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Integration of needle-free jet injection with advanced electroporation delivery enhances the magnitude, kinetics, and persistence of engineered DNA vaccine induced immune responses

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ABSTRACT

The combination of optimized DNA constructs, improved formulations and advanced *in vivo* electroporation (EP) has been shown to generate potent and efficacious immune responses in the clinic. Needle-free jet injection has also been reported to improve DNA vaccine delivery over standard needle and syringe in clinical trials. Here we investigated the impact of combined jet injection and EP (Jet-EP) delivery on muscle transfection efficiency and DNA vaccine immunogenicity in rabbits and nonhuman primates (NHPs) compared to jet injection alone. Our results show that the addition of EP significantly enhanced *in vivo* DNA transfection efficiency of rabbit muscle over jet injection alone. Jet-EP delivery augmented the rate and magnitude of DNA vaccine induced humoral and cellular responses over jet injection alone in both rabbits and NHPs. Jet-EP delivery also resulted in higher proportions of polyfunctional antigen specific T cells producing IFNγ, IL-2, and/or TNFα. Elevated antibody levels were sustained nine months post immunization in NHPs immunized with a DNA vaccine using Jet-EP delivery, far outperforming jet delivery alone. Our results provide proof-of-concept that addition of advanced EP to needle-free jet injection delivery improves *in vivo* DNA transfection efficiency, increasing the magnitude, rate and duration of cellular and humoral immune responses to DNA vaccines. This combination likely has significant advantages in important vaccine and immunotherapy settings.

1. Introduction

Historically, naked DNA vaccine delivery by standard syringe and needle injection has induced at best weak immunity in nonhuman primates (NHPs) and humans [1,2]. However, significant optimizations in the design, formulation and delivery of DNA vaccines have generated impressive preclinical results that have successfully translated to the clinic, resulting in the recent first-in-class demonstration of clinical efficacy by an engineered human papillomavirus (HPV) DNA vaccine delivered by *in vivo* electroporation (EP) for the treatment of cervical intraepithelial neoplasia [3], as well as induction of rapid and potent immunity by a synthetic prME Zika DNA vaccine delivered by advanced EP [4]. This vaccine induced immune responses in 100% of vaccinees in a phase I safety and immunogenicity clinical trial in which 95% of vaccinated subjects studied demonstrated induction of Zika virus inhibiting antibodies based on an *in vitro* neuronal cell assay and 100% of studied clinical subjects developed protective antibodies in passive transfer studies [4].

One major inflection point for the success of DNA vaccines in the clinic was the addition of enhanced delivery technologies to significantly improve uptake of plasmid DNA vaccines. Transfection efficiency can be improved using both chemical and physical means. Chemical approaches include DNA complexing agents made of cationic lipids or polymers, which may be combined with other materials to form micro- or nanoparticles designed for cellular uptake, endosomal escape, and nuclear delivery [5,6]. Chemical approaches are commonly used for efficient *in vitro* transfection, but have been less successful *in vivo*, likely due to the complex biological environment affecting the stability and dispersion of the formulation, thus limiting the transfection efficiency of these systems [7,8]. Multiple physical mechanisms have also been

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developed to assist with DNA vaccine administration, including jet injection, microneedle delivery [9], sonoporation [10] and EP. Of these chemical and physical methods, EP has had the most widespread success for *in vivo* DNA vaccine delivery. Current *in vivo* EP technology applies a series of very short electrical pulses at the DNA injection site to induce transient permeability of cell membranes, resulting in up to 1000-fold increases in transfection efficiency [11,12] and superior immune responses over standard needle-syringe injection delivery [13,14]. Accordingly, these better designed, more potent formulations of DNA vaccines delivered using advanced EP can generate antibody responses comparable to viral-based vectors, and have been shown to consistently drive strong cellular immune responses in large animals and humans [15–18] while avoiding anti-viral vector immunity, thus allowing multiple and repeating treatments. Multiple phase I and phase II clinical trials employing CELLECTRA® EP have reported strong immunogenicity data [4,18–21], and recently, a therapeutic HPV DNA vaccine delivered by this device was shown to be effective at reducing or eliminating precancerous lesions and viral loads in patients with HPV-related cervical intraepithelial neoplasia in a randomized controlled phase 2b study [3]. This was the first randomized controlled trial demonstrating the clinical efficacy of an EP-enhanced DNA vaccine.

Needle-free jet injection is an alternative physical delivery mechanism to parenterally administer medicines into patients without using a conventional needle to pierce the skin. Jet injectors function by propelling liquid through a small orifice under high pressure, creating a fluid stream capable of puncturing skin, effectively dispersing and depositing the injected material at a desired depth for intramuscular, subcutaneous, or intradermal delivery of therapeutic products. Compared to needle and syringe delivery, jet injections do not generate any sharps waste, help patients overcome needle phobia, and are faster to administer. Modern jet injectors use disposable parts which contact the patient, eliminating the historical risk of cross-contamination between treatments. Previous clinical trials reported that DNA vaccines delivered by intramuscular needle-free jet injection are more immunogenic compared to standard syringe-and-needle injection, possibly due to broader dispersion of the injectate [22–24]. However, jet injection-delivered DNA vaccine immune responses can be inconsistent in human trials [24] and are typically of low magnitude [22]. Additionally, the longevity of immune responses following needle-free DNA vaccination is unknown. We hypothesized that the addition of adaptive EP could potentially overcome the observed inconsistencies of needle-free delivery for DNA vaccines related to duration, breadth and magnitude of immune response. Previous studies have shown that EP can enhance jet delivered plasmid gene expression in porcine skin [25] as well as DNA vaccine-induced immune responses in mice [26]. However, there are no reports of combination jet and EP delivery studies performed in higher order animal species where, unlike in mice, effective DNA vaccine delivery through only an injection methodology is a significant hurdle. Because EP delivery has been shown to generate durable, high-magnitude immune responses as well as potent T cell responses in large animals [27] and humans [13], it is well-suited to address the potential shortcomings of jet injection alone. From a product development perspective, the disposable components used for both jet delivery and electroporation can be combined into a single disposable component, streamlining device design and potentially improving usability. Therefore, it is important to first identify whether the combination of jet delivery and EP indeed confers an immunological advantage over jet delivery alone, and whether the reported superiority of jet delivery compared to needle-and-syringe holds true with the addition of EP.

We sought to determine the feasibility of a combination needle-free jet injection and *in vivo* EP system for delivering DNA vaccines with robust immune responses. Here, we compared the immunogenicity of a Zika DNA vaccine in rabbits and nonhuman primates when delivered by needle-free jet injection alone or in combination with EP. The results of the study support that the addition of EP substantially improves the immune potency and longevity of immune responses by jet injected DNA vaccines.

2. Material and methods

2.1. Plasmid DNA and delivery devices

The pZIKV plasmid DNA construct encodes consensus full-length Zika virus (ZIKV) precursor of membrane (prM) and envelope (E) proteins that were genetically optimized and sub-cloned into a modified pVax1 mammalian expression vector, as previously described [28,29]. The pGFP plasmid DNA construct encodes GFP reporter gene subcloned into the modified pVax1 mammalian expression vector.

Intramuscular needle-free jet injections were performed using the Biojector® B2000 delivery device with #4 cartridges (manufactured by Bioject, now part of Inovio Pharmaceuticals). The B2000 is a CO_2-powered jet injector capable of injecting up to 1.0 mL, with variable injection depths controlled by cartridge nozzle geometry. The #4 cartridge was selected for optimal intramuscular delivery in previous rabbit studies (data not shown). Intramuscular EPs were performed using the clinical CELLECTRA® EP device (Inovio Pharmaceuticals), designed to deliver three pulses at a constant current of 0.5A.

2.2. Animals and vaccinations

Female New Zealand white rabbits (2–3 kg) were purchased from Charles River laboratories (Wilmington, MA) and acclimated for 1 week before experimentation under standard conditions. Rhesus macaques weighing 2.25–6.25 kg were individually housed and acclimated for 4 weeks before experimentation under standard conditions. All animals were housed at BTS Research (San Diego, CA) and all housing, handling and treatment protocols were approved and handled according to the standards of the Institutional Animal Care and Use Committee.

Rabbit immunizations: Rabbits (n of 5 per group) were injected with 1 mL of 1 mg/mL pZIKV into the quadriceps muscle using Biojector® B2000 alone (Jet), Jet with intramuscular EP immediately following plasmid injection (Jet-EP), or standard needle-and-syringe injection with intramuscular EP immediately following plasmid injection (EP). Muscles were immobilized by the operator during injection and EP procedures to ensure co-localization. Rabbits were immunized on weeks 0, 3, and 6 for total three immunizations. To assess the humoral response post each immunization, sera were collected on weeks 0, 3, 6, and 9 for ELISAs.

NHP immunizations: Rhesus macaques (n of 4 or 5 per group, mixed male and female randomized by weight and sex) were injected with 1 mL of 1 mg/mL pZIKV into the quadriceps muscle using Jet or Jet-EP as described above. NHPs were immunized on weeks 0, 3, and 6 for total three immunizations. Sera were collected on weeks 0, 2, 5 and 8, as well as 9 months post final immunization for ELISAs. Whole blood samples were collected into BD Vacutainer CPT tubes on weeks 0, 2, 5, and 8 for PBMC IFNγ ELISpot analysis.

2.3. GFP expression in rabbit quadriceps muscle

Rabbits (n of 4 per group) were injected bilaterally with 1 mL of 0.5 mg/mL pGFP into the quadriceps muscle using Jet or Jet-EP.

After 72 hours, animals were euthanized and quadriceps were collected. Muscles were cut perpendicular to the injection plane into 3 mm thick sections. GFP expression in the tissue was detected using a FluorChem R Imager (Protein Simple, San Jose, CA) and fluorescent intensity was analyzed using ImageJ software.

2.4. Enzyme-linked immunosorbent assays (ELISAs)

ELISAs were performed to determine sera antibody binding titers. Nunc ELISA plates were coated with 1 μg/mL recombinant ZIKV envelope protein (Meridian Life Science, Memphis, TN) in DPBS overnight at 4 °C. Plates were washed three times then blocked with 3% BSA DPBS with 0.05% Tween 20 for 2 hours at 37 °C. Plates were then washed and incubated with serial dilutions of rabbit or NHP sera and incubated for 2 hours at 37 °C. Plates were again washed and then incubated with HRP conjugated-species specific secondary antibodies and incubated for 1 hour at 37 °C. After final wash plates were developed using SureBlue TMB 1-Component peroxidase substrate as substrate and the reaction stopped with TMB stop reagent (KPL, Gaithersburg, MD). Plates were then read at 450 nm within 30 minutes using a SpectraMax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, CA).

2.5. Enzyme-linked immunospot (ELISpot) assays

NHP whole blood samples were collected into BD Vacutainer CPT tubes and PBMCs were isolated by centrifugation according manufacturer's instructions, then resuspended in RPMI1640 media supplemented with 10% FBS (R10). NHP interferon (IFN)γ ELISpot assays were performed using commercial Mabtech IFNγ ELISpot kits (Mabtech, Sweden). Briefly, 96-well ELISpot plates precoated with capture antibody were blocked with R10 medium overnight at 4 °C. The following day, 200,000 NHP PBMCs in R10 media were added to each well and incubated at 37 °C in 5% CO_2 the presence of peptide pools consisting 15-mers overlapping by 9 amino acids and spanning the length of ZIKV-prME protein, DMSO (negative control), or PMA plus ionomycin (positive control). After 18–20 hours, plates were washed and developed according to the manufacturer's protocols, and IFNγ positive spots were counted be an automated ELISpot reader (CTL, Shaker Heights, OH). Antigen-specific responses were determined by subtracting the number of spots in DMSO-treated from peptide-treated wells. Results are shown for individual animal spot-forming units (SFU)/10^6 PBMCs obtained for triplicate wells.

2.6. Intracellular Cytokine Staining (ICS)

NHP PBMCs isolated as described above were stimulated for 6 hours at 37 °C with ZIKV-prME peptide pools plus anti-CD28/49d co-stimulatory antibody (BD, San Jose, CA) or DMSO (negative control) in the presence of Brefeldin A-containing protein transport inhibitor (BD). Stimulated cells were stained with fixable viability dye (ThermoFisher Scientific, Waltham, MA) and antibodies against surface markers. Cells were then fixed in 4% paraformaldehyde and permeabilized with Perm/Wash Buffer (BD) according to manufacturer's instructions before staining for intracellular cytokines. Cells were analyzed using an LSR Fortessa (BD), and data was evaluated with FlowJo software (Tree Star, San Carlos, CA). Antigen-specific responses were determined by subtracting the values of DMSO-treated from peptide-treated samples. The following antibodies used for staining were obtained from BD Biosciences, Biolegend, Beckman Coulter or ThermoFisher Scientific: CD3-APC-Cy7 (SP34), CD4-BV650 (OKT4), CD8-BUV737 (SK1), CD14-BV421 (M5E2), CD16-BV421 (3G8), CD19-Pacific Blue (J3-119), TNFα-FITC (Mab11), IL-2-PECF594 (MQ1-17H12) and IFNγ-AF700 (B27).

2.7. Statistical analyses

GraphPad Prism 7.02 was used to analyze and plot the data. Box-and-whisker graphs plot all data points with whiskers down to minimum and up to maximum values and boxes extending from 25th to 75th percentiles. Where appropriate, the statistical difference between immunization groups at each time point was assessed using nonparametric Mann-Whitney test or one-way ANOVA using Dunn's post-test, each with $p < 0.05$ was defined as significant.

3. Results

3.1. Integrated Jet-EP delivery enhances plasmid gene expression in rabbit muscle

As previously discussed, needle-free jet injection improves plasmid gene expression *in vivo*. We therefore investigated whether jet injection could be integrated with intramuscular EP to further enhance plasmid DNA delivery in a rabbit model. Rabbits received intramuscular (IM) injections of GFP reporter plasmid delivered by needle-free jet injection alone (Jet), or integrated with CELLECTRA® EP (Jet-EP). Muscles were harvested 72 hours post-treatment and GFP expression quantified by fluorescence imaging. Jet-EP combination-treated muscles had significantly increased GFP expression by greater than four-fold as compared to Jet alone (1.71 ± 0.46 vs. 0.37 ± 0.15 mean ± SD fluorescence units; Fig. 1A). Jet-EP delivery significantly increased the area, brightness, and depth of GFP expression throughout several muscle layers (Fig. 1C), while Jet only delivery resulted in minimal GFP expression within a small area of muscle (Fig. 1B). These results suggest integrated Jet-EP improves the uptake and consistency of plasmid DNA delivery compared to Jet injection alone in higher order muscle tissue.

3.2. Integrated Jet-EP delivery enhances DNA vaccine induced humoral responses in rabbits

The expression of a DNA reporter construct appeared consistently higher when using Jet-EP over Jet injection alone. To assess the impact of increased expression on immune responses, we next investigated the effect of Jet-EP delivery of a DNA vaccine in a rabbit model. Rabbits received three IM immunizations spaced three weeks apart with a Zika virus DNA vaccine (pZIKV) delivered by Jet injection, Jet-EP, or standard needle-and-syringe injection followed by EP (n of 5 each). Humoral immune responses in rabbits were measured at week 0 and three weeks post each immunization by Zika envelope binding ELISA. Jet-EP delivery resulted in significantly stronger humoral responses as compared to Jet alone (9,810 ± 2,340 vs 630 ± 180 final mean ± SE endpoint titers [EPTs]; Fig. 2). Jet-EP delivery also significantly enhanced the kinetics of DNA vaccine-induced immune responses, with 3/5 (100 ± 31 mean ± SE EPTs) seroconverted rabbits after first and 5/5 (1,990 ± 867) after second immunization using Jet-EP, as compared to 3/5 seroconverted rabbits after first and second immunization (50 ± 27 and 910 ± 789, respectively) with Jet injection alone (Fig. 2). There were no significant differences in antibody binding EPTs at any timepoint post immunization between Jet-EP and standard needle-EP deliveries (9,810 ± 2,340 and 6,750 ± 3,118, respectively), indicating that the advantage of jet delivery over conventional needle might become insignificant with the addition

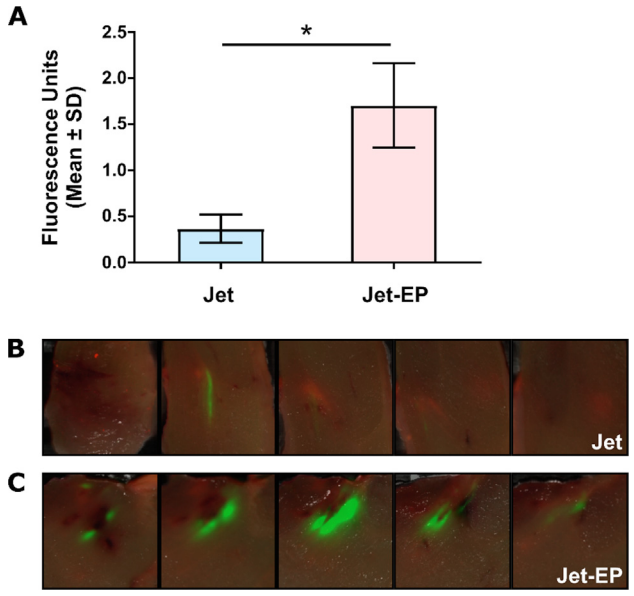

Fig 1. Plasmid gene expression with Jet versus integrated Jet-EP delivery in rabbit muscle. Rabbits were injected bilaterally with pGFP reporter gene plasmid into the quadriceps muscles via Jet or Jet-EP delivery and muscles collected at 72 h for GFP protein measurement by fluorescent imaging. Mean total tissue fluorescence intensities (A). Representative GFP images of a Jet alone (B) or Jet-EP (C) treated muscle cut into 3 mm thick sections perpendicular to injection sites. * Indicates significant difference between treatment groups as described in the methods.

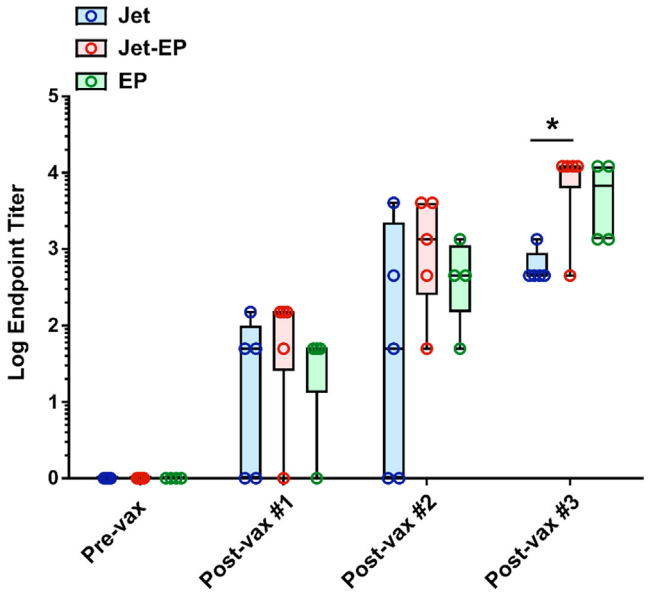

Fig 2. Rabbit humoral immune responses following Zika DNA vaccination via Jet versus integrated Jet-EP delivery. Rabbits were immunized with pZIKV DNA vaccine delivered by Jet alone, Jet-EP, or standard needle-and-syringe injection followed by EP (EP), and ZIKV envelope binding antibodies were measured by ELISA as described in the materials and methods. Lines indicate median and circles represent individual binding IgG endpoint titers (EPTs). * Indicates significant difference between treatment groups as described in the methods.

of EP due to the significant improvement in DNA vaccine delivery improvement by EP.

3.3. Integrated Jet-EP delivery enhances DNA vaccine induced humoral responses in nonhuman primates

Given the positive results in rabbits, we next evaluated Jet-EP delivery on DNA vaccine-induced immune responses in nonhuman primates (NHPs). NHPs received three IM immunizations spaced three weeks apart with pZIKV delivered by Jet injection (n of 5) or Jet-EP (n of 4). Humoral immune responses were measured at week 0 and two weeks post each immunization by Zika envelope binding ELISA. As shown in Fig. 3, the positive impact of integrated Jet-EP delivery was even greater on NHP humoral responses than in the rabbit model. Jet-EP delivery resulted in a significantly stronger humoral response as compared to Jet alone (post-third immunization mean ± SE EPTs of 19,575 ± 9,758 vs. 840 ± 802; Fig. 3). Jet-EP delivery also significantly enhanced the kinetics of DNA vaccine-induced immune responses, with binding antibody titers detected in all 4/4 NHPs after single immunization with Jet-EP (Fig. 3A, C), and only 1/5 seroconverted NHPs after first and 2/5 after second immunization with Jet injection alone (Fig. 3A, B). Jet-EP delivered humoral immune responses were long-lasting with binding titers maintained in all 4/4 NHPs at nine months post final immunization that were significantly greater than NHPs in the Jet alone group (300 ± 87 vs 41 ± 29). Therefore, integrated Jet-EP delivery significantly increases the rate, magnitude and duration of DNA vaccine induced antibody titers in NHPs when compared with Jet alone.

3.4. Integrated Jet-EP delivery enhances DNA vaccine induced cellular responses in nonhuman primates

While the reagents are unavailable to measure cellular responses in rabbits, T cell responses were measured in NHPs at week 0 and two weeks post immunization by IFNγ ELISpot to Zika envelope peptides. As shown in Fig. 4A, Jet-EP delivery resulted in greater T cell responses as compared to Jet injection alone after first (508 ± 268 vs. 40 ± 14 mean ± SE IFNγ spot-forming units/10^6 PBMCs [SFUs]), second (1,350 ± 168 vs 471 ± 152), and third (3,214 ± 724 vs. 567 ± 167) immunizations. These increased cellular immune responses were statistically significant at all timepoints post immunization (Fig. 4). Interestingly, cellular immune responses were boosted after each immunization for Jet-EP treated NHPs (Fig. 4A, C), but plateaued between second and third immunization with Jet alone (Fig. 4A, B).

To further assess the phenotype of the DNA vaccine-induced T cell response, multiparametric flow cytometry was used to measure the production of IFNγ, interleukin-2 (IL-2), and tumor necrosis factor alpha (TNFα) cytokines of $CD4^+$ or $CD8^+$ T cells following antigen-specific stimulation of PBMCs. The proportion of antigen-specific $CD4^+$ T cells producing IFNγ, IL-2, and/or TNFα was significantly increased in NHPs immunized with Jet-EP delivery as compared to Jet alone (Fig. 5A). Polyfunctional $CD8^+$ T cells producing IFNγ, IL-2, and/or TNFα were also observed, although at similar levels between Jet-EP and Jet alone treatment groups (Fig. 5B). Overall, integrated Jet-EP delivery significantly increased the rate and magnitude of DNA vaccine induced T cell responses over Jet injection alone in NHPs.

4. Discussion

Development and optimization of an appropriate delivery system has played an instrumental role in bringing DNA vaccines to clinical relevance. As one example, *in vivo* EP is one of the most potent and consistent delivery modalities for enhancing the immune potency of DNA vaccines in the clinic and the use of this technology resulted in the first demonstration of clinical efficacy combining a DNA vaccine against HPV with EP [3]. Needle-free jet delivery is another example of a physical delivery method and has been reported to enhance DNA vaccine immunogenicity by increasing fluid dispersion. We believe that the studies outlined in this publication are the first report that EP provides

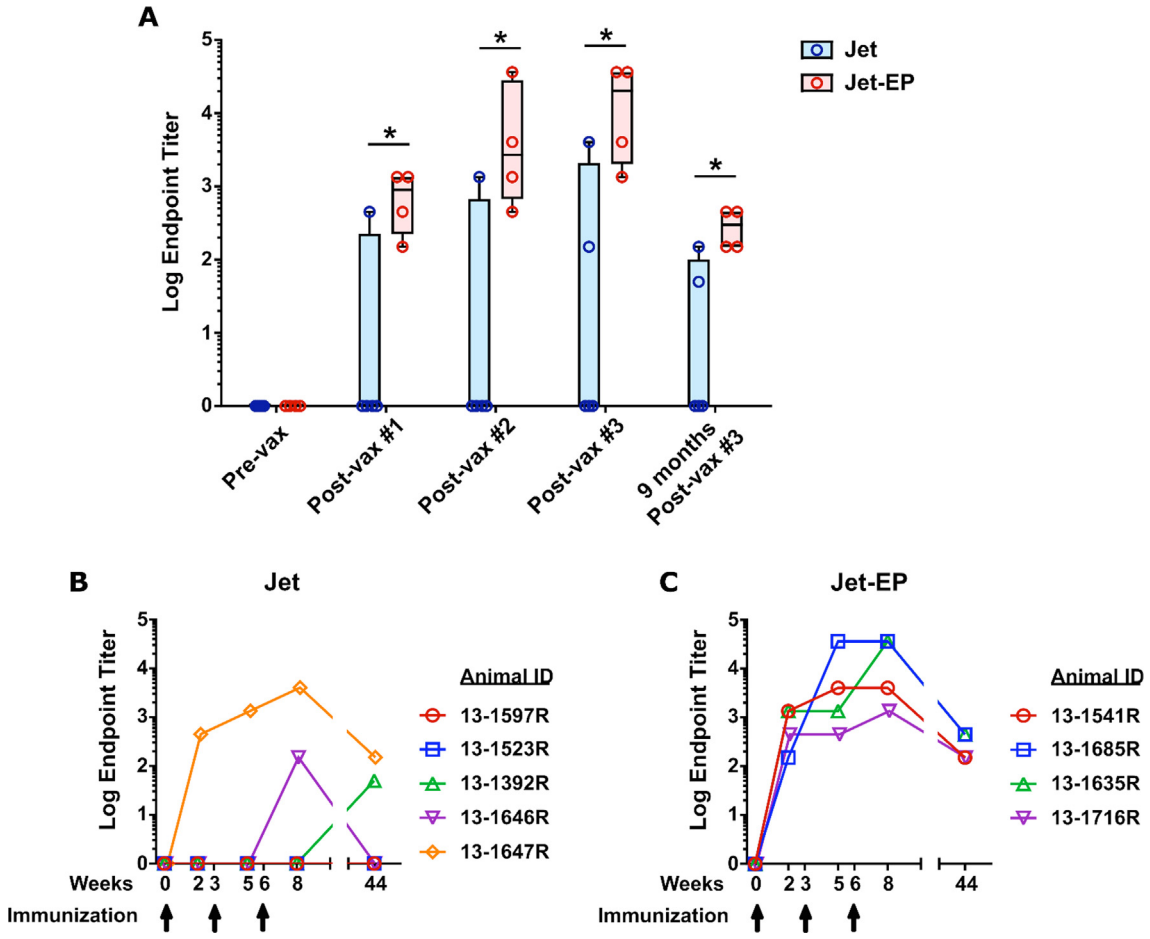

Fig 3. NHP humoral immune responses following Zika DNA vaccination via Jet versus integrated Jet-EP delivery. NHPs were immunized with pZIKV DNA vaccine delivered by Jet alone or Jet-EP, and ZIKV envelope protein binding antibodies were measured by ELISA as described in the materials and methods. (A) Direct comparison of treatment groups in which lines indicate median and circles represent individual binding IgG endpoint titers (EPTs). DNA vaccine-induced antibody timecourse for individual NHPs in Jet (B) or Jet-EP (C) delivery groups. * Indicates significant difference between treatment groups as described in the methods.

an immunological benefit to intramuscular needle-free jet injection for DNA vaccine delivery in NHPs. Furthermore, we show in a rabbit model that both needle-free jet injection and needle-and-syringe injection are comparable when combined with EP.

In a rabbit model, both standard EP and combined Jet-EP delivery of a DNA vaccine exhibited superior magnitude and kinetics of immune responses compared to jet delivery alone. Previous studies have reported the superiority of jet delivery of DNA vaccines compared to needle-and-syringe in the absence of EP [30], but this effect was not observed in this rabbit study upon the addition of EP. These results suggest that EP is the dominant mechanism of *in vivo* DNA delivery. A future device prototype integrating jet injection and EP may simplify the delivery workflow and increase the ease of device use in the clinic. A needle-free jet device without EP has been used in a Zika virus DNA vaccine clinical trial with consistent but low antibody titers when the DNA dose was split amongst multiple administration sites [22]. A weaker response was seen in the clinic when a single administration site regime was followed [22]. This clinical data mirrors our observations in the current study that a Zika DNA vaccine administered using needle-free jet injection alone at a single site in NHPs resulted in low seroconversion rates. However, combining jet injection delivery with intramuscular EP vastly enhanced both the level and rate of antibody responses in NHPs even with a single administration site. Importantly, this enhancement persisted for at least up to nine months after the final vaccination (Fig. 3). To our knowledge, the durability of immune responses for a DNA vaccine delivered by a needle-free jet injection device has not been previously reported and therefore the enhanced durability of Jet-EP delivery compared to jet delivery alone in this study was encouraging. The cellular response measured by IFNγ ELISpot and ICS demonstrated that jet injection alone was able to elicit low-level antigen specific T cells in NHPs after the second immunization. However, the integrated Jet-EP delivery significantly boosted T cell responses at each time point for all animals, providing a more consistent, potent as well as durable immune response. Importantly, Zika virus infections have been shown to elicit T cell responses in humans, which are thought to contribute to protection alongside the humoral response [31]. Considering the lack of established clinical correlates of protection for Zika virus infection, the combined cellular and humoral immune responses induced by the Jet-EP delivery system represent a promising step forward towards a clinically-relevant DNA vaccine against Zika virus. Additional studies of this combined delivery approach in the context of prophylactic and therapeutic vaccine development is warranted.

To our knowledge, only two clinical comparisons of jet versus EP deliveries on DNA vaccine immunogenicity have been performed [23,32]. Both clinical studies evaluated HIV vaccination regimens using a DNA prime/MVA (modified vaccinia Ankara viral vector) boost strategy, which would influence final immune response outcomes. Immune responses were also measured after completion of DNA prime vaccination but before viral vector boost,

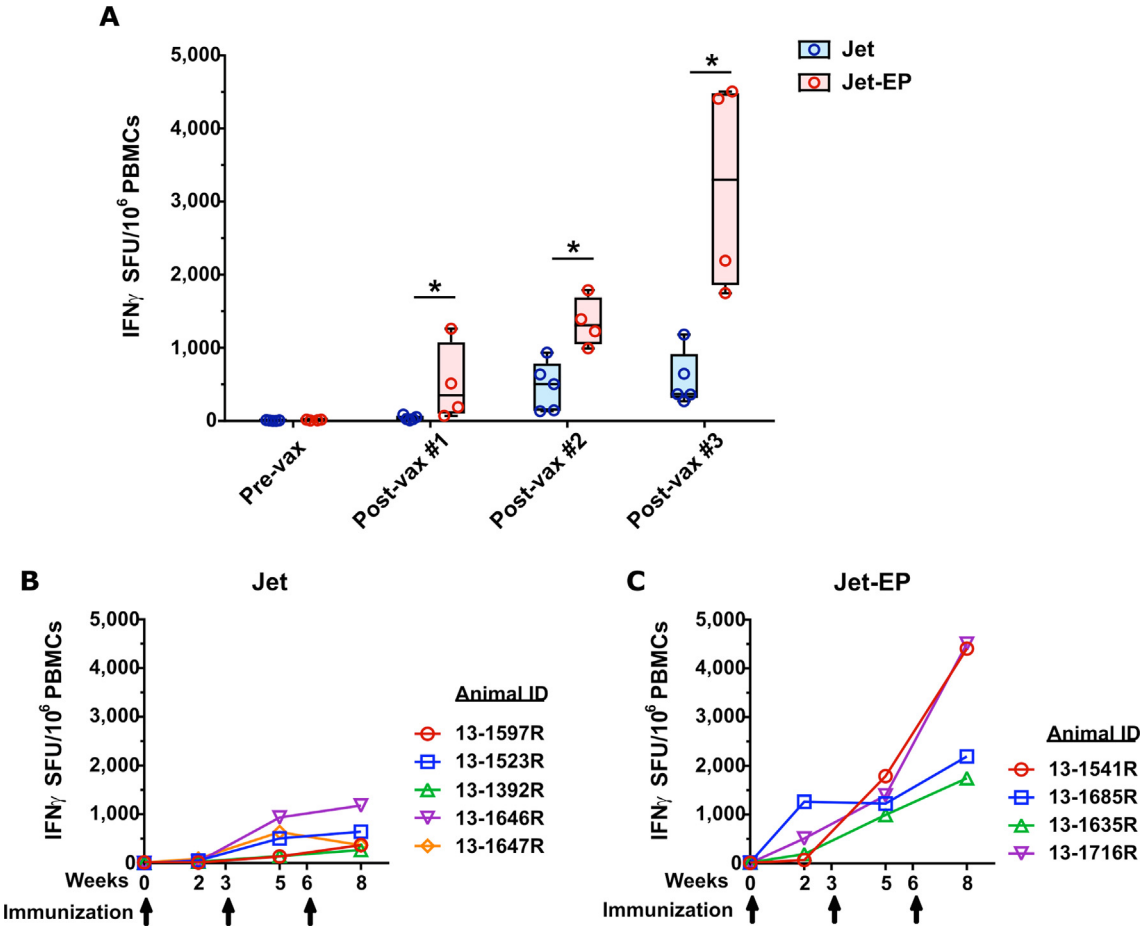

Fig 4. NHP cellular immune responses following Zika DNA vaccination via Jet versus integrated Jet-EP delivery. NHPs were immunized with pZIKV DNA vaccine delivered by Jet alone or Jet-EP, and specific cellular responses to ZIKV envelope peptides were measured by IFNγ ELISpot as described in the materials and methods. (A) Direct comparison of treatment groups in which lines indicate median and circles represent individual binding IFNγ spot-forming units (SFUs) per million PBMCs. DNA vaccine-induced IFNγ SFU timecourse for individual NHPs in Jet (B) or Jet-EP (C) delivery groups. * Indicates significant difference between treatment groups as described in the methods.

and in general the response rates were too low to demonstrate significant differences in both trials. In the first trial, Nilsson et al. delivered DNA vaccines intradermally (as opposed to intramuscularly as in this manuscript) by jet injection alone, or jet injection followed by intradermal EP [23]. T cell response rates after DNA prime were elevated in the EP cohort compared to the jet delivery alone cohort, although this difference was not significant (33% vs 14%, $p = 0.6158$) and there were no differences in immune responses after MVA boost. Additionally, Babiuk et al. also investigated intradermal vaccination comparing jet versus needle delivery with and without EP in pigs and found that only groups receiving EP had significantly increased antibody titers compared to baseline [25]. The evaluation of intradermal jet delivery with or without EP is currently ongoing and although out of scope for these current studies, will be the subject of a future manuscript. The evaluation of intradermal jet delivery with or without EP is currently ongoing and although out of scope for these current studies, will be the subject of a future manuscript. In the second clinical trial, Ake at al. compared intramuscular delivery using jet injection alone, or needle-and-syringe delivery followed by EP [32]. In this study, T cell responses were measured two weeks following DNA prime and prior to viral vector boost, but response rates were overall very low after DNA prime (7% in jet delivery cohort and 10% in electroporation delivery cohort), and similar to the Nilsson study, there were no significant differences in immune responses after MVA boost. In both of these clinical examples, it is likely that the interaction between the DNA vaccination and the viral vector boost was influential in the observed final immune responses, and therefore it is not possible from either of these trials to evaluate immunogenicity of DNA vaccination alone. In contrast, our study exclusively investigates the immune response kinetics of DNA vaccination using intramuscular jet delivery with and without electroporation.

A key factor governing the success of intramuscular DNA vaccines in the clinic is the successful transfection of myocytes and other resident cell types. Further, the expression of the encoded antigens and subsequent trafficking by APCs to the lymph nodes is critical to induce immune responses. The primary mechanism of the improved immune responses using Jet-EP compared to needle-free jet delivery alone appears to be an increase in the breadth of muscle cell transfection, which is consistent with our analysis of reporter construct expression in rabbit muscle. These observations provide strong evidence that the stronger and more durable immune responses in groups receiving EP are linked to this more widespread transfection. Additionally, EP itself has been proposed to act as a physical adjuvant to further boost cellular and humoral immune responses [33]. The repeated boost effect on cellular immune responses is most pronounced in animals receiving EP while repeated administration of jet alone provided a modest boost, suggesting that EP is primarily driving this boosting effect. Needle-free jet injection is thought to augment the immune response to DNA vaccines by improving the spatial distribution of drug within tissue, by creating localized tissue damage, or by physically disrupting cell membranes due to the high-speed fluid

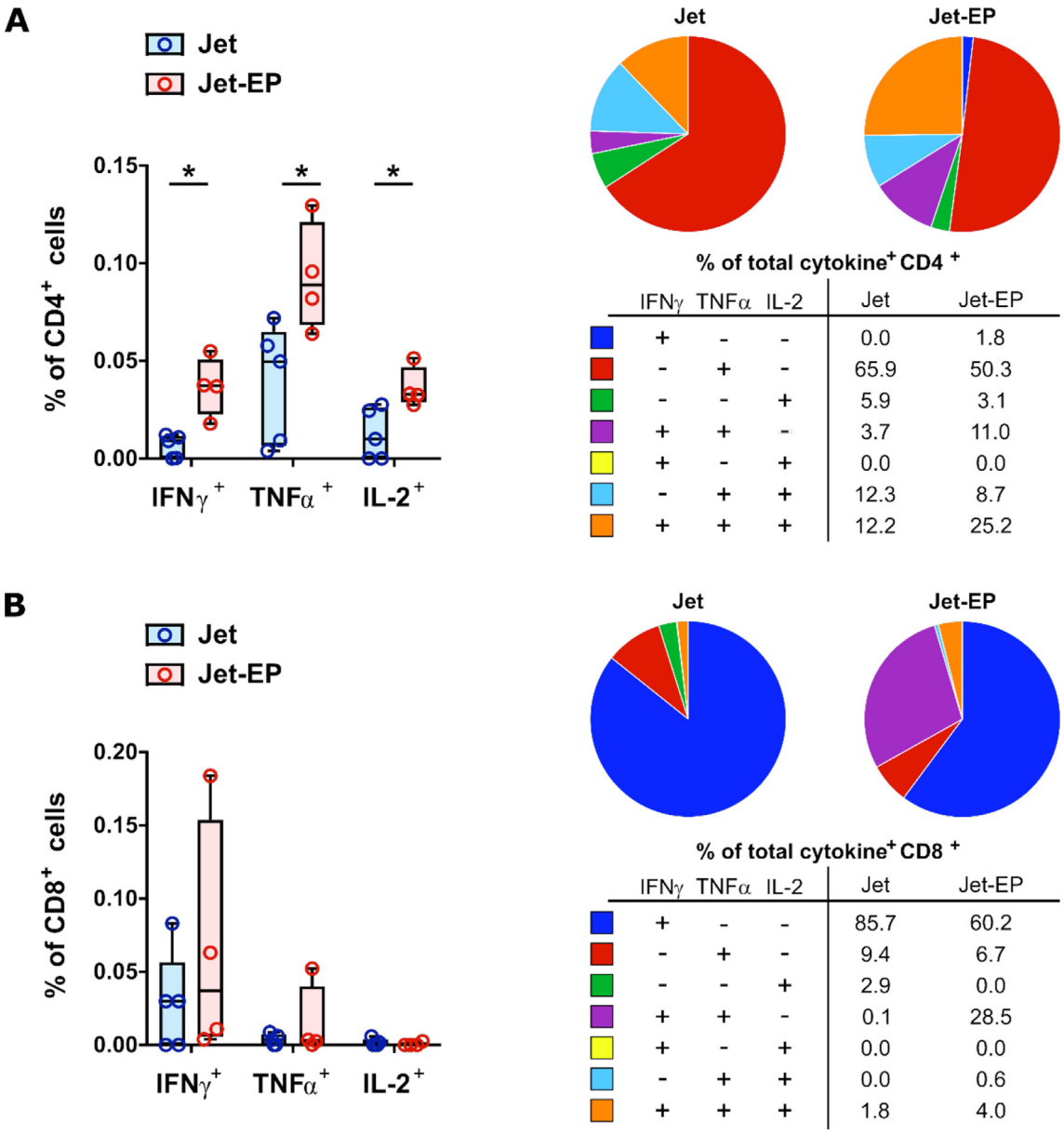

Fig 5. Flow cytometric analysis of NHP T cell phenotypes following Zika DNA vaccination via Jet versus integrated Jet-EP delivery. Multiparametric flow cytometry was used to measure IFNγ, IL-2, and TNFα positive $CD4^+$ or $CD8^+$ T cells following antigen-specific stimulation of PBMCs collected two weeks after third immunization. Percent of IFNγ, IL-2, and/or TNFα positive (left panel) and proportion of single, double, or triple cytokine positive (right panel) of total cytokine positive $CD4^+$ (A) or $CD8^+$ (B) T cells. * Indicates significant difference between treatment groups as described in the methods.

stream [34]. The novel combination of EP and needle-free jet injection therefore is a promising method for DNA vaccination because their separate mechanisms of actions, when combined, may be synergistic. Future studies will seek to confirm and quantify this synergy.

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Author disclosure statement

J.J., S.J.R., P.B., P.F., K.G., B.K.L., D.W., A.K., E.S., J.M., L.M.H., and K. E.B. are employees of Inovio Pharmaceuticals and as such receive salary and benefits, including ownership of stock and stock options. K.M. reports receiving grants from DARPA and receiving consulting fees from Inovio related to DNA vaccine development. D.B.W. has received grant funding, participates in industry collaborations, has received speaking honoraria, and has received fees for consulting, including serving on scientific review committees and board services. Remuneration received by D.B.W. includes direct payments or stock or stock options, and in the interest of disclosure he notes potential conflicts associated with this work with Inovio and possibly others. In addition, he has a patent on DNA vaccine delivery pending to Inovio.

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