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A novel adjuvant G3 induces both Th1 and Th2 related immune responses in mice after immunization with a trivalent inactivated split-virion influenza vaccine

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ABSTRACT

A preferred adjuvant should promote both Th1 and Th2 responses. However, most adjuvants in common use are biased towards a Th2-driven response. Therefore, the ability of a novel saponin-based adjuvant G3 to inducing balanced Th1 and Th2 responses in BALB/c mice immunized with a split trivalent seasonal influenza vaccine was evaluated in comparison to that of the adjuvant $Al(OH)_3$. Clear differences in the IgG profiles induced by G3, $Al(OH)_3$ or non-adjuvanted vaccine were recorded. Both adjuvants enhanced high and similar levels of the Th2 associated IgG1 subtype compared to mice given vaccine alone. Only G3 enhanced the IgG2a subclass reflecting a Th1 response, whereas $Al(OH)_3$ even abrogated the IgG2a production. Accordingly, G3 enhanced the production of IL-2 and IFN-γ and also of IL-2/IFN-γ double secreting cells, emphasizing the strong Th1 driving effect of G3. Only $Al(OH)_3$ increased splenocyte production of IL-17. Taken together, the results indicate a strong propensity for G3 to induce both Th1 and Th2 driven immune responses.

1. Introduction

Non-replicative vaccines are in general poorly immunogenic therefore demanding immune enhancement i.e., adjuvants. The most used adjuvants are aluminium compounds, being the market leader for both animal and human vaccines, with documented effects and safety profiles [1]. However, aluminium compounds have limitations, mainly providing antibody-mediated immunity in accordance with a T helper cell type 2 (Th2) profile [1,2]. Early experiments showed that the inability of aluminium salts to induce cytotoxic T lymphocytes (CTL) was overcome by incorporation of a *Quillaja saponaria* Molina component i.e., QS-21 [3]. Likewise, purified viral envelope proteins incorporated into a matrix of the *Quillaja* component A (Quil A), cholesterol and phospholipids, i.e. ISCOMs, induced $CD8^+$ antigen-specific CTLs [4,5]. The relationship between adjuvant activity, composition and side effects of *Quillaja* saponins has been extensively evaluated focusing on QS-21 and ISCOMs [6–10]. In parallel, many other adjuvant formulations and delivery systems are being designed to improve the immune response to a variety of antigen constructs [11,12].

G3, a new generation of adjuvant consisting of two components, i.e., Quil A and cholesterol, is formulated with a new artificial membrane technique (Patent No. WO 2013/051994). The around 20 nm sized G3 particles are formed via an initial hydrogen bond between the hydroxyl group of cholesterol and the carboxyl group in glucuronic acid in the Quil A micelle and a subsequent hydrophobic interaction between sterol and the triterpene of Quil A. G3 combined with proteins from a split influenza vaccine induced high levels of antibodies and the further addition of a third component, a diterpene, induced $CD8^+CD3^+$ T-cells and protection against challenge with a H1N1 virus lacking compatible HI and VN antigenic determinants [13]. That study also clearly demonstrated the versatility of the G3 technology by the incorporation of an amphipathic diterpene molecule into the G3 nanoparticle.

For full use of the versatility of G3 we need a better understanding of its immune modulating capacity, including B and T-cell responses. Here we aimed at exploring the immune profile induced by a split influenza vaccine as antigen when adjuvanted with G3 in comparison to the adjuvant aluminium hydroxide. The B and T cell responses were examined by serology and by FluoroSpot analysis on single or dual cytokine secreting splenocytes obtained from mice one week after the booster immunizations.

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2. Material and Methods

2.1. Antigen and adjuvants

A split trivalent (A/California/7/2009 (H1N1) pdm09-derived strain; A/Texas/50/2012 (H3N2)-derived strain; B/Massachusetts/2/2012) influenza (Flu) vaccine (Vaxigrip 2014/2015; Sanofi Pasteur MSD, Diegem, Belgium) was used for immunization. One μg Flu antigen was either mixed with a saponin adjuvant "G3" consisting of cholesterol and Quil A (Brenntag Biosector A/S, Fredrikssund, Denmark) formulated in 20 nm particles (MoreinX AB, Uppsala, Sweden) or with an Aluminium hydroxide gel (Alhydrogel® adjuvant 2%; Invivogen, San Diego, CA, USA), according to the manufacturer's instructions.

2.2. Mice and immunizations

Female BALB/c mice were housed at Karolinska Institutet, Stockholm, Sweden and handled in accordance with the guidelines of the Swedish Ethical Committee for Animal Protection. The mice were divided into four groups (n = 6) and immunized subcutaneously with 200 μl volumes of; Flu in 3 μg G3 (Flu-G3); Flu in 100 μl Alhydrogel® (Flu-Al$(OH)_3$); Flu in PBS (Flu) or only PBS. The mice were immunized twice, 5 weeks apart. Sera were collected before first immunization and after 2, 5 and 6 weeks. Spleens were collected 7 days after the last immunization.

2.3. ELISA

Flat bottom Maxisorp plates (Nunc) were coated over night at 4 °C with 0.12 μg antigen (Vaxigrip 2015–2016) in 100 μl 50 mM Tris-Cl (pH 9.5) per well. After blocking (1% milk powder in PBS) for 1 h at RT, sera were added at 1:100, 1:1 000, 1:10 000 and 1:100 000 dilutions and incubated for two hours at RT. Thereafter, HRP-conjugated anti-mouse IgG1 or IgG2a (Zymed, 1:1000), or total Ig (Dako, 1:1000) was added and incubated overnight at 4 °C before developed with 100 μl/well of TMB substrate (Sigma-Aldrich, St. Louis, MO, USA). The reactions were stopped after 10 min with 100 μl 1 M HCl and A_{450} determined in an ELISA reader (Multiscan Ex, Labsystems). Wells were washed (3x) with PBS containing 0.05% Tween 20 between each step.

2.4. Hemagglutination-inhibition assay (HI)

The HI assay followed established protocols, using round bottomed microtiter plates (NunClon, Sigma-Aldrich) and 0.5% chicken erythrocytes (Håtunalab AB, Bro, Sweden) but using split virus vaccine, instead of intact virus particles. Four HA units were used to determine the HI titre.

2.5. Preparation of mouse splenocytes

Spleens were minced and passed through a cell strainer (BD/Falcon, Becton Drive Franklin Lakes, NJ, USA). The splenocytes were washed twice in cell culture medium (RPMI 1640, 10% heat-inactivated fetal calf serum (FCS), 1 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.5 mM HEPES; Invitrogen Life Technologies, Carlsbad, CA, USA), counted, and checked for viability using a Guava ViaCount Assay (Guava Technologies, Hayward, CA, USA) before dilution to appropriate cell concentrations.

2.6. ELISpot and FluoroSpot

Secretion of IL-4 was analyzed in ELISpot while simultaneous secretion of IFN-γ/IL-2, IFN-γ/IL-5 and IFN-γ/IL-17 was analyzed using the FluoroSpot assay. The tests were set up in duplicates under sterile conditions. Plate washing volumes were 200 μl and all other steps 100 μl if nothing else is stated. Low fluorescent 96-well PVDF membrane plates (Cat. No. S5EJ104I07; Millipore, Bedford, MA, USA) were pre-wetted with 15 μl/well of 35% ethanol in water for no more than one minute, and washed five times with sterile water. The plates were coated with monoclonal antibodies, mAbs (15 μg/ml of each mAb in sterile PBS) for the following cytokines and cytokine combinations; IL-4 (mAb 11B11), IFN-γ/IL-2 (mAbs AN18/1A12), IFN-γ/IL-5 (mAbs AN18/TRFK5), IFN-γ/IL-17 (mAbs AN18/IL17-I) (Mabtech, Nacka Strand, Sweden) and incubated at 4 °C overnight. Thereafter, the plates were washed five times with sterile PBS followed by 1 h blocking in cell culture medium (200 μl/well). The blocking medium was removed and fresh medium with or without stimuli was added (50 μl/well) followed by addition of fresh splenocytes (100 μl/well; 250 000 cells/well for antigen-specific analysis (Flu 1 μg/ml) and medium control and 100 000 cells/well for polyclonal (ConA 5 μg/ml) activated cells). After incubation at 37 °C and 5% CO_2 for 40 h, cells were removed by washing the plates five times with PBS in an automated ELISA washer (Bio-Tek Instruments Inc., Winooski, VT, USA). Detection antibodies diluted in PBS with 0.1% bovine serum albumin (PBS/BSA); biotinylated anti-IL-4 (1 μg/ml; mAbs BVD6-24G2), BAM-tagged anti-IFN-γ (mAb R4-6A2 diluted 1:200), biotinylated antibodies to IL-2 (mAb 5H4), IL-5 (mAb TRFK4), IL-17 (mAb MT2270) all diluted to 2 μg/ml (Mabtech) were added to the plates and incubated for 90 min at RT. The plates were washed as described above and enzyme- or fluorophore-conjugated reagents; Streptavidin-ALP diluted 1:1000, anti-BAM-640 and Streptavidin-550 (SA-550) both diluted 1:200 in PBS/BSA were added. After incubation at RT for 1 h plates were washed as above and 100 μl of BCIP/NBT substrate (ELISpot) or 50 μl fluorescence enhancer (FluoroSpot) was added to each well. Substrate was developed for approximately 15 min at RT after which the plates were washed extensively in tap water and left to dry. The enhancer was incubated for 15 min before discarded and after removal of the plate underdrain the plates were left to dry protected from light. Plates were read and analyzed in an ELISpot/FluoroSpot reader system (iSpot Spectrum, AID, Strassberg, Germany). To obtain the number of cytokine-secreting cells specifically activated by antigen, the number of spots in control wells with only cells in medium were subtracted.

2.7. Statistical analysis

Statistical differences between experimental groups were determined using the Mann-Whitney test (Prism.5.0 Graph Pad Software).

3. Results

3.1. G3 and Al$(OH)_3$ adjuvants enhance different serum IgG subclasses

Flu-specific antibodies were detected in all three groups of immunized mice. The Flu-Al$(OH)_3$ group displayed slightly less total Ig than the Flu-G3 group but more than the group receiving Flu without adjuvant (Fig. 1A). The groups of mice given Flu adjuvanted with G3 or Al$(OH)_3$ responded with similar levels of IgG1 but showed high variation within each group (Fig. 1B). For most of these mice the IgG1 response, as well as the HI titers (data not shown) were higher than those for mice receiving non-adjuvanted antigen. Mouse No. 6 in the Flu-G3 group and mice Nos. 5 and 6 in the Flu-Al$(OH)_3$ group were outliers in their respective group displaying lower IgG1 titers than the other mice. This pattern was also evident in their total Ig response. G3 enhanced

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