Original Article

Comparative analysis of enzymatically produced novel linear DNA constructs with plasmids for use as DNA vaccines

AA Walters1,3, E Kinnear1, RJ Shattock1, JU McDonald1, LJ Caproni2, N Porter2 and JS Tregoning1

The use of DNA to deliver vaccine antigens offers many advantages, including ease of manufacture and cost. However, most DNA vaccines are plasmids and must be grown in bacterial culture, necessitating elements that are either unnecessary for effective gene delivery (for example, bacterial origins of replication) or undesirable (for example, antibiotic resistance genes). Removing these elements may improve the safety profile of DNA for the delivery of vaccines. Here, we describe a novel, double-stranded, linear DNA construct produced by an enzymatic process that solely encodes an antigen expression cassette, comprising antigen, promoter, polyA tail and telomeric ends. We compared these constructs (called ‘Doggybones’ because of their shape) with conventional plasmid DNA. Using luciferase-expressing constructs, we demonstrated that expression levels were equivalent between Doggybones and plasmids both in vitro and in vivo. When mice were immunized with DNA constructs expressing the HIV envelope protein gp140, equivalent humoral and cellular responses were induced. Immunizations with either construct type expressing hemagglutinin were protective against H1N1 influenza challenge. This is the first example of an effective DNA vaccine, which can be produced on a large scale by enzymatic processes.

INTRODUCTION

In the 1990s, it was demonstrated that when antigen-encoding nucleic acid is introduced into the body it can invoke an immune response against the encoded antigen.1 DNA vaccines have many advantages over traditional strategies: they are relatively safe compared with attenuated vaccines, they are stable and can be stored frozen or lyophilized, they are easy to manufacture and can stimulate cell-mediated immune responses.2 Although there are DNA vaccines licensed for veterinary use3 and DNA vaccines have been shown to be effective in numerous models, currently there are no DNA vaccines licensed for human use.4,5 The main hurdle regarding the latter two points.6

Conventionally DNA vaccines are produced as plasmids grown in genetically modified bacteria, normally Escherichia coli. Such plasmids must contain, alongside the gene of interest, a bacterial origin of replication and a selective gene, normally encoding antibiotic resistance, to maintain the persistence of the plasmid in the bacterium.7 Although DNA vaccines have proven to be safe in a number of animal models and early-phase clinical trials, there is a concern that the antibiotic resistance genes in the DNA vaccine may be conveyed to pathogenic bacteria or microflora. The use of antibiotic resistance genes has previously been negated through the use of RNAi ‘RNA-OUT’ as a counter selectable marker or auxotrophic selection.8,9 Such methods can increase production efficiency by decreasing the metabolic burden on the bacteria.10 However, the reliance on bacteria as a method to produce the vaccine means there is a small chance of recombination events within the plasmid, leading to the loss of the antigenic determinant.11

To avoid problems with recombination events, extensive quality controls at a molecular level need to be installed on a batch-by-batch basis. In addition, the presence of endotoxin within the system means that, after extraction, DNA must be cleaned and further quality control measures applied.12

The use of minimal DNA constructs, for example, minicircles, small circular fragments of DNA derived from a larger plasmid13 or minimalistic immunologically defined expression vectors,14 that only encode an antigen expression cassette (promoter, antigen and polyA region), goes some of the way to solve the problem of extraneous elements. These vectors have been shown to be immunogenic, inducing both a cellular and humoral response, but both of these vectors require a bacterial fermentation step.15 Synthesizing the vaccine entirely in vitro, with the absence of a bacterial step, would ensure uniformity between batches and increase their readiness for good manufacturing practice production. Recently, the molecular tools by which to do this have become available in the form of enzymes derived from bacteria and bacteriophages. Using a proprietary method from Touchlight Genetics, covalently closed linear DNA constructs can be made synthetically, which permits rapid vaccine design and manufacture. The DNA vectors produced by the process are referred to as ‘Doggybones’ (DBs) owing to their proposed shape and are synthesized in controlled batch reactions.

The aim of this study was to characterize DB constructs for use as DNA vaccines and to compare them with plasmids. We demonstrated that DBs are comparable to conventional plasmids in terms of expression and immunogenicity. This study therefore concludes that DBs could be a suitable replacement for plasmid DNA for gene delivery in the context of DNA vaccines.

1Mucosal Infection & Immunity Group, Section of Infectious Diseases, Department of Medicine, Imperial College London, St Mary’s Campus, London, UK and 2Touchlight Genetics Ltd., Leatherhead Food Research Institute, Leatherhead, Surrey, UK. Correspondence: Dr J Tregoning, Mucosal Infection & Immunity Group, Section of Infectious Diseases, Department of Medicine, Imperial College London, St Mary’s Campus, London W2 1PG, UK. E-mail: john.tregoning@imperial.ac.uk

2Current address: The Jenner Institute, University of Oxford, Oxford OX3 7DQ, UK.

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RESULTS

Production of linear closed DNA constructs

Linear DNA constructs were produced using the outlined mechanism (Figure 1a). The process is composed of two steps; first plasmid DNA that has the sequence for the antigen flanked by telRL sites is amplified by rolling circle replication using phi29 DNA polymerase from Bacillus subtilis phage phi29, resulting in the production of long concatamers. The protelomerase TelN (from E. coli phage N15) then cleaves the concatamers into strands containing a single cassette and seals the ends with a short hairpin loop. The constructs are composed of a linear double-stranded region with an antigen expression cassette, encoding the sequences for the cytomegalovirus immediate early promoter plus enhancer, the gene of interest and the SV40 late polyA tail, flanked by single-stranded telomere ends (Figure 1b). In the initial round of amplification, plasmid DNA is used as a template, but this is then selectively digested with restriction enzymes and then exonuclease III. In subsequent rounds of amplification the DB itself can be used as the template.

To confirm that hairpin loops had formed, DNA migration was compared on denaturing or native gels. A representative DB construct (DB GL derived from pGL DOG containing a luciferase cassette) was compared with linear DNA (PCR GL) encoding the same sequence but derived through PCR. The only structural difference is that the DBs contain covalently closed telomere ends, whereas the PCR products contained open ends. On the native gel, the constructs migrated at a similar speed reflecting the similarity in size (2.4 kb for DB GL and 2.7 kb for PCR GL) (Figure 1c). However, on a denaturing gel the DB became a large open single-stranded circular structure and thus appeared larger than the equivalent linear PCR construct, which became single DNA strands (Figure 1d). A similar observation was made when a plasmid containing the telRL sites was linearized by protelomerase TelN—resulting in hairpin-ended DNA or restriction endonuclease—resulting in open-ended DNA (Figures 1c and d).

The plasmid and DB constructs that were used in subsequent imaging studies, were characterized using dynamic light scattering over a pH gradient. Both plasmid and DB show similar trends and...
were at their largest (Figure 1e) and most cationic (Figure 1f) at low pH. They became more condensed and more negatively charged as the solution became more basic. The two constructs behaved remarkably similar given the differences in structure and molecular weight.

Comparative expression from plasmid or DB constructs
To compare expression levels, we made plasmid and DB constructs that encoded the firefly luciferase gene and transfected CHO-K1 cells. In tests to compare delivery of both DNA constructs by electroporation (EP), both the DB and the plasmid were transfected with equal efficiency (Figure 2a). Owing to the novelty of the DB structure, we wished to determine whether there were differences in the mechanisms by which transfection reagents package the DNA. We compared a liposome formulation (Lipofectamine), a polycationic complex formulation (PEI) and a branched dendrimer formulation (Polyfect). We reasoned that, having been optimized for circular supercoiled plasmids, the reagents may compact linear DNA constructs differently, leading to complexes with differing physical parameters and capacity to transfect. Although Lipofectamine and Polyfect have manufacturer optimized protocols, PEI-mediated transfection was optimized to balance nitrogen to phosphate ratios, an 8:1 PEI:DNA ratio gave optimum expression with both constructs (data not depicted). We observed comparable levels of expression of luciferase with DB and plasmid with either Lipofectamine (Figure 2b) or PEI (Figure 2c), but when Polyfect was used as the transfection reagent, plasmid DNA had significantly higher transfection efficiency then DB at 48 h ($P < 0.01$, Figure 2d). To define why different reagents behaved differently, we measured the size (Figure 2e) and charge (Figure 2f) of the DNA-transfection reagent complexes at pH 7.

**Figure 2.** Doggybone and plasmid have similar expression levels in vitro. The efficiency of transfection of 1 μg Doggybone (○) or plasmid (□) encoding luciferase in CHO-K1 by electroporation (a), Lipofectamine (b), PEI (c) or Polyfect (d) was tested. Light emission was recorded as relative light units (RLU). The size (e) and the charge (f) of complexes formed by each transfection reagent at pH 7 was characterised by dynamic light scattering. Bars/points represent mean of $n = 3$ replicates ± s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, calculated using analysis of variance and Bonferroni’s post test.
complexes using dynamic light scattering. Lipofectamine-plasmid constructs had a significantly more negative charge than Lipofectamine-DB constructs ($P < 0.01$), but this appears not to alter the capacity to transfecct cells, however, Polyfect-plasmid constructs were significantly larger ($P < 0.01$) than Polyfect-DB constructs suggesting, for this reagent, the secondary structure of DNA had an important role. We wished to confirm the expression data using a different gene readout system and observed similar transfection efficiency between DB and plasmids expressing the red-fluorescent protein tdTomato (Supplementary Figure 1).

Having validated gene expression from DBs in vitro, we sought to confirm these observations in vivo. To this end mice were immunized with luciferase-expressing constructs intramuscularly (IM) with EP. Light emitted after intraperitoneal luciferin injection was measured by in vivo imaging and images overlaid with X-ray images of the same animals to indicate the position of expression, background photon detection was eliminated by the concurrent imaging of control, untransfected animals (Supplementary Figure 2). Luciferase gene expression was detectable in vivo from both the plasmid and the DB constructs (Figure 3a). Relative expression levels were quantified and there were no significant differences between DB and plasmid (Figure 3b), however, there was a trend for reduced expression from the DB constructs on day 7. As DBs are smaller than plasmid DNA, in a mass-matched study, more gene copies are introduced, therefore we compared mass- and molarity-matched in vivo luciferase expression from the two constructs (Figure 3c). There was a trend toward reduced expression in expression after DB transfection compared with the molarity-matched plasmid group. In these studies, we show that genes were expressed from DBs and plasmid at comparable levels in vitro and in vivo.

Comparative immunogenicity of plasmid- and DB-expressed antigens

To test the immunogenicity of DB-delivered genes, HIV clade C MWS2 gp140 (the envelope protein gp120 and the transmembrane protein gp41) and hemaglutinin (HA) from H1N1 influenza (A/England/195/2009) were chosen as model antigens. For the HIV gp140 studies, we used a prime boost regime of four IM DNA immunizations with EP 2 weeks apart followed by a single IM protein boost. EP was used as it gives a significantly enhanced

Figure 3. In vivo assessment of DNA construct expression levels. BALB/c mice ($n = 5$) were injected IM with 25 μg Doggybone ($\bullet$) or plasmid ($\square$) encoding luciferase by electroporation. At days 2, 3 and 7 after inoculation, expression was visualized using a Carestream in vivo imaging system after intraperitoneal injection of Rediject luciferin, one representative image shown per time point (a). Luciferase levels from $n = 22$ animals were quantified as relative light units (RLU) using the Carestream analysis software (b), points represent individual mice, the line represents mean ± s.e.m. Mice were injected with 50 μg Doggybone and compared with a mass-matched plasmid (50 μg) or concentration matched plasmid (85 μg) expressing luciferase and expression was measured by in vivo imaging over time (c), points represent mean of $n = 4$ mice ± s.e.m. NS denotes $P > 0.05$ analyzed by analysis of variance and Bonferroni's post test.
response to in vivo DNA delivery. All immunizations were well tolerated and no adverse reactions were observed.

Both DB and plasmid groups developed comparable gp140-specific immunoglobulin (IgG) levels with the group mean at 734 and 675 ng/ml, respectively; these were significantly greater than the group receiving protein alone but not significantly different from each other (Figure 4a). Interestingly, it was only after the protein boost that an antibody response could be detected. Cellular responses against peptide pools comprising peptides derived from the N terminus (pool 1) or C terminus (pool 2) of CN54 gp140 were measured using interferon-γ secretion as a readout. As with humoral responses, we observed a comparable cellular response to both the DB and the plasmid. The plasmid group had an average of 191 and 306 interferon-γ-spot-forming units for peptide pools 1 and 2, respectively, whereas the DB group had an average of 154 and 280 spot-forming units (Figure 4b). There were negligible amounts of cells secreting interferon-γ in the phosphate-buffered saline (PBS)-primed group; two animals were excluded from the DB group because the ELISPOT wells were too numerous to count.

DNA constructs expressing influenza HA were tested for their ability to protect mice from challenge with a homologous H1N1 influenza strain (A/England/195/2009). Mice received three IM DNA immunizations with EP 2 weeks apart before intranasal infection with H1N1. Prechallenge HA-specific IgG could be detected in both the DNA-vaccinated groups but not in the control group (Figure 4c). The DB and plasmid protected animals from weight loss after infection, whereas the PBS control group lost significantly more weight at days 6 and 7 after infection (P < 0.01, Figure 4d). Viral RNA could be recovered from the lungs of every mouse in the group receiving PBS, whereas no virus could be isolated from the lungs of animals receiving either DNA construct signifying protection (Figure 4e). After challenge, there were higher levels of HA-specific lung IgA in the DB and plasmid groups than the PBS control group (Figure 4f). We show that the DB and the plasmid DNA both induce an immune response to an antigen, and when used as a vaccine can protect from viral infection.

**DISCUSSION**

The objective of this study was to compare closed linear DNA generated through enzymatic processes with circular, bacterially derived plasmids, for use as DNA vaccines. In this study, we did not observe any difference between the expression levels of luciferase when delivered as a DB or a plasmid. Although we studied the role of DBs as DNA vaccines, the equivalence in expression levels with the plasmid suggests that these constructs could also be used for other gene delivery purposes. There are several factors that contribute to the expression levels from transfected DNA constructs; these include construct size, DNA topology with linear DNA transfecting less well than supercoiled plasmid and the number of CpG motifs affecting the time it takes for the immune system to recognize and thus clear the

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**Figure 4.** In vivo immunogenicity assessment of DNA constructs expressing HIV gp140. BALB/c mice (n = 5) were inoculated four times IM with electroporation with 25 μg gp140 encoding plasmid (□). Doggybone (●) or phosphate-buffered saline (PBS) (▲) as a negative control. At day 56, all groups received 10 μg of recombinant gp140 IM. Serum was collected on day 70 and gp140-specific IgG levels were tested using enzyme-linked immunosorbent assay (a). On day 70, splenocytes were taken and restimulated with synthetic peptides representing the N (pool 1) or C (pool 2) terminus of the gp140 protein, interferon-γ was measured using ELISPOT (b). BALB/c mice (n ≥ 4) were inoculated IM with electroporation three times with 25 μg influenza HA encoding plasmid (□). Doggybone (●) or PBS (▲) as a negative control. At day 42, mice were infected with 10^5 plaque-forming unit H1N1 influenza intranasally. Antibody undefined was calculated on d41 before infection (c). Weight loss was monitored following infection (d); lung viral load (e) and lung IgA (f) were measured on day 7 after infection. Points represent individual animals, lines represent mean ± s.e.m. **P < 0.01, ***P < 0.001, analyzed by analysis of variance and Bonferroni’s post test. For panel d, points represent mean of n = 5 animals ± s.e.m. **P < 0.01, ***P < 0.001 Doggybone compared with the PBS group; **P < 0.01, ***P < 0.001, plasmid compared with the PBS group.
transfected cells. The equivalence in response between the DB and plasmid reflects the balance between a number of these factors. For example, although DBs contain less DNA per unit weight as plasmids, they occupy the same space in physiological environments probably owing to supercoiling of the plasmids. Differences were observed between Polyfect (based upon dendrimers complexing the DNA) and the charged reagents, PEI and Lipofectamine, which may reflect the differences in charge and structure of the plasmid and DB. Another linear minimal construct, the minimalistic immunologically defined expression vector, also has comparable expression constructs, the minimalistic immunologically defined expression vector, also has comparable expression in vitro and in vivo as plasmid DNA. 14,27 Future work will investigate formulating DB to improve expression levels, for example, formulating with PEI as has previously been performed with minicircles. 28,29

We also observed an equivalent immune response to antigens expressed from either DB or plasmid. Using influenza challenge, we were able to demonstrate that the immune response to DNA vaccine alone was protective and that there were no differences between plasmid and DB. Immunization induced both a cellular and a humoral response, although for the HIV antigen, protein boosting was required for an antibody response. In part, the poor antibody response to the gp140 vaccination may be due to the enzyme-linked immunosorbent assay which, because of reagent availability, was coated with a different clade C gp140–CN54 to the vaccine antigen MWS2, reducing the sensitivity. However, it has been previously observed that DNA vaccines induce a better cellular response than humoral response, and we observed a strong cellular response in our studies. The use of DNA vaccines in humans has so far had limited success, with poor translation from preclinical models to clinical trials. However, the use of EP as in the current study has been shown to considerably improve the immunogenicity of a DNA vaccine. 15 Increased immunogenicity with electroporated DNA vaccines is associated with increased expression of the delivered DNA and potentially also increased inflammation due to the tissue damage after the application of the electric field. 12

Until now the most promising candidates for minimal DNA constructs were minicircles. These are circular plasmids containing only the gene of interest and the necessary regulatory elements that are excised from a larger parental plasmid grown in bacteria. 13 Minimalistic immunologically defined expression vectors are probably the second most prominent candidate, comprised of linear constructs sealed with hairpin loops that are derived from a plasmid with restriction endonucleases and sealed enzymatically. 13 Although these constructs address many of the concerns outlined by the food and drug administration, such as integration, allergy and the presence of antibiotic resistance genes, the fact that they are still produced in bacteria represents a potential regulatory concern. 34 Here, we describe a third candidate minimal DNA construct for gene delivery, which does not require a bacterial production step—Doggybone DNA. In much the same way as PCR allowed for amplification of specific DNA through the actions of enzymes, the DB process allows for the production, en masse, of good manufacturing practice standard DNA constructs for use as gene delivery vectors from a template.

MATERIALS AND METHODS

DNA constructs

The proTLx expression plasmid consisted of the cytomegalovirus immediate early promoter plus enhancer, a multiple cloning site and an SV40 late polyadenylation signal all flanked by two telRL sequences, the site of protelomerase TelN recognition and cleavage. The plasmid backbone contained an ampicillin resistance gene and the pUC origin of replication. The Luciferase gene from Photorhabdus pyralis (firefly), the gene encoding gp140 from HIV-1 Clade C MSW2 and the hemaglutinin gene from H1N1 Influenza (A/England/195/2009) were cloned (including a GCCACC Kozak sequence) into the HindIII and EcoRI sites on the proTLx base plasmid by restriction digestion. Plasmids were maintained in recombinant deficient E. coli strains. For use in vivo, a plasmid was prepared from E. coli using an EndoFree Giga kit (Qiagen, Manchester, UK). Recombinant plasmids were verified by restriction endonuclease digestion and sequencing.

Purification of TelN protelomerase

The gene encoding protelomerase TelN, from E. coli phage N15, was cloned into pHQE-30 UA (Qiagen) under the control of the isopropyl-β-d-thiogalactopyranoside-inducible promoter from phage T5. The enzyme was overproduced in E. coli M15 pREP4 cells (Qiagen), with an N-terminal 6 x His histidine tag, and purified on a HiTrap column (GE Healthcare, Chalfont St Giles, UK) using a linear gradient of imidazole (0–500 mM) and standard purification techniques. The eluted protein was buffer exchanged into 10 mM Tris-HCl pH 7.4, 75 mM NaCl, 50% (v/v) glycerol, 1 mM dithiothreitol and 0.1 mM EDTA and stored at a concentration of 15 μM at –20°C.

Preparation of closed linear DNA (DBs)

Plasmid was prepared using a miniprep kit (Qiagen) and used as a template for rolling circle amplification by the method described in the patent EP2391731. 14 The template plasmid containing the gene of interest flanked by telRL sites was mixed with custom primers (50 μM), and the template was denatured by heating to 95°C. To initiate rolling circle amplification from the denatured template, the reaction was first mixed with reaction buffer (30 mM Tris-HCl pH 7.4, 5 mM (NH4)2SO4, 30 mM KCl, 7.5 mM MgCl2, 2 mM dithiothreitol) and then 2 mM dNTPs (BioLine, London, UK) were added together with 2000 units of Phi29 DNA polymerase (Lucigen, Middleton, WI, USA) and 4 units of thermostable pyrophosphatase (Lucigen). Upon mixing, the reaction was incubated at 30°C for 18h with custom primers (2 μM) and 2 mM dNTPs. In order to produce DBs by cleaving at the telRL sites and capping the double-stranded ends, the protelomerase TelN (1 μM) was added to the reaction and the reaction mixture was incubated at 70°C for 10 min. The amplified concatamers were processed by adding 2 x unit excess of ApoLI restriction enzyme (NEB) and 1.5 M excess amounts of the single-turnover TelN protelomerase. This cleaved the concatamers to produce DB DNA encompassing the expression cassette containing luciferase, gp140 or HA and a second DB consisting of the backbone components, which was also cleaved by ApoLI to produce three fragments. These backbone fragments were digested by the addition of 500 U mg−1 DNA exonuclease III. The reaction was monitored until all the backbone fragments had been removed by exonuclease. The DB DNA was purified using Cleanup Maxi columns (A&A Biotechnology, Gdynia, Poland) following the manufacturer’s instructions, and eluted into Tris EDTA buffer at pH 8 or H2O. The resulting elutions were concentrated using 50 MWCO Amicon columns (Millipore, Bedford, MA, USA) to a final concentration of 1 ng ml−1.

DNA hairpin assay

Two different telRL-containing plasmids were constructed to investigate the ability of TelN to cleave DNA to produce the hairpin ends. The commercially available Luciferase-encoding plasmid pGL4.13 (Promega, Madison, WI, USA) was modified to include two telRL sequences flanking the Luciferase cassette. It was hereafter named pGL DOG with the telRL expression region was created. pUC18 containing the ability of TelN to cleave DNA to produce the hairpin ends. The commercially available Luciferase-encoding plasmid pGL4.13 (Promega, Madison, WI, USA) was modified to include two telRL sequences flanking the Luciferase cassette. It was hereafter named pGL DOG with the telRL expression region was created. pUC18 containing the luciferase-encoding plasmid pGL4.13 (Promega, Madison, WI, USA) was modified to include two telRL sequences flanking the Luciferase cassette. It was hereafter named pGL DOG with the telRL expression region was created. pUC18 telRL was created by the addition of a telRL site into the HindIII and BamHI sites of pUC18 (Thermo Scientific, Waltham, MA, USA).

DB and linear DNA constructs were run on native and denaturing gels. Native gel: 100 ng of each sample mixed with 6 x gel loading buffer (Sigma, Gillingham, UK) was run on a 0.8% agarose in Tris-acetate-EDTA gel buffered in 1 x Tris-acetate-EDTA. Denaturing gel: 500 ng of each sample was mixed with 6x denaturing sample buffer (300 mM NaOH, 6x EDTA, 18% Ficoll, 0.15% bromocresol green and 0.25% xylene cyanol). The samples were then run on a 1% agarose (in H2O) at 10 V in denaturing running buffer (50 mM NaOH, 0.1 M EDTA) overnight on ice to minimize overheating. Once sufficiently separated, the gel was neutralized for 30 min in 1 M Tris-HCl pH 7.6, 1.5 M NaCl. Both gels were stained for DNA with ethidium bromide and visualized under ultraviolet light.
DNA construct characterization by dynamic light scattering
DNA size and charge were calculated using dynamic light scattering on a Zetasizer Nano (Malvern Instruments, Malvern, UK) in a disposable zeta cell. DNA or DNA complexes were first diluted to 2 μg ml⁻¹ in filter-sterilized degassed water. Samples were read at 25 °C with a back scatter set at 173°, the average of 20 readings in triplicate was taken in each case. Data were analyzed using Malvern instruments Zetasizer software 7.01.

In vitro transfection
CHO-K1 cells were grown to 95% confluence in compete Dulbecco’s modified Eagle’s medium before being transfected with 1μg of luciferase or tdTomato-expressing plasmid or DB. DNA was delivered by EP using a Nucleofector II device with a cell line transfection kit T (Lonza, Slough, UK) or using the following transfection reagents: Lipofectamine 2000 (Invitrogen, Paisley, UK), Polyfect (Qiagen) or PEI (Polysciences, Warrington, PA, USA). Each was used according to the supplier’s specification. Luciferase expression; at the appropriate juncture, media was removed, cells were lysed using luciferase lysis buffer (Promega) and frozen for 24 h. Expression was visualized after the addition of luciferase assay substrate (Promega) and light emission was read using a FluorStar OPTIMA multilable plate reader (BMG Labtech, Offenbach, Germany) and recorded as relative light units (RLU). tdTomato red-fluorescent protein expression was imaged using a Nikon Eclipse TE2000-S inverted microscope (Nikon, Tokyo, Japan) under bright light and a TxRed filter set (excitation 540–580 nm and emission 600–660 nm) at 24 and 48 h after transfection.

Animals
Female BALB/c mice were obtained from Harlan Scientific (Gwennap, UK) and used at 6–8 weeks of age. All procedures undertaken had been approved by the local ethics review board and performed by personal licenses under the appropriate project license. Experiments carried out were in accordance with the Animals (Scientific Procedures) Act 1986.

DNA injections
Mice were injected IM into the anterior tibialis with 25 μg plasmid or DB in 50 μl of sterile PBS followed by EP. Two lots of five pulses of 150 V with switched polarity between pulses were delivered using a CUY21 EDIT system (BEX, Tokyo, Japan). For the HIV gp140 immunization study, mice received a prime boost regime of four IM DNA immunizations with EP 2 weeks apart followed by a single IM protein boost of CN54 gp140 protein (10 μg). For the influenza study, mice received three IM DNA immunizations with EP 2 weeks apart before infection with H1N1.

In vivo imaging
After transfection with plasmid or DB encoding luciferase, expression was visualized using an In vivo FX pro (Bukker BioSpin, Coventry, UK) following intraperitoneal injection of RedJect D-Luciferin (Perkin Elmer, Waltham, MA, USA) in accordance with the manufacturer’s specification. Light emission was measured for 4 min without binning and an X-ray was taken for 30 s; the two images were overlaid using Carestream MI SE software. Relative luminescence was quantified by using the software’s region of interest analysis function, with background levels set using control, untransfected animals.

Antigen-specific enzyme-linked immunosorbent assay
A quantitative assay was used to determine serum antibody levels adapted from Donnelly et al. Ninety-six-well plates were coated with 1 μg ml⁻¹ gp140 or HA1 (A/England/195/2009, Invitrogen) and blocked with 1% bovine serum albumin. A dilution series of recombinant murine IgG was used on each plate as a standard to quantify the antigen-specific antibodies. Sera were diluted 1:500, 1:5000 and 1:50 000 to ensure the absorbance reading measured fell within the linear range of the standard curve. Bound IgG was detected by incubation for 2 h at 37 °C with horseradish peroxidase-conjugated goat anti-mouse IgG (AbD Serotec, Kidlington, UK). Plates were washed and developed with 50 μl TMB/E substrate and the reaction was terminated by the addition of 50 μl of 2 M H₂SO₄ and read at A₄50. For IgA measurements on lung mash supernatants, a similar protocol was performed using biotinylated anti-IgA and detected using horseradish peroxidase-streptavidin, compared with a standard curve of recombinant murine IgA.

Cellular Assays
Splenocytes were isolated by mechanical dissociation through sterile nylon mesh, followed by red blood cell lysis with ammonium chloride. The splenocytes were cultured for 72 h at 2.5 × 10³ cells per well in the presence of HIV gp140 CN54 peptides divided into two pools as has previously been described and 2 μg ml⁻¹ anti CD28 (BD Pharmingen, Oxford, UK), Concanavalin A (ConA, Sigma) and culture media were used as the positive and negative controls, respectively. ELISPOT assays were performed using a commercial kit from MABTECH (Nacka Strand, Sweden) following the manufacturer’s recommendations. The spots were counted using the AID ELISPOT reader ELR03 (Autoimmune, Diagnostika, Straßberg, Germany).

Influenza Infection
H1N1 Influenza strain (A/England/195/2009) was grown in Madin-Darby Canine Kidney cells, in serum-free Dulbecco’s modified Eagle’s medium supplemented with 1 μg ml⁻¹ trypsin. The virus was harvested 3 days after inoculation and stored at –80 °C. Viral undefined and lung viral load were determined by the plaque assay as previously described. Mice were infected intranasally with 5 × 10⁴ plaque-forming unit virus. Weight was measured daily to monitor disease severity. The harvesting of lung tissues was carried out as previously described. For the preparation of lung mash supernatants, lungs were homogenized through 100 μm cell strainers (BD Pharmingen) and washed through with a 1-ml volume of RPMI five times; following centrifugation this supernatant was retained for viral load analysis. After the removal of the supernatants from all tissues, cells were treated with ammonium-chloride-potassium lysing buffer for 5 min and they were resuspended in RPMI. Cell viability was assessed by trypan blue exclusion, and total cell numbers were counted by disposable multowell hemocytometer.

Statistical analysis
The appropriate statistical test was performed using GraphPad prism 5.01 (GraphPad Software Inc, La Jolla, CA, USA). On the basis of the type of data either a two-way analysis of variance with Bonferroni’s post test or a student’s t-test was performed.

CONFLICT OF INTEREST
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Supplementary Information accompanies this paper on Gene Therapy website (http://www.nature.com/gt)