics, stability, preliminary safety and mechanism, and the synergistic effect of a combined adjuvant of ginsenoside Rg1 mixed with the homemade MF59-like NE or with two different antigens, i.e., HBsAg or influenza virus H1N1 haemagglutinin (HA), to provide data and support for the development of new combined adjuvants. We also delineated and demonstrated how the same combined adjuvant exhibited synergistic immune enhancement or opposite effects on different pathogen antigens.

Methods

Adjuvants

NE (3.9% squalene, 0.47% polysorbate and 0.47% Sorbitan Trioleate) was homemade, and Ginsenoside Rg1 (purity 99.44%, HPLC) was purchased from Chengdu MUST Bio-Technology Co., Ltd. (Chengdu, China).

Antigens

Hepatitis B surface antigen (HBsAg) was purchased from the Beijing Institute of Biological Products, Ltd. (Beijing, China). Inactivated influenza vaccine (split virion, H1N1, A/Brisbane/2/2018) was purchased from the Changchun Institute of Biological Products, Ltd. (Changchun, China).

Sample preparation

NE and Ginsenoside Rg1 (100 μg/ml target concentration) were mixed 1:1 v/v with different antigens, HBsAg or HA (5 or 15 μg/ml target concentration).

Reagents

RDE (□) (340122) was purchased from Japan Bio Research Co., Ltd. HRP Conjugated Goat anti-Mouse IgG1 and IgG2a were purchased from Bethyl Laboratories, Inc. (Montgomery, TX, USA). SPF chicken blood was purchased from Beijing Merial Vital Laboratory Animal Technology Co., Ltd. All other chemical reagents are products of analytically pure grade.

Mice and method of euthanasia

Eight-week-old, specific pathogen free (SPF) female BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and randomly divided into several different groups, with starting weights between 16–18 g. The experiment personnel only knows the number of each group, but does not know the specific substance to which each group is immunized. The Animal Ethics Committee of the National Vaccines and Serum Institute (AECNVSI) approved the animal experiments and all procedures. Mice were euthanized via an intraperitoneal injection of 80 mg/kg sodium pentobarbital (Serva, Germany) and painless blood collection.

Grouping and immunization procedures

For the HBsAg (S) antigen, the mice were divided into 4 groups of 11 mice: S+$Al(OH)_3$, S+NE, S+NE+Rg1 and S+Rg1. Mice were immunized at weeks 0 and 3 via intramuscular injection, and blood was collected from 7 mice per group 3 weeks after immunization boosting for the quantitative detection of anti-HB antibodies and from 4 mice for IgG1 and IgG2a antibody subclass detection using enzyme-linked immunosorbent assay (ELISA). One week after immunization boosting, the spleens of 4 mice per group

were collected for enzyme-linked immunospot assay (ELISpot) and intracellular cytokine staining (ICS) testing using fluorescence-activated cell sorting (FACS).

For the HA antigen, the mice were divided into 5 groups: HA, HA+NE, HA+NE+Rg1, HA+Rg1 and NS control. Mice were immunized at weeks 0 and 4 via intramuscular injection, eight mice per group were tested for HAI antibodies, and 10 mice per group were challenged with 10 LD_{50} of the A/Puerto Rico/8/34(H1N1) influenza strain.

Serum antibodies and cell-mediated immune response

Serum anti-HBs and IgG1 and IgG2a antibody levels were determined using indirect ELISA. Specific T-cell responses to HBsAg were measured using ELISpot and ICS. Influenza H1N1-specific antibodies were titrated via haemagglutination inhibition (HAI).

Mouse protection against influenza infection

To investigate the protective efficacy of different adjuvants for influenza virus challenge, 50 mice were randomly divided into 5 groups (10 mice per group) and challenged with 10 LD_{50} of the A/Puerto Rico/8/34(H1N1) influenza strain after the administration of 400 μl of 0.4% sodium pentobarbital on Day 14 after the immunization boost. The mortality and body weight of the challenged mice were monitored for the subsequent 14 days. Mice that lost greater than 35% body weight were humanely euthanized.

Safety and preliminary mechanism of action

The safety of the combined adjuvant NE+Rg1 was evaluated using haemolysis and coagulation experiments with rabbit red blood cells, and the preliminary mechanism of action of the NE+Rg1 was evaluated via the stimulation of TLR4 and TLR7 on HEK293 cells.

Physiochemical characteristics and the stability

The particle size, PdI and Zeta potential of NE+Rg1 at different time points (weeks 3, 8, and 12) at 37℃ were determined to delineate the physical and chemical characteristics and stability. Particle size, PdI and Zeta potential were determined using the ZetaSizer Nano ZS90 dynamic light scattering instrument (Malvern Instruments, UK).

Statistical methods

GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA) was used for data analyses and statistical tests. Means were compared using non-parametric tests (Mann-Whitney test) and one-way analysis of variance (ANOVA) followed by a Tukey-Kramer post hoc test using a 95% confidence interval. Tests were considered significant when $P < 0.05$.

Results

Antibody responses to HBsAg

For the immunopotentiation of the combined NE+Rg1 and HBsAg adjuvant, the humoural immunity results 3 weeks after 1 dose showed that the S+NE+Rg1 group showed a significantly higher mean serum antibody level than the S+ Al and S+Rg1 groups (Mann-Whitney test, P =0.0128 and P =0.0003, respectively). IgG1 in serum showed similar results, and the serum antibody level of the S+NE +Rg1 group was higher than the S+ Al group (P=0.0013). The IgG2a level was significantly higher in the S+NE +Rg1 group than the S+ Al (P < 0.001) and S+NE (P=0.0081) groups. Therefore, NE and Rg1 showed a certain synergistic immune-enhancing effect for the hepatitis B virus, and the higher IgG2a levels suggested that Rg1 enhanced cellular immunity for HBsAg to a certain extent (Figure 2).

Figure 2. Serum anti-HBs and IgG1 and IgG2a antibody levels of mice immunized with HBsAg combined with different adjuvants. One dose of 0.5 μg HBsAg per mouse was intramuscularly injected, and serum was collected 3 weeks after immunization. Quantitative detection of anti-HBs (mIU/ml) and IgG1 and IgG2a (ng/ml) antibodies was performed using commercially available ELISA kits. Ninety-six well plates were coated with 1 μg/ml HBsAg overnight, and the plate was blocked with 2% bovine serum albumin (BSA) for 1 h then washed 5 times with 1‰ Tween 20-PBS. The initial series of diluted mouse serum was added and incubated at 37℃ for 1 h. After washing, HRP-labelled sheep anti-mouse IgG1 or IgG2a antibodies (1:10,000 dilution) were added, and incubated at 37℃ for 1 h. A chromogenic solution was added and incubated at 37℃ for 10 min and the termination solution was added. The absorbance value at dual wavelengths of 450/630 nm was read. Data are presented as the means ± SEM and are representative of one of two independent experiments with similar results. Note: * indicates P≤0.05, ** indicates P≤0.01 and *** indicates P≤0.001.

Cell-mediated immune response to HBsAg

To assess the ability of NE+Rg1 to elicit a cellular immune response when administered intramuscularly with HBsAg, NE+Rg1-induced IL-2 and IFN-γ (as markers of the Th1 response) secreting cells in the spleen were detected using ELISpot. As shown in Figure 3a, mice vaccinated with S+NE+Rg1 produced higher counts of specific IFN-γ- and IL-2-expressing cells than NE-vaccinated mice (P=0.0286), and the SFCs of the NE+Rg1 group were significantly higher than aluminium hydroxide (P=0.0286). The results of ICS were similar to ELISpot. IFN-γ and IL-2 expression in $CD8^+$ T cells of the NE+Rg1 group showed a slightly higher enhancement than the NE group, but the difference was not statistically significant. However, the effect was stronger than the aluminium hydroxide adjuvant (IFN-γ, P=0.0286; IL-2, P=0.0265, Figure 3b).

Figure 3. Specific T-cell responses measured using ELISpot and ICS in mice immunized with HBsAg combined with different adjuvants. Seven days after the immunization boost, mice were sacrificed. Splenocytes were isolated from the spleen and stimulated with a specific S peptide (S28-39, IPQSLDSWWTSL, H-2d restricted). Briefly, for ICS, 250 μl of flow fluid (2% FBS PBS) was added to each well of the 96-well cell plate, and centrifugation was performed at 200 g for 5 min. Then, 50 μl of FITC-CD8 (BD, USA) and PerCP-CD3 (BD, USA) dyes were added to each well for surface dyeing. After

centrifugation at 200 g for 5 min, 250 μl of flow fluid was added to each well, and 100 μl Fixation/Permeabilization solution (BD, USA) was added each well. The reaction was performed at 4℃ for 20 min in the dark. Then, 50 μl of PE-IFN-γ and APC-IL-2 dyes (BD, USA) diluted in Perm/Wash buffer (BD, USA) were added to each well. After washing, 200 μl of flow solution was added to each well, and the results were detected using flow cytometry (BD FACS Canto™ II, USA). Data are presented as the means ± SEM and are representative of one of two independent experiments with similar results.

HAI titre and protection against influenza infection

The HAI results showed that the HA+NE serum inhibitory titre was significantly higher than the single HA (approximately 8 times) group after 1 dose of immunization, and there was no significant difference with NE+Rg1. After immunization with 2 doses, the HA+NE and NE+Rg1 groups had similar HAI titres, which were approximately 16 times higher than the single HA group, but these differences were not statistically significant. However, the HAI titre of the NE+Rg1 group was significantly higher than the HA+Rg1 group (P 0.001). After the 10 LD_{50} challenge, the mice were monitored for toxicity signs and body weight changes continuously for 14 days. As shown in Figure 5, the survival rates of the HA, HA+NE, HA+NE+Rg1, HA+Rg1 and NS groups were 0%, 100%, 100%, 30% and 0%, respectively on day 14. The weight change curve revealed that weights in the single HA and HA+Rg1 groups decreased to a very low point around the 7th day then gradually increased, but weights in the NE+Rg1 and NE groups gradually recovered after a slight weight loss. The NE+Rg1 group did not show a significant advantage over the NE group, but the former group showed more drastic fluctuations in body weight. These results showed that NE and Rg1 had no synergistic immune enhancement or protective effect against the influenza HA antigen, which is not consistent with the observed results of the NE+Rg1 in combination with HBsAg. The results indicate that the combination of the same adjuvant with different antigens induces different or opposite results.

Figure 4. The serum HAI titre and challenge and the survival rate post-challenge. The mice were intramuscularly injected at weeks 0 and 4, and serum was collected at weeks 0, 4 and 6. The immune dose of HA was 1.5 μg/dose, and Rg1 was 50 μg/dose(a). Mice were challenged with 10 LD_{50} of the A/Puerto Rico/8/34 influenza strain after administration of 400 μl of 0.4% sodium pentobarbital on Day 14 after the immunization boost. The mortality and body weight of the challenged mice were monitored for the subsequent 14 days. Mice that lost greater than 35% body weight were humanely euthanized (b).

Safety and preliminary mechanism of action

The results of haemolysis and coagulation experiments showed that NE and Rg1+NE had no haemolytic effect on 2% rabbit red blood cells, which preliminarily supports the safety of NE and Rg1+NE for haemolysis.

To detect the mechanism of action of the adjuvant effect of ginsenoside Rg1+NE, we hypothesized that the structure or functional group of Rg1 was the same or similar to the structural or chemical groups that act on TLR4. Many vaccine adjuvants induce the production of interferon and other inflammatory cytokines via the activation of TLRs.[14] Therefore, we evaluated the activation of TLR4 and TLR7 to

determine whether ginsenoside Rg1 and NE had an adjuvant effect via activation of TLR4 and TLR7 receptors. Because NE is milky white in appearance, it may interfere with the absorption value to some extent. Therefore, NE and NE+Rg1 were diluted into different multiples. As shown in Figure 5, the absorbance results showed that the A_{630} values of NE (500×, 5000×, 50000×), NE+Rg1 (50× and 1 μg/ml) and Rg1 (1 μg/ml, 100 ng/ml, 10 ng/ml) were not different than NS. However, the absorbance value induced by 100 ng/ml LPS was close to 1.0 (hTLR-4), and the absorbance induced by 100 ng/ml R848 was over 2.0 (hTLR7). NE and Rg1 did not activate HEK293 hTLR-4 or hTLR-7 cells to secrete the SEAP enzyme, and the substrate did not turn blue. These results indicate that NE and Rg1 are not ligands of TLR4 or TLR7.

Figure 5. Effects on the surface receptors TLR4 and TLR7. HEK293 hTLR-4 and hTLR-7 cells were added to 96-well cell culture plates at a concentration of 4.0×10^4/well and 180 μl/well. The samples were added as 20 μl/well, cultured at 37℃ with 5% CO_2 for 20 h, and the absorbance at a 630-nm wavelength was detected. The final concentrations were 100 ng/ml R848, 100 ng/ml LPS, and 1 μg/ml, 100 ng/ml or 10 ng/ml for Rg1-1, Rg1-2 and Rg1-3, respectively, and 500-, 5000- and 50000-times dilutions of NE-1, NE-2 and NE-3, respectively. In NE+Rg1, NE was diluted 50 times, and the concentration of Rg1 was 1 μg/ml.

Physiochemical characteristics and stability

The results of particle size, PdI and Zeta potential at 37℃ at different times (3, 8 and 12 weeks) showed that NE+Rg1 displayed relatively stable physicochemical characteristics (Figure 6). These results suggest that the combined adjuvant may be stored stably at 4℃ for a long time.

Discussion

The MF59 emulsion has been used in human influenza vaccines for 2 decades, and extensive data show that it has a stronger protective effect than adjuvant-free influenza vaccines and it increases the breadth and duration of the immune response in people of different ages [15,16]. However, serum protection against drifting viruses was as low as 20% in older subjects, which fails to meet the serum protection criteria set by the Committee for Medicinal Products for Human Use (CHMP) [17]. Compared to adjuvant-free vaccines, MF59 enhanced immunogenicity against seasonal and pandemic influenza strains, and the MF59 adjuvant vaccine induced a longer cross-reactive immune response and increased the duration of the immune response compared to the non-adjuvanted vaccine[18]. Nassim et al.[19] reported that a single dose of 3.75 μg MF59 adjuvant adult influenza vaccine provided effective protection for up to 10 months. These responses are related to the mechanisms of action of MF59, which includes humoural and cell-mediated multiple immune responses. Therefore, MF59 adjuvant influenza vaccine provided a higher and broader antibody response to antigen-drift influenza virus, which makes it a strong candidate for seasonal influenza vaccination programmes in vulnerable populations, such as infants and the elderly[20], and, it reduced hospitalization rates for influenza-related serious complications in the elderly[21]. From a cost-benefit perspective, influenza vaccines with adjuvants effectively saved antigens and achieved a higher cost-benefit ratio and should be considered a preferential choice for older adults

aged 65 years or older[22]. Ginsenoside had strong efficacy and a good safety in antitumour and other aspects[23].

The present study showed that ginseng saponin Rg1 had no haemolytic effect and exhibited good safety when combined with NE. NE+Rg1 induced stronger humoural immunity and cellular immune response than aluminium hydroxide when combined with the HBsAg antigen, and it induced higher IgG2a, IFN-γ and IL-2 levels than single NE, which means that it may induce a more balanced Th1/Th2 response that compensates for the weak Th1-type response induced by NE alone. However, NE and Rg1 had no obvious synergy when used in combination with influenza HA antigen, in terms of HAI titre and the protection of challenge, and the mechanism of action is unknown. This is similar to our previous findings that there was no synergistic effect of chitosan combined with c-di-GMP on HBsAg in mice by intranasal immunization (data not shown). Therefore, it is suggested that specific adjuvants with specific antigens initiate and enhance specific arms of the immune responses to specific antigens.

The successful application of $AS01_B$ in the shingles vaccine suggests that combined adjuvants are the future trend in vaccine development. However, each adjuvant combination or formulation has a fixed effect on a particular pathogen. For example, VZV gE combined with the $AS01_B$ adjuvant system yielded the strongest gE-specific $CD4^+$ T cell responses and strong gE-specific antibody responses[24]. $AS01_B$ stimulates the release of IFN-γ from $CD4^+$ T cells[25], and it provided unprecedented protection against herpes zoster in older adults regardless of age at vaccination and was efficacious in immune-suppressed populations, which is likely explained by the synergistic action of the components of the adjuvant system [24]. Although the results obtained in this experiment are limited to only one animal strain, it highlights that we should choose different adjuvants or adjuvant combinations, including different formulations or proportions, to induce the most suitable arm of the immune response and immune level for specific pathogens.

Conclusions

In summary, our data demonstrated that NE and NE+ginsenoside Rg1 produced a range of immunological adjuvant effects in HBsAg- or HA-immunized mice, giving rise to very distinct immunological signatures for different pathogen antigens. A synergistic effect was found in combination with the HB antigen, but no synergistic effect was found with the influenza virus haemagglutinin antigen. Adjuvant activity is a result of multiple factors, and the enhanced immune response obtained with one antigen cannot as a rule be extrapolated to another antigen[26]. Therefore, it is suggested that researchers should consider the specific pathogen antigen and the specific immune response required when selecting and evaluating adjuvant combinations.

List Of Abbreviations

HBsAg: Hepatitis B surface Antigen

HA: Haemagglutinin

HAI: HAemagglutination Inhibition

SPF: Specific Ppathogen Free

ANOVA: One-way Analysis of Variance

SFCs: Spot Forming Cells

NE: Nano Emulsion

ELISA: Enzyme-Linked ImmunoSorbent Assay

AECNVSI: Animal Ethics Committee of the National Vaccines and Serum Institute

ELISpot: Enzyme-Linked ImmunoSpot Assay

ICS: Intracellular Cytokine Staining

Declarations

Ethics approval and consent to participate

The Animal Ethics Committee of the National Vaccines and Serum Institute (AECNVSI) approved all the animal experiments and all procedures.

Consent to publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed in the current study are all presented within the manuscript, and are also available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

JX, PS and ZW conceived the experiments; PS, ZW, JL, DW, ZZ, SH, YH, YW and SL carried out the experiments. PS and ZW performed the writing, statistical analysis and formal analysis. JX reviewed the manuscript. All authors read and approved the final manuscript.

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Figures

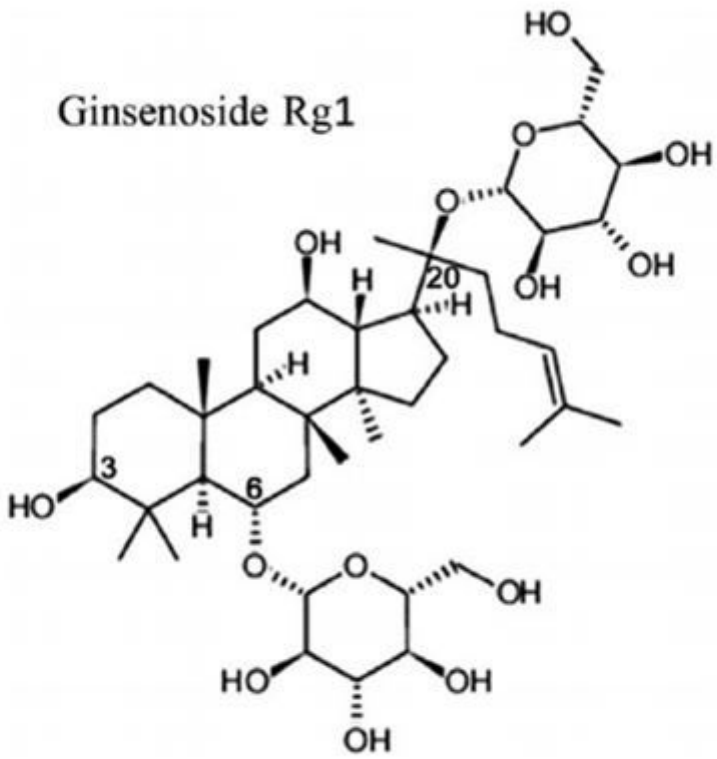

Figure 1

Chemical structure of ginsenoside Rg1

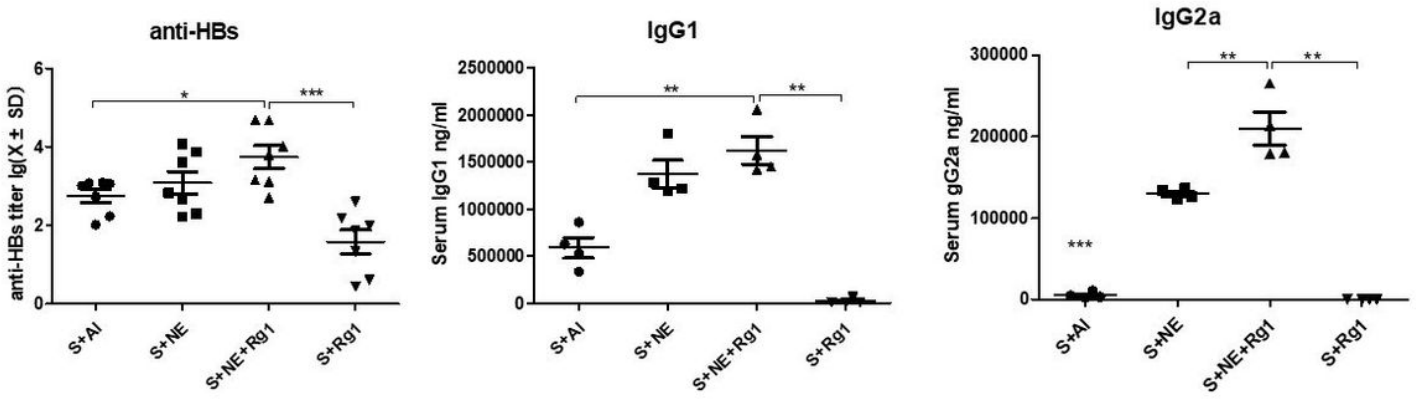

Figure 2

Serum anti-HBs and IgG1 and IgG2a antibody levels of mice immunized with HBsAg combined with different adjuvants

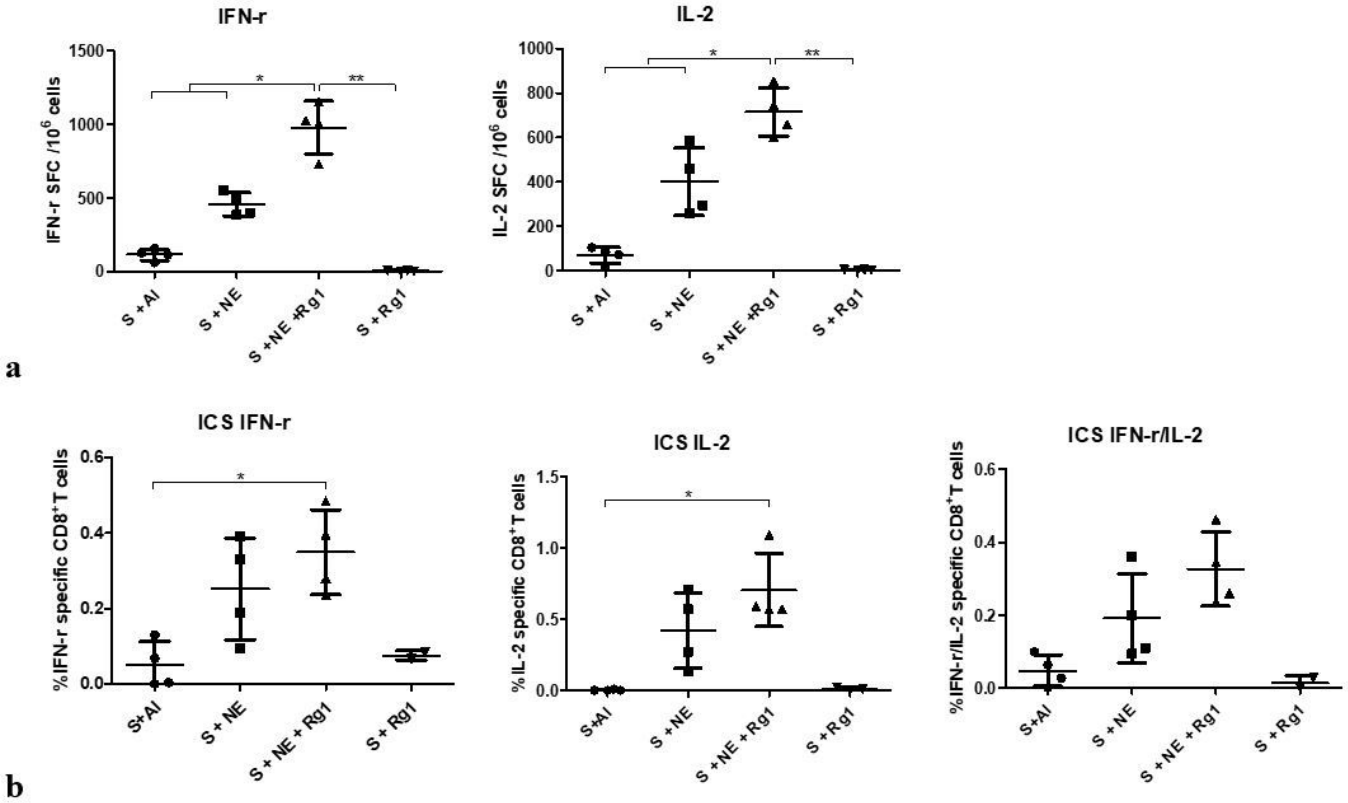

Figure 3

Specific T-cell responses measured using ELISpot and ICS of mice immunized with HBsAg combined with different adjuvants

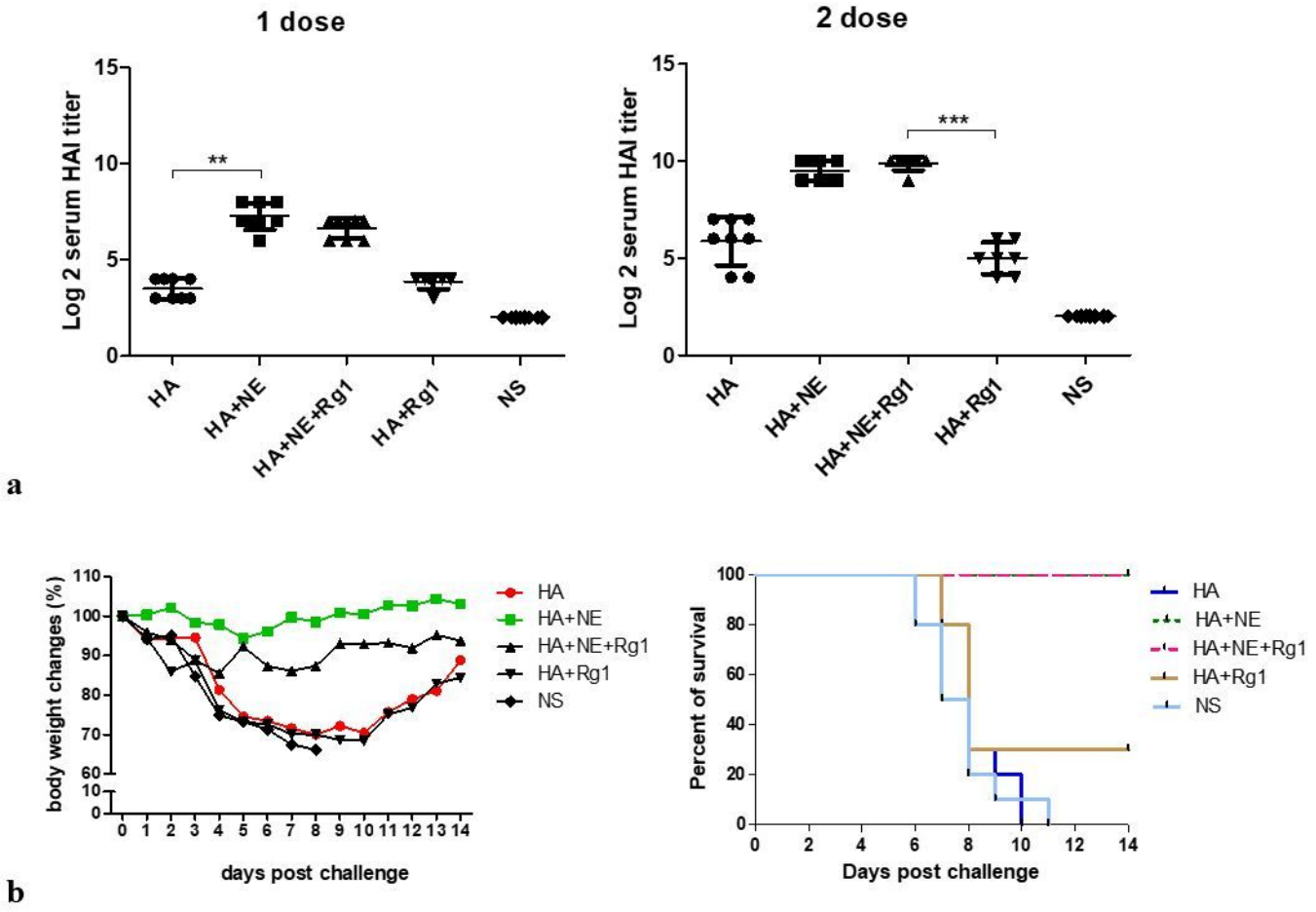

Figure 4

The serum HAI titre and challenge and survival rate post-challenge

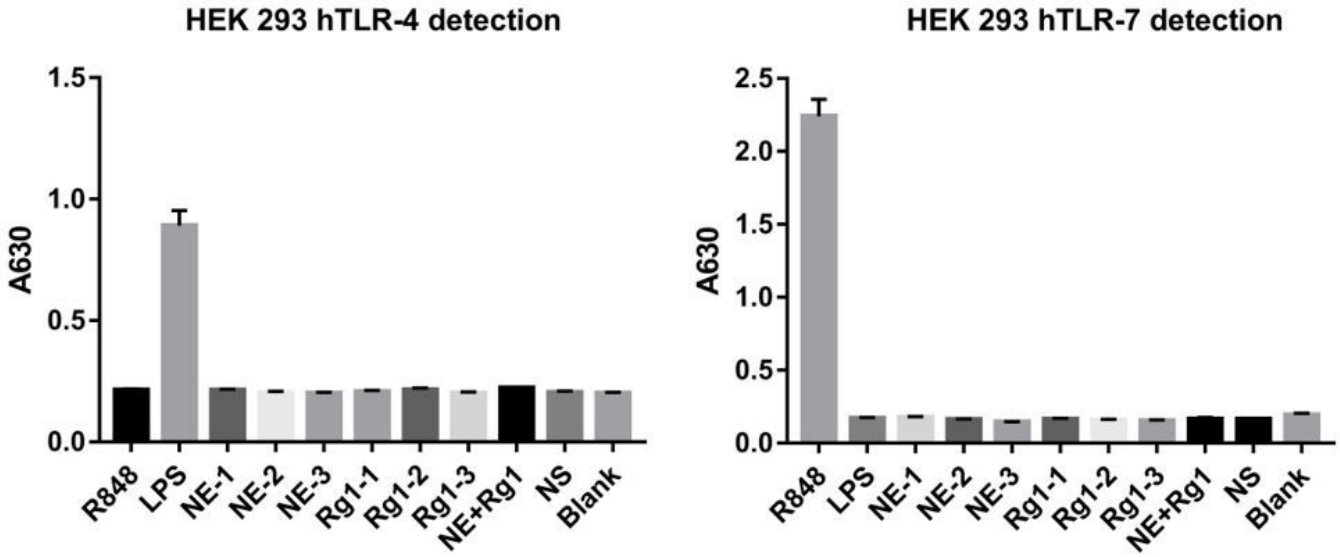

Figure 5

Effects on the surface receptors TLR4 and TLR7

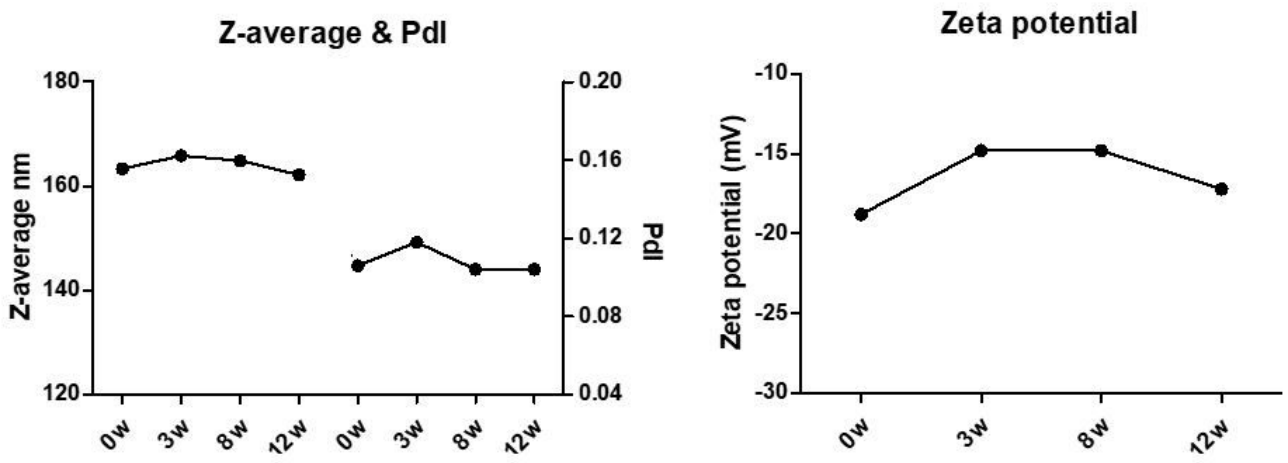

Figure 6

Particle size, PdI and Zeta potential of NE+Rg1 at different time points at 37°C

Supplementary Files

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- ARRIVEchecklist.docx