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Non-linear enhancement of mRNA delivery efficiencies by influenza A derived NS1 protein engendering host gene inhibition property

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ABSTRACT

Nucleic acid induced immunogenicity remains a significant impediment in biomedical therapeutics because the innate immune system is a complex network overlaid with functional redundancies. Herein we report that non-structural protein 1 (NS1), an immune evasion protein derived from influenza A virus, when co-delivered in mRNA format is a potent mRNA transfection enhancer without toxicity. Transfection enhancement is mediated by NS1's effector domain through inhibition of IRF3 and PKR, activators of early anti-viral responses as well as CPSF30, a non immunostimulating protein. Importantly, host gene inhibition mediated via CPSF30 inhibition is a highly effective immune evasion mechanism because it blocks de novo gene expression non-specifically and inhibits global anti-viral responses during mRNA transfection. We show that only NS1 with CPSF30 inhibition property can enhance modified mRNA transfections. Furthermore, transfection efficiency of unmodified mRNA, if co-delivered with NS1-TX91 mRNA, can exceed that of modified mRNA in HepG2, RAW 264.7 and HeLa cells. The novel impact of NS1-TX91 lays the foundation of a virus inspired immune evasion genes co-delivery approach that can address problems arising from RNA immunogenicity for non-vaccine mRNA therapeutics in an affordable and scalable way. It is also transferable to applications that benefits from active inhibition of material-induced immunogenicity.

1. Introduction

Material induced immunogenicity is a well-known delivery barrier in the biomedical field as it generates undesirable side effects that reduce therapeutic efficacy and biocompatibility. This problem is more significant for nucleic acid therapeutics because of complex immunological safeguards that prevent the expression of exogenous nucleic acid. For example, *in vitro* transcribed mRNA is recognized by the cell's pattern recognition receptors (PRR) leading to a limited number of early interferon (IFN) independent anti-viral responses [1], one of which includes the production of IFN [2]. IFN then triggers a full range of anti-viral responses which include the upregulation of protein kinase RNA activated (PKR) [3] and 2′,5'-oligoadenylate synthetase (OAS) [4] responsible for translation inhibition and enhanced mRNA degradation, respectively. Recognizing the adverse impact of anti-viral responses, delivery strategies are targeted at minimizing PRR engagements in an effort to reduce IFN production. For example, capped mRNAs synthesized using expensive modified nucleotides reduce activation of cytoplasmic PRRs such as retinoic acid-inducible gene I (RIG-I), PKR and OAS [3,5]. HPLC purification removes short RNA fragments, which are efficient activators of endosomal PRRs such as TLR3. Hence, an ideal "de-immunized" mRNA will require modified mRNA synthesis followed by HPLC purification, which is expensive and difficult to scale up. Hence, a gap remains in this area as we continue to discover alternative approaches that can address these early anti-viral responses in an affordable and practical way.

In this study, we demonstrate a virus inspired delivery approach that could address this gap. It entails co-delivery of mRNA encoding an immune evasion gene that engenders a potent immune evasion mechanism that blocks global anti-viral responses which include but are not limited to those activated by the above mentioned PRRs. We first show that non-structural protein 1 (NS1) derived from influenza A virus (IAV) enhances mRNA transfection by antagonizing interferon production. Through gain and loss of function amino acid substitutions, we determined that transfection

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enhancement is mediated by NS1's effector domain through inhibition of interferon regulatory factor 3 (IRF3), protein kinase RNA activated (PKR) and cleavage and polyadenylation factor subunit 30kda (CPSF30). In particular, we illustrate that host gene inhibition mediated by NS1's inhibition of CPSF30 partially blocks de novo gene expression, and is unusually effective for *in vitro* mRNA transfection enhancement because only NS1 engendering the CPSF30 inhibition property can further enhance modified mRNA transfections. In addition, we also show that transfection performance of unmodified mRNA can exceed that of modified mRNA in HepG2, RAW 264.7 and HeLa cells if NS1 mRNA is co-delivered.

2. Materials and methods

2.1. Cells and reagents

BJ fibroblasts, HepG2, HeLa, RAW264.7 and Vero cells were purchased from the American Type Culture Center (ATCC) and cultured in DMEM growth medium supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. Primary human keratinocytes and melanocytes were gifts from Dr. Carlos Clavel from A*STAR Institute of Medical Biology. BJ ISRE reporter cells were generated by transducing BJ fibroblasts with lentivirus containing pGreenfire-ISRE-GFP/luciferase gene (Systems Bioscience). Transduced cells were selected with 600 μg/ml geneticin for 7 days. Primary keratinocytes were cultured in DermaLife K Medium Complete Kit (Axil Scientific) while primary melanocytes were cultured in Medium 253 supplemented with Human Melanocyte Growth Supplement-2 (Thermoscientific). Resazurin stock solution was prepared by dissolving 1 g of resazurin sodium salt (MP Biomedicals) in 100 mL sterile PBS and filtered through a 0.22 μm filter. Resazurin working solution (Alamar Blue reagent) was freshly prepared prior to each experiment by diluting stock solution with growth medium at a ratio of 1:250. DMEM with high glucose, penicillin-streptomycin 100 × solution, fetal bovine serum (FBS) and trypsin-EDTA (10× solution) were purchased from Hyclone, GE Healthcare Life Sciences. Stemfect mRNA transfection reagent (Cat# 00-0069) was purchased from Stemgent and used according to manufacturer's protocol. Steady-GLO luciferase reagent and GLO lysis buffer were purchased from Promega.

2.2. Cloning of NS1 genes

Plasmids containing CAAGS promoter and open reading frame of H5N1 subtype A/Hong Kong/156/1997 (HK97; representative H5N1 before 2004); A/Vietnam/1203/2004 (VN04; representative H5N1 after 2004); H1N1 subtype A/Puerto Rico/8/1934 (PR8; lab adapted H1N1 model virus); A/Texas/36/1991 (TX91; human seasonal-like H1N1 virus before 2009); A/California/04/2009 (CAL; human seasonal-like H1N1 after pandemic outbreak in 2009); A/Shanghai/2013 (SH1; chicken H7N9 during 2013 bird flu outbreak in China); PR8 mutant PR8R38AK41A, c-terminal truncated PR8(1-73), SH1 mutant S103FI106M, were gifts from Aldofo Garcia-Sastre reported in previous publications [6,7]. PR8 mutants PR8S103FI106M and PR8I123A124A were synthesized as g-blocks from IDT DNA. The ORF of NS1 plasmids were amplified by PCR (primers 5′ GGG GCGGCCGC TCAAACTTCTGACCTAATT 3′ and 5′ GGG GTCGAC GCCACC ATGGATCCAAACACTGT 3') and cloned between SaI-I and Not-I sites of pGEM4Z-A64 vector containing a T7 promoter, poly(A) tail (A64) and does not contain any enhancement sequences (e.g. β-globin sequences) within mRNA's untranslated regions (3′ and 5′ UTRs). Cloning was verified by restriction digestion and sequencing.

2.3. In vitro transcription

In vitro transcription was performed as previously described [8,9]. Briefly, plasmid containing a T7 promoter and polyA tail (64 residues) was linearized and used as template for *in vitro* transcription in the presence of anti-reverse cap analogue (ARCA, NEB) according to manufacturer's protocol with a capping efficiency of ~80% (based on 4:1 ratio of ARCA cap to GTP). Where applicable, pseudouridine triphosphate and/or 5-methylcytidine triphosphate (Trilink Biotech) completely replaced uridine triphosphate (UTP) and cytidine triphosphates (CTP). IVT mRNA was purified with RNEasy kit (Qiagen), quantified by spectrophotometry and analyzed by agarose gel electrophoresis to confirm the synthesis of full-length mRNA. *In vitro* transcription kits: Ψ+m5C double modified mRNA was synthesized using Ambion's MEGAscript T7 Transcription Kit (AM1333), while Ψ - single modified and unmodified mRNAs were synthesized using NEB's T7 High Yield RNA Synthesis kit (E2040S) according to manufacturer's protocol.

2.4. In vitro transfection

All transfections in this study was performed using Stemfect mRNA transfection reagent according to manufacturer's protocol. BJ cells were seeded at a density of 3×10^4 cells/well on 48-well plates. For NS1 screening, cells were transfected with 100 ng of NS1 mRNA and 100 ng luciferase mRNA per well. Cells were incubated for 18 h before analyzing for cell viability and luciferase expression. Experiments were independently repeated 6 times with the same outcome.

For all zero-sum dosing transfections, cells seeded on 48 well plates were transfected with 120ng/well of total mRNA containing different mass ratios of NS1 mRNA or GFP mRNA (as control) and luciferase mRNA. Cells were incubated for 18 h before analyzing for cell viability and luciferase expression. All experiments were independently repeated 4-8 times with the same outcome.

For host gene inhibition study, cells seeded on 48 well plates were pre-transfected with 100ng/well of ψ-modified mRNA encoding NS1 (PR8, PR8S, TX91) or ψ-modified luciferase mRNA (as control) or both at a mass ratio of 1:1. After 4 h of incubation time, media was aspirated and cells were washed once with PBS before transfecting them with 100ng/well of GFP plasmid (pGFP). GFP expression was assayed by flow cytometry after 40 h post addition of pGFP nanoparticles. Gating was applied to GFP + cells and respective mean fluorescence intensities were normalized to that of non-transfected cells. Transfection efficiency of initial mRNA transfection based on %GFP + cells (transfected for the first time) is ~90–98% (Supplementary Fig. 4A). Transfection efficiency of follow up pGFP transfection based on %GFP + cells (performed 4 h after initial mRNA transfection) is ~20% (Supplementary Fig. 4B). All experiments were independently repeated 3 times with the same outcome.

For primary cells transfection, 1.2×10^4 keratinocytes or melanocytes were seeded overnight on 96 well plates. Cells were transfected with 200 ng of ψ-modified luciferase mRNA and NS1-TX91 mRNA at ratios 1:0 and 4:1. Cells were incubated for 18 h before analyzing for cell viability and luciferase expression. All experiments were independently repeated twice with the same outcome.

For comparison of transfection efficiency between unmodified and modified formats, HepG2 and BJ fibroblasts were seeded overnight on 96 well plates at ~1.2×10^4 cells per well. Cells were transfected with unmodified (UM) NS1-TX91 mRNA + unmodified luciferase mRNA (mass ratio 1:1) and modified GFP mRNA + modified luciferase (mass ratio 1:1, both ψ- (SM) and ψ/m5C- (DM) formats). Cells were incubated for 18 h before analyzing