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704/DNA vaccines leverage cytoplasmic DNA stimulation to promote anti-HIV neutralizing antibody production in mice and strong immune response against alpha-fetoprotein in non-human primates

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Genetic immunization is an attractive approach for prophylactic and therapeutic vaccination using synthetic vectors to deliver antigen-encoding nucleic acids. Recently, DNA delivered by a physical means or RNA by liposomes consisting of four different lipids demonstrated good protection in human phase III clinical trials and received Drugs Controller General of India and US FDA approval to protect against COVID-19, respectively. However, the development of a system allowing for efficient and simple delivery of nucleic acids while improving immune response priming has the potential to unleash the full therapeutic potential of genetic immunization. DNA-based gene therapies and vaccines have the potential for rapid development, as exemplified by the recent approval of Collategene, a gene therapy to treat human critical limb ischemia, and ZyCoV, a DNA vaccine delivered by spring-powered jet injector to protect against SARS-CoV2 infection. Recently, we reported amphiphilic block copolymer 704 as a promising synthetic vector for DNA vaccination in various models of human diseases. This vector allows dose sparing of antigen-encoding plasmid DNA. Here, we report the capacity of 704-mediated HIV and anti-hepatocellular carcinoma DNA vaccines to induce the production of specific antibodies against gp120 HIV envelope proteins in mice and against alpha-fetoprotein antigen in non-human primates, respectively. An investigation of the underlying mechanisms showed that 704-mediated vaccination did trigger a strong immune response by (1) allowing a direct DNA delivery into the cytosol, (2) promoting an intracytoplasmic DNA sensing leading to both interferon and NF-κB cascade stimulation, and (3) inducing antigen expression by muscle cells and presentation by antigen-presenting cells, leading to the induction of a robust adaptive response. Overall, our findings suggest that the 704-mediated DNA vaccination platform is an attractive method to develop both prophylactic and therapeutic vaccines.

INTRODUCTION

Nucleic acid-based vaccination represents one of the most promising and attractive methods to prevent or cure infectious diseases or cancers.[1,2] The administration of nucleic acid-based vaccines to patients results in the endogenous production of immunogenic proteins that mimic the antigens produced during the natural disease.[3] Although similar in their action mechanisms, DNA- and mRNA-based vaccines differ by the cellular compartment in which they need to be distributed to initiate their therapeutic effect.[4] Efficient delivery of DNA into the cell nucleus and mRNA into the cell cytoplasm is critical, but several challenges remain to allow sufficient nucleic acid internalization while targeting the appropriate cell compartment. In addition, the immunogenicity of nucleic acid-based vaccines needs to be improved to fully unleash their full potential.[3] DNA vaccines are composed of a simple molecule of closed and circular double-stranded plasmid DNA (pDNA) containing a gene encoding an antigen of interest under the control of a strong promoter.[5] This technology is inexpensive, simple to manufacture and transport, stable at unfrozen temperatures, and easy to administer, and it demonstrates excellent safety during clinical trials.[3,6] However, despite promising results of DNA vaccines observed in humans, leading to its approval for vaccination against COVID-19 by the Drugs Controller General of India, several limitations remain. The amount of DNA administered by injection needs to be dramatically lowered (2 mg of DNA per injection, corresponding to 0.03 mg DNA/kg), and a simpler delivery system than electroporation or Biojector must be designed for the use of DNA vaccines at a world scale. Usually, the poor efficiency of DNA vaccines in humans is attributed to insufficient antigen production in patients because of the lower dose of pDNA that can be administered in humans during clinical trials (0.014–0.07 mg DNA/kg for an adult) compared with the effective dose

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used in small animals (1–4 mg DNA/kg).[7,8] Therefore, research groups have focused on developing pDNA that enhances antigen expression and innovative approaches to improving DNA delivery into the targeted cells to improve DNA vaccine immunogenicity.[9–14]

Getting high antigen expression from the DNA administered is unlikely to alleviate DNA vaccine limitations alone. Similar to what happens during pathogen infection, a vaccine's activation of innate sensors to trigger an immediate "danger signal" is a prerequisite to induce a strong and long-lasting immune response.[15] This is a critical step to create a local pro-inflammatory environment necessary for proper recruitment and activation of immune cells to fight an invading pathogen or a tumor. As such, an ideal DNA vaccine formulation should not only deliver the DNA encoding the antigen to the targeted cells but also efficiently activate the appropriate cellular danger sensors for an optimal immune response priming. Those DNA sensors, such as cGAS-STING (cyclic GMP-AMP synthase-stimulator of interferon genes)[16] and DHX (DEAH box nucleic acid helicase),[17] are located mainly in the cell cytoplasm and detect the abnormal presence of DNA outside of the cell nucleus. In addition, DNA can be recognized in endosomes by a unique sensor, TLR9 (Toll-like receptor 9),[18] although it has been demonstrated that TLR9 is not essential to induce immune responses following DNA vaccine injection.[19] Therefore, direct delivery of DNA to the cell nucleus periphery to efficiently stimulate cytoplasmic DNA sensors and facilitate rapid expression of the immunogenic protein represents a promising strategy to address current DNA vaccine challenges.

Our team reported using a tetrafunctional amphiphilic block copolymer, called 704, to enhance cellular uptake of DNA through direct delivery into the cytoplasm, bypassing endosomal pathways.[20] This star-shaped copolymer comprises four hydrophobic-hydrophilic blocks (polyethylene oxide-polypropylene oxide [PEO-PPO]) centered on an ionizable core (ethylenediamine) and spontaneously autoassembled in aqueous solutions with DNA. In preclinical models, 704-based formulations promoted target protein production at the injection site.[20,21] In addition, we demonstrated 704's unique ability to improve DNA vaccine immunogenicity against hepatocellular carcinoma (HCC),[22] allergic asthma,[23] colorectal cancer,[24] *Mycobacterium abscessus*-induced cystic fibrosis,[25] a transposase-derived protein encoded by human neogenes,[26] and Zika virus.[27]

Therefore, the potential of this 704/DNA vaccine prompted us to explore whether it could also be applied to HIV infection by inducing the production of functional antibodies against HIV envelope proteins. We first investigated if 704 tetrafunctional block copolymer could decrease the necessary amount of DNA to the nanogram dose in mice for an efficient HIV vaccination. Then, we investigated whether 704 could induce an efficient humoral immune response against a quasi-homologous protein in non-human primates. Finally, we focused on elucidating the mechanisms used by this tetrafunctional block copolymer to improve DNA vaccine efficiency dramatically.

RESULTS

704-mediated DNA vaccination induces high levels of antibodies against HIV envelope proteins with potent neutralizing activity

The HIV envelope protein gp120 is responsible for virus entry into cells through interaction with the CD4 receptor. Blocking this binding is a promising strategy to prevent HIV infection; therefore, we investigated the potential of 704/DNA vaccines to promote the efficient production of gp120-specific neutralizing antibodies. Two plasmids with similar backbones were designed, encoding either gp120 (CH505 gp120)[28] or gp145 (CH505 gp145),[29] an uncleaved version of gp120 fused with gp41 providing more relevant epitopes to elicit broad neutralization.[30] Mice were injected intramuscularly (i.m.) every 3 weeks with various concentrations of DNA (0.2–50 μg) encoding either gp120 or gp145; then, the production of binding and neutralizing antibody was assessed on day 84 using anti-gp120 ELISAs and antibody blocking assays (Figure 1). Although unformulated DNA successfully induced the production of high antibody titers against gp120 with both plasmids at the higher concentration (50 μg per muscle, i.e., 1 mg DNA/kg), low concentrations failed to trigger any humoral-mediated immune response (Figures 1A and 1B). Interestingly, not only did 704-mediated DNA vaccination induce high levels of anti-gp120 antibody in serum, but only 0.2 μg of DNA (0.01 mg DNA/kg) was necessary to induce a potent antibody production with both plasmids. Similar results were observed with the neutralizing activity of the produced antibody (Figures 1C–1F). 704/gp120 pDNA elicited the most robust neutralizing antibody response compared with unformulated DNA, with as low as 0.2 μg of DNA (Figures 1C–1E). Surprisingly, although 704-mediated gp145 DNA vaccination performed better with low DNA concentrations (0.2–1 μg), we observed a dose-dependent neutralizing activity with similar blocking efficacies between formulated and unformulated DNA for 5 and 50 μg (Figures 1D and 1F). Collectively, these results suggest that 704-mediated gp120 or gp145 DNA vaccination can generate a robust anti-gp120 antibody production while requiring up to 250-fold lower DNA dose than unformulated plasmid. Furthermore, sera of mice immunized with 704/DNA were capable of blocking both a key epitope of HIV neutralization (CD4) and a confirmed broad neutralizing antibody (CH106).

AFP-encoding 704/DNA vaccines triggered efficient humoral immune responses in non-human primates

To further test the efficacy and safety of 704/DNA-mediated vaccination, three non-human primates (Amy, Sharon, and Floor) received three i.m. immunizations (days 0, 28, and 56) of 704-formulated DNA encoding human alpha-fetoprotein (hAFP) (250 μg per *tibialis*), a self-antigen expressed in HCC sharing more than 98% homology with the macaque AFP (Figures 1G and 1H). Blood serum analysis revealed that all macaques produced anti-hAFP antibodies after only two i.m. injections, with Amy and Sharon reaching similar antibody levels after 110 days (Figure 1G). Surprisingly, Floor demonstrated a lower anti-hAFP

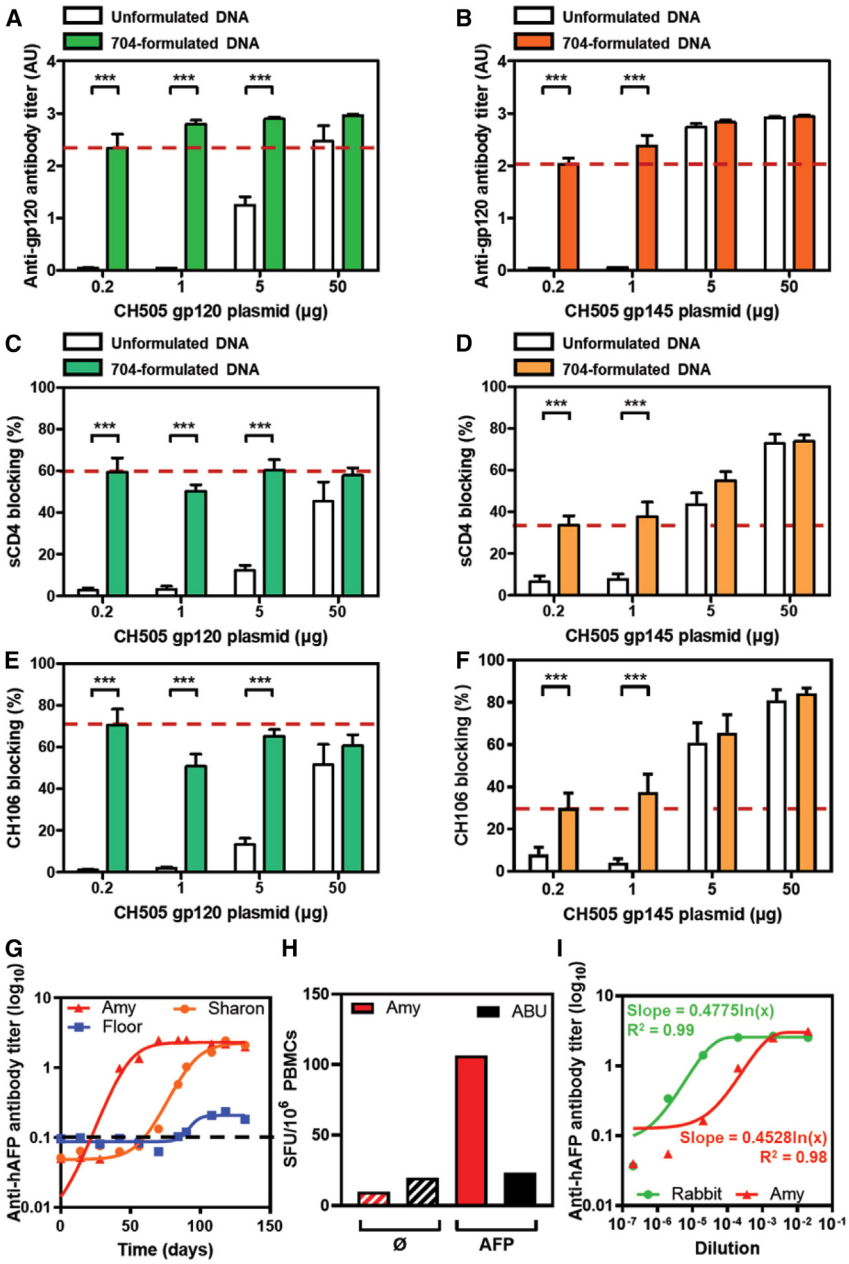

Figure 1. 704-mediated DNA vaccination induced strong functional antibody production against HIV envelope proteins in mice and homolog hAFP in *Macaca fascicularis*

Mice were injected i.m. at days 0, 21, 42, and 63 with 0.2, 1, 5, or 50 µg of CH505 TF gp120 plasmid DNA (A, C, and E) or CH505.M11 gp145 plasmid DNA (B, D, and F) unformulated (white) or formulated (green and orange) with 704. (A and B) Humoral immune response was determined at day 84 by ELISA for anti-gp120 or anti-gp145 total IgG. (C–F) At day 84, sera were collected and assayed for the ability to block the binding of sCD4 and broad neutralizing antibody CH106 to gp120. Dashed line, antibody titer or percentage of blocking obtained with 0.2 µg of plasmid DNA formulated with 704. (G and H) Three *Macaca fascicularis* named Floor, Amy, and Sharon were injected i.m. at days 0, 21, and 56 with 250 µg of hAFP plasmid DNA formulated with 704. (G) Humoral immune response was determined as a function of time by ELISA for anti-hAFP total IgG for the three macaques. Dashed line, assay background. (H) Cellular immune response was measured by ELISPOT, using media or hAFP antigens. (I) Serum titration assay with day 84 immunized macaque Amy and day 43 immunized rabbit with five consecutive injections every week of 2 mg of hAFP plasmid DNA formulated with 704. Humoral response was determined using the same ELISA but with species-specific peroxidase detection antibodies. Data are shown as the mean ± SEM (n = 8). Data were analyzed using Mann-Whitney test (comparison with unformulated plasmid DNA), ***$p < 0.001$. Doses are given per *tibialis anterior*.

antibody titer in the collected blood. In addition, we tested the cellular immune response induced in Amy. Peripheral blood mononuclear cells (PBMCs) were isolated at day 150 and incubated with medium alone or hAFP peptides. The frequency of PBMCs capable of producing an IFNγ (interferon-γ) response to stimulation was 5-fold higher than that of the unvaccinated control (Abu), suggesting that the 704/DNA vaccines triggered both arms of the adaptive immune response. As expected, no safety issues emerged during the full duration of the protocol, and all macaques remained healthy. To deepen our characterization of the anti-hAFP antibody response observed in macaques, a head-to-head serum titration of sera from a non-human primate and an immunized rabbit was performed. Two different secondary peroxidase-conjugated antibodies were used (anti-rabbit and anti-monkey IgG); therefore, the comparison was made using the slope of the anti-hAFP antibody titer as a function of the serum dilution curve (Figure 1H). Strikingly, similar slopes were observed between immunized rabbit (s = 0.04775ln(x)) and immunized monkey sera (s = 0.4528ln(x)), although rabbit AFP has only 78% homology with hAFP. Altogether, these data demonstrate that 704-based DNA vaccines are highly immunogenic and can induce efficient antibody production in macaques against a highly homologous protein.

704-mediated DNA delivery induces high cytokine and interferon production

Triggering an efficient innate immune response upon vaccination is critical to promote durable immune memory.[31] Innate immune recognition and immunogenicity of DNA-based vaccines are mediated mainly by cellular danger sensors,[27] leading to IFN and pro-inflammatory cytokine production.[32] Therefore, we next evaluated the

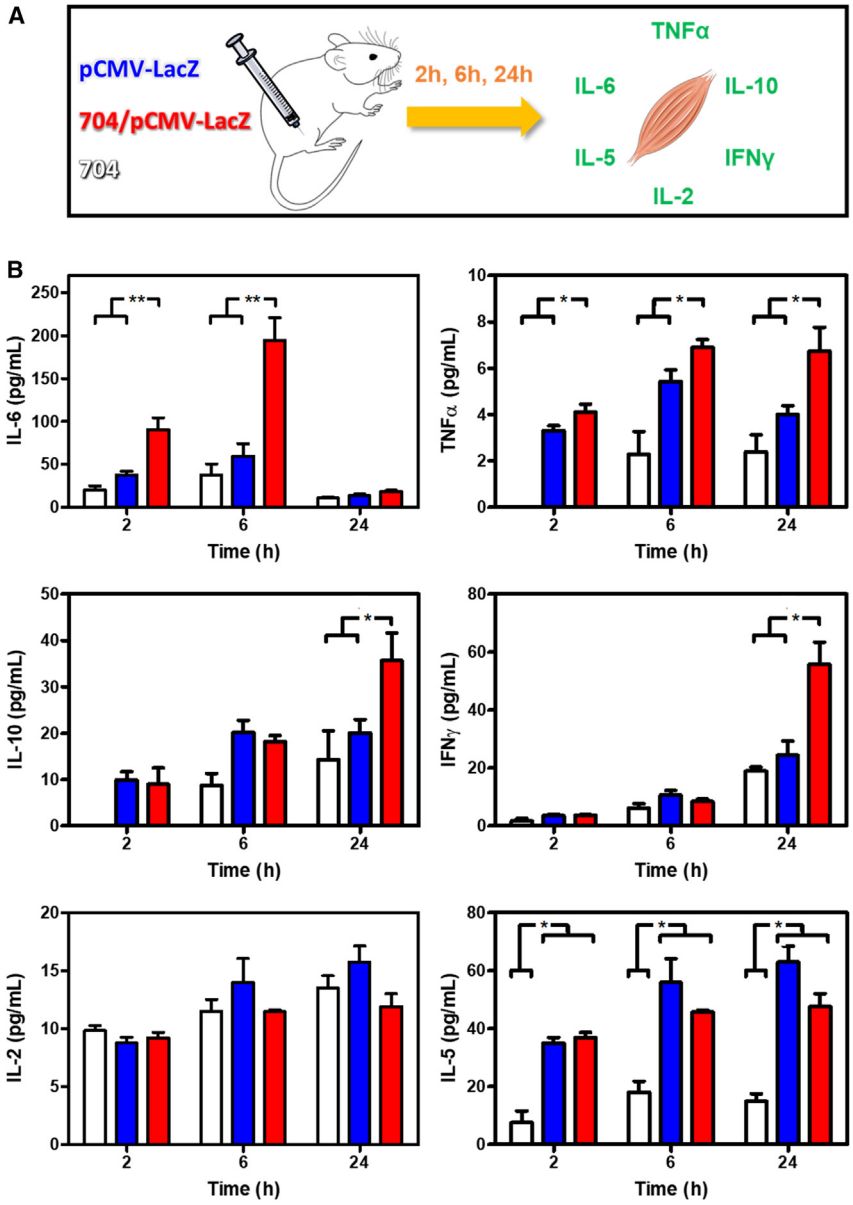

Figure 2. Vaccination with 704 enhances DNA recognition by cellular danger sensors resulting in improved cytokine and interferon production

(A) Schematic depicting the experimental protocol. Mice (n = 6 per group) were injected intramuscularly with unformulated 704 (white, negative control) or 2 μg of 704-free (blue) or 704-formulated (red) pCMV-LacZ. Muscles were explanted 2, 6, and 24 h post-injection for cytokine analysis. (B) Cytokine secretion level into the injected *tibialis anterior* measured by FlowCytomix. Values are shown as the mean ± SEM. Data were analyzed using Mann-Whitney test, *$p < 0.05$ and **$p < 0.01$. Doses are given per *tibialis anterior*.

capacity of 704 to enhance danger sensor activation by DNA (Figure 2A). Mice were injected i.m. with 2 μg of 704-free or 704-formulated pCMV-LacZ pDNA. After 2, 6, or 24 h, the injected *tibialis anterior* muscles were explanted, and the kinetics of cytokine and IFN production was analyzed (Figure 2B). Unformulated 704 was used as a control. In comparison with 704-free DNA, DNA formulated with 704 induced higher levels of IL-6 and TNF-α production from 2 h post-injection, which persisted for 6 h for IL-6 and 24 h for TNF-α. Similarly, increased production of IFNγ and IL-10 was observed 24 h post-injection. Interestingly, both 704-free and 704-formulated DNA induced a high level of IL-5 production 2 h post-injection, while no change in IL-2 levels was observed. As expected, the injection of unformulated 704 did not induce a significant change in immunomodulatory molecule production. Taken together, the results indicate that 704 can promote the innate immune response mediated by the pDNA recognition by danger sensors.

704/DNA vaccine-mediated humoral immune response is dependent on antigen expression by muscle fibers

Efficient DNA delivery into the targeted cells to express the antigen of interest represents a critical step for DNA vaccine efficiency.[33,34] This includes muscle cells but also antigen-presenting cells (APCs) such as dendritic cells (DCs). To better understand our vaccine system, we characterized the intensity of the humoral immune response induced by 704-mediated DNA vaccination as a function of the targeted cells expressing the antigen of interest. To this aim, first a pCMV-lacZ pDNA was modified with muscle-specific miRNA133a-3p targeting sites (pCMV-LacZ-miR133a-3p) to inhibit β-galactosidase (β-Gal) expression in muscle fibers.[35] Similarly, a pCMV-lacZ pDNA was genetically engineered with DC-specific miRNA 142a-3p targeting sites (pCMV-LacZ-miR142a-3p) to suppress β-Gal expression in DCs (Figure 3A).[36] As expected, the β-Gal production in the muscles of mice injected with miR133a-3p was dramatically decreased compared with miR142a-3p after 7 days (Figure 3B). In contrast, immortalized DCs (JAWSII) transfected with miR142a-3p displayed a 4-fold reduction in β-Gal production compared with miR133a-3p (Figure 3C), validating the function of the plasmid constructs. Next, mice were injected i.m. at days 0 and 21 with pCMV-LacZ-miR133a-3p or pCMV-LacZ-miR142a-3p formulated with 704, and the humoral immune response was measured at day 42 by quantifying anti-β-Gal antibody titers using ELISA (Figures 3D and 3E). Surprisingly, while 704/miR142a-3p DNA vaccination induced strong anti-β-Gal antibody production, 704/miR133a-3p DNA-based vaccine failed to induce a robust humoral immune response, with antibody titers

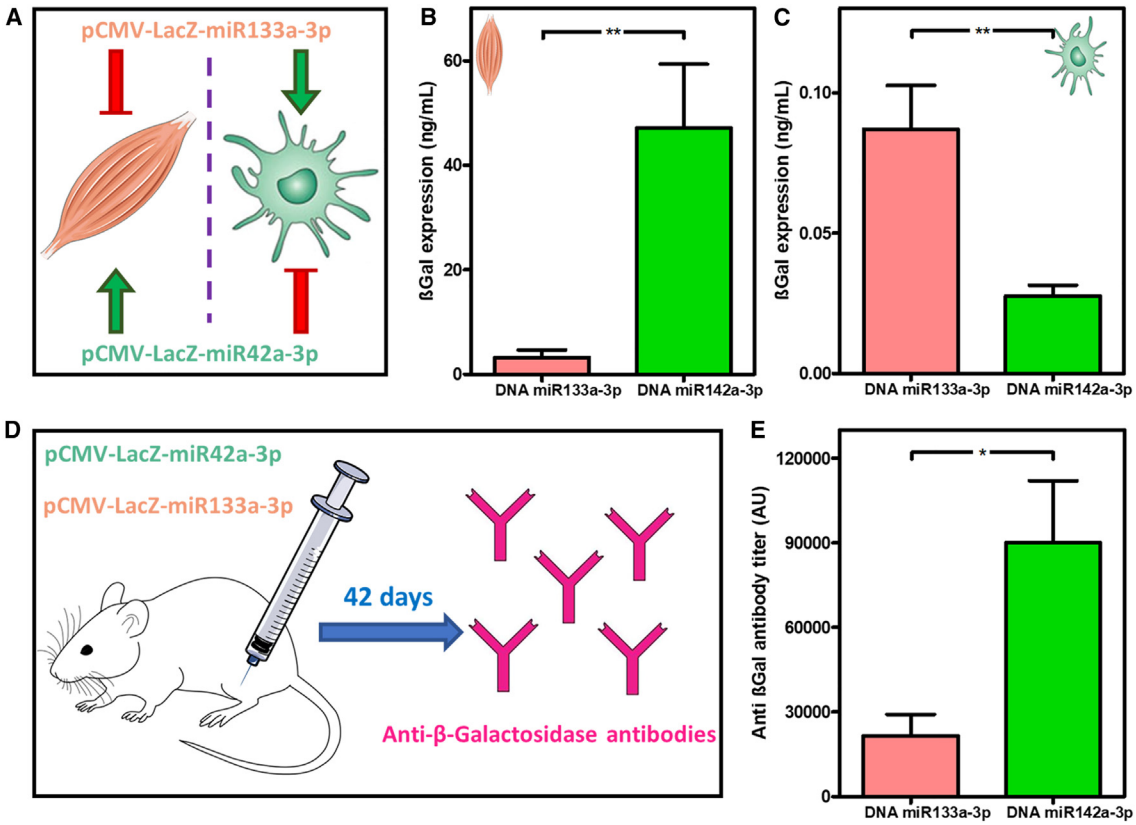

Figure 3. Antigen expression by muscle fibers is a key component to trigger an efficient 704-mediated DNA vaccination

(A) Schematic depicting the targeted expression of pCMV-LacZ miR133a-3p (pink) and pCMV-LacZ miR142-3p (green). (B) Mice (n = 3 per group) were injected intramuscularly with 2 μg of pCMV-LacZ miR133a-3p or 2 μg of pCMV-LacZ miR142-3p formulated with 704. The expression level of β-galactosidase in *tibialis anterior* (n = 6 muscles) was determined 7 days post-injection. (C) JAWSII cells were transfected with 500 ng of pCMV-LacZ miR133a-3p or 500 ng of pCMV-LacZ miR142-3p complexed with DOSK at CR = 5. Expression level of β-galactosidase was determined 24 h post-transfection. (D) Schematic depicting the immunization protocol. (E) Mice (n = 5 per group) were injected intramuscularly with 2 μg of pCMV-LacZ miR133a-3p or 2 μg of pCMV-LacZ miR142-3p formulated with 704. The humoral immune response was determined at day 42 by measuring the titer of anti-β-Gal-specific antibody in serum. Values are shown as the mean ± SEM. Data were analyzed using Mann-Whitney test, *$p < 0.05$ and **$p < 0.01$. Doses are given per *tibialis anterior*.

4-fold lower (Figure 3E). Collectively, these results indicate that antigen expression by muscle fibers is crucial in inducing an effective 704-mediated DNA vaccination. In addition, our data show that antigen expression by DCs contributes only partially to the immunization process but may have a synergistic effect with expression of the antigen in muscle fibers.

704-mediated delivery promotes DNA but not RNA adjuvant capacity

Immunogenicity of DNA- and RNA-based vaccines is mainly driven by their recognition by specific innate immune sensors in the endosomal or cytoplasmic compartment. Double-stranded DNA or unmethylated CpG motifs are detected by DNA sensors.[37] Double-stranded RNA, GU-rich sequences, and 5′-triphosphate are recognized by RNA sensors.[38–40] Therefore, we next investigated the immune stimulation induced by DNA or RNA after 704-mediated delivery. Mice were injected i.m. with 10 μg of either non-coding pVAX pDNA or non-coding unmodified mRNA, formulated with 704. The expression of genes responsible for pro-inflammatory cytokine production was quantified in the injected muscles 6 and 24 h post-injection (Figure 4A). After 6 h, formulated DNA was able to induce a high expression of IL-6, IL-10, TNF-α, and IFNγ compared with 704/RNA (Figure 4B), which lasted for at least 24 h. In addition, 704/DNA injection triggered a strong expression of IL-2 after 24 h. Interestingly, even though formulated RNA did not induce the expression of pro-inflammatory cytokines, a slight and transitory increase in IL-5 expression was observed 6 h post-injection. To go further, we compared the humoral immune responses induced by 704/DNA and 704/RNA vaccines. Mice were injected i.m. at days 0 and 21 with 10 μg of either plasmid (pCMV-LacZ) or mRNA (mRNA-LacZ) encoding β-Gal, formulated with 704. The β-Gal expression (six muscles per group; n = 3 mice) was analyzed 7 days after the first injection, and the humoral immune response was evaluated in the remaining mice at day 42 (Figures 4A–4D). With similar levels of β-Gal produced (Figures 4C), 704-mediated RNA vaccination failed to induce an efficient anti-β-Gal antibody production compared with the 704/DNA

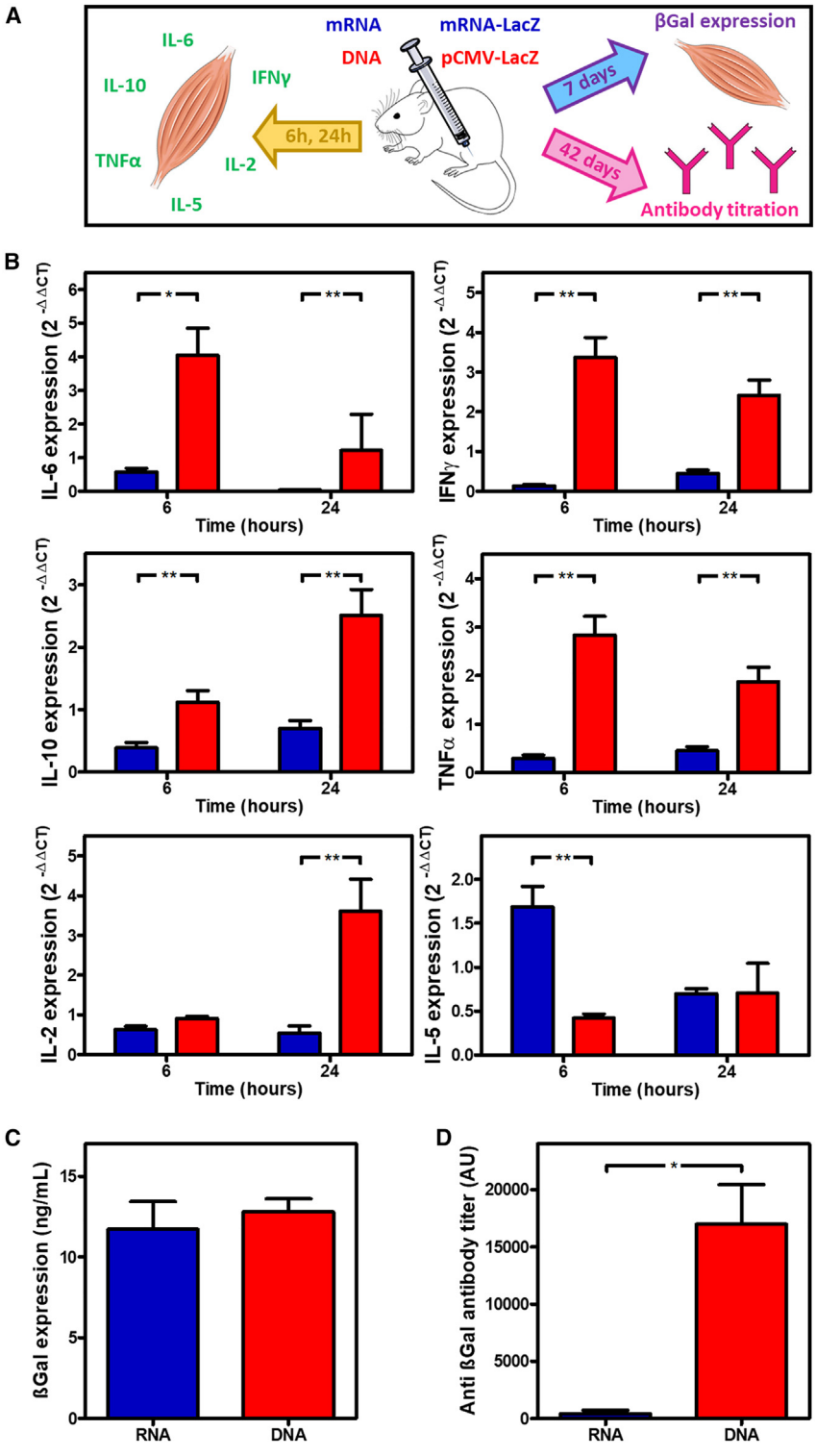

Figure 4. 704 promotes DNA but not RNA recognition for an efficient innate and adaptative immune responses priming

(A) Schematic representation displaying the experimental protocol used to determine the immunogenicity and efficiency of 704-mediated RNA and DNA vaccination. (B) Mice (n = 6 per group) were injected intramuscularly with 10 μg of non-coding mRNA (blue) or non-coding pVax DNA (red) formulated with 704. Cytokine expression levels into *tibialis anterior* (n = 6 muscles) were determined 6 or 24 h after injection by qRT-PCR analysis and normalized against the expression levels of HPRT (housekeeping gene). Data are expressed as the $2^{-\Delta\Delta CT}$ formula. (C and D) Mice (n = 8 per group) were injected intramuscularly with 10 μg of mRNA LacZ (blue) or pCMV-LacZ DNA (red) formulated with 704. (C) The expression level of β-galactosidase in *tibialis anterior* (n = 6 muscles) was determined 7 days post-injection. (D) The humoral immune response was determined at day 42 by measuring the titer of anti-β-Gal-specific antibody in serum. Values are shown as the mean ± SEM. Data were analyzed using Mann-Whitney test, *p < 0.05 and **p < 0.01. Doses are given per *tibialis anterior*.

vaccination control. Taken together, these results demonstrate that the immunostimulation induced by DNA is a key element of 704-mediated vaccination efficiency.

704/DNA vaccine mechanism is dependent on MyD88 and STING pathways

Several studies have reported that unmethylated CpG DNA motifs and classical B-form double-stranded DNA are potent immune stimulators via the recruitment of the adaptor molecules MyD88 and STING, respectively.[41,42] Thus, we investigated which sensing pathways were responsible for 704/DNA vaccine efficacy. MyD88$^{-/-}$ and STING$^{-/-}$ mice were immunized using 704-mediated β-Gal pDNA vaccination, and their humoral immune responses were analyzed on day 42. Wild-type, TLR3$^{-/-}$ (double-stranded RNA sensor), and MAVS$^{-/-}$ (adaptor molecules of RIG-1 RNA sensor) mice were also vaccinated and used as controls. Compared with wild-type controls, mice lacking MyD88 (Figure 5A) and STING (Figure 5B) demonstrated a dramatic reduction in antibody titers by 5- and 3-fold, respectively. As expected, mice knocked out for the RNA sensors TLR3 and MAVS did not display any change in the humoral immune response induced by 704-based DNA vaccines (Figure 5C). Next, we assessed the expression level of genes involved in immune responses in the injected *tibialis anterior* of STING$^{-/-}$ and wild-type mice 6 h after the first 704/DNA vaccine injection. The expression profiles of pro-inflammatory cytokines (IL-6, TNF-α, and IFNγ), an anti-inflammatory cytokine (IL-10), and MyD88-dependent DNA sensors responsible for CpG recognition (DHX9 and DHX36) were

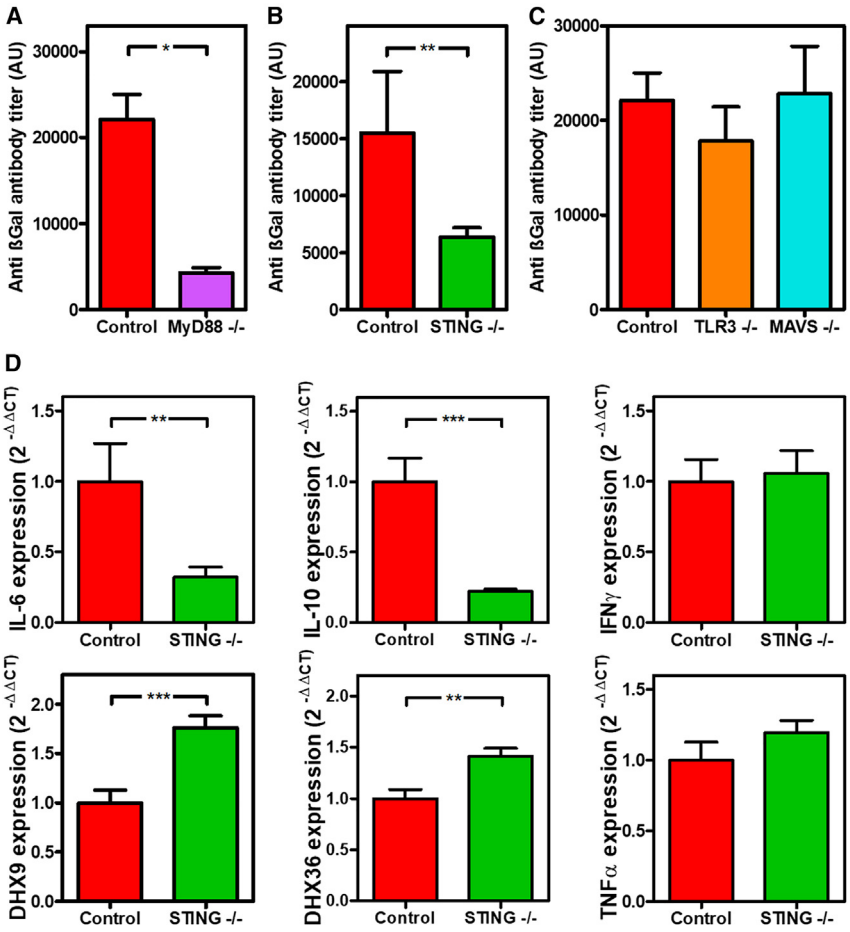

Figure 5. DNA sensing pathways are involved in 704/DNA vaccine

(A–C) Mice (n = 6 per group) that were wild type (control), Myd88$^{-/-}$, STING$^{-/-}$, TLR3$^{-/-}$, or MAVS$^{-/-}$ were injected intramuscularly with (B) 1.5 μg or (A and C) 50 μg of pCMV-LacZ formulated with 704. The humoral immune response was determined at day 42 by measuring the titer of anti-β-Gal-specific antibody in serum. (D) Mice (n = 5 per group) that were wild type (control) or STING$^{-/-}$ were injected intramuscularly with 1.5 μg of pCMV-LacZ formulated with 704. Cytokine expression levels into *tibialis anterior* (n = 10 muscles per group) were determined 6 h after injection by qRT-PCR analysis and normalized against the expression levels of HPRT (housekeeping gene). Data are expressed as the $2^{-\Delta\Delta CT}$ formula. Values are shown as the mean ± SEM. Data were analyzed using Mann-Whitney test, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.01$. Doses are given per *tibialis anterior*.

evaluated (Figure 5D).[43] In the absence of STING expression, the expression of both IL-6 and IL-10 was markedly reduced compared with control mice. Although no differences were observed regarding IFNγ and TNF-α expression, STING$^{-/-}$ mice surprisingly showed a higher level of DHX9 and DHX36 expression than the control mice. Altogether, these results suggest that both MyD88 and STING pathways are involved and complementary in 704/DNA vaccine immunogenicity and may have a synergistic effect.

To deepen our analysis, the influence of CpG motifs within the plasmid backbone on the 704/DNA vaccine was next evaluated. Mice were vaccinated with a 704/DNA vaccine containing either (1) a β-Gal plasmid rich in unmethylated CpG motifs (88 CpG dinucleotides; CpGHigh) or (2) a β-Gal plasmid free of unmethylated CpG motifs (CpGLow). As expected, the vaccine formulation containing the CpGHigh plasmid induced higher levels of IL-5, IL-6, IL-10, and TNF-α secretion at the injection site as soon as 2 h after injection compared with the CpG-free formulation (Figure 6A). Strikingly, this persisted for at least 6 h for IL-5 and TNF-α. Interestingly, the secretion of IL-2 and IFNγ was independent of the presence of unmethylated CpG motifs on the plasmid backbone. Antibody titers after 42 days were in agreement with cytokine secretion results. For similar β-Gal production by the muscle fibers (Figure 6B), CpGHigh plasmid-based vaccination induced 3-fold higher antibody titers compared with CpGLow plasmid (Figure 6C). Collectively, these results indicate that the 704/DNA vaccine platform efficacy relies on CpG motif recognition by specific DNA sensors triggering an efficient humoral immune response.

704-mediated vaccination efficiency relies on direct delivery of DNA into the cytosol

To substantiate these observations, we investigated which DNA sensors were involved in 704/DNA immunogenicity. TLR9 has been described as involved in pDNA-based vaccines; therefore, we first tested the role of this endosomal DNA sensor in 704/DNA vaccination.[17] Mice were injected with several formulations containing 704/CpGHigh pDNA vaccine alone or supplemented with either a TLR9 agonist (ODN1826) or an antagonist (ODN2088) (Figure 7A). Interestingly, the addition of an endosomal TLR9 agonist or antagonist did not modulate the humoral immune response 42 days after the first injection compared with regular 704/DNA vaccines. To further confirm these results, we subsequently evaluated the contribution of endocytosis on muscle fiber transfection and immune stimulation induced by 704/DNA vaccination (Figure 7B). Tmem176b-deficient mice (Tmem176b$^{-/-}$) were used for this study, as the lack of this non-selective cation channel hampers phagosomal pH regulation, preventing endocytosis.[44] Wild-type or Tmem176b$^{-/-}$ mice were injected i.m. with (1) pGWIZ-Luc pDNA formulated with 704, and analyzed for luciferase production in the injected muscles after 7 days, or (2) pCMV-LacZ DNA formulated with 704 (at days 0 and 21) and then evaluated for the humoral immune response induced at day 42 (Figure 7B). Strikingly, despite the lack of endocytosis capability of Tmem176b$^{-/-}$ mice, they displayed similar expression levels of luciferase after 7 days as well as identical antibody titers after 42 days

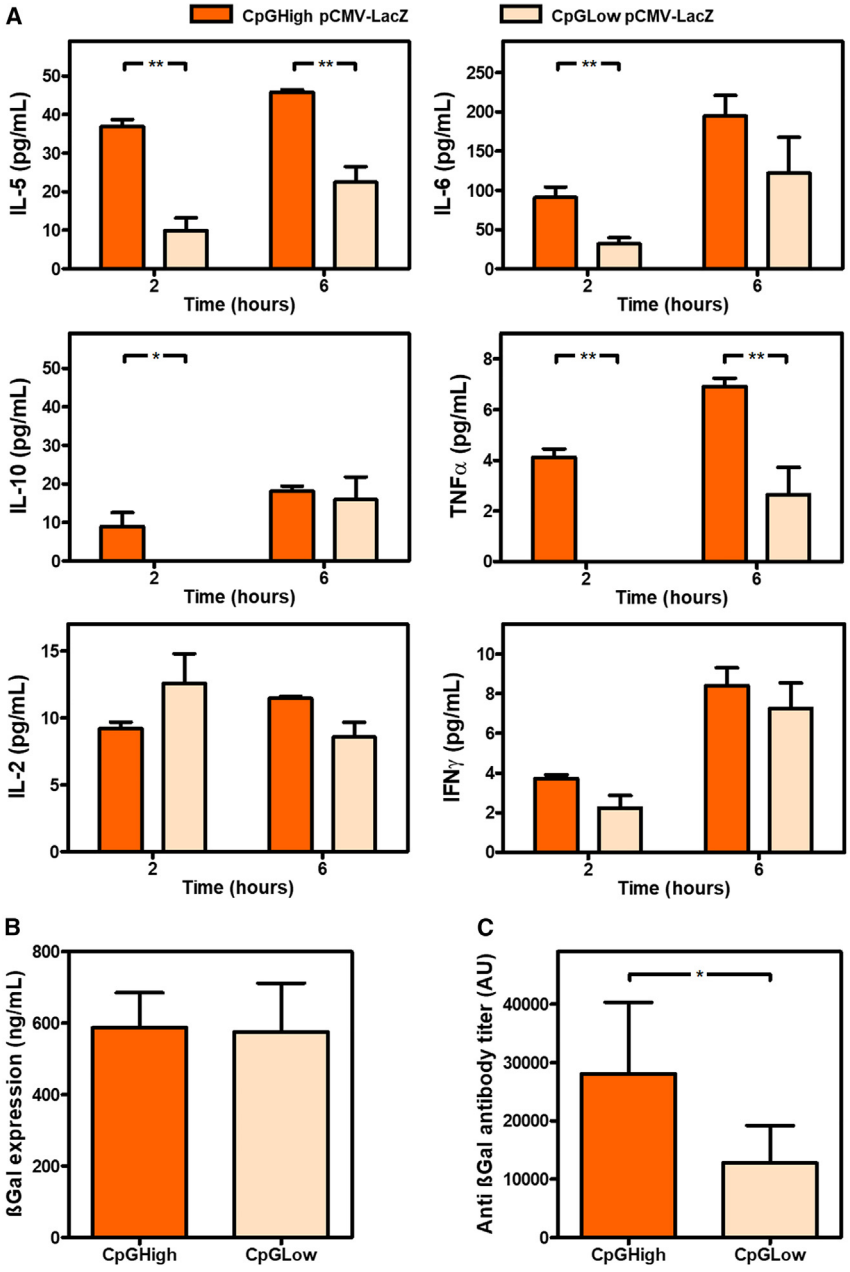

Figure 6. CpG recognition boosts 704/DNA vaccination

(A and B) Mice (n = 12 per group) were injected intramuscularly with 2 μg of pCpGHigh-LacZ (orange) or 2 μg of pCpGLow-LacZ (beige) formulated with 704. (A) Cytokine secretion levels into *tibialis anterior* (n = 6 muscles per group) were quantified 2 or 6 h post-injection by FlowCytomix. (B) β-Gal expression in muscles (n = 6 per group) 7 days after injection. (C) The humoral immune response (n = 6 mice per group) was determined at day 42 by measuring the titer of anti-β-Gal-specific antibody in serum. Values are shown as the mean ± SEM. Data were analyzed using Mann-Whitney test, *p < 0.05 and **p < 0.01. Doses are given per *tibialis anterior*.

compared with wild-type mice. Taken together, these data indicate that 704-mediated DNA vaccination is independent of endosomal internalization and endosomal DNA recognition by the host cells.

Another possibility is that the CpG motif detection occurs in the cytoplasmic compartment of cells. Therefore, the role of the cytoplasmic DHX9 and DHX36 DNA sensors in 704/DNA vaccine immunogenicity was next investigated. Mice were injected with various vaccine formulations, and the production of antigen (day 7) and antibody titers (day 42) were assessed. Formulation 1 contained a CpGHigh plasmid and an mRNA encoding DHX9 and DHX36, formulated with 704. Formulation 2 contained a CpGHigh plasmid and a non-coding mRNA, formulated with 704 (control for formulation 1). Formulation 3 contained a CpGLow plasmid and an mRNA encoding DHX9 and DHX36, formulated with 704. Formulation 4 contained a CpGLow plasmid and a non-coding mRNA, formulated with 704 (control for formulation 3). As expected, the four different formulations induced similar levels of antigen production (Figure 7C). Strikingly, overexpressing DHX9 and DHX36 markedly increased (+75%) the antibody titers in animals vaccinated with the plasmid rich in CpG motifs (formulation 1 vs. formulation 2) (Figure 7D). However, the increased expression of these DNA sensors did not influence the humoral immune response in animals vaccinated with CpGLow plasmids (formulation 3 vs. formulation 4). To strengthen our observations, we tested the impact of DHX9- and DH36-mediated DNA recognition on the efficacy of the standard 704/pCMV-LacZ vaccine used throughout this study. Mice were vaccinated with 2 or 5 μg of pCMV-LacZ DNA formulated with 704, with or without mRNAs encoding DHX9 and DHX36 (Figure 7E). Coherently, overexpressing DHX9 and DHX36 significantly improved the humoral immune response of mice 42 days after the first injection, independent of the DNA dose injected. As expected, similar results were obtained with a plasmid coding for the murine antigen mAFP (Figure S1), suggesting that such improvement of the immune response is independent of the antigen expressed. Collectively, these data indicate that 704 not only can carry DNA into the cytosol by a direct delivery mechanism but also allows for efficient stimulation of intracytoplasmic DNA sensors responsible for 704/DNA vaccine immunogenicity.

DISCUSSION

A key challenge for the utilization of DNA in more licensed animal and human vaccines relies on decreasing the amounts of DNA

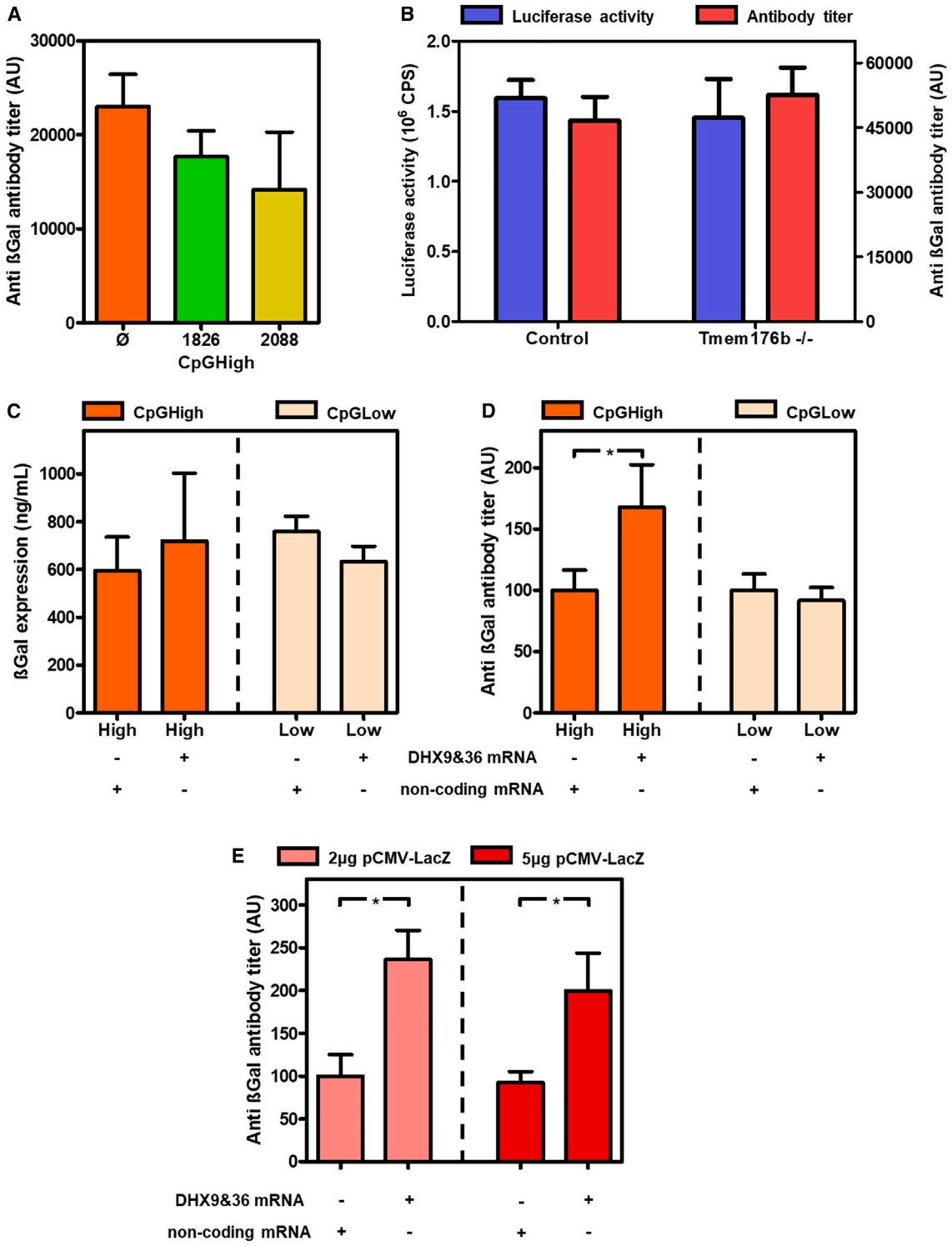

Figure 7. Direct delivery of DNA mediated by 704 is responsible for the induction of a strong humoral immune response

(A) Mice (n = 5) C57BL/6 were injected intramuscularly with 704/DNA vaccines containing 5 μg of pCpGHigh DNA alone (orange) or mixed with either ODN 1826 (agonist of TLR9, green) or ODN 2088 (antagonist of TLR9, yellow). The humoral immune response was determined at day 42 by measuring the titer of anti-β-Gal-specific antibody in serum. (B) Mice (n = 5) that were wild type (control) or Tmem176b$^{-/-}$ were injected intramuscularly with 10 μg of pGWIZ-Luc (blue) or 10 μg of pCMV-LacZ formulated with

(legend continued on next page)

required per injection while boosting their immunogenicity. This could be overcome by improving the DNA delivery, using more potent vectors, while stimulating the proper DNA sensors within the targeted cells for a synergizing effect on the immune response. Herein, we demonstrated the capacity of the 704/DNA vaccine platform to induce robust humoral immune responses against both HIV envelope proteins in mice and hAFP in *Macaca fascicularis*, via direct delivery and targeting of DNA sensors within the cell cytoplasm.

704-based vaccination has already been used against several diseases in mice, such as asthma,[22,23,45] HCC,[20] and *Mycobacterium abscessus*,[24,46] or to induce antibody production against human neogenes.[25,47] Protective immunity was also shown against ZIKV infection in 704-vaccinated mice.[26] In this work, 704-mediated vaccination was able to produce neutralizing antibodies that block the binding of CD4 and the broad neutralizing CH106 antibody to gp120 HIV envelope protein with only 0.2 μg of plasmid per injection. This was 250-fold lower than the 50 μg unformulated plasmid required to generate the same humoral immune response. In the *Macaca fascicularis* experiment, 704-based vaccines led to the production of high antibody titer against the quasi-homologous (more than 98%) AFP with as few as 250 μg of the plasmid, as well as a cellular immune response. This indicates that the effective dose of DNA when formulated with 704 is as low as 0.01 mg/kg compared with the 3 mg/kg with naked DNA. This represents a significant improvement compared with current DNA vaccination strategies tested in humans, as both electroporation and PharmaJet require 2 mg of DNA per dose, which represents a dose three times higher.[48,50]

Resident immune cells at the injection have been described as one of the key components for generating a protective humoral immune response. APCs such as DCs can be locally transfected with the delivered pDNA, be stimulated, and then present the newly produced antigens of interest via their major histocompatibility complex (MHC) I or II.[49] However, the importance of muscle cells in the DNA-based vaccination efficiency has been overlooked and is still actively discussed.[51] With 704/DNA vaccines, our experiments showed that the expression of the antigen by muscle fibers was critical to inducing antibody production, as exemplified by the poor immune response induced by the plasmid bearing miR133a targeting sites. Nonetheless, we observed only a partial suppression of antibody production in our study, confirming the implication of other cells expressing the antigen. For instance, several papers highlighted the crucial implication of antigen expression in DCs for an efficient DNA vaccination.[51] Therefore, it is likely that antigen expression in DCs could explain this persisting antibody production. However, it does not seem to be the main mechanism, as the use of a plasmid with miR142a targeting sites did not hamper 704-mediated DNA vaccination. This suggests the presence of two distinct mechanisms as a function of the cell type expressing the antigen. We hypothesize that (1) the antigen expression by muscle fibers leads to its secretion and cross-presentation by the APCs recruited at the injection site and represents the main component of 704/DNA-mediated humoral immune response, while (2) the antigen can also be expressed by DCs, leading to their direct presentation to other immune cells. Therefore, the immune response induction used by the 704 block copolymer seems to significantly differ from electrotransfer, the gold standard for DNA vaccination. Indeed, electrotransfer allows antigen expression only in muscle cells, leading to immune response induction through only an APC-mediated indirect cross-priming mechanism. This limitation is inherent to the electrotransfer method, as the parameters used for optimal elongated-shape muscle cell transfection (e.g., voltage, pulse duration, and repetition frequency) are drastically different from those used for round-shaped cells such as APCs.[52,53] Therefore, 704-mediated DNA transfer into cells for immunization can be more suitable for allowing antigen expression in a wide range of cells, including APCs.

Multiple strategies have been used to enhance the poor immunogenicity of conventional DNA vaccines. Adjuvants are commonly used to achieve a strong innate immune stimulation, leading ultimately to a robust adaptative immune response against the antigen encoded by the pDNA.[54] However, an ideal DNA-based vaccine should effectively stimulate by itself the pattern recognition receptors (PRRs) responsible for DNA recognition. DNA sensor stimulation can efficiently induce the secretion of several pro-inflammatory cytokines and IFNs, as well as allowing for efficient recruitment of immune cells. Although 704 block copolymers do not possess any inherent immunostimulatory properties,[21] our results clearly show that 704/DNA formulations induce a high level of cytokine and IFN secretion into the injected *tibialis anterior* compared with DNA alone. Considering that 704 is already demonstrated as potent for enhancing DNA delivery to various organs,[20,23,45,55–58] this strongly suggests that the superior innate immune stimulation achieved by 704-mediated vaccines is due to a better DNA delivery and recognition by cellular DNA sensors. Interestingly, in our study, we observed an influence of the nucleic acid nature on 704-formulated nucleic acid immunogenicity. Using unmodified mRNA stimulating endosomal TLR7/8 RNA sensors, we expected to observe efficiency of RNA vaccination comparable to that of a DNA vaccine for the same antigen expression levels. However, 704-mediated RNA vaccination failed to trigger an efficient innate immune stimulation. Although these results highlight that 704-mediated vaccination efficiency is limited to DNA-based vaccines, they also provide useful insight into other applications of the

704 (red). Luciferase activity (blue) in *tibialis anterior* (n = 10 muscles) was determined at day 7 and the humoral immune response (n = 5) (red) was determined at day 42. (C and D) Mice (n = 12) were injected twice intramuscularly (days 0 and 21) with 704/DNA vaccines containing 5 μg of pCpGHigh (orange) or 5 μg of pCpGLow (beige) mixed with either 20 μg of mRNA encoding DHX9&36 or non-coding mRNA. (C) Expression level of β-Gal in *tibialis anterior* (n = 6 muscles) was determined at day 7 and (D) the humoral immune response (n = 9) was determined at day 42. (E) Mice (n = 8) were injected twice intramuscularly (days 0 and 21) with 704/DNA vaccines containing 2 (pink) or 5 μg (red) of pCMV-LacZ formulated with either 20 μg of mRNA encoding DHX9&36 or non-coding mRNA. Humoral immune response was determined at day 42. Values are shown as the mean ± SEM. Data were analyzed using Mann-Whitney test, *$p < 0.05$. Doses are given per *tibialis anterior*.

amphiphilic block copolymer 704. For instance, in the context of therapies requiring low or no immune stimulation such as gene therapy, 704-mediated RNA delivery may be a safe and promising strategy.

STING- and MyD88-dependent pathways are responsible for the activation of signaling cascades involved in DNA recognition.[41,42] STING contributes to cytosolic DNA recognition, while MyD88 is involved in signaling after CpG motif recognition. In this work, we demonstrated their crucial role in 704/DNA vaccination efficiency. The 704/DNA vaccine capacity to promote antibody production was dramatically hampered in mice knocked out for MyD88, but also in mice lacking STING expression. More interestingly, while STING$^{-/-}$ mice had an impaired immune gene expression in muscles 6 h after vaccination, we observed the overexpression of DHX9 and DHX36, two MyD88-dependant DNA sensors responsible for CpG motif recognition.[43] This suggests not only that both STING and MyD88 are crucial for optimal immune priming by 704/DNA vaccines, but also that these two pathways are compensating for each other. Experiments using plasmids rich in or free of CpG motifs within their non-coding segment confirmed this hypothesis. While the 704/CpGLow DNA formulation induced a moderate humoral immune response, the 704/CpGHigh DNA vaccine demonstrated an increased secretion of Th2 and pro-inflammatory cytokine secretion after injection followed by a better humoral immune response after 42 days. Overall, we believe that the 704/DNA immunogenicity is induced by a synergistic recognition of DNA by receptors involved in MyD88 and STING pathways.

Finally, we demonstrated the importance of the location and type of receptors to unleash the full potential of 704-formulated nucleic acid-based vaccines. We previously reported that 704 promotes direct delivery of nucleic acids within the cytosol of skeletal muscle cells and improves plasmid expression. This is most likely because nuclei are anchored below the plasma membrane in muscle fibers, resulting in very close proximity of both nuclear and plasma membranes. This particular topology greatly facilitates the transfer of the DNA molecules from the cytoplasm to the nuclei after 704-mediated direct delivery across the plasma membrane. However, we could not exclude that some non-encapsulated or released DNA from the polyplexes could also interact with the cells and be endocytosed. Therefore, we investigated the contribution of TLR9-mediated immune stimulation on the 704/DNA vaccine efficiency. In our study, both the TLR9 agonist ODN1826 and the TLR9 antagonist ODN2088 failed to induce any immunomodulation. This indicates that the DNA detection after 704-mediated delivery is independent of the intraendosomal receptor. This agrees with the lack of immunogenicity of unmodified mRNA delivered by 704, as the recognition of such RNA also happens in the endosomal compartment. Furthermore, DNA expression and humoral immune response have been maintained in Tmem176b$^{-/-}$ mice impaired for endocytosis, at a level similar to that of wild-type mice. Furthermore, increasing the expression of DHX9 and DHX36 confirmed these observations. For a similar antigen expression, they led to a dramatic increase in the humoral immune response in the mice vaccinated with 704 vaccines composed of "classical" or CpG-enriched plasmids compared with CpG-free. Considering that (1) we have already reported the direct delivery of DNA within the cytosol of cells by amphiphilic block copolymer[19] and (2) that STING and Myd88 are also adaptive molecules for cytosolic DNA sensors, we strongly believe that 704/DNA vaccine's strong immunogenicity, efficacy, and long-lasting immune response rely on its intrinsic ability to deliver DNA directly within the cell cytosol to stimulate intracellular DNA sensors. This clearly contrasts with the DNA delivery by electroporation, which promotes the formation of vesicle-like structures containing DNA molecules at the cell membrane.[14]

In conclusion, the present work has demonstrated the capacity of 704-mediated DNA vaccination to induce high specific antibody titers and cellular immune response against HIV envelope proteins with a minimal amount of DNA and to quasi-homologous antigen in non-human primates. We characterized the immune mechanisms underlying 704/DNA vaccine immunogenicity, relying on direct delivery of DNA into muscle cells and also DC cytosol to efficiently stimulate the intracytoplasmic DNA sensors while allowing a strong antigen expression. The copolymer 704 may prove to be an ideal synthetic vector for the future development of DNA vaccines, both for immunotherapy and for infectious diseases. Moreover, the codelivery of mRNA encoding DNA sensors as "adjuvants" can unleash the full potential of the vaccine formulation, while minimizing side effects

MATERIALS AND METHODS

Animal procedures

Murine experiments

MyD88$^{-/-}$, Sting$^{-/-}$, TLR3$^{-/-}$, MAVS$^{-/-}$, Tmem176b$^{-/-}$, and C57BL/6 female mice 8 weeks of age (Elevage Janvier, Le Genest, France) were housed under conventional conditions according to Institut National de la Santé et de la Recherche Médicale (INSERM) guidelines. All animal experiments were performed in accordance with the recommendations of the French Ministry of Higher Education and Research and approved by an animal experimentation ethics committee under reference APAFIS#7897. For i.m. DNA/RNA vaccination, mice were anesthetized with isoflurane (1%–3% for maintenance, up to 5% for induction) in oxygen from a precision vaporizer (Tem Sega, Pessac, France), then different DNA/RNA-polymer formulations were injected into both *tibialis anterior* muscles using an Insumed Pic Indolore 30G syringe (Artsana, Grandate, Italy). Two sites were injected per animal, so DNA doses are given per *tibialis anterior*. In all cases, the injection volume was 50 μL per injection site. After vaccination, serum samples were collected prior to the first and third weeks after the last immunization to analyze the humoral immune response.

Rabbit experiments

Six female New Zealand White albino rabbits 6 months of age were housed and immunized five times every week with 2 mL of 704 formulated DNA encoding hAFP at 1 mg/mL (Agro-Bio, La Ferte Saint Aubin, France). At day 43, serum samples were collected.

Non-human primate experiments

Three female *Macaca fascicularis* of Mauritius origin (housed in an Association for Assessment and Accreditation of Laboratory Animal

Care [AAALAC] accredited facility, following institutional animal care and use committee [IACUC] approved procedures) were injected in both *tibialis anterior* muscles with 1 mL of 704-formulated DNA encoding hAFP at 0.25 mg/mL. The animals were immunized at days 0, 28, and 56. After vaccination, serum samples were collected prior to the first injection and every 2 weeks after until day 132, to analyze the humoral response.

Nucleic acid preparation and formulation

The pCMV-LacZ plasmid (Clontech, St Germain en Laye, France), encodingβ-Gal under the control of the human cytomegalovirus (CMV) immediate-early gene promoter, was used as antigen. pCMV-LacZ-miR133a-3p and pCMV-LacZ-miR142a-3p were constructed by cloning four miRNA 133a-3p targeting sequences (forward, cagctggtt gaggggaccaaa; reverse, tttggtcccctcaaccagctg) or four miRNA 142a-3p targeting sequences (forward, tccataaagtaggaaacactaca; reverse, tgtagtgtttcctactttatgga) into the 3′ UTR of the LacZ gene, which inhibit respectively the expression on muscular fibers or DCs.

pCpGrich-LacZ was constructed by cloning the LacZ gene from pCpGfree-LacZ into the pCpGrich-mcs backbone (InvivoGen, San Diego, CA, USA). pCpGrich-LacZ contains 88 CpG dinucleotides motifs, whereas pCpGfree-LacZ has none. Both plasmids encode β-Gal under the control of the CMV enhancer and the human elongation factor 1α core promoter. The CH505 TF gp120 plasmid and CH505.M11 gp145 plasmid encoding respectively the gp120 and gp145 HIV envelope proteins were kindly supplied by Barton Haynes (Duke University, Durham, NC, USA). The pCMV-hAFP plasmid is a pVAX1 plasmid (Thermo Fisher Scientific, Les Ulis, France) encoding hAFP. pGWIZ-Luc (Genlantis, San Diego, CA, USA) is a plasmid encoding the luciferase reporter gene under the control of the CMV immediate-early gene promoter.

All plasmids were prepared and purified using EndoFree plasmid purification columns (Qiagen, Courtaboeuf, France) and were confirmed to be free of endotoxin contamination (endotoxin <0.1 EU/μg pDNA) by the *Limulus* amebocyte lysate assay (Lonza, Clermont-Ferrand, France). The non-coding pVAX DNA was purchased from Thermo Fisher Scientific (Les Ulis, France).

The non-coding unmodified mRNA was purchased from TriLink BioTechnologies (San Diego, CA, USA). The mRNA-LacZ, mRNA-DHX9, and mRNA-DHX36 (TriLink BioTechnologies) are unmodified ARCA capped mRNAs, coding respectively for β-Gal protein and DHX9 and DHX36 DNA sensors.

The TLR9 agonist ODN1826 and antagonist ODN 2088 were purchased from InvivoGen.

The tetrafunctional block copolymer 704 was kindly supplied by InCell-Art (Nantes, France).

pDNA, mRNA, and ODNs were formulated immediately prior to i.m. injection as previously described.[12]

Cell culture conditions and transfection

Immature DCs (JAWSII; CRL-11904, ATCC, Rockville, MD, USA) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS; Eurobio, Courtaboeuf, France), 100 U/mL penicillin (Life Technologies, Villebon sur Yvette, France), 10 μg/mL streptomycin (Life Technologies), and 5 ng/mL murine GM-CSF (Life Technologies).

Twenty-four hours prior to transfection, cells were seeded in 24-well culture plates at a density of 60,000 cells in 1 mL of complete medium and incubated at 37°C in a humidified 5% CO_2/95% air-containing atmosphere. Thirty minutes before transfection, medium was removed and replaced by 400 μL of complete medium. One hundred microliters of lipoplexes was prepared by mixing two equal solutions containing 0.5 μL pCMV-LacZ-miR142a-3p and the ionizable lipid DOSK (dioleyl-succinyl-kanamycin) at a charge ratio (±) of 5 and then deposed in each well.

β-galactosidase expression

For *in vitro* β-Gal expression experiments, cells were washed after 24 h of incubation at 37°C, 5% humidified CO_2, with PBS and lysed in Reporter lysis buffer 1× (Promega, Charbonnieres, France) supplemented with 1 tablet per 50 mL of complete Mini Protease inhibitor cocktail (Roche, Meylan, France) according to the manufacturer's recommendations. After an overnight freezing cycle, cell lysates were centrifuged at 10,000 rpm for 5 min.

For *in vivo* β-Gal expression experiments, *tibialis anterior* muscles were dissected 7 days after i.m. injection and immediately frozen in liquid nitrogen. Then the muscles were sheared by an Ultra-Turrax T25 Basic (IKA, Lille, France) in Reporter lysis buffer (Promega) supplemented with protease inhibitor cocktail (Roche).

The expression was then quantified in cell lysates and muscle extracts using the Beta-Glo assay system (Promega) according to the manufacturer's protocol.

Measurement of the immune response

Cytokine expression was determined by qRT-PCR analysis. Briefly, muscles were sampled as described above. Total RNA was extracted from muscles with TRIzol (Life Technologies) according to the manufacturer's protocol. qRT-PCR was then realized in two phases: first, reverse transcription of RNA in cDNA was conducted using High Capacity cDNA reverse transcription kits (Life Technologies) on a GeneAmp PCR System 9700 thermal cycler (Life Technologies). Then, real-time PCR was effected using TaqMan gene expression assays on a StepOnePlus real-time PCR system (Life Technologies). Probes used to amplify specific gene products from murine cDNA (Life Technologies) were, for IL-2, Mm00434256_m1; for IL-5, Mm00439646_m1; for IL-6, Mm00446190_m1; for IL-10, Mm00439614_m1; for DHX9, Mm00456021_m1; for DHX36, Mm00661002_m1; for TNF-α, Mm00443260_g1; for IFNγ, Mm01168134_m1; and for HPRT (housekeeping gene), Mm00446966_m1.

Cytokine secretion was measured on muscle extracts by FlowCytomix multiplex technology (Ebioscience, Paris, France). Muscles were explanted and frozen in liquid nitrogen. Then, the muscles were transferred in 2 mL tubes containing PBS supplemented with protease inhibitor cocktail (Roche) (1mL/tube/muscle), homogenized using a TissueLyser II (Qiagen) with two cycles of 3 min frequency 30, and the muscle lysates underwent 5 freeze/thaw cycles to maximize cytokine extraction. Cytokines were measured in lysates using a Mouse Th1/Th2 FlowCytomix 10-plex RTU kit (Ebioscience, Paris, France) on a BD FACSVerse (BD Biosciences, Rungis, France).

Humoral immune responses were measured by ELISA. For murine sera, 96-well plates (Nunc Maxisorp, Roskilde, Denmark) were coated overnight at 4°C with 5 μg/mL recombinant β-Gal or 2 μg/mL gp120 protein in 50 mmol/L $NaHCO_3$ (pH 9.5) and then blocked for 1 h at room temperature with PBS 0.05% Tween 20, 1% bovine serum albumin, before diluted sera were distributed in triplicates. Plates were incubated at 37°C for 90 min, then β-Gal-specific IgG was detected using peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, Newmarket, UK) diluted 1/5,000 in PBS 0.05% Tween 20, 1% bovine serum albumin. Plates were washed three times in PBS 0.05% Tween 20 between steps, and peroxidase activity was revealed with 1 mg/mL *ortho*-phenylenediamine in pH 5 citrate buffer. Reactions were stopped by addition of 1 mol/L H_2SO_4, then absorbance (A) was measured at 492 nm. For non-human primate and rabbit serum analysis by ELISA, the same protocol as described above was used, except that 96-well plates were coated with 1 μg/mL recombinant hAFP (Thermo Fisher Scientific, Courtaboeuf, France) and hAFP-specific IgG was detected using peroxidase-conjugated goat anti-monkey IgG (Bio-Rad, Oxford UK) and goat anti-rabbit (Jackson ImmunoResearch, Newmarket UK) diluted 1/100 and 1/5,000, respectively, in PBS 1% bovine serum albumin. Anti-gp120 and anti-hAFP titers were determined by measuring the absorbance at 490 nm of sera diluted at 1/2,000 and 1/100, respectively. Anti-β-Gal titers were determined using cascading dilution from 1/800 to 1/1,638,400 for each sample and then by tracing a linear trend curve in the linear portion of the curve between 0 and 1 of absorbance to determine the theoretical dilution value for absorbance = 1 (ThDiA1) and calculated by 1/ThDiA1. A standard β-Gal-specific mouse serum was included in each ELISA plate to normalize results. As a measurement of serum antibody quality, mouse samples were assessed for their ability to block the binding of soluble CD4 (sCD4) to the CD4 binding site (CD4bs) of CH505 TF gp120 *env* protein, a key epitope of HIV neutralization, and to block the binding of a known bNab CH106, as follows: 384-well ELISA plates (Costar 3700) were coated with 30 ng/well of CH505 TF gp120 *env* overnight at 4°C and blocked with assay diluent (PBS containing 4% [w/v] whey protein/15% normal goat serum/0.5% Tween 20/0.05% sodium azide) for 1 h at room temperature. All assay steps were conducted in assay diluent (except the substrate step) and incubated for 1 h at room temperature followed by washing with PBS/0.1% Tween 20. Samples diluted 1/50 and appropriate reference wells were incubated, washed, and followed with biotinylated-CH106 detected by streptavidin-horseradish peroxidase at 1:30,000 (Thermo Scientific) followed by TMB substrate (KPL). For sCD4 blocking, a saturating concentration of sCD4 (Progenics Pharm) was added following the sample incubation step. Binding of sCD4 was then detected with biotin-anti-CD4 mouse Mab OKT4 (eBioscience cat. no. 13-0048) followed by streptavidin-HRP. Serum from non-immunized animals was used as a negative control. Plates were read at 450 nm. Percentage blocking was calculated as follows: 100 − ((sample triplicate mean/0% blocking control mean) × 100).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (La Jolla, CA, USA). Values are expressed as the mean ± SEM. Significant differences between groups are indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.01$ by Mann-Whitney test.

Data availability

All data are available in the article. Correspondence and requests for materials should be addressed to B.P. Reprints and permissions information is available.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2023.04.029.

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AUTHOR CONTRIBUTIONS

T.C., T.H., and B.P. conceived and designed the experiments. T.C. and T.H performed the experiments. T.C., T.H., and B.P. analyzed the data and wrote the manuscript. T.C., T.H., and B.P. conceived the figures. All authors discussed the results, commented on, and proofread the manuscript. The principal investigator is B.P.

DECLARATION OF INTERESTS

B.P. owns stock in In-Cell-Art, which commercializes tertafunctional amphiphilic block copolymers and is an inventor on several patents ans patent applications related to use of block copolymers for DNA and RNA delivery.

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