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Review

Innate sensing of mRNA vaccines

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With the recent success of mRNA vaccines and the approval of several RNA oligonucleotide therapeutics, RNA holds great promise for future drug development. The rise of RNA therapeutics has been enabled by the tremendous progress in our understanding of the sophisticated cellular mechanisms that disarm potentially dangerous exogenous RNA and safeguard RNA homeostasis. Exogenous RNA, such as an mRNA vaccine when injected, faces an intricate system of immune-sensing receptors, restriction factors, and nucleases referred to as nucleic acid immunity. A careful analysis of the functional interaction between the innate response to mRNA, the efficacy to translate the encoded protein antigen, and the quality of the resulting adaptive immunity bears great potential for further improvement of mRNA vaccines and RNA therapeutics for various clinical applications. In this review, we summarize the most recent efforts to advance mRNA vaccines by capitalizing on recent insight in innate RNA sensing.

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Introduction

In 1989, Charlie Janeway coined the famous term "the dirty little secret of immunologists", emphasizing that minute hardly detectable amounts of microbial by-products trigger the innate immune system to costimulate the formation of antigen-specific adaptive immunity [1]. While the structural identity and the receptor-mediated recognition of diverse microbial by-products such as lipopolysaccharide are well-characterized by now, and the necessity of defined adjuvants in protein-antigen-based vaccines is commonly accepted, the astonishing triumph of mRNA vaccines in the Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic may provoke the question of the 'dirty little secret' involved in those vaccines. Just as has been done for conventional vaccines, the exact definition of the molecular substrate, the mechanisms of detection, and the functional consequences for the resulting adaptive immunity is not only urgently required but also bears great potential for future development of RNA therapeutics in general. In principle, both the intended constituents of the vaccine preparation (mRNA and lipid nanoparticles), and unintended contaminants generated during the manufacturing process (e.g. RNA by-products), can contribute to the induction of costimulatory activity of the innate immune system. One might predict that only a well-defined innate immune stimulus in mRNA vaccines will finally suffice the quality requirements of future mRNA vaccines.

The innate immune system has developed highly sophisticated mechanisms to detect invading pathogens and damage based on the presence of foreign nucleic acids (NA) or of modified or displaced self-NA [2–6]. As with any exogenous NA, therapeutic RNA is confronted with this highly efficient detection and defense system, termed *nucleic acid immunity* [3]. Broadly, one can divide this system into three functional classes of genes, which identify, neutralize, and amount an immune response to self- or modified NA. The first group of genes are *immune-sensing receptors* that are characterized by their ability to induce cytokine production. The second group, antiviral restriction factors, directs restricting the function of the NA recognized. A third group of genes, the nucleases and enzymes of NA metabolism, participates in these processes not only by degrading NA, but by generating degradation products that function as ligands for immune-sensing receptors, such as TLR8 and TLR9 [7–10]. All three groups, immune-sensing receptors, restriction factors, and nucleases, are tightly intertwined and cooperate to initiate the most appropriate defense. On the other hand, dysregulation of this system can cause pathologies and disease (Figure 1).

A number of discoveries in the field of NA immunity have prepared the ground for the success of mRNA vaccines to contain the SARS-CoV-2 pandemic. One key

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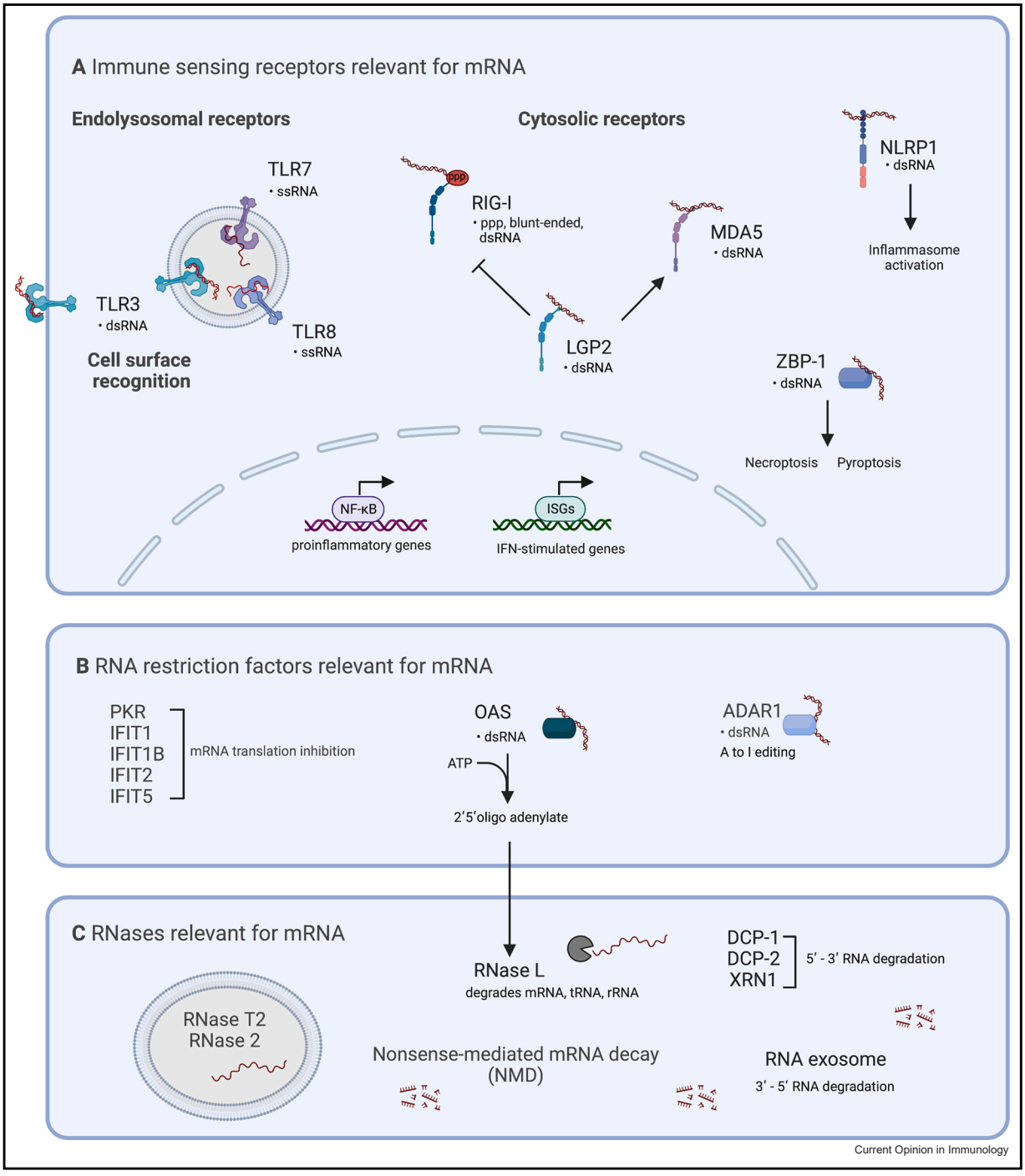
Figure 1



Overview of mechanisms participating in nucleic acid immunity. **(a)** RNA immune-sensing receptors are characterized by their ability to activate immune signaling pathways that induce cytokine release. Depending on the type of receptor and the cell type in which the receptor is expressed, these cytokines include type-I IFN (IFN-α, IFN-β), interferon-inducible genes (ISGs), and inflammatory cytokines and chemokines. **(b)** RNA-restriction factors exhibit direct effects on the RNA detected or induce cell-autonomous responses that are geared toward specific or global inhibition of translation. **(c)** RNases both degrade RNA and to generate degradation products recognized by the immune-sensing receptor TLR8.

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feature is the 5′-triphosphate end of RNA generated by *in vitro* transcription (IVT), and which is detected by retinoic acid-inducible gene-I (RIG-I) if presented as double-stranded blunt-end RNA [11,12], and ignored by RIG-I if the N1 position of the 5′-end is methylated [13]. A second key structural feature is double-stranded (ds)RNA, present in mRNA preparations either as stretches of dsRNA within the secondary structure of the single-stranded(ss) molecule, or as unintended by-products of the *in vitro* transcription process. DsRNA detection is length-dependent and mediated by the immune-sensing receptors TLR3, melanoma differentiation-associated protein 5 (MDA5), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) Family Pyrin Domain Containing 1 (NLRP1), and multiple restriction factors, including Protein kinase R (PKR), Oligoadenylate synthetase (OAS), and Adenosine deaminase acting on RNA (ADAR1) [2,14,15]. Consequently, purification protocols have been established to remove unintended RNA contaminants in mRNA preparations [16–18]. A third molecular key component of mRNA is the uridine-based recognition of unmodified RNA by TLR7 and TLR8 [9,10,19–21]. The answer is the replacement of uridine in RNA by pseudouridine or N1-methyl-pseudouridine in mRNA vaccines, which not only substantially reduces immune sensing but also enhances RNA stability and translation efficiency [22,23].

In general terms, innate sensing of mRNA is determined by the structure and the concentration of mRNA itself and by the content of contaminating unintended by-products. In the following paragraphs, we outline the recent efforts to improve mRNA vaccines by i) characterizing the innate and adaptive immunological consequences of current mRNA vaccines, by ii) adapting the sequence and structure to achieve better mRNA designs, by iii) optimizing the *in vitro* transcription-based manufacturing process, by iv) developing better purification procedures, and by v) adding defined innate immune-adjuvant activities.

Characterization of the innate and adaptive immune response to current mRNA vaccines

Few studies have analyzed the properties of the approved COVID-19 mRNA vaccines (BNT162b2 and mRNA-1273) to activate innate immunity *in vivo*. In one recent study [24], the authors performed a comprehensive profiling of the innate and adaptive immune responses of 56 volunteers vaccinated with the Pfizer–BioNTech mRNA vaccine (BNT162b2). Besides the expected robust induction of a SARS-CoV-2-specific adaptive immunity with neutralizing antibodies (IgG) and antigen-specific CD4 and CD8 T cells after the second dose, bulk mRNA sequencing of whole blood revealed the induction of antiviral and interferon-response modules (type-I interferon response, RIG-I-like signaling, and viral sensing) already one day after the first vaccination, clearly indicating a direct innate immune activation by the mRNA vaccine. The nearly fourfold increase of differentially expressed genes after the second vaccination as compared with the first injection included a broader innate response. However, the results suggested that Interferon-γ (IFN-γ) released by SARS-CoV-2- specific T cells upon secondary antigen contact was responsible for the increased broader response after the boost, but identity and localization of IFN-γ-releasing T cells remained open in that study.

In a follow-up study [25••], the authors further characterized the immune response to the mRNA vaccine BNT162b2 in mice. Here, they identified CD8 T cells in draining lymph nodes and NK cells as the major producers of circulating IFN-γ and thus responsible for the enhanced and broader innate immune activation after the boost vaccination. Interestingly, the use of a panel of knockout mice revealed that induction of adaptive immunity to BNT162b2 was dependent on MDA5 signaling but not TLR2, TLR3, TLR4, TLR5, TLR7, inflammasome activation, or necroptosis or pyroptosis cell death pathways. While activation of MDA5 explains the early type-I IFN-driven innate response observed in this study, species differences of innate immune receptors between mouse and human need to be considered. For example, the observation in this study in mice that inflammasome activation is not required for the induction of adaptive immunity, demonstrates that murine NLRP1B is not critically involved in this animal model, but does not exclude a contribution of the newly identified dsRNA sensor NLRP1 in humans [26].

Of note, in humans, it has been observed that inappropriately high innate immune activation as reflected by clinical signs such as severe headache, fatigue, chills, and injection-site pain might limit dosing and thus efficacy of mRNA vaccines [26–28]. Nonetheless, less pronounced innate immune responses relative to the dose administered have been seen in phase-I clinical studies of candidate mRNA vaccines in which uridine has been replaced by N1-methylpseudouridine [29]. Pseudouridine has been reported to reduce recognition by a number of innate receptors and restriction factors, including TLR7 and TLR8 [22,30], TLR3 [31], RIG-I [11,32] MDA5 [33], PKR [34], and OAS [35]. With the recent insight of the role of RNase T2 and RNase 2 in generating RNA-degradation products for TLR8 activation [9,10], and with the observation that MDA5 at least in mice is required for the induction of adaptive

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immunity [25••], the impact of N1-methylpseudouridine on the recognition by the different innate receptors, especially MDA5, needs to be revisited.

Optimization of sequence and structure of mRNA

The general building blocks of a mRNA as used in current mRNA vaccines comprise a 5′- cap1 structure (a N7-methylguanosine linked to the first nucleotide through a 5′–5′ triphosphate bond and a 2′-O-methyl group at the first nucleotide) to escape from RIG-I recognition, a 5′-untranslated region (UTR), the coding sequence (CDS) for the desired antigen, a 3′-UTR and a poly-A tail. The mRNA synthesis is performed from a DNA template that is composed of at least the 5′- and 3′-UTRs and the CDS [36–38]. UTRs contain regulatory elements to recruit cellular factors to the 5′- and 3′-end of the mRNA [39]. Changes of the UTRs in sequence and structure have been introduced to enhance translation efficacy [40,41•], in-cell stability, and the ribosomal load of mRNA [41•]. In one approach, the RNA sequencing-based platform Pooled Evaluation of mRNA in-solution Stability, and In-cell Stability and Translation RNA-seq (PERSIST-seq) was developed, which enables parallel evaluation of the effects of changes of UTR and CDS on mRNA-translation efficiency and stability inside cells, as well as stability in solution *in vitro*. The authors generated a library of hundreds of mRNAs with variable 5′- or 3′-UTR designs. They found that the commonly used uridine modifications pseudouridine and N1-methylpseudouridine improved stability of mRNAs in solution and in cells. The authors derived design rules for highly structured mRNAs with improved stability. *In-cell stability* was identified as a significant, independent characteristic connected to high translational efficacy of a mRNA, and 'superfolder' RNA structures were revealed to be optimal both in terms of stability and expression [41•]. However, from an immunological perspective, it is important to note that this study used transfected HEK293T cells throughout, cells that are known to lack functional nucleic acid immune-sensing pathways such as MDA5, TLR3, TLR7, and TLR8, as well as the DNA sensing pathway cyclic GMP-AMP synthase (cGAS)/Stimulator of interferon genes (STING), all features that render HEK293T exceptionally amenable to transfection with nucleic acids. However, as a direct consequence of this amenability, this screening platform misses numerous aspects of the innate recognition properties of mRNA, a feature that highly structured 'superfolder' mRNA in their study most likely exhibits and which ultimately will determine translation efficacy and induction of adaptive immunity in an organism *in vivo*.

Optimization of the noncoding regions of a mRNA vaccine was examined in two other recent studies that used the relevant *in vivo* readouts in mice (ACE2 transgenic mice) and nonhuman primates (cynomolgus macaques). They demonstrated that integrating 5′-UTR HSD17B4- and 3′-UTR PSMB3 elements followed by a histone stem-loop motif and a poly-A sequence greatly improved the protective efficacy of a nonmodified mRNA SARS-CoV-2 vaccine (CV2CoV) [42,43] Interestingly, the mRNA vaccine with the optimized noncoding region, CV2CoV, induced a higher type-I IFN response (IFNα2a, IP-10, and MIP-1) in the sera of nonhuman primates at 24 h after the first injection as compared with the original mRNA CVnCoV [43]. Obviously, the higher innate immune response seen with the changes in the noncoding region did not negatively affect but may have even contributed to the better vaccine outcome [29].

Another study investigated the effects of primary sequence and structural stability of a mRNA on protein translation [44]. Using combined computational sequence design and global modified nucleotide substitution in different mRNAs (Enhanced green fluorescent protein (eGFP), human erythropoietin (hEPO), and Luc), they found that a reduced secondary structure within a 5′-leader region (5′-UTR and about 10 codons of the CDS) and an increased secondary structure in the rest of the mRNA (CDS and 3′-UTR) correlated with high protein translation. The authors also concluded that the secondary structure of mRNA is an important determinant of both mRNA stability and translation efficacy, although the structure proposed differs from [41•]. Furthermore, they found a roughly equivalent positive impact of secondary structure and codon optimization on translation efficacy. Although the two main nucleotide modifications used, N1-methylpseudouridine and 5-methoxy-uridine, reduced innate immune activation as shown by others, again, innate immunity possibly affecting translation efficacy was not addressed in that study. Therefore, despite the recent insight into the impact of sequence and structure of mRNA on stability and translation efficacy, current information on the impact of GC content, codon usage, and secondary structure on innate immune recognition remains incomplete (Figure 2).

Improvement of RNA quality through refinements of the *in vitro* transcription process

Inherent features in the enzymatic process of IVT provide a source of unintended contaminating RNA molecules that can form dsRNA, the prototypic nucleic acid ligand for detection by the innate immune system [2,12]. Thus, it has been a goal in the development of mRNA vaccines to find methods to reduce the content of such contaminating RNA molecules [45,46•]. Numerous protocols for the generation of *in vitro*-transcribed

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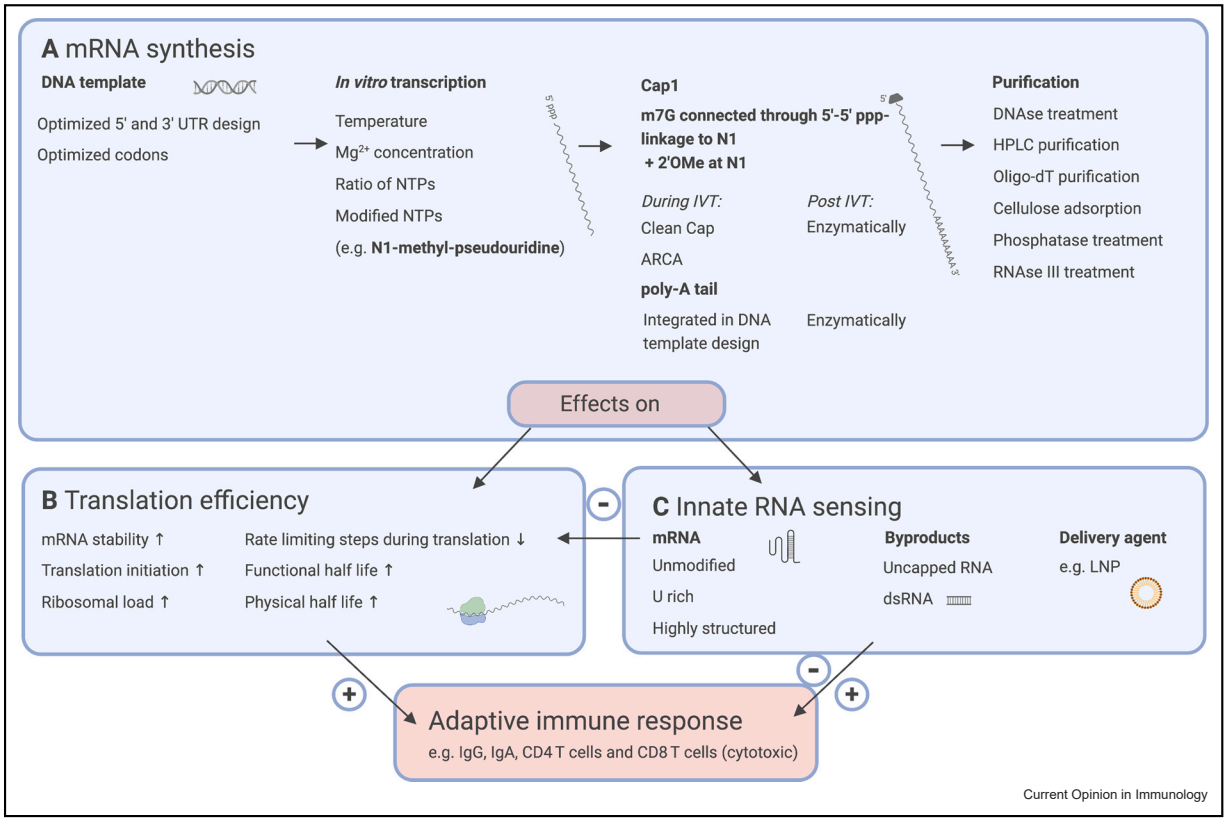
Figure 2



Overview of the various steps of the mRNA-synthesis process and their impact on the translation efficiency and innate immune activation and the resulting adaptive immune responses. **(a)** mRNA synthesis is performed from a DNA template that is optimized in its 5′- and 3′-UTR design and coding sequence. *In vitro* transcription generates the mRNA molecule. Various input factors, for example, temperature or nucleoside triphosphate (NTP) composition, have been shown to influence the quality of the resulting product. To disguise the mRNA as 'endogenous mRNA', a Cap1 (a N7-methylguanosine linked to the first nucleotide through a 5′–5′ triphosphate bond and a 2′-O-methyl group at the first nucleotide) and a poly-A tail are attached to the mRNA. Removal of dsRNA and uncapped by-products is facilitated through several purification methods. **(b)** The translation efficiency determines the amount of antigen translated from the mRNA. The process is influenced by multiple factors, for example, mRNA stability and translation initiation. **(c)** Innate RNA sensing of the mRNA, the by-products resulting from the manufacturing process, and the delivery agent all affect directly the translation efficiency and the adaptive immune response.

Table 1

Parameters affecting RNA purity.

In vitro transcription conditions	dsRNA by-product	References
DNA-template concentration ↗	-	[33]
Temperature ↑	↓	[47••]
NTP concentration ↗	-	[33]
Mg^{2+} concentration ↓	↓	[33]
NaCl concentration ↗	?	[33,48]
Ψ, m1Ψ, 5mC	↓	[33]
m6A	↑	[33]

Increased (↑), decreased (↓), varied (↗), no effect (-), controversial (?).

mRNA are available from the literature. However, to date, it remains unclear how the different steps and conditions of the enzymatic mRNA-synthesis procedure influence the final RNA product with respect to innate immune sensing. Clearly, the analytical methods to assess innate immune-activation properties of RNA products from IVT need to be further refined and standardized, as well (Table 1).

In principle, scalable enzymatic mRNA synthesis is not particularly challenging. Current efforts to further improve the manufacturing process aim at the production of high-quality mRNA with minimal contaminating RNA by-products resulting from T7 RNA polymerase (RNAP) activity, the most common RNA polymerase. A recent publication analyzed the role of the cytosolic innate immune receptors MDA5 and RIG-I in detecting mRNA and the RNA by-products [33]. They used HEK293T cells transiently transfected with RIG-I or MDA5- expressing plasmids and determined the induction of IFN-β levels in response to different IVT-RNA products. As expected, treatment of the IVT-RNAs with calf intestinal phosphatase (CIP), which

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removes the 5′-triphosphate, abrogated activation of RIG-I but not activation of MDA5. To further investigate the RIG-I-independent MDA5 stimulatory activity, they included RNase treatment, native Polyacrylamide gel electrophoresis (PAGE), and MDA5–adenosine triphosphatase (ATPase) analysis, and found that mainly dsRNA by-products, and less so the secondary structure of the primary mRNA, are responsible for MDA5 activation. Interestingly, modified NTPs incorporated into the IVT-RNAs were found to affect the level of dsRNA by-products. IVT-RNA generated with pseudouridine, N1-methyl-pseudouridine, and 5-methylcytidine (5mC) all reduced dsRNA by-product formation, while synthesizing IVT-RNA in the presence of N6-methyladenosine (m6A) induced almost the same levels of dsRNA by-products than IVT-RNA generated with unmodified nucleotides. Finally, IVT-reaction conditions were studied. While changing the concentration of the RNAP, of the DNA template, of NTPs, and of NaCl concentration had little effect on the level of dsRNA by-products, decreasing the $MgCl_2$ concentration in fact reduced the formation of dsRNA by-products [33]. The authors concluded that I) lowering the Mg^{2+} concentration during the IVT reduces dsRNA by-products responsible for MDA5 activation, and II) CIP treatment eliminates uncapped 5′-triphosphate ends responsible for RIG-I activation.

Another approach was the use of a commercially available, thermostable version of RNAP allowing to raise the temperature during IVT from the commonly used 37–50 °C [47••]. The RNA produced under these high-temperature IVT conditions contained substantially less dsRNA by-products as evidenced by denaturing gel analysis of the IVT products, intact mass spectrometry, and dsRNA immunoblot. The authors reported two main sources of dsRNA by-products, one arising from 3′ extension of the run-off product, and one formed by the production of antisense RNAs. They demonstrate that high temperature predominantly reduced the 3′-extension of the run-off product. Furthermore, they show that RNAs synthesized with the thermostable RNAPs exhibit fully functional translation in cells *in vivo* and a reduced innate immune response in dendritic cells *in vitro*. They concluded that the combination of template-encoded poly-A tailing and high-temperature IVT reduces the formation of both types of dsRNA by-products (antisense RNAs and run-off RNA), and thus might alleviate the need for postsynthesis purification.

The authors of another study demonstrated that 3′-extended by-products can be diminished by the addition of a DNA oligonucleotide, so-called capture DNA, that is complementary to the 3′-end of the expected run-off RNA in order to prevent self-primed extension [49]. Reduced self-primed extension by the addition of capture DNA was confirmed for conditions commonly applied to achieve high RNA yields. To avoid the generation of impurities from primed synthesis at the 3′-end of the capture DNA, the authors replaced the 3′-sugar hydroxyl of the 3′-terminal base of the capture DNA with an amino group. Although in this work evidence is presented for templates encoding different RNA sequences, the applicability of this approach to long sequences such as protein-encoding mRNA awaits confirmation.

In a separate study, the same lab proposed the use of high-salt concentrations to destabilize RNAP-promoter DNA binding and to reduce RNAP–RNA-product rebinding known to cause unwanted by-products during IVT [48]. The authors cotethered the RNAP and a DNA-promotor strand in close proximity on magnetic beads and carried out IVT under high-salt conditions, resulting in increased yields of highly pure RNA in the case of relatively short RNAs (24 and 34 nucleotides). Longer RNAs such as mRNAs have not been included in these analyzes.

A frequently studied way of changing IVT conditions is to replace the most common natural nucleotides (A, U, G, C) by modified nucleotides, for example, pseudouridine (Ψ), N1-methyl-pseudouridine (m1Ψ), 2-thiouridine (s2U), N6-methyladenosine (m6A), and 5mC. Such substitutions are known to affect the activation of immune-sensing receptors [11,22,23,31,34,35,50], the abundance of mRNA impurities [17,18,33,51••], and the efficiency of protein translation [23,31,34,35]. The covariance of these three aspects complicates the identification of primary and secondary effects of modified nucleotides. Furthermore, recent work introduced phosphothioate (PS) modifications into the backbone of mRNA by substituting NTPs with nucleoside triphosphate αS (NTPαS) [52•]. The authors analyzed transcription and translation of the PS-modified mRNAs in an *E. coli*-based cell-free translation system called PURE. Introduction of all 16 combinations of the four NTPαS during transcription from a DNA template for Flag-tagged epidermal growth factor (FLAG-EGF) mRNA decreased the RNA yield as assessed by denaturing PAGE analysis. On the other hand, Western Blot analysis using an antiFlag M2 revealed a similar or higher yield of FLAG-encoded protein by the PS-modified mRNAs for twelve out of sixteen combinations of four NTP and four NTPαS compared with the non-modified mRNA, with the best protein yield for the monosubstituted PS-modified mRNAs. The monosubstituted PS-modified mRNAs encoding firefly luciferase and AcGFP yielded higher protein levels than the unmodified mRNA. These results demonstrate that the introduction of NTPαS is a promising approach, especially in a biological environment in which stability of the mRNA is more relevant than in a cell-free translation system. However, the impact of NTPαS on IVT-

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associated RNA by-products and on the activation of innate immune receptors awaits further investigation.

Improvement of purification procedures

The field of mRNA vaccine research is well aware that the IVT process leads to a mRNA product that is contaminated by unwanted dsRNA by-products that are responsible for innate immune activation [16–18]. A recent study demonstrated that a combination of advanced engineering of the process and the use of 1-methylpseudouridine has the potential to substantially improve the translation and immune-activation properties of a therapeutic mRNA product [51••]. In this study, a classical process using equimolar levels of NTPs and standard affinity purification with OligodT-affinity chromatography was compared with an improved process with a custom undisclosed NTP ratio and OligodT-affinity chromatography in combination with ion-pair Reversed phase-high-performance liquid chromatography (RP-HPLC) to ensure further removal of any residual dsRNA impurities. The mRNA product resulting from the advanced protocol (m1Ψ, custom NTP ratio, and extra purification) showed the lowest immunogenicity *in vitro* and *in vivo*, while on the other hand, the mRNA containing canonical NTPs strongly activated innate immunity, regardless of the purification process.

Besides chromatography methods, another strategy to eliminate impurities of double-stranded by-products of mRNA preparations is the treatment with RNase III that cleaves dsRNA. In one study that uses mRNA encoding a chimeric antigen receptor (CAR) to generate CD19 CAR T cells for the treatment of B-cell leukemia, the authors incorporated 1- methylpseudouridine and applied RNase III to eliminate dsRNA contaminants [53]. They observed a twofold increase of CAR surface expression and of cytotoxic killing of leukemia cells, a reduced expression of checkpoint regulators, and a robust CAR T-cell-mediated suppression of leukemic burden *in vivo*. This study presents a notable example in which mRNA is used to achieve the translation of a certain functional protein for a nonvaccine purpose. Of note, unlike for a vaccine in which a certain amount and quality of innate immune activation is even required for proper function, in situations such as the generation of CAR T cells or protein-replacement therapies, innate immune activation needs to be eliminated as much as possible.

An inherent disadvantage of RNase III is the cleavage not only of dsRNA by-products but of stretches of dsRNA within the secondary structure of the intended mRNA molecule itself. Therefore, in a follow-up study, RNase-III treatment was replaced by a mRNA-purification method that is based on adsorption of dsRNA by-products to cellulose [18].

Not only dsRNA is an immune-stimulatory by-product of the IVT. Incomplete capping leads to 5′-ppp and 5′-pp ends that are sensed by RIG-I [12,54–56]. In a recent comprehensive study [57••], mRNA constructs with three distinct cap structures (anti-reverse cap analog (ARCA), 2′-O-methylated ARCA, and CleanCap), with or without additional phosphatase treatment, as well as the impact of HPLC purification, were investigated along with the use of three different uridine modifications and one cytidine modification. Innate immune activation was examined by the expression of the activation marker CD80 and by the release of cytokines, including Tumor necrosis factor alpha (TNF-α), Interleukin-6 (IL-6), and IFN-β in primary human macrophages. Consistent with the literature, HPLC purification led to the reduction of dsRNA content and reduced the innate immunogenicity of the RNA product. However, conversion of the 5′-ppp/pp into 5′-OH only modestly affected mRNA expression and immune activation. Interestingly, among the four nucleotide modifications tested, 5-methoxy-uridine (mo^5U) outperformed other modifications up to 4-fold regarding translation of the encoded protein and reduced innate immune activation. However, the superior effect of mo^5U in this study has only been shown in macrophages and needs to be confirmed in other cell types and *in vivo*.

The addition of defined innate immune-adjuvant activities

A recent report demonstrated that in mice, the induction of adaptive immunity by one of the approved SARS-CoV-2 mRNA vaccines depends on the activation of MDA5 [25••]. Nonetheless, it is clear that in future mRNA vaccines, a functionally relevant drug activity such as an adjuvant effect, needs to be quantitatively and qualitatively well-characterized. Consequently, efforts are being made to improve mRNA vaccines by adding molecular components that provide well-defined adjuvant functions, which preferably are directly attached to the mRNA to facilitate codelivery to the same antigen-presenting cell.

One approach was to create highly immunogenic dsRNA by hybridization with complementary RNA [58]. For this purpose, in one study, two dsRNA formulations were tested, one dsRNA formulation generated by adding an antisense(as) strand complementary to the coding region of the mRNA (asRNA), and another dsRNA formulation by adding a strand complementary to the poly-A tail (pU). Both featured an 18-base 5′-overhang sequence. While the full-length asRNA drastically reduced efficacy of translation, the use of pU limiting the hybridized

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portion to the poly-A region of the mRNA resulted in efficient translation accompanied by strong immune stimulation through TLR3 and RIG-I activation. Finally, *in vivo* mouse immunization experiments using ovalbumin demonstrated that the mRNA:pU design significantly enhanced the intensity of specific cellular and humoral immune responses, as compared with single-stranded mRNA. However, in this experimental setup, the authors did not use modified nucleotides or a cap structure, and the delivery agent and the purification process were distinct from current mRNA-vaccine designs.

In a follow-up study (so far only available on bioRxiv), the authors enhanced the immunostimulatory properties of mRNA by tethering short 24-base-pair dsRNA with a 5′-triphosphate blunt end to the mRNA via hybridization of a 17-mer single-strand 5′-overhang complementary to the sequence of the coding mRNA, a feature that the authors termed dsRNA tooth [59]. In this approach, the potency of RIG-I stimulation is controlled by tuning the number of such dsRNA teeth. By screening different combinations, the authors developed constructs that potently activated dendritic cells with minimal influence on antigen- translation efficiency. Ova-encoding mRNA constructs in conjunction with encapsulation into low immunogenic mRNA carriers improved the potential to induce Ova-specific cytotoxic immune responses and to increase survival in a Ova-expressing melanoma model of lung metastasis.

Another group reported the development of targeted stimulation of a specific innate immune receptor through tethering of selective small-molecule agonizts to the 3′-UTR of IVT mRNA [60]. They generated 2′-O-methyl RNA/DNA chimeric oligonucleotides complementary to the 3′-UTR of IVT mRNA with a 5′-thiol group and a covalently linked NeutrAvidin, a design that is normally used for monovalent *multiply-labeled tetravalent RNA imaging probes*. The oligonucleotides were then incubated with one of the two biotinylated small-molecule Toll-like receptor (TLR) agonizts PAM2CSK (TLR2) or CL264 (TLR7). TLR agonist-tethered oligonucleotides were then hybridized to IVT mRNA, and naked mRNA was injected i.m. into mice without a carrier for delivery. With this approach, the authors observed a local innate immune response at the injection site and a robust antigen-specific T-cell-mediated and humoral immunity. Thus, the data provided evidence that oligonucleotide-mediated tethering of TLR agonizts to IVT mRNA represents a promising strategy to improve vaccine efficacy.

Conclusions and outlook

Besides increasing the general potency of mRNA vaccines, engineering the appropriate functional qualities of mRNA to achieve the preferred outcomes for different clinical situations offers great potential for future development. One such outcome is the generation of a predominant antibody response to primarily provide antibody-mediated protection from bacterial and viral infections. The design and composition of future mRNA vaccines may allow the modulation of the predominant Ig type (e.g. IgG vs. IgA), the localization of protective antibodies (e.g. mucosa), as well as the prolonged longevity of the response. Moreover, in other clinical situations such as cancer, a predominantly cytotoxic T-cell response might be preferred. Moreover, while the immune toxic side effects of a mRNA vaccine need to be avoided in prophylactic settings, a certain degree of immune toxicity might be tolerated or even desired in therapeutic ones. Continued investigation into nucleic acid immunity is likely to provide the molecular arsenal to achieve those different desired activities as the result of an informed and targeted development process. The potential of structural adaptations has already been evidenced in the early stages of mRNA-vaccine development. However, the full potential of structural refinement of mRNA-vaccine design has clearly not been leveraged to date.

Even if our understanding has tremendously improved over the last two decades, important questions remain to be solved. One burning question is to understand how sequence- and modification-dependent secondary-structure formation of the intended mRNA molecule itself determines the recognition by the receptors of nucleic acid immunity. As straightforward as such an analysis sounds, it is not at all trivial. Endpoints of such analyzes are cytokine or interferon-stimulated gene induction, the effects of restriction factors on translation efficacy, and RNA stability. Not only do these three readouts form a complex, functionally intertwined network (e.g. translation inhibition through type-I IFN), the main variables to be studied, the sequence and modifications, also directly affect the ribosomal-translation process itself. Thus, as great as the potential for the improvement of mRNA vaccines is, the potential for misinterpretation and wrong conclusions from such studies remains high as well. However, although we find ourselves on a scientifically challenging road, it clearly leads to even better ground for the future RNA therapeutics.

Conflict of interest

GH is inventor on patents covering RIG-I.

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