



## The effect of gamma-irradiation conditions on the immunogenicity of whole-inactivated Influenza A virus vaccine



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### ABSTRACT

Gamma-irradiation, particularly an irradiation dose of 50 kGy, has been utilised widely to sterilise highly pathogenic agents such as Ebola, Marburg Virus, and Avian Influenza H5N1. We have reported previously that intranasal vaccination with a gamma-irradiated Influenza A virus vaccine ( $\gamma$ -Flu) results in cross-protective immunity. Considering the possible inclusion of highly pathogenic Influenza strains in future clinical development of  $\gamma$ -Flu, an irradiation dose of 50 kGy may be used to enhance vaccine safety beyond the internationally accepted Sterility Assurance Level (SAL). Thus, we investigated the effect of irradiation conditions, including high irradiation doses, on the immunogenicity of  $\gamma$ -Flu. Our data confirm that irradiation at low temperatures (using dry-ice) is associated with reduced damage to viral structure compared with irradiation at room temperature. In addition, a single intranasal vaccination with  $\gamma$ -Flu irradiated on dry-ice with either 25 or 50 kGy induced seroconversion and provided complete protection against lethal Influenza A challenge. Considering that low temperature is expected to reduce the protein damage associated with exposure to high irradiation doses, we titrated the vaccine dose to verify the efficacy of 50 kGy  $\gamma$ -Flu. Our data demonstrate that exposure to 50 kGy on dry-ice is associated with limited effect on vaccine immunogenicity, apparent only when using very low vaccine doses. Overall, our data highlight the immunogenicity of influenza virus irradiated at 50 kGy for induction of high titre antibody and cytotoxic T-cell responses. This suggests these conditions are suitable for development of  $\gamma$ -Flu vaccines based on highly pathogenic Influenza A viruses.

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### 1. Introduction

Emergence of Highly Pathogenic Avian Influenza (HPAI) strains, H5N1, H5N6, H7N9, and H9N2, represent major health concerns due to the risk of worldwide pandemics [1]. Since 2003, the World Health Organisation (WHO) reported over 800 cases of human infection with avian H5N1, with an average mortality rate of 53% [2]. Most infections with H5N1 occur via infected poultry, though rare clusters of human-human transmission have been reported between family groups in Thailand [3,4], Indonesia [5], Turkey [6], and Vietnam [4]. HPAI may gain mutations to facilitate aerosol transmission between humans, as notably, a mere 5 mutations in a laboratory H5N1 strain allowed efficient aerosol transmission

between ferrets [7–9]. Existing inactivated Influenza vaccines induce strain-specific antibody responses, hence protective efficacy against emerging seasonal and pandemic strains is limited [12,13]. We reported the possible use of gamma-irradiated Influenza A virus ( $\gamma$ -Flu) as a vaccine candidate capable of inducing cross-protection against seasonal and pandemic virus strains [10,11,16].

To ensure sterility of irradiated influenza materials, the concept of Sterility Assurance Level (SAL) has been adopted and a value of  $10^{-3}$  or  $10^{-6}$  (one in a thousand or million chance of having live micro-organisms after treatment) has been arbitrarily determined and widely accepted [19]. The Australian Department of Agriculture recently considered an irradiation dose of 50 kGy as mandatory for sterilisation of highly pathogenic agents [14,15]. Considering the risk of avian Influenza pandemics, inclusion of HPAI strains may be warranted in future  $\gamma$ -Flu preparations; hence vaccine irradiation dose may be increased to meet the safety requirement. However, increasing irradiation dose may affect vac-

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cine efficacy. In addition, while damaging effect of  $\gamma$ -irradiation is dose-dependent [17,18]; the extent of structural damage is influenced by irradiation temperature [20,21,36–38]. Importantly, protein antigenicity is better maintained when virus samples are irradiated on dry-ice (DI) [23]. In the current study, we investigated the effect of irradiation dose and temperature on the immunogenicity of  $\gamma$ -Flu.

## 2. Materials and methods

### 2.1. Ethics statement

This study was conducted in strict accordance with Australian Code of Practice for Care and Use of Animals for Scientific Purposes (7th edition [2004], 8th edition [2013]) and South Australian Animal Welfare Act 1985. Experimental protocol approved by Animal Ethics Committee at The University of Adelaide (S-2013/014 & S-2016/036).

### 2.2. Cells & viruses

Influenza A virus [A/Puerto Rico/8/34 (H1N1) (A/PR8)] was grown in allantoic cavity of 10-day-old embryonated chicken eggs. Eggs injected with  $10^3$  TCID<sub>50</sub> A/PR8, incubated for 48 h at 37 °C, and chilled at 4 °C overnight. Allantoic fluid harvested, pooled and stored at –80 °C. Virus stock titrated in Madin-Darby Canine Kidney (MDCK) cells using TCID<sub>50</sub> assay [24] and estimated as  $1.5 \times 10^6$  TCID<sub>50</sub>/mL. Virus stock concentrated using chick erythrocytes (cRBCs) as previously described [25]. Concentrated A/PR8 stock titre estimated as  $2 \times 10^8$  TCID<sub>50</sub>/mL. For Haemagglutination Assay, live or irradiated stocks were serially diluted in PBS using 96-well round-bottom plate and 0.8% cRBCs in PBS added. Plates were incubated at 4 °C and haemagglutination patterns analysed 24 h later.

### 2.3. Vaccine preparations

A/PR8 stocks inactivated by exposure to  $\gamma$ -radiation from <sup>60</sup>Co irradiation facility at Australian Nuclear Science and Technology Organisation (ANSTO), either on dry-ice or at room temperature. Sterility confirmed by passages as recommended by WHO [26]. Lack of detectable HA activity, as measured by Haemagglutination assay, in allantoic fluid from 3 passages indicated complete loss of virus infectivity.

### 2.4. Transmission Electron Microscopy (TEM)

Irradiated A/PR8 ( $\gamma$ -A/PR8) samples loaded into 3 mm formvar-amorphous carbon-coated copper grids and left for 2 min. Excess solution removed by blotting. Samples stained with 2% Uranyl Acetate for 2 min, then blotted and left to dry at RT for 10 min before visualisation with FEI Tecnai G2 Spirit Transmission Electron Microscope (Adelaide Microscopy, University of Adelaide).

### 2.5. SDS-PAGE

Irradiated and control samples heat-treated at 85 °C for 20 min. Viral proteins separated by electrophoresis on Pre-Cast NuPAGE Novex 4–12% Bis-Tris gel (Thermo Fisher Scientific), then stained with Coomassie Brilliant Blue. Novex Sharp Pre-Stained Protein Standards (Thermo Fisher Scientific) used for MW comparison.

### 2.6. Mice & treatment

Six-week-old female wild-type BALB/c mice (H-2<sup>d</sup>) supplied by Laboratory Animal Services, University of Adelaide. Mice were anaesthetized intraperitoneally (IP) with 10  $\mu$ L/gram body weight ketamine anaesthetic (1% xylazine, 10% ketamine in sterile H<sub>2</sub>O), and vaccinated intranasally (IN) with one or two doses 14 days apart of  $\gamma$ -A/PR8. Control animals treated with PBS. 21 days post-vaccination, animals were anaesthetised, challenged IN with A/PR8 ( $1.6 \times 10^2$  TCID<sub>50</sub>/mouse), and monitored for 3 weeks for clinical symptoms and weight loss. Animals were culled if they lost 20% of starting body weight.

### 2.7. Measurement of influenza-specific antibody responses

Blood samples collected from all mice via submandibular bleeding 20 days post-vaccination and serum levels of A/PR8-specific IgG were determined by ELISA as described previously [27]. Absorbance measured at 450/620 nm using Biotrack II plate reader, end point titres expressed as reciprocal of the last dilution where OD value  $\geq$  cut-off value. Cut-off value was determined as mean  $+(3 \times \text{S.D.})$  of OD values of samples from control mice.

### 2.8. In vitro neutralisation assay

96-well tissue-culture plates seeded with  $6 \times 10^4$  MDCK cells/well. A/PR8 activated by treatment with 2  $\mu$ g/mL TPCK-trypsin (Sigma-Aldrich) for 30 min at 37 °C. Heat-inactivated sera were serially diluted, mixed with A/PR8 (diluted in allantoic fluid + 4  $\mu$ g/mL TPCK-trypsin) in 1:1 ratio, and incubated for 1 h at 37 °C. Mixture added to MDCK monolayers at MOI of 0.1 and incubated for 2 h at 37 °C. Then, inoculum was removed, monolayers washed with PBS and returned to incubator for 22 h in serum-free media. Monolayers washed, fixed and permeabilised with acetone/methanol (1:1 ratio) at 4 °C and incubated with polyclonal murine anti-A/PR8 sera (generated as previously described [28]) for 1 h at 4 °C. Alexa-Fluor<sup>®</sup> 488 goat anti-mouse IgG (H + L) (Life Technologies) added for 1 h at 4 °C. Nuclei stained with DAPI (1  $\mu$ g/mL) for 30 min at room temperature (RT). Images acquired using a Nikon TiE inverted fluorescence microscope and analysed using NIS elements software (Tokyo, Japan).

### 2.9. Cytotoxic T-cell assay

Mice primed by intravenous injection of live or  $\gamma$ -A/PR8. 6 days later, target splenocytes from naive mice labelled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (0.125 mM) or CellTrace™ Far-Red DDAO-SE (2  $\mu$ M, Thermo Fisher Molecular-Probes). CFSE population pulsed with Influenza A nucleoprotein peptide (GL Biochem (Shanghai) Ltd, sequence: TYQR-TRALV). Target cells mixed at 1:1 ratio (CFSE/CellTrace Red) and adoptively transferred into primed mice using intravenous injection ( $10^7$  cells/mouse). 24 h later, mice sacrificed, spleens harvested, processed to single-cell suspensions, and analysed using FACS (LSRII, BD Biosciences). Data analysed using FlowJo (Treestar Incorporated). Specific lysis as follows: lysis [%] =  $[1 - (\% \text{primed pulsed targets} / \% \text{primed non-pulsed targets}) / (\% \text{unprimed pulsed targets} / \% \text{unprimed non-pulsed targets})] \times 100$ .

### 2.10. Statistical analysis

Quantitative results expressed as mean  $\pm$  SEM. Unpaired Student's *t*-test used for comparison of data from two separate groups, and One-way ANOVA used for comparison of data from 3 or more groups. Statistical analysis performed using GraphPad Prism 6, ver-

sion 6.0d (GraphPad Software, La Jolla, CA, USA).  $P$  values  $< 0.05$  (95% confidence) considered statistically significant.

### 3. Results

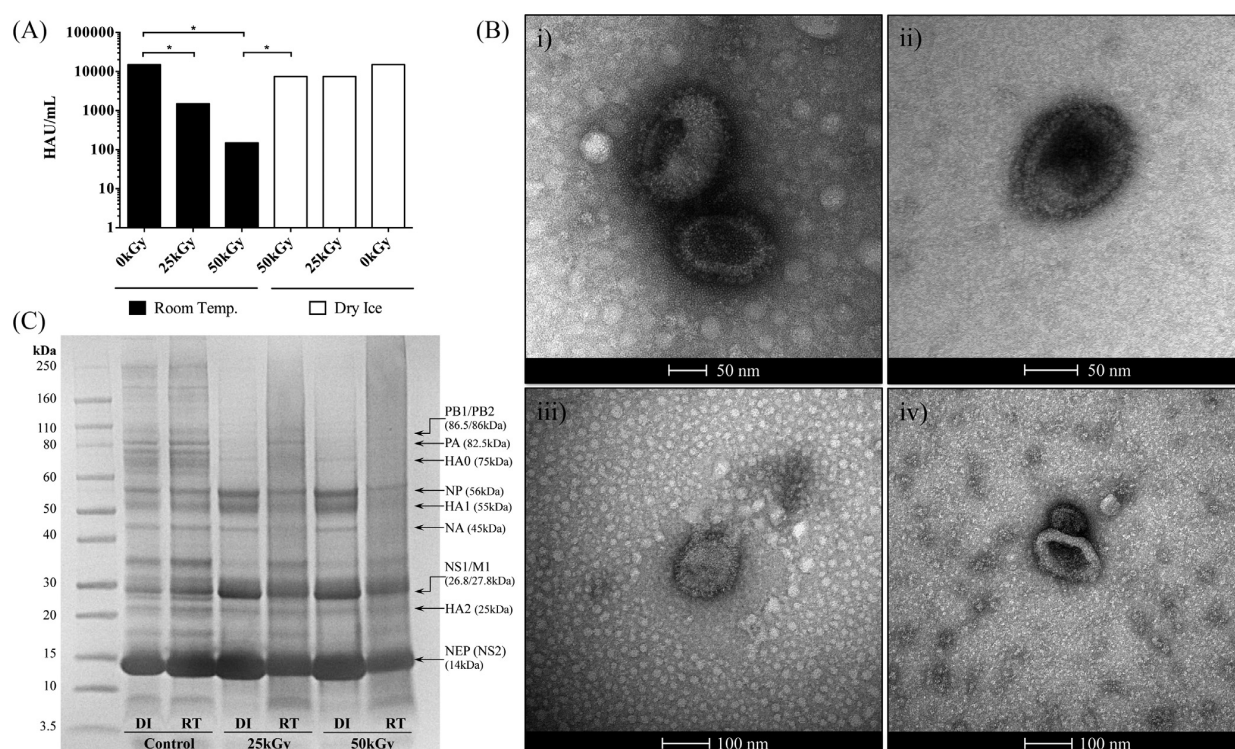
#### 3.1. The effect of irradiation conditions on HA titres and virion morphology

A/PR8 virus samples were exposed to 25 or 50 kGy of  $\gamma$ -radiation, either at room temperature (RT) or on dry-ice (DI). Haemagglutination assay used to determine the effect of irradiation conditions on HAU titres. Fig. 1A shows RT irradiation resulted in 90% and 99% loss of HAU titres for 25 and 50 kGy respectively. In contrast, only 50% loss in HAU was detected after irradiation on DI, regardless of irradiation dose. Considering that HAU titre is dependent on binding of HA to cRBCs, loss in HAU titres after irradiation at RT may be associated with structural damage. Therefore, TEM was used to visualise overall structure of irradiated viruses (Fig. 1B). Samples irradiated on DI show a more intact virus structure compared to preparations irradiated at RT. It should be noted that DI samples were resolved at 220,000 $\times$  magnification, whilst clear resolution for RT samples could only be visualised at lesser magnification of 135,000 $\times$ . Additionally, we detected dark aggregates in RT images, indicating potential formation of protein aggregates or split viral particles. We used SDS-PAGE to compare integrity of viral proteins in irradiated samples and non-irradiated controls. All major viral proteins were visible in control non-irradiated samples (Fig. 1C). The three influenza polymerase

proteins resolved as two bands, with PB1 and PB2 migrating together to form a less defined band. Bands consistent with molecular weights for NP and M1 were visible for all samples. Importantly, uncleaved (HA0) and cleaved (HA1, HA2) forms of HA were present in control samples and DI irradiated samples. In contrast, HA0 and HA1 bands appeared faint in RT irradiated samples, consistent with reduced HAU titres. Furthermore, lanes related to RT samples showed an increase in smearing of proteins as opposed to formation of discrete bands, indicative of increased protein damage. Considering the significant reduction in HA titre and the observed effect on virion structure and protein integrity (Fig. 1), RT samples were considered inappropriate vaccine preparations and excluded from further testing.

#### 3.2. Estimating $D_{10}$ value and SAL

Sterility of 25 and 50 kGy-irradiated materials was confirmed using 3 passages in 10-day-old embryonated eggs. Additionally, to test whether these doses met the internationally accepted SAL, aliquots of cRBC-concentrated live virus were subjected to different irradiation doses and tested for reduction in virus titre using TCID<sub>50</sub> assay. We detected a log-linear relationship between the increased irradiation dose and the associated reduction in virus titre (Fig. 2). Based on this linear inactivation curve ( $R^2 = 0.9511$ ), we estimated a  $D_{10}$  value of 2.04 kGy. To calculate SAL, we considered the linear inactivation curve, the  $D_{10}$  value, the initial titre of  $2 \times 10^8$  TCID<sub>50</sub>/mL, and the need for 11 or 14 log<sub>10</sub> titre reductions to achieve SAL of  $10^{-3}$  or  $10^{-6}$ , respectively. Therefore, SAL for our



**Fig. 1.** The effect of irradiation dose and temperature on the structure of Influenza A virus. (A) A/PR8 samples exposed to 25 or 50 kGy of  $\gamma$ -irradiation either on dry-ice (DI) or at room temperature (RT). 0 kGy samples were used as controls and virus titre was estimated using haemagglutination assay and expressed as HAU/mL. Each column indicates mean value of quadruplicates  $\pm$  SEM, and data analysed by One-way ANOVA ( $P < 0.05$ ). (B) Transmission Electron Microscopy (TEM) used to visualise morphology changes following irradiation on dry-ice using 25 kGy (i) or 50 kGy (ii), or RT using 25 kGy (iii) or 50 kGy (iv). Virus preparations negatively stained with 2% uranyl acetate and visualised using FEI Tecnai G2 Spirit Transmission Electron Microscope. (C) SDS-PAGE of heat-lysed Influenza preparations, both irradiated samples and non-irradiated controls. Influenza proteins labelled according to their known MW from UniProtKB database (*Influenza A virus (strain A/Puerto Rico/8/1934 H1N1)*), and according to those identified by Shaw et al. [47].



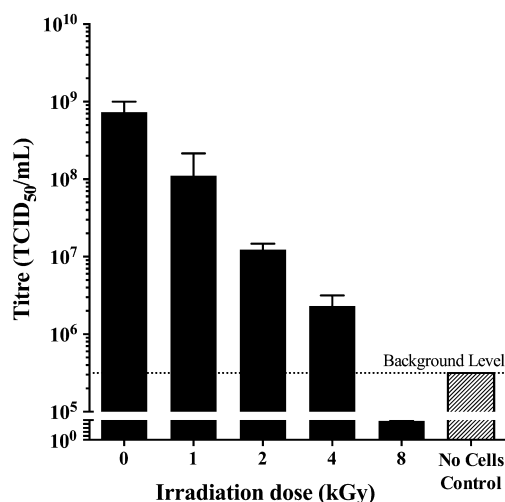


Fig. 2. Inactivation curve of A/PR8 following exposure to different doses of  $\gamma$ -rays. Samples of A/PR8 virus were exposed to increasing doses of  $\gamma$ -irradiation, and virus infectivity was determined by TCID<sub>50</sub> assay. Background level was measured by the binding of cRBCs to A/PR8 after incubation for 5 days in the absence of MDCK cells.

vaccine could be calculated using the formula “SAL = D<sub>10</sub> value  $\times$  11” or “SAL = D<sub>10</sub> value  $\times$  14”, giving 22.4 kGy or 28.6 kGy for SAL value of 10<sup>-3</sup> or 10<sup>-6</sup>, respectively. This indicates that materials exposed to 25 or 50 kGy meet the internationally accepted SAL for  $\gamma$ -irradiated pathogens. In fact, 50 kGy represents a much higher dose than required to achieve sterility, representing about 24 log<sub>10</sub> reductions in virus titre. Importantly, we confirmed complete inactivation of our preparations in accordance with WHO recommended method of two passages in 10-day-old embryonated eggs [26], and performed 3 passages with no detectable HA in allantoic fluid.

### 3.3. The effect of irradiation dose on induction of protective immunity

To study the effect of irradiation dose on vaccine efficacy, mice were vaccinated with 25 or 50 kGy  $\gamma$ -A/PR8 irradiated on dry-ice. Serum samples were taken on day 20 post-vaccination and analysed for Flu-specific IgG titres using ELISA. Fig. 3A shows both preparations induced elevated A/PR8-specific IgG titres in serum following mucosal vaccination compared to controls and titres for 25 and 50 kGy  $\gamma$ -A/PR8 vaccinated groups were comparable. Additionally, vaccinated and control animals were challenged IN with lethal A/PR8 21 days post-vaccination, and monitored for clinical symptoms and weight loss. Fig. 3B shows  $\gamma$ -A/PR8 irradiated with either 25 or 50 kGy induced 100% protection against lethal IN challenge and vaccinated mice did not show any weight loss compare to controls. Our data indicate that both preparations (25 and 50 kGy) are highly immunogenic and show comparable protective efficacies when using 32  $\mu$ L/mouse of undiluted vaccine preparation ( $6.4 \times 10^6$  TCID<sub>50</sub> equivalent/mouse).

### 3.4. The effect of vaccine dose on vaccine efficacy

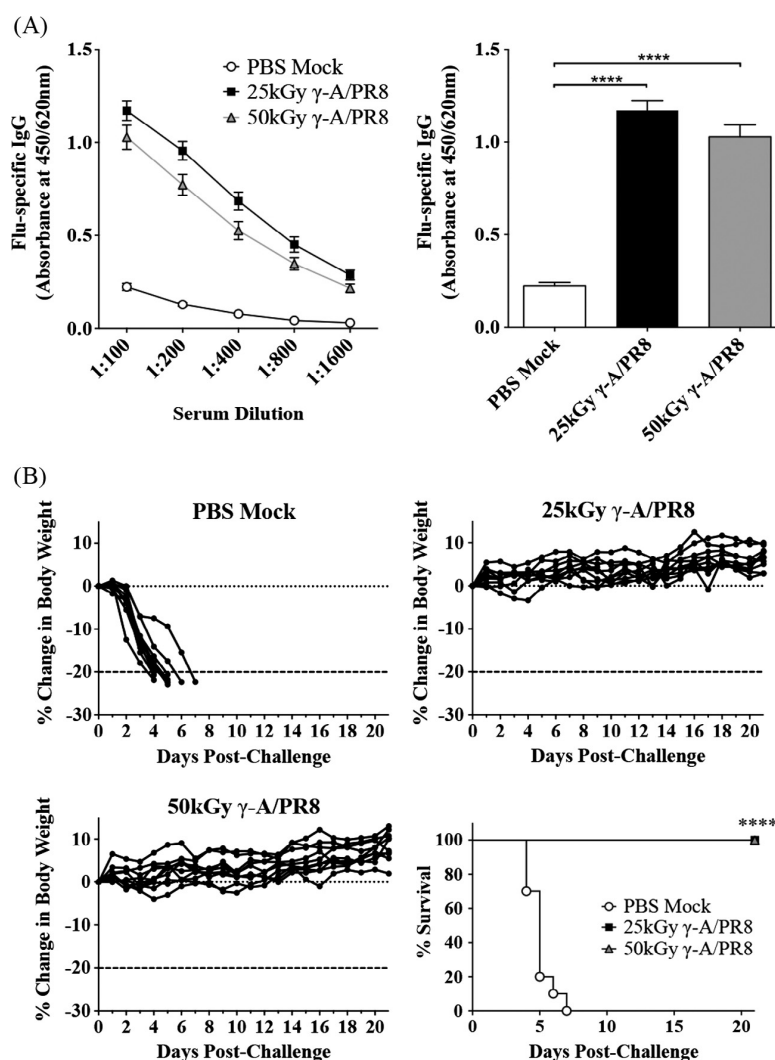
Our data and published studies [21,29–34] clearly indicate that structural damage could be controlled using freezing irradiation conditions. Nonetheless, increased exposure to  $\gamma$ -rays may be associated with reduced vaccine efficacy, albeit to a limited extent. To test this, mice were vaccinated with a single intranasal dose of  $\gamma$ -A/PR8, using either one-half or one-eighth of the dose used in Fig. 3. Protective efficacy was monitored following challenge with live A/PR8. Fig. 4A shows that vaccination with reduced doses of 25 or

50 kGy  $\gamma$ -A/PR8 resulted in 100% survival. However, we observed some weight loss (~10%) in animals vaccinated with one-eighth dose of 50 kGy  $\gamma$ -A/PR8 prior to full recovery. No weight loss was observed for the other vaccinated groups. We also analysed antibody responses; whilst all reduced doses induced seroconversion following intranasal vaccination, we detected a 50% reduction in IgG titres in serum samples from mice vaccinated with 50 kGy  $\gamma$ -A/PR8 compared to samples from mice vaccinated with 25 kGy  $\gamma$ -A/PR8. However, this reduction did not reach statistical significance (Fig. 4B). This indicates that while 50 kGy  $\gamma$ -A/PR8 appears to be immunogenic and confers high protective efficacy, exposure to 50 kGy may be associated with some damage to viral proteins. As such, this may have affected antibody responses and the ability of  $\gamma$ -A/PR8 to induce protection without weight loss, when using a reduced antigen dose.

To further investigate the effect of high radiation dose on  $\gamma$ -A/PR8, we employed a two-dose vaccination strategy using a very low vaccine dose of  $5 \times 10^4$  TCID<sub>50</sub> equivalent/mouse, approximately one-hundredth of the dose used in Fig. 3. A single vaccination with this reduced vaccine dose was not sufficient to induce protective immunity against lethal challenge, regardless of irradiation dose (Fig. 5A). Consequently, mice were vaccinated with two doses of  $5 \times 10^4$  TCID<sub>50</sub> equivalent, two weeks apart, followed by a lethal A/PR8 challenge 3 weeks later. When considering the two-dose strategy, vaccination with 25 kGy  $\gamma$ -A/PR8 resulted in significant protection (50% survival) following homotypic challenge. In contrast, two-dose vaccination with 50 kGy  $\gamma$ -A/PR8 was not associated with any protection. Interestingly, analysing antibody titres in serum harvested 24 h pre-2nd vaccination and 24 h pre-challenge showed both 25 and 50 kGy  $\gamma$ -A/PR8 induced comparable Flu-specific IgG titres (Fig. 5B). Additionally, *in vivo* CTL assay was performed to determine whether the observed difference in protection was due to T-cell mediated mechanisms rather than antibody responses. As shown in Fig. 5C, 50 kGy  $\gamma$ -A/PR8 induced slightly less potent CTL responses against nucleoprotein peptide (NPP) pulsed target cells compared to both 25 kGy  $\gamma$ -A/PR8 and live virus control, however this trend was not statistically significant.

### 3.5. Neutralising antibody responses induced by $\gamma$ -A/PR8

Antibody levels detected using ELISA in immune sera from mice vaccinated with 25 and 50 kGy  $\gamma$ -A/PR8 did not differ significantly, despite observed differences in protective efficacies (Fig. 5). Therefore, we investigated whether high radiation dose affected the quality of humoral responses rather than quantity. Serum samples from mice vaccinated with  $6.4 \times 10^6$  TCID<sub>50</sub> and 2 doses of  $5 \times 10^4$  TCID<sub>50</sub>  $\gamma$ -A/PR8 were tested using an *in vitro* neutralisation assay to quantify neutralising antibody responses. MDCK cells were infected with sera-treated A/PR8 at MOI of 0.1. PBS-treated A/PR8 was used as a virus-only control. Fluorescent staining of infected monolayers showed incubation of A/PR8 with sera from control mice (mock-sera) did not affect the ability of A/PR8 to infect MDCK cells, as infectivity for both mock-sera treated virus and the virus-only control were comparable. Importantly, A/PR8 treatment with immune sera from mice vaccinated with  $6.4 \times 10^6$  TCID<sub>50</sub> of 25 and 50 kGy  $\gamma$ -A/PR8 showed complete abrogation of virus infectivity (100% neutralisation), indicating that both vaccines induced strong neutralizing antibody responses (Fig. 6A). Different serum dilutions (1:20, 1:40 and 1:80) were also tested, and no difference in virus neutralisation was detected. Conversely, when testing serum samples from mice vaccinated with  $5 \times 10^4$  TCID<sub>50</sub>  $\gamma$ -A/PR8, we detected differences in virus neutralisation between immune sera from mice vaccinated with 25 versus 50 kGy  $\gamma$ -A/PR8. Importantly, serum samples from both 25 and 50 kGy  $\gamma$ -A/PR8 vaccinated groups induced significant virus neu-



**Fig. 3.** Intranasal vaccination with 25 kGy and 50 kGy  $\gamma$ -A/PR8 induces homotypic protection against lethal challenge. Mice vaccinated IN with  $6.4 \times 10^6$  TCID<sub>50</sub> equivalent/mouse of  $\gamma$ -A/PR8 vaccine irradiated with 25 or 50 kGy on dry-ice. (A) Serum samples harvested on day 20 post-vaccination were analysed for Flu-specific IgG by direct ELISA using serial serum dilutions, and 1:100 dilution of immune sera was selected for statistical analysis. (B) Weight loss of vaccinated mice following IN challenge on day 21 with lethal dose of A/PR8, and percentage survival. Data compiled from two independent experiments (n = 10). Data analysed by (A) One-Way ANOVA and (B) Fisher's Exact test (\*\*\*\*P < 0.0001).

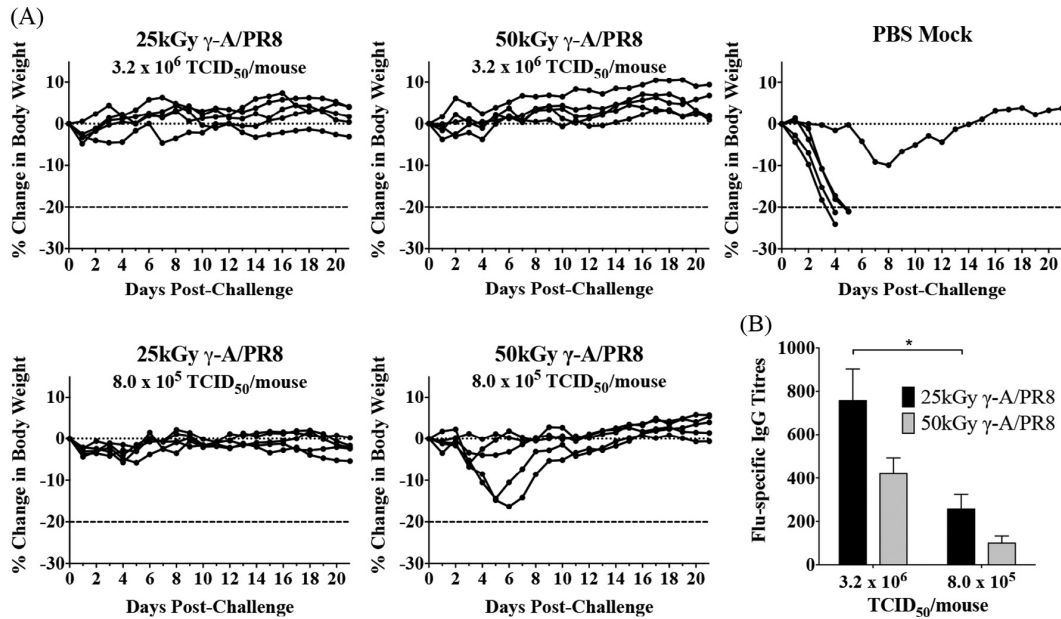
tralisation when compared to control samples. However, neutralisation ability between samples from vaccinated groups was different, as sera from the 25 kGy  $\gamma$ -A/PR8 vaccinated group appeared to be ~2.3-fold more effective at neutralizing A/PR8 (determined by difference between means of normalized FITC fluorescence) when compared to sera from 50 kGy  $\gamma$ -A/PR8 vaccinated mice (Fig. 6B).

#### 4. Discussion

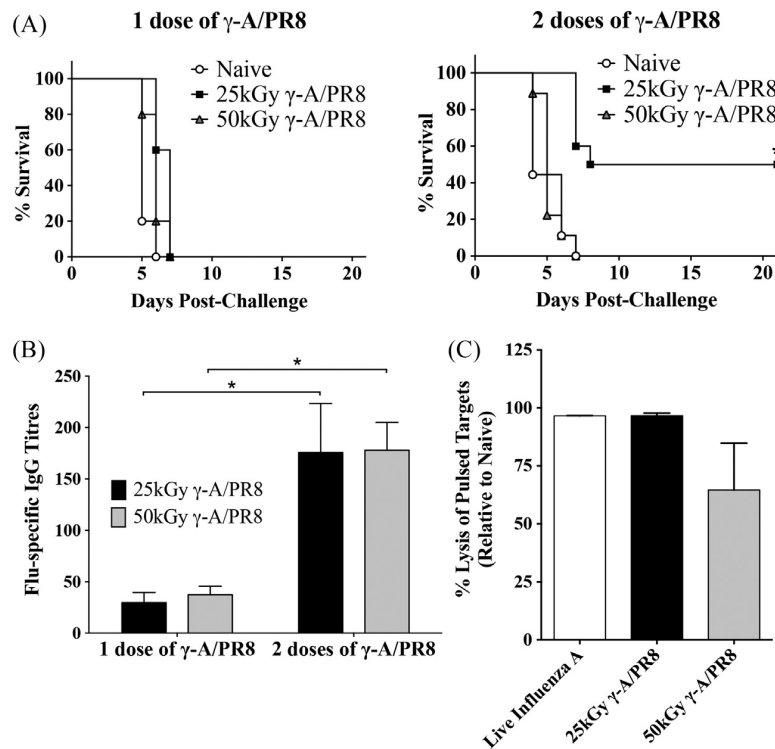
Rapid emergence of HPAI strains highlights the urgent need to develop safe vaccines capable of providing protection against circulating as well as emerging pandemic Influenza A viruses. We reported previously that vaccination with  $\gamma$ -Flu confers protection against lethal homotypic and heterosubtypic Influenza A virus challenges, including HPAI H5N1 [10,35]. Considering the risk of a worldwide pandemic, inclusion of HPAI virus strains may be desirable in future clinical developments of  $\gamma$ -Flu. To comply with

safety regulations regarding irradiation of pathogenic agents, 50 kGy may be considered. Therefore, we estimated the irradiation dose required to achieve a SAL of  $10^{-3}$  or  $10^{-6}$ , and investigated the effect of high irradiation dose and temperature conditions on  $\gamma$ -Flu efficacy.

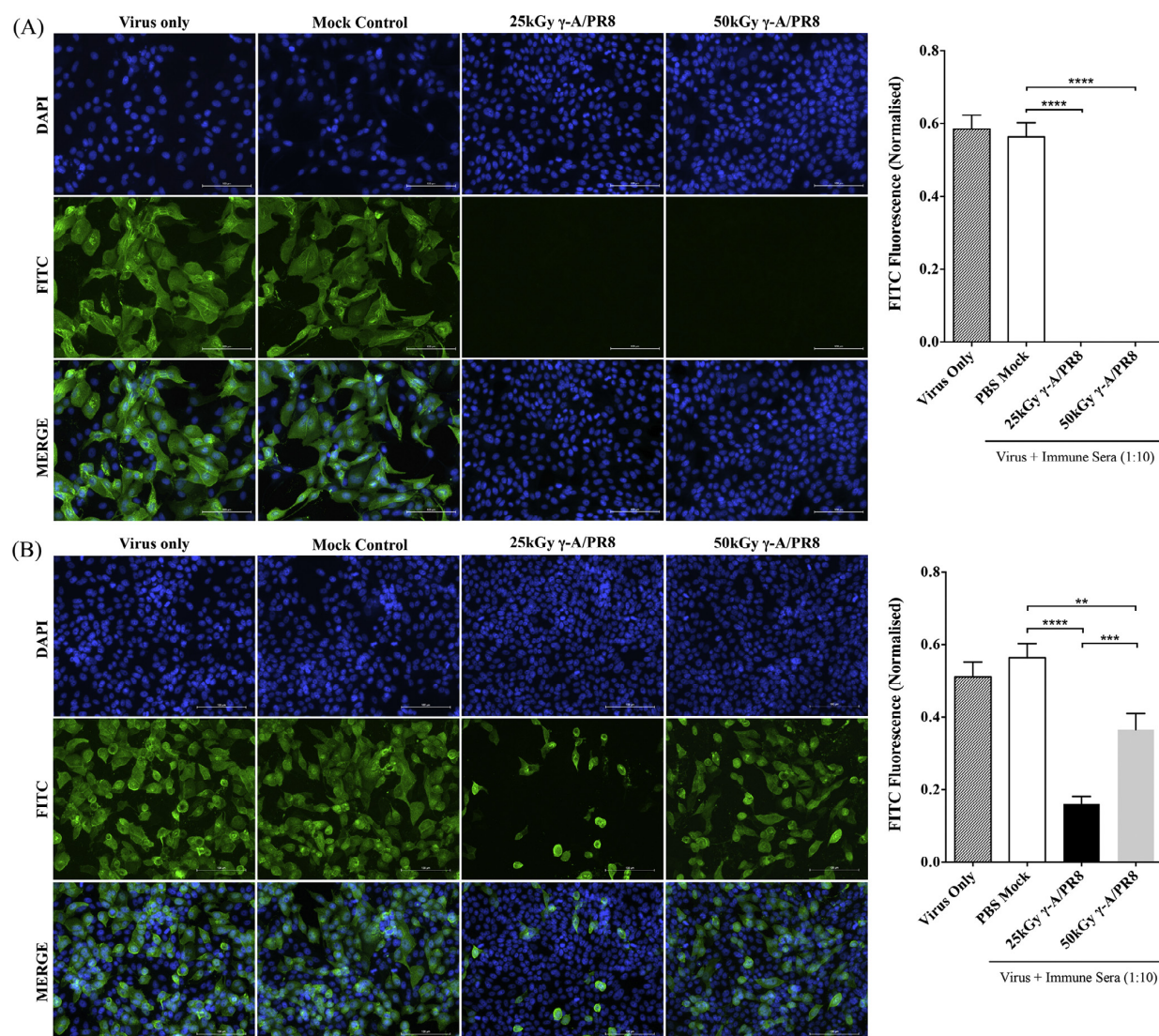
It has been reported previously that freezing target materials at ultra-low temperatures during irradiation reduces free radical formation and consequently minimizing the indirect damage to proteins [21,33]. For example,  $\gamma$ -irradiation of frozen plasma samples has been effective in sterilizing HIV virus with minimal impact on functionality of coagulation factors [40]. Furthermore, irradiation of freeze-dried materials was associated with maintained protein biological activity even after exposure to 45 kGy [39]. Our data indicate that we could maintain surface protein functionality and viral morphology by irradiating frozen materials in contrast to irradiation at RT. Interestingly, Feng et al. [22] used SDS-PAGE to demonstrate the decrease in the abundance of Murine Norovirus-1 capsid protein VP1 as irradiation dose increased. We



**Fig. 4.** The efficacy of  $\gamma$ -A/PR8 vaccine. Mice vaccinated IN with  $3.2 \times 10^6$  or  $8.0 \times 10^5$  TCID<sub>50</sub> equivalent/mouse of  $\gamma$ -A/PR8 vaccine irradiated on dry-ice. (A) Weight loss of vaccinated mice following IN challenge with lethal dose of A/PR8. (B) Serum samples harvested on day 20 post-vaccination were analysed for Flu-specific IgG by direct ELISA. Absorbance readings at 450/620 nm of naïve sera used to calculate relative IgG titres. Data presented as mean  $\pm$  SEM, and analysed by One-Way ANOVA (\*,  $P < 0.05$ ).



**Fig. 5.** Enhanced protective efficacy of 25 kGy  $\gamma$ -A/PR8 compared to 50 kGy when using low vaccine dose. (A) Mice vaccinated IN with either a single dose of  $\gamma$ -A/PR8 irradiated on dry-ice ( $5 \times 10^4$  TCID<sub>50</sub> equivalent/mouse), or two doses administered two weeks apart. 3 weeks post-vaccination, mice were challenged IN with lethal dose of A/PR8, and monitored for survival. Data presented as survival rate ( $n = 10$ ), and analysed by Fisher's exact test ( $P < 0.05$ ). (B) Serum samples harvested via submandibular bleed 24 h pre-challenge from all mice, and tested for Flu-specific IgG using direct ELISA. Absorbance readings at 450/620 nm of naïve sera used to calculate relative IgG titres. Data presented as mean  $\pm$  SEM, significance determined by One-Way ANOVA ( $P < 0.05$ ). (C) CTL-mediated killing of NPP pulsed target splenocytes 24 h after adoptive transfer into mice primed with live influenza, 25 kGy or 50 kGy  $\gamma$ -A/PR8 (dry-ice irradiated). Percentage killing determined in relation to unprimed controls, data presented as mean  $\pm$  SEM and analysed by One-Way ANOVA.



**Fig. 6.** *In vitro* A/PR8 neutralisation by immune sera from vaccinated mice. Mice vaccinated IN with (A)  $6.4 \times 10^6$  TCID<sub>50</sub>, or (B) two doses of  $5 \times 10^4$  TCID<sub>50</sub> equivalent  $\gamma$ -A/PR8 irradiated on dry-ice. Serum samples harvested via submandibular bleed on day 20 post-vaccination, and neutralisation efficacy of naïve (PBS mock-vaccinated) and  $\gamma$ -A/PR8-vaccinated immune sera at 1:10 dilution determined by *in vitro* neutralisation assay. After incubation with sera-treated virus, A/PR8 positive MDCK cells visualised using Nikon TiE inverted fluorescence microscope, with DAPI channel detecting cell nuclei, and FITC channel detecting A/PR8. Scale bar = 100  $\mu$ m. FITC fluorescence quantified using NIS elements software, and normalised using corresponding quantified DAPI fluorescence. Data presented as mean  $\pm$  SEM (n = 8), and significance determined by One-Way ANOVA (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ).

showed a similar trend for  $\gamma$ -A/PR8 samples irradiated at RT as we detected increased protein smearing with higher irradiation dose, as opposed to formation of many discrete bands (Fig. 1C). In contrast, materials irradiated on dry-ice showed discrete protein bands, particularly key proteins NA, HA1, and NP (45, 55, and 56 kDa respectively) that were maintained even following exposure to 50 kGy. In addition, RT samples failed to induce flu-specific serum antibody responses and sufficient homotypic protection (data not shown). Thus, we concluded that irradiation at RT is not suitable for vaccine development.

To ensure our dry-ice preparations satisfied requirements for internationally accepted standards [15,41–43], we established the killing curve of A/PR8 using vaccine samples irradiated at different doses. Our data show a clear log-linear relationship between increased irradiation doses and the associated reduced virus titre. This mathematical relationship was used to calculate a  $D_{10}$  value

of 2.04 kGy and a SAL value of 22.4–28.6 kGy. Therefore, 25 kGy sufficiently complies with guidelines of the International Atomic Energy Agency (IAEA) and International Standards Organisation (ISO). It is important to note that guidelines related to SAL should be accompanied by approved sterility tests, which we performed based on WHO recommendations. While SAL for our preparation is achieved using 25 kGy, 50 kGy may still be desirable to inactivate HPAI. Interestingly, 50 kGy is reported as the lowest dose capable of inactivating Venezuelan equine encephalitis virus (VEEV) [44], and an exposure to 50 kGy on dry-ice was reported to reduce  $\gamma$ -VEEV antigenicity and epitope integrity [45]. In contrast, our data show vaccination with 25 or 50 kGy dry-ice  $\gamma$ -A/PR8 resulted in significantly elevated A/PR8-specific IgG titres and 100% protection against lethal challenge.

Despite high efficacy of 50 kGy  $\gamma$ -A/PR8, we further investigated whether exposure to high irradiation dose affected vaccine



immunogenicity, and whether increased vaccination dose could overcome such effects. To address these possibilities, we used reduced  $\gamma$ -A/PR8 vaccine doses. Remarkably, these doses conferred 100% protection against lethal challenge. We did notice that animals vaccinated with  $8 \times 10^5$  TCID<sub>50</sub> equivalent/mouse (one-eighth dose) of 50 kGy  $\gamma$ -A/PR8 lost some weight prior to full recovery, in contrast to animals vaccinated with the same dose of 25 kGy  $\gamma$ -A/PR8. This minor difference in vaccine efficacy was confirmed using a severely reduced vaccine dose ( $5 \times 10^4$  TCID<sub>50</sub> equivalent/mouse) administered using a two-dose strategy. While 25 and 50 kGy  $\gamma$ -A/PR8 induced comparable levels of A/PR8-specific IgG, we detected a significant difference in A/PR8 neutralisation by immune sera associated the difference in vaccine protective efficacy using this low-dose setting. Interestingly, a study investigating  $\gamma$ -irradiation of allergens showed irradiation with 15 kGy abolished binding of IgE from allergic individuals to allergen proteins [46]. High irradiation potentially damaged allergen epitopes, consequently affecting antibody binding. Similarly, antibodies induced by 50 kGy-treated virus samples may recognise slightly damaged epitopes rather than native epitopes, hence live virus epitopes are less well recognised and consequently virus neutralisation is reduced. Importantly, our data indicate that using higher vaccination doses could overcome the reduced efficacy of 50 kGy  $\gamma$ -A/PR8, as equal and highly effective virus neutralisation for 25 and 50 kGy  $\gamma$ -A/PR8 was observed when using high vaccine dose. Additionally, both 25 and 50 kGy  $\gamma$ -A/PR8 preparations induced CTL responses against internal Influenza peptides that resembled CTL activity induced by live virus.

## 5. Conclusion

Overall, our data show no detectable difference in performance between 25 and 50 kGy  $\gamma$ -A/PR8 when using standard doses. Reduction in 50 kGy  $\gamma$ -A/PR8 efficacy is only apparent when using intentionally low vaccination dose, which is not relevant to a clinical setting nor for future  $\gamma$ -Flu development. This study has demonstrated the suitability of using freezing conditions for  $\gamma$ -irradiation of viruses to produce inactivated vaccines that elicit strong protective immunity. This supports the use of 50 kGy for developing future  $\gamma$ -Flu vaccines that include HPAI virus strains.

## Author contributions

MA, SCD & TRH conceived and designed the study. SCD, JL, EVS, RB & JD performed experiments and prepared reagents. SCD, EVS & JD performed statistical analyses. MA, SCD, JD, TRH, JCP & SRM wrote the manuscript.

## Conflict of interest

The authors have no conflicting financial interests, except MA is head of the vaccine research group at the University of Adelaide and the Chief Scientific Officer of Gamma Vaccines Pty Ltd. TRH is the Executive Chairman of Gamma Vaccines Pty Ltd. This does not alter adherence to policies on sharing data and materials.

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