Immunology Research and Perspectives

https://irp.cultechpub.com/irp

Cultech Publishing

Review

N1-Methylpseudouridine as a Molecular Regulator of mRNA Vaccine Immunogenicity and Translation Efficiency

Shoaib Manzoor[1,*], Sidra Haseen[1], Umar Javaid[2], Rutab Rizwan[3]

[1]Department of Microbiology, University of Jhang, Punjab, Pakistan

[2]Green International University, Lahore, Pakistan

[3]University of Central Punjab, Lahore, Pakistan

[*]Corresponding author: Shoaib Manzoor, shoaibmanzoor064@gmail.com

Abstract

The messenger RNA (mRNA) vaccine platform has enabled rapid development of preventive and therapeutic immunotherapies, yet unmodified mRNA inherently activates innate immune sensors, triggering interferon responses that destabilize mRNA and impair protein translation. This review critically examines the chemical and structural characteristics of N1-methylpseudouridine (m1Ψ) and evaluates its impacts on innate immune recognition, mRNA stability, and translational efficiency. Incorporation of m1Ψ alters base-pairing and stacking interactions, reducing recognition by pattern-recognition receptors including Toll-like receptors and RIG-I-like receptors. Recent mechanistic studies have revealed that m1Ψ evades immune detection through impaired endolysosomal processing by RNase T2 and phospholipase D enzymes, providing a molecular basis for its reduced immunogenicity. Compared with other modified nucleosides such as pseudouridine (Ψ) and 5-methylcytidine (m5C), m1Ψ demonstrates superior suppression of innate immune activation and enhanced protein expression. However, emerging evidence suggests context-dependent effects, including potential concerns regarding 100% m1Ψ substitution in cancer applications that warrant careful consideration. Recent advances in understanding m1Ψ-mediated translation dynamics—including ribosome profiling and cryo-electron microscopy studies—have revealed that this modification directly modulates elongation kinetics and initiation efficiency in a sequence-dependent manner, challenging earlier assumptions that its effects are solely immune-mediated. Despite these advances, critical knowledge gaps remain regarding dose-dependent effects, long-term adaptive immune outcomes, and optimization for self-amplifying RNA platforms. This review synthesizes current mechanistic understanding of m1Ψ as a molecular regulator, evaluates competing hypotheses in the field, and identifies priority areas for future investigation that address the translational potential and limitations of this modification.

Keywords

N1-methylpseudouridine, mRNA vaccines, Innate immune recognition, Translation efficiency, RNA stability, Nucleoside modification

Article History

Received: 25 February 2026 Revised: 07 April 2026

Accepted: 06 May 2026 Available Online: 09 May 2026

1. Introduction: The Rationale for a New Synthesis

Innate immune sensors, including pattern recognition receptors (PRRs), detect unmodified mRNA as foreign [1]. These receptors—primarily Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs)—recognize specific RNA features and initiate inflammatory responses. Activation leads to type I interferon and pro-inflammatory cytokine production, which can substantially decrease mRNA translation efficiency and stability, resulting in reduced antigen expression and diminished vaccine efficacy [1,2]. This innate immune hyperactivation poses a fundamental barrier to effective mRNA vaccination.

To address this limitation, researchers have developed nucleoside-modified mRNAs that minimize recognition by innate immune sensors while preserving translational capacity. Among various modifications N1-methylpseudouridine (m1Ψ)—where the nitrogen atom at position 1 of pseudouridine (Ψ) is methylated—has emerged as particularly significant. The foundational discovery by Karikó and Weissman in 2005, demonstrating that Ψ-modified mRNA exhibits reduced immunogenicity, was recognized with the Nobel Prize in Chemistry in 2023, underscoring the fundamental importance of nucleoside modifications in therapeutic mRNA development [2].

Why another review? Several comprehensive reviews have addressed nucleoside modifications in mRNA therapeutics [3-5]. However, three developments necessitate a new synthesis. First, landmark mechanistic studies published in 2024-2026 have fundamentally transformed our understanding of how m1Ψ operates—including cryo-electron microscopy structures of m1Ψ-modified ribosome complexes [6], elucidation of endolysosomal processing mechanisms [7], and direct demonstration of translation modulation independent of immune effects [6]. Second, emerging controversies regarding the optimal degree of modification—particularly the debate over whether 100% m1Ψ substitution is universally beneficial or potentially problematic in certain contexts such as cancer immunotherapy [8]—require critical evaluation. Third, the field has reached an inflection point where descriptive observations must give way to mechanistic interpretation to guide rational design of next-generation mRNA therapeutics.

This review therefore aims to: (1) critically evaluate the mechanistic evidence for m1Ψ function, weighing competing hypotheses where they exist; (2) synthesize recent structural and biophysical data that explain m1Ψ's properties at atomic resolution; (3) assess unresolved controversies including dose-dependent effects and context-specific outcomes; and (4) identify priority research directions grounded in evidence rather than speculation.

Scope and methodology: A systematic literature search was conducted using PubMed, Web of Science, and Google Scholar databases (January 2005-March 2026). Search terms included combinations of: "N1-methylpseudouridine," "m1Ψ," "mRNA vaccine," "nucleoside modification," "innate immunity," and "translation efficiency." Inclusion criteria encompassed peer-reviewed original research articles and systematic reviews from established journals. Preclinical studies employing rigorous mechanistic approaches (structural biology, genetic knockout models, primary human cells) were prioritized over descriptive studies using transformed cell lines alone. Preprints were included only when they provided unique mechanistic insights subsequently validated in peer-reviewed literature.

2. Chemical and Structural Basis of m1Ψ Function: Mechanistic Insights from Biophysical Studies

2.1 Structural Properties and Thermodynamic Consequences

N1-methylpseudouridine is a chemically modified nucleoside derived from Ψ through methylation at the N1 position. Understanding its structural properties requires comparison with canonical uridine and its precursor Ψ. Uridine consists of a uracil base attached to ribose via an N-C glycosidic bond. Ψ differs fundamentally: the uracil base is bound to ribose through a carbon-carbon (C-C) bond, introducing greater planarity, rigidity, and an additional hydrogen-bond donor at the N1 position [9]. m1Ψ preserves the C-C bond of Ψ but replaces the N1 hydrogen with a methyl group, resulting in distinct biochemical properties [9].

Canonical uridine base-pairs with adenosine using two hydrogen bonds. Ψ enhances RNA stability by serving as an additional hydrogen-bond donor at N1 and through non-canonical stabilizing interactions [9,10]. The methyl group at the N1 position in m1Ψ blocks this donor capability, reducing hydrogen-bonding capacity compared to Ψ, although base pairing through the uracil moiety is maintained [9]. This alteration in hydrogen bonding potential has important implications for how m1Ψ-modified RNAs interact with both innate immune sensors and the translational machinery.

Landmark biophysical studies: Foundational work by Davis and colleagues established that Ψ increases base stacking stability compared to uridine, with NMR measurements demonstrating that Ψ forms more stable base stacking arrangements that propagate throughout the helix to stabilize neighboring purine nucleosides [11]. Kierzek and coworkers subsequently provided comprehensive thermodynamic data revealing that Ψ can stabilize RNA duplexes when replacing uridine, with the extent of stabilization depending on sequence context and the specific base pair formed [12].

More recently, Dutta and colleagues employed computational approaches combined with experimental validation to demonstrate that m1Ψ provides context-dependent stabilization of base stacking interactions compared to both uridine and Ψ, presumably resulting from increased molecular polarizability due to the presence of the methyl group [10]. Their

results indicate that the methyl group enhances hydrophobic surface area and van der Waals forces, providing additional stacking contributions estimated at 0.3-0.7 kcal/mol beyond Ψ [10]. Molecular dynamics simulations further suggest that m1Ψ-containing RNAs exhibit reduced conformational entropy at modified positions while maintaining overall structural integrity [13].

The combination of altered hydrogen bonding and enhanced base stacking modifies RNA folding patterns. Ψ promotes general structural stability, enhancing stem-loop formation and tertiary motif stability [9]. m1Ψ maintains these stacking interactions while exhibiting decreased hydrogen bonding at the modification site, which can subtly affect local RNA structure and flexibility [9-11,13]. These structural effects provide the physical basis for both the reduced immune recognition and altered translation dynamics observed with m1Ψ-modified mRNAs.

2.2 Synthesis and Manufacturing Considerations

Recent advances in synthetic chemistry have enabled efficient production of m1Ψ triphosphate (m1ΨTP) at preparative scales. A chemoenzymatic approach combining biocatalytic cascade rearrangement of uridine with selective N1-methylation using dimethyl sulfate achieves ~200 mg scale production of isolated m1ΨTP with 68% yield and approximately three-fold less waste compared to traditional chemical routes [14]. This method utilizes *Saccharomyces cerevisiae* uridine 5'-monophosphate kinase for ATP-dependent phosphorylation and *Escherichia coli* acetate kinase for conversion to the triphosphate, enabling scalable manufacturing of this critical mRNA vaccine building block [14]. Despite these advances, the absence of standardized production guidelines, high costs of cGMP-grade reagents for in vitro transcription, and need for robust scale-up capabilities remain significant barriers [15]. Supply chain limitations and evolving regulatory frameworks further complicate clinical translation of modified mRNA therapeutics.

3. Innate Immune Recognition and Modulation by m1Ψ: Molecular Mechanisms and Controversies

3.1 Pattern Recognition Receptors for RNA

Endosomal TLRs–specifically TLR3, TLR7, and TLR8–together with cytoplasmic RNA helicases known as RLRs, including RIG-I and MDA5, serve as key PRRs that recognize foreign RNAs [16,17]. TLR3 primarily recognizes double-stranded RNA (dsRNA) within endosomes, while TLR7 and TLR8 identify single-stranded RNA (ssRNA) containing uridine-rich sequences [16-18]. RIG-I functions as a cytoplasmic sensor of short dsRNA or viral RNA bearing 5'-triphosphate termini, and MDA5 senses longer dsRNA typically generated during viral replication [16,17]. Upon sensing foreign RNA, these PRRs trigger signal cascades activating adaptor proteins–mitochondrial antiviral-signaling protein (MAVS) for RLR sensing; TIR-domain-containing adapter-inducing interferon-β (TRIF) or myeloid differentiation primary response 88 (MyD88) for TLR sensing–and kinases, followed by transcription factors including interferon regulatory factor 3 (IRF3), IRF7, and nuclear factor kappa-B (NF-κB) [19-21].

3.2 The RNase T2/PLD Mechanism: A Paradigm Shift in Understanding Immune Evasion

A landmark study by Bérouti and colleagues in 2025 fundamentally transformed our understanding of how modified nucleosides evade immune detection [7]. Prior to this work, the field understood that Ψ and m1Ψ reduced TLR activation, but the molecular mechanism remained obscure. The investigators systematically dissected the endolysosomal processing pathway required for TLR7 and TLR8 activation.

They demonstrated that the enzyme RNase T2 cleaves RNA at uridine motifs, with highest affinity for GU sequences. This cleavage generates guanosine 2′,3′-cyclic monophosphate (2′,3′-cGMP)-terminated RNA fragments that interact with pocket 2 of TLR7. Subsequently, phospholipase D enzymes (PLD3 and PLD4) liberate the terminal 2′,3′-cGMP to interact with pocket 1 of TLR7, enabling full receptor activation [7].

Critically, when RNA contained Ψ or m1Ψ in place of uridine, RNase T2 could not cleave the RNA, preventing generation of immunostimulatory fragments. Moreover, PLD3/4 exhibited weaker affinity for Ψ- or m1Ψ-containing RNA compared to uridine-RNA, further reducing enzymatic activity. In primary human monocytes, uridine-containing in vitro transcripts elicited TLR8-dependent inflammatory responses characterized by tumor necrosis factor (TNF) and interleukin-6 (IL-6) secretion, while Ψ- or m1Ψ-containing transcripts did not. Similarly, plasmacytoid dendritic cells mounted type I interferon responses against uridine-RNA but not modified RNA, and this response was abolished in RNase T2-deficient cells [7].

These findings have profound implications. First, they establish that the immune evasion properties of Ψ and m1Ψ operate through a specific enzymatic mechanism–resistance to endolysosomal processing–rather than simply passive avoidance of receptor binding. Second, they explain why endogenous RNAs containing Ψ (the most abundant mammalian mRNA modification) avoid triggering autoimmunity. Third, they provide a mechanistic framework for optimizing future modifications: candidates that resist RNase T2 cleavage and PLD engagement will likely exhibit reduced immunogenicity.

3.3 Differential Effects on TLR and RLR Pathways

Recent studies have elucidated differential effects of Ψ and m1Ψ on innate immune sensors. Ψ-containing RNA avoids detection by TLR7/8 through the RNase T2/PLD mechanism described above [7]. m1Ψ similarly evades nuclease processing, resulting in minimal TLR7/8 activation under most conditions. In the cytosol, both Ψ and m1Ψ modifications strongly reduce RIG-I signaling without substantially impeding MDA5 activation, demonstrating distinct effects on different PRR families [22].

A note on terminology: It is important to emphasize that m1Ψ does not "disable" interferon signaling as sometimes suggested in the literature. Rather, it reduces PRR activation and downstream IFN induction to levels that permit efficient translation while retaining sufficient immune engagement for vaccine efficacy. This distinction is critical for understanding both the benefits and limitations of the modification.

3.4 Context-Dependent Effects: The Cancer Controversy

A provocative hypothesis has emerged regarding the potential downside of complete m1Ψ substitution. Rubio-Casillas and colleagues reviewed evidence suggesting that adding 100% m1Ψ to mRNA vaccines in a melanoma model stimulated cancer growth and metastasis, while non-modified mRNA vaccines induced opposite results [8]. They propose that the immune-suppressive effects of complete m1Ψ substitution might impair anti-tumor immunity, raising the question of whether lower percentages of modification might be optimal for cancer applications.

This hypothesis remains controversial and requires rigorous testing. Critics note that the melanoma model studies cited have not been independently replicated, and the clinical success of m1Ψ-modified vaccines in preventing COVID-19 (including in cancer patients) suggests acceptable safety profiles. However, the therapeutic context differs fundamentally from prophylaxis: cancer vaccines aim to stimulate immunity against tumors, and excessive suppression of innate activation might counteract this goal. As Bartok and colleagues note in their commentary, mRNA-based anti-tumor vaccination, particularly of cold tumors, could conceivably benefit from stronger activation of multiple immune receptors to counteract the anti-inflammatory tumor microenvironment [23,24].

This debate highlights an important principle: the optimal degree of nucleoside modification likely depends on application context. For protein replacement therapies where minimal immune activation is paramount, complete m1Ψ substitution may be ideal. For cancer vaccines seeking to break immune tolerance, partial modification or even unmodified formulations might prove superior. CureVac's CVGBM cancer vaccine uses unmodified mRNA with promising phase 1 results as a monotherapy, and BioNTech is investigating non-modified mRNA in its iNest platform [23,24].

3.5 Dose-Dependent Effects and Innate Immune Tolerance

Comparative studies in non-human primates have revealed nuanced differences in immune activation profiles between modified and unmodified mRNA formulations. Engstrand and colleagues evaluated high-dose unmodified and m1Ψ-modified mRNA vaccination using a Gag-antigen model [1]. Unmodified mRNA induced higher levels of IFNα and interleukin-7 (IL-7) compared to m1Ψ-mRNA, even at substantially lower doses. Conversely, m1Ψ-mRNA application resulted in increased IL-6 and TNF release, attributable in part to the higher lipid nanoparticle (LNP) content required for equivalent mRNA dosing, as LNPs themselves can induce these cytokines independently of TLR activation [1].

Notably, unmodified RNA induced a tolerizing effect upon repetitive application, with reduced differential gene expression after the fifth immunization compared to the first. In contrast, high-dose m1Ψ-mRNA maintained consistent immune activation without evidence of tolerance [1]. These findings suggest that the choice of modification influences not only the magnitude but also the kinetics and adaptability of immune responses. These findings are summarized schematically in Figure 1, which contrasts the mechanistic outcomes of m1Ψ-modified mRNA versus unmodified uridine-rich RNA.

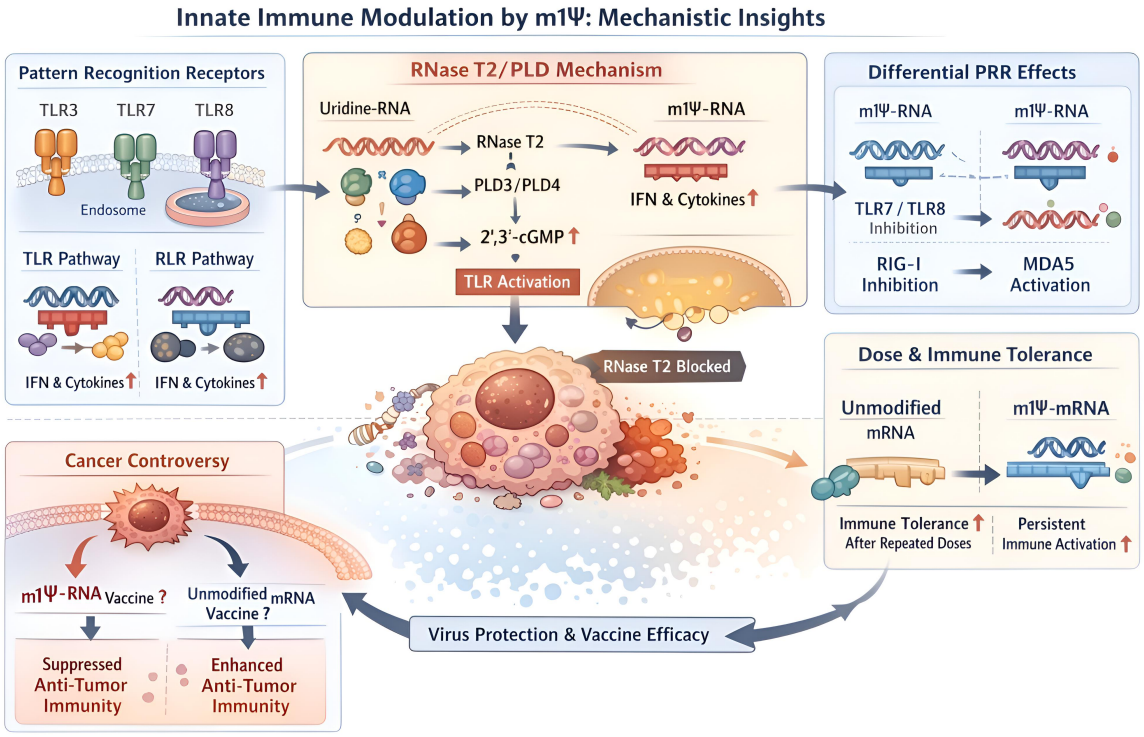

Figure 1. A summary diagram illustrating the mechanistic insights into how m1Ψ-modified mRNA modulates innate immune activation. The modification reduces the innate immune response and prevents RNA degradation, which contrasts with unmodified uridine-RNA that is rapidly processed, inducing strong immune activation and inflammation.

4. Effects on mRNA Stability and Translation Efficiency: Direct Mechanistic Insights

4.1 Enhancement of mRNA Stability

N1-methylpseudouridine decreases mRNA degradation, increases nuclease resistance, and extends the intracellular half-life of mRNA, favoring more sustained antigen generation in vaccines and therapeutics [11,25]. These effects are achieved through chemical reinforcement of the RNA backbone. m1Ψ enhances base stacking and reduces the likelihood of enzymatic cleavage, making RNA less flexible and less susceptible to nuclease attack. Studies with nucleobase-modified nucleic acids have demonstrated that larger and denser modifications, such as m1Ψ, inhibit nuclease binding and reduce degradation, thereby extending RNA half-life even under high-nuclease cellular conditions [25].

The incorporation of m1Ψ into mRNA protects the transcribed message against both exonuclease and endonuclease activity. Compared to unmodified RNA, m1Ψ-modified message exhibits prolonged stability, enabling continuous translation and sustained antigen supply [12,26]. Furthermore, m1Ψ-modified RNAs better evade innate immune sensors, eliminating activation of stress-related mRNA decay pathways. By reducing stress signaling, m1Ψ further extends transcript duration and antigen presence in target cells [27].

4.2 Direct Modulation of Translation Dynamics: Beyond Immune Effects

A longstanding assumption in the field held that m1Ψ's enhancement of translation resulted indirectly from reduced immune activation—by preventing the antiviral state that induces translational arrest, the modification allowed more efficient protein production. However, groundbreaking work published in 2026 has fundamentally revised this view.

Rozman and colleagues employed ribosome profiling at subcodon resolution to demonstrate that m1Ψ increases ribosome density on synthetic mRNAs, leading to higher protein production independent of innate immune activation or eukaryotic translation initiation factor 2α (eIF2α) phosphorylation [6].

Cryo-electron microscopy structural studies revealed that m1Ψ alters interactions within the ribosomal decoding center, providing a mechanistic basis for slowed elongation [6]. The modification directly slows ribosome movement in defined sequence contexts while simultaneously promoting translation initiation. Furthermore, by introducing synonymous recoding that disrupts modification-mediated changes in elongation, the authors showed that m1Ψ-dependent enhancement of protein output is modulated by codon composition. The impact of m1Ψ is strongest in mRNAs containing non-optimal codons with uridines at the wobble position, suggesting that strategic codon optimization can maximize the translational benefits of this modification [6].

At the atomic level, Monroe and colleagues used fully reconstituted bacterial translation systems to reveal that m1Ψ does not substantially change the rate constants for amino acid addition by cognate tRNAs or termination by release factors [28]. However, m1Ψ can subtly modulate the fidelity of amino acid incorporation in a codon-position and tRNA-dependent manner both in vitro and in human cells [28]. Computational modeling demonstrates that altered energetics of mRNA:tRNA interactions largely account for the context dependence of low-level miscoding observed on Ψ- and m1Ψ-containing codons [28]. Importantly, studies examining all three stop codons (UAA, UAG, UGA) reveal that m1Ψ modification does not impede translation termination, with observed rate constants for peptide release on UAA and m1ΨAA codons being equivalent (~0.1 s^{-1}) [28,29].

These findings have profound implications for mRNA design. They suggest that the benefits of m1Ψ are not uniform across all sequence contexts but can be optimized through codon selection. mRNAs containing non-optimal codons—particularly those with uridines at the wobble position—stand to benefit most from m1Ψ modification, while those already optimized for rapid translation may show smaller enhancements.

4.3 Integration: A Two-Component Model

Synthesizing these mechanistic insights, we can now propose a two-component model for m1Ψ function. First, the modification reduces innate immune recognition through both the RNase T2/PLD evasion mechanism [7] and reduced RIG-I activation [22], preventing the establishment of an antiviral state that would otherwise induce translational arrest and mRNA degradation. Second, m1Ψ directly modulates ribosome dynamics in a sequence-dependent manner, slowing elongation at specific codons while promoting initiation, ultimately increasing ribosome density and protein output [6].

This dual mechanism explains why m1Ψ consistently outperforms other modifications and why its effects are most pronounced in specific sequence contexts. It also provides a rational framework for designing optimized mRNAs that leverage both components.

5. Comparative Analysis of Nucleoside Modifications

Synthetic mRNA typically employs three major RNA modifications: m1Ψ, Ψ, and 5-methylcytidine (m5C). While all enhance protein production and decrease immune stimulation, they differ substantially in immunosuppressive potency and translation enhancement capacity [30-32]. A detailed comparison of the three major nucleoside modifications is presented in Table 1.

Table 1. Comparison of commonly used nucleoside modifications in synthetic mRNA.

Feature	N1-Methylpseudouridine (m1Ψ)	Pseudouridine (Ψ)	5-Methylcytidine (m5C)
RNase T2 cleavage resistance	High [7]	High [7]	Not established
PLD3/4 affinity reduction	High [7]	High [7]	Not established
TLR7/8 activation	Very low [7]	Very low [7]	Moderate [31]
RIG-I activation	Low [22]	Low [22]	Moderate [31]
Translation enhancement mechanism	Direct + immune-mediated [6]	Primarily immune-mediated [33]	Modest
Ribosome density effect	Increased [6]	Moderate [33]	Limited
Sequence-dependent modulation	Strong [6]	Weak [28]	Not established
Cellular toxicity	Lowest [30]	Low [30]	Moderate [31]
saRNA compatibility	Demonstrated [34]	Demonstrated [34]	Demonstrated [34]
Clinical preference	Licensed vaccines	Preclinical	Emerging

m1Ψ demonstrates superior immunosuppression compared to Ψ and m5C, explained in part by the RNase T2/PLD evasion mechanism [7]. Incorporation of m1Ψ into mRNA leads to marked reduction in detection by both endosomal and cytoplasmic RNA sensors [30]. Ψ also inhibits immune sensing relative to unmodified uridine, though less effectively than m1Ψ in some contexts [31,35]. m5C exhibits moderate immune-sensing inhibition but is generally less potent than either m1Ψ or Ψ [31,36].

Regarding translation enhancement, m1Ψ surpasses both Ψ and m5C through the recently elucidated direct mechanism [6]. While Ψ improves translation compared to unmodified mRNA, protein yields typically remain lower than with m1Ψ, and the translational effects of m5C are modest compared to both m1Ψ and Ψ [37].

Recent advances in self-amplifying RNA (saRNA) platforms have expanded the utility of modified nucleosides. Contrary to earlier assumptions that modified nucleosides were incompatible with saRNA replication, multiple modified nucleosides including m5C have been successfully incorporated into saRNA at 100% substitution, conferring innate immune evasion and enhanced expression potency [34]. This discovery considerably broadens the scope of saRNA for vaccines with increased potency and entry into previously inaccessible cell types.

6. Functional Role in Approved mRNA Vaccines: Evidence and Interpretation

The use of m1Ψ in clinical mRNA vaccines is based on the fact that it can stabilize the message and suppress innate immune response [1,38]. The change is important to ensure that cytoplasmic RNA sensors do not recognize the RNA as foreign and thus trigger antiviral reaction that would otherwise cause the destruction of mRNA or inhibit translation. As a result, the m1Ψ-modified vaccines are characterized by increased cellular half-lives and translation rates, which increase antigen production [1,38].

Safety data reveal that m1Ψ reduces systemic inflammation. In vivo animal studies comparing uridine-based and m1Ψ-based vaccines demonstrate reduced signatures of inflammatory genes and decreased cytokine levels with m1Ψ modification leading to increased tolerability [1]. This lowered reactogenicity does not affect the efficacy of the vaccine but still keeps the capacity to produce certain immunological reactions.

The m1Ψ-modified mRNA vaccines have proven to be clinically successful when compared to unmodified formulations. Both Pfizer-BioNTech BNT162b2 and Moderna mRNA-1273 vaccines, which also use m1Ψ, had more than 90% efficacy against symptomatic COVID-19 infection [1]. Conversely, the CVnCoV vaccine of CureVac, which employed unmodified mRNA and used the same LNP formulation, only had a 47% efficacy [1,39]. However, this difference should not be credited to m1Ψ only. Gebre and colleagues showed that further optimization of non-coding regions in CV2CoV had analogous reactions in preclinical non-human primate frameworks to BNT162b2, suggesting that sequence optimization, as well as untranslated region design, are additionally significant in vaccine performance [39]. The use of m1Ψ is now incorporated into CureVac's investigational influenza and SARS-CoV-2 vaccines (such as CV0501, CV0601, and CV0701) [1].

In terms of immunogenicity enhancement, m1Ψ allows stable and efficient expression of antigens in host cells, which provokes strong humoral and cellular immune reactions. As an illustration, dual mRNA vaccines expressing SARS-CoV-2 nucleoprotein and spike protein with m1Ψ modification have shown higher cytokine expression, dendritic and natural killer cells activation, and overall coverage of variants of the virus [40]. Outside of COVID-19, m1Ψ-modified mRNA vaccines are in progress against other infectious disease targets, such as Andes virus and influenza, taking advantage of the versatility of the nucleoside modification platform [1,22].

7. Limitations, Knowledge Gaps, and Competing Hypotheses

Despite significant advances, several questions regarding m1Ψ's mechanisms and effects remain unanswered, and competing hypotheses require resolution through targeted experimentation.

7.1 The 100% Modification Debate

As discussed in Section 3.4, a fundamental controversy centers on whether complete m1Ψ substitution is universally optimal or potentially detrimental in certain contexts. The hypothesis advanced by Rubio-Casillas and colleagues–that 100% m1Ψ might suppress anti-tumor immunity in cancer vaccine applications [8]–stands in tension with the established benefits for infectious disease vaccines. Resolution requires: (1) Direct comparative studies in tumor models using identical mRNA sequences differing only in modification percentage. (2) Mechanistic investigation of how modification level affects antigen-presenting cell activation and T cell priming. (3) Clinical trials in cancer patients comparing partially and fully modified formulations

7.2 Long-Term Immune Modulation

Questions remain regarding how m1Ψ incorporation affects the durability and quality of immune responses. While m1Ψ reduces innate sensing and systemic inflammation, resulting in superior antigen-specific antibody responses compared to unmodified uridine, the mechanisms by which this modification influences long-term adaptive immunity–including memory B and T cell formation–remain incompletely understood. The effects of m1Ψ-containing mRNA vaccines on immunoglobulin G (IgG) Fc glycosylation patterns and antibody effector functions over time are also not fully characterized [1,41]. Comparative studies of antibody durability between modified and unmodified mRNA vaccines suggest that while initial antibody titers may be higher with m1Ψ modification, the rate of decline over 4-6 months requires booster doses, similar to other platforms [1,42].

7.3 Dose-Dependent Effects and Thresholds

Although m1Ψ enhances RNA stability and translation, potentially enabling reduced vaccine doses without compromising immunogenicity, the extent to which varying doses of m1Ψ-containing mRNA alter innate immune stimulation and adaptive immune quality remains inadequately defined. Studies in non-human primates using high-dose regimens (160 μg unmodified mRNA versus 400-800 μg m1Ψ-mRNA) reveal complex dose-response relationships, with unmodified mRNA inducing higher IFNα despite much lower dose [1]. The threshold between sufficient innate sensing for effective antigen presentation and excessive sensing that would desensitize expression is finely balanced. How dose variations impact this equilibrium and subsequently affect the breadth and magnitude of adaptive immunity has not been thoroughly investigated.

7.4 Adaptive Immune Response Questions

Recent studies indicate that unmodified mRNA induces higher interferon-gamma (IFNγ) release from CD8+ T cells, while m1Ψ-mRNA demonstrates better induction of CD4+ memory cells, though sample sizes in non-human primate studies remain too small for definitive conclusions [1]. The role of m1Ψ in T cell memory development and immune gene expression modulation in lymphoid and peripheral tissues is partially understood. Furthermore, the impact of m1Ψ on vaccine-induced polyclonal immunity and interactions between m1Ψ and other vaccine components, including LNPs and RNA caps, require additional study [1,40,43].

8. Future Perspectives: Evidence-Based Priorities

Future research on m1Ψ must move beyond descriptive observation toward hypothesis-driven mechanistic investigation. Based on the current evidence base, we identify the following priorities:

Priority 1: Resolve the modification percentage controversy. Direct comparative studies in appropriate disease models (infectious disease versus cancer) using identical mRNA sequences with varying m1Ψ content (0%, 25%, 50%, 75%, 100%) are urgently needed. These studies should assess not only protein expression and immunogenicity but also therapeutic efficacy in relevant disease models.

Priority 2: Elucidate sequence-dependent translation modulation. The finding that m1Ψ effects are strongest in mRNAs containing non-optimal codons with uridines at the wobble position [6] provides a rational basis for sequence optimization. Systematic investigation of codon-context effects across the entire genetic code will enable predictive design of maximally efficient mRNAs.

Priority 3: Investigate combinatorial modifications. Coupling m1Ψ with other nucleotides—including Ψ, m5C, and modified cap structures—may enable precise control over mRNA stability, translation rate, and immune evasion [44,45]. The recent demonstration that multiple modified nucleosides can be incorporated into saRNA at 100% substitution [34] opens new avenues for combinatorial optimization.

Priority 4: Determine m1Ψ effects on saRNA platforms. Understanding how m1Ψ influences RNA replication, antigen production stability, and immune cell activation in saRNA contexts will be essential for next-generation vaccine design [46-48].

Priority 5: Develop predictive computational models. AI and machine learning approaches trained on systematic datasets of m1Ψ-containing mRNA sequences could predict optimal codon usage, secondary structure effects, and translation efficiency [15]. Such models would accelerate design and reduce empiricism.

Priority 6: Enhance delivery vehicles for modified RNAs. LNPs specifically optimized for m1Ψ-modified RNAs may improve targeting, reduce required doses, and enhance therapeutic windows. Recent studies on freeze-drying of mRNA-LNP formulations have shown promise for enhancing long-term stability and enabling distribution without cold-chain requirements [49-51].

9. Conclusion

N1-methylpseudouridine is one of the most impactful nucleoside modifications that enhances mRNA vaccination by lowering immune response, stabilizing transcripts, and improving translation efficiency. Recent mechanistic advances have transformed our perception of how this modification functions. The RNase T2/PLD evasion mechanism explains its immunomodulatory actions at molecular resolution . Direct translation modulation via altered ribosome dynamics confirms that the advantages of m1Ψ are not limited to immune evasion . These insights can be used to rationally design optimized mRNA sequences that maximize the benefit of m1Ψ modification in translation.

The clinical significance of m1Ψ has been emphasized by comparisons of modified and unmodified mRNA vaccines; m1Ψ-based formulations have shown better efficacy and safety profiles, though confounding factors should be taken into consideration. This modification has become the standard for existing mRNA vaccines and is being extended into various therapeutic uses such as cancer immunotherapy, protein replacement, and gene editing.

Nevertheless, significant controversies and knowledge gaps remain. The question of whether 100% m1Ψ substitution is always optimal or potentially harmful in cancer therapies requires rigorous experimental resolution. The effects on adaptive immune memory, dose-dependent responses, and long-term antigen-presenting cell and T cell responses demand further research. The finding that translation modulation is sequence-dependent [6] offers an opportunity for optimization but also a challenge in generalizing design rules.

By focusing on these priorities with hypothesis-based mechanistic studies, the translational and immunological potential of m1Ψ can be maximized, enabling safer, more effective, and adaptable mRNA vaccines and therapeutics for infectious diseases, cancer, and beyond. Further development of mRNA technology, grounded in nucleoside modification principles, will continue to revolutionize medicine, offering rapid and flexible platforms to combat emerging health threats and chronic diseases alike.

Conflict of Interest

The authors declare no conflict of interest.

Generative AI Statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Reference

[1] Engstrand O, Joas G, Miranda MC, Yan X, Lenart K, Cerveira RA, et al. Immunogenicity and innate immunity to high-dose and repeated vaccination of modified mRNA versus unmodified mRNA. Molecular Therapy Nucleic Acids, 2025, 36, 102588. DOI: 10.1016/j.omtn.2025.102588

[2] Karikó K, Buckstein M, Ni H, Weissman D. Suppression of RNA recognition by toll-like receptors: The impact of nucleoside modification and the evolutionary origin of RNA. Immunity, 2005, 23(2), 165-175. DOI: 10.1016/j.immuni.2005.06.008

[3] Nelson J, Sorensen EW, Mintri S, Rabideau AE, Zheng W, Besin G, et al. Impact of mRNA chemistry and manufacturing process on innate immune activation. Science Advances, 2020, 6(26), eaaz6893. DOI: 10.1126/sciadv.aaz6893

[4] Li S, Luo P, Fan J, Li Y, Tu J, Long X. RNA modifications in health and disease. MedComm, 2025, 6(9), e70341. DOI: 10.1002/mco2.70341

[5] Ho LLY, Li R, Zhang Y. Pseudouridine and N1-methylpseudouridine as potent nucleotide analogues for RNA therapy and vaccine development. RSC Chemical Biology, 2024, 5(5), 418-425. DOI: 10.1039/d4cb00022f

[6] Rozman B, Broennimann K, Shanmugha Rajan K, Nachshon A, Saha C, Arazi T, et al. N1-Methylpseudouridine directly modulates translation dynamics. Nature, 2026, 651, 533-541. DOI: 10.1038/s41586-025-09945-5

[7] Bérouti M, Wagner M, Greulich W, Piseddu I, Gartig J, Hansbauer L, et al. Pseudouridine RNA avoids immune detection through impaired endolysosomal processing and TLR engagement. Cell, 2025, 188(18), 4880-4895. DOI: 10.1016/j.cell.2025.05.032

[8] Rubio-Casillas A, Cowley D, Raszek M, Uversky VN, Redwan EM. Review: N1-methyl-pseudouridine (m1Ψ): Friend or foe of cancer? International Journal of Biological Macromolecules, 2024, 267(Pt 1), 131427. DOI: 10.1016/j.ijbiomac.2024.131427

[9] Shen LX, Cai Z, Tinoco I. RNA structure at high resolution. FASEB Journal, 1995, 9(11), 1023-1033. DOI: 10.1096/fasebj.9.11.7544309

[10] Dutta N, Deb I, Sarzynska J, Lahiri A. Structural and thermodynamic consequences of base pairs containing pseudouridine and N1-methylpseudouridine in RNA duplexes. bioRxiv, 2023. DOI: 10.1101/2023.03.20.533518

[11] Davis DR. Stabilization of RNA stacking by pseudouridine. Nucleic Acids Research, 1995, 23(24), 5020-5026. DOI: 10.1093/nar/23.24.5020

[12] Kierzek E, Malgowska M, Lisowiec J, Turner DH, Gdaniec Z, Kierzek R. The contribution of pseudouridine to stabilities and structure of RNAs. Nucleic Acids Research, 2014, 42(5), 3492-3501. DOI: 10.1093/nar/gkt1330

[13] Šponer J, Leszczynski J, Hobza P. Thioguanine and thiouracil: Hydrogen-bonding and stacking properties. The Journal of Physical Chemistry A, 1997, 101(49), 9489-9495. DOI: 10.1021/jp9720404

[14] Pfeiffer M, Krammer L, Zöhrer J, Breinbauer R, Nidetzky B. Integrated chemoenzymatic synthesis of the mRNA vaccine building block N1-Methylpseudouridine triphosphate. Angewandte Chemie International Edition, 2025, 64(34), e202506330. DOI: 10.1002/anie.202506330

[15] Jung SJ, Seo JJ, Lee S, Hyun SI, Lee JE, Lee S, et al. RNA stability enhancers for durable base-modified mRNA therapeutics. Nature Biotechnology, 2025. DOI: 10.1038/s41587-025-02891-7

[16] Kawai T, Akira S. Innate immune recognition of viral infection. Nature Immunology, 2006, 7(2), 131-137. DOI: 10.1038/ni1303

[17] Said EA, Tremblay N, Al-Balushi MS, Al-Jabri AA, Lamarre D. Viruses seen by our cells: The role of viral RNA sensors. Journal of Immunology Research, 2018, 2018, 1-14. DOI: 10.1155/2018/9480497

[18] Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science, 2004, 303(5663), 1526-1529. DOI: 10.1126/science.1093620

[19] Carty M, Guy C, Bowie AG. Detection of viral infections by innate immunity. Biochemical Pharmacology, 2020, 183, 114316. DOI: 10.1016/j.bcp.2020.114316

[20] Nan Y, Nan G, Zhang YJ. Interferon induction by RNA viruses and antagonism by viral pathogens. Viruses, 2014, 6(12), 4999-5027. DOI: 10.3390/v6124999

[21] Lutz J, Lazzaro S, Habbeddine M, Schmidt KE, Baumhof P, Mui BL, et al. Unmodified mRNA in LNPs constitutes a competitive technology for prophylactic vaccines. NPJ Vaccines, 2017, 2(1), 29. DOI: 10.1038/s41541-017-0032-6

[22] Prokhorova D, Matveeva A, Zakabunin A, Ryabchenko A, Stepanov G. Influence of N1-methylpseudouridine in guide RNAs on CRISPR/Cas9 activity. International Journal of Molecular Sciences, 2023, 24(23), 17116. DOI: 10.3390/ijms242317116
[23] Bartok E, Hartmann G. Immune sensing mechanisms that discriminate self from altered self and foreign nucleic acids. Immunity, 2020, 53(1), 54-77. DOI: 10.1016/j.immuni.2020.06.014
[24] Bartok E, Zillinger T, Hartmann G. To modify or not to modify–That is still the question for some mRNA applications. Molecular Therapy Nucleic Acids, 2025, 36(3), 102655. DOI: 10.1016/j.omtn.2025.102655
[25] Gu R, Oweida T, Yingling YG, Chilkoti A, Zauscher S. Enzymatic synthesis of nucleobase-modified single-stranded DNA offers tunable resistance to nuclease degradation. Biomacromolecules, 2018, 19(8), 3525-3535. DOI: 10.1021/acs.biomac.8b00816
[26] Gaddam D, Stevens N, Hollien J. Comparison of mRNA localization and regulation during endoplasmic reticulum stress in Drosophila cells. Molecular Biology of the Cell, 2012, 24(1), 14-20. DOI: 10.1091/mbc.e12-06-0491
[27] Parr CJC, Wada S, Kotake K, Kameda S, Matsuura S, Sakashita S, et al. N1-Methylpseudouridine substitution enhances the performance of synthetic mRNA switches in cells. Nucleic Acids Research, 2020, 48(6), e35. DOI: 10.1093/nar/gkaa070
[28] Monroe J, Eyler DE, Mitchell L, Deb I, Bojanowski A, Srinivas P, et al. N1-Methylpseudouridine and pseudouridine modifications modulate mRNA decoding during translation. Nature Communications, 2024, 15(1), 8119. DOI: 10.1038/s41467-024-51301-0
[29] Svidritskiy E, Korostelev AA. Mechanism of translation termination. Nature Structural & Molecular Biology, 2018, 25, 577-586. DOI: 10.1038/s41594-018-0086-5
[30] Loomis KH, Lindsay KE, Zurla C, Bhosle SM, Vanover DA, Blanchard EL, et al. *In vitro* transcribed mrna vaccines with programmable stimulation of innate immunity. Bioconjugate Chemistry, 2018, 29(9), 3072-3083. DOI: 10.1021/acs.bioconjchem.8b00443
[31] Andries O, Mc Cafferty S, De Smedt SC, Weiss R, Sanders NN, Kitada T. N1-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice. Journal of Controlled Release, 2015, 217, 337-344. DOI: 10.1016/j.jconrel.2015.08.051
[32] Finol E, Krul SE, Hoehn SJ, Lyu X, Crespo-Hernández CE. The mRNACalc webserver accounts for the N1-methylpseudouridine hypochromicity to enable precise nucleoside-modified mRNA quantification. Molecular Therapy Nucleic Acids, 2024, 35(2), 102171. DOI: 10.1016/j.omtn.2024.102171
[33] Svitkin YV, Cheng YM, Chakraborty T, Presnyak V, John M, Sonenberg N. N1-methyl-pseudouridine in mRNA enhances translation through eIF2α-dependent and independent mechanisms by increasing ribosome density. Nucleic Acids Research. 2017, 45(10), 6023-6036. DOI: 10.1093/nar/gkx135
[34] Nucleoside Modified Self-Amplifying RNA for Vaccines and in situ Therapeutics. Boston University Events Calendar, 2024. Available from: https://www.bu.edu/eng/news-events/events-calendar/ (accessed on 01 March 2026).
[35] Anderson BR, Muramatsu H, Nallagatla SR, Bevilacqua PC, Sansing LH, Weissman D, et al. Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation. Nucleic Acids Research, 2010, 38(17), 5884-5892. DOI: 10.1093/nar/gkq347
[36] Kormann MSD, Hasenpusch G, Aneja MK, Nica G, Flemmer AW, Herber-Jonat S, et al. Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. Nature Biotechnology, 2011, 29(2), 154-157. DOI: 10.1038/nbt.1733
[37] Gilbert WV, Bell TA, Schaening C. Messenger RNA modifications: Form, distribution, and function. Science, 2016, 352(6292), 1408-1412. DOI: 10.1126/science.aad8711
[38] Bonam SR, Hazell NC, Mathew MJ, Liang Y, Zhang X, Wei Z, et al. Innate and adaptive immune parameters following mRNA vaccination in mice. Vaccines, 2024, 12(5), 543. DOI: 10.3390/vaccines12050543
[39] Gebre MS, Rauch S, Roth N, Yu J, Chandrashekar A, Mercado NB, et al. Optimization of non-coding regions for a non-modified mRNA COVID-19 vaccine. Nature, 2022, 601, 7893, 410-414. DOI: 10.1038/s41586-021-04231-6
[40] Xu S, Yang K, Li R, Zhang L. mRNA vaccine era-mechanisms, drug platform and clinical prospection. International Journal of Molecular Sciences, 2020, 21(18), 6582. DOI: 10.3390/ijms21186582
[41] Reinig S, Kuo C, Wu CC, Huang SY, Yu JS, Shih SR. Specific long-term changes in anti-SARS-CoV-2 IgG modifications and antibody functions in mRNA, adenovector, and protein subunit vaccines. Journal of Medical Virology, 2024, 96(7), e29793. DOI: 10.1002/jmv.29793
[42] Heine A, Juranek S, Brossart P. Clinical and immunological effects of mRNA vaccines in malignant diseases. Molecular Cancer, 2021, 20(1), 1-14. DOI: 10.1186/s12943-021-01339-1
[43] Roy K, Gao J. mRNA-LNP formulation challenges and solutions. Expert Opinion on Drug Delivery. 2025. In press.
[44] Lundstrom K. Latest development on RNA-based drugs and vaccines. Future Science OA, 2018, 4(5), FSO300. DOI: 10.4155/fsoa-2017-0151
[45] Maruggi G, Ulmer JB, Rappuoli R, Yu D. Self-amplifying mRNA-based vaccine technology and its mode of action. Current Topics in Microbiology and Immunology, 2021, 440, 31-70. DOI: 10.1007/82_2021_233
[46] Schmidt C, Schnierle BS. Self-amplifying RNA vaccine candidates: Alternative platforms for mRNA vaccine development. Pathogens, 2023, 12(1), 138. DOI: 10.3390/pathogens12010138
[47] Yıldız A, Răileanu C, Beissert T. Trans-amplifying RNA: A journey from alphavirus research to future vaccines. Viruses, 2024, 16(4), 503. DOI: 10.3390/v16040503
[48] Yang R, Cui J. Advances and applications of RNA vaccines in tumor treatment. Molecular Cancer, 2024, 23(1), 1-19. DOI: 10.1186/s12943-024-02141-5
[49] Khan FH, Baudin F, Perumal AS, Kamen AA. Freeze-drying of mRNA-LNPs vaccines: A review. Vaccines, 2025, 13(8), 853. DOI: 10.3390/vaccines13080853
[50] Youssef C, Hitti J, Puppin Chaves Fulber J, Khan MFH, Sudalaiyadum Perumal A, Kamen AA. Preliminary evaluation of formulations for stability of mRNA-LNPS through freeze-thaw stresses and long-term storage. 2025. DOI: 10.20944/preprints202505.2093.v2
[51] Khan FH, Perumal AS, Kamen AA. Investigation on a freeze-drying process for long-term stability of mRNA-LNPs. Vaccines, 2026, 14(3), 242. DOI: 10.3390/vaccines14030242