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Combined biolistic and cell penetrating peptide delivery for the development of scalable intradermal DNA vaccines

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ABSTRACT

Physical-based gene delivery *via* biolistic methods (such as the Helios gene gun) involve precipitation of nucleic acids onto microparticles and direct transfection through cell membranes of exposed tissue (*e.g.* skin) by high velocity acceleration. The glycosaminoglycan (GAG)-binding enhanced transduction (GET) system exploits novel fusion peptides consisting of cell-binding, nucleic acid condensing, and cell-penetrating domains, which enable enhanced transfection across multiple cell types. In this study, we combined chemical (GET) and physical (gene gun) DNA delivery systems, and hypothesized the combination would generate enhanced distribution and effective uptake in cells not initially transfected by biolistic penetration. Physicochemical characterization, optimization of bullet contents and transfection experiments *in vitro* in cell monolayers and engineered tissue demonstrated these formulations transfected efficiently, including DC2.4 dendritic cells. We incorporated these formulations into a biolistic format for gene gun by forming fireable dry bullets obtained *via* lyophilization (freeze drying). This system is simple and with enhanced scalability compared to conventional methods to generate bullets. Flushed GET bullet contents retained their ability to mediate transfection (17-fold greater and 13-fold greater reporter gene expression than standard spermidine bullets in the absence and presence of serum, respectively). Fired GET bullets *in vitro* (in cells and collagen gels) and *in vivo* (mice) showed increased reporter gene transfection compared to untreated controls, whilst maintaining cell viability *in vitro* and having no obvious toxicity *in vivo*. Lastly, a SARS-CoV-2 plasmid DNA vaccine with spike (S) protein-receptor binding domain (S-RBD) was delivered by gene gun using GET bullets. Specific T cell and antibody responses comparable to the conventional system were generated. The non-physical and physical combination of GET-gold-DNA carriers using gene gun shows potential as an alternative DNA delivery method that is scalable for mass deployable vaccination and intradermal gene delivery.

1. Introduction

The COVID-19 pandemic demonstrated the progressive evolution of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants, and in response, the necessity for continuous vaccine development to meet the demands of fluctuating immune profiles in humans. COVID-19 led to the launching of the world's first human-approved DNA vaccine using plasmid DNA (pDNA) delivered *via* a needle-free injection device. This provided proof-of-principle evidence that DNA vaccines are effective, safe, and immunogenic in humans, albeit less protective than mRNA vaccines [1,2]. Thus, further research to improve DNA design, delivery and translation remains important for DNA-based therapeutics including vaccines.

Efficient DNA delivery systems are vital for successful transfection *in vivo*. These molecular vehicles are responsible for packaging, protecting and transporting pDNA into target cells and mediating nuclear localization [3]. Naked DNA is highly susceptible to nuclease degradation and electrostatic binding with plasma proteins leading to decreased bioavailability. Moreover, the bulky size of pDNA requires its compaction to improve cellular entry and trafficking. The complexity of

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extracellular and intracellular barriers encountered by DNA therapeutics limits their efficacy and utility in the clinic, and there is still no effective injectable formulation that mirrors lipid nanoparticles used for mRNA delivery. Conventional parenteral routes of administration for vaccines (*e.g.* intramuscular, subcutaneous, intradermal) are less effective for DNA vaccines, thus these routes still require device-assisted or physical-based administration methods [4,5]. The mechanisms of cellular entry of these devices can be broadly generalized as physically-mediated (*e.g.* biolistic devices, needle-free injection systems, DNA tattooing, suction-mediated delivery) and transient energy potential (*e.g.* electroporation, sonoporation, photoporation) [6–8]. This study focuses on biolistic delivery or particle-mediated epidermal delivery (PMED) using the Bio-Rad Helios gene gun, wherein DNA is adhered onto gold particles and fired to deliver DNA directly through cell membranes [9,10].

The spermidine bullet system used for the Helios gene gun approach has excellent *in vitro* and *in vivo* transfection [10–12] and is the current gold-standard gene gun bullet formulation. However, each bullet has an optimal loading capacity of only 1–2.5 μg DNA per 0.5 mg gold, and increasing DNA loading ratio results in microparticle aggregation without improvement in transfection efficiency [10–13]. As doses in the order of hundreds of micrograms are needed to generate significant immune-responses in humans [1,2], further optimization of DNA vaccine delivery is needed to translate this platform from bench to bedside.

Non-viral or 'chemical' delivery systems contain cellular uptake-enhancing moieties that are safer, more versatile and easier to manufacture compared to viral vectors such as adenovirus [3]. They also could avoid the problematic generation of anti-vector antibodies, which preclude repetitive usage of viral vectors [14–16]. The glycosaminoglycan (GAG)-binding enhanced transduction (GET) peptide system exploits cell modified penetrating peptides (CPPs) for enhanced cell binding, uptake, and transfection [17]. FGF2B-LK15-8R (FLR), a second generation GET peptide, consists of: a Fibroblast growth factor (FGF2)-heparan sulphate GAG cell-binding domain which facilitates membrane docking; LK15, an amphipathic DNA-complexing domain with endosomal escape capabilities; and Octa-arginine (8R), a cell-penetrating domain that enhances endocytosis [17]. GET functions as an intracellular delivery vehicle for different conjugated cargoes, enables high transfection even in difficult-to-transduce cells, and has been used for *in vivo* bone regeneration, lung gene delivery, magnetofection and delivery of peptides from proteins such as insulin [17–32].

We hypothesized that if the cell uptake benefits of GET could be married with the distribution of biolistic approaches, this could yield a viable approach to generate a more effective and dose adaptable system for DNA vaccination. We methodically formulated physical biolistic transfection with non-physical GET-mediated delivery in a fireable system. GET peptide was exploited to complex DNA with standard gold microcarriers for use in the standard gene gun biolistic format. We demonstrated that this was effective with or without gene gun-mediated delivery *in vitro* and that it could be used with experimental pDNA vaccines *in vivo* targeted for SARS-CoV-2 immunity. With the increasing demand for novel vaccine technologies, we argue that a combined chemical and physical system could be a route for more effective and deployable vaccines and a variety of other DNA therapeutics requiring administration to the skin such as for wound healing.

2. Material and methods

2.1. Materials

A complete list of materials is provided in the supplemental materials section.

2.2. Plasmid preparation

Plasmids encoding reporter genes for *Gaussia* luciferase (GLuc) and firefly luciferase (FLuc) under the control of a cytomegalovirus promoter (pCMV) as described in detail in previous studies [20,29,30] were purified using a Maxi-prep kit (Qiagen, UK) according to the manufacturer's protocol. A SARS-CoV-2 spike protein receptor binding domain (S-RBD) expression plasmid (pVAX1-S-RBD) that was previously tested and provided by Scancell Ltd. UK was used as a model vaccine [33].

2.3. GET-gold-DNA formulation determination

GET-DNA complexes were formulated at various nitrogen to phosphate (N:P) charge ratios (CR) of GET peptide (SynPeptide) to DNA (CR 5:1, 10:1, and 50:1, referred to as CR 5, 10 and 50 respectively, Table S1). pDNA was added to GET and incubated at room temperature for 15 min to allow for electrostatic complexation. Gold microcarriers (1.0 μm) were weighed at 0.5 mg per 1 μg pDNA using a microscale and added to the GET-pDNA complexes. After a 15-min incubation period, 100 μL of calcium chloride ($CaCl_2$, 1 M) was added dropwise while continuously mixing using a vortex. The gold slurry was allowed to precipitate at room temperature for 10 min then was centrifuged for 15 s at 16,000 *g* using a microcentrifuge. The supernatant was discarded, and the pellet was washed with 200 μL nuclease-free water (NFW) once then was resuspended in 200 μL NFW. The final suspension was used for 96-well plate transfection experiments.

2.4. Spermidine-gold-DNA bullet preparation

Spermidine-DNA complexes were formed and precipitated onto gold microcarriers as detailed in the Helios gene gun instruction manual (BioRad Laboratories Inc., USA). The required gold microparticles (0.5 mg gold per bullet) and 100 μL of 0.05 M spermidine were mixed using a vortex mixer and sonicator, then the DNA (DNA loading ratio (DLR) 2) was added into the gold slurry. Precipitation of DNA-gold complexes was done by adding 100 μL of $CaCl_2$ (1 M) dropwise with continuous moderate mixing using the vortex. After 10 min, the supernatant was discarded, and the pellet was washed thrice with 200 μL absolute ethanol. After the final wash, the pellet was resuspended in 200 μL of 0.03 mg/mL polyvinylpyrrolidone (PVP) in absolute ethanol, transferred to a 15 mL Falcon tube and topped up to a final volume of 1 mL with PVP in absolute ethanol. Tefzel tubing was inserted in the tubing prep station (BioRad Laboratories Inc., USA) and dried by purging with nitrogen. The spermidine-gold-DNA slurry was mixed and immediately transferred into the Tefzel tubing using a syringe. The Tefzel tube was loaded into the tubing support cylinder and allowed to settle for 15 min. After careful aspiration of the liquid, the Tefzel tube was rotated for 5 min under 4 L per minute of nitrogen to evenly coat the gold mixture along the wall and dry it. It was then cut into 0.5-inch. bullets using the tubing cutter. The spermidine bullets were then stored in a desiccated container at 4°C.

2.5. GET-gold-pDNA bullet preparation

GET-DNA complexes were formulated at CR 50 by mixing 4 μg pDNA (1 μg/μL) and 3.4 μL FLR peptide (10 mM) [29] to a total volume of 100 μL NFW. After incubation at room temperature for 15 min, gold microcarriers were added to the GET-DNA complexes, precipitated with $CaCl_2$, and washed as described above. A range of GET-gold-DNA formulations with various DNA-LR (2, 4, 8 μg DNA / 1 mg gold) were generated for testing, wherein each final bullet contained 0.5 mg gold. Freezing media was prepared by combining D-mannitol (80 mg/mL), sucrose (18 mg/mL), glycerol (2 mg/mL) diluted with NFW to reach a total volume of 100 mL for a 2× working solution. The final pellet was resuspended in 1:1 ratio of NFW and freezing media to a total volume of 25 μL. Tefzel tubing was pre-cut into 0.5-inch pieces using the tubing cutter. The GET-gold-DNA solution was briefly vortexed and sonicated, then was pipetted into the pre-cut Tefzel tubes. The bullets were snapped frozen by immersion in liquid nitrogen and lyophilized (VirTis SP

Scientific) following the manufacturer's protocol. The chamber was tightly sealed under constant vacuum pressure and temperature was maintained at -40°C throughout the 16 h lyophilization process. The lyophilized bullets were evaluated for uniformity of cake-like appearance then stored at -20°C.

2.6. Extraction of bullet contents

Both GET and spermidine bullet contents were flushed from the Tefzel casing using 25 μL of NFW and briefly sonicated for 10 s when necessary. Final flushed bullet contents were vortexed to achieve a homogenous suspension.

2.7. Physicochemical analysis of bullet contents

GET and spermidine bullet contents were flushed with 700 μL NFW for measurement of size and zeta potential using Malvern Nanosizer Nano ZS. For each sample, both size and zeta potential were measured thrice to estimate the error in each measurement. Particle size and polydispersity index (PDI) were measured using dynamic light scattering. Briefly, 700 μL of samples were loaded into a dust-free cuvette then loaded into the machine. The temperature was set at 25 °C and equilibration time of 60 s in between readings. The samples were retrieved for subsequent zeta potential measurement. Particle zeta potential was measured using electrophoretic light scattering. Briefly, the above samples were further diluted by adding 100 μL NFW then the new total volume of 800 μL per sample were pipetted into a capillary zeta cell. The parameters were set at a maximum run of 20 cycles and the process was repeated for each individual sample.

2.8. DNA quantity and quality determination

The quantity of DNA flushed from spermidine or GET bullets was analyzed by UV spectroscopy using NanoDrop (NanoDrop 2000 spectrophotometer, Thermo Scientific) prior to downstream experiments. The quality and integrity of the pDNA was assessed using gel electrophoresis. Conditions tested included, naked DNA and spermidine or GET bullet contents with or without 0.08% (*w/v*) sodium dodecyl sulphate (SDS). To evaluate the protective effect of GET against DNAse, increasing concentration of DNAse I from 0, 0.0025, 0.025 and 0.25 units/μL were added to DNA samples for 10 min at 37 °C. Samples for elution were prepared by adding appropriate volumes of samples with the gel loading dye (with and without SDS) diluted to a total volume of 24 μL using NFW. Agarose gels at 1% (*w/v*) were prepared using 1× Tris-acetate-EDTA (TAE) buffer and ethidium bromide and run with 1 kb DNA ladder at 100 V for 1 h and then the gels were digitally imaged using a Fujifilm LAS-4000 gel imager.

2.9. Protein content determination

Protein content quantification was assessed by using a Pierce bicinchoninic acid (BCA) kit following the manufacturer's instruction manual. Conditions assessed were GET bullet contents, GET protein alone, DNA alone and freezing media. Briefly, 25 μL of samples were added into non-tissue culture 96-well plates in triplicate then incubated with 200 μL of BCA reagent for 30 min at 37 °C. The 96-well plate was centrifuged at 200 *g* for 5 min and 100 μL of samples were transferred onto a new plate. A dilution series of bovine serum albumin (BSA) was prepared in duplicate to generate a standard curve as per the instruction manual. Absorbance was read at 562 nm using a microplate reader (Infinite 200 Pro, Tecan).

2.10. Cell culture

Dendritic cells (DC2.4) were cultured in RPMI-1640 complete media (RPMI supplemented with 10% fetal bovine serum (FBS), 50 units/mL of penicillin/streptomycin, 2 mM L-glutamine, 1 mM non-essential amino acids and 0.2 mM 2-mercaptoethanol) in T75 flasks and incubated at 37 °C and 5% CO_2. The cells were passaged every 3 to 4 days when 70 to 90% confluency was reached. Cells were seeded at a density of 1×10^4 cells per well on 96-well plates, 1×10^6 cells per well on 6-well plates, and 2×10^6 per collagen gel model for all transfection experiments. On the day of experiment, old media was discarded and replaced by fresh cell culture media with or without FBS depending on experimental design.

2.11. Conventional cell transfections

GET-DNA complexes at CR 5, 10 and 50, with and without gold, were formulated as described (Section 2.3). DC2.4 cells were seeded and incubated overnight then the media was replaced with 50 μL fresh RPMI with or without FBS prior to transfection. GET-DNA ± gold complexes were added into 96-well plates at 0.125 μg pDNA per well and incubated at 37 °C overnight. Supernatants were collected after 24 h for assessment of transfection efficiency using luciferase assay (section 2.14).

2.12. Preparation of 2D and 3D collagen gel models

DC2.4 were seeded at a density of 1×10^6 cells per well on a 6-well plate to generate 2D monolayers. Cell culture plates were incubated for 48 h before conducting gene gun transfection experiments. 3D collagen models made of collagen type 1 were generated using cell strainers as scaffold. Components for one collagen gel are described in Table S2. The collagen mix was scaled up depending on the number of models needed for each experiment and kept on ice to prevent gelling. The DC2.4-collagen suspension was quickly mixed and immediately spread evenly onto inverted cell strainers, ensuring full coverage of the surface area. The cell strainers were placed inside individual wells of a 6-well plate and incubated for 30 min at 37 °C and 5% CO_2 until the collagen set. After the incubation period, collagen gel models were immediately transfected using the gene gun (Fig. S2).

2.13. Gene gun mediated transfection in 2D and 3D models

Gene gun equipment (Helios gene gun system; BioRad Laboratories Inc., USA) was set up according to the manufacturer's instruction manual. The gene gun was first connected to a helium source using a helium hose and regulated at 200 pounds per square inch (psi). The gene gun was activated by firing 1 to 2 pre-shots using an empty cartridge holder in place. The 6-well plates containing the cells were placed inside the biosafety cabinet and old media were aspirated. The bullets were loaded into the barrel chamber, locked in place, and the gene gun was positioned properly directly on top of the target cells making sure there was even contact across the target area. After bombardment of the cells, 1 mL of fresh growth media was added into 2D monolayers, while 2 mL of fresh growth media was added into 3D collagen gels. Cells were incubated for 24 h at 37 °C and 5% CO_2.

2.14. In vitro reporter gene expression assay

Gaussia luciferase luminescence was measured using the BioLux *Gaussia* luciferase assay kit [30]. Triplicate 10 μL samples of supernatants were added into white 96-well plates. GLuc assay buffer solution was prepared by 1:100 dilution of substrate to assay buffer, then 50 μL was added into each well. A luminometer (Infinite 200 Pro, Tecan) was used to measure luminescence, which was expressed as relative light units (RLU) alone or per μg protein (determined by BCA assay). Firefly luciferase luminescence was measured using FLAR-1 firefly luciferase assay (Prolume, USA). Cells in 6-well plates were scraped and centrifuged at 500 *g* for 5 min. Supernatants were discarded and 350 μL of firefly assay solution was added to the pellet. After 5 min of incubation in the dark, 100 μL was pipetted in triplicate into white 96-well plates.

Luminescence was measured as for GLuc.

2.15. Cell viability assay

PrestoBlue assay was used to measure cell metabolic activity after transfection of GET-gold-DNA complexes. PrestoBlue reagent was prepared by 1:10 dilution with Hank's Balanced Salt Solution. Media was aspirated and PrestoBlue reagent was added into the wells depending on well surface area (50 μL for 96-well plates, 1 mL for 6-well plates containing 2D monolayers, 1.5 mL for 6-well plates containing 3D collagen gel models). The cells were incubated until a colour change was visually detected in the untreated control cells. Supernatants (50 μL) were transferred into a black 96-well plate in triplicate. Fluorescence was measured using a microplate reader (Infinite 200 Pro, Tecan) at Ex/Em 560/590 nm.

2.16. Animal experiments

All experiments were performed at the animal facility of Nottingham Trent University, Nottingham, UK under a Home Office approved project license (PP2706800). As the purpose of the experiments was to directly compare the spermidine and GET bullets, only female Balb/c mice (Charles River UK) were used to minimise variation, allowing the minimal number of mice to be used per group (based on prior experience of individual variation of responses to gene gun immunisation).

2.17. In vivo bioluminescent tracking and imaging

A total of five groups of two mice received gene gun-fired spermidine and GET bullets, intradermal flushed spermidine and GET bullet contents, or received no treatment. *In vivo* imaging was done 24 and 48 h after administration of the complexes. Mice were anesthetized and 3 mg of D-luciferin (Perkin Elmer, XenoLight RediJect D-luciferin) in 100 μL PBS was injected intraperitoneally. Dorsal and ventral images were taken within 20 min of D-luciferin injection. Mice were killed by a schedule 1 method on day 1 and day 2 post-*in vivo* imaging then tissues (skin at administration site, spleen, draining lymph node and liver) were dissected for *ex vivo* imaging.

2.18. Vaccination study

Three mice per group were immunized with pVAX1-S RBD delivered using spermidine bullet (group A) and GET bullet (group B). Three doses were intradermally administered using the gene gun on days 1, 8, and 15. On day 21, the mice were killed by a schedule 1 method and whole blood samples were taken and the spleens removed for analysis of immunological responses by ELISA (section 2.20) and ELISpot (section 2.19).

2.19. ELISpot

S-RBD specific interferon gamma (IFNγ) response by T cells was tested using ELISpot Flex Mouse IFN gamma alkaline phosphatase (ALP) kit (Mabtech, UK) [33]. Splenocytes were immediately extracted by flushing spleens with a needle and syringe containing 10 mL RPMI complete media. The cells were centrifuged at 300 *g* for 10 min and the remaining pellet was resuspended in RPMI complete media. ELISpot assay was performed following the manufacturer's protocol. Briefly, ELISpot plates (96- well MAIP multiscreen plates, Millipore) were coated with capture antibody at 0.5 μg/mL per well overnight at 4°C, washed four times with sterile phosphate-buffered saline (PBS), then blocked with 100 μL RPMI overnight at room temperature. Splenocytes were added at a density of 5×10^6 per well, stimulated by addition of lipopolysaccharide (LPS, Sigma-Aldrich, UK) as positive control and S-RBD peptide pool (JPT Innovative Peptide Solutions, USA) at 1 μg/mL per well in triplicate, then incubated for 40 h at 37°C. Unstimulated controls containing splenocytes with media alone were included. Biotinylated detection antibodies at a concentration of 0.05 μg/mL per well was added and incubated for 2 h at room temperature. Plates were washed with PBS with Tween20 (0.05 *w/v*) 5 times in between each development step. Streptavidin-alkaline phosphatase at 50 μL was added followed by incubation for 1.5 h at room temperature. Substrate was added and allowed to develop until distinct spots were observed (5 min). The substrate reaction was stopped by placing the plates under running tap water and dried plates were read using an automated plate reader (ImmunoSpot ELISpot reader, Cellular Technologies Ltd., UK).

2.20. ELISA

Presence of antibodies to the SARS-CoV-2 spike RBD in mouse sera was determined by ELISA [33]. Pooled whole blood from immunized and naive mice was allowed to clot for 30 min and centrifuged at 1000 *g* for 10 min to obtain serum. Serial dilution of the serum using PBS with 2% BSA (PBS-BSA) was done ranging from 1:100 down to 1:100,000. Serial dilution of S-RBD neutralizing antibody (SinoBiological, UK) was prepared in triplicate to generate a standard curve. All samples were added in triplicates into high protein-binding ELISA plates (Nunc Immuno F96 MaxiSorp, Thermo Fisher) that were pre-coated with S1 protein (GenScript, UK) at 200 ng/well for the S-RBD antibody assay or GET peptide for the GET antibody assay. Blocking was done for one hour using casein blocker (Thermo Fisher Scientific). Anti-mouse IgG (Fc specific) biotin (Sigma-Aldrich, UK, 1:2000) was added and incubated for one hour, followed by streptavidin-HRPO conjugate (Invitrogen, UK, 1:1500) for another hour. Washing with PBS was done in between steps. Development was done by adding TMB core plus substrate (BioRad, UK) and reaction was stopped using 1 M sulphuric acid after 5 min. Plates were read at 450 nm using a spectrophotometer.

2.21. Statistical analysis

A total of three biological replicates were performed for all *in vitro* experiments, wherein each biological replicate had three technical replicates. The animal experiments were done once. Normally distributed data from independent groups were all presented as mean ± standard deviation (SD). Grouped data were analyzed using parametric tests (one-way ANOVA and two-way ANOVA). Post-hoc pairwise analyses were performed whenever necessary. A p-value of <0.05 was considered statistically significant throughout this paper. All statistical analyses were computed using GraphPad Prism 9 software.

3. Results

3.1. In vitro testing of GET-gold-DNA formulations

The GET peptide was complexed with pDNA then precipitated onto gold to form GET-gold-DNA complexes (Fig. 1A and B). Various GET-DNA charge ratios (CR 5, 10 and 50 GET:pDNA ratio) were evaluated in the presence or absence of gold microcarriers in DC2.4 cells with or without serum (fetal calf serum, FBS) supplementation.

In the absence of the gold microcarrier, GET-DNA at CR 10 without serum yielded significant reporter gene expression (Fig. 1C, p-value <0.01) while retaining cell viability (Fig. 1D). However, in the presence of serum, GET-DNA at CR 50 was observed to have a higher luciferase expression than CR 10 (Fig. 1C). Notably, poor cell viability (Fig. 1D) regardless of the presence of serum was observed for GET at CR 50. Thus, GET at CR 50 in the absence of gold microcarrier yielded poor transfection and largely non-viable cells.

The addition of gold to GET-DNA CR 50 in the presence of serum led to a significantly higher transfection efficiency (Fig. 1C, p-value 0.004). Cell viability was maintained when GET-DNA CR 50 was bound to gold regardless of serum status. Therefore, the GET-gold-DNA CR 50 was selected for incorporation into the gene gun bullet system.

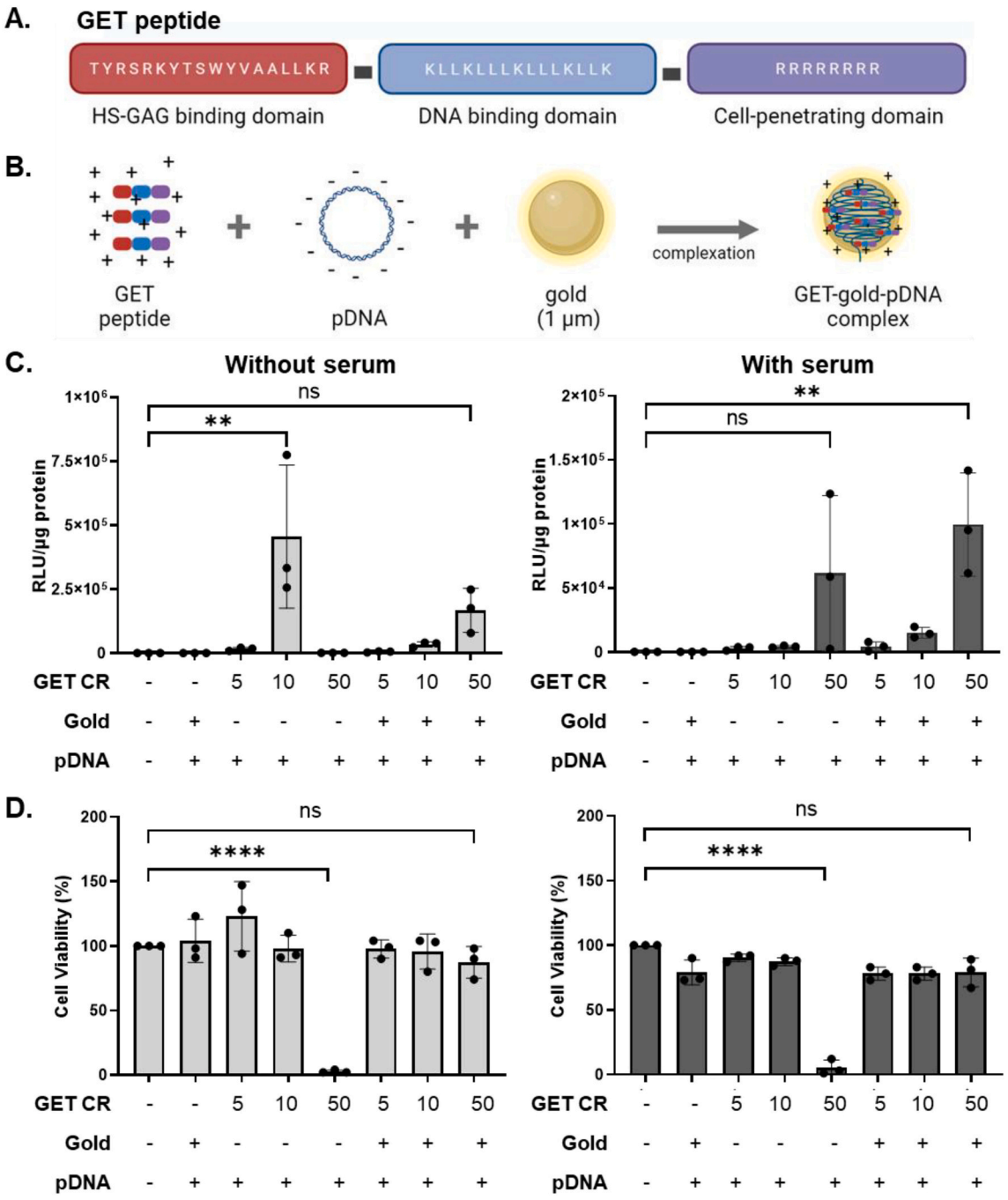


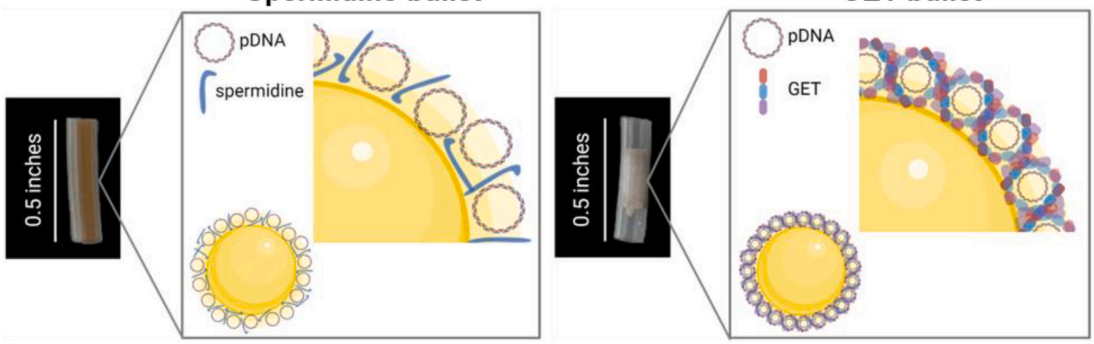
Fig. 1. GET-gold complex formulation *in vitro.* A. Illustration of GET (glycosaminoglycan-binding enhanced transduction) peptide domains. B. Cationic GET peptide electrostatically binds to anionic DNA to form nanoparticles, which are then precipitated onto gold microcarriers. pGLuc was delivered to DC2.4 cells using different GET-DNA charge ratios (CR) ± gold. C—D. *Gaussia* luciferase expression (relative light units per microgram of protein, RLU/μg) was analyzed after 24 h followed by cell viability using PrestoBlue. GET-gold-DNA at CR 50 successfully transfected DC2.4 cells in the presence of serum whilst maintaining cell viability. All formulations contained 0.125 μg pDNA per well of a 96-well plate, and gold complexes were formulated at 0.5 mg gold per 1 μg pDNA. $N = 3$ biological replicates, each point represents the mean of technical triplicates, bar graphs and error bars represent mean ± SD, ***p*-value <0.01, ****p-value <0.0001, statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

3.2. Bullet adaptation and physicochemical characterization

Gold standard spermidine bullets were used as the positive control for all gene gun-related experiments. For the GET bullets, GET peptide was used in place of spermidine peptide to complex DNA. The conventional spermidine bullet manufacturing procedure was adapted to generate an alcohol- and PVP-free, bullet machine-independent process, which relies on simple complexation, pipetting and freeze drying. These GET bullet prototypes have a different physical appearance, compared to conventional bullets (freeze-dried cake *versus* tube bound track) (Fig. 2A).

Extracted spermidine and GET bullet contents when resuspended were observed to have different physicochemical properties. Spermidine bullets were 901.8 ± 276 nm, non-uniform in size (PDI 0.7) and negatively charged, while GET bullets were more compacted (264.6 ± 55 nm), more uniformly sized (PDI 0.3), and positively charged (Fig. 2B).

A.



B.

	Z-average [nm]	Polydispersity index	Zeta Potential [mV]
Spermidine bullet	901.8 ± 276	0.7 ± 0.1	- 5.9 ± 3.7
GET bullet	264.6 ± 55	0.3 ± 0.0	24.9 ± 3.3

C.

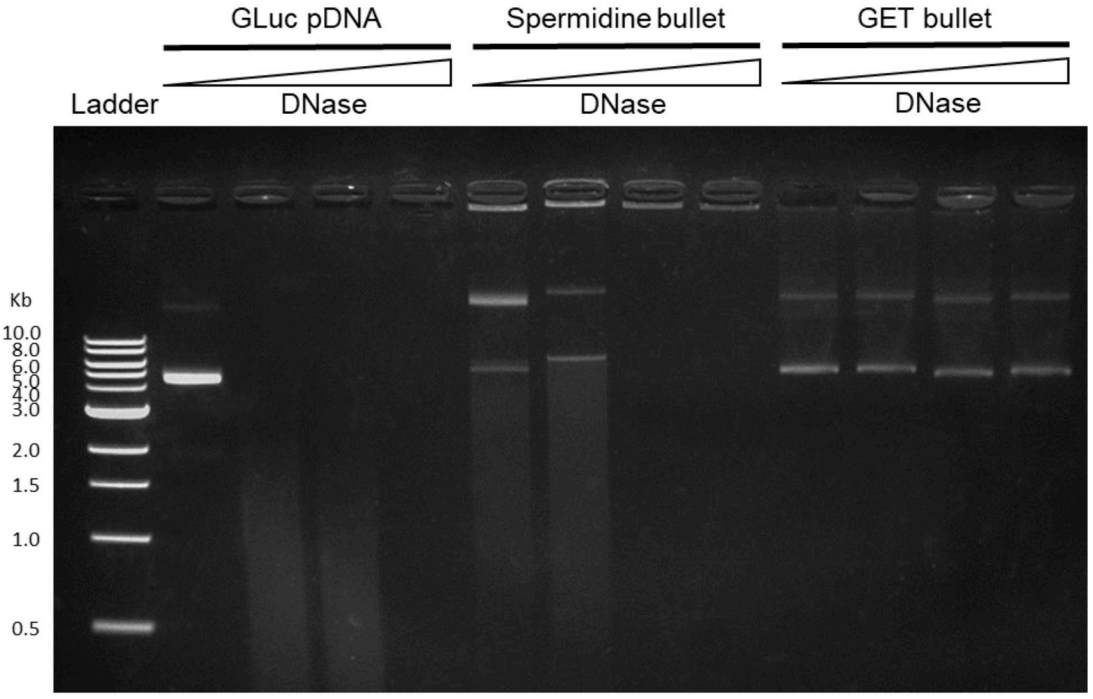


Fig. 2. Comparison of physicochemical properties of spermidine and GET bullets. A. Representative image of both bullet systems. B. GET bullet complexes were smaller, more uniform in size and remained positively charged. Spermidine bullet complexes were bigger, highly dispersed and negatively charged. $N = 3$ independent repeats, values represent mean ± SD. C. Both bullet contents were initially incubated with increasing doses of DNAse I (0, 0.0025, 0.025, 0.25 units/μL) followed by decomplexation using SDS (0.08% *w/v*) to assess integrity of DNA while within the nanocomplexes. GET bullet complexes protected the DNA against increasing doses of DNAse unlike spermidine bullets or 'naked' GLuc pDNA.

Nuclease protection capacities of both bullet systems were assessed by incubation with increasing doses of DNAse, then disruption by SDS to release DNA, and analysis of DNA integrity using electrophoresis. Spermidine bullet formulation did not protect against DNase degradation whilst GET bullets were protected (Fig. 2C).

3.3. Bullet content chemical transfection assessment in vitro

The GET bullet manufacturing protocol subjected the complexes to multiple potentially disruptive processes such as centrifugation, washing and freeze drying. Bullet content formulations were extracted by simple flushing and brief sonication then analyzed for transfecting capabilities.

GET bullet contents were instantly and completely ejected, unlike those of spermidine bullets, which required brief sonication to strip the gold particles off the PVP adhesive (Fig. 3A). Extracted GET bullet contents retained the ability of GET to chemically transfect cells in both absence and presence of serum (Fig. 3B, p-value <0.0001 and p-value <0.01, respectively), unlike spermidine bullets. Both formulations had little effect on cell viability (Fig. 3C).

3.4. Optimization of GET bullet formulation

DNA-gold microcarrier formulations rely on DNA loading ratio (DNA-LR), the amount of DNA loaded per mg of gold, and the microcarrier loading quantity (MLQ), the amount of gold (in grams) delivered per bullet shot. Conventional spermidine bullets typically use DNA-LR of 2 and MLQ of 0.5, which theoretically delivers 1 μg DNA per 0.5 mg gold

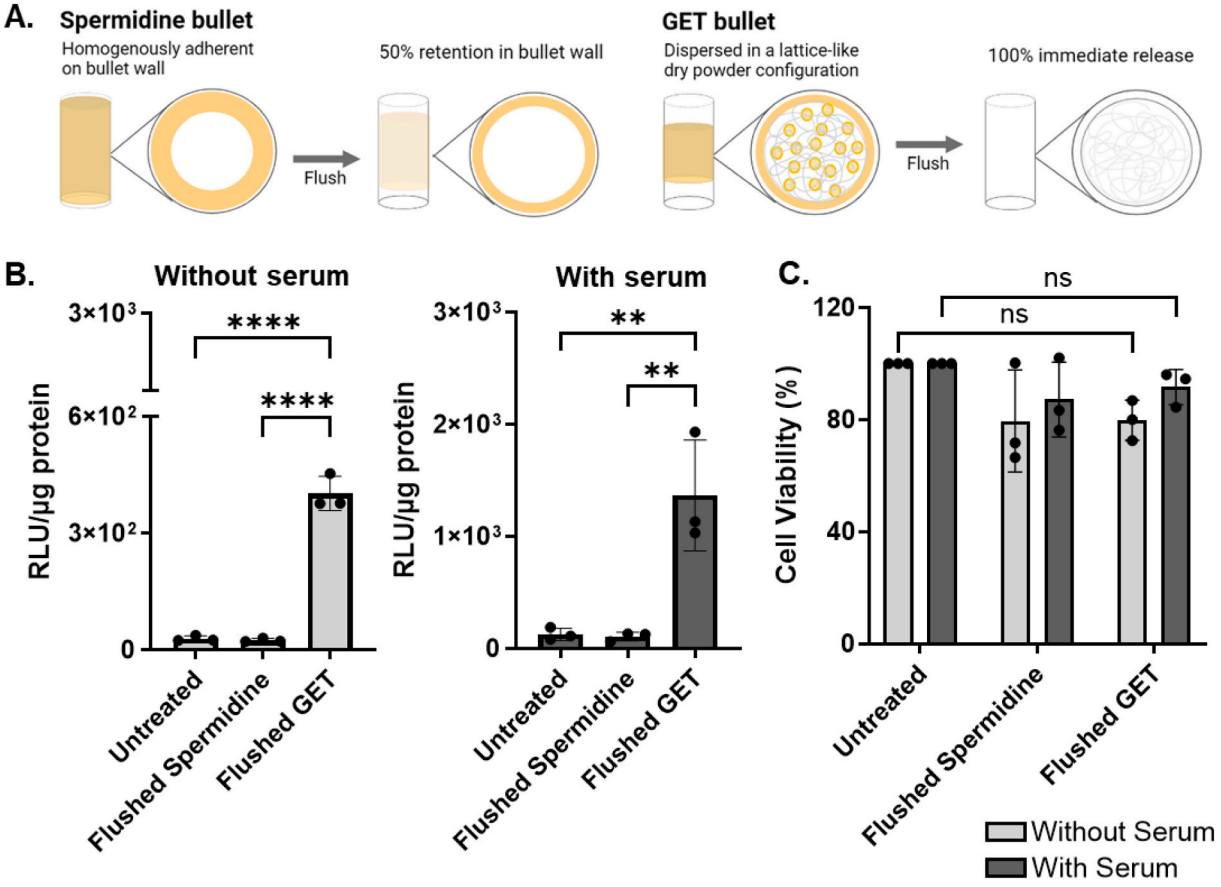


Fig. 3. Lyophilized GET bullet contents had higher transfection efficiency compared to spermidine bullets. A. Flushed GET bullets had 100% immediate release. Flushing of spermidine bullet content required further sonication to release the residuals adherent on the bullet wall. B—C. Extracted GET bullet contents significantly improved transfection compared to flushed spermidine bullet in the presence of serum without decreasing cell viability. Each bullet was loaded with 1 µg pDNA and 0.5 mg gold. N = 3 biological replicates, each point represents the mean of technical triplicates, bar graphs and error bars represent mean ± SD, ***p*-value <0.01, ****p-value <0.0001, statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test for the luciferase assay and two-way ANOVA followed by Dunnett's multiple comparisons test for the cell viability assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

per bullet [12]. We first generated GET bullets with 1 µg DNA to replicate these values.

The DNA content of both spermidine and GET bullets was analyzed using agarose gel electrophoresis. Analysis of the GET bullet manufacturing procedure revealed a loss of approximately 65% of loaded DNA during the centrifugation and washing steps (Fig. 4A and B). Furthermore, protein quantification of the GET peptide in the bullets revealed that the loss of GET peptide resulted in a final CR of 5 instead of the initial CR of 50 (Fig. 4C).

To compensate for DNA loss, GET bullets were generated using increasing DNA concentrations (DNA-LR 2, 4 and 8, which was equivalent to 1, 2 and 4 µg DNA loaded per bullet) while maintaining the MLQ of 0.5 mg gold per bullet. Increasing the DNA-LR proportionately increased the final total DNA in the bullet leading to approximately 1 µg DNA in GET bullets with a DNA-LR of 8, which is comparable to spermidine bullets (Fig. 4B).

Intactness or tightness of the complexes formed in GET bullets was assessed by incubating formulations in the presence of SDS, a disrupting agent. DNA species were observed to be released from spermidine bullets, even in the absence of SDS. In contrast, for GET bullets, DNA release was only observed after incubation with SDS (Fig. 4D). This demonstrated that GET bullets retain the property of more conventional GET formulations that strongly bind and complex to DNA electrostatically and require chemical disruption to release DNA.

In summary, a DNA-LR of 8 per 1 mg gold was observed to approximate standard 1 µg spermidine bullets; hence we used this optimized GET bullet version for the subsequent *in vitro* and *in vivo* experiments.

3.5. Firing of GET bullets via gene gun in vitro

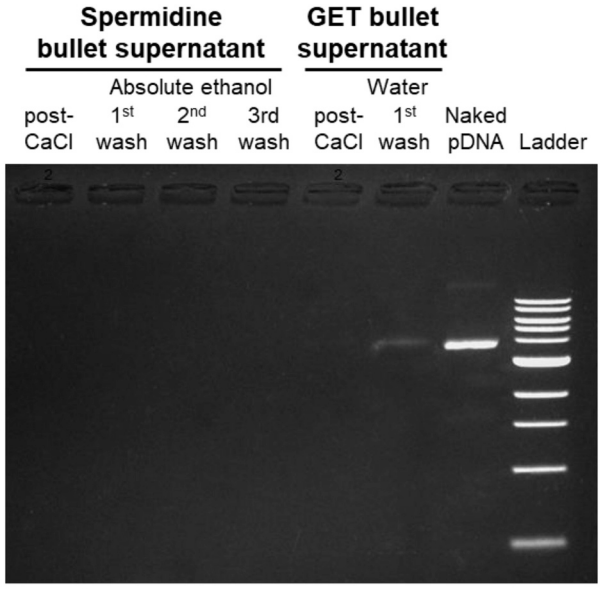
We devised the concept of combining chemical-mediated transfection capabilities of GET with the physical-mediated transfection of gene gun delivery (Fig. 5A). Both bullet systems were evaluated using the gene gun *in vitro* to assess the effect of combining chemical and physical mechanisms of transfection.

Extracted GET bullet contents outperformed extracted spermidine bullet contents in chemical-mediated transfections, but the opposite was observed during firing of the bullets. Preliminary GET bullets using the original DNA-LR of 2 failed to significantly increase reporter gene expression compared to untreated cells (Fig. 5B and C). After optimization of the GET bullet using a DNA-LR of 8, both secreted *Gaussia* luciferase (from pGLuc) and a further intracellular reporter, firefly luciferase (from pFLuc) expression was significantly higher (*p*-value 0.01 and p-value 0.013, respectively) compared to negative control (Fig. 5D and E). This showed that GET bullets, unlike conventional spermidine bullets, could be fired and also have significant transfection when incubated with cells through GET-mediated delivery.

3.6. Reporter gene expression assessment of fired GET bullets in vivo

We delivered FLuc-expressing formulations (1 µg DNA) by gene gun to the ventral skin of mice. *In vivo* imaging was conducted ventrally and

A.



B.

	DNA -LR	Theoretical amount of DNA (ng)	Measured amount of DNA (ng)
Naked DNA			1,000
Spermidine bullet	2	1,000	848 ± 57
GET bullet	2	1,000	366 ± 22
	4	2,000	490 ± 22
	8	4,000	967 ± 137

C.

	Total protein (μg)	N:P charge ratio
FLR alone	738 ± 33	50
GET bullet	68 ± 5	4.6 (~5)

D.

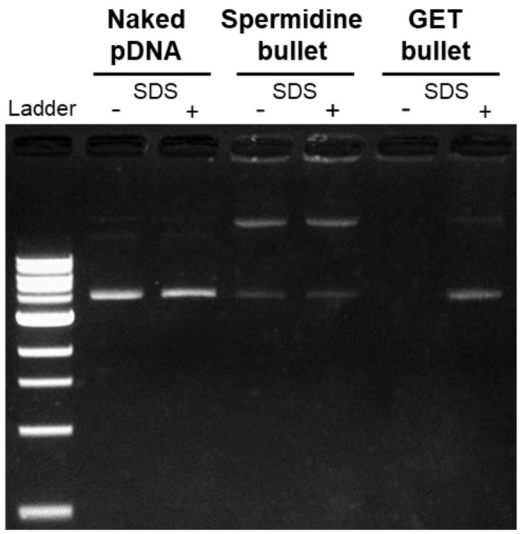


Fig. 4. Optimization of GET bullets. A. Supernatants collected during the manufacturing processes of both spermidine and GET bullets were analyzed for presence of DNA. Plasmids were detected in the wash step of the GET bullet indicating loss of DNA. B. Increasing the DNA loading ratio (DNA-LR) from 2 to 4 or 8 proportionally increased the DNA in each GET bullet detected using NanoDrop and agarose gel. DNA-LR 8 (4 μg DNA/0.5 mg gold per bullet) was determined to be the optimal initial DNA content loaded to attain the target 1 μg DNA per bullet dose. C. GET peptide quantification using Pierce BCA protein assay showed a final net CR of 5 from the original CR 50, indicating loss of peptides during the bullet manufacturing process. Values represent the mean of 3 independent repeats ± SD. D. GET-DNA complex is stable and required presence of a disrupting agent SDS for dissociation unlike spermidine-DNA complex. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dorsally. Previously it has been observed that both ventral and dorsal site gene expression results from conventional spermidine bullet firing, providing indirect evidence of migration of transfected cells dorsally from the transfection site. We dissected selected organs (draining lymph nodes, skin, spleen and liver) to determine whether the transfected cells homed specifically to these sites. Gene gun-mediated transfection *in vivo* showed high levels of firefly luminescence for both spermidine bullets and GET bullets at the site of administration (Fig. 6A to 6C, ventral), which peaked at 24 h and persisted at lower levels for 48 h. Dorsal images for both systems detected luminescence at 24 h which disappeared by 48 h, with spermidine bullets appearing to have higher drainage or migration of transfected cells into lymph nodes compared to GET bullets (Fig. 6A to 6C, dorsal). Both intradermally administered flushed spermidine and GET bullet contents (Fig. S4 A and B respectively) did not show significant transfection after adjusting for background levels. Expression of FLuc in skin removed from the delivery site was detected *ex vivo* (Fig. 6A to 6C, *in vivo*) but not in the spleen, liver, and lymph nodes.

3.7. Vaccination using GET bullets with SARS-CoV2 S-RBD expression plasmid DNA

The GET bullet system was adapted to deliver pVAX1-S-RBD as a DNA vaccination strategy (a spike-only version of a previously trialled pDNA COVID-19 vaccine) [33]. S-RBD specific binding antibodies were observed for both bullet systems (until 1/100,000 dilution) (Fig. 7A). Area under the curve (AUC) was 1.5-fold higher (p-value 0.0001) for spermidine bullets (AUC = 114,383) than for GET bullets (AUC = 76,759) (Fig. 7B).

S-RBD specific T cell response was detected in both bullet systems, with spermidine bullet eliciting a significantly higher T cell response compared to GET bullet (Fig. 7C, p-value 0.0002). Antibody responses were also assessed against the GET peptide delivery system and found to be negligible (Fig. S4).

4. Discussion

4.1. GET-gold-DNA CR 50 formulation significantly transfects in vitro

GET-DNA formulations at various charge ratios, as free complexes or bound to gold microcarrier, were tested in dendritic cells (DC2.4) in the absence and presence of serum. DC2.4 cells were selected to represent antigen presenting cells (APCs), key target cells *in vivo* for vaccines. The formulations were assessed in the absence and presence of serum as multiple electrostatic interactions and inhibitory substances abundant in serum can directly interfere with the nanocomplexes and the protein

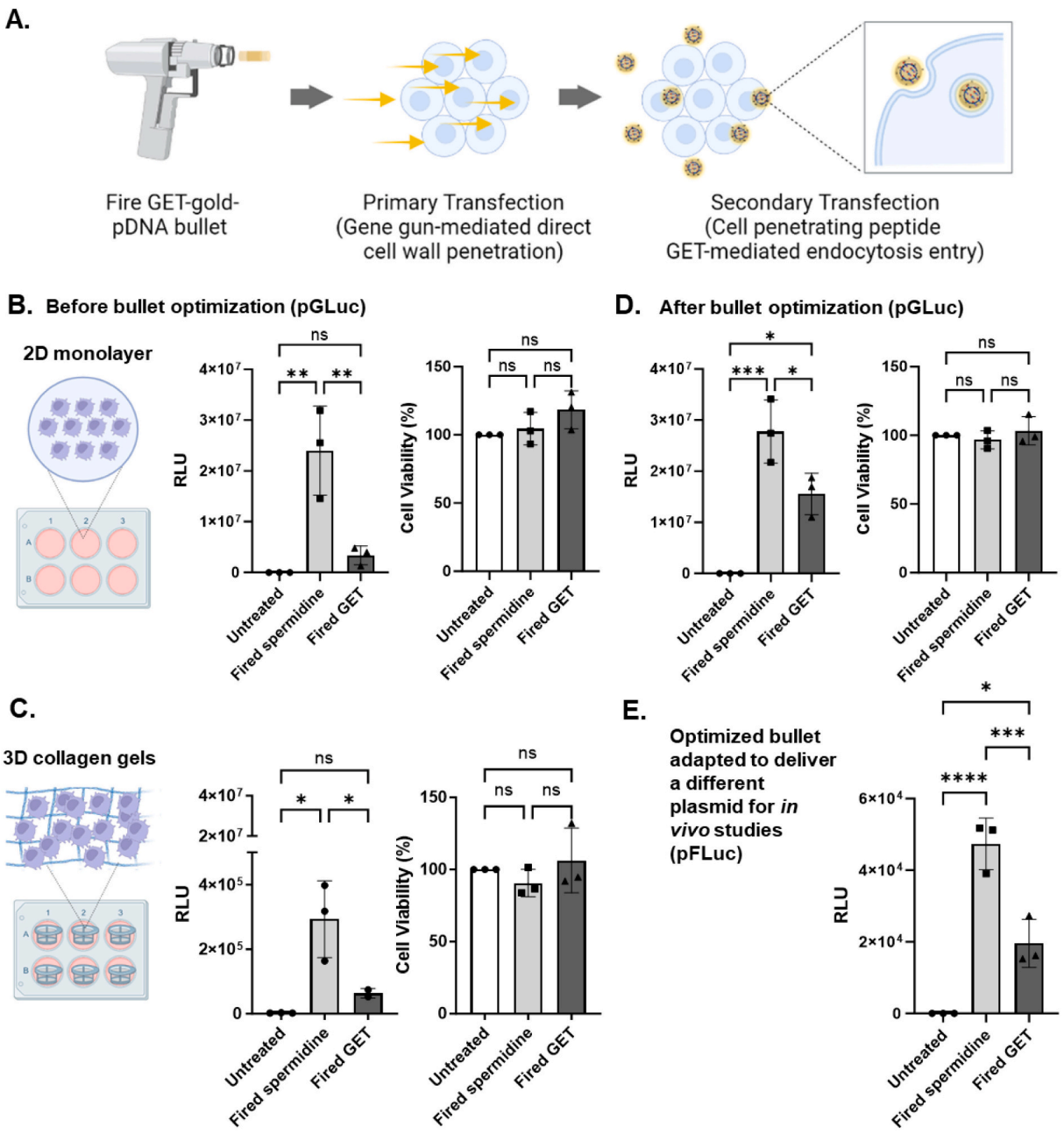


Fig. 5. Firing of GET bullets *in vitro*. A. GET-gold biolistic proposed dual mechanism of transfection. B—C. Initial GET bullet prototypes had higher transfection compared to untreated however did not reach statistical significance in both monolayer and multi-layered *in vitro* models. D. Optimized GET bullets containing similar 1 μg DNA content to spermidine bullet showed improved transfection efficiency compared to untreated control while maintaining good cell viability. E. Adaptation of the optimized GET bullets to deliver a different plasmid likewise resulted to improved transfection compared to untreated control. $N = 3$ biological replicates, bar graphs and error bars represent mean ± SD, *p-value <0.05, statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

corona generated can prevent transfection [17,22,34]. GET peptides are positively charged due to their polyarginine domain while many serum proteins are negatively charged. The interference generated by binding of serum proteins to GET-DNA nanocomplexes might explain the overall lower transfection levels observed across different GET-DNA CRs in the presence of serum [22]. Consistent with other cell penetrating peptide transfection studies using cationic-rich sequences, the presence of serum decreases transfection efficiency [23,35–37].

The absence of gold microcarrier affected both transfection efficiency and cell viability of the GET-DNA at different charge ratios *in vitro*. Without both gold and serum, GET-DNA at CR 10 transfected DNA the highest while maintaining cell viability. The addition of serum decreased the transfection capabilities of CR 10 but improved CR 50. At very high charge ratios, the absence of both steric hindrance provided by the gold and interference by serum proteins meant that a surplus of free cationic peptides likely led to both poor transfection and significant cell cytotoxicity. Upon addition of serum, the negatively charged serum proteins are likely to interact with the excess cationic GET peptide of CR 50 and lead to an overall decrease in net positive charge. Serum-inactivation of free GET peptide resulted in a small improvement in transfection efficiency at high charge ratios, however, GET-DNA at CR 50 without gold significantly affected cell viability making this formulation unacceptable to use.

Gold-GET-DNA at CR 50 provided significantly higher luciferase

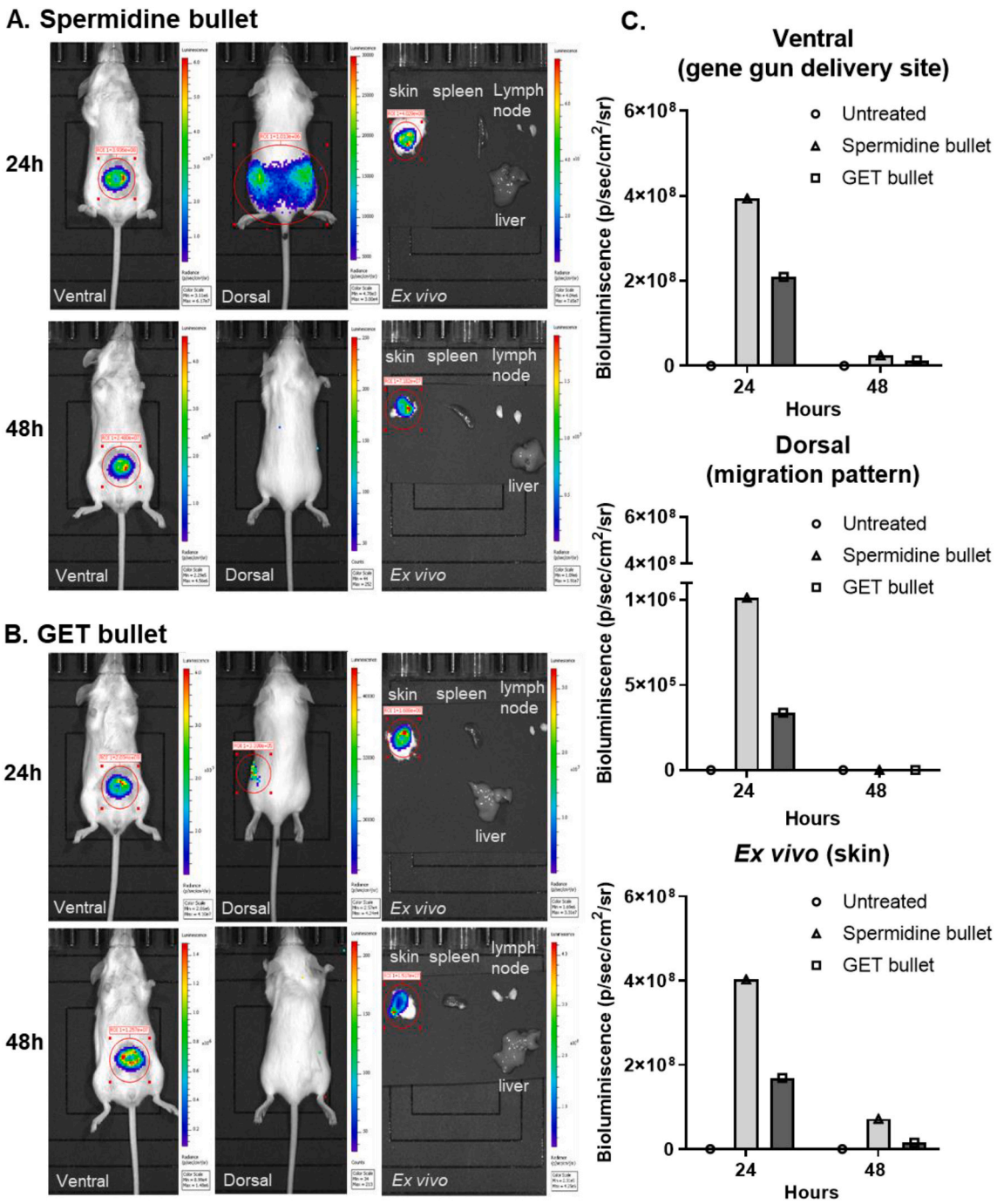


Fig. 6. *In vivo* reporter gene (Firefly luciferase, pFLuc) detection of GET and spermidine bullets using IVIS imaging system. A-B. Both spermidine and GET bullet systems (1 μg DNA) demonstrated high bioluminescence at the ventral site and *ex vivo* skin (administration site) at 24 h. C. Dorsal migration pattern of bioluminescence was more prominent in the spermidine bullet system. At the 48-h timepoint, signal detection was lower at the dorsal site compared to ventral administration and skin site suggesting transient migration but continuous delivery site reporter gene expression in both bullet systems. $N = 2$ mice were used for each group in this experiment.

levels while maintaining cell viability. In line with the above results, the improved transfection can be attributed to the steric hindrance effect generated by the addition of 1 μm gold microcarriers that led to decreased inhibitory electrostatic interactions with the serum proteins. Similarly, the lower overall net cationic charge of gold-bound GET-DNA CR 50 compared to free GET-DNA CR 50 without gold is likely to have led to the improvement in cell viability seen. Our results are supported by a study on spermidine bullets that showed the presence of a microcarrier such as gold protects the DNA-spermidine complexes and improves stability and thereby transfection efficiency [10]. Moreover, consistent with multiple studies of GET peptide bound to a magnetic iron-oxide particle, transfection efficiency was influenced by charge ratio and the presence of serum [17,22]. The presence of the iron-oxide carrier also improved GET transfection in serum when compared to free unbound GET complexes [17,22]. In summary, GET-gold-DNA at CR 50 significantly improved reporter gene expression even in serum-containing environments.

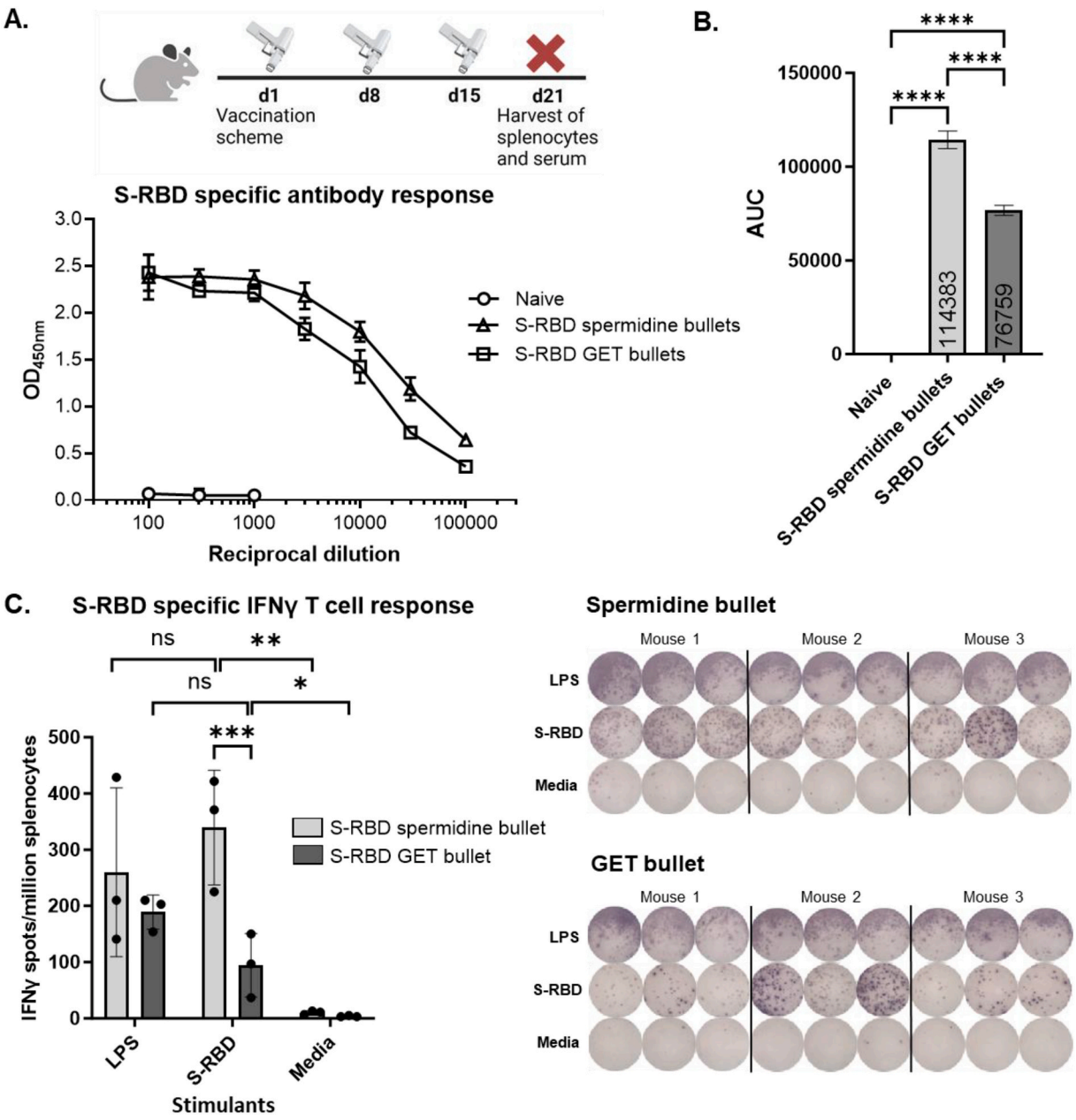


Fig. 7. *In vivo* vaccination study against SARS-CoV2 using pVAX-S-RBD (spike protein receptor binding domain) GET bullets. A. S-RBD specific binding antibodies were detected in both spermidine and GET bullets (1 μg DNA). B. The area under the curve (AUC) for S-RBD specific binding antibodies obtained with the GET bullet was 1.5 times lower than with the spermidine bullet. ****p-value <0.0001. N = 3 mice per group, individual points represent pooled sera per group, error bars represent SD. C. S-RBD GET bullets successfully elicited T cell-mediated IFNγ response against the RBD domain of spike protein after 3 doses of vaccination in mice, albeit at lower levels than standard spermidine bullets. Lipopolysaccharide (LPS) as stimulant for positive control, sterile media for negative control. ***p-value <0.001, **p-value <0.01, *p-value <0.05, N = 3 mice per group, individual points represent each mouse, bar graphs and error bars represent mean ± SD.

4.2. GET-gold-DNA can be adapted into a fireable bullet format

Integrating the GET-gold-DNA into the gene gun bullet system required modification and tailoring of the conventional spermidine bullet protocol. PVP with absolute ethanol is used as diluent in the spermidine bullet to facilitate evaporation and allow adherence to the bullets. However, GET peptide is not compatible with absolute ethanol hence the diluent was replaced with equal ratios of nuclease free water and freezing media containing polysaccharides. The water was then removed using sublimation *via* lyophilization. Due to these differences, the GET bullet prototype had a more delicate lattice-like configuration macroscopically and was easier to eject from the bullets compared to spermidine bullets using PVP as an adhesive. In summary, GET bullets were successfully generated by using a simpler, more scalable alternative technique.

Physicochemical characterization of the bullet formulation contents is a crucial step for both quality control and understanding how physical parameters of nanoparticles affect transfection. The observable difference of the *Z*-average (or size) between the GET and spermidine bullet may be related to the differences in overall compactness of the DNA packaging by both peptide systems at the selected concentration. GET bullet contents were smaller, more uniform in size, and positively charged, while spermidine bullet contents were larger, less uniform in size, and negatively charged. Previous physical characterization of naked GET-DNA complexes showed 3-fold smaller sized nanoparticles (<100 nm) [30] compared to our results, however another study of GET-

DNA bound to larger iron-oxide particles showed similarly larger sized nanoparticles (300 nm). [22,31] This suggests that physical characteristics are influenced not only by which peptide and what concentration is used, but also the coupling to a larger carrier particle.

DNA packaging is tighter and more stable in GET peptide complexes compared to spermidine. The physicochemical parameters were further corroborated functionally by a degradation challenge using increasing concentrations of DNAse. GET bullet contents, by virtue of having less exposed DNA due to tighter DNA packaging, was not degraded by DNAse prior to disruption of complexes and release of DNA using SDS, unlike spermidine bullets. The additional upper band and smear seen in both bullet samples suggested a different pDNA conformation after release from the gold microcarrier. This difference could be due to partially nicked or refolded DNA resulting from the precipitation or dissociation from the gold microcarrier, decomplexation (from spermidine/GET) or the bullet cartridge itself. This finding is similar to a study on DNA integrity from extracted bullet contents using electrophoresis, which concluded that DNA integrity was affected by the bullet making procedure, as well as during decomplexation from the gold [10]. Notably, similar results were obtained in another study where GET-DNA complexes protected against DNAse degradation and upon decomplexation of the particles, DNA degradation was seen as a smear, shift or loss of bands [29,30]. In summary, DNA in GET bullets is packaged efficiently and protected from DNAse degradation.

4.3. Chemical-mediated transfection is retained in GET bullet contents

Extracted DNA-GET bullet contents retained their transfection efficiency significantly better than extracted spermidine bullet contents. This observed transfection effect can be explained by the physicochemical analysis previously discussed, where GET bullet contents remained small, uniform, and positively charged, all of which are features ideal for cell uptake by endocytosis as shown in other studies [22,23,30]. In addition, the cell-penetrating nature of GET peptide, unlike spermidine, facilitates the chemical-mediated transfection of the flushed GET bullet contents. This indicated a potential secondary transfection mechanism, supplementing the primary gene-gun mediated transfection mechanism when bullets are fired. Lastly, these findings indirectly demonstrated that the highly efficient transfecting capabilities of the gold standard spermidine gene gun bullets are entirely dependent on gene gun-related physical transfection mechanisms rather than any inherent chemical transfection-related mechanisms and endocytotic uptake. Other spermidine-based transfection studies, where spermidine-DNA alone led to inefficient DNA transfection but was improved when spermidine-DNA was complexed to lipids corroborate this finding [38,39]. In summary, GET bullet contents retained chemical-mediated transfection capabilities, potentially augmenting physical-mediated transfection, which are lacking in conventional gene gun bullets.

4.4. DNA content can be optimized in GET bullets

Particle-mediated delivery systems have unique parameters to account for amount of DNA (DNA-LR) and particle carrier loaded in grams (MLQ) per bullet. The typical DNA-LR range for spermidine bullets is 1–5 μg DNA per 1 mg gold [12] but can be increased up to DNA-LR of 10 with an MLQ of 0.25–0.5 (equivalent to 250 mg to 500 mg gold per bullet) [10,40]. Using a higher DNA-LR and MLQ causes microparticle agglomeration and cytotoxicity likely due to DNA-gold clumping generated during the spermidine bullet process [10,12,40]. This highlights the DNA dosing limitation of the spermidine bullet system because increasing beyond DNA-LR of 5 (2.5 μg DNA per spermidine bullet) results in a formulation unsuitable for cartridge preparation [12]. Furthermore, increasing DNA loading onto spermidine gold bullets does not translate to increase reporter gene expression likely due to microcarrier saturation and clumping of gold microcarriers [10,40]. With these factors in mind, we used the standard recommended spermidine bullet formulation as the positive control, while maintaining within the ranges during GET bullet optimization.

Initial loaded DNA content (theoretical DNA amount) may differ from the final DNA content after the bullet making procedure. Optimized GET bullets reached the 1 μg target DNA content after increasing the DNA LR to 8, which was equivalent to 4 μg DNA per 0.5 mg gold per bullet. The use of absolute ethanol and PVP in the standard spermidine bullet protocol enabled improved adherence of DNA onto the surface of the gold microcarrier. The GET bullet complexes were more loosely adherent on the gold surface likely due to the different strategy of loading the bullet by precipitation. These factors explain the higher percentage loss of both DNA and peptide in GET bullets, and highlights the importance of improving adhesion of GET-DNA onto the microcarrier. Other studies attempting to increase DNA in the spermidine-gold bullets led to similar DNA loading limitations seen with the GET-gold bullet, wherein only a limited amount of DNA can be loaded until it hits a plateau or saturation for a constant amount of gold microcarriers [10,12,40]. In summary, loss of DNA in GET bullets due to suboptimal adhesion onto the gold surface can be compensated by increasing the DNA loading ratio. Assessing the amount of DNA in bullets through random batch sampling is important to ensure accurate amounts of DNA within the bullets. DNA that was not loaded could be recovered using column purification, this meaning that DNA is not wasted in this process.

Lastly, the GET bullets used in this study were manufactured using precipitation with $CaCl_2$ as the mechanism of DNA adhesion onto the microcarrier [12]. GET-DNA was previously shown to adhere onto iron oxide particles *via* functional groups and facilitated transfection, but as a liquid formulation [17,22,31]. Alternative strategies such as electrostatically binding DNA on the microcarrier surface using functional groups (*i.e.* carboxyl group, amino group, polyethylenimine) in the format of a GET bullet are currently being explored.

4.5. Gene gun fired GET bullets transfect cells in vitro

Gene gun-fired GET bullets significantly improved reporter gene expression compared to untreated controls, but were inferior to spermidine bullets in both 2D monolayers and 3D collagen gel models. Given that both bullet systems had similar DNA quantity, fired under similar experimental conditions, and contained the same plasmid construct, we attributed the lower transfection levels of fired GET bullets to two main reasons. First, the less adherent and fragile floss-like structure of the GET bullet might have led to unaccountable DNA losses during the firing procedure, leading to an overall lower number of complexes reaching the contact surface area. Multiple biolistic studies involving different sizes of gold loaded with spermidine-DNA showed that transfection levels are affected by density, size of the particles, surface contact area and depth of penetration [10,41,42]. Other studies also show that the quantity of intact DNA complexes directly delivered intracellularly is what makes particle-mediated gene gun delivery more efficient compared to other DNA delivery routes. [4,43,44]

Second, GET packaging of the DNA is tighter than spermidine packaging resulting to lower unloading of the DNA in the cytoplasm and subsequent entry into the nucleus. This is consistent with other GET-DNA complex studies wherein a charge ratio of 5 and above resulted in complete complexation of DNA [31] and inability to be degraded by DNAse [30]. An intermediate stability is ideal for optimal gene expression because unstable complexes are susceptible to rapid DNA degradation, but overly stable complexes restrict DNA release and subsequent transcription and translation.

4.6. Gene gun fired GET bullets transfect in vivo

Fired GET bullets containing firefly luciferase reporter gene generated high luminescence levels *in vivo*. Both bullet systems had high

reporter gene expression levels at the ventral administration site within 24 h that persisted at 48 h suggesting continuous expression locally in the directly transfected surface area. Meanwhile, luminescence at the dorsal site was transient in both bullet systems and disappeared by 48 h suggesting transitory migration *via* the lymphatic system. GET bullets had lower luminescence in the dorsal images suggesting either less efficient lymph node draining or lower transfection levels compared to standard spermidine bullets.

In vivo bioluminescent imaging studies involving gene gun mediated spermidine-DNA bullets in mice also showed similar levels and kinetics of reporter gene expression at the skin site of administration, peaking in 24 h then gradually decreasing over 3 to 5 days [40,45]. Other *in vivo* studies using GET-DNA also showed successful gene transfection in 24 to 48 h [30]. In summary, GET bullets facilitate gene expression *in vivo* within 24 to 48 h on the site of administration.

4.7. DNA vaccine delivered via GET bullets elicited antigen-specific antibody and T cell responses

GET bullets delivering a plasmid encoding the S-RBD domain of SARS-CoV-2 generated both T-cell specific and antibody immune responses against the target antigen. The ideal immune response against an infectious disease agent such as SARS-CoV-2 is a combination of protective antibody response and T cell response to address both extracellular and intracellular viruses respectively [46,47].

The magnitude of S-RBD binding antibodies induced by GET bullets was around 1.5-fold lower compared to the spermidine bullets. This corresponded with the slightly lower transfection efficiency and migratory capabilities of the GET bullet compared to the spermidine bullet in the IVIS analyses of FLuc reporter delivery. Despite the lower effective DNA delivery of GET bullets, a strong antibody immune response was still generated. Results from a DNA vaccine against influenza assessing effects of administration routes including gene gun showed that transfection efficiency does not determine vaccination efficiency [4]. This is further supported by the observation that even as little as nanogram amounts of DNA administered by particle-mediated gene gun delivery elicited similar antibody responses when compared to delivery of higher quantities of DNA *via* the same route [5]. Taken together, this provides evidence that GET bullets can both deliver DNA vaccines and generate a robust humoral response.

S-RBD specific T cell responses were elicited by the GET bullet, but these were also lower than those obtained with spermidine bullets. Studies of particle-mediated DNA vaccine delivery *via* gene gun using influenza models revealed that intracellular delivery of DNA into appropriate cells was necessary for an effective major histocompatibility complex (MHC) class 1 antigen presentation needed for a robust CD8 T cell response [44]. In addition, multiple studies showed that route of administration greatly influenced T cell responses, wherein particle-mediated delivery *via* gene gun resulted in higher T cell responses compared to parenterally-delivered DNA vaccines [4,5,44,48,49]. Direct delivery of DNA vaccines intracellularly within target cells is necessary for a robust T cell response at DNA-sparing doses [4,5,44] and is best facilitated by particle-mediated DNA vaccination using the gene gun. In summary, improving DNA loading in GET bullets and decreasing tightness of the nanocomplex by altering charge ratios may improve T cell responses by virtue of higher intracellular delivery and unloading of DNA for antigen transcription.

Importantly, GET-gold-DNA vaccine complexes generated negligible anti-vector antibodies. Testing antibody response against vector or delivery vehicles is important to determine potential limitations in efficacy of repeated doses for multiple immunisation schemes or future administration of other vaccines using the same delivery system. Anti-vector antibodies may lead to rapid clearance of the therapeutic agent from the circulation [50]. Likewise, testing for a T cell response against the vector may also be important to detect T cell competition between epitopes within the vector and antigen that may decrease overall T cell responses [51,52]. Lack of standardization of processes and tools to assess immunogenicity of non-viral vectors or its components remain to be addressed in future studies. However, it was clear that GET peptides generated negligible GET-related antibody responses in the bullet format.

Other *in vivo* studies using GET-DNA and GET-peptide complexes resulted in improved bone defect regeneration [30], gene delivery in lungs [29], and lowering of blood sugar after delivery of insulin [32]. Taken together, although GET bullets generated slightly lower *in vivo* expression and immune responses than spermidine bullets, it represents an effective system that has other benefits such as ease of manufacture and scalability compared to conventional biolistic bullets. However, further improvements in DNA adherence and loading of GET bullets are needed for it to become transformative for DNA delivery.

5. Conclusion

We have demonstrated that the GET peptide system can be successfully incorporated into existing approaches for biolistic delivery. Fired GET bullets successfully transfected cells both in *in vitro* and *in vivo*, generating both SARS-CoV-2 S-RBD specific T cell and antibody responses in mice. Future strategies to improve the adherence of GET-DNA onto the carrier surface and to increase the DNA concentration in these bullets beyond the current limitations of the gene gun approach should further improve the efficacy of the system. Exploring combinations of chemical transfection with other physical delivery methods such as using a different microcarrier, biolistic system, and microneedle patches for intradermal deposition are on-going. Effective DNA delivery and transgene expression in the skin with a pain-free device will have applications beyond vaccination. Future work could explore GET bullet use in regenerative medicine of the skin such as wound healing and scar aesthetics, as well as topical disease treatment such as oncology and monogenic skin disorders. This translatable and effective augmented biolistic method should now be explored as an alternative to conventional approaches to nucleic acid delivery.

Author contributions

J.M.D. and J.E.D. conceived and initiated the project; R.B·S., G.L., V. B., J.M.D. and J.E.D. designed the experiments; R.B.S. and G.L. conducted the experiments; J.M.D. and J.E.D. supervised the study; R.B.S, J. M.D. and J.E.D. wrote the manuscript. All authors approved the final manuscript.

CRediT authorship contribution statement

Roizza Beth So: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation. **Gang Li:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Victoria Brentville:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Janet M. Daly:** Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis. **James E. Dixon:** Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2024.01.031.

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