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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) – thereby influencing cytokine responses and the balance between immunogenicity and reactogenicity

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[2]. 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. Additionally, Ψ substitution diminishes activation of PKR and OAS, thus preventing translation shutdown and RNA degradation.

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. Ongoing research is refining the use of Ψ and m1Ψ to optimize vaccine design, striking a balance between robust immune protection and safety.

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INTRODUCTION

Messenger RNA (mRNA) vaccines have emerged as a powerful platform for preventing infectious diseases and other therapeutic applications. A fundamental challenge in developing mRNA-based therapeutics is the innate immune system's propensity to recognize exogenous RNA as a danger signal. When unmodified mRNA is introduced into cells, it can trigger pattern-recognition receptors (PRRs) such as RIG-I-like receptors (RLRs) in the cytosol and Toll-like receptors (TLRs) in the endosome, leading to production of type I interferons and pro-inflammatory cytokines. While an appropriate innate response is essential for mounting adaptive immunity, excessive or premature activation can be detrimental – it may halt antigen production, cause systemic inflammation, and reduce the overall efficacy of the vaccine(1,2). Mammalian cells have evolved ways to distinguish "self" RNA from "non-self." Notably, endogenous RNAs (e.g., cellular mRNAs, rRNAs, tRNAs) often contain modified nucleosides that dampen innate sensor activation, whereas pathogenic or in vitro transcribed RNAs lack these modifications(3). This has led to the insight that incorporating specific nucleotide modifications into synthetic mRNAs can shield them from immune detection, thereby improving their performance as vaccines.

Pseudouridine (Ψ), the C5-glycoside isomer of uridine, is the most common RNA base modification in nature, found in tRNA, rRNA, snRNA, and even mRNA(4). In pseudouridylation, the uracil base is rotated and attached to the ribose via a carbon-carbon bond, rather than the normal nitrogen-carbon bond, resulting in a uridine isomer with unique properties. This isomerization creates an additional hydrogen bond donor at the N1 position (–N1H) of the base (5). Consequently, Ψ can form additional or more substantial base-pair interactions; it behaves as a "universal base" that not only pairs with adenosine (A) but can also wobble-pair with other bases (G, U, or C) in specific contexts. The presence of Ψ generally stabilizes RNA secondary structure through increased base stacking and pairing, typically raising the melting temperature of RNA duplexes. These chemical features inspired the landmark hypothesis that substituting Ψ for uridine in synthetic mRNA could reduce its visibility to RNA-sensing PRRs without compromising its coding capacity (6).

N1-methylpseudouridine (m1Ψ) is a further modified form of pseudouridine in which the N1 hydrogen (the very group conferring Ψ's extra hydrogen bond donor) is replaced by a methyl group(7). m1Ψ is naturally found in certain rRNAs and tRNAs, installed by dedicated methyltransferases. Chemically, adding a methyl to N1 of Ψ eliminates the additional H-bond donor, restricting m1Ψ to standard Watson-Crick base pairing (with A) and preventing the wobble pairing that Ψ permits. Both Ψ and m1Ψ can thus stabilize RNA structure, but m1Ψ may alter local RNA dynamics differently due to its fixed N1 methylation. The development of m1Ψ was driven by

the search for Ψ analogues that might further improve mRNA performance as a drug or vaccine molecule(8). Indeed, m1Ψ has been shown to outperform Ψ in specific contexts; for example, in transfected mammalian cells and mice, m1Ψ-modified mRNA produced higher protein levels and elicited lower innate immune activation than Ψ-modified mRNA *[18]*. For this reason, the two most widely used COVID-19 mRNA vaccines (Pfizer-BioNTech's BNT162b2 and Moderna's mRNA-1273) both employ complete substitution of uridine with m1Ψ in their mRNA payloads (8,9).

This review will discuss in depth how Ψ and m1Ψ modifications influence the detection of mRNA by key innate sensors, including RIG-I, MDA5, TLR3, TLR7, and TLR8, as well as 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 discuss how these modifications modulate other RNA-activated pathways, such as protein kinase R (PKR) and 2′-5′ oligoadenylate synthases (OAS), which influence mRNA stability and translation. Finally, we consider the immunological implications of using modified versus unmodified mRNA, including the balance between vaccine immunogenicity and reactogenicity, as well as emerging strategies to optimize mRNA vaccine design.

RESULTS AND DISCUSSIONS

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

RIG-I-like receptors (RLRs) and Toll-like receptors (TLRs) are the principal families of PRRs that detect RNA within cells. RLRs are cytosolic sensors; RIG-I (retinoic acid-inducible gene I) primarily recognizes short double-stranded RNA (dsRNA) with a 5′ tri- or diphosphate end and an unmethylated 5′ cap. In contrast, MDA5 (melanoma differentiation-associated protein 5) recognizes long dsRNA (typically >0.5–1 kb) irrespective of sequence[20][21]. Upon binding RNA, RIG-I and MDA5 undergo conformational changes and oligomerize (forming filament-like assemblies in the case of MDA5). They then activate the adaptor MAVS, leading to downstream signaling that triggers the transcription factors IRF3/7 and NF-κB, culminating in the production of type I interferons (e.g., IFN-α/β) and pro-inflammatory cytokines (e.g., IL-6, TNF-α) (7, 10, 11). RIG-I and MDA5 thus serve as a first line of defense against cytoplasmic viral RNA. However, in the context of mRNA delivery, unintended activation of these sensors can severely limit protein expression by inducing an antiviral state within the transfected cell.

Toll-like receptors 3, 7, and 8 (TLR3, TLR7, TLR8) are endosomal sensors that detect RNA taken up via endocytosis. TLR3 binds to double-stranded RNA (a molecular pattern associated with viruses) in the acidified endosomal lumen[24]. Upon binding dsRNA segments (≥90 bp), TLR3 dimerizes and signals through the adaptor TRIF, activating IRF3 and NF-κB, similar to RLRs (7, 12). TLR7 and TLR8 detect single-stranded RNA, especially uridine-rich or GU-rich sequences, in endosomes of specialized immune cells. Human TLR7 is abundantly expressed in plasmacytoid dendritic cells (pDCs), where its activation by

RNA leads to robust production of type I interferons. TLR8 is expressed in monocytes and macrophages, and its activation tends to produce inflammatory cytokines, such as TNF-α and IL-12. Notably, TLR7 and TLR8 each employ a two-step ligand binding mechanism, featuring two cooperative binding pockets that accommodate RNA degradation products. One pocket of TLR8 binds a uridine moiety, and the other binds an RNA oligonucleotide; together, these ligands induce TLR8 dimerization and signaling (7,13). TLR7 similarly requires a small molecule (2′,3′-cyclic GMP) and an RNA oligonucleotide for full activation. In essence, TLR7/8 are wired to sense RNA breakdown products that typically arise when endosomal RNases digest RNA(7). This intricacy ensures that the TLRs respond to RNA only after it has been taken into endosomes and processed, thereby helping to discriminate between extracellular/self RNA and pathogenic RNA.

When a conventional unmodified mRNA is delivered (e.g., via lipid nanoparticles) into the body, some fraction of that RNA will inevitably encounter these sensors. For example, incompletely capped or uncapped transcripts or double-stranded byproducts can activate RIG-I; any long double-stranded regions or contaminant dsRNA can engage MDA5 and TLR3; and fragments of mRNA that end up in endosomes (due to extracellular release or imperfect delivery) can trigger TLR7/8 if rich in uridines(1). The consequences of such activation include the rapid induction of interferon-stimulated genes, the secretion of IFN-α/β and inflammatory cytokines, the maturation of dendritic cells, and, in some cases, the activation of inflammasomes (via secondary sensors, such as NLRP3). From a vaccine standpoint, moderate activation of these pathways can be beneficial as an adjuvant, promoting antigen presentation and a potent T-cell and B-cell response. However, excessive innate activation is problematic: high levels of IFN-I can shut down protein synthesis via PKR and other effectors, thereby curtailing antigen production from mRNA (13). Strong inflammatory responses can cause fever and other systemic side effects, or even acute pathological reactions. Moreover, chronic activation of these pathways could raise safety concerns, including the potential for autoimmunity.

It was in this context that researchers hypothesized nucleoside modifications might be the key to “tune” the innate immunogenicity of mRNA. They noted that vertebrate RNA (which typically does not provoke TLR7/8 in the same way as bacterial RNA) is enriched in modified nucleosides. For instance, transfer RNA and ribosomal RNA – which do not usually trigger TLRs – contain many Ψ, 2′-O-methylated bases, etc. In vitro-transcribed RNA containing specific modified nucleosides is inert to human TLR3, TLR7, and TLR8, whereas the same RNA with unmodified bases is highly stimulatory. Human dendritic cells exposed to modified RNA (containing Ψ, 5-methylcytidine, 2-thiouridine, or other modifications) produced substantially less TNF-α, IFN-α, IL-6, and co-stimulatory markers than those exposed to unmodified RNA(14). This finding suggests that our innate immune system has essentially evolved to “ignore” modified-self RNA while reacting to unmodified RNA as a sign of infection or cellular damage [7]. Below, we examine how pseudouridine and N1-methylpseudouridine facilitate immune evasion in the context of each primary sensor.

Modulation of Endosomal TLR Activation by Ψ and m1Ψ

One of the most significant impacts of pseudouridine on innate immunity is its ability to suppress TLR7/8-mediated sensing of RNA. Unmodified single-stranded RNA, especially with repeating uridines, is a potent agonist for human TLR7 and TLR8. In fact, synthetic RNA oligonucleotides or viral RNAs rich in uridylates can trigger these receptors

to drive robust cytokine secretion (pDCs releasing IFN-α via TLR7; monocytes releasing IL-6/TNF via TLR8) (15). By replacing uridine with pseudouridine in mRNA, this stimulation is dramatically reduced. It was first reported that RNA containing Ψ does not activate TLR7 or TLR8 in human immune cells. A follow-up study in mice demonstrated that unmodified mRNA was significantly more immunogenic in vivo than Ψ-modified mRNA, resulting in higher levels of inflammatory cytokines and immune cell activation. Conversely, the Ψ-modified mRNA was better tolerated; however, it was conjectured that an exogenous adjuvant might be needed to compensate for the lower innate stimulation if used in a vaccine (5,16). (In practice, the lipid nanoparticle delivery system provides adjuvant activity for the COVID-19 vaccines, obviating the need for additional adjuvants.)

Mechanistically, how does pseudouridine prevent TLR7/8 activation? This remained somewhat mysterious for years, but recent breakthroughs have elucidated two complementary mechanisms:

Impaired Generation of TLR Ligands: Endosomal RNases (especially RNase T2 and 3′ exonucleases like PLD3/PLD4) digest RNA into smaller fragments that serve as the actual TLR7/8 ligands. RNase T2 preferentially cleaves after uridines in specific sequence contexts, producing uridine-containing dinucleotides that bind TLR8(11). Pseudouridine modification makes RNA a poor substrate for these nucleases. RNase T2 and PLD3/4 are unable to efficiently process Ψ-containing RNA, thereby failing to generate the canonical uridine-rich fragments that typically activate TLR7 and TLR8. Mass spectrometry confirmed that Ψ-modified RNA remains largely intact or yields abnormal digestion products in endosomes(17). The likely reason is that Ψ increases the structural rigidity of RNA (favoring a persistent A-form helix), and the altered geometry or base presentation interferes with RNase binding and cleavage. As a result, key ligands like 2′,3′-cGMP (for TLR7) and uridine-containing oligoribonucleotides (for TLR8) are not sufficiently produced from Ψ–mRNA. In elegant experiments, RNase T2 knockout cells failed to respond to unmodified mRNA (confirming that those fragments are essential for sensing). In contrast, the response to Ψ–mRNA was minimal to begin with. Thus, pseudouridine provides an upstream block by obstructing the normal enzymatic generation of TLR activators(5).

Reduced TLR Binding and Activation: Pseudouridine also directly diminishes the ability of TLR7/8 to recognize RNA. TLR8 has two binding pockets: one accommodates a uridine base (or uridine nucleoside), and the other binds a short RNA oligomer. Biochemical assays indicate that pseudouridine is a poor agonist for TLR8's uridine-sensing pocket. In vitro, uridine (as UMP or other analogs) can induce conformational dimerization of TLR8 – a hallmark of its activation – whereas pseudouridine fails to trigger efficient dimerization (18). A recent study demonstrated that while uridine and m1Ψ both induce robust TLR8 dimerization, pseudouridine induces minimal TLR8 activity on its own. This suggests that TLR8's ligand-binding site has specificity for the uridine base in its native orientation; the isomerized base (Ψ) is not well-recognized, possibly due to the different placement of functional groups. Consistently, when pseudouridine-containing RNA fragments were tested, they showed only weak agonistic activity unless supplemented with other co-stimulatory ligands[48]. Likewise, TLR7 disregards pseudouridine-containing oligonucleotides – even if the auxiliary ligand (cGMP) is present, a Ψ-rich short RNA does not fully activate TLR7. In essence, the presence of Ψ in RNA fools these receptors into treating the RNA as if it were self (benign) RNA(19,20).

The net result of these mechanisms is that mRNAs incorporating pseudouridine are essentially "invisible" to TLR7 and TLR8. Empirically, Ψ-modified mRNA fails to induce TLR7/8-driven cytokines in human immune cells. For example, primary human monocytes, plasmacytoid DCs, or macrophage models exposed to Ψ-modified IVT RNA secrete little to no interferon or inflammatory cytokines, whereas unmodified IVT RNA triggers the secretion of robust IFN-α, IL-6, TNF-α, and other cytokines (18). This immune evasion is a cornerstone of current mRNA vaccine design, as it permits the encoded antigen to be produced in host cells with minimal interruption by the innate immune system. Indeed, the success of COVID-19 mRNA vaccines has been attributed in large part to the use of pseudouridine; this modification "turned out to be game-changing" by enabling high efficacy without excessive inflammation.

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. Structurally, this makes sense: pseudouridine's extra N1H donor (absent in uridine) is what probably prevents it from fitting optimally in TLR8's uridine-binding site. 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). As a result, TLR8 can recognize m1Ψ as if it were a normal uridine ligand.

The finding that m1Ψ can still trigger TLR8 has important implications for vaccine reactogenicity and immune response. On the one hand, m1Ψ confers mRNA superior translational capacity and stability (discussed later), but on the other hand, it may contribute to specific inflammatory responses. This could be a trade-off: m1Ψ vaccines might induce a touch more innate immune activation (potentially contributing to strong adaptive immunity or, conversely, to side effects) compared to a hypothetical fully Ψ-modified vaccine. In the context of the approved vaccines, any TLR8 activation by m1Ψ is likely mitigated by the limited exposure of mRNA to endosomes (since efficient delivery aims for cytosolic release) and by the presence of the 5′ cap and other modifications(22). Nonetheless, developers must consider that m1Ψ, while largely immunosilent, is not completely inert. Future design of mRNA therapeutics may explore combinations of Ψ and m1Ψ, or other modifications, to fine-tune this balance (21).

Significantly, TLR3 (which senses dsRNA) is also affected by nucleoside modifications. Karikó et al. noted that RNA containing pseudouridine did not activate TLR3 in human cells. Since TLR3 binding depends on the dsRNA structure rather than specific sequence motifs, the mechanism here may relate to RNA secondary structure formation. Modified bases, such as Ψ or 2-thiouridine, can alter the stability and conformation of double-stranded regions, potentially reducing the ability of TLR3 to bind to its ligand. Indeed, one study found that incorporating certain modifications (e.g., 2-thio-U or m6A) in dsRNA reduced TLR3 signaling(1,3). Although pseudouridine was not explicitly tested in that particular case, it is likely to have a similar effect, given Karikó's results. However, it should be noted that TLR3 is less easily fooled if long perfect dsRNA segments are present;

thus, the more practical way to avoid TLR3 activation in mRNA production is to minimize dsRNA contaminants (e.g., through high-performance liquid chromatography purification of IVT RNA to remove duplex byproducts(15,23)). In summary, pseudouridine and m1Ψ primarily guard against TLR7/8, and to a lesser extent, TLR3, thereby preventing aberrant endosomal PRR activation and excessive cytokine release during mRNA vaccination(13).

Effects of Modified Nucleosides on RIG-I and MDA5 Recognition

In the cytosol, the RLR family, especially RIG-I, represents a significant barrier to exogenous RNA. A key strategy in mRNA vaccine design has been to evade RIG-I activation, since RIG-I engagement can rapidly induce an antiviral state that would shut down the translation of the mRNA. The incorporation of pseudouridine and related modifications has proven highly effective at reducing RIG-I's responsiveness. RIG-I recognizes RNA featuring a 5′-triphosphate and double-stranded structure, such as short blunt-ended dsRNAs, or hybridized regions of single-stranded RNA with a tri- or diphosphate end(12). Although vaccine mRNAs are typically capped (avoiding a 5′-PPP motif) and delivered with minimal double-stranded regions, in vitro transcription can produce small amounts of uncapped transcripts or duplex impurities that activate RIG-I. Moreover, single-stranded mRNA can fold into short hairpins that may be sensed by RIG-I (7,24).

Studies have demonstrated that RNAs containing pseudouridine or m1Ψ have a greatly diminished ability to activate RIG-I signaling. Introducing modified nucleotides (Ψ, m5C, etc.) into short RNAs prevents RIG-I from undergoing the conformational changes required for downstream signaling(25,26). In essence, RIG-I may still bind to the RNA. Still, the presence of modifications in the RNA duplex interferes with RIG-I's precise molecular interactions and its ATP-driven translocation or oligomerization on the RNA. Biochemically, RIG-I's helicase domain and C-terminal domain sense the shape and chemical features of the RNA duplex termini. Modifications like pseudouridine could subtly alter the geometry of the RNA backbone or base stacking, such that RIG-I does not recognize it as a normal ligand. Supporting this, Pseudouridine and m1Ψ strongly reduce RIG-I's downstream activity, whereas MDA5 remains fully able to detect dsRNA even if modified[4]. This distinction likely arises because MDA5 binds along the length of long dsRNAs in a largely sequence-agnostic manner; a few modified bases do not prevent MDA5 from coating an extended duplex. RIG-I, however, is acutely sensitive to the structure of the RNA end and the first few base pairs where it initiates binding(19,27). Thus, modified nucleotides at or near the 5′ end or within short duplex regions can derail RIG-I activation, whereas MDA5 will still detect a long double helix (modified or not) by sheer length of dsRNA. Empirically, this means Ψ/m1Ψ substitution suppresses RIG-I-mediated interferon induction but cannot eliminate responses to long dsRNA contaminants (which instead engage MDA5). Vaccine manufacturers have addressed the latter by improving IVT reactions and purification to minimize dsRNA impurities(27). In combination, high purity and nucleoside modification render RIG-I and MDA5 activation by therapeutic mRNAs negligible.

Comparisons of modified and unmodified mRNAs provide an illustrative example of the importance of RIG-I evasion. One study found that removing or reducing cell-intrinsic innate sensing (through nucleoside mods and cap modifications) led to dramatically higher protein expression and altered adaptive immune outcomes. In mice, an unmodified mRNA vaccine elicited stronger immediate interferon responses, which in some cases translated to

different magnitudes of T cell responses. In contrast, a modified mRNA (m1Ψ, cap1) produced less interferon and relied more on the antigen itself for immunogenicity(22,28). In cancer vaccine models, unmodified mRNA has shown an intriguing advantage: the enhanced type I IFN production can bolster anti-tumor immunity by "hotting up" the tumor microenvironment. Sittplangkoon et al. (2022) demonstrated that an unmodified mRNA-LNP vaccine induced substantially more IFN-α and activated dendritic cells more efficiently than m1Ψ-modified mRNA, resulting in better $CD8^+$ T cell responses and tumor control in a mouse melanoma model(10). Notably, blocking the type I IFN receptor abrogated the therapeutic efficacy of the unmodified mRNA vaccine, underscoring that innate IFN was a key driver of its antitumor effect. This finding underlines that while modified nucleosides are generally preferable for non-cancer vaccines (to avoid hyperactivation), there are scenarios where the inherent adjuvanticity of unmodified mRNA is desirable. Indeed, some cancer vaccine trials are now exploring unmodified mRNA, leveraging its strong RIG-I/TLR activation to enhance immunogenicity (29).

From a mechanistic viewpoint, pseudouridine's ability to blunt RIG-I activation also intertwines with other innate pathways. Protein kinase R (PKR) is a cytosolic enzyme that binds long dsRNA and, upon activation, phosphorylates the translation initiation factor eIF2α, causing a shutdown of protein synthesis. Unmodified IVT mRNA can activate PKR if it forms even transient dsRNA structures or if some double-stranded byproducts are present. However, pseudouridine substitution in mRNA was found to diminish PKR activation, thereby preventing the translation block that would normally occur(16). Pseudouridine-containing mRNAs did not induce appreciable PKR phosphorylation, unlike unmodified mRNAs, which readily activated PKR and led to reduced protein output(5). This is likely because pseudouridine-modified transcripts have a lower tendency to form the secondary structures that PKR binds, or PKR's binding affinity is reduced by the presence of Ψ in those structures. The outcome is a significantly higher translational capacity for modified mRNA, as observed in cell cultures and mice (7).

Similarly, pseudouridine helps mRNA escape the 2′-5′ oligoadenylate synthase (OAS) pathway. OAS proteins detect dsRNA and produce 2′-5′ linked oligoadenylates that activate RNase L, an endoribonuclease that cleaves single-stranded RNAs in the cell (both viral and cellular). In unmodified mRNA transfection, if any dsRNA regions or duplex byproducts activate OAS, RNase L can be triggered to destroy the mRNA payload. Remarkably, pseudouridine-modified mRNA was shown to resist OAS activation and RNase L-mediated cleavage(30). Nucleoside modifications (Ψ, 2-thio-U, etc.) in mRNA lead to far less activation of OAS1 and consequently less RNA degradation by RNase L. This provides yet another layer of increased stability for the mRNA within the cell, ensuring it survives long enough to be translated into an antigen. It is noteworthy that OAS, like MDA5, primarily senses the presence of dsRNA, rather than specific sequences, so complete avoidance of OAS may rely on both chemical modifications and purification of the mRNA to remove double-stranded regions (24,25). Nonetheless, the modifications significantly raise the threshold at which these antiviral enzymes are activated.

In summary, pseudouridine and N1-methylpseudouridine modifications recalibrate the innate immune sensing of mRNA on multiple fronts. They prevent aberrant TLR7/8 activation in endosomes, dramatically reduce RIG-I recognition in the cytosol, and mitigate secondary RNA-responsive pathways, such as PKR and OAS, that could otherwise inhibit protein expression. MDA5 and TLR3 remain potentially activatable by long dsRNA; hence,

careful manufacturing and design (e.g., avoiding long inverted repeats in the mRNA sequence and removing dsRNA contaminants) are employed in concert with nucleoside modification to achieve an optimal outcome.

Immunogenicity and Safety Implications for mRNA Vaccine Design

The use of pseudouridine and N1-methylpseudouridine has been pivotal in balancing the immunogenicity and safety profile of mRNA vaccines. By suppressing excessive innate immune sensing, these modifications enable the mRNA to persist and translate efficiently, resulting in higher antigen levels and more robust adaptive immune responses for a given dose (26). Indeed, the stunning immunogenic efficacy of the first mRNA COVID-19 vaccines (over 90% protection) was achieved with modified mRNA that elicited potent neutralizing antibody and T cell responses while maintaining an acceptable reactogenicity profile. Early attempts with unmodified mRNA vaccines often encountered problems. CureVac's initial COVID-19 vaccine candidate (CVnCoV, which contained unmodified uridine) showed markedly lower efficacy in trials, which many experts attributed to insufficient protein expression due to innate RNA sensing and a resultant blunted immune response (21). CureVac subsequently switched to incorporating m1Ψ in their next-generation vaccine (CV2CoV), aligning with the approach of other successful mRNA vaccines(18). This exemplifies the critical role nucleotide chemistry plays in the real-world performance of mRNA vaccines.

On the other hand, completely ablating innate recognition is not always desirable. A degree of PRR activation can act as an intrinsic adjuvant, recruiting and activating antigen-presenting cells. The mRNA vaccine platform can be tuned for non-pandemic vaccines or cancer immunotherapies; some developers are exploring partial uridine substitution or even unmodified mRNAs to boost immunostimulation in situ [63]. For example, BioNTech has reported a trial of a colorectal cancer mRNA vaccine using unmodified mRNA in a lipoplex formulation, aiming to exploit the stronger innate signals to inflame the tumor microenvironment and enhance T-cell priming (22). As noted, studies have found that unmodified mRNA can drive more potent type I IFN-dependent T cell responses in tumor models. The trade-off is that unmodified mRNAs come with higher reactogenicity and lower translation efficiency, which might be acceptable in oncology (where some fever and cytokine release can be tolerated), but less so in prophylactic vaccines for healthy individuals (27).

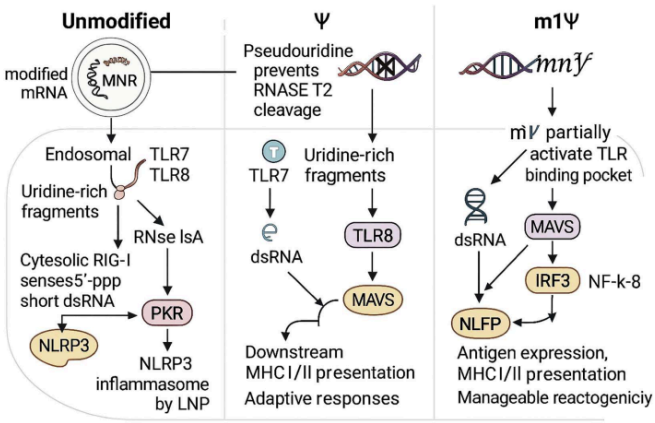

Figure 1. Molecular mechanisms of innate immune sensing in unmodified, pseudouridine (Ψ), and N1-methylpseudouridine (m1Ψ) mRNA vaccines. Unmodified mRNA activates Toll-like receptor (TLR)7/8, retinoic acid-inducible gene I (RIG-I), protein kinase R (PKR), and 2′-5′-oligoadenylate synthetase (OAS), leading 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.

The current consensus in vaccine design is that pseudouridine- or N1-methylpseudouridine-modified mRNAs strike a favorable balance: they avoid triggering most TLRs and RIG-I, thus minimizing side effects and maximizing protein yield, while still inducing sufficient danger signals to stimulate adaptive immunity, partly thanks to the immune-active delivery vehicle. In fact, the lipid nanoparticles used for mRNA delivery have their own adjuvant properties – they can induce transient inflammation (e.g., via NF-κB and inflammasome activation in innate cells), which likely complements the low-level innate sensing of the modified mRNA to drive vaccine responses. The result is a focused immune activation: the LNP and perhaps a small amount of RNA sensing provide maturation signals to dendritic cells. At the same time, the mRNA directs the production of the antigen in those cells. This synergy leads to robust $CD4^+$ and $CD8^+$ T cell responses and antibody production, as observed with m1Ψ-modified COVID vaccines (8). Reactogenicity events, such as injection-site pain, transient fever, or fatigue, are manageable and attributed mainly to innate immune activation (e.g., cytokines like IFN and IL-6). Interestingly, such events might be slightly more pronounced with m1Ψ (due to TLR8 detection) than they would be with pure Ψ. However, direct comparisons in humans are lacking. It's worth noting that even with modifications, some innate sensing still occurs; for instance, a fraction of cells transfected with modified mRNA will produce interferon (though less than with

unmodified). This residual sensing can be beneficial, as interferon can upregulate MHC and co-stimulatory molecules, enhancing T cell activation. The key is that it does not reach a level that dramatically suppresses vaccine antigen synthesis (31).

Looking forward, researchers are investigating additional or alternative modifications that might further optimize immune modulation. N1-methylpseudouridine has proven exceptionally useful, but as we learned, it doesn't eliminate TLR8 activity. One candidate might be Ψ itself (without TLR8 activation) combined with 5-methylcytidine (to aid RNA stability), an approach used in some pre-2015 studies (9). Other naturally occurring modified uridines (e.g., 2-thiouridine or dihydrouridine) or totally synthetic analogues are being tested for their ability to evade sensors. Each modification must be weighed for its effect on RNA structure, translational fidelity, and immunogenicity. There is also interest in selectively modulating innate pathways – for example, incorporating a specific pattern into the mRNA that deliberately activates a mild TLR response as an adjuvant, while keeping RIG-I and PKR largely inactive. This could involve using a partial substitution strategy, where most uridines are Ψ/m1Ψ, for high translation efficiency. Still, a few strategic uridine motifs are left unmodified or replaced with another analog to provide a small TLR7 stimulus. The field of RNA adjuvant design is nascent but promising(11).

Another consideration is the potential impact of PRR evasion on repeated dosing and tolerance. If an mRNA is too stealthy, repeated administration might not cause any "alarm" in the immune system and could risk being viewed as tolerogenic. However, this seems unlikely, given that even modified mRNA/LNPs cause some inflammation and cell death, which in turn recruits immune responses (10). On the contrary, excessive innate activation with the first dose could desensitize cells or trigger negative feedback (e.g., high IFN can induce suppressors of cytokine signaling proteins), which would dampen responses to booster doses. In that regard, the moderated innate activation with m1Ψ vaccines may favor strong responses upon boosting(29).

Finally, understanding how pseudouridine mediates immune evasion also has implications for the safety of RNA therapeutics and autoimmunity. The fact that our immune receptors ignore pseudouridylated RNA is thought to be a mechanism to prevent autoimmunity – our own non-coding RNAs (rich in Ψ) do not trigger TLRs. In contrast, foreign RNAs do (with unmodified U)(16). This concept underscores why modified mRNA therapeutics are less likely to cause off-target immune activation. It also suggests that using Ψ/m1Ψ could mitigate risks of interferon-related autoimmune reactions (for instance, reducing the chance of an mRNA therapy exacerbating conditions like lupus, which are driven by innate sensing of self-RNA). Indeed, Bérouti et al. conclude that their findings "provide a molecular basis for self-avoidance by RNA-sensing TLRs" – in other words, pseudouridine is a marker of self that TLRs have learned to ignore (5).

In conclusion, the incorporation of pseudouridine and N1-methylpseudouridine in mRNA vaccines represents a sophisticated means to modulate innate immunity. These modifications enable vaccine developers to manipulate the RIG-I and TLR pathways, thereby dialing down the interferon and inflammatory responses that would otherwise hinder the vaccine, while still engaging the immune system in a controlled manner. The resulting vaccines can be both highly immunogenic and safe, as exemplified by the successful COVID-19 mRNA vaccines(4). Ongoing research is refining this balance – exploring how different patterns of RNA modification influence the quality of adaptive immunity (antibody levels, T helper polarization, memory responses) and the spectrum of

cytokines induced. By leveraging the growing understanding of RNA–PRR interactions and RNA chemistry, next-generation mRNA vaccines and therapeutics will be further optimized for efficacy with minimal side effects, potentially including personalized tuning of innate activation based on the disease context (infectious disease vs. cancer vs. tolerogenic therapies). What remains clear is that the discovery of Ψ and m1Ψ as mRNA building blocks has been a turning point in nucleic acid medicine, illuminating a path to harness the power of the immune system with precision (2).

CLINICAL IMPLICATION

The Clinical Implications section is where the authors interpret the study's findings in terms of their impact on real-world clinical practice or health outcomes. This section answers the question "So what?" by explaining how the results can influence healthcare practices, diagnostic approaches, treatment decisions, or public health strategies. In medical laboratory studies, the clinical implications often discuss how new diagnostic methods, biomarkers, or findings can improve patient care or clinical procedures.

LIMITATIONS

The Limitations section is where the authors acknowledge the weaknesses or constraints of their study. It is essential to present these limitations transparently, allowing readers to understand the context of the research. It provides insight into factors that may influence the results, such as sample size, methodology, or external variables that were not controlled for in the study. Being transparent about the limitations demonstrates scientific integrity and helps readers understand how these weaknesses may affect the interpretation or generalizability of the findings.

CONCLUSIONS

Pseudouridine and N1-methylpseudouridine have revolutionized the design of mRNA vaccines by reconciling two conflicting requirements: evading destructive innate immune detection while still provoking a proper immune response. These modified nucleosides reshape the interaction between mRNA and the host's innate sensors. Ψ-modified mRNA is essentially invisible to TLR3, TLR7, and TLR8 due to impaired endosomal RNA degradation and poor receptor binding, which explains the significantly reduced interferon and cytokine induction compared to unmodified RNA (1,15). In the cytosol, Ψ and m1Ψ prevent the aberrant activation of RIG-I and related antiviral effectors, ensuring that the mRNA can direct robust protein expression without premature shutdown (21,32). The substitution of uridine with m1Ψ in COVID-19 vaccines exemplifies how harnessing these modifications yields a potent vaccine that is highly immunogenic yet well-tolerated. At the same time, insights from human and animal studies remind us that some level of innate stimulation – whether via residual PRR activation or the adjuvant effect of delivery vehicles – remains essential for optimal vaccine performance. The nuanced difference between Ψ and m1Ψ (with m1Ψ retaining some TLR8 agonism) highlights that we can further fine-tune mRNA's immunological profile by choice of modifications(18).

Moving forward, the principles learned here open up opportunities beyond vaccines. mRNA therapeutics for protein replacement or gene editing can employ nucleoside modifications to minimize immunogenicity. Conversely, mRNA cancer vaccines might intentionally incorporate defined, unmodified motifs to amplify immune activation in the tumor. The evolving toolkit of RNA modifications and a deeper understanding of PRR signaling pathways will enable the rational design of mRNA molecules for various purposes – achieving the desired immune outcome, whether it be silencing or stimulation. In summary, pseudouridine and N1-methylpseudouridine play a crucial role as key modulators of innate immunity in mRNA technology, underpinning the success of current mRNA vaccines and informing the development of the next generation of RNA-based medicines. These findings also reinforce a broader biological principle: chemical modifications of RNA are a natural means by which self/non-self discrimination is enforced in our immune system, a concept that researchers have cleverly leveraged to create safer and more effective therapeutics.

CONFLICT OF INTEREST

All financial, commercial, or other relationships that may be perceived by the academic community as potentially representing a conflict of interest must be disclosed. If no such relationship exists, authors will be asked to confirm the following statement:

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