

Strong Immune Responses Induced by Direct Local Injections of Modified mRNA-Lipid Nanocomplexes

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***In vitro* transcribed mRNAs hold the promises of many medical applications in disease prevention and treatment, such as replacement or supplement of missing or inadequately expressed endogenous proteins and as preventive vaccines against infectious diseases, therapeutic vaccines, or other protein-based biopharmaceuticals for cancer therapy. A safe and efficient delivery system for mRNA is crucial to the success of mRNA therapeutic applications. In this study, we report that InstantFECT, a liposome-based transfection reagent, can pack pseudouridine-incorporated mRNA into nanocomplexes that are highly efficient in mediating *in vivo* transfection in multiple organs after local delivery. High levels of expression of EGFP and luciferase reporters after intratumoral and intramuscular injections were observed, which lasted for up to 96 hrs. Immunogenicity of antigens encoded by mRNA delivered with nanocomplex was investigated by subcutaneous delivery of modified mRNAs encoding *Staphylococcus aureus* adenosine synthase A (AdsA) and a model tumor-associated antigen ovalbumin (OVA). Strong T cell responses were provoked by both mRNAs delivered. Therapeutic and protective treatment with the OVA mRNA-liposome nanocomplex significantly inhibited B16-OVA tumor progression and increased mouse survival. There was no sign of obvious toxicity related to the treatment both in tissue culture and in mice. An intravenous injection of the same dosage of the modified mRNA-lipid nanocomplex showed minimal transfection in major organs, indicating an excellent safety feature as the gene transfer occurred only at the injection sites, whereas intravenous (i.v.) injection with the same amount of mRNA complexed with a commercial transfection reagent Trans-IT showed luciferase expression in the spleen. In summary, InstantFECT cationic liposomes provide a safe and efficient *in vivo* locoregional delivery of mRNA and could be a useful tool for basic research and for the development of mRNA-based therapies.**

INTRODUCTION

Recombinant protein-based biopharmaceuticals are becoming ever important for both therapeutic and preventive applications. An alternative approach is to introduce DNA or RNA to cells that direct the synthesis of proteins in targeted cells. Both viral and non-viral

vectors have been developed to deliver foreign genes in the forms of DNA or RNA to the cells. The viral vectors carry certain risks of reverting to the wild-type and may even be mutagenic. These viral vectors are complicated and tedious to be prepared and are highly immunogenic.¹ On the other hand, non-viral vectors represented by cationic liposomes and polymers, can deliver biologically active agents, such as plasmid DNA, small interfering RNA (siRNA), mRNA, and proteins to cells through a simple process called transfection, which is highly efficient *in vitro*.² Nucleic acids form complexes with transfection reagents through electrostatic interactions to form either lipoplexes or polyplexes depending on the packing material used and are subsequently taken up by cells through endocytosis.³ Compared to viruses, these have the advantages of simple usage, ease of preparation and production scale-up and are less immunogenic. However, they are generally inefficient in various *in vivo* applications.⁴

Due to ubiquitous presence of RNase activities, ordinary mRNAs have very short half-lives, limiting their applications.^{5,6} Self-replicating RNA viral elements have been used to significantly prolong the half-life of RNA, but it is a rather complicated system and involves viral RNA polymerase that could be antigenic or harmful to the host cells.^{7,8} The incorporation of nucleotide analogs such as pseudouridine greatly improves the stability of mRNA and simultaneously reduce its immunogenicity. This paves the road for a practical use of mRNA technology.^{9,10} The importance of 5' cap structures, poly(A) tail, and 3' and 5' untranslated regions (UTRs) for mRNA stability and efficiency of translation has been well recognized.^{8,11,12}

Naked mRNA to some degree is able to cross the lipid bilayer to reach cytoplasm for translation¹³ and has already been used for

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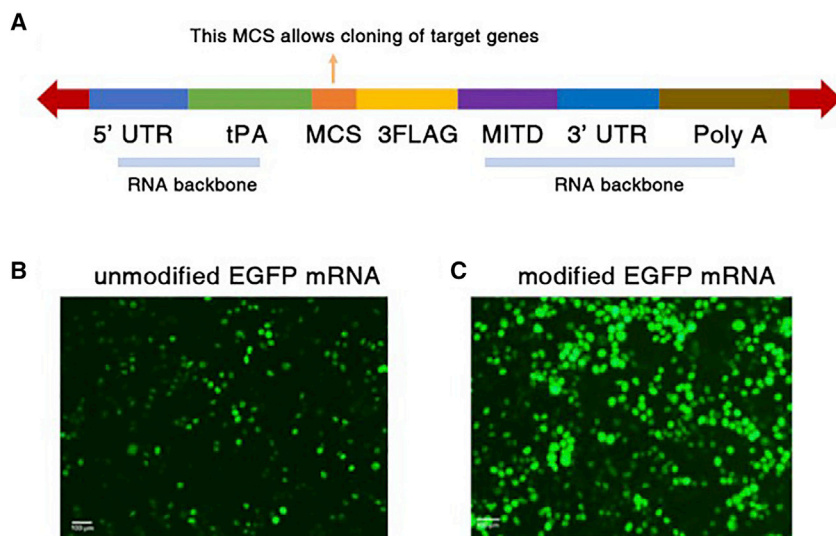


Figure 1. Improved Expression of EGFP from Base-Modified mRNA

(A) The key elements of the mRNA plasmid backbone construct used in the experiment. 3XFLAG, three copies of flag tags for the detection of expressed protein; tPA, tissue plasminogen activator; MITD, MHC I-targeting domain; UTRs, untranslated regions, poly(A), 130 bases in length. (B) HEK293 cells transfected with 500 ng of unmodified cap0 EGFP mRNA. (C) HEK293 cells transfected with 500 ng of pseudouridine cap1 modified EGFP mRNA. All these transfections are with commercial messenger MAX liposome. Scale bar, 100 μ m.

intranodal delivery of tumor-specific antigens to elicit a robust T cell response.¹⁴ Physical delivery methods like gene gun or *in vivo* electroporation also showed efficient delivery of mRNA to mice, but limited efficacy was found when applied in humans or other large animals.^{15,16}

Various carriers have been used to enhance the uptake and expression of mRNA to increase the efficacy of vaccines.^{17,18} Intradermal injections of mRNA encoding tumor antigens complexed with protamine are already in clinical trials.¹⁹ Some commercially available lipid-based transfection reagents like Lipofectamine (Invitrogen) or Trans-IT (Mirus) work efficiently in cell transfection *in vitro* but are highly toxic for *in vivo* delivery of mRNA.⁹ The lipid nanoparticles (LNPs) are made up of pH-sensitive ionizable cationic lipids and neutral helper lipids by microfluidic mixing, which self-assembles with RNA molecules into 100- to 300-nm nanoparticulate structures.²⁰ Systemically delivered mRNA-LNPs mainly target liver due to spontaneous binding with apolipoprotein E, which serves as a natural ligand for hepatocytes. When delivered intradermally, subcutaneously, or intramuscularly, it leads to a prolonged expression of proteins in transfected cells and mostly restricted to the site of injection.²¹ This could act as a depot from which mRNA is used locally or enters the circulation.^{22,23}

The preparation of LNPs, however, requires a set of delicate and expensive instruments, a quite complicated recipe of lipidic components, and some skills to accomplish. A simple protocol that allows an easy preparation of an mRNA-containing nanocomplex that can mediate an efficient mRNA transfection and protein expression after local delivery would be in high demand for many research areas, including research and development of vaccines.

Here, we present InstantFECT, a simple liposome system that can form a distinct nanocomplex with pseudouridine-modified

mRNA, mediate highly efficient and reproducible *in vivo* transfection, and result in a prolonged antigen expression that lasts for up to 4 days after local administration via intratumoral, subcutaneous, and intramuscular routes. Robust humoral and cytotoxic immune responses were observed after vaccinations with modified mRNA nanocomplex encoding adenosine synthase A (AdsA) from *Staphylococcus aureus*²⁴ and ovalbumin (a model antigen for the development of therapeutic cancer vaccine).

RESULTS

mRNA Construct and Its Synthesis

mRNA was transcribed *in vitro*, with its uridine residues partially substituted with N-methyl pseudouridine (TriLink) to gain resistance to degradation by RNases and enhance the translation.⁹ The mRNA was 5'-capped with a cap0 structure by vaccinia-virus-capping enzyme and then further modified to cap1 structure with 2'O methyl transferase. The superior protein expression from cap1-pseudouridine-modified mRNA to unmodified mRNA has been shown previously²⁵ and is also consistent with our EGFP expression data, indicating the importance of these modifications in the overall protein expression (Figures 1 and S2). These modifications increased biological stability and translation efficiency and reduced the immunogenicity of mRNA. There are a few other elements that help in the efficient expression and enhance the immunogenicity of the expressed protein. These include 3' and 5' UTRs, which are known to be important for translation, tPA (tissue plasminogen activator),²⁶ which helps in excreting the mature protein product out of the cell^{27,28} to be detected by the major histocompatibility complex class II (MHC II) pathway, and MITD (MHC I targeting domain), which directs the protein product to MHC I pathway.²⁹

InstantFECT alone and the InstantFECT-mRNA nanocomplex were negatively stained and examined with transmission electron microscopy (TEM) (Philips CM100) (Figure 2A). While the image of liposomes showed many vesicles with some aggregates, the liposome-mRNA nanocomplex appeared to be well separated individual structures of oval or spherical shape and 100–200 nm in diameter. There were apparent hairy structures adherent to and extending from the surface of these nanostructures.

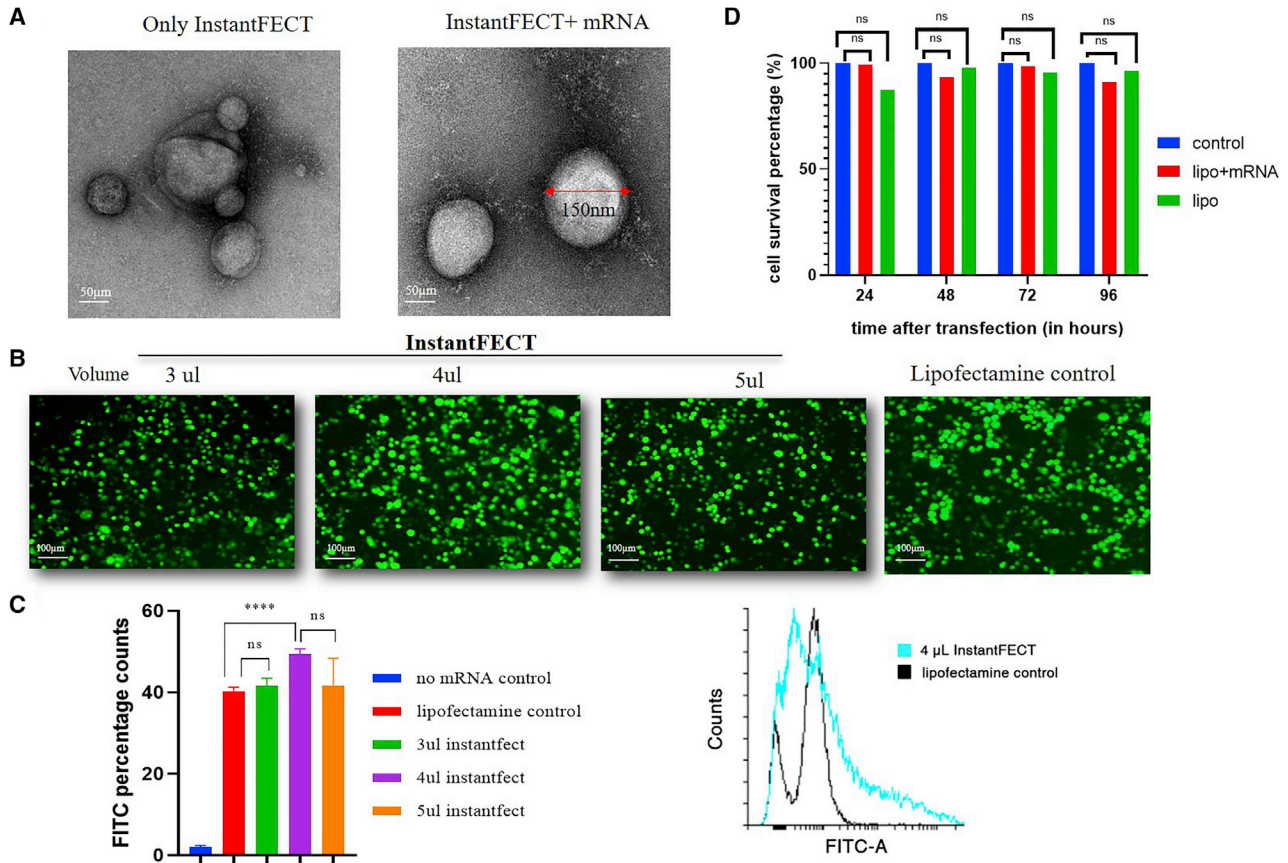


Figure 2. InstantFECT Shows High Transfection Efficiency without Noticeable Cytotoxicity

(A) TEM images of the InstantFECT liposome and mRNA nanocomplex. They seem to be more stable when complexed with mRNA and are around 100–200 nm in diameter. (B) *In vitro* transfection results of modified EGFP mRNA with 3, 4, and 5 μ L of InstantFECT to HEK293 cells compared to commercial messenger MAX lipofectamine transfection of EGFP mRNA. Scale bar, 100 μ m. (C) Flow cytometry results showing % EGFP-expressing cells transfected with EGFP mRNA complexed with either Lipofectamine or different volumes of InstantFECT (left) and the distribution patterns of cell population expressing EGFP fluorescence at different intensities from Lipofectamine and 4 μ L of InstantFECT-treated groups. (D) XTT assay plot showing the viability of HEK293 cells after 24, 48, 72, and 96 h of treatment with 500 ng EGFP mRNA with 4 μ L InstantFECT or InstantFECT alone. Scale bar, 100 μ m. The control stands for untreated cells. One-way ANOVA test, ns (non-significant difference), $p > 0.05$, significant differences, **** $p < 0.01$

The expression of EGFP with pseudouridine-incorporated mRNA was examined in HEK293 cells by transfection of mRNA-cationic liposome complexes. First, to optimize the amount of the liposome used, different volumes of liposome (2–5 μ L) were used with a fixed amount of mRNA (500 ng). The cells were incubated with these complexes for 24 h and observed for EGFP expression under fluorescence microscopy (Figure 2B) followed by flow cytometry (Figures 2B and 2C). The percentage of cells that showed GFP signals was ~37.5% with 3 μ L of messenger-MAX Lipofectamine, while by applying 3–5 μ L of InstantFECT, a transfection efficiency of 37.5% to 42.6% could be achieved. This indicated that the transfection efficiency with InstantFECT is comparable with that of the commercialized mRNA transfection reagent messenger-MAX Lipofectamine (Figure 2C).

To check the effect of InstantFECT on the viability of mammalian cells, HEK293 cells were transfected with 4 μ L of the liposomes alone

or 4 μ L liposome with 500 ng of EGFP mRNA. After 24, 48, 72, and 96 h of transfection, XTT (sodium 3'-[1-(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) assay was performed to check the cell viability. The data indicated that the treatment of the liposome-alone as well as mRNA-liposome nanocomplexes had minimal toxicity to treated cells (Figure 2D).

InstantFECT Mediated *In Vivo* mRNA Delivery

For the *in vivo* delivery, five mice were injected with 5 μ g of modified luciferase mRNA complexed with different volumes of InstantFECT. The mice were anaesthetized 24 h after injection and were given luciferin substrate just before viewing them in the *in vivo* imager for non-invasive longitudinal monitoring of gene expression patterns in live animals. The results showed the highest level of luciferase activity with the mRNA complexes prepared from 4 μ L InstantFECT

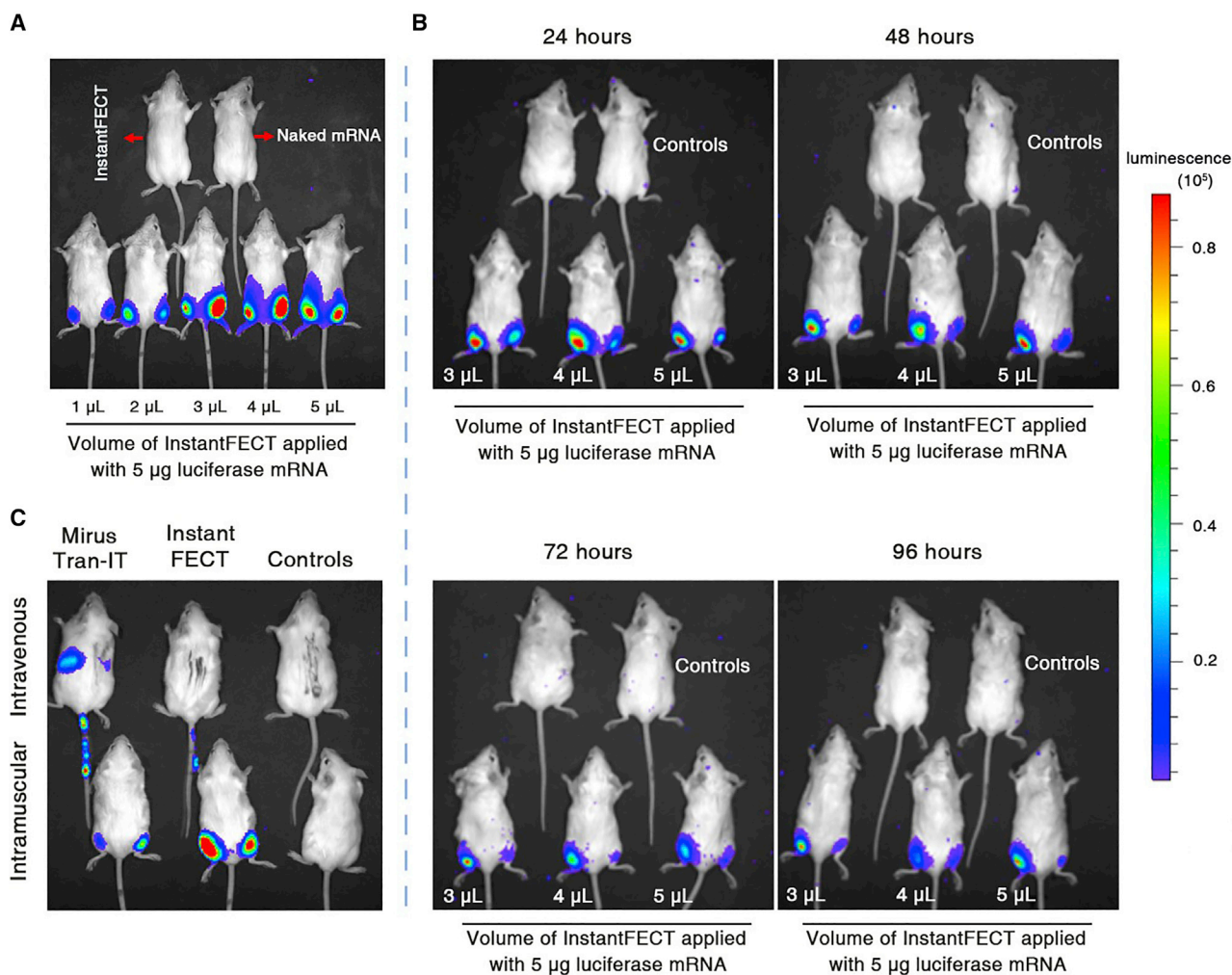


Figure 3. *In Vivo* mRNA Transfection via i.m. and i.v. Routes with InstantFECT and Trans-IT

(A) 5 μ g of modified luciferase mRNA alone or the same amount of mRNA complexed with indicated amounts of InstantFECT liposome were intramuscularly injected in duplication to each side of the hind legs. *In vivo* imaging was performed in anaesthetized mice 24 and 48 h after the injection. One mouse received InstantFECT (4 μ L) injection and served as the control. (B) Five μ g of the modified luciferase mRNA was complexed with 3, 4, and 5 μ L of InstantFECT and the nanocomplexes were injected into mice by i.m. delivery. Mice were anaesthetized 24, 48, 72, and 96 h after injection, and *in vivo* imaging was performed. Controls include liposome alone (top left) and naked mRNA alone (top right). (C) Comparison of *in vivo* transfection results for mRNA-Trans-IT and mRNA-InstantFECT. 5 μ g of luciferase mRNA was complexed with 3 μ L of Trans-IT or 4 μ L of InstantFECT and used for each delivery i.v. or i.m. Controls received liposome injections only.

(Figure 3A). The duration of expression from mRNA delivered by InstantFECT *in vivo* is shown in Figure 3B. The levels of luciferase activity were the highest at 24 h following the transfection, very high at 48 h, and remained high even 96 h after injection. It should be noted that intramuscular (i.m.) injections with neither naked mRNA nor InstantFECT alone resulted in any significant levels of bioluminescence signals.

The transfection efficiency after i.m. delivery of the luciferase mRNA-InstantFECT nanocomplex was directly compared to that of the mRNA nanocomplex prepared with a commercial liposome, Trans-IT (Mirus). As shown in Figure 3C, the bioluminescence signal reflecting luciferase activity from mRNA-InstantFECT-treated mice

was orders of magnitude higher than that of mRNA-Trans-IT nanocomplex (note that the pseudocolor was in log scale). It is interesting to note that muscles of each mouse transfected with the mRNA-InstantFECT nanocomplex showed center areas with the highest luciferase activities, which were surrounded by weaker and somewhat diffused peripheral areas, some of which were substantially extended away from the center, while for the mRNA-Trans-IT nanocomplex-transfected mice, the areas that showed luciferase activities were more restricted to the center, with little diffused peripheral areas (Figures 3B and 3C). It is obvious that i.m. injection of mRNA-InstantFECT nanocomplex led to substantially higher levels of transgene expression and wider transfected area than that of the mRNA-Trans-IT nanocomplex.

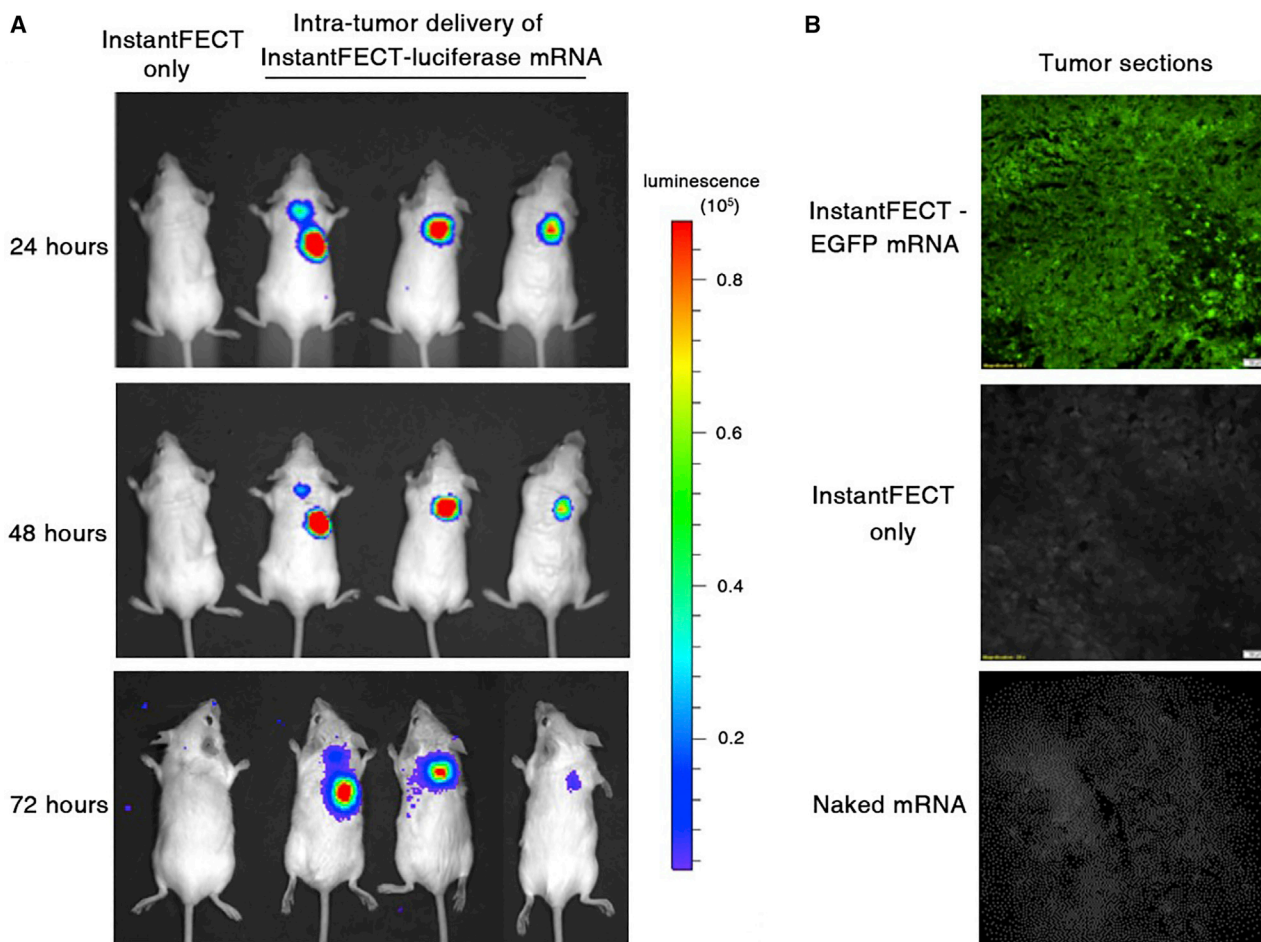


Figure 4. Expression of Reporters after Intratumor Delivery of mRNA-InstantFECT Nanocomplex

(A) 5 μ g of luciferase mRNA complexed with 4 μ L of InstantFECT was injected into s.c.-implanted 4T-1 mouse breast cancer. Luciferase was highly expressed in the tumors for at least 3 days. (B) Intense and widespread EGFP expression found in cryo-sections from solid tumors 24 hr after transfection with 5 μ g of EGFP mRNA complexed with 4 μ L of InstantFECT. Scale bar, 100 μ m

InstantFECT was also compared with Trans-IT for *in vivo* delivery with luciferase mRNA via intravenous route. The mice were tail-vein injected with 5 μ g of luciferase mRNA, complexed with either InstantFECT or Trans-IT, and were viewed for luminescence 24 h after delivery as described before; the results showed that the most noticeable locations of luciferase expression were parts of the tails near the injection sites for both formulations. However, a modest level of luciferase expression was also noticed in the spleen of mRNA-Trans-IT-transfected mice, while the mouse received mRNA-InstantFECT didn't show any significant expression anywhere except in the tail.

Intratumoral Delivery of mRNA

We performed intratumoral delivery of luciferase mRNA complexed with InstantFECT liposomes by direct injection into a subcutaneously implanted 4T-1 tumor (mouse breast cancer cell line). Bioluminescence measurements indicated that luciferase was highly expressed in tumors that lasted for at least 3 days (Figure 4A). To examine

the extent of reporter expression morphologically in solid tumors after transfection, a similar transfection was performed with a nanocomplex containing 5 μ g of modified EGFP mRNA. The tumors were excised 24 h after delivery and fixed with freshly prepared 4% paraformaldehyde (PFA), and cryo-sectioning was performed. The sections were examined under fluorescence microscope (Figure 4B). Intense and widespread EGFP expression was observed in the entire view field, indicating that the mRNA nanocomplex was well spread within the tumor tissue. Both controls with naked mRNA or liposomes alone did not show any significant signal.

Immunogenicity and mRNA-Based Adsa Vaccination with Nanocomplex

To test whether the local delivery of mRNA-based vaccine by InstantFECT can provoke systemic immune response against pathogens of infectious diseases, we immunized mice with modified mRNA encoding Adsa antigen in the form of the InstantFECT

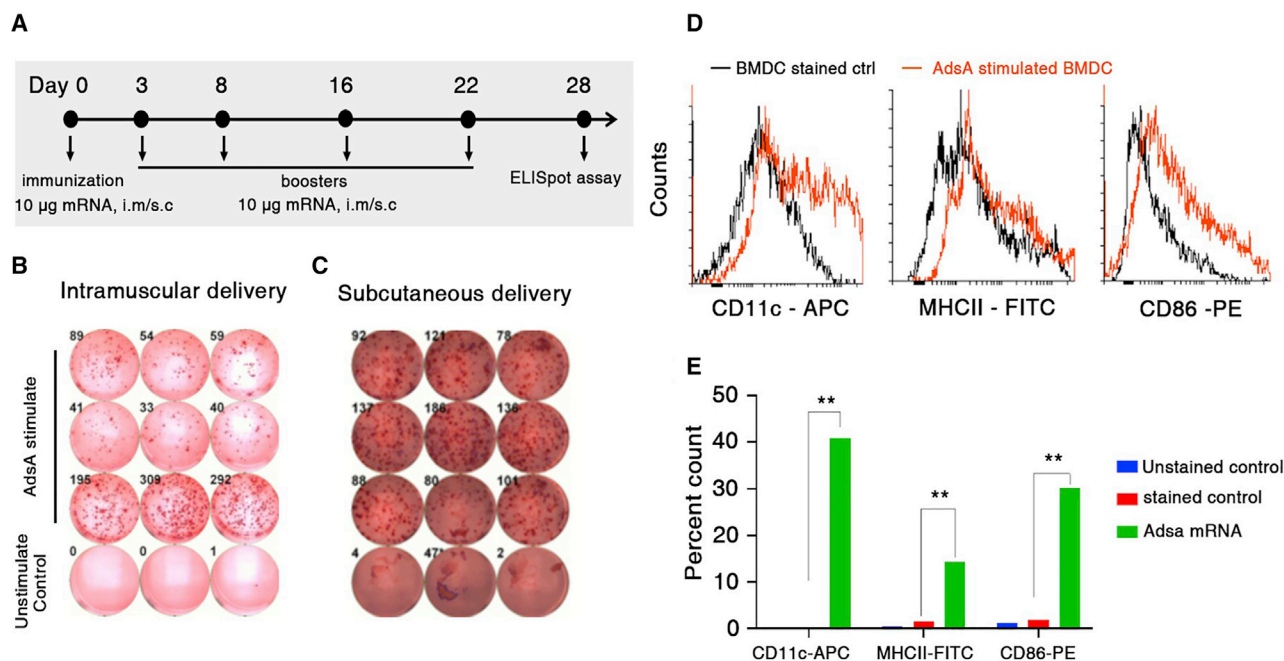


Figure 5. Adsa mRNA-InstantFECT Nanocomplex Provoked Specific T Cell Response and Induced Dendritic Cell Maturation

(A) A schedule for vaccination with Adsa-modified mRNA-InstantFECT nanocomplex. $n = 3$ for both i.m. and s.c. groups. (B) Interferon γ ELISPOT results of splenocytes from the Adsa mRNA i.m. vaccination group. (C) Interferon γ ELISPOT results of splenocytes from the Adsa mRNA s.c. vaccination group. Positive control was inducer ionomycin; negative control was media only. (D) Flow cytometry profiles of three different markers of dendritic cell maturation for bone marrow dendritic cells transfected with Adsa mRNA nanocomplex and un-transfected bone marrow DCs. (E) Quantitative analysis of % bone marrow dendritic cells positive for three different markers of dendritic cell maturation (CD11c, MHCII, and CD86) after different treatments. The graphs were standardized with unstained control (cells without any transfection and staining, not shown in graph) and compared with BMDC-stained controls (cells without any transfection but stained with three DC markers). Adsa-stimulated BMDCs were the test group, which were transfected with Adsa mRNA and also stained with three DC maturation markers. One-way ANOVA test showed significant differences, $**p < 0.01$.

nanocomplex. The Adsa antigen serves as a vaccine against a serious infectious agent, *S. aureus*, and a peptide-based vaccine is currently under clinical trial.²⁴ Two groups of three 5- to 6-week-old BALB/c mice were challenged with the initial priming shots on days 0, 3, and 8, followed by two booster shots on days 16 and 22 by i.m. or subcutaneous (s.c.) routes with InstantFECT nanocomplex containing Adsa mRNA, respectively. For the immunization process, 10 µg of Adsa mRNA was complexed with 4 µL of the InstantFECT liposome just prior to use, and 100 µL was injected i.m. to the thigh muscles of mice or s.c. on each of the right and left flank to the mice. Control groups had the liposome alone, prepared in the same solution as that of the nanocomplex. Seven days after the last injection, splenocytes were collected and an interferon γ enzyme-linked immunospot (ELISPOT) assay was performed to quantify immune responses on a per-cell basis (Figure 5A). Strong antigen-specific T cell responses were observed on splenocytes collected from mice that had either i.m. or s.c. immunization with Adsa mRNA delivered with InstantFECT (Figures 5A–5C).

Dendritic cell maturation is a key step for immune activation, and mature dendritic cells (DCs) are professional antigen-presenting cells that effectively take up, process, and present antigens. We tested whether the mRNA-InstantFECT complex can actively induce DC

maturation. We transfected bone marrow DCs (BMDCs) with modified Adsa mRNA-InstantFECT nanocomplex. 24 h later, transfected cells were collected and stained with antibodies specific to markers of DC maturation like CD11c, CD86, and mouse MHC II (Table 1). The flow cytometry results showed that the mRNA transfection led to DC maturation featured by increased expression of DC maturation markers CD11c, CD86, as well as MHC II known to be important to the activation of the immune system (Figures 5D and 5E).

Therapeutic and Prophylactic Effects of the mRNA Nanocomplex against B16-OVA Melanoma

To test the possible use of mRNA-based vaccination with tumor antigens for cancer immunotherapy, we used a murine melanoma variant B16-OVA, that expresses ovalbumin (OVA) as a model. Modified OVA mRNA (encoding 249–339 amino acids of OVA) complexed with InstantFECT served as a therapeutic (Figures 6D–6F) or prophylactic (Figures 6G–6I) vaccine against a B16-OVA transplant in C57BL/6J mice.

First, interferon γ ELISPOT was performed with splenocytes isolated from C57BL/6J mice that were immunized with the OVA mRNA-InstantFECT nanocomplex. A strong T cell response was observed against both OVA (385-amino-acid-long sequence, InvivoGen) and

Table 1. The Markers Used for Assessing the DCs' Maturation

Maturation Marker	Fluorochrome	Function
CD11c	APC	on immune stimulus presented on mature DCs, CD8 ⁺ , and NK cells
CD86	PE	increased on immune stimulus on T cells, B cells, DCs
MHC II	FITC	on immune stimulus presented on APCs

DC, Dendritic cells; NK, Natural Killer cells; APCs, Antigen presenting cells.

MHC I-restricted OVA peptide epitope (SIINFEKL, InvivoGen) (Figures 6B and 6C). We then conducted immunotherapy with therapeutic vaccination against an established B16-OVA tumor using the OVA mRNA-InstantFECT nanocomplex. Figure 6E showed that the tumor progression was dramatically suppressed in mice immunized with the OVA mRNA-InstantFECT vaccine, compared to the mice in the control group that were injected with InstantFECT alone. By day 40 post-tumor-implantation, all of the animals in the control group died, while all the vaccinated mice survived. These mice remained tumor-free until the experiment was ended 3 months after the tumor implantation (Figures 6E and 6F). Similarly, prophylactic vaccination with the OVA mRNA-InstantFECT nanocomplex prior to tumor implantation provided a long-term protection to mice against B16-OVA melanoma (Figure 6H). All the mice vaccinated with OVA mRNA survived till the experiment was ended 3 months after tumor implantation, while all the mice from the control group died by day 37 after tumor challenge (Figure 6I), indicating that a strong immunity was established by mRNA vaccination, which conferred a lasting protection against tumor progression.

Treatment-Related *In Vivo* Toxicity

In a total of ~50 mice that received multiple doses of i.m., s.c., or intratumoral injections with the mRNA-liposome nanocomplex, none of the treated mice showed any sign of obvious toxicity either in the treated area or systemically as reflected with body weight losses or changes in general health condition, suggesting that the vaccination via multiple routes was safe to the animals (Figure S3).

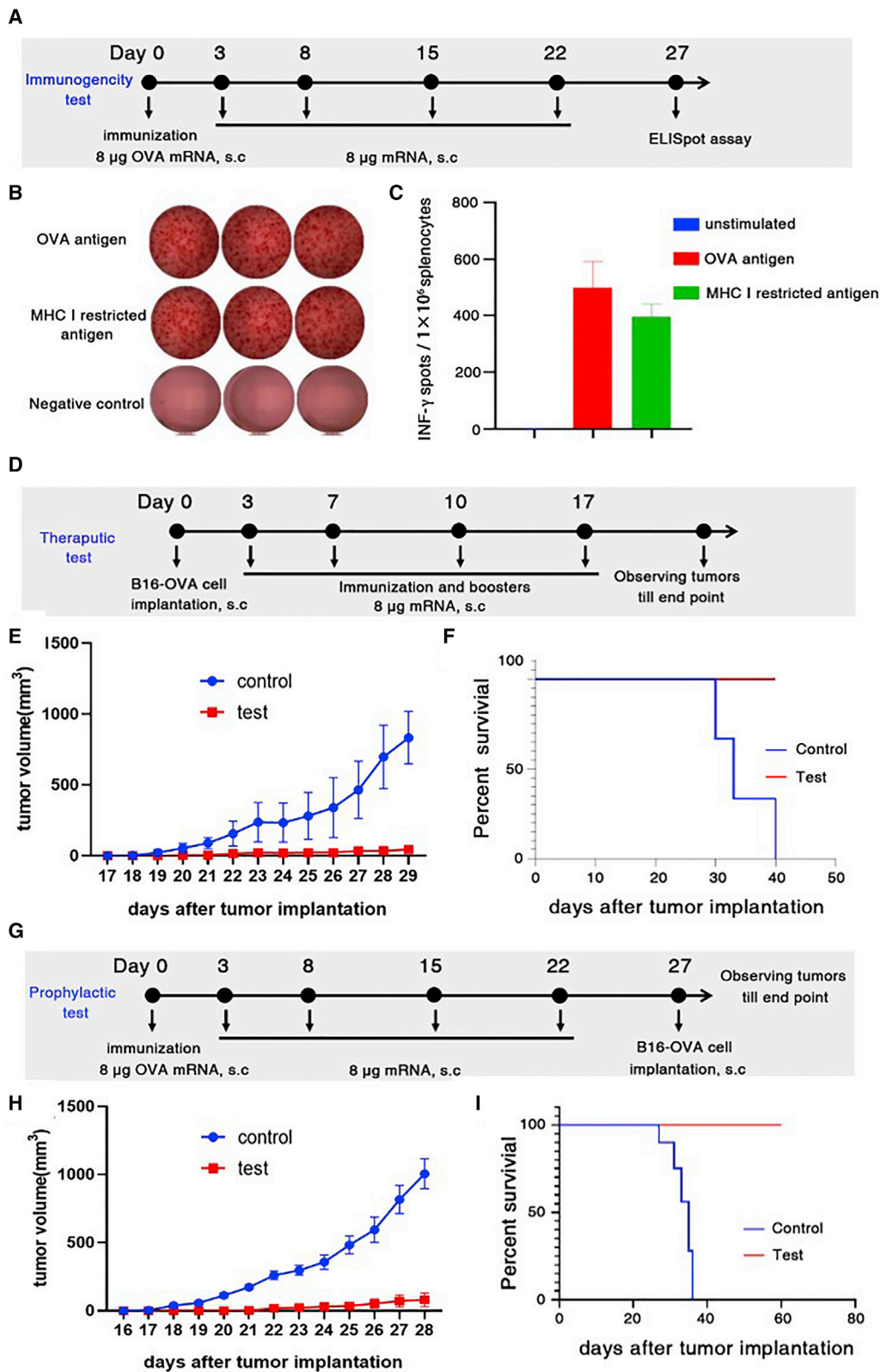
DISCUSSION

Plasmid DNA, mRNA, and siRNA are promising biological agents useful for positively or negatively modulating a particular gene expression. As nucleic acids are fragile molecules that work intracellularly, a carrier is absolutely required to provide full protection of nucleic acids in the process of delivery from enzymatic degradations and to gain access to cytoplasm in order to exert their activities. Most synthetic nucleic acid delivery agents are either liposomes or polymers that carry cationic charges, which are essential to condense nucleic acids into nanoparticles and to trigger cellular uptake through charge-charge interactions. However, cationic charged nanoparticles tend to bind biomolecules in blood, on cell surface, or in extracellular matrix, practically immobilizing these nanoparticles at the injection site in solid tissues, thus limiting their *in vivo* transfection efficiency.

InstantFECT liposome transfection reagent is quite different from the other reagents on the market in two aspects: it mediates efficient transfection over a broad liposome-to-nucleic acid ratio, even when nucleic acid is in excess, and it shows quite a tolerance toward interference of serum components during transfection (X.G., unpublished data). In this work, we found that the optimal ratio (0.5 µg mRNA/4 µL InstantFECT) that worked the best for *in vitro* transfection was quite different from the optimal ratio (5–10 µg mRNA/4 µL InstantFECT) for *in vivo*. TEM images show that mRNA-InstantFECT nanocomplexes prepared at the optimal ratio for *in vivo* transfection are well separated individual particles with 100–200-nm sizes. These nanocomplexes have visible strands extending out from the liposome surface, which resembles an mRNA-coating layer, and therefore the nanocomplex should be negatively charged. At this ratio, both free mRNA and nanocomplex co-existed in the mixture. The control experiment failed to show that mRNA alone had any significant transfection in solid tissues, such as muscles and tumors (Figures 3 and 4), therefore, it is most likely that the nanocomplex exerted transfection activity. Bioluminescence results revealed that the site of injection of the luciferase mRNA-InstantFECT nanocomplex had a bright center and a large, but less intensive, peripheral area (Figure 3), suggesting that these nanocomplex are quite diffusible in solid tissues like muscle. The pattern of widespread EGFP expression in cryo-sections of 4T-1 breast tumor after intratumoral injections of the mRNA-InstantFECT nanocomplex provided further evidence that nanocomplexes are indeed rather readily diffusible from the injection site in solid tissues, like a tumor. It is reasonable to assume that relatively small size and net negative charges of the mRNA-coated nanocomplex contributed to a good diffusibility in solid tissues and the excellent *in vivo* transfection results.

Safety is a critical aspect for any *in vivo* application, and it would be undesirable if any transfection occurs away from the injection site. To test that possibility, a full dose of luciferase mRNA-InstantFECT nanocomplex was injected intravenously via tail vein. The luciferase activity was followed. The results showed that 24 h later, the tail near the injection site is the only part where some weak signals can be identified. On the other hand, animals that received intravenous (i.v.) injection of the same amount of mRNA complexed with Trans-IT had signals clearly detectable in the tail and at the location of the spleen. The data suggest that for *in vivo* mRNA transfection with InstantFECT, even if any leakage does occur at the injection site and some nanocomplexes enter the bloodstream, the likelihood of transfection at vital organs would be minimal.

Currently, subunit vaccines are mostly protein-, peptide-, or polysaccharide-based and suffer from problems such as a tedious research and development process, difficulty in manufacturing process, and stability issues during storage and field application. They usually rely on an insoluble adjuvant, such as aluminum hydroxide hydrate, for a rapid, potent, and sustained immune response, although it is well known that aluminum hydroxide hydrate preferably activates Th2 immunity, rather than MHC I-restricted cytotoxic T cells.³⁰ *In vitro* transcribed mRNAs are well suited for vaccine development because



(legend on next page)

they provide high level, transient expression of antigens for adaptive immunity. Their research and development processes are rapid, and manufacturing processes can be readily implemented and modified according to the need. An advantage of mRNA-based vaccine is that mRNA has intrinsic adjuvant activities. It activates the Toll-like receptor-3 (TLR-3), TLR-7, and TLR-8 signaling pathways, provokes innate immune responses, and upregulates interferon production. In addition, poly I:C (Polyinosinic:polycytidylic acid)³¹ and other designed double-stranded RNA analogs³² have been developed as nucleic acid adjuvants to boost vaccine response.

mRNA vaccines are known to promote immune cell recruitment, DC maturation, and antigen presentation and work synergistically to establish an antigen-specific adaptive immunity.^{12,33} Therapeutic vaccination of mRNA-based neoantigens have demonstrated potent and durable clinical benefits against melanoma.^{13,34} Here, we showed that i.m. and intratumoral injections of the modified mRNA nanocomplex led to a widespread and high-level expression of reporter proteins that lasted for at least 96 h (Figure 4). The prolonged expression of antigens after i.m. and s.c. injections along with the self-adjuvant activity of mRNA could have worked synergistically to promote the maturation of DCs (and antigen presentation), the expansion of T cells, and antigen-specific cytotoxic T cell responses. This resulted in excellent immune responses against *S. aureus* antigen and a model tumor antigen OVA of B16-OVA melanoma. Strong antitumor activities accompanied with a 100% survival rate over an extended period (at least 3 months) were observed in tumor-bearing animals after preventive or therapeutic vaccinations.

In summary, we provide some insights to an effective *in vivo* transfection system for local administration of pseudouridine-modified mRNA in the form of mRNA-coated InstantFECT nanocomplex. A high level and prolonged expression were routinely achieved with minimal toxic effects. The transfection by mRNA-InstantFECT nanocomplex was able to induce DC maturation and invoke strong T cell responses against *S. aureus* AdsA and OVA, a model tumor-associated antigen. Moreover, the vaccination with the OVA mRNA-InstantFECT nanocomplex led to a dramatic antitumor therapeutic effect against B16-OVA melanoma in a mouse model, suggesting the potential application of mRNA-lipoplex in efficient and safe delivery of therapeutic agents *in vivo*. This *in vivo* transfection system is expected to be widely applicable for basic research, as well as for the development of many nucleic-acid-based therapeutic approaches.

MATERIALS AND METHODS

Transfect Reagent Preparation

InstantFECT was donated by PGR-Solutions (Pittsburgh, PA, USA). The invention disclosure that led to InstantFECT has been published.³⁵ It was received in a dried film packed in a glass vial, accompanied with a vial of reconstitution solution. After adding the recommended amount of the reconstitution solution to the dried film, it was set for a full minute and then vortexed for a full minute to rehydrate the lipid film into a slightly translucent liposome suspension that is ready to use.

mRNA Synthesis

TMV 5' and 3' UTR regions were inserted to pUC57 plasmid vector, and a copy of poly(A) tail (130 bases in length) was then inserted behind the 3' UTR to yield the DNA backbone for our mRNA, which we called pRV (Puc-57 recombinant vector) plasmid. Luciferase, EGFP, AdsA, and OVA genes were separately inserted into the pRV backbone to obtain pRV-luciferase, pRV-EGFP, pRV-AdsA, and pRV-OVA plasmids. We also fused in frame an htPA (human tissue plasminogen activator) tag²⁶ and a 3× FLAG tag to the 3' end of the mRNA coding sequence (Figure S1). The htPA tag facilitates the secretion of the target protein from the cells, while the FLAG tag helps detection of the expressed protein in cell extracts by western blot.

The *in vitro* transcription of luciferase, EGFP, AdsA, and OVA mRNA from corresponding plasmid DNAs was performed by using a T7-HiScribe mRNA synthesis kit (NEB). The mRNAs with partial uridine base substituted were synthesized by *in vitro* transcription (IVT) with a portion of UTP substituted with N-methyl pseudouridine triphosphate (TriLink BioTechnologies). In a typical preparation, the mixture contains plasmid DNA (1 μg), NTPs [nucleoside triphosphates] (7.5 mM adenosine triphosphate [ATP], 7.5 mM cytidine triphosphate [CTP], 7.5 mM guanosine triphosphate [GTP], 5 mM uridine triphosphosphate [UTP], and 2.5 mM N-methyl pseudouridine), T7 polymerase mix (2 μL), and T7 buffer mix (2 μL) was kept at 37°C for 2–3 h. The mRNA product was purified by LiCl precipitation, followed by 70% ethanol washes. The modified IVT mRNA was then 5'-capped using the vaccinia-virus-capping system (NEB). After purification by LiCl precipitation, the 5'-cap-modified IVT mRNA was stored at –20°C. For OVA mRNA, the region encoding 241–339 amino acids was chosen, which contains the MHC I- and MHC II-restricted epitopes of OVA antigen.

TEM of Liposomes and Nanocomplex

Liposomes alone or the mRNA-InstantFECT nanocomplex were adsorbed on copper grids and negatively stained with uranyl acetate.

Figure 6. Modified OVA-mRNA InstantFECT Nanocomplex Mediated Immunotherapy for B16-OVA Melanoma

(A) Timeline of vaccination with OVA mRNA-InstantFECT nanocomplex. (B) Interferon γ ELISPOT assay with splenocytes from mice received s.c. immunizations for T cell response against stimulant of OVA antigen and MHC I-restricted OVA peptide epitope. (C) Quantification of ELISPOT results for T cell response against stimulant of OVA antigen and MHC I-restricted OVA peptide epitope. (D) Timeline for tumor immunotherapy using therapeutic vaccination with OVA-mRNA nanocomplex for B16-OVA melanoma. (E) Tumor growth of B16-OVA from therapy and the control groups. (F) Survival curve for the OVA mRNA-InstantFECT nanocomplex vaccinated and control groups (n = 5; control, InstantFECT only). (G) Timeline for prophylactic vaccination with OVA mRNA-InstantFECT nanocomplex. (H) Tumor growth of B16-OVA from animals that received prophylactic vaccination prior to tumor implantation and InstantFECT-only control group. (I) Survival curve of mice bearing B16-OVA tumor in OVA-mRNA-vaccinated mice and the control mice (n = 5; control, InstantFECT only).

The samples were examined with the help from the electron microscopy unit of HKU.

HEK293 Transfection

Cells were cultured in complete DMEM media with 10% FBS (fetal bovine serum) and 1% PS (penicillin-streptomycin) with normal passages. On the day of transfection, a 100-mm cell culture dish of 293 cells with ~70% confluency was first rinsed once with 0.9% NaCl, and the excess of 0.9% NaCl solution was removed. It was then rinsed once with 1× trypsin-EDTA, and the excess of 1× trypsin-EDTA was quickly removed. The cells were returned to the incubator and set for 3 min at 37°C. A cell suspension was made in completed DMEM media with 10% FBS.

Transfection was conducted in a 96-well plate with a simplified “co-seeding” protocol, a range of liposome (1–5 µL) was added to a set of wells, and 50 µL of DMEM media without FBS was added to each well to dilute InstantFECT liposome. 50 µL of serum-free medium containing 500 ng of EGFP mRNA was added to each well with liposome-media solution and then mixed several times with a multi-channel pipet to form the mRNA nanocomplex. 100 µL of HEK293 suspension in DMEM media with 10% FBS was added per well and then mixed. The cells were then incubated at 37°C with 5% CO₂. 24, 48, and 72 h after transfection, cells are observed under a fluorescence microscope, and then a cell suspension was prepared for flow cytometry analysis.

Flow Cytometry Analysis

24, 48, and 72 h after transfection, cells in 96-well plate in triplicates were washed with PBS and trypsinized. The replicates of each sampling group were pooled together after trypsinization. They were centrifuged and then resuspended in 1% BSA in PBS and analyzed by flow cytometry (Canto II analyzer, BD Biosciences).

For the BMDC maturation study, the cells were washed with FACS buffer after collection and stained with CD11c-allophycocyanin (APC), CD86-phycoerythrin (PE), and MHC II-fluorescein isothiocyanate (FITC) antibodies (BioLegend) diluted in FACS buffer. The cells were then stained for 20–30 min at 4°C, protected from the light, washed with fluorescence-activated cell sorting (FACS) buffer before the final resuspension and flow analysis.

XTT Assay

24, 48, and 72 h after transfection, 50 µL of 300 µg/mL XTT solution (Sigma-Aldrich) was added to each of the wells and incubated for 4–5 h at 37°C with 5% CO₂. 5 mL of the labeling reagent and 100 µL of the electron coupling reagent were freshly mixed, from which 50 µL of the mixture was added to each well of the 96-well plate. The cell viability was calculated from absorbance at optical density 450 nm.

TCA Precipitation and Western Blot

Cells were transfected with AdSA or OVA mRNA and InstantFECT. 24 h later, the supernatant from cell culture was collected, and 0.4 vol-

ume of 100% TCA was added and incubated for 15–24 h at 4°C and then centrifuged (4,000 rpm, 15 min, 4°C) to precipitate proteins in the supernatant. The precipitates were resuspended in PBS. The cells were lysed at 4°C with RIPA (Radioimmunoprecipitation assay) buffer (140 mM NaCl, 25 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1% Triton X-100, 0.1% SDS, 0.1% protease inhibitors cocktail, MilliQ water, and pH adjusted to 7.4) for 1 h with shaking and centrifuged (15,000 rpm, 15 min, 4°C); the cytosol fraction was collected and stored. The samples of supernatant and cytosol fraction were separated on a SDS-PAGE gel (12%) and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was then blocked in 5% milk in TBST (Tris-buffered saline, 0.1% Tween 20) for 1 h and incubated with rabbit primary anti-FLAG antibody (in 5% milk TBST) at 4°C on a shaker overnight. The next day, the membrane was washed four times with TBST and was incubated with anti-rabbit immunoglobulin G-horseradish peroxidase (IgG-HRP) (in 5% milk TBST) for 1 h at room temperature (RT) on a shaker. It was again washed five times with TBST and then developed with the Immobilon western chemiluminescent HRP substrate (Merck).

Animal Models

BALB/c and C57/BL-6J mice (5–6 weeks old) were provided by the Laboratory Animal Unit of HKU and were kept with free access of water and food according to animal Ethics committee, CULATR (Committee on the Use of Live Animals in Teaching and Research) rules. The B16-OVA cell line was kindly provided by Prof. Li Quan of the Chinese University of Hong Kong (CUHK). B16-OVA cells (2×10^5 in 100 µL) were injected into the right flank of C57/BL6-J mice. Tumors started to appear after ~17 days. If untreated, the tumors grew to >300 mm³ by day 20, and ulcers with bleeding occurred in high frequency in the next 6–7 days, and the experiment had to be terminated. For the 4T-1 tumor model, 5×10^4 cells in 100 µL were injected s.c. to BALB/c mice. Tumors were palpable by day 10, and tumors got ulcerated in the next 10 days. 4T-1 cell line was originally derived from 410.4 mammary tumour of MMTV+ BALB/c mice fostering on C3H mice (BALB/BfC3H). 4T-1 is resistant to 6-thioguanine. The cells were obtained from lab stock.

Local and Systemic Administration of mRNA-Liposome Complex

5 µg of luciferase mRNA was mixed with 70 µL of DMEM media (without FBS and PS), and 20 µL of 150 mM NaCl was added, followed by 4 µL of InstantFECT liposome. This mixture was vortexed for 5–10 s and injected i.m. into mice within 10–15 min after the preparation. Each muscle was injected with 50 µL of the mixture.

For i.v. injection with Mirus Trans-IT, 5 µg of luciferase mRNA was mixed with 70 µL of DMEM media (without FBS and PS), and 20 µL of 150 mM NaCl was added, followed by 3 µL of Trans-IT mRNA boost solution and 2 µL of Trans-IT mRNA reagent. This mixture was vortexed for 5–10 s and injected i.v. into mice within 10–15 min after the preparation.

For intratumoral delivery, 5 µg of luciferase mRNA was mixed with 70 µL of DMEM media (without FBS and PS), and 20 µL of 150 mM NaCl was added, followed by 4 µL of InstantFECT liposome. This mixture was vortexed for 5–10 s and injected intratumorally to mice within 10–15 min after the preparation.

In Vivo Imaging

24, 48, and 72 h after injection, mice were anaesthetized with ketamine and dopamine anesthetic mix (as recommended by the CULATR, 25:1 ratio), and 100 µL of luciferin substrate (30 mg/mL, Gold Biotechnology) was injected intraperitoneally into the mice. Within 5 min of substrate delivery, mice were viewed under the *in vivo* imager (IVIS SPECTRUM) and luciferase signals were detected.

Cryo-sectioning

Tissues were harvested and fixed overnight at 4°C with gentle shaking in 4% formaldehyde freshly prepared from polyformaldehyde. The next day, the tissue blocks were washed three times for 5 min each with PBS then transferred to 30% sucrose-PBS cryo-protectant solution, kept at 4°C with gentle shaking. The sucrose medium was changed after 24 h and kept overnight at 4°C with gentle shaking. The tissue blocks were embedded in OCT-optimal cutting temperature compound (Tissue-Tek) at –20°C. Cryostat sections with at 15–20 µm in thickness were made and mounted on gelatin-coated slides, air-dried at RT for 30 min, then mounted with ProLong anti-fade mounting media (Thermo Fisher) and visualized using a fluorescence microscope under FITC excitation spectrum (450–495 nm).

Detailed mRNA Vaccination Procedure

For AdSA mRNA vaccination, 10 µg of AdSA mRNA was diluted with serum-free medium, to which 20 µL of 0.9% NaCl was added, and 4 µL of InstantFECT was added into the solution. The final mixture was pipetted several times and was injected as soon as possible into the mice. Here, 5- to 6-week-old female BALB/c mice were injected with a total of 100 µL (2 × 50 µL) of the mRNA-InstantFECT nanocomplex via i.m. (on the right and left thigh muscle) or s.c. (on each of the right and left flank) routes. The injections were done on days 0, 3, 8, 16, and 22. On day 28, spleen cells were collected for ELISPOT assay.

Similar nanocomplexes were prepared with OVA mRNA (8 µg) and InstantFECT (4 µL); 50 µL of the mixture was delivered s.c. to each mouse on right and left flank. Here, 5- to 6-week-old female C57BL/6J mice in groups of five were used. The timeline for performing the ELISPOT assay was also the same as AdSA mRNA vaccination. For prophylactic vaccination, mice received five s.c. injections of the OVA mRNA-InstantFECT nanocomplex starting from day 1. The mice were challenged with 2 × 10⁵ B16-OVA tumor cells on day 27, and tumor growth was monitored. The control group received the same amount of InstantFECT injections by s.c. delivery. For therapeutic vaccination, on day 0 mice were injected with 2 × 10⁵ B16-OVA tumor cells and the OVA mRNA-InstantFECT nanocomplex by s.c. on days 3, 7, 10, and 17, and the tumor size was measured till the end point.

Interferon γ ELISPOT Assay

Six 5- to 6-week-old BALB/c were injected i.m. and s.c. with either AdSA or OVA mRNA on days 0, 3, 8, 16, and 22. On day 28, mice were sacrificed, and spleen cells were collected from each of them. 100 µL of the spleen cells (5 × 10⁴) were then incubated in the 96-well interferon γ ELISPOT plate (R&D Systems), which was hydrated 30 min prior to the experiment with 200 µL of DMEM medium without FBS. To these, 0.5–2 µg of the corresponding MHC I-restricted peptide for AdSA, OVA, or the positive inducer ionomycin (10 µL/well) were added. The cells were incubated for 20 h at 37°C with 5% CO₂. The media was discarded and wells were washed with the washing buffer, followed by incubation with the anti-interferon γ biotinylated antibody for 1 h at RT. After washing, it was incubated with streptavidin-HRP followed by washing and the addition of HRP substrates, incubated for 30 min for color to develop, then it was washed and dried.

BMDC Collection

Femurs and tibiae of 5- to 6-week-old mice were collected and isolated from the surrounding muscle mass. Intact bones were kept in PBS for 4–5 min, and then the ends of the bones were cut off with scissors, and bone marrow was flushed out with PBS using a 17G–20G needle. After washing and red blood cells (RBC) lysis, cells were collected and seeded in a 10-cm Petri dish with DC medium containing RPMI-1640 (Gibco) with 1% PS antibiotic (Gibco), 2-mercaptoethanol (50 µM), and 10% heat-inactivated FBS, supplemented with 20 ng/mL GM-CSF (granulocyte monocyte colony stimulating factors) (PepRotech). The medium was changed every 2 days. On day 7, only the floating cells (immature cells) were collected and transfected with the mRNA-InstantFECT complex. After 18–24 h of transfection, all cells were collected and processed for flow cytometry.

All the graphs and statistical representations were done by using GraphPad Prism. Results were presented as mean ± SD or mean ± SEM. One-way ANOVA test was used when both time and treatment were considered. Survival rate was analyzed with the log rank test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2019.12.044>.

AUTHOR CONTRIBUTIONS

S.A. did most of the experiments and wrote the paper. Q.L., and N.Z. provided help with the experiments and editing the paper. X.G. and J.-D.H. designed and directed the project and wrote the paper.

CONFLICTS OF INTEREST

X.G. co-invented InstantFECT transfection technology and has interests of marketing this reagent. The rest of authors declare no competing interests.

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