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Adjuvanticity of a Synthetic Phosphatidylinositol Dimannoside to a Subvirion Influenza Vaccine in an Influenza Mouse Model

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Abstract

Dose-sparing is one of the key strategies to rapidly develop and supply vaccines to combat emerging strains of influenza virus. The objective of this study was to evaluate the adjuvanticity of a synthetic phosphatidylinositol dimannoside (PIM_2) and/or aluminium hydroxide to a subvirion influenza vaccine (APR8). Mice were immunised with a suboptimal dose vaccine alone or formulated with PIM_2, aluminium hydroxide or the two combined. Immunised mice were challenged with a lethal dose APR8 virus. PIM_2 significantly enhanced virus-specific T cell cytotoxicity and immunised mice had significantly reduced pulmonary virus load. Aluminium hydroxide adjuvant significantly boosted virus-specific humoral and cellular responses and conferred improved protection in mice against a lethal challenge. The magnitude of enhanced immune responses and protection by the addition of aluminium hydroxide to the vaccine was over 10-fold more. Aluminium hydroxide conferred stronger adjuvanticity to the vaccine compared to PIM_2. The adjuvanticity of aluminium hydroxide was further augmented in combination with PIM_2 boosting virus-specific T cell cytotoxicity and resulting in a significantly lower lung virus load compared to that for vaccine containing a 10-fold higher antigen dose. The addition of adjuvants to an influenza vaccine could substantially lower the antigen doses required and more doses of efficacious influenza vaccine would be produced with limited antigen supply, such as in the early phase of an influenza pandemic.

Keywords: Adjuvants; Phosphatidylinositol dimannosides; Aluminium hydroxide adjuvant; Influenza virus; Vaccination; Mouse model

Abbreviations

IFV: Influenza Virus; PIM_2: Phosphatidylinositol Dimannoside

Introduction

Mycobacteria are a key component in Freund's complete adjuvant, which is believed to be one of the most effective adjuvants in stimulating both humoral and cellular immune responses [1]. Various constituents of mycobacteria including cell wall components have been shown to induce a wide range of immune responses [2,3]. Phosphatidylinositol mannosides (PIMs) are a cell wall lipid anchor motif of larger mycobacteria cell wall constituents, lipoglycans, including lipoarabinomannans (LAMs) and lipomannans (LMs) [4]. PIMs have been shown to exert an immune modulatory effect on macrophages similar to that of LAMs and LMs [2]. Natural PIMs usually occur with two (PIM_2) or six mannose (PIM_6) residues attached to their inositol component [2]. We have shown that a synthetic phosphatidylinositol dimannoside (PIM_2) compound has immune modulatory activities [5] and the synthetic PIM_2 and its analogues enhanced efficacy of a mycobacterial protein-based vaccine and promoted a Th1 type immune response in a tuberculosis mouse model [6].

Aluminium containing adjuvants have been the most widely used adjuvants since 1926 [7]. The low-cost aluminium-containing adjuvants have a good track record of safety, and have shown adjuvanticity with a variety of antigens. However, the adjuvanticity of aluminium hydroxide for influenza antigens in humans remains uncertain [8-10]. In a mouse model, aluminium hydroxide was shown to significantly augment antibody responses to a whole-virus H5N1vaccine and enhance protection against lethal challenge with homologous and heterologous H5N1 viruses [11]. In an A/PR/8/34 (H1N1) mouse model, although aluminium hydroxide was shown to boost antibody response to a whole-virus vaccine, it was not associated with enhanced protection [12]. These results suggest the use of aluminium hydroxide adjuvant alone may not be sufficient and a combination of adjuvants could be beneficial.

The objective of the current study was to investigate the adjuvanticity of a synthetic PIM_2 and its combination with aluminium hydroxide to a subvirion influenza vaccine in a mouse model. A suboptimal vaccine dose was first determined allowing for the detection of protection-enhancing adjuvants to the vaccine. The adjuvanticity of PIM_2, aluminium hydroxide and their combination to the suboptimal vaccine was then investigated. Our results showed that PIM_2 enhanced antigen-specific T cell cytotoxicity, and aluminium hydroxide adjuvant augmented antigen-specific humoral and cellular immune responses conferred extra protection in mice against a lethal influenza challenge.

Materials and Methods

Animals, virus, subvirion vaccine and adjuvant

Female BALB/c mice (5-6 weeks old) were supplied by AgResearch Ruakura Small Animal Facility. All procedures involving the experimental use of animals were approved by the Grasslands Animal Ethics Committee, Palmerston North, New Zealand.

Influenza virus A/PR/8/34 (H1N1, APR8) was prepared as previously described [13]. To prepare the subvirion vaccine, influenza virus in the allantoic fluid was inactivated with a final concentration of 0.1% formaldehyde and incubated at 37°C for 60 min prior to purification. The inactivated influenza virus was then purified using step-wise discontinuous sucrose density gradients as described by Arora et al. [14]. The purified influenza virus in PBS was split by treatment with Triton X100 [15]. Triton X100 was subsequently removed using Pierce Detergent Removal Spin Columns (Thermo Scientific, Rockford, IL, USA) and filtered through 0.2 μm filter. The protein concentration of the subvirion influenza antigens was determined by Bio-Rad DC protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The concentration of the haemagglutinin proteins in the subvirion preparation was estimated based on density of the protein bands after SDS-PAGE separation under the reduced condition.

Aluminium hydroxide gel (1.3%; Abbreviated to Al in the paper), was purchased from Sigma (Sigma-Aldrich, Co., St Louis, MO, USA). Synthetic dipalmitoyl phosphatidylinositol dimannoside (PIM$_2$) were prepared by modification of previously described methods [5] that included a purification step involving a Phenomenex Fusion-RP column.

Dose-dependent protection trial against a challenge with 100 MLD$_{50}$

A vaccine dose-dependent protection trial was undertaken in mice against a challenge with 100 MLD$_{50}$ (mouse LD$_{50}$) APR8. Mice (n=10 per group) were immunised intramuscularly three times at weekly intervals with 50 μl of the subvirion IFV vaccine containing 5, 1, 0.2 or 0.02 μg of HA equivalent antigens in phosphate buffered saline (PBS). Control mice were sham injected with 50 μl PBS in a similar manner. Two weeks after the third vaccination, mice were challenged intranasally with 100 MLD$_{50}$ (200 TCID$_{50}$) APR8 in 20 μl PBS under anaesthesia [13]. Clinical manifestations and bodyweight were observed at least once daily for 2 weeks post challenge. Mice with more than 20% weight-loss were deemed terminal for the infection and were euthanised by CO$_2$ inhalation under anaesthesia (Ketamine/Xylazine, approximately 80 μg/4 μg per gram bodyweight).

Adjuvant trials in mice: Vaccine at doses of 0.02 and 0.2 μg provided partial protection against the lethal challenge from the dose-dependent trial (see results for details). The vaccine dose of 0.02 μg HA subvirion (1V) was selected to study the adjuvanticity of PIM$_2$, aluminium hydroxide or their combination. Mice were immunised with nonadjuvanted 1V vaccine (1V), or adjuvanted with aluminium hydroxide (1V-Al), or with PIM$_2$ (1V-PIM$_2$) or with the combination of aluminium hydroxide and PIM$_2$ (1V-Al-PIM$_2$). A nonadjuvanted 10-fold vaccine dose (0.2 μg HA, 10V) group and a PBS control group (PBS) were also included in the study (Table 1).

Mice were immunised three times at weekly intervals (Figure 1). Trial-1 and Trial-2 mice were challenged intranasally with 100 MLD$_{50}$ (200 TCID$_{50}$) APR8 in 20 μl PBS under anaesthesia 2 weeks after the third vaccination [13]. Trial-1 mice were observed and weighed daily for 2 weeks post-challenge. Trial-2 mice were euthanised at 3 days post infection (dpi) and lung lobes were collected. The weight of the right and left lung lobes were recorded. The right lung lobes were stored at -80°C for virus titration and pulmonary cytokine detection (see below), and the left lobe was preserved in 10% buffered formalin for histopathology (see below). Trial-3 mice were euthanised 2 weeks after the third vaccination and the spleens were collected to prepare splenocytes for the measurement of antigen-specific cellular responses (see below). A blood sample was also collected from the Trial-3 mice prior to the euthanasia. Serum samples were separated and stored at -20°C until assayed.

Group	APR8 subvirion vaccine	Adjuvant	
		Aluminium hydroxide	PIM$_2$
PBS	-	-	-
10V	0.2	-	-
1V	0.02	-	-
1V-Al	0.02	200	-
1V-PIM$_2$	0.02	-	35
1V-Al-PIM$_2$	0.02	200	35

Table 1: Vaccine composition for each vaccination group (μg/50 μl dose).
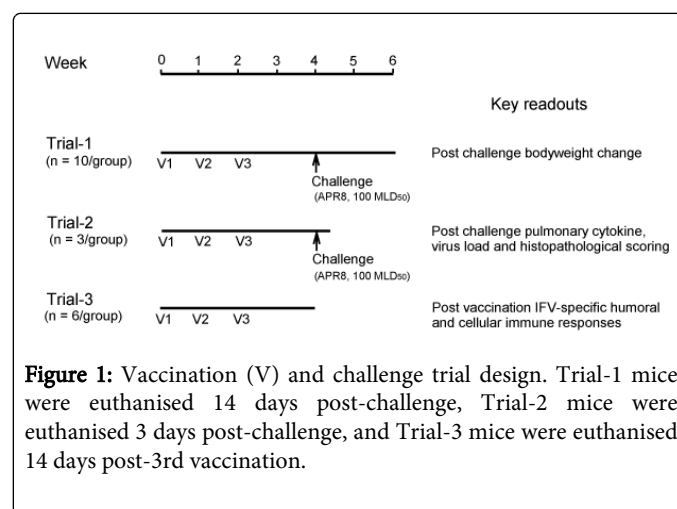

Figure 1: Vaccination (V) and challenge trial design. Trial-1 mice were euthanised 14 days post-challenge, Trial-2 mice were euthanised 3 days post-challenge, and Trial-3 mice were euthanised 14 days post-3rd vaccination.

Titration of influenza virus in lungs

The titre of influenza virus in the supernatants was determined in MDCK cells using chicken red blood cell haemagglutination endpoint method as described previously [13] and expressed as TCID$_{50}$/ml of influenza virus in 10% lung tissue preparations (equivalent to TCID$_{50}$/0.1 g of lung tissues).

Measurement of the pulmonary cytokine TNF and IFNs

The level of pulmonary cytokines (10% lung tissue extracts) was measured in lungs collected from Trial-2 mice on 3 dpi. The level of

IFN-α was assayed using Mouse IFN-alpha Platinum ELISA (eBioscience, Bender MedSystems Gmbh, Vienna, Austria) and TNF and IFN-γ was measured using BD Cytometric Bead Array Mouse Th1/Th2 Cytokine Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. The theoretical limit of detection of the cytokines was described in the instructions (IFN-α: 7.48 pg/ml, IFN-γ: 2.5 pg/ml and TNF: 6.3 pg/ml).

Histopathology

The formalin fixed left lung lobes were embedded in paraffin, sectioned at 4 μm and stained with haematoxylin and eosin. The lung sections were examined by a veterinary pathologist without knowledge of animal's treatment. Pathological changes of bronchi, bronchioles, interstitium and lung parenchyma were scored. The subjective grading of lesions was based on the severity of the change within affected areas and the extent of tissue affected by the change. Scores were 0 (none)=no tissue affected, 1 (mild)<25% affected, 2 (moderate)=25-50% affected, 3 (marked)=50-75% affected and 4 (severe)>75% affected.

Measurement of antigen-specific IgG1 and IgG2a by ELISA

The level of IFV-specific IgG1and IgG2a in sera collected from Trial-3 mice 2 weeks after the 3rd immunisation was detected by ELISA in a manner similar to that previously described [13]. Goat-anti-Mouse-IgG1-HRP (1:2500 dilution) or Goat-anti-Mouse-IgG2a-HRP (1:2500 dilution) (Immunology Consultants Laboratory, Inc., Portland, OR, USA) was used in the assay for antigen-specific IgG1 or IgG2 detection. Antibody titre was defined as the reciprocal of the highest serum dilution having an OD_{490} reading more than mean $\pm$ 3 standard deviations of OD_{490} readings of the negative control (1:100). Titres were expressed as log_{10}-transformed data. Samples were arbitrarily given a titre of 10 if their OD_{490} readings were lower than that of the negative control at 1: 100 dilution (mean $\pm$ 3 standard deviations of OD_{490} readings).

Antigen-specific cytotoxic T lymphocyte (CTL) killing assay

Antigen-specific cytotoxic activity was determined using a chromium release assay with non-infected or IFV-infected 3T3 cells as target cells. Cells were labelled with 100 μCi of $NaCrO_4$ (Perkin-Elmer, Melbourne, Australia) in medium (RPMI 1640/10% FCS) for 1 h at 37°C in atmosphere of 5% CO_2 in air. Labelled cells were washed three times with medium and added at a concentration of 5000 cells/well in round-bottom microtiter plates (Nunc, Roskilde, Denmark). Single-cell splenocyte suspensions were prepared by passaging spleen tissue homogenates through an 80-gauge wire mesh sieve. Splenic red blood cells were lysed using a solution of 17 mmol/L Tris-HCl and 140 mmol/L NH_4Cl. Such effector cells were added at various effector to target cell (E:T) ratios in a final volume of 200 μl/well. Plates were incubated for 4 h at 37°C. Maximum chromium release was induced by addition of 10% Triton-X, and spontaneous release was assessed by incubating target cells without effector cells. Then, supernatants were harvested, and the released ^{51}Cr was measured with a scintillation counter. Counts from triplicate wells were averaged, and the percentage of specific lysis was calculated as follows: % specific lysis=100 $\times$ (experimental release-spontaneous release)/(maximum release-spontaneous release). Spontaneous release was less than 15% of the maximum release in all assays.

Statistical analysis

Comparisons of bodyweight changes were performed using a repeated measurements model with fixed effects of treatment group, days post-infection and their interaction, and uniform correlation within each animal. P-values for pairwise comparison were produced with 'fdr' adjusted method [16]. The titres of influenza virus and levels of cytokines in lungs and pathological scores of lungs were analyzed using the nonparametric Kruskal-Wallis test. The levels of influenza-specific IgG1 and IgG2a in sera were analyzed using Fisher's pairwise comparison. The mice survival curves were compared using the Log-Rank method. Data were analyzed using statistical software R2.15.2 or Minitab 15.1.0.0. (LEAD Technologies Inc, State College, PA, USA). $P<0.05$ was considered statistically significant.

Results

Suboptimal protective vaccine dose

The vaccine dose to be used in the adjuvant study was first titrated in mice. A vaccine dose that provided partial protection against a lethal challenge would be the most appropriate dose to evaluate adjuvanticity, as it provides a margin of protection which could be improved upon. All of the PBS control mice (10/10) succumbed to the infection by 6 dpi, while mice (10/10) immunised with the subvirion vaccine dose of 5 or 1 μg HA were fully protected from the lethal challenge. In contrast, 6/10 or 2/10 mice immunised with the subvirion vaccine dose of 0.2 or 0.02 μg HA survived the challenge, respectively, providing 60% and 20% protection against the lethal challenge. The dose of 0.02 μg HA subvirion vaccine was selected for the subsequent adjuvant study.

Protection enhancement by adjuvant

Following the lethal influenza challenge, the PBS control mice had a significant loss in weight from 3 dpi and declined further thereafter, compared to that of the immunised groups ($P<0.05$) (Figure 2a). While there was no significant difference in weight-loss in mice between 1V and 1V-PIM$_2$ group, mice immunised with the aluminium hydroxide adjuvanted 1V vaccine (1V-Al) had significantly less weight-loss ($P<0.01$) than those immunised with nonadjuvanted 1V or 10V vaccines on 4, 5 and 6 dpi, or on 5 and 6 dpi, respectively (Figure 2a). Indicating enhanced protection was conferred from the addition of aluminium hydroxide to the vaccine. When compared to mice in 1V-Al group, mice in the 1V-Al-PIM$_2$ group appeared to have a trend of reduced weight-loss, albert not statistically significant (Figure 2a). In the PBS control group, one, eight and one mice were euthanised on welfare grounds on 4, 5 and 6 dpi, respectively, while 3/10, 1/10 or 2/10 mice in 1V, 10V or 1V-PIM$_2$ group were euthanised between 5 and 7 dpi, respectively (Figure 2b). All mice (10/10) immunised with 1V-Al or 1V-Al-PIM$_2$ survived the lethal challenge and had an improved survival rate although not statistical significantly, compared to those immunised with non-adjuvanted 1V vaccine ($P=0.067$, Log-Rank method, Figure 2b).

PIM$_2$ and aluminium hydroxide enhance virus clearance in lungs

The impact of immunisation with vaccines formulated with different adjuvants on the pulmonary virus load was assessed by measuring the titre of influenza virus in right lung lobes of the Trial-2 mice, collected at 3 dpi. The pulmonary influenza virus load of mice in

10V, 1V-Al, 1V-PIM$_2$ and 1V-Al-PIM$_2$ group were significantly lower than that in 1V group (P<0.05, Figure 3). The addition of aluminium hydroxide to the 1V vaccine resulted in a $10^{2.75}$ fold reduction in pulmonary influenza virus titre in 1V-Al group (median $10^{2.00}$ TCID$_{50}$/ml) compared to those in the nonadjuvanted 1V group (median $10^{4.75}$ TCID$_{50}$/ml). While there was no significant difference in the pulmonary influenza virus titre between 10V and 1V-Al mice, mice in the 1V-Al-PIM$_2$ group had significantly lower pulmonary influenza virus compared to 10V mice (P<0.05), suggesting there was a synergistic effect of PIM$_2$ and aluminium hydroxide to the vaccine. The median total lung weight was not significantly different among the different groups of mice (data not shown).
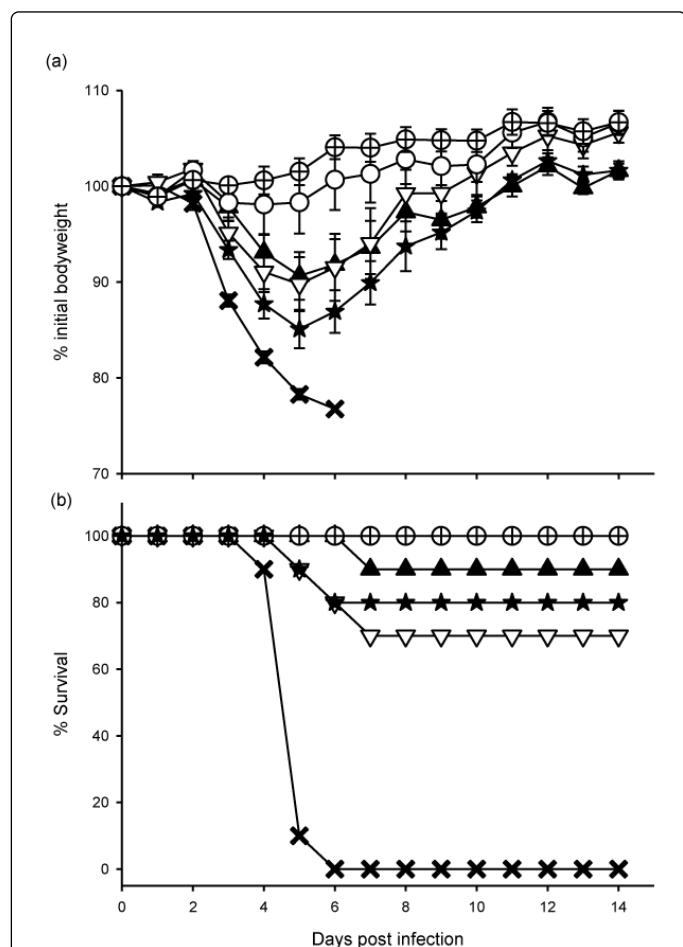

Figure 2: Clinical manifestations of the adjuvant Trial-1 mice following a lethal dose of influenza virus challenge. Mice (n=10 per group) were intramuscularly injected with a subvirion influenza vaccine (APR8), with or without adjuvant, three times at weekly intervals and were challenged intranasally with 100 MLD$_{50}$ APR8 two weeks after the third vaccination. Mice were observed daily post challenge for bodyweight (a, mean ± SEM), and survival (b). There were 6 groups of mice, PBS control (), 10V (), 1V (), 1V-Al (), 1V-PIM$_2$ (★) and 1V-Al-PIM$_2$ (⊕). Please refer to Table 1 for vaccine formulations.
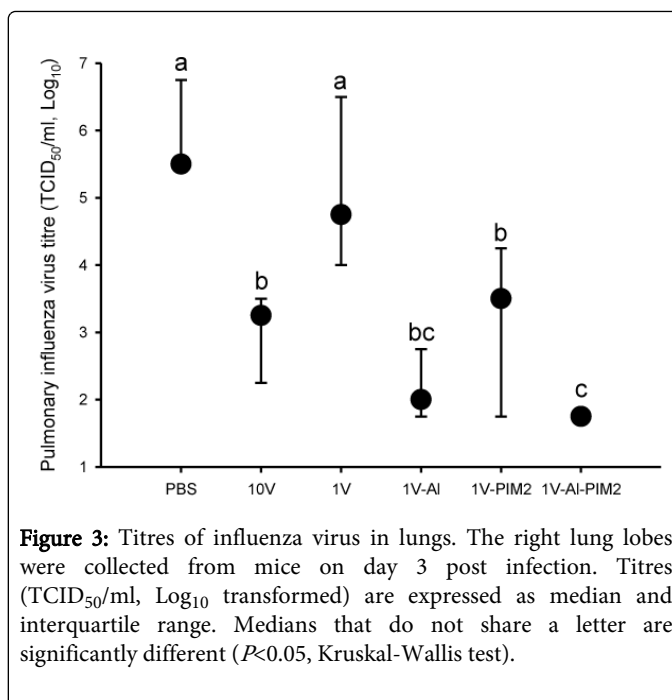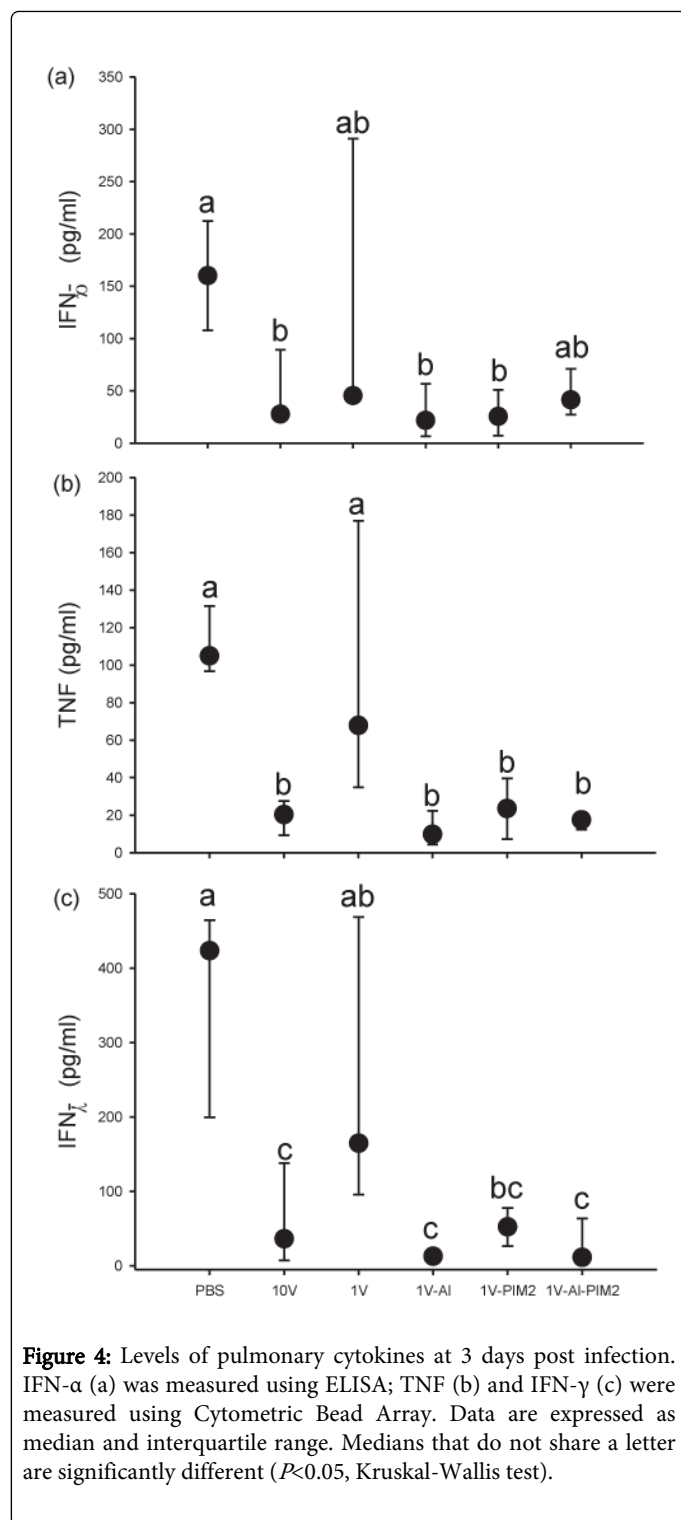

Figure 3: Titres of influenza virus in lungs. The right lung lobes were collected from mice on day 3 post infection. Titres (TCID$_{50}$/ml, Log$_{10}$ transformed) are expressed as median and interquartile range. Medians that do not share a letter are significantly different (P<0.05, Kruskal-Wallis test).

Pulmonary cytokines in mice at day 3 dpi

The impact of immunisation of vaccines formulated with different adjuvants on the level of pulmonary cytokines was evaluated by measuring the level of TNF, IFN-α and IFN-γ in right lung lobes of the Trial-2 mice, collected at 3 dpi. Mice immunised with 10V, 1V-Al and 1V-PIM$_2$ had significantly lower levels of pulmonary IFN-α compared to the PBS control mice (Figure 4a). Mice immunised with PIM$_2$ or aluminium hydroxide adjuvanted 1V (1V-PIM$_2$ or 1V-Al) had significantly lower level of pulmonary TNF than those immunised with nonadjuvanted 1V (P<0.05, Figure 4b). Mice in the PBS control group and 1V group had significantly higher levels of pulmonary IFN-γ than those in groups of 1V-Al, 1V-Al-PIM$_2$ and 10V (P<0.05, Figure 4c). The pulmonary levels of TNF (Spearman's rank correlation coefficient, ρ=0.850) and IFN-γ (ρ=0.849) were significantly positively correlated with the pulmonary influenza virus titres (P<0.001).

Histopathology

For histopathological examinations, the proportion of affected bronchioles was scored as 0-10 (0-100% proportion affected) and the degree of severity of exudate (0-4), infiltration of mononuclear cells (0-4), neutrophils (0-4) in the lumen and necrosis (0-4), hyperplasia (0-4) of bronchiolar epithelium were scored with a maximum of combined score of 30. Mice immunised with 10V or aluminium hydroxide adjuvanted 1V vaccines (1V-Al, 1V-Al-PIM$_2$) had significantly reduced proportion of affected bronchioles, bronchiolar exudation, neutrophil infiltration in bronchiolar epithelium and bronchiolar epithelial necrosis compared to those immunised with nonadjuvanted 1V or 1V-PIM$_2$ (P<0.05, Table 2). There was no significant difference in these parameters between 1V and 1V-PIM$_2$ groups. The maximum scores for pathological changes in bronchioles, interstitium (data not shown) and lung parenchyma (data not shown) were 30, 12 and 20, respectively.

Figure 4: Levels of pulmonary cytokines at 3 days post infection. IFN-α (a) was measured using ELISA; TNF (b) and IFN-γ (c) were measured using Cytometric Bead Array. Data are expressed as median and interquartile range. Medians that do not share a letter are significantly different ($P<0.05$, Kruskal-Wallis test).

The maximum total pathological score for each animal was 62. Mice immunised with 10V, 1V-Al or 1V-Al-PIM$_2$ had significantly lower total pathological scores compared to those immunised with 1V or 1V-PIM$_2$ ($P<0.05$, Table 2). In addition, there was a negative monotonic correlation between the total pulmonary pathological score and the bodyweight-loss of Trial-2 mice at 3 dpi (Spearman's rank correlation, $\rho=-0.556$, $P=0.017$).

Aluminium hydroxide adjuvant boosts antigen-specific IgG1 and IgG2a titres

The influence of PIM$_2$ and aluminium hydroxide on IFV-specific antibody responses to the influenza vaccine was measured in Trial-3 mice at 2 weeks post the third vaccination. While there was no significant difference in IFV-specific antibody titres between 1V and 1V-PIM$_2$ group, mice immunised with aluminium hydroxide adjuvanted 1V vaccines (1V-Al and 1V-Al-PIM$_2$) had significantly higher titre of IFV-specific IgG1 and IgG2a compared to those immunised with nonadjuvanted 1V ($P<0.05$, Figure 5). The addition of aluminium hydroxide to the 1V vaccine (1V-Al) augmented 20-fold or 5-fold more of IFV-specific IgG1 titre in mice compared to those immunised with the nonadjuvanted 1V or 10V vaccine. There was no significant difference in the ratio of IgG1/IgG2a antibody titre in mice among the immunised groups ($P>0.05$) (data not shown).

Group	Affected bronchiole (%)	Bronchiolar exudate	BE-neutrophil infiltration	BE-necrosis	Total pathological score
PBS	60.0[a], 55.5-62.5	3.0[a], 3.0-4.0	4.0[a], 3.0-4.0	2.0[a], 2.0-4.0	38.6[a], 31.6-38.6
10V	10.0[b], 3.6-38.9	1.0[b], 0.0-1.0	1.0[b], 1.0-2.0	1.0[bc], 1.0-1.0	11.0[b], 10.1-28.4
1V	50.0[a], 34.6-56.3	3.0[a], 2.0-3.0	3.0[ab], 2.0-3.0	2.0[ab], 1.0-4.0	27.5[a], 25.4-35.6
1V-Al	5.0[b], 0.00-23.1	0.0[b], 0.0-1.0	1.0[c], 0.0-3.0	0.0[cd], 0.0-1.0	18.2[b], 9.0-20.1
1V-PIM2	48.0[a], 7.1-50.0	3.0[a], 2.0-3.0	3.0[abc], 1.0-3.0	2.0[ab], 1.0-3.0	31.5[a], 20.1-32.5
1V-Al-PIM2	4.6[b], 4.6-10.7	1.0[b], 1.0-1.0	1.0[c], 1.0-1.0	0.0[d], 0.0-0.0	10.1[b], 6.1-20.1

Table 2: Bronchiole pathological scores (median, interquartile range) of the left lung collected from Trial-2 mice three days post influenza virus challenge (refer to Figure 1 for vaccination history). Medians that do not share a letter are significantly different ($P<0.05$, Kruskal-Wallis test). BE: Bronchiolar Epithelium.

Aluminium hydroxide and PIM2 boost antigen-specific cytotoxic T cell activity

IFV-specific Cytotoxic T Lymphocytes (CTL) are important effector cells in controlling virus infection. The impact of PIM2 and aluminium hydroxide on the splenic IFV-specific CTL killing capacity was measured in the Trial-3 mice using chromium release assay at 2 weeks after the third vaccination. The splenic IFV-specific T cell cytotoxicity was measured at effector:target ratios of 5:1, 10:1, 20:1 and 40:1, and 20:1 ratio appeared optimal (Figure 6). Influenza virus-specific T cell cytotoxicity appeared in a vaccine dose-dependent manner, as mice immunised with 10V had significantly higher antigen-specific CTL cytotoxicity compared to those immunised with 1V ($P<0.05$; Figure 6). The addition of PIM2 to 1V vaccine (1V-PIM2) significantly enhanced the IFV-specific splenic T cell cytotoxicity in the immunised mice compared to those immunised with nonadjuvanted 1V ($P<0.05$; Figure 6). Mice immunised with 1V-Al had significantly enhanced IFV-specific splenic T cell cytotoxicity compared to those immunised with 1V, 10V or 1V-PIM2 ($P<0.05$;

Figure 6). Moreover, the addition of PIM2 to the 1V-Al significantly further enhanced antigen-specific T cell cytotoxicity in immunised mice compared to that in the 1V-Al mice (P<0.05; Figure 6), suggesting an additive adjuvant effect of PIM2 and aluminium hydroxide in enhancing antigen-specific CTL killing activity.
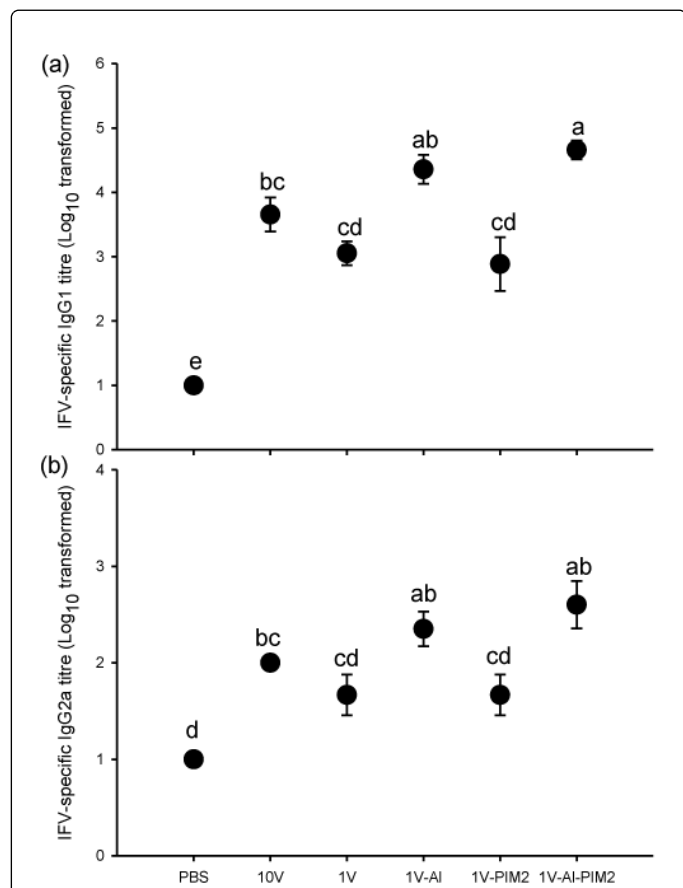

Figure 5: Antigen-specific antibody IgG1 (a) and IgG2a (b) titre in serum collected 2 weeks after the third vaccination of Trial-3 mice. Antibody titre were Log_{10} transformed and expressed as mean ± SEM (n=6). Means that do not share a letter are significantly different ($P<0.05$, Fisher's pairwise comparison).

Discussion

Synthetic PIM_2 has been shown to improve the efficacy of a mycobacterial protein-based vaccine and induced a Th1 type immune response in a tuberculosis mouse model, when PIM_2 was mixed with an oil/water adjuvant Emulsigen^{TM} [6]. In the current study, the adjuvanticity of PIM_2 to a split influenza vaccine was investigated in two formulations, PIM_2 was used alone (1V-PIM_2) or PIM_2 was mixed with aluminium hydroxide (1V-Al-PIM_2). Mice immunised with 1V-PIM_2 had significantly enhanced the virus-specific CTL activity and had significantly lower pulmonary virus load than those immunise with non-adjuvanted 1V. Natural PIMs are Toll-like receptor (TLR) 2 agonists, can activate nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and mitogen-activated protein kinase of murine macrophages stimulating TNF production in these cells [17,18]. The immune enhancement of PIM_2, when used alone, did not offer demonstrable

additional protection to the 1V influenza vaccine against a lethal challenge in the current study.

Several studies have shown that aluminium hydroxide adjuvant enhances immune responses to influenza vaccines in mice [12,19], but whether the improved immune responses would offer extra protection against viral challenge remained uncertain. A suboptimal dose of vaccine was used in the current adjuvant study, which provided the opportunity for adjuvants to enhance protection. By using this sensitive system, it was demonstrated that not only did aluminium hydroxide adjuvant significantly augment the antigen specific-antibody and CTL responses, it conferred significant extra protection in mice against a lethal challenge with reduced weight-loss, reduced pulmonary virus load and lung pathology compared to those immunised with nonadjuvanted 1V vaccine.
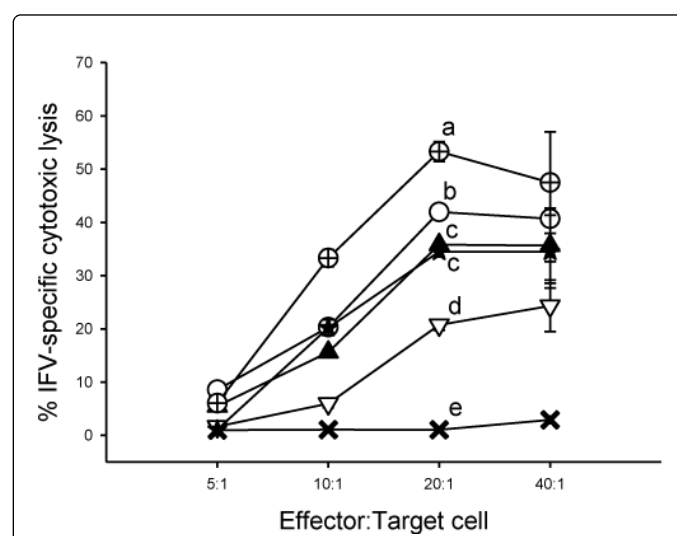

Figure 6: Antigen-specific splenic T cell cytotoxicity. Splenocytes were isolated from Trial-3 mice 2 weeks post the third vaccination. Splenic antigen-specific cytotoxic activity was determined using a chromium release assay with non-infected and IFV-infected 3T3 cells as target cells at four Effector:Target ratios. There were 6 groups of mice, PBS control (), 10V (), 1V (), 1V-Al (), 1V- PIM_2 (★) and 1V-Al- PIM_2 (⊕). Data are expressed as mean ± SEM (n=6). Means that do not share a letter are significantly different ($P<0.05$, Fisher's pairwise comparison).

Mice immunised with 1V-Al-PIM_2 had significantly augmented CTL activities compared to those vaccinated with 1V-Al, and the combination was the only vaccine to have significantly lower lung virus load compared to the vaccine containing 10-fold higher antigen dose. This suggested an additive effect of PIM_2 and aluminium hydroxide to the vaccine. While weight-loss was observed in 1V-Al mice at 3, 4 and 5 dpi, no obvious weight-loss was observed in 1V-Al-PIM_2 mice in these days. The combination of different adjuvants targeting different immune pathways has received increased attention in recent years [20]. Examples of adjuvant combination include the addition of the TLR4 agonist MPL (3-O-desacyl-4-monophosphoryl lipid A, purified from Gram-negative bacterium Salmonella minnesota) with aluminium salt (Adjuvant System 04, AS04) which was shown to significantly enhance the adjuvanticity of aluminium salt [21] and this adjuvant combination is licenced for use in humans. Another example is the combination of aluminium hydroxide with

TLR2 ligand, Pam3CSK4, which when used with a virosomal vaccine against respiratory syncytial virus in mice providing improved protection [22]. In the current study, mice immunised with the subvirion vaccine supplemented with PIM_2 enhanced IFV-specific splenic T cell cytotoxicity. Effector CD8+ T cells are critical for the effective resolution of influenza virus infection via direct lysis of infected cells [23]. In addition to serving as TLR2 agonists, natural PIMs activate human and murine CD1d-restricted NKT cells, thereby triggering antigen-specific IFN-γ production and cell-mediated cytotoxicity [24].

In summary, the synthetic PIM_2 was a weaker adjuvant to a split subvirion influenza vaccine compared to aluminium hydroxide in this study. The combination of PIM_2 and aluminium hydroxide saw an additive adjuvant effect to the vaccine. PIM_2 enhanced virus-specific CTL activity and lowered pulmonary virus load. More studies are required to investigate if other PIMs or PIM_2 analogues would have stronger adjuvanticity to the split influenza vaccine than PIM_2.

Acknowledgement

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