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PSEUDOURIDINE AND N1-METHYLPSEUDOURIDINE IN mRNA VACCINES MODULATE RETINOIC ACID INDUCIBLE GENE I (RIG-I) AND TOLL-LIKE RECEPTORS (TLR) ACTIVATION

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Abstract

Background: The advent of mRNA vaccines has underscored the significance of nucleoside modifications in regulating innate immune recognition. Pseudouridine (Ψ) and its derivative, N1-methylpseudouridine (m1Ψ), are widely used to enhance the efficacy and safety of mRNA vaccines**.**

Objective: This review examines how Ψ and m1Ψ alter the activation of key innate immune sensors, including RIG-I, MDA5, and endosomal Toll-like receptors (TLR3, TLR7, TLR8).

Methods: A narrative review was conducted using PubMed, Scopus, and Web of Science databases, focusing on studies evaluating the molecular and immunological effects of Ψ and m¹Ψ on innate immune sensors.

Discussion: Incorporation of Ψ or m1Ψ in mRNA markedly suppresses activation of TLR3, TLR7, and TLR8. Ψ-containing RNA avoids detection by TLR7/8 via two mechanisms: it resists endosomal nuclease digestion into immunostimulatory fragments and is poorly recognized by the TLR7/8 ligand-binding sites. m1Ψ similarly evades nuclease processing yet, unlike Ψ, can directly activate TLR8. In the cytosol, Ψ/m1Ψ modifications strongly reduce RIG-I signaling without impeding MDA5.

Conclusions: By limiting innate PRR activation, these modifications increase the translation and stability of mRNA vaccines while reducing inflammatory interferon responses. This immune evasion is crucial for the high efficacy and tolerability of current mRNA vaccines; however, a trade-off exists between minimizing reactogenicity and the adjuvant benefits of innate stimulation.

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INTRODUCTION

Messenger RNA vaccines have become an essential platform for infectious disease prevention. A central obstacle in the development of mRNA therapeutics is the innate immune system's tendency to recognize exogenous RNA as a danger signal (1,2). Unmodified mRNA activates cytosolic receptors such as RIG I and MDA5, as well as endosomal receptors including TLR3, TLR7 and TLR8, which induces type I interferons and pro inflammatory cytokines (3). Although limited innate activation can support adaptive immunity, excessive signalling inhibits translation, reduces antigen production and ultimately diminishes vaccine efficacy (4).

A major advance in the field was the recognition that endogenous RNAs contain numerous nucleoside modifications that attenuate innate immune sensing. In contrast, in vitro transcribed mRNA lacks these modifications and is therefore more immunostimulatory (5). This insight has motivated the incorporation of modified nucleosides into synthetic mRNA. Among these, pseudouridine and N1 methylpseudouridine have become especially important because they preserve coding capacity while reducing activation of RNA sensing pathways. Their widespread use in current mRNA vaccines underscores their biological and translational significance (6).

Despite substantial progress, important gaps remain in understanding how individual nucleoside modifications influence discrete components of the innate immune system (7). Many studies demonstrate that pseudouridine and N1 methylpseudouridine reduce interferon responses and enhance protein expression, yet the specific ways in which these modifications alter recognition by RIG I, MDA5, TLR3, TLR7 and TLR8 are not fully defined. Their effects on other RNA activated pathways, including PKR and OAS, also require clearer mechanistic delineation. (8,9).

This review will discuss in depth how Ψ and m1Ψ modifications influence the detection of mRNA by key innate sensors, RIG-I, MDA5, TLR3, TLR7, and TLR8, and the downstream signaling pathways. We will first summarize the roles of these PRRs in sensing RNA and initiating immune responses. We then examine the molecular mechanisms by which Ψ and m1Ψ modifications alter RNA–PRR interactions, drawing on evidence from cell-based assays, animal studies, and human vaccine data. We also address how these modifications modulate other RNA-activated pathways such as protein kinase R (PKR) and 2′-5′ oligoadenylate synthases (OAS), which affect mRNA stability and translation. Finally, we consider the immunological implications of using modified vs. unmodified mRNA, including the balance between vaccine immunogenicity and reactogenicity, and emerging strategies to optimize mRNA vaccine design.

DISCUSSION

Innate Immune Sensors of RNA: RIG-I, MDA5, and TLRs.

RIG I like receptors and Toll like receptors are the principal pattern recognition receptors that detect intracellular RNA. RIG I recognizes short double stranded RNA with 5′ tri or diphosphate ends or unmethylated caps, whereas MDA5 detects long double stranded RNA regardless of sequence (20,21). RNA binding promotes conformational activation and oligomerization of these receptors, which then signal through MAVS to activate IRF3, IRF7 and NF κB, resulting in type I interferon and pro inflammatory cytokine production (7, 10, 11). Although these sensors provide essential antiviral defense, their

activation during mRNA delivery induces an antiviral state that can suppress translation and reduce therapeutic efficacy.

TLR3, TLR7 and TLR8 detect RNA within endosomes. TLR3 binds double stranded RNA and signals through TRIF to induce IRF3 and NF κB activation [24]. TLR7 and TLR8 recognize single stranded RNA enriched in uridine or GU rich motifs and are predominantly expressed in plasmacytoid dendritic cells, monocytes and macrophages. (7, 12). These receptors require ligand cooperation, in which uridine containing degradation products and short oligonucleotides jointly induce receptor activation. This mechanism ensures that TLR7 and TLR8 respond primarily to processed RNA within endosomes, thereby helping to distinguish self RNA from pathogenic RNA. (7,13).

Unmodified in vitro transcribed mRNA can inadvertently activate these sensors. Incompletely capped transcripts or double stranded byproducts stimulate RIG I, long duplex regions or contaminant dsRNA engage MDA5 and TLR3, and uridine rich RNA fragments that enter endosomes activate TLR7 and TLR8 (1). Such activation leads to rapid interferon responses, cytokine secretion and dendritic cell maturation. Although moderate activation can enhance vaccine responses, excessive signaling suppresses protein synthesis through PKR and other interferon stimulated pathways and can produce systemic inflammation or other adverse effects (13).

These challenges motivated the hypothesis that nucleoside modification could modulate innate sensing. Endogenous RNAs are enriched in modified nucleosides, which reduce TLR and RLR stimulation, whereas unmodified RNAs are strongly immunogenic (14). In vitro transcribed RNA containing pseudouridine, 5 methylcytidine, 2 thiouridine or related modifications elicits markedly reduced cytokine production by human dendritic cells compared with unmodified RNA (7). The following sections examine how pseudouridine and N1 methylpseudouridine enable modified mRNA to evade each major class of innate RNA sensors.

Modulation of Endosomal TLR Activation by Ψ and m1Ψ

Pseudouridine markedly reduces TLR7 and TLR8 mediated sensing of RNA. Unmodified single stranded RNA, particularly uridine rich sequences, is a potent agonist of human TLR7 and TLR8, driving IFN α secretion by plasmacytoid dendritic cells and IL 6 or TNF α production by monocytes (15). Substitution of uridine with pseudouridine strongly suppresses this response. Early studies showed that Ψ containing RNA fails to activate TLR7 or TLR8 in human immune cells, and mouse studies demonstrated that unmodified mRNA is substantially more immunogenic in vivo than Ψ modified mRNA, producing higher cytokine levels and greater immune activation, whereas Ψ modified RNA is better tolerated (5,16). In current vaccines, lipid nanoparticles provide sufficient adjuvant activity, reducing concerns about the lower innate stimulation of Ψ modified mRNA.

Two principal mechanisms underlie the suppression of TLR7/8 signaling by pseudouridine. The first is impaired generation of TLR ligands. Endosomal RNases, including RNase T2 and 3′ exonucleases such as PLD3 and PLD4, normally degrade RNA into uridine containing fragments that activate TLR7 and TLR8 (11). Pseudouridine containing RNA is a poor substrate for these enzymes, preventing the formation of canonical ligands. Mass spectrometry shows that Ψ modified RNA remains largely intact or yields atypical digestion products within endosomes (17), likely due to increased RNA rigidity and altered base presentation. Consistently, RNase T2 knockout cells fail to respond

to unmodified RNA, confirming the necessity of RNase derived fragments, while Ψ modified RNA elicits minimal signaling even in wild type cells (5).

The second mechanism is reduced receptor binding and activation. TLR8 contains distinct binding pockets for a uridine moiety and an RNA oligomer. Biochemical assays show that pseudouridine is a poor agonist for the uridine specific pocket and does not efficiently induce TLR8 dimerization, in contrast to uridine or N1 methylpseudouridine (18). Pseudouridine containing RNA fragments display weak TLR8 agonism unless supported by additional co ligands (18). Similarly, TLR7 does not fully activate when presented with Ψ rich oligonucleotides, even in the presence of the auxiliary ligand cGMP (19,20). These data indicate that pseudouridine disrupts proper ligand recognition, causing TLR7 and TLR8 to interpret the RNA as self like rather than pathogenic.

Together, these mechanisms render pseudouridine modified mRNA largely invisible to TLR7 and TLR8. In human immune cells, Ψ modified in vitro transcribed RNA induces little to no IFN α, IL 6, TNF α or related cytokines, whereas unmodified RNA triggers robust cytokine secretion (18). This immune evasion is central to mRNA vaccine functionality, enabling sustained antigen expression with minimal inflammatory interference. The success of COVID 19 mRNA vaccines has been attributed in significant part to the adoption of pseudouridine, which proved essential for achieving high efficacy with acceptable reactogenicity.

N1-methylpseudouridine and TLR activation

A surprising nuance revealed by recent work is that N1-methyl-Ψ does not fully recapitulate pseudouridine's TLR-evasive behavior. While m1Ψ-modified RNA is similarly resistant to RNase T2/PLD digestion (because it is structurally very similar to Ψ), the methyl group on N1 alters its interaction with TLR8. Notably, m1Ψ retains substantial stimulatory activity for TLR8 – nearly equivalent to uridine in some assays(18,19). In Bérouti's study, m1Ψ could bind in TLR8's uridine-specific pocket and promote receptor dimerization just as an unmodified uridine would. Thus, m1Ψ is not "neglected" by TLR8 in the way pseudouridine is. But in m1Ψ, that N1H is replaced by a methyl, effectively making m1Ψ resemble uridine in terms of hydrogen-bond donors/acceptors on the Watson-Crick face (21).

The observation that m1Ψ can activate TLR8 has implications for vaccine reactogenicity and immune responses. Although m1Ψ enhances mRNA translation and stability, it may still contribute to limited inflammatory signaling through TLR8 (18,19). In current vaccines, this effect is likely modest because efficient delivery minimizes endosomal exposure and the 5′ cap and other regulatory features further dampen sensing (22). Thus, m1Ψ is largely immunosilent but not completely inert, and future mRNA designs may combine Ψ, m1Ψ, or additional modifications to optimize this balance (21).

Nucleoside modifications also influence TLR3, which recognizes double stranded RNA. Karikó et al. showed that pseudouridine containing RNA does not activate TLR3 in human cells, possibly because modified bases alter the stability or conformation of dsRNA regions required for TLR3 engagement. Consistently, certain modifications such as 2 thio U or m6A reduce TLR3 signaling in structured RNAs (1,3). Although pseudouridine was not tested in that study, its effects likely parallel these findings. Nevertheless, TLR3 remains responsive to long, well formed dsRNA, making control of dsRNA contaminants during mRNA production essential; purification methods such as HPLC effectively reduce these

byproducts (15,23). Overall, Ψ and m1Ψ primarily suppress TLR7 and TLR8, with secondary effects on TLR3, thereby limiting excessive endosomal PRR activation during mRNA vaccination (13).

Effects of Modified Nucleosides on RIG-I and MDA5 Recognition

In the cytosol, RIG I is a major barrier to exogenous RNA, as its activation rapidly induces an antiviral state that suppresses mRNA translation. Incorporating pseudouridine or related modifications markedly reduces RIG I responsiveness. RIG I detects 5′ triphosphate RNA and short dsRNA structures, including uncapped transcripts or duplex impurities that may arise during in vitro transcription, as well as short hairpins formed by single stranded mRNA (7,12,24). Modified nucleotides such as Ψ or m1Ψ greatly diminish RIG I signaling by preventing the conformational changes required for activation (25,26).

RIG I may still bind RNA, but alterations in RNA geometry and base stacking interfere with ATP driven translocation and oligomerization. In contrast, MDA5 readily detects long dsRNA even when modified, as its recognition depends on extended duplex length rather than nucleotide identity (4,19,27). As a result, Ψ and m1Ψ suppress RIG I driven interferon induction but cannot block MDA5 activation by long dsRNA contaminants, which must instead be minimized through improved IVT and purification methods (27).

Comparisons of modified and unmodified mRNAs highlight the functional importance of RIG I evasion. Reducing intrinsic sensing through nucleoside and cap modifications increases protein expression and alters adaptive immunity (22,28). Unmodified mRNA produces stronger early interferon responses, which can enhance or reshape T cell immunity. In cancer models, the stronger innate signaling of unmodified mRNA can be beneficial: unmodified mRNA LNPs induce higher IFN α, more potent dendritic cell activation, and improved $CD8^+$ T cell responses, resulting in superior tumor control in mice (10). Blocking type I IFN abrogates this benefit, demonstrating that innate IFN is central to this antitumor effect. Consequently, although modified nucleosides are optimal for minimizing reactogenicity in prophylactic vaccines, unmodified mRNA retains value in therapeutic cancer settings (29).

Pseudouridine also reduces activation of PKR, which phosphorylates eIF2α and halts translation when triggered by dsRNA. Unmodified mRNAs can activate PKR through transient secondary structures or dsRNA byproducts, whereas pseudouridine containing transcripts produce minimal PKR phosphorylation and maintain higher translational output in cells and mice (5,7,16). Similarly, pseudouridine helps mRNA evade the OAS–RNase L pathway. OAS proteins detect dsRNA and generate 2′ 5′ oligoadenylates that activate RNase L, degrading intracellular RNA. Modified mRNA (Ψ, 2 thio U, etc.) induces far less OAS1 activation and RNase L mediated cleavage, thereby improving intracellular mRNA stability (24,25,30). Avoiding OAS activation requires both nucleoside modification and removal of dsRNA contaminants during mRNA production.

In summary, pseudouridine and N1 methylpseudouridine attenuate multiple innate sensing pathways: they suppress TLR7 and TLR8 in endosomes, reduce RIG I detection in the cytosol, and limit activation of PKR and OAS. MDA5 and TLR3 remain responsive to long dsRNA, making careful sequence design and purification essential complements to nucleoside modification for optimizing mRNA vaccine performance (13).

Immunogenicity and Safety Implications for mRNA Vaccine Design

Pseudouridine and N1-methylpseudouridine are central to achieving the balance between immunogenicity and safety in mRNA vaccines. By limiting excessive innate sensing, these modifications enhance mRNA stability and translation, yielding higher antigen expression and stronger adaptive immune responses at a given dose (26). The high efficacy of the first COVID-19 mRNA vaccines, which generated potent neutralizing antibodies and T cell responses with acceptable reactogenicity, relied on such modified mRNAs. In contrast, early unmodified mRNA vaccines, such as CureVac's CVnCoV, showed substantially lower efficacy, likely due to restricted protein expression caused by innate sensing (21). CureVac subsequently adopted m1Ψ for their next-generation vaccine (CV2CoV), paralleling other successful platforms (18), underscoring the importance of nucleoside chemistry in clinical performance.

However, complete suppression of innate signaling is not always optimal. Moderate PRR activation can provide intrinsic adjuvant activity by recruiting and activating antigen presenting cells. For therapeutic cancer vaccines or certain non-pandemic applications, developers are investigating partial uridine substitution or fully unmodified mRNA to amplify local immunostimulant (63). BioNTech, for example, has tested an unmodified mRNA vaccine for colorectal cancer using a lipoplex system designed to enhance tumor site inflammation and T cell priming (22). Unmodified mRNA can elicit stronger type I IFN dependent T cell responses in tumor models, though at the cost of higher reactogenicity and reduced translation efficiency (27). Such tradeoffs may be acceptable in oncology but are less suitable for prophylactic vaccination in healthy individuals.

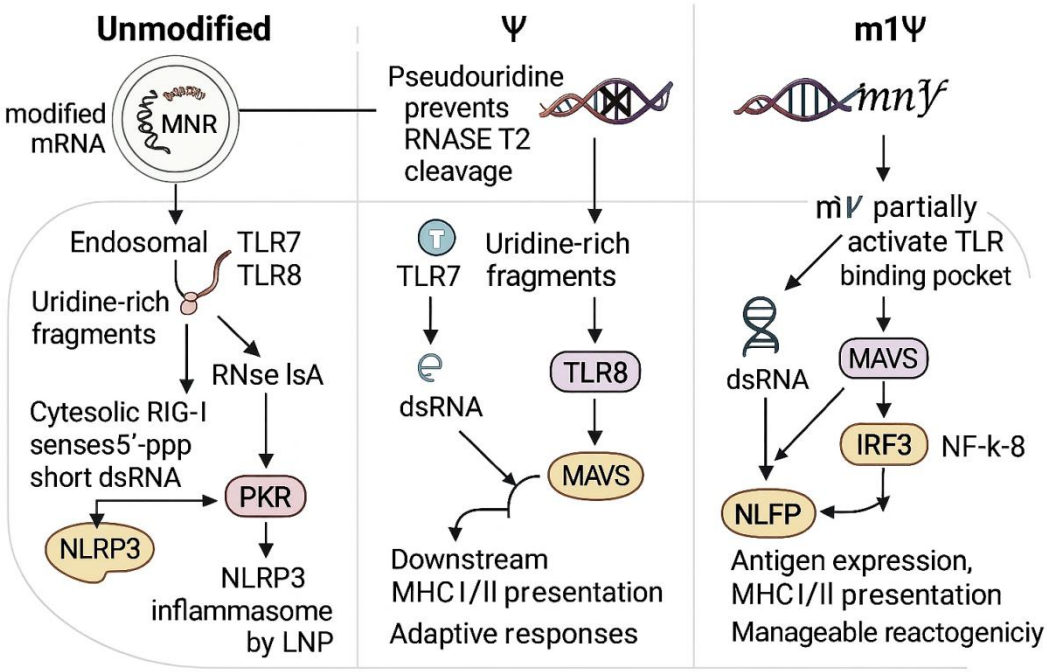

Figure 1. Molecular mechanisms of innate immune sensing in unmodified, pseudouridine (Ψ), and N1-methylpseudouridine (m1Ψ) mRNA vaccines. Unmodified mRNA activates Toll-like receptor (TLR)7/8, retinoic acid-inducible gene I (RIG-I), protein kinase R (PKR), and 2′-5′-oligoadenylate synthetase (OAS), leading to interferon (IFN-α/β) and cytokine (IL-6, TNF-α, IL-1β) release, translation inhibition via eIF2α phosphorylation,

and RNA degradation by RNase L. Pseudouridine (Ψ)-modified mRNA resists RNase T2 cleavage, suppresses TLR7/8 and RIG-I activation, and avoids PKR and OAS pathways, enabling efficient antigen expression. N1-methylpseudouridine (m1Ψ)-modified mRNA shows similar suppression but partially activates TLR8, maintaining high antigen production with tolerable reactogenicity. Antigen expression is presented via major histocompatibility complex (MHC) class I and II, stimulating $CD8^+$ and $CD4^+$ T cells and driving antibody production.

Pseudouridine and N1-methylpseudouridine allow mRNA vaccines to balance immunogenicity and safety by preventing excessive activation of TLRs and RIG-I while maintaining efficient antigen expression. Lipid nanoparticles (LNPs) used for delivery provide additional adjuvant activity through NF-κB and inflammasome pathways, supplying maturation signals to dendritic cells that complement the low-level innate sensing of modified mRNA. This synergy supports strong $CD4^+$ and $CD8^+$ T cell responses and antibody production, as shown for m1Ψ-modified COVID-19 vaccines (8). Reactogenicity such as injection-site pain or transient fever arises mainly from cytokine induction (e.g., IFN, IL-6) and may be slightly more pronounced with m1Ψ due to residual TLR8 activity, although direct human comparisons are lacking. Importantly, even modified mRNA elicits some interferon production, which can enhance antigen presentation without significantly suppressing translation (31).

Efforts are underway to refine these modifications further. Although m1Ψ is highly effective, it does not eliminate TLR8 activation, motivating exploration of combinations such as Ψ with 5-methylcytidine (9) or other natural and synthetic uridine analogues. Each modification must be assessed for effects on RNA structure, translational fidelity, and innate sensing. Partial substitution strategies may also be employed: most uridines are replaced with Ψ or m1Ψ for efficient translation, while select uridine motifs remain unmodified to provide controlled TLR7 stimulation. Such approaches represent the emerging field of RNA-based adjuvant design (11).

Innate modulation also affects repeated dosing. Excessive innate activation after the first dose may induce negative feedback mechanisms that dampen responses to boosters, whereas the moderated signaling produced by m1Ψ vaccines may favor robust recall immunity (29). Concerns about over-suppression of innate cues are mitigated by the fact that modified mRNA/LNPs still induce some inflammation and cell death, which help maintain immunogenicity (10).

The immune-evasive properties of pseudouridine also have implications for RNA therapeutic safety and autoimmune disease. Endogenous RNAs enriched in Ψ do not activate TLRs, whereas foreign RNAs containing unmodified U do (16). This supports the idea that Ψ functions as a molecular marker of "self." As Bérouti et al. note, their findings "provide a molecular basis for self-avoidance by RNA-sensing TLRs" (5). Incorporating Ψ or m1Ψ may therefore reduce the risk of interferon-driven autoimmune exacerbation.

In conclusion, pseudouridine and N1-methylpseudouridine enable precise tuning of RIG-I– and TLR-mediated sensing, allowing strong adaptive responses with minimal inflammatory suppression. The success of COVID-19 mRNA vaccines demonstrates the effectiveness of this strategy (4). Ongoing research aims to optimize modification patterns to shape antibody quality, T-helper polarization, memory formation, and cytokine profiles, with potential customization depending on the therapeutic context—infectious disease, cancer immunotherapy, or tolerance induction. The discovery and application of Ψ and

m1Ψ represent a turning point in nucleic acid medicine, enabling targeted immune engagement with reduced side effects (2).

CLINICAL IMPLICATION

The findings of this review highlight the clinical relevance of nucleoside modifications, particularly pseudouridine and N1-methylpseudouridine, in the development of safe and effective mRNA vaccines. By suppressing aberrant activation of innate immune receptors such as RIG-I and TLR7/8, these modifications enhance mRNA stability, translation efficiency, and antigen expression. Clinically, this translates into vaccines that provide strong protective immunity with reduced systemic reactogenicity, a balance that was critical to the success of COVID-19 mRNA vaccines. Understanding these mechanisms is essential for guiding future vaccine development, including boosters, therapeutic cancer vaccines, and RNA-based treatments for genetic and infectious diseases. Moreover, the modulation of PRR pathways provides a framework for tailoring immune responses to specific clinical contexts, ensuring both efficacy and safety in patient care.

LIMITATIONS

This review is limited by several methodological and evidence-based constraints. First, the selection of studies is inherently biased toward topics with extensive published data, resulting in an overrepresentation of findings from COVID 19 mRNA vaccines. Although these studies provide valuable mechanistic insights, they may not generalize to other disease contexts, delivery systems, or dosing regimens.

Second, while pseudouridine and N1 methylpseudouridine consistently suppress innate immune sensing in preclinical systems, human data regarding repeated or long-term administration remain scarce. Most mechanistic conclusions rely on in vitro experiments or animal models, which do not fully recapitulate human immunogenetics, innate to adaptive signaling interactions, or interindividual variability. Furthermore, the interaction between nucleoside modifications, lipid nanoparticle formulations, and host genetic background is not yet fully understood, raising the possibility of context specific effects that current datasets cannot resolve. These limitations highlight the need for broader comparative studies across mRNA platforms, systematic evaluation outside the COVID 19 setting, and continued clinical monitoring to define the safety and immunological consequences of repeated dosing in humans.

CONCLUSIONS

Pseudouridine and N1 methylpseudouridine have reshaped mRNA vaccine design by enabling evasion of excessive innate sensing while preserving the capacity to induce protective immunity. These modifications reduce activation of TLR3, TLR7, and TLR8 by limiting endosomal RNA degradation and weakening receptor engagement, resulting in lower interferon and cytokine induction than unmodified RNA (1,15). In the cytosol, Ψ and m1Ψ dampen RIG I and related antiviral pathways, supporting sustained protein expression without premature shutdown (21,32). Their use in COVID 19 vaccines demonstrates how nucleoside chemistry yields potent yet well tolerated immune responses. At the same time, residual innate activation from delivery systems or limited PRR engagement remains essential for vaccine efficacy, and the differing TLR8 activity of Ψ and m1Ψ highlights the possibility of fine-tuning immunogenicity (18).

These insights extend beyond vaccines. Modified mRNA can minimize immunogenicity in protein replacement or gene editing therapies, whereas cancer vaccines may benefit from retaining select unmodified motifs to enhance local immune activation. The expanding repertoire of RNA modifications and deeper understanding of PRR signaling will enable more precise design of mRNA suited to specific immunological goals. Overall, pseudouridine and N1 methylpseudouridine serve as central modulators of innate immunity in mRNA technology, contributing to the success of current vaccines and guiding future RNA based therapeutics, while illustrating how natural RNA modifications underpin self-versus non self-discrimination in immunity.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

ES conceived the study, drafted the manuscript, and integrated revisions. FK and NC contributed to literature review, data interpretation, and manuscript writing. MM and PI provided critical input on immunological and molecular aspects and revised the manuscript for intellectual content. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work.

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The authors used ChatGPT (OpenAI, version GPT-5) to assist in improving grammar of the manuscript.

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