Min-Immune™ Gold-Mediated dsRNA Depletion Restores Expression and Viability of VEE Self-Amplifying RNA

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Introduction

Self-amplifying RNAs (saRNAs) are engineered derivatives of positive-sense single-stranded RNA viruses in which viral structural protein-coding sequences are replaced with a gene of interest, while nonstructural proteins required for RNA replication including the viral RNA-dependent RNA polymerase (RdRP) are retained. Following delivery, saRNAs undergo intracellular replication, amplifying the RNA template and enabling sustained transgene expression from low input doses[1]. These properties have established saRNAs as a promising platform for vaccines and gene-based therapeutics. Kostaive is now an approved COVID 19 vaccine that uses self-amplifying mRNA to enhance antigen expression, exemplifying the promise of saRNA technology for vaccines and gene-based therapeutics.

A major limitation of saRNA systems is activation of host innate immune responses. *In vitro* transcribed saRNA generates double-stranded RNA (dsRNA) intermediates and other pathogen-associated molecular patterns that are recognized by pattern recognition receptors, including Toll-like receptors (TLR3, TLR7 and TLR8), MDA5, RIG-I, PKR and the OAS pathway[2]. Activation of these pathways induces type I interferon (IFN) signaling and antiviral programs that reduce RNA stability and translation, limiting transgene expression[3, 4]. While beneficial for vaccines, excessive IFN responses can be detrimental in other applications where sustained expression is needed.

Strategies to mitigate innate immune sensing without impairing replication remain limited. Unlike non-replicating mRNA, incorporation of modified nucleotides (e.g., pseudouridine or N1-methyl-pseudouridine) is generally incompatible with efficient saRNA function, as these modifications can impair RdRP activity[5]. Consequently, efforts have focused on minimizing dsRNA contaminants generated during *in vitro* transcription.

Here, we evaluate the impact of dsRNA removal on saRNA performance using the Min-Immune™ Gold purification module. A 9-kb capped and polyadenylated saRNA encoding green fluorescent protein (GFP) was used as a model construct. RNA integrity was assessed by denaturing agarose gel electrophoresis, and functional output was quantified by GFP expression following transfection into cultured cells. Activation of dsRNA-responsive innate immune pathways was measured by RT-qPCR. Together, these studies define the extent to which dsRNA depletion enhances saRNA expression while attenuating innate immune activation.

Methods and Results

saRNA Template and *In Vitro* Transcription

A plasmid encoding a Venezuelan equine encephalitis (VEE) virus-based self-amplifying RNA expressing green fluorescent protein (GFP) (T7-VEE-GFP; NovoPro Bioscience[6], was used as the transcription template. The 11.5-kb plasmid was linearized with *Mlu* I (New England Biolabs) and purified by phenol-chloroform extraction followed by salt/EtOH precipitation. Linearized DNA was used for *in vitro* transcription with the T7 mScript™ Complete Standard mRNA Production System (CELLSCRIPT™) according to the manufacturer's protocol, yielding a 9,696-nt saRNA containing a Cap 1 structure and poly(A) tail.

Double-stranded RNA contaminants were removed using the Min-Immune™ Gold dsRNA Removal module of T7 mScript Complete Standard mRNA Production system according to the manufacturer's instructions. Purified saRNA was subsequently analyzed for integrity.

Cell Culture and Transfection

HEK293 cells were maintained in complete growth medium at 37°C with 5% CO_2. Cells were seeded at a density of 8×10^5 cells per well in 6 well plates, 24 h prior to transfection. For each well, 1 µg saRNA was transfected using 2.5 µL Lipofectamine™ MessengerMAX™ (Thermo Fisher Scientific) diluted in 100 µL Opti-MEM™ I Reduced Serum Medium, following the manufacturer's protocol.

GFP expression was assessed 48 h post-transfection. Fluorescence images were acquired using identical exposure settings across all conditions.

Innate Immune Activation Assay

THP-1 cells were used to assess activation of dsRNA-responsive innate immune pathways. Cells were transfected with 2 µg saRNA using 3.5 µL Lipofectamine™ MessengerMAX™. At 28 h post-transfection, cells were harvested, washed and total RNA was isolated using the MasterPure™ Complete DNA and RNA Purification Kit (Biosearch Technologies).

For gene expression analysis, 25 ng RNA per reaction was analyzed by one-step RT-qPCR using the iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad) on a CFX96 real-time PCR system (Bio-Rad). Twelve primer sets targeting dsRNA-responsive genes were used. Reactions were performed in technical replicates. Gene expression was normalized to GAPDH and calculated relative to mock-transfected controls using the ΔΔCq method.

Figure 1. The Min-Immune™ enzymatic removal of dsRNA clips some of the capped and tailed 10 kb transcript. Shortened Min-Immune™ Gold treatment times, or reaction times, or treatment incubation times also result in some clipping of this saRNA with more of the full length saRNA remains at 30- and 15-minutes.

Lane M, RNA Millennium™ Markers, **Lane 1**, VEE-GFP saRNA, **Lane 2**, VEE-GFP saRNA after the standard 60-minute Min-Immune™ reaction, **Lane 3**, 30-minute reaction, **Lane 4**, 15-minute reaction.

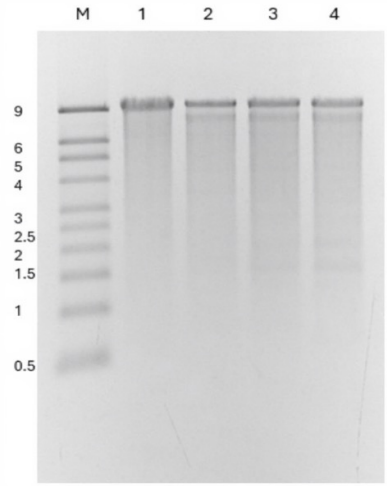


Figure 2. Fluorescence and corresponding phase contrast images of HEK293 cells 48 h post transfection with VEE-GFP self-amplifying RNA (saRNA). dsRNA removal improves saRNA mediated GFP expression and cell viability in HEK293 cells.

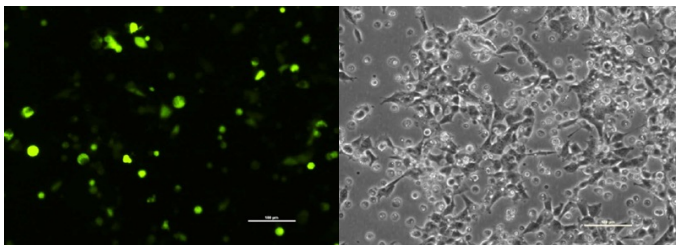


Figure 2A. Untreated saRNA shows GFP expression but is associated with pronounced cytotoxicity and disrupted cell morphology.

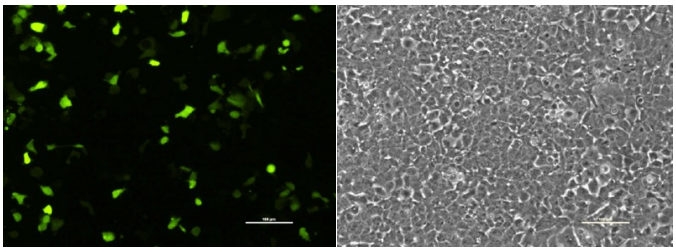


Figure 2C. HEK293 cells transfected with VEE-GFP saRNA treated for 15-minutes similarly exhibit strong GFP expression and maintain healthy cell morphology, indicating that short dsRNA removal treatment is sufficient.

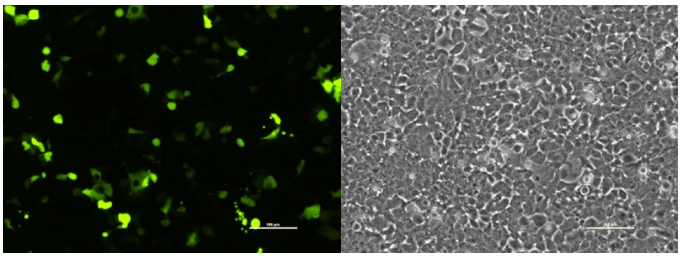


Figure 2B. HEK293 cells transfected with VEE-GFP saRNA subjected to a 60-minute dsRNA removal treatment display robust GFP expression with healthy, confluent cell morphology.

Figure 3. Fluorescence images of THP-1 monocytes 28 h post transfection with VEE-GFP self-amplifying RNA (saRNA). **dsRNA removal enhances saRNA mediated GFP expression in THP-1 monocytes.**

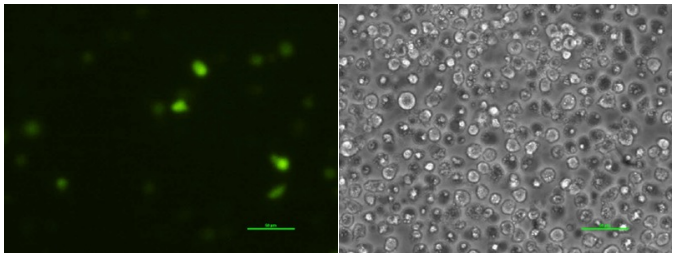


Figure 3A. THP-1 monocytes transfected with untreated VEE- GFP self amplifying RNA (saRNA) show very weak GFP expression at 28 h post transfection.

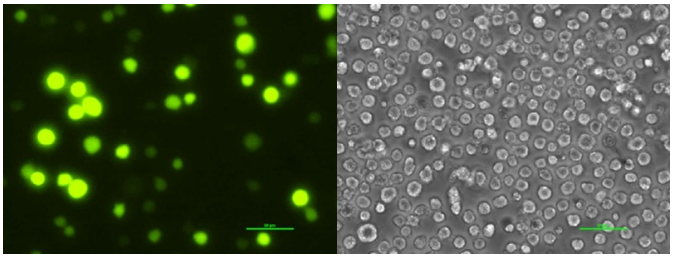


Figure 3B. THP-1 monocytes transfected with VEE-GFP saRNA subjected to a 60-minute dsRNA removal treatment exhibit increased GFP expression with preserved cell morphology.

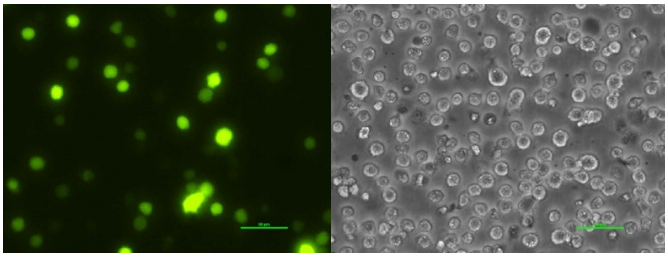


Figure 3C. THP-1 monocytes transfected with VEE-GFP saRNA treated for 15-minutes also show enhanced GFP expression compared to untreated saRNA, indicating that short dsRNA removal treatment is sufficient.

RT-qPCR

To evaluate innate immune activation, THP-1 monocytes were transfected with untreated or dsRNA-depleted VEE-GFP saRNA (15-minute or 60-minute treatment). THP-1 cells were chosen for their robust innate immune response and full complement of Toll-like receptors. At 28 hours post-transfection, total RNA was isolated and expression of twelve dsRNA-responsive genes — RIG-I, MDA5, OAS1, TLR3, TLR7, TLR8, TNF, PKR, NLRP3, IFNB, and IL-6 — was quantified by one-step RT-qPCR. Expression was normalized to GAPDH and reported relative to mock-transfected controls (ΔΔCq method).

Untreated saRNA induced strong upregulation of multiple dsRNA sensors and innate immune effectors, with TLR3, TLR7, TLR8, and TNF showing the most pronounced responses (Fig. 4A). Both 15-minute and 60-minute dsRNA removal treatments reduced expression of these markers to near-baseline levels, comparable to mock controls.

IFNB and IL-6 responded with substantially greater magnitude than the other measured targets and are shown separately (Fig. 4B). Untreated saRNA induced ~50-fold and >120-fold relative expression, respectively. Both dsRNA removal treatments suppressed this response to near-baseline levels — indicating effective abrogation of type I interferon and inflammatory cytokine signaling regardless of treatment duration.

Figure 4A. RT qPCR analysis of innate immune gene expression in THP-1 cells following VEE-GFP transfection with or without dsRNA removal.

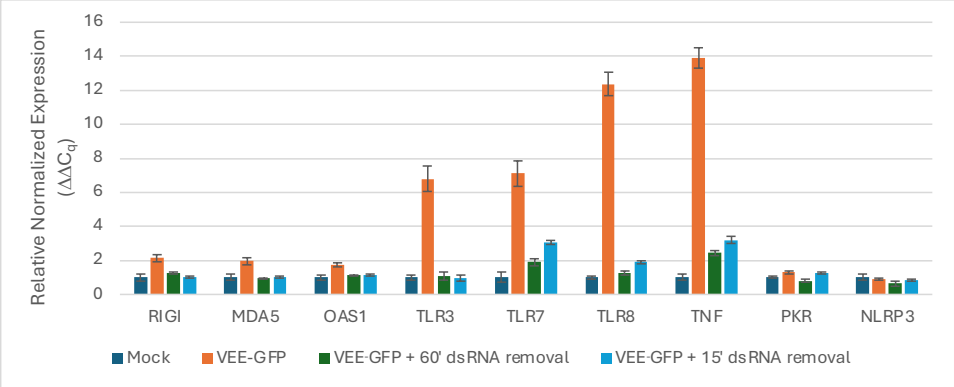
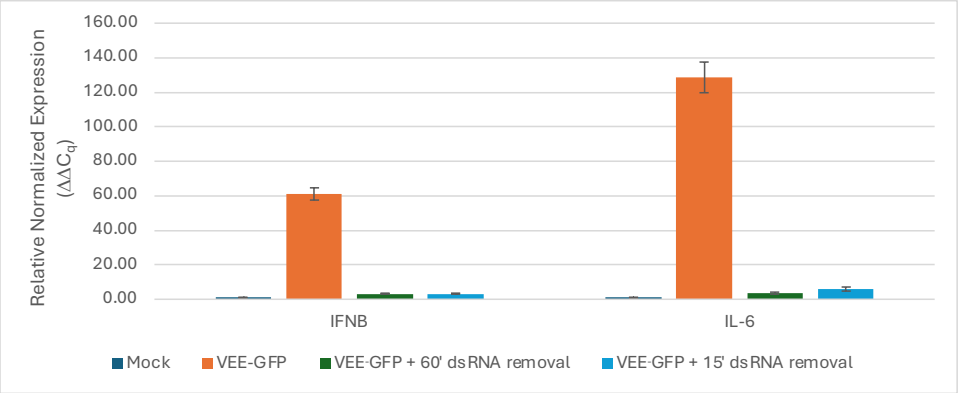


Figure 4B. RT qPCR analysis of IFNB and IL-6 expression following VEE-GFP transfection with or without dsRNA removal.



Conclusion

These data demonstrate that dsRNA contaminants are the primary drivers of innate immune activation and impaired expression following VEE-GFP self-amplifying RNA (saRNA) transfection. In THP-1 monocytes, untreated saRNA elicits strong innate immune responses and negligible GFP expression, while dsRNA removal restores immune gene expression to near baseline levels. Both 15-minute and 60-minute treatments are sufficient to achieve this effect, indicating rapid and effective depletion of immunostimulatory dsRNA species.

Consistent with reduced immune activation, dsRNA depleted saRNA shows improved functional performance, including enhanced GFP expression and preserved viability in HEK293 cells and restored expression in THP-1 monocytes. Min-Immune™ Gold efficiently eliminates dsRNA by-products from saRNA transcripts, mitigating dsRNA mediated immune responses and improving saRNA activity. Although minor transcript clipping is observed following Min-Immune™ Gold treatment overall gains in immune compatibility and expression efficiency represent a clear net benefit, supporting Min-Immune™ Gold as a robust approach for saRNA manufacturing.

Key Findings:

- Untreated VEE-GFP saRNA induced strong upregulation of dsRNA sensors and cytokines in THP-1 cells.
- IFNB and IL-6 hyper-induction was nearly eliminated by dsRNA removal.
- Both 15- and 60-minute dsRNA-removal treatments sharply reduced innate immune gene expression to near-baseline.
- dsRNA impurities are the primary drivers of saRNA-triggered innate immune activation.

References

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