ORIGINAL ARTICLE

Increase in DNA vaccine efficacy by virosome delivery and co-expression of a cytolytic protein

Tessa Gargett[1,5], Branka Grubor-Bauk[1], Darren Miller[2], Tamsin Garrod[1], Stanley Yu[1], Steve Wesselingh[3], Andreas Suhrbier[4] and Eric J Gowans[1]

The potential of DNA vaccines has not been realised due to suboptimal delivery, poor antigen expression and the lack of localised inflammation, essential for antigen presentation and an effective immune response to the immunogen. Initially, we examined the delivery of a DNA vaccine encoding a model antigen, luciferase (LUC), to the respiratory tract of mice by encapsulation in a virosome. Virosomes that incorporated influenza virus haemagglutinin effectively delivered DNA to cells in the mouse respiratory tract and resulted in antigen expression and systemic and mucosal immune responses to the immunogen after an intranasal (IN) prime/intradermal (ID) boost regimen, whereas a multidose ID regimen only generated systemic immunity. We also examined systemic immune responses to LUC after ID vaccination with a DNA vaccine, which also encoded one of the several cytolytic or toxic proteins. Although the herpes simplex virus thymidine kinase, in the presence of the prodrug, ganciclovir, resulted in cell death, this failed to increase the humoral or cell-mediated immune responses. In contrast, the co-expression of LUC with the rotavirus non-structural protein 4 (NSP4) protein or a mutant form of mouse perforin, proteins which are directly cytolytic, resulted in increased LUC-specific humoral and cell-mediated immunity. On the other hand, co-expression of LUC with diphtheria toxin subunit A or overexpression of perforin or NSP4 resulted in a lower level of immunity. In summary, the efficacy of DNA vaccines can be improved by targeted IN delivery of DNA or by the induction of cell death in vaccine-targeted cells after ID delivery.

DNA vaccines are appealing vaccine candidates as they are simple and inexpensive to manufacture and also highly stable. These characteristics make DNA vaccines an ideal technology particularly for use in developing countries where inexpensive vaccines are required most urgently. In practical terms, vaccines may be required to elicit systemic or mucosal immune responses.

However, the administration of DNA vaccines to mucosal surfaces is generally ineffective because the DNA is rapidly degraded before it is taken up by epithelial cells.[1,2] Nevertheless, mucosal delivery induces a more relevant immune response at sites where transmission of pathogens, including human immunodeficiency virus (HIV), is common. Novel vaccine strategies to elicit protection against pathogens that are transmitted by contact with mucosal surfaces should therefore focus on inducing systemic and mucosal immune responses by delivering antigen to the mucosa. By targeting mucosal surfaces,[3] intranasal (IN) immunisation generates immune responses not only in distant mucosal tissues (for example, cervicovaginal mucosa) but also systemically. Consequently pan-mucosal immune responses generated at distal sites such as the

genital tract represent an important strategy for the prevention of sexually transmitted infections.[4] Influenza virus virosomes have been used previously to deliver peptide or protein[5,6] and DNA vaccines[7,8] in an attempt to increase the efficacy of delivery and are attractive because ~95% of injected DNA remains extracellular[9] and consequently is degraded and fails to contribute to vaccine efficacy, whereas DNA complexed with polyethyleneimine has been used for IN delivery to improve uptake and overcome mucosal degradation of the DNA.[10] Therefore, virosome encapsulation may offer an improved delivery strategy for a mucosa-targeted vaccine. Influenza virosomes use a combination of lipid molecules and influenza envelope proteins (including hemagglutinin), maintain membrane binding and endosomal membrane fusion properties of the virus and provide targeted delivery to cells. In addition, several other strategies have been used to increase the cellular uptake of DNA after vaccination, including the use of gene guns and more recently, electroporation (reviewed in Saade and Petrovsky[11]). Intradermal (ID), rather than intramuscular, delivery of DNA is more effective,[12] likely because the dermis/subdermis contain a higher proportion of antigen-presenting

[1]Discipline of Surgery, The University of Adelaide, Basil Hetzel Institute, Adelaide, South Australia, Australia; [2]Division of Information Technology, Engineering and the Environment, School of Engineering, University of South Australia, Adelaide, South Australia, Australia; [3]South Australian Health and Medical Research Institute, North Terrace, Adelaide, South Australia, Australia and [4]Inflammation Biology Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia
[5]Current address: Experimental Therapeutics Laboratory, Hanson Institute, Royal Adelaide Hospital, Adelaide, South Australia, Australia.
Correspondence: Professor EJ Gowans, Discipline of Surgery, The University of Adelaide, Basil Hetzel Institute, 37a Woodville Road, Woodville South, South Australia 5011, Australia.
E-mail: eric.gowans@adelaide.edu.au
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cells, including dendritic cells (DC), than muscle.[13] DC are the major antigen-presenting cells able to prime naïve T cells, which is the primary aim of vaccination. Although direct presentation of epitopes results from endogenous expression of the immunogen in DC, cross presentation is likely to represent a more common pathway to induce immunity to viruses which do not directly infect DC.[14] Thus, a successful DNA vaccine delivery must target DC and/or otherwise

Figure 1 (**A**) *In vivo* expression of LUC on day 3 post vaccination. C57Bl/6 mice received a single IN vaccination of naked or virosome-encapsulated DNA encoding LUC. Luminescence was detected by the IVIS live imager and quantified by Living Image software. (a) Empty pVAX vector, (b) 100 μg pVax LUC, (c) 20 μg pVAX LUC, (d) Liposome plus 20 μg pVAX LUC, E) Virosome plus HA plus 20 μg pVAX LUC. (**B**) The graph shows mean photons s^{-1} (±s.e.m.) and each point represents a single mouse.

direct the immunogen to cross-presenting DC. Cellular necrosis has recently been identified as a key pathway that allows endogenous antigen to be taken up by DC for cross presentation, and therefore the induction of cell death following vaccination has the potential to enhance vaccine antigen cross presentation.[15,16] A previous study[17] used Fas to induce apoptosis and improve the immune response to an HIV-1 Env DNA vaccine, although the genes were co-administered on separate plasmids, with the result that cell death was not specifically targeted to antigen-positive cells. Similarly, prior intramuscular administration of cardiotoxin before administration of a DNA vaccine was used to induce sterile inflammation and DC recruitment at the site of vaccination.[18] These studies indicate that a vaccination strategy, which induces cell death, may enhance inflammation and subsequent vaccine efficacy.

Consequently, the aims of this study were to identify new techniques to improve DNA vaccination and to this end we examined the efficacy of DNA vaccination after (i) IN delivery by influenza virosomes to protect DNA from degradation and (ii) ID delivery of vaccines that encode an immunogen and a cytolytic or toxic protein to increase the efficacy of DNA vaccination by inducing cell death in vaccine-targeted cells, resulting in cross presentation of the immunogen.

RESULTS

Mucosal immunity after IN delivery of DNA

Preliminary *in vitro* studies showed that haemagglutinin (HA)-containing virosomes delivered DNA to HEK293T cells equally effectively as commercially available transfection reagents and was more effective than these agents in delivering DNA to the mucin-producing A549 cell line (data not shown). IN delivery of the virosomes to C57Bl/6 mice showed that HA+ virosomes were able to effectively deliver DNA to the nasal epithelia (Figure 1), as determined by live imaging of luminescence, whereas naked DNA was unable to do so. However, there was considerable mouse–mouse variability in luciferase (LUC) expression, which may be due to the administration technique, which involves placing drops of liquid on the nares and allowing the mouse to inhale. The level of anaesthesia affects the respiration rate and the efficiency of vaccine delivery, and lightly anesthetised mice may swallow, rather than inhale, an aliquot of the vaccine dose. Pretreatment of the nasal passages with lysophosphatidycholine, a surfactant that has been used to improve IN delivery by others,[17] did improve transfection efficiency (data not
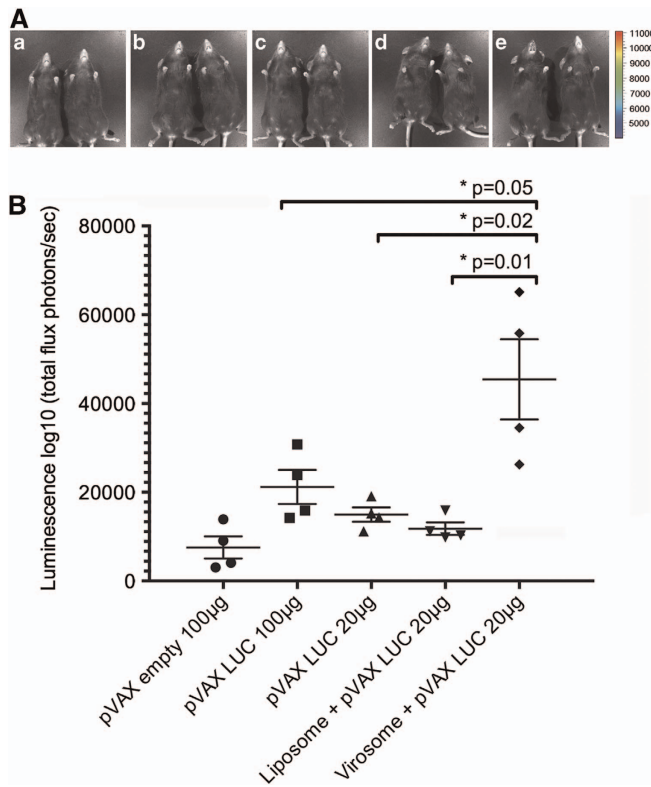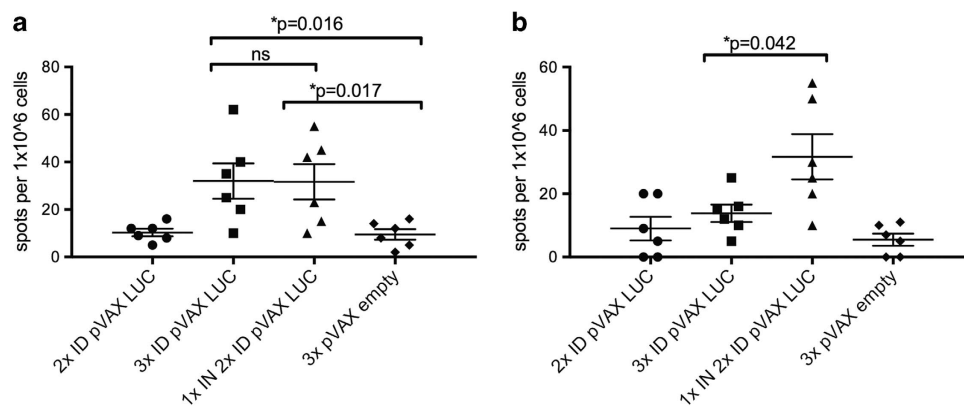

Figure 2 Systemic and mucosal T-cell responses to LUC. IFN-γ ELIspot was performed with cells restimulated with a single immunodominant LUC peptide. SFU for cells from (**a**) spleens and (**b**) vaginal-draining lymph nodes from vaccinated mice. Graphs show mean SFU per 10^6 cells (±s.e.m.), and each point represents a single mouse. Significant differences were determined by the Kruskal–Wallis multiple comparison test.

shown); however, variability within groups was still observed. Live imaging also showed that HA was necessary for effective delivery of the DNA, as virosomes in which the HA was substituted with bovine serum albumin failed to result in detectable luminescence (data not

shown). However, an IN boost with DNA in HA+ virosomes failed to result in the expression of LUC, most likely because the mice developed robust anti-HA antibody responses after the initial vaccination (data not shown).

As a result of the failure of the IN boost to induce systemic and mucosal immune responses to LUC, the mice received an IN prime followed by two ID booster injections at 4-week intervals, and the responses were compared with those after three ID injections. The results of this experiment showed that mice that received the $3 \times$ ID injections developed a mean anti-LUC antibody titre of 1/6998 while the IN prime, $2 \times$ ID boost group developed a mean titre of 1/2573, although this difference was not significant. In contrast, the anti-LUC titre in the bronchial lavage from the $1 \times$ IN, $2 \times$ ID group was fourfold higher (1/11 vs 1/2.6) than that in the $3 \times$ ID group while the titre in the vaginal lavage was approximately twofold higher (1/7.5 vs 1/4). The cell-mediated immune response was also examined by ELIspot analysis of interferon-γ-secreting cells in the splenocyte population and in cells recovered from the vaginal-draining lymph nodes. There was no significant difference in the systemic (splenocytes) responses induced by $3 \times$ ID or $1 \times$ IN/$2 \times$ ID regimens, (Figure 2a) whereas, in contrast, mice which were vaccinated with the $1 \times$ IN/$2 \times$ ID regimen showed a significant increase in the frequency of interferon-γ-secreting cells in the lumbar and caudal/sacral lymph nodes representing the vaginal-draining lymph nodes (Figure 2b). Cells recovered from the draining lymph nodes around the nose (cervical) were also examined, but these results were not significant. Thus, the mucosal cell-mediated immunity was significantly higher (5.5-fold after subtracting the background) in the $1 \times$ IN/$2 \times$ ID vaccinated mice compared with the $3 \times$ ID vaccination. Collectively, these data show that the addition of HA to the virosomes was necessary to deliver DNA to the mouse respiratory tract and an IN prime was necessary to induce humoral and cell-mediated immunity in the vaginal mucosa.
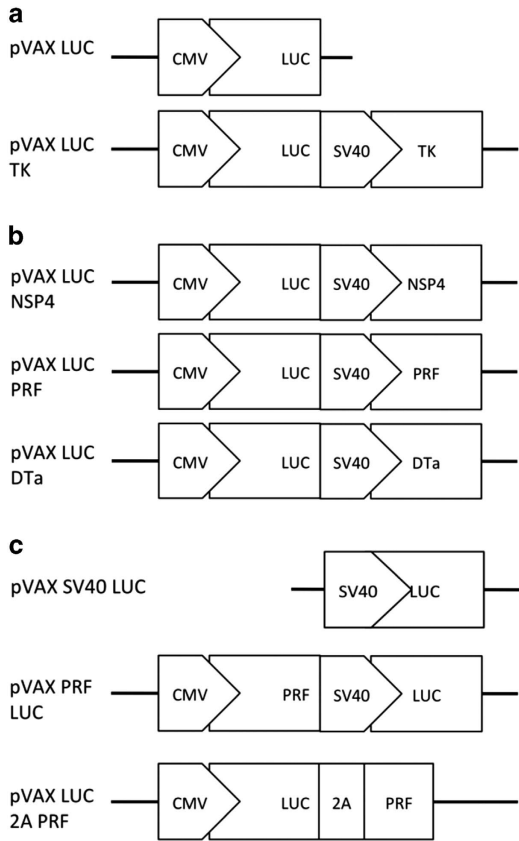

Figure 3 A schematic diagram of the plasmid constructs. The constructs were organised into three groups as noted in the figure. (**a**) TK constructs, (**b**) cytolytic gene constructs in which the cytolytic gene expression was controlled by the SV40 promoter and (**c**) cytolytic gene constructs in which the cytolytic gene expression was controlled by the CMV promoter.

Expression of different genes that induce cell death results in different cell death kinetics *in vitro*

As an alternative strategy to increase the efficacy of DNA vaccination, we wished to compare the efficacy of several proteins that induce cell
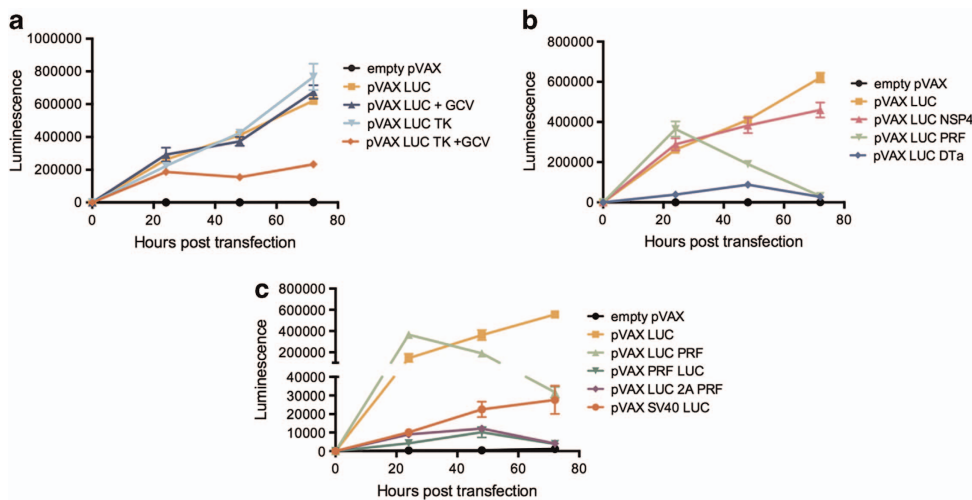

Figure 4 The induction of HEK293T cell death by cytolytic gene expression as determined by LUC expression. The cells were transfected with the different DNA constructs as noted in **a**, **b** and **c**, and LUC expression used as a measure of cell viability compared with the control pVAX LUC. **c** was published originally in reference number 21.
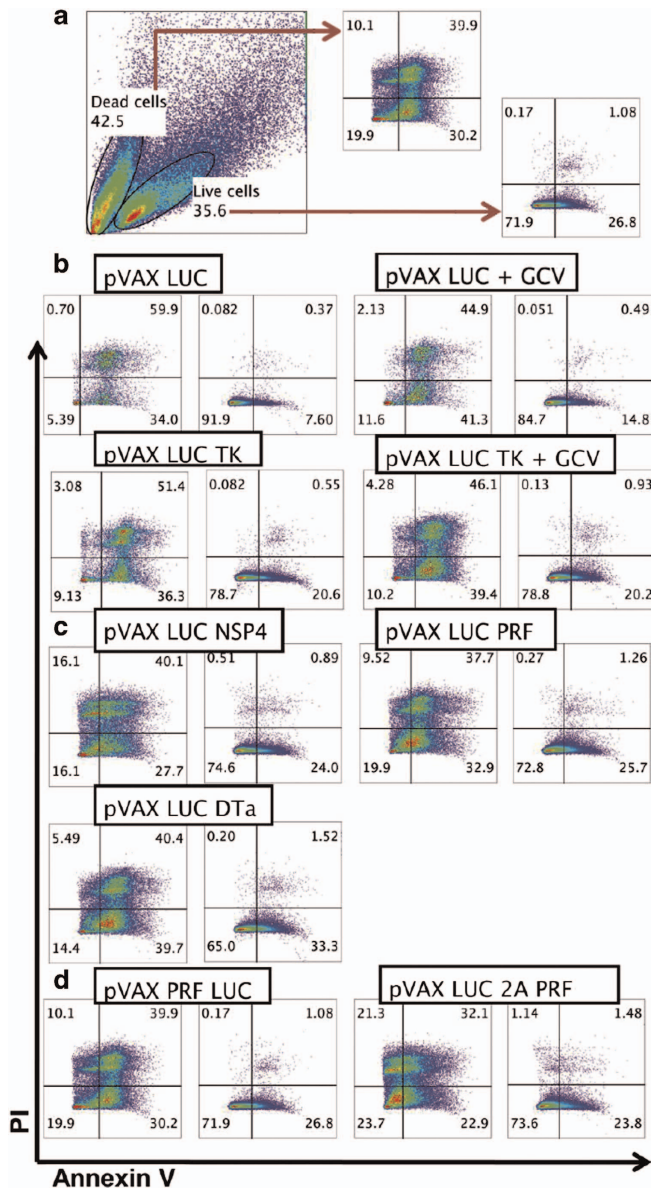
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Figure 5 Markers of cell death after transfection of HEK293T cells with the different DNA constructs, as determined by Annexin V and PI staining. (a) The gating strategy for the Annexin V (x axes) and PI (y axes) staining analysis. Two panels are shown for each construct; the left panel represents cells in the dead cell gate and the right panel represents cells in the live cell gate. (b) pVAX LUC, pVAX LUC TK, pVAX LUC + GCV and pVAX LUC TK + GCV, (c) pVAX LUC NSP4, pVAX LUC PRF and pVAX LUC DTa representing cytolytic genes expressed from the SV40 promoter and (d) pVAX PRF LUC and pVAX LUC 2A PRF representing cytolytic genes expressed from the CMV promoter.

death by different mechanisms when co-expressed with LUC as an immunogen, to examine the hypothesis that lytic or necrotic cell death would result in cross presentation of the immunogen and thus increase the efficacy of vaccination. The herpes simplex virus thymidine kinase (TK), the rotavirus non-structural protein 4 (NSP4),[19] diphtheria toxin subunit A (DTa) and a modified form of mouse perforin (PRF)[20] were examined. A schematic of the different constructs is shown in Figure 3. We have previously shown that the SV40 promoter is 10-fold less efficient than the cytomegalovirus (CMV) promoter,[21] while the foot and mouth disease virus 2A

protease self-cleaves resulting in equimolar concentrations of LUC and PRF.

Initially, induced cell death was examined by LUC expression in transfected cells and loss of LUC expression accepted as an indication of cell death. As proof of concept, cells which were transfected with DNA encoding LUC and TK ± the addition of ganciclovir (GCV) showed no evidence of cell death unless GCV was added to the culture (Figure 4a). We then examined the effect of co-expression of NSP4, DTa or PRF with LUC from constructs, which encoded the cytolytic/apoptotic genes under the control of the SV40 promoter. The results of this experiment showed that DTa was highly toxic as levels of LUC were always low when compared with the control, so that the luminescence was reduced by 90% at 48 h post transfection and none was detected at 72 h (Figure 4b). PRF reduced luminescence by 50% at 48 h and by 100% at 72 h; however, the kinetics of LUC expression differed markedly to that after co-expression of DTa. In contrast, NSP4 showed little toxicity (Figure 4b). The effect of expression of PRF from the CMV promoter, rather than the SV40 promoter was then examined; this showed that the kinetics of LUC expression were similar to that after DTa expression (Figure 4c) and confirmed that higher levels of PRF killed cells more rapidly.

The mechanism of cell death after cytolytic/apoptotic gene expression *in vitro*
We then wished to determine the mechanism of cell death as it has been suggested that necrotic cells are more immunogenic than apoptotic cells.[22] HEK 293 T cells were transfected with the different DNA constructs and 72 h later, cells that appeared in the live and dead cell gates by flow cytometry were examined by Annexin V and propidium iodide (PI) staining. The majority of cells in the dead cell gate were Annexin V$^+$, PI$^+$ (Figure 5), whereas the majority of cells in the live cell gate were Annexin V$^-$, PI$^-$. A proportion (around 20%) of cells after transfection with each construct was apoptotic, but a variable proportion, ranging from 32–60% was considered to be necrotic (either Annexin V$^+$ PI$^+$ or AnnexinV$^-$ PI$^+$). This population is likely to represent secondary necrotic cells that develop markers of necrosis in a time-dependent manner. The staining revealed that, of the dead cells observed following TK/GCV expression, 39% were apoptotic and 50% were necrotic, whereas the NSP4 induced 28% apoptosis and 56% necrosis, PRF induced 33 and 47%, respectively and DTa, 40 and 45%. The highest proportions of dead cells were detected after transfection with plasmids, which encoded PRF under the control of the CMV promoter. In summary, the expression of NSP4 and PRF resulted in the highest proportion of PI$^+$ cells, suggesting necrotic or lytic cell death, whereas DTa and TK/GCV preferentially induced apoptotic cell death.

The effect of cytolytic gene expression on LUC expression *in vivo*
To examine the effect of cytolytic/apoptotic gene expression on LUC expression *in vivo*, the expression of LUC was examined in vaccinated mice by live imaging. The efficacy of *in vivo* delivery of DNA encoding LUC was first examined by direct imaging after ID injection of either naked DNA or HA + virosome-encapsulated DNA but no difference was detected (data not shown), showing that naked DNA was equally effective as virosome-encapsulated DNA in this setting. We therefore decided to use naked DNA to conduct *in vivo* experiments to test the effect of induction of cell death following DNA vaccination. In contrast to the effect *in vitro*, expression of TK/GCV had no effect on LUC expression, most likely because TK/GCV only kills actively dividing cells (Figure 6a). DTa and PRF, however, accelerated the loss of LUC-positive cells; DTa resulted in a 10-fold reduction in the initial
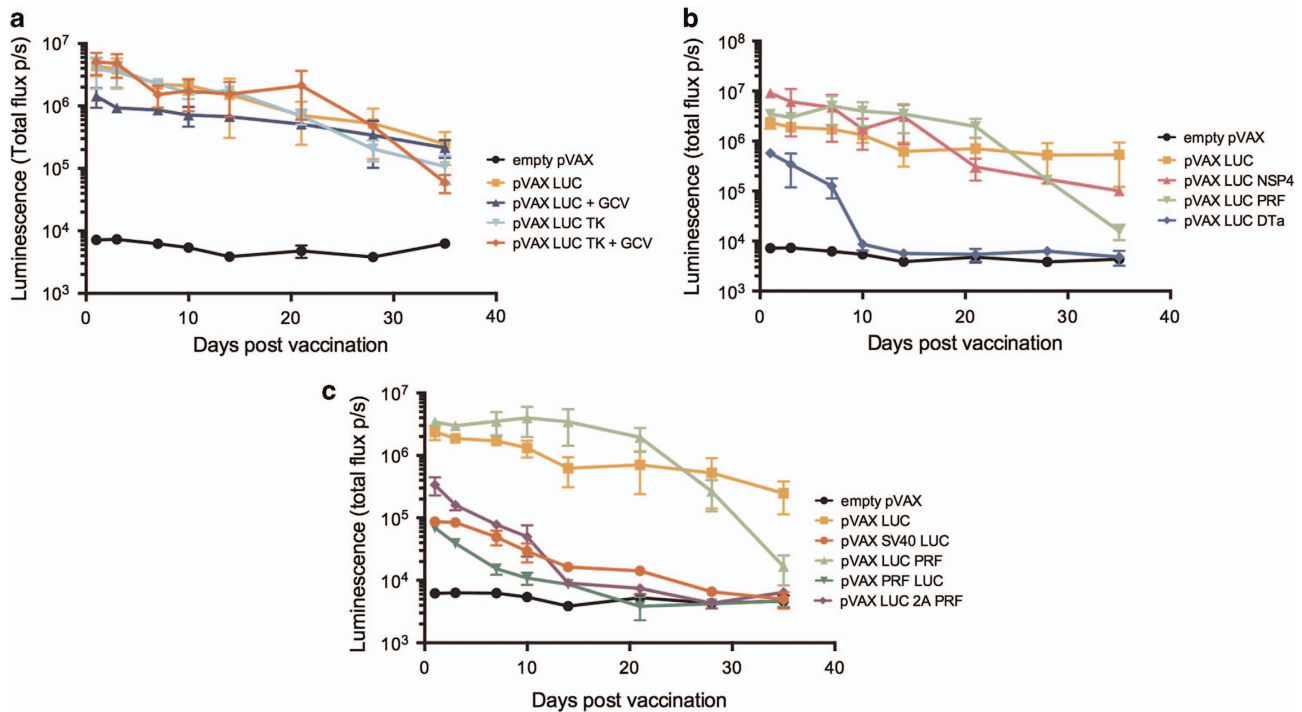
Figure 6 *In vivo* cell death as determined by LUC expression post vaccination. C57Bl/6 mice were vaccinated with 50 µg DNA and luminescence detected by IVIS imaging at intervals post vaccination. (**a**) pVAX LUC, pVAX LUC TK, pVAX LUC + GCV and pVAX LUC TK + GCV, (**b**) pVAX LUC NSP4, pVAX LUC PRF and pVAX LUC DTa representing cytolytic genes expressed from the SV40 promoter and (**c**) pVAX PRF LUC and pVAX LUC 2A PRF representing cytolytic genes expressed from the CMV promoter. Graphs show the mean luminescence ± s.e.m. for five mice as measured by the IVIS live imager. **c** was published originally in reference number 21.

level of LUC expression and in a rapid loss of LUC-positive cells, whereas PRF showed a slower loss (Figure 6b). Consistent with the *in vitro* data, expression of PRF from the CMV promoter resulted in a rapid loss of LUC-positive cells (Figure 6c).

The effect of cytolytic gene expression on the LUC-specific immune response

The above results showed that expression of DTa and PRF from the CMV promoter resulted in greater cell death than expression of NSP4 or TK (+ GCV) and this might be expected to result in higher levels of immune activation. To examine this, the LUC-specific immune response was measured in vaccinated mice. The antibody response was assessed by enzyme-linked immunosorbent assay, and, consistent with the fact that TK/GCV had no effect on LUC expression, expression of TK/GCV failed to increase the anti-LUC antibody titre over that elicited by the canonical vaccine (Figure 7a). In contrast, mice vaccinated with DNA encoding LUC PRF showed a trend towards increased IgG titres, but this was not significant after statistical analysis (Kruskal–Wallis and Wilcoxon post test; Figure 7b). Mice vaccinated with the DTa construct developed 70-fold lower IgG titres than the control mice (Figure 7b), although this difference was not statistically significant.

The cell-mediated immune responses were also measured by stimulation of splenocytes with a C57BL/6 immunodominant LUC peptide.[23] The results of this analysis showed that expression of NSP4 and PRF (from the SV40 promoter) increased the cell-mediated immune responses (Figures 7c and d), although the results were not statistically significant, whereas expression of DTa and PRF (the latter from the CMV promoter) resulted in a lower cell-mediated response.

DISCUSSION

In this study, we examined two different strategies to increase the efficacy of DNA vaccines. The first involved encapsulating the DNA in HA + virosomes, which bind to sialic acid receptors and deliver it to respiratory epithelial cells after IN delivery. The second strategy induced cell death in vaccine-targeted cells after ID vaccination by the inclusion of a gene encoding cytolytic or toxic proteins in the DNA vaccine and evaluated the resultant immune responses. The uptake of viral antigen-positive dead or dying cells by DC and other antigen-presenting cells represents a fundamental mechanism to elicit immunity against pathogens that do not intrinsically infect DC and we plan to exploit this natural mechanism to increase DNA vaccine efficacy.

DNA contained in virosomes with HA embedded in the lipid membrane was able to prime the immune system to elicit mucosal humoral and cell-mediated immunity after ID booster injections. The induced immune response to HA reflects the immunity induced to recombinant virus vectors after multiple doses, necessitating the development of heterologous vector vaccination regimens. However the IN/ID regimen with a DNA vaccine, developed in this model system, will overcome this limitation, and has the potential to elicit robust mucosal immunity. Although the responses were low, LUC is known to be a poor T-cell immunogen,[24] and it is likely that the responses to a more immunogenic protein will be higher. Our IN/ID strategy is similar to an IN/intramuscular strategy described previously.[25]

We also examined the ability of cytolytic or apoptotic genes to increase the immune response to an immunogen encoded in a bicistronic plasmid. We hypothesised that antigen-positive, necrotic cells would release damage-associated molecular patterns along with
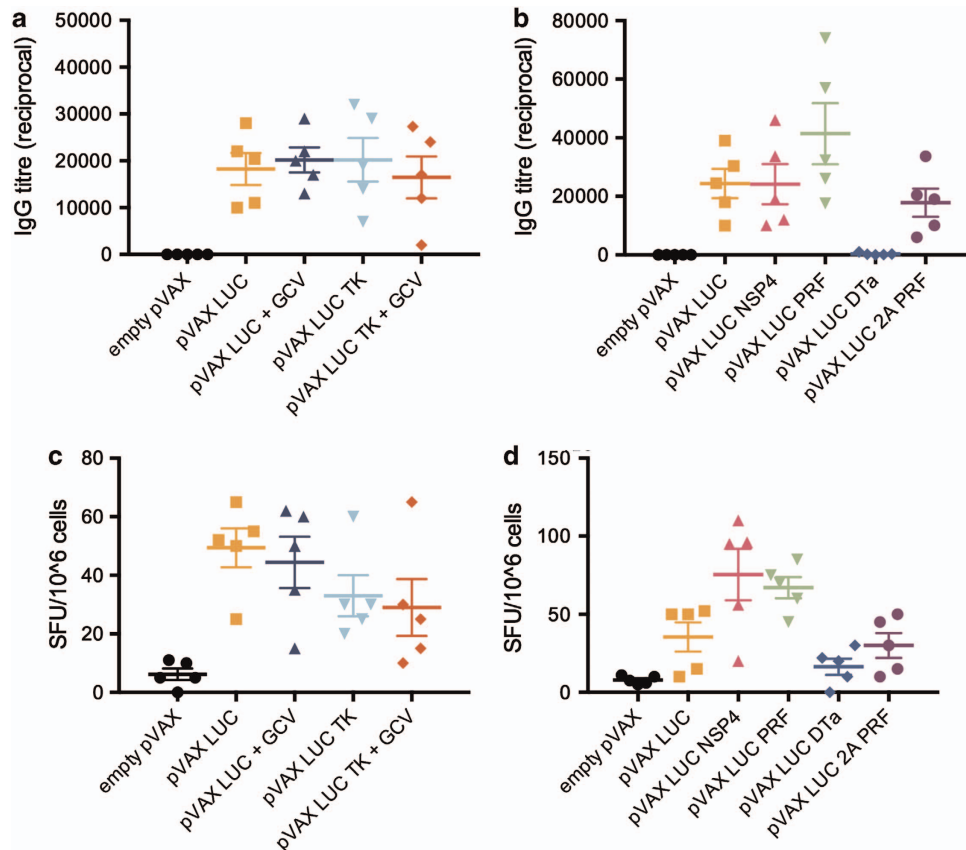
Figure 7 Humoral and cell-mediated responses to LUC in mice vaccinated with the different DNA constructs. IgG titres were determined by enzyme-linked immunosorbent assay in serum from mice vaccinated with (**a**) the LUC ± TK constructs or (**b**) LUC + the directly cytolytic genes. Graphs show mean reciprocal IgG titre, ± s.e.m. IFN-γ-secreting cells after restimulation of splenocytes with an immunodominant epitopes as detected by ELIspot in mice vaccinated with (**c**) the LUC ± TK constructs or (**d**) LUC + the directly cytolytic genes. Graphs show meant SFU per 10^6 cells, ± s.e.m.

the antigen that would be phagocytosed by circulating DC and result in cross presentation of the antigen and maturation of the DC. The results of the study showed that, of the proteins controlled by the SV40 promoter, NSP4 and PRF increased the immune responses to LUC, whereas TK/GCV and DTa, and PRF driven from the CMV promoter, either had no effect or decreased the immune responses. The data suggest that there is a threshold of expression of the immunogen that must be achieved before necrosis is induced in the vaccine-targeted cells. As F-actin exposed on necrotic cells is recognised by CLec9A on DC,[26] it is likely that NSP4 and PRF expression (from the SV40 promoter) increased the frequency of cross-presenting DC and that this represented the mechanism of increased immune responses. Furthermore, these studies with the model antigen LUC provide a greater understanding of the effect of different cytolytic proteins and we have shown that co-expression of PRF with a highly immunogenic viral antigen, HIV gag,[21] not only resulted in increased levels of gag-specific cell-mediated immunity but also in greater levels of protection against challenge with a chimeric HIV, EcoHIV, that replicates in mice.[21,27]

Although the potential of DNA vaccines has not been realised in humans, our previous studies[21,28] and this study have outlined different strategies to increase efficacy that might be combined in the case of mucosal vaccines to elicit robust mucosal immunity. We have also shown that PRF when co-expressed with the hepatitis C virus NS3 protein results in greatly increased cell-mediated immune responses in large adult pigs (Grubor-Bauk, in preparation), suggesting that the strategy may be equally effective in humans.

METHODS

Virosome preparation
The virosomes were prepared essentially as described[7] using the cationic lipid DOTAP, with one major difference, as recombinant HA (Protein Sciences, Meriden, CT, USA) rather than HA from egg-derived influenza virus was added to the DNA–DOTAP suspension. The preparation was pelleted (30 K r.p.m., 1.5 h at 4 °C) on to a 50% sucrose cushion to remove free HA from liposome-associated HA and the liposomes collected from the interface.

DNA vaccines
All DNA vaccines were prepared by standard molecular biology methods. The vaccines were based on the plasmid pVAX (Invitrogen, Melbourne, VIC, Australia) and encoded firefly LUC under the control of the CMV promoter. DNA constructs (Figure 3) designed to induce cytolysis in vaccine-targeted cells contained the LUC gene controlled by the CMV promoter, and various cytolytic genes controlled by the SV40 promoter or alternatively as a LUC-cytolytic polyprotein controlled by the CMV promoter that was designed to autocleave by including the FMDV2a protease, essentially as described.[21] The constructs were sequenced to confirm authenticity and endotoxin-free preparations were used for vaccination.

Vaccination and sample collection
Six to eight-week-old female C57Bl/6 mice, bred in specific pathogen-free conditions, were purchased from the University of Adelaide and housed at the Women's and Children's Hospital PC2 animal facility. Ethical approval was obtained from the Women's and Children's Health Network and the University of Adelaide Animal Ethics Committee. The mice were anaesthetised with domitor/ketamine for all procedures. ID injections were performed by delivery of the DNA to the ear. IN vaccination was performed by delivery of DNA in

50 µl saline essentially as described.[29] Blood was collected by retro-orbital bleed. Lavage samples were collected using phosphate-buffered saline containing 0.5 mM phenylmethanesulfonyl fluoride as a wash. Vaginal lavage was performed by inserting a pipette tip into the vaginal opening and repeatedly pipetting a volume of 50 µl in and out. Bronchial lavage was performed by opening the skin covering the thorax, tying off the trachea and 50 µl of phosphate-buffered saline/phenylmethanesulfonyl fluoride injected into the trachea. The lavage was collected in an eppendorf tube placed over the nose of the mouse. Splenocytes were prepared as described[21] and immunocytes from draining lymph nodes were prepared in a similar manner, essentially by gentle passage through a 70-µm cell strainer. The cells were resuspended in RPMI culture media before analysis. Live imaging was performed using a Xenogen IVIS 200 live imaging system (Caliper Life Sciences). The mice were injected with D-luciferin either by the intraperitoneal route or by 50 µl applied to the nares, rested for 10 min and then placed in the imaging chamber.

ELIspot

Briefly, 5×10^5 splenocytes were added to duplicate wells and stimulated with 4 µg ml^{-1} LUC peptide for 40 h at 37 °C, essentially as described.[21] Spots were formed by the addition of BCIP/NBT (Sigma, Castle Hill, NSW, Australia) and left to develop in the dark for up to 30 min. Plates were read on an AID EliSpot Reader (Autoimmun Diagnostika, Strassberg, Germany) and analysed by the AID EliSpot software (Autoimmun Diagnostika). The average number of spots (spot-forming units; SFU) from the negative control was subtracted from each stimulated sample and the data adjusted to SFU per 10^6 splenocytes.

Flow cytometry

To assess if suicide gene candidates induced necrosis or apoptosis, HEK293T cells were plated in 24-well plates and transfected with plasmid DNA as described above. Cells were collected at 72 h time points and stained with mouse Annexin V-FITC (BD #556420) and PI (BD #556463) according to the BD staining protocol. Cells were analysed on a BD FACS Canto and analysed with FlowJo software, essentially as described.[21]

Statistical analysis

Throughout the study, statistical analysis was performed by Kruskal–Wallis multiple comparison with Wilcoxon post-tests, to allow for nonparametric data and to correct for multiple comparisons. Data in graphs are presented as the mean $+ / -$ s.e.m. Data analysis and generation of graphs were performed using Graphpad Prism 5.0b and SAS Version 9.3, with assistance from the Data Analysis and Management Centre, University of Adelaide. Nonparametric Kruskal–Wallis test was used to compare the difference between the multiple vaccine groups. If the global test showed significant difference between the groups, then Wilcoxon tests were performed to compare the *post hoc* difference between groups.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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