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Strategies for controlling the innate immune activity of conventional and self-amplifying mRNA therapeutics: getting the message across

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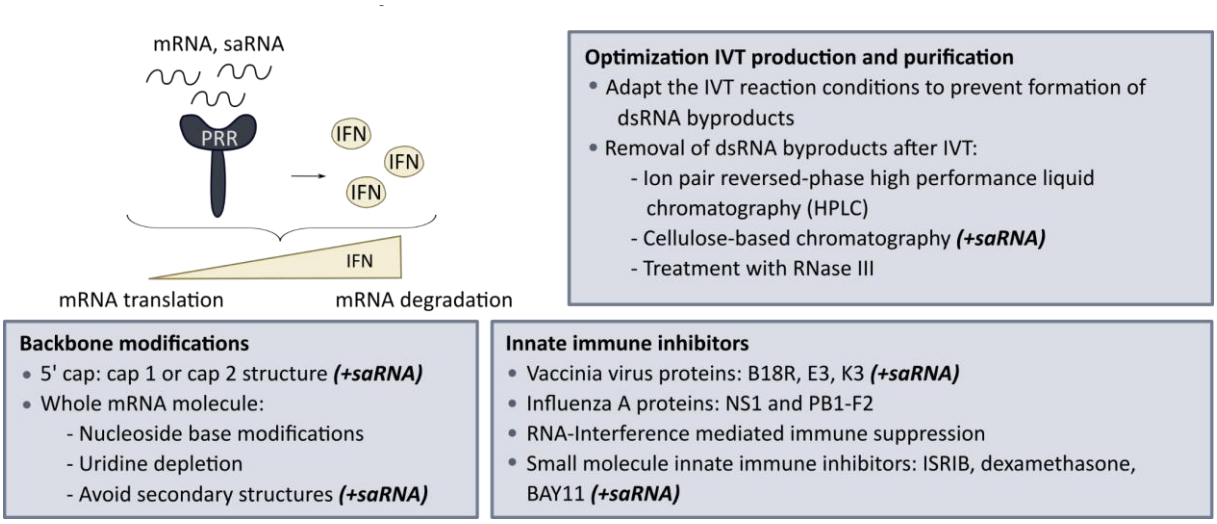
Abstract

The recent approval of messenger RNA (mRNA)-based vaccines to combat the SARS-CoV-2 pandemic highlights the potential of both conventional mRNA and self-amplifying mRNA (saRNA) as a flexible immunotherapy platform to treat infectious diseases. Besides the antigen it encodes, mRNA itself has an immune-stimulating activity that can contribute to vaccine efficacy. This self-adjuvant effect, however, will interfere with mRNA translation and may influence the desired therapeutic outcome. To further exploit its potential as a versatile therapeutic platform, it will be crucial to control mRNA's innate immune-stimulating properties. In this regard, we describe the mechanisms behind the innate immune recognition of mRNA and provide an extensive overview of strategies to control its innate immune-stimulating activity. These strategies range from modifications to the mRNA backbone itself, optimization of production and purification processes to the combination with innate immune inhibitors. Furthermore, we discuss the delicate balance of the self-adjuvant effect in mRNA vaccination strategies, which can be both beneficial and detrimental to the therapeutic outcome.



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1. Introduction

The recent FDA approval of the first messenger RNA (mRNA) vaccine formulations, developed by Pfizer/BioNTech and Moderna, to control the worldwide COVID-19 pandemic, created a new wave of attention for mRNA-based therapeutics [1]. Currently, several other mRNA-based COVID-19 vaccine formulations are still under investigation, containing either conventional non-amplifying mRNA or self-amplifying mRNA (saRNA) [2–5]. These successful outcomes are the result of a long and tedious journey that started in 1961 when conventional mRNA was first discovered, followed by successful pre-clinical experiments in 1990 [6–8]. In contrast, the larger saRNA gained a lot of attention, mainly for vaccination purposes as first reported in 1994 due to is self-replicating ability [2,3]. The latter results in enhanced and prolonged protein expression which overcomes the often transient nature of conventional mRNA therapeutics [2–4]. Besides its use in vaccination against infectious diseases or cancer, mRNA is currently explored for many other non-immunotherapy applications, including protein replacement therapy, cell reprogramming and gene editing [7,9,10]. To date, a variety of mRNA formulations for different therapeutic indications have entered clinical trials, studying their safety and efficacy [2,9,11].

Several concerns, such as extra- and intracellular instability, intracellular delivery and inherent immunogenicity, delayed the translation of mRNA therapeutics to the clinic. Over the last few years, substantial progress was made in the design of the mRNA molecule itself and the development of appropriate delivery vehicles to overcome these concerns [9,12]. First, stability issues are tackled by modifications of the mRNA backbone structure, such as 5' capping, 5' and 3' untranslated regions (UTR) optimization and poly(A) tail addition [10,13]. Second, the development of an optimal carrier has been a continuous research focus to improve both the stability and cellular uptake efficiency of mRNA. Both viral and non-viral carriers have been tested, where non-viral nanoparticles have emerged as a safer alternative to their viral counterparts, with lipid-nanoparticles being the most clinically advanced [10,11,14,15]. An overview of the available carrier systems, their composition and the administration route is outside the scope of this review but is excellently summarized elsewhere [9,11].

A third but very important factor influencing the effect of mRNA therapeutics is the inherent innate immune-stimulating activity of mRNA, which can either support or hamper the therapeutic outcome. Non-immunotherapy-based applications suffer from this intrinsic innate immune stimulation as it induces an overall cellular antiviral state in most cell types promoting mRNA degradation and inhibiting the translation process. On the contrary, vaccination purposes might benefit from additional innate immune stimulation. However, this concept is rather complex and mainly depends on finding the optimal balance between the different factors involved [16,17]. The innate immune activation by mRNA therapeutics may also result in side effects ranging from flu-like symptoms to risks of autoimmune diseases [18]. Hence, there is a high need to control and fine-tune inherent innate immunogenicity to align it with the anticipated therapeutic effect and to guarantee the safety of mRNA therapeutics. In this review, we will provide an extensive overview of mRNA-induced innate immunity and more specifically the strategies to control these responses. First, we will present the structural differences between conventional and self-amplifying mRNA, each with their advantages and limitations. Second, we will provide a concise overview of the cellular pathways leading to this inherent innate immune stimulating activity. Third, an in-depth discussion of the current strategies explored to control mRNA immunity will be provided, together with their influence on the safety and efficacy of conventional mRNA and saRNA-based therapeutics for different applications. Generally, these strategies can be divided in three main categories, including modifications to the backbone structure, optimization of the production and purification processes, and the use of innate immune inhibitors as

a supplement therapy. Finally, given the more ambiguous situation for mRNA-based vaccines, we will provide a concise overview of the current knowledge and discussion regarding the influence of mRNA-induced immune responses on vaccine efficiency.

2. mRNA versus self-amplifying mRNA

2.1. Conventional mRNA

Messenger RNA (mRNA) is a fairly simple and relatively small single-stranded structure of around 1000 – 5000 nucleotides. It is typically made up of five elements that are critical for its expression: a cap structure (m7GpppN), a 5' untranslated region (5' UTR), an open reading frame (ORF) encoding the gene of interest (GOI) flanked by the start and stop codon, a 3' untranslated region (3' UTR) and a poly(A) tail at the 3' end of about 100 – 250 adenosine residues. In living cells, mRNA is transcribed from DNA by RNA polymerase, spliced and subsequently transported from the nucleus to the cytosol. Then, the ribosomes are recruited, resulting in the direct but transient expression of the encoded protein, during the life span of the mRNA molecules (**Figure 1, left**) [19].

In analogy to naturally produced mRNA, synthetic mRNA is prepared by *in vitro* transcription (IVT) of a linearized plasmid DNA (pDNA) or PCR template containing the gene of interest and a promoter region for a bacteriophage T7, SP6 or T3 RNA polymerase. To start the IVT reaction, also the four ribonucleotide triphosphates (NTPs), a ribonuclease (RNase) inhibitor (to inactivate contaminating RNase), a pH buffer and Mg^{2+} as co-factor for the RNA polymerase should be provided. The transcription is initiated by binding of RNA polymerase to its promoter sequence, after which it links ribonucleotides together as it moves along the DNA template to form the complementary mRNA strand. The RNA transcript is completed when the enzyme runs off at the end of the template (runoff transcription), ready for the next round. At the end, the DNA template is fragmented by treatment with a DNase and the produced mRNA is purified from the remaining reaction compounds, such as the RNA polymerase and the unincorporated NTPs [20].

The simplicity of the mRNA construct and its relatively small size compared to saRNA are key features promoting the use of conventional mRNA. Due to its short half-life and inherent instability, however, only low and transient protein production levels could be achieved. To date, remarkable improvements in mRNA translation efficiency and stability have been made through sequence optimization, the incorporation of chemically modified nucleosides, and several advanced purification strategies. Yet, protein expression remains proportional to the number of mRNA molecules successfully delivered to the cytosol of the cells and relatively high doses are often required to obtain significant therapeutic effects [3,13,21–23].

2.2. Self-amplifying mRNA

When compared to conventional, non-amplifying mRNA, saRNA is a more complex and considerably larger (9000 – 12000 nucleotides) structure as besides the basic elements found in an mRNA molecule (a cap, 5' UTR, an ORF with GOI, 3' UTR and poly(A) tail), saRNA also contains the coding sequences of a viral replicase complex, a genomic and a subgenomic (SG) promoter [3,20,24]. Most saRNAs are based on the genome of alphaviruses such as the Sindbis virus (SINV), Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEEV). Alphaviruses are a group of small, enveloped positive-stranded RNA viruses producing considerable amounts of subgenomic RNA encoding for viral structural proteins during their natural replication cycle in the host cell cytosol [25]. saRNAs benefit from the self-replicating ability of these alphaviruses by retaining their non-structural proteins (nsP1-4), which include the replication machinery, while replacing the viral structural proteins by the GOI, which renders the mRNA incapable of producing infectious viral particles [3]. Although saRNA clearly

differs structurally from conventional mRNA, it is produced in the same manner during an IVT reaction as described above [3,20].

Unlike conventional mRNA, the GOI cannot be immediately translated from the originally delivered saRNA molecule upon entry in the host cell. As depicted in **Figure 1 (right)**, after cytosolic delivery, the host cell machinery is immediately engaged for the translation of nsP1-4, which together make up the replicase complex. This complex attaches to the plasma membrane where so-called spherules, i.e. membrane-associated structures, are formed in which replication takes place protected from cellular defense mechanisms. In a first phase, the translated nsP1-4 polyprotein is cleaved by nsP2 to generate the early replication complex (replicase), which consists of the nsP1-3 polyprotein and associated nsP4 (**Figure 1, A**), and transcribes the original saRNA into its complementary negative-sense RNA strand (**Figure 1, B**). In a later phase, the whole replication complex is cleaved into the individual nsPs that join together to form the cleaved replicase. Now, the negative-sense RNA strand serves as a template for the production of new copies of the original genomic RNA but also for the production of a high amount of shorter, SG RNAs from a 26S SG promoter (**Figure 1, C**). Subsequently, high levels of the protein of interest are translated from these SG RNAs (**Figure 1, D**) [3,26,27]. Overall, it is clear that the replicase complex is a multifunctional complex, in which each of the non-structural proteins has their own specific function. nsP1 is an enzyme required for 5' capping of viral RNA and serves as an anchor to tether the replicase complex to the plasma membrane. nsP2 has helicase activity to unwind the RNA duplex during replication, but also has an important protease activity cleaving the polyprotein into individual nsPs. The function of nsP3 is not completely understood yet, but it is certainly an essential compound in the replicase complex. nsP4 is an RNA-dependent RNA polymerase and structures the replicase complex [28].

Due to its auto-replicative ability, a higher protein expression level can be maintained and also the duration of protein expression is significantly extended [3,22,23]. For example, Brito *et al.* evaluated the expression of a cationic nanoemulsion-formulated saRNA in non-human primates after intramuscular delivery using secreted alkaline phosphatase (SEAP) as reporter gene. Expression from saRNA reached its peak expression 3 days post-administration and remained measurable at least 14 days after injection [29]. Recently, Leyman *et al.* found that conventional mRNAs reached their maximum expression 1 day after intradermal electroporation in pigs, followed by a steady decrease in expression until day 6, whereas the expression of saRNA was similar to that of conventional mRNA at day 1, but peaked at day 6 and persisted until day 12 [30]. Additionally, in the vaccination context, Vogel *et al.* showed that saRNAs can be delivered at lower concentrations than unmodified conventional mRNA while still achieving an equivalent protection against influenza viruses [31]. However, in comparison to conventional mRNA, saRNA is a more complex and much larger molecule, thus complicating its delivery. Recent efforts to reduce its size resulted in the generation of trans-amplifying RNAs in which the saRNA is split into two transcripts, one encoding nsP1-4 and the other encoding the GOI as a "transreplicon" [32,33]. Beissert *et al.* found that mRNA translation from such a trans-amplifying RNA vaccine was as efficient as its saRNA counterpart and an influenza-hemagglutinin encoding trans-amplifying mRNA successfully induced protective immune responses in mice upon intradermal injection [33]. Nevertheless, the mRNA coding for the replicase is still longer than 7 kb, which is considerably larger than the typical conventional mRNA and will therefore also require delivery vectors with a high loading capacity for efficient RNA delivery. Furthermore, saRNA not only encodes the protein of interest but also viral nsPs. These viral proteins might potentially be immunogenic, which may limit the repeated use of saRNA-based therapeutics. However, little information is available regarding the immunogenicity of the replicase complex itself and additional studies should be conducted to further explore this [3,20,21,23].

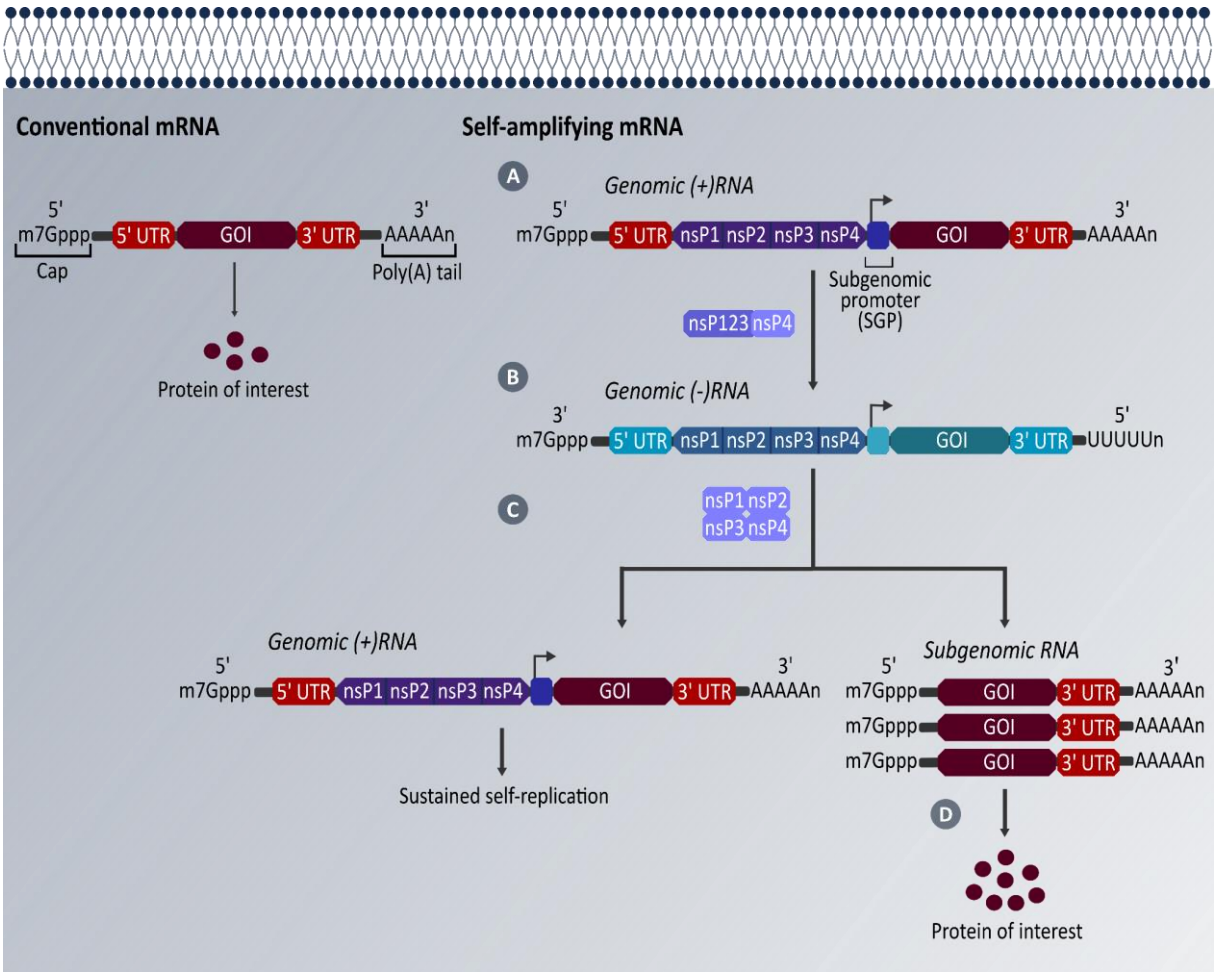


Figure 1: Schematic representation of the structure of conventional mRNA and the structure and intracellular amplification of self-amplifying mRNA (saRNA). (Left) Conventional mRNA consists of five critical elements: a 5' cap structure (m7GpppN), a 5' untranslated region (5' UTR), the gene of interest (GOI), a 3' untranslated region (3' UTR) and a poly(A) tail. After conventional mRNA delivery into the cell cytosol, the encoded protein is produced directly. (Right) saRNA consists of a 5' cap structure, a 5' UTR, the sequences of viral non-structural proteins (nsP1-4), a subgenomic promoter (SGP), the GOI, a 3' UTR and a poly(A) tail. (A) When saRNA arrives in the host cell cytosol, nsP1-4 is translated and forms the early replication complex (B) that generates a complementary negative-sense RNA strand from the original saRNA. (C) In a later phase, the early replication complex is cleaved into the individual nsPs. These form the cleaved replicase that uses the negative-sense RNA strand as a template to generate new copies of the original genomic RNA and also recognizes the SGP and triggers the production of an enormous amount of subgenomic RNAs, (D) which eventually leads to the production of the protein of interest.

3. Innate immunity stimulation

Our innate immune system has evolved to recognize non-self molecules, such as viral single-stranded (ss) and double-stranded (ds) RNA, initiating a series of signaling pathways to fight the pathogen. Both conventional mRNA and saRNA are potent activators of the innate immune system, leading to interferon (IFN) secretion which hampers RNA translation and promotes mRNA degradation [10,16]. saRNA might be somewhat more immunogenic than conventional mRNA, as during self-replication in the host cell's cytosol dsRNA amplification intermediates are formed, which can be recognized as foreign [5,21,22]. Moreover, to retain saRNA functionality, some commonly applied strategies to reduce the innate immune-stimulating activity of conventional mRNA, like nucleoside base modification and sequence alteration (see Section 4.1.), are not tolerated. Below, we will provide a concise overview of the initiated pathways and factors involved in this innate immune-stimulating process.

Activation of the innate immune system can be initiated by stimulation of pattern recognition receptors (PRRs) through binding with pathogen-associated molecular patterns (PAMPs). The mRNA binding PRRs can be classified according to their cellular locations as the cytosolic PRRs and endosomal PRRs. In the cytosol, the PRRs include the nucleotide oligomerization domain like receptors (NLRs) and the RIG-I-like receptors (RLRs). In the endosomes, the PRRs are represented by the toll-like receptors (TLRs). Upon mRNA recognition, a complex series of interacting signaling pathways is initiated, eventually leading to the production of type I IFNs and pro-inflammatory cytokines as presented in **Figure 2** [10,16,24,34–36].

All TLRs which have been identified in humans, differ in their cellular location and ligand specificity. Endosomal mRNA-binding TLRs are TLR3, binding to dsRNA, and TLR7/8, which recognize uridine-rich ssRNA (**Figure 2**). Stimulation of TLR3 and TLR 7/8 leads to the activation of the adaptor proteins, TIR-domain-containing adaptor-inducing IFN-β (TRIF) and myeloid differentiation primary response gene 88 (MYD88) respectively. Subsequently, signaling pathways involving several mediators, such as TRAF proteins and the IkB kinase (IKK) complex, are initiated, resulting in the activation and nuclear import of the transcription factors IRF3, IRF7 and NF-κB. Ultimately, this coordinates the production of type I IFNs, a subset of interferon stimulated genes (ISGs) and pro-inflammatory cytokines, such as IL-6 and IL-12 [10,16,24,34,36–38]. The RLRs, RIG-I and melanoma differentiation-associated protein 5 (MDA-5), are RNA helicases both composed of three domains: the caspase activation and recruitment domain (CARD), the DExD/H box RNA helicase domain and a regulatory domain [39,40]. In general, it is considered that activation of RIG-I is mediated by short dsRNA containing a 5’ di- or triphosphate end, while MDA-5 is activated by recognition of long dsRNA (**Figure 2**) [10,16,24,35]. Stimulation of both receptors leads to conformational changes which induce interaction of the CARD domain with the mitochondrial antiviral-signaling (MAVS) protein. Upon complex formation, a cascade of signaling pathways is initiated, finally resulting in transcription factor activation and transport to the nucleus [10,16,24,35,36,39]. In contrast to the aforementioned RLRs, the precise role of a third member of the RLR family, laboratory of genetics and physiology 2 (LGP2), is less clear. LGP2 does not possess a CARD domain and is suggested to be involved in the regulation of RIG-I- and MDA-5-signaling. It is generally considered that LGP2 stimulates MDA-5 signaling, as recently confirmed by Duic *et al.*, while it is suggested to block RIG-I signaling [10,16,39–41]. Another recent study by Sanchez and colleagues showed the involvement of the IFN-inducible dsRNA-dependent protein kinase activator A (PACT), and more specifically the PACT-LGP2 association, in the regulatory role of LGP2 [39]. Nonetheless, a lot of controversy remains and further in-depth studies are needed to precisely reveal the underlying molecular mechanisms of these regulatory effects [10,39–41]. A fourth cytosolic PRR suggested to be associated with mRNA-mediated immunity is the NOD2 receptor, belonging to the NLR family (**Figure 2**). Sabbah *et al.* showed that ssRNA activation of the cytoplasmatic NOD2 receptor leads to the MAVS-interaction-mediated triggering of the transcription factor IRF3, eventually inducing IFN-β secretion [10,16,24,35,40,42].

Once type I IFNs are expressed, they are transported to the extracellular environment where they can react with interferon-α/β receptors (IFNARs) in an auto- or paracrine fashion (**Figure 2**) [10]. Type I IFNs are a family composed of many different members, of which IFN-α and IFN-β are the most characterized. They are recognized by a heterodimeric IFNAR consisting of the low-affinity IFNAR1 and high affinity IFNAR2 [34,43,44]. However, De Weerd *et al.* showed that specifically IFN-β can form a functional complex with IFNAR1, independent of IFNAR2, which can lead to unconventional signal transduction and selective gene expression [43,45,46]. Activation of the IFNAR1-IFNAR2 complex results in the initiation of the Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway. The Janus kinases JAK1 and TYK2 phosphorylate the STAT1 and STAT2 proteins, resulting in their dimerization and binding to IRF9 (**Figure 2**). This three-component complex, also called IFN-stimulated gene factor 3 (ISGF3), migrates to the nucleus where it binds to promotor IFN-stimulated response elements (ISREs) leading to the transcription of ISGs. The latter encode a plethora

of proteins involved in the innate immune pathways described above or in direct anti-viral responses [10,34,43,44,47,48]. Also other STAT proteins can be phosphorylated and other complexes, such as STAT1 homodimers, can be formed that bind to IFN-γ-activated site (GAS) elements in the promotor region of ISGs instead. Researchers suggest that activation of different STAT molecules can lead to different responses. For instance, it has been suggested that STAT3 would be involved in hampering type I IFN responses, rather than stimulating them [34,43,44,47–49]. Which STAT proteins are activated upon IFN-recognition by the IFNARs would be determined by different factors such as cell type and relative STAT protein levels [43,47,48]. Moreover, it is suggested that several other STAT-independent pathways are important in the IFN-signaling cascade and a combination is needed to induce IFN-I responses. Precise molecular mechanisms of both STAT (in)dependent signaling and complexity of these interactions still remain to be fully elucidated [34,43,44,47,48].

Upon expression of ISGs, several anti-viral responses can be initiated. Two important ISGs shown to be involved in this anti-viral responses upon dsRNA recognition, are dsRNA-dependent protein kinase (PKR) and 2'-5'-oligoadenylate synthetase (OAS) [10,24]. PKR is a 68 kDa protein that can hamper mRNA translation through phosphorylation of the eukaryotic translation initiation factor 2 alpha subunit (eIF2α) (**Figure 2**). Furthermore, several reports have shown that PKR can activate NF-κB, which is captured in the cytoplasm through binding of the IkBα protein. Dissociation of these two factors allows transportation of NF-κB to the nucleus where it is able to perform its role as a transcription factor. Precise mechanisms are still under investigation, but the NF-κB activating action of PKR Is suggested to be mediated by IKK complex interaction and to be independent of its kinase activity [10,50–53]. The OAS pathway is one of the first defined antiviral pathways following type I IFN stimulation. Upon dsRNA recognition, OAS produces 2'-5'-linked oligoadenylates which activate the RNase L enzyme, an endoribonuclease that leads to the degradation of ssRNA (**Figure 2**) [10,54,55]. ADAR1 is another IFN-inducible dsRNA binding protein belonging to the adenosine deaminase acting on RNA (ADAR) family. ADAR1 can convert adenosine to inosine which induces mismatch base-pairing resulting in destabilization of dsRNA. Evidence suggests that ADAR1 is involved in suppression of RLR, PKR and OAS pathways, mediated by several processes which remain to be fully explained. This pro-viral activity is useful to inhibit autoimmunity caused by host dsRNA recognition but should be controlled during viral infection [10,53,56]. Of note, some ISGs can act as negative feedback regulators of the type I IFN immune response. For example, USP18 is known to prevent IFNAR activation as shown by Honke *et al.* where USP-18 secreting $CD169^{+}$ macrophages displayed a lower type I IFN sensitivity upon viral infection [38,57].

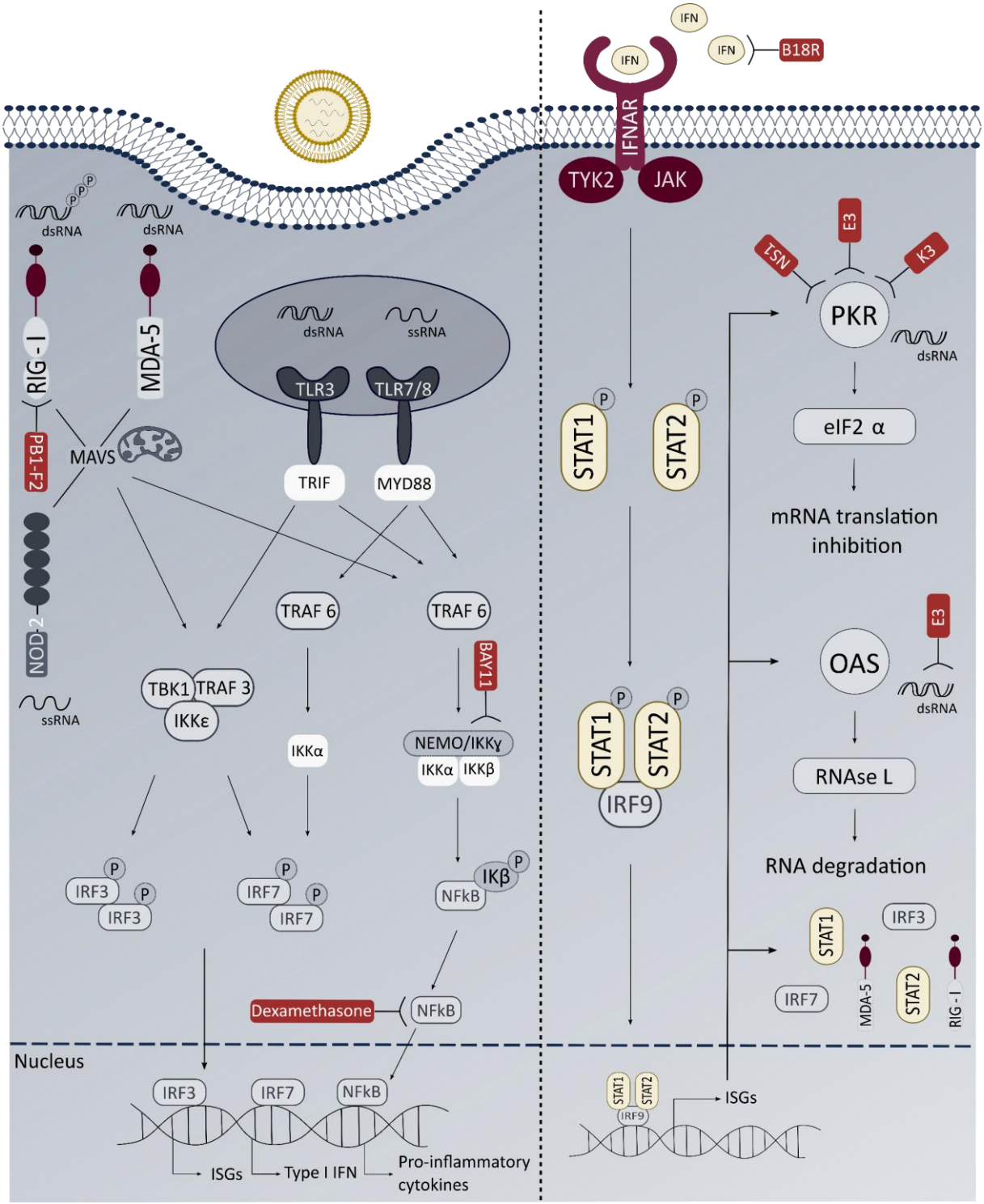


Figure 2: Innate immune pathways stimulated upon mRNA delivery. Simplified representation of mRNA recognition by both cytosolic and endosomal pattern recognition receptors (PRRs) leading to production of type I interferons (IFN) and pro-inflammatory cytokines. Subsequent activation of the interferon-α/β receptor (IFNAR) results in Janus Kinase - signal transducer activator of transcription (JAK-STAT) signaling leading to interferon stimulated genes (ISGs) production. Finally, PKR and OAS activation can result in diminished mRNA translation and enhanced mRNA degradation, respectively. Site of action of the inhibitors, discussed in Section 4.3., is visualized. Abbreviations: dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; TLR, Toll-like receptor; MDA-5, melanoma differentiation-associated protein 5; RIG-I, retinoic-acid-inducible gene I; MAVS, mitochondrial adaptor molecule; TRIF, TIR-domain-containing adaptor inducing IFN-b; MyD88, myeloid differentiation primary response gene 88; TRAF, TNF receptor-associated factor; IKK, IkB kinase; TBK1, TANK-binding kinase 1; IRF, interferon-regulatory factor; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-kB;

TYK2, tyrosine kinase 2; ISGs, Interferon Stimulated Genes; PKR, dsRNA-dependent protein kinase; EIF2a, eukaryotic translation initiator factor 2; OAS, 20-50-oligoadenylate synthetase.

4. Strategies to control innate immune activation upon conventional and self-amplifying mRNA delivery

As described above, the innate immune-stimulating activity affects both the translation and integrity of conventional and self-amplifying mRNA. For non-immunotherapy applications, mRNA-induced immune stimulation is highly unwanted and should be avoided completely. In contrast, for vaccination applications, the situation is more ambiguous and mRNA-induced immune stimulation should rather be controlled than completely eliminated as discussed further in Section 5. Below, we will present an extensive overview of the strategies and methods that are currently being used to avoid or control the innate immune-stimulating activity of mRNA and saRNA, as presented in **Figure 3**.

4.1. Modifications to the mRNA backbone itself

The first strategy to limit mRNA-induced immunity, comprises the modifications on the level of the pDNA template or the IVT mRNA or saRNA molecule itself, as summarized in **Figure 3A**.

4.1.1.5' Cap

The 5' cap structure (m7GpppN) is a regular characteristic of eukaryotic mRNAs, including viral RNAs such as those from alphaviruses [58]. It is typically composed of an N7-methylated guanosine linked to the first nucleotide of the RNA via a reverse 5' to 5' triphosphate bridge (cap 0) (m7G(5')pppN1pN2p). In humans and other higher eukaryotes, the standard cap 0 structure is further modified to a cap 1 (m7G(5')pppN1mpN2p) or cap 2 (m7G(5')pppN1mpN2mp) structure by 2'-O-methylation of the first or both nucleotide riboses, respectively [59]. The 5' cap structure is known to be required in various processes throughout the lifecycle of an mRNA, including pre-mRNA splicing, nuclear export, polyadenylation, initiation of translation by binding to the eukaryotic initiation factor (eIF) 4E and protection from 5' to 3' exonuclease cleavage. Moreover, a cap 1 or cap 2 structure may also prevent recognition of exogenous RNA by innate immune sensors, as it mimics the 5' cap structure of eukaryotic mRNAs [58]. Recent studies found that a cap 1 structure is crucial to block RIG-I activation, whereas cap 0 and uncapped (5' ppp) dsRNAs bind to RIG-I with similar affinity (**Figure 2**) [59,60]. Also, the cap 1 structure revealed to be important to evade recognition by MDA-5 [61] and the interferon-induced protein with tetratricopeptide repeats (IFIT) family of restriction factors [62]. Subsequently, as mRNA with other cap structures interact differently with PRRs, the cap structure can also influence protein expression from the IVT mRNA construct [63].

These cap structures can either be incorporated during the IVT production process of mRNA or saRNA (co-transcriptional capping) or after initial mRNA synthesis using recombinant vaccinia virus-derived capping enzymes (post-transcriptional capping) [13]. Up till now, the most reported cap analogues for co-transcriptional capping are the anti-reverse cap analogues (ARCAs) (3'-O-Me-m7G(5')ppp(5')G), which contain an additional methyl group on the 3' position of the last nucleoside compared to cap 0 to prevent incorporation of the 5' cap in the reverse orientation. However, the ARCA is not the method of choice for 5' capping with regard to evading innate immune stimulation, as it generates an immunogenic cap 0 structure and results in a capping efficiency of only 70% due to competition with GTP nucleotides for incorporation into the mRNA. So, 30% of the IVT mRNA remains uncapped and contains a 5' triphosphate at its 5' end, which introduces additional immunogenicity and instability [13,24,64]. This fraction of uncapped mRNA can be reduced by phosphatase treatment to remove the triphosphates at the 5' ends and avoid recognition by PRRs [65]. Quite recently, trimer analogues, such as Cleancap, have been introduced as an attractive alternative to ARCA for co-transcriptional capping with incorporation of a natural cap 1 or cap 2 structure and an increased capping efficiency of 90 –

99%, which is of interest to generate less immunogenic mRNA constructs [16,66]. Interestingly, Trilink Biotechnologies also provides the CleanCap Reagent AU that has been specifically designed for saRNAs based on the genomes of positive-sense alphaviruses. Post-transcriptional capping is carried out using vaccinia virus-derived capping enzymes and can generate cap 0 and cap 1 structures. Although enzymatic capping can be highly efficient (100%) and can generate natural cap 1 structures, there is often a high variability in efficiency. Also, co-transcriptional capping is simpler, cheaper and easier to control compared with post-transcriptional enzymatic capping reactions [13,24,67].

4.1.2.Untranslated regions

The 5' and 3' UTRs in mRNA are important regulators of mRNA stability and translational efficiency [68,69]. As the composition of the UTR sequences does not directly influence the immunogenicity of the conventional IVT mRNA or saRNA construct, we will just give a brief overview of UTRs that are often used [13]. A more in depth discussion can be found in the review of Kuhn *et al.* [70]. 3' UTRs of α- and β-globin mRNAs are popular sequences, often incorporated in IVT mRNAs to increase its stability and translation [71–73]. The stabilizing effect of human β-globin 3' UTRs sequences can even be further improved by incorporating two β-globin 3' UTRs in a head-to-tail orientation [74]. Other similar efficacious UTR sequences include the 5' UTR of human heat shock protein 70 [75], the UTRs of albumin [76] and viral UTRs from VEEV [77] and SINV [78]. Recently, Sahin's group used a SELEX (systematic evolution of ligands by exponential enrichment) to identify new 3' UTR sequences that augment protein expression from IVT mRNA. In total 64 double (d)UTR combinations were tested and the dUTRs carrying the mtRNR1 (mitochondrially encoded 12S rRNA) element in combination with AES (amino-enhancer of split) or human β-globin were found to be superior to the standardly used double β-globin in mRNA translation in human dendritic cells. Intravenous injection of liposome-formulated mRNA with an AES-mtRNR1 3' UTR in mice was found to increase protein expression and the induction of antigen-specific T cells. For the reprogramming of human fibroblasts, mRNA containing the AES-mtRNR1 3' UTR outperformed mRNA with other 3' UTR variants [79]. Also for saRNA vaccines, the 5' and 3' UTRs can be optimized to improve translation, which is based on the evolution of naturally occurring alphaviruses [23].

4.1.3.Poly(A) tail

The 3' end of the conventional IVT mRNA or saRNA is decorated by a poly(A) tail, which plays a key role in regulating mRNA stability. A long poly(A) tail is known to have a stabilizing function and naturally has a length of approximately 250 adenosine ribonucleotides in mammalian cells. In addition, the poly(A) tail is important for RNA translation by supporting the formation of a closed-loop state, which also impedes deadenylation and mRNA degradation, through association of poly(A)-binding proteins (PABPs) with both the poly(A) tail and the 5' cap [80,81]. A poly(A) tail is added to the IVT mRNA or saRNA either directly from the encoding DNA template (allowing precise control over the number of adenosines to incorporate) or post transcriptionally by using a recombinant poly(A) polymerase. However, the latter is not regularly done because of the high variability in poly(A) tail length generated between batches [9]. Most research focusses on estimating the impact of the length of the poly(A) tail on RNA translation and stability, but there is no general rule about its length on RNA translation. Some reported that longer poly(A) tails increase protein expression from mRNA in multiple cell types [74,82,83], whereas other suggested that longer poly(A) tails (≥100 adenosines) are not necessarily better [81,84]. In contrast, the impact of poly(A) tail length on the immunogenicity of both non-amplifying and self-amplifying mRNA is less intensively studied. For example, one study of Koski *et al.* provided evidence that the immune-stimulatory activity of IVT mRNA could be lowered by enzymatic 3'-polyadenylation with a minimum of 150 adenosines, as increasing the length of the poly(A) tail generates mRNA with a reduced relative uridine content or shielded uridines in the sequence [85,86].

4.1.4.Nucleoside base modifications

The use of modified nucleoside analogues is probably the most applied approach to prevent IVT mRNA from intracellular recognition by PRRs and consequently evade innate immunity. To date, 143 modified nucleosides have been characterized in endogenous RNAs and differences in nucleoside modifications can enable PRRs to detect foreign RNA of invading pathogens [87]. For example, RNA modifications are more abundant in mammalian rather than in bacterial mRNA and a subset of nucleoside modifications is unique to either bacterial or mammalian RNA [87,88]. Therefore, it is not surprising that unmodified IVT mRNA has a stronger innate immune-stimulating activity and nucleoside base modifications have been introduced to silence recognition by innate immune sensors [88,89].

Pioneering work in this area was done by Karikó *et al.* who demonstrated in primary dendritic cells (DCs) that upon mRNA transfection, TLR3, TLR7 and TLR8 activation could be reduced or completely eliminated with mRNA containing the following modifications either separately or in combination: 5-methylcytidine (m5C), N^6-methyladenosine (m^6A), 5-methyluridine (m5U), 2-thiouridine (s2U) or pseudouridine (Ψ) [88]. Especially replacement of uridine by Ψ seemed very promising, as it was shown to not only reduce the immunogenicity of mRNA, but to also enhance its translational capacity both *in vitro* and *in vivo* [90]. This increase in translational efficiency was later on shown to be aided by a reduced detection by PKR, a reduced PKR-mediated phosphorylation of eIF2α [91,92], a reduced OAS-mediated activation of RNase L and an increase in resistance to RNase L-mediated degradation [93] (Section 3). Later on, many other studies were published in favor of modified mRNA, demonstrating its vast potential for therapeutic applications. For example, Kormann and colleagues found that replacement of 25% of uridine and cytidine with s2U and m5C synergistically diminished binding to PRRs, decreased innate immune activation and increased protein expression of erythropoietin (EPO)-encoding mRNA *in vitro* and *in vivo* in mice after intramuscular injection [94]. In a similar study by Karikó *et al.* on EPO-encoding mRNA it was shown that HPLC-purified (Section 4.2.3), codon-optimized (Section 4.1.5), Ψ-modified mRNA was clearly superior to its unmodified counterpart in inducing protein expression and limiting innate immune activation in mice, leading to increased erythropoiesis [95]. Mahiny *et al.* showed that m5C/s2U-modified mRNA encoding a zinc finger nuclease (ZFN) induced a more robust protein expression in mouse lungs than unmodified mRNA and corrected SP-B deficiency in mice [96]. Moreover, in 2015, Andries *et al.* reported on replacing uridine by N^1-methylpseudouridine (m1Ψ) to reduce the intracellular innate immunity of mRNA. This modification seemed to increase translational capacity even more compared to Ψ-modified or m5C/Ψ-modified mRNA *in vitro* and *in vivo* [97]. Similarly, Devoldere *et al.* screened 16 different modifications in human retinal cells (*in vitro*) and found that m1Ψ-modified mRNA had the highest translational capacity of all modified mRNAs tested and also induced protein expression in bovine retinal explants and mouse retinas (*in vivo*) [98].

Although these findings suggest that mRNA modification is an inevitable prerequisite for the development of non-immunogenic, mRNA-based protein therapies, some other studies have been published that contradict this approach. For example, researchers of CureVac demonstrated that Ψ incorporation in sequence-optimized (Section 4.1.5), HPLC-purified (Section 4.2.3) mRNA reduced protein expression in HeLa cells and in mice after lipid nanoparticle (LNP)-mediated delivery. In contrast, when the mRNA sequence was not optimized, Ψ substitution did enhance protein production [76]. Kauffman *et al.* found that Ψ-modification of mRNA had no significant impact on protein expression and immunogenicity in mice when delivered systemically with liver-targeting C12-200 LNPs [99]. Hence, it is important to realize that mRNA modification does not necessarily increases protein expression and can even decrease it. This discrepancy may arise from variations in delivery system, route of administration, the level of innate immune sensing in the targeted cell types and mRNA properties like different UTRs, sequence optimization (Section 4.1.5) and HPLC purification (Section 4.2.3) [21,24,99]. Also, there is no control over the location where modified nucleosides are inserted in the mRNA strand. It has been shown that incorporation of modified nucleosides in translation-

enhancing elements, such as UTRs and internal ribosomal entry sites (IRESs), impair their functionality [76].

In accordance with conventional IVT mRNA, also IVT saRNA is recognized as foreign by innate immune sensors. However, in contrast to conventional mRNA, the usage of modified nucleosides is not considered as an option to reduce its immune-stimulating activity. The effect of incorporating modified nucleosides into saRNA would already be lost after the first round of amplification. Moreover, it is expected to impair the self-replicating capacity of saRNA in the target cells [22].

4.1.5.Open reading frame

So far, we have discussed adaptations to the cap structure, UTRs, poly(A) tail and nucleotides of the IVT mRNA, but an important missing element in this overview is the ORF. This protein encoding region of conventional mRNA can be modified through codon optimization, in which rare codons are replaced by frequently used synonymous ones that have abundant cognate tRNA in the cytosol without altering the amino acid sequence of the encoded protein. Codon optimization is applied to increase mRNA stability and translation efficiency, but cannot be applied in general for mRNA-based therapeutics, as some proteins require slow translation for their proper folding, which is ensured by less frequent codons [13,100,101]. In addition, as codon optimization is not used as a tool to directly modulate the inherent immunogenicity of IVT mRNA, we will not discuss this topic further in detail, but some other reviews are available describing this thoroughly [102–104]. Furthermore, codon optimization also includes the replacement of GC-poor codons in the ORF by synonymous GC-rich codons, as this has been shown to increase steady-state mRNA levels *in vitro* [105] and protein expression *in vivo*, while reducing innate immunogenicity [76] as explained below.

In contrast to codon optimization, the following adaptation clearly affects the immune-stimulating activity of IVT conventional mRNA. The immunogenicity of IVT mRNA can be eliminated by minimizing or completely eliminating uridine content, as uridine-rich regions are known potent activators of several RNA sensors, including TLR7, TLR8 and RIG-I [106–109]. In addition, uridine depletion is not necessarily restricted to the ORF of the mRNA but can be applied over the whole mRNA molecule. Minimizing or eliminating uridine content can be accomplished in several ways [86]. First of all, the IVT mRNA can be rendered completely non-immunogenic by replacing all uridines with Ψ, as was done for example in a study of Karikó and colleagues [95]. Secondly, uridine can also be replaced for 25% by s2U, resulting in mRNA with some residual immunogenicity as shown by Kormann *et al.* [94]. Thirdly, as also mentioned previously, the length of the poly(A) tail can be increased to diminish the relative uridine content or to shield the uridines present in the mRNA sequence [85]. Lastly, the uridine content in the IVT mRNA can be decreased by enrichment of the GC content. For example, Thess and colleagues designed an unmodified EPO-encoding mRNA by selecting GC-rich codons for each amino acid. This rendered the mRNA non-immunogenic and increased protein expression levels even more than its Ψ-modified counterpart. Also, LNP-mediated, intravenous delivery of their EPO-encoding mRNA construct in pigs and non-human primates resulted in a significant increase in serum EPO levels and reticulocyte counts as well as an elevation of the hematocrit, without detectable immunogenicity [76].

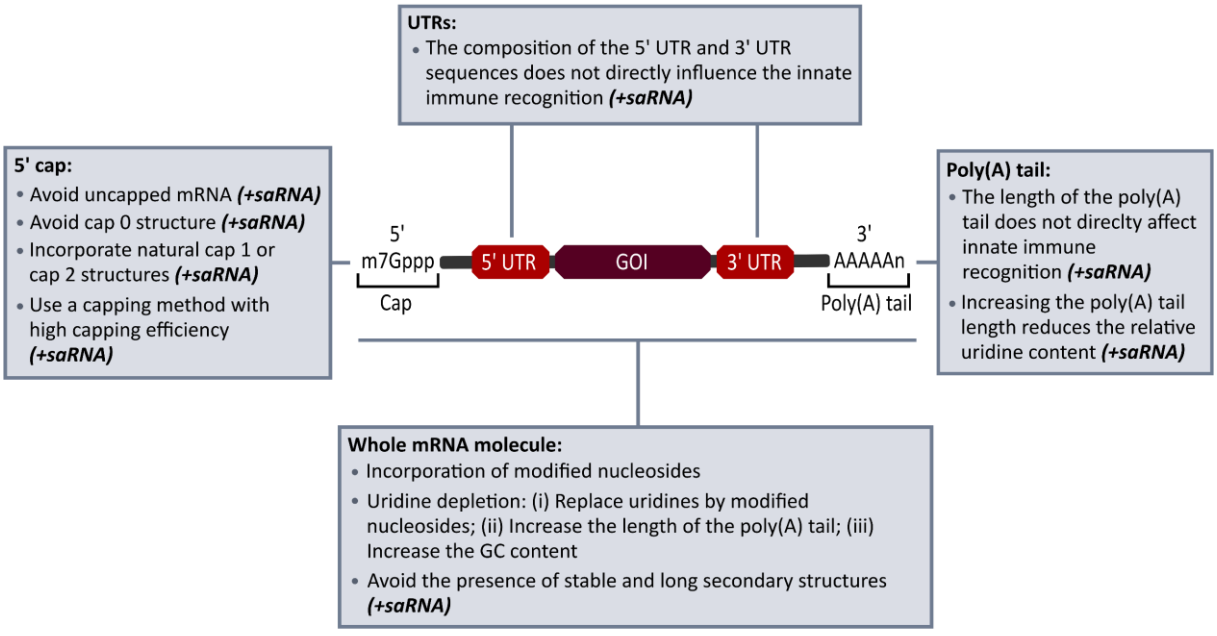
Distinct from conventional mRNA, the ORF of saRNA encodes four, viral nsPs essential for its self-replicating capacity. These nsPs are important controlling elements with regard to the saRNA biology and the host cell response [110]. Therefore, it might be interesting to introduce mutations in the nsPs to adapt both parameters. For example in the past, several groups reported on amino acid substitution mutations in the nsP2 protein to decrease the cytopathic effect of alphavirus-based saRNAs [111–114]. Recently, Li *et al.* used an *in vitro* evolution approach in IFN-competent cells to identify mutations within the VEE nsPs that promote *in situ* expression of subgenomic RNA. Five mutations in nsP2 and nsP3 were found to have a significant impact on saRNA persistence and gene expression levels. Additionally, they checked whether these mutations change the innate IFN response to saRNA, which

might declare their findings. Nevertheless, the best performing saRNAs in terms of protein expression were not those with the lowest interferon response [110]. Hence, mutations to the nsPs proteins can be considered to lower the innate immune-stimulating activity of saRNA but this is not straightforward and more research is still required.

4.1.6.mRNA secondary structures

Lastly, in several parts of the mRNA or saRNA, including the UTRs and the ORF, the mRNA secondary structure can have a significant impact on mRNA translation and immune-stimulating activity [16,115]. These secondary structures in the single-stranded mRNA molecules can be easily formed through complementary self-interactions [116]. First of all, highly stable secondary structures in the 5' UTR and the first 10 codons of the ORF should be avoided as they are known to reduce translation initiation efficiency and therefore overall protein production [117–119]. In contrast, Mauger *et al.* reported that an increased secondary structure, which was varied by the incorporation of modified nucleosides, in the ORF downstream of the first 30 nucleotides and in the 3' UTR correlated with increased protein expression. They demonstrated that introducing modified uridines, such as the use of m1Ψ, stabilizes mRNA secondary structures, by forming more stable base pairs than uridine [119]. These structural changes might not only enhance the functional half-life of mRNA, but was recently also found to be a key determinant for mitigating hydrolytic degradation of mRNA [120]. However, highly stable and long secondary structures in the whole construct are unwanted and should be avoided as they can be recognized as dsRNAs by PRRs [16,115].

A.



B.

Optimization IVT production and purification:
- Adapt the IVT reaction conditions to prevent formation of dsRNA byproducts
- Removal of dsRNA byproducts after IVT:
  - Ion pair reversed-phase high performance liquid chromatography (HPLC)
  - Cellulose-based chromatography *(+saRNA)*
  - Treatment with RNase III

C.

Innate immune inhibitors:
- Vaccinia virus proteins: B18R, E3, K3 *(+saRNA)*
- Influenza A proteins: NS1 and PB1F2
- RNA-Interference mediated immune suppression
- Small molecule innate immune inhibitors: ISRIB, dexamethasone, BAY11 *(+saRNA)*

Figure 3: Schematic overview of strategies to control innate immune activation upon mRNA and/or *(+saRNA)* delivery: A) Modifications to the mRNA backbone itself. Conventional mRNA contains a 5' cap structure (m7GpppN), a 5' untranslated region (5' UTR), the gene of interest (GOI), a 3' untranslated region (3' UTR) and a poly(A) tail. This figure indicates which adaptations can be made to each of these regions to suppress innate immune recognition. B) Optimizing *in vitro* transcription (IVT) production and purification of mRNA and saRNA. During IVT, immunogenic byproducts, including dsRNAs, are formed next to the desired mRNA strand. To limit the dsRNA amount and reduce innate immune stimulation, their formation during IVT can be prevented or they can be removed after IVT. C) Innate immune inhibitors. A concise overview is given of innate immune inhibitors that have been used to reduce innate immune stimulation upon mRNA and/or saRNA delivery.

4.2. Optimizing *in vitro* transcription (IVT) production and purification of mRNA and saRNA

4.2.1. General IVT production and purification of mRNA and saRNA

Both conventional mRNA and saRNA are synthesized by IVT of a linearized pDNA or PCR template, as described in Section 2.1. During IVT, also some byproducts are formed including short ssRNAs produced by abortive initiation events and dsRNAs, which have been identified as major triggers for the innate immune pathway by binding to TLR3, MDA-5 and RIG-I (**Figure 2**) [5,24,121,122]. Hence, mRNA or saRNA purification is a vital step to remove immunogenic byproducts and ensure a safe and fully functional end product (**Figure 3B**). Simple purification methods, such as LiCl or alcohol-based precipitation and silica membrane columns, are often used on small laboratory scale and efficiently remove free NTPs, proteins, salts and to a lesser extent short RNAs [20,123]. However, when highly pure mRNA should be obtained for GMP production, chromatographic approaches are typically used as a first choice [20], high performance liquid chromatography (HPLC) [124,125], anion-exchange chromatography [126,127], size-exclusion chromatography [128,129], and affinity chromatography with immobilized oligo(dT) [130]. Of interest, these chromatographic methods might be applicable for saRNA purification as well, but this is anything but straightforward due to its larger and more complex structure. At the moment, no commercially viable scalable purification process has been disclosed so far for saRNA purification [3,5].

4.2.2. Prevention of immunogenic dsRNA byproduct formation during IVT

Recent studies revealed two main mechanisms through which dsRNA byproducts are formed. Both are based on aberrant activity of the RNA polymerase besides its normal function as DNA-dependent RNA polymerase, transcribing mRNA from a pDNA template. First of all, T7 RNA polymerase has an obscure RNA-dependent RNA polymerase activity [131–134]. The synthesized RNA (runoff transcript) can serve as a template for T7 RNA polymerase leading to 3' extension of the runoff transcript if there is sufficient 3' complementarity to fold back on itself in *cis*, which results in the formation of intramolecular RNA duplexes [134]. In addition, during initial transcription, RNA polymerase produces short abortive RNA byproducts from 5 to 11 nucleotides in length [122,135]. When these short fragments anneal to complementary sequences in the runoff transcript (in *trans*), this can prime complementary RNA synthesis from the primary transcripts resulting in the generation of dsRNA contaminants [136]. Secondly, the T7 RNA polymerase has promoter-independent activity and could wrongly initiate transcription from a promoter-less DNA end, resulting in the formation of an antisense RNA molecule that is fully complementary to the intended sense transcript. Hybridization of these two RNA molecules leads to the formation of long dsRNA contaminants in the IVT reaction mixture [137].

In a recent study, Mu *et al.* showed that for a few specific templates the formation of dsRNA species can be reduced during IVT by lowering Mg^{2+} concentrations or by using modified nucleosides [137]. However, since Mg^{2+} is a crucial element during IVT, reducing its concentration also affects the total mRNA yield, which is undesirable in the production of large quantities of mRNA. Another strategy to reduce the amount of dsRNA byproducts is the use of a thermostable T7 RNA polymerase in the IVT

reaction. Wu *et al.* demonstrated that performing the IVT reaction of a couple of mRNAs at 50 °C with thermostable T7 RNA polymerases reduced the 3' extension of the runoff product, and subsequently the formation of dsRNA byproducts without loss of the functional runoff transcript. Furthermore, the presence of a template-encoded poly(A) tail showed to reduce antisense dsRNA byproducts, but not the amount of 3' extended dsRNA byproducts [138]. In another study of Gholamalipour *et al.*, the addition of a DNA oligonucleotide complementary to the 3' end of the expected runoff RNA has shown to restrain the production of 3' extended dsRNA byproducts during an IVT reaction of mRNA [139]. However, this DNA oligonucleotide needs to be removed again after IVT, requiring another enzymatic step, which is unfavorable as it complicates the production process. It should be noted that to date, the concept of diminishing dsRNA formation by adaptation of several IVT reaction components has not been described specifically for saRNA and the optimal reaction conditions may differ from those for conventional mRNA due to the structural differences between both mRNA types.

4.2.3.Removal of dsRNA byproducts from IVT mRNA and saRNA

An alternative approach to reduce the number of dsRNA contaminants is to eliminate dsRNA byproducts after completion of the IVT reaction. The vast majority of known purification techniques for mRNA and saRNA as mentioned in Section 4.2.1 are ineffective in the removal of dsRNAs. In this context, a major breakthrough was achieved when Karikó and colleagues reported that dsRNA impurities could be eliminated from IVT mRNA by ion pair reversed-phase HPLC based on the longer retention time of dsRNAs. They demonstrated that reversed-phase HPLC purification eliminated the residual immune stimulating activity of Ψ- or m5C/Ψ-modified mRNA, rendering a completely non-immunogenic mRNA. In addition, reversed-phase HPLC purification of both modified and unmodified mRNA improved protein production significantly in primary cells [121]. In a recent study, Nelson *et al.* highlighted the importance to combine different strategies to eliminate innate immune recognition of IVT mRNA. They modified the mRNA by replacing uridine by m1Ψ and removed dsRNAs by reversed-phase HPLC purification and by alteration of some IVT components including the use of a custom NTP ratio instead of equimolar levels of each NTP. The combination of both resulted in the creation of an IVT mRNA nearly indistinguishable of injected PBS with regard to the innate immune activation in two mouse strains, whereas both approaches on their own were less effective at reducing immunogenicity [140]. Despite the clear potential of ion pair reversed-phase HPLC to remove dsRNA byproducts and reduce the immunogenicity of IVT mRNA, the need for expensive and specialized instrumentation, the long purification time, the use of toxic acetonitrile as eluent and the low recovery rate of only 50% impedes the cost effectiveness of the mRNA production and makes it less suitable for scaling up the process [121,136].

Of interest, Baiersdörfer *et al.* reported on a cellulose-based chromatographic method that removes at least 90% of the dsRNA contaminants, based on the selective binding of dsRNA to cellulose in an ethanol-containing buffer. This new purification method is similarly effective to remove dsRNA contaminants as reversed-phase HPLC, but on the contrary is fast, cost effective, scalable and has a relatively high recovery rate between 65% and 85%. Upon cellulose-based chromatography, m1Ψ-containing IVT mRNA no longer induced IFN-α secretion *in vivo* when injected intravenously as lipoplex. In addition, elimination of dsRNA byproducts by cellulose-based purification showed to increase the *in vivo* translatability of IVT mRNA to a similar extent as reversed-phase HPLC purification, suggesting that these methods are equally effective and interchangeable for the removal of dsRNA contaminants from conventional IVT mRNA [136].

A third and last strategy to eliminate dsRNA byproducts after completion of the IVT reaction, is treatment of mRNA with RNase III, which specifically recognizes and cleaves dsRNAs of approximately 22 nucleotides in length. A recent *in vivo* study showed an improvement in cytotoxic killing of leukemia cells by chimeric antigen receptor (CAR)-expressing T cells generated with IVT mRNA, which was digested with RNase III [141]. The main disadvantage of this technique is the risk on erroneous cleavage

by RNase III of double-stranded secondary structures formed by ssRNA. Moreover, an additional purification step is required to remove RNase III from the end product [136].

In contrast to conventional mRNA, saRNA cannot be purified from dsRNAs by ion pair reversed-phase HPLC, as shear forces would cause the longer saRNA to break. On the contrary, cellulose-based chromatography of saRNA has already been reported to efficiently remove dsRNA byproducts from saRNA in a recent study by Zhong and colleagues. They demonstrated that cellulose purified saRNA elicited a significantly lower type I IFN response in IFN-β reporter mice after intradermal electroporation in comparison to saRNA purified on silica-based columns, which was however not reflected by an increase in protein translation. Nevertheless, a clear improvement in efficiency of a saRNA vaccine against Zika virus was noticed, characterized by a triplication in antibody titers, a doubling of the cellular immune responses and an increase in seroconversion rate from 62,5% to 100% [142]. Furthermore, no studies have been published yet describing RNase III treatment for saRNA to remove dsRNA contaminants, but most likely this technique would also work for saRNA.

4.3. Innate immune inhibitors

As mentioned above, several backbone modifications to reduce innate immune stimulation, including nucleoside base modification and sequence alteration, cannot be applied for saRNA, as this would interfere with its functionality. Also for conventional mRNA, nucleoside modifications could possibly alter mRNA secondary structures, translation and protein folding. Therefore, the use of innate immune inhibitors is gaining attention to modulate the immune-stimulating activity of both conventional and self-amplifying mRNA (**Figure 3C**). Over the last few years, many inhibitors have been screened for both conventional and self-amplifying mRNA but, although they often possess a clear mode of action, not every inhibitor tested showed potential as an appropriate adjuvant [10,21,24,143,144]. In this section, we will provide a comprehensive overview of the most important results obtained up to date.

4.3.1.Viral immune evasion mechanisms

Viruses have developed a plethora of intelligent mechanisms to avoid recognition and killing by the host's immune system, such as evading PRR detection, inhibiting IFN mediated effects and hampering cytokine function [145]. Inspired by these viral escape mechanisms, several viral proteins have been evaluated as potential innate immune inhibitors to improve mRNA translation.

4.3.1.1. Vaccinia virus proteins: B18R, E3 and K3

Vaccinia viruses (VVs) belong to the Poxviridae family, which are a family of large and double-stranded DNA viruses, primarily known for their use in smallpox vaccine formulations. The genome can be divided in a fixed central region and the lesser conserved terminal regions. The latter are responsible for encoding proteins involved in virulence intensity and immune response regulation such as B18R, E3 and K3 which have been evaluated for their role in IFN interference [146,147].

B18R is a 60-65 kDa glycoprotein encoded by the Western Reserve strain of the VVs and acts as a decoy receptor to neutralize type I IFNs (**Figure 2**). As such, the activation of IFNAR and subsequent pathway signaling is inhibited, resulting in reduced mRNA degradation and enhanced translation. This VV protein can be present in its soluble form or bound to the cell surface and is reactive towards human, cow, rabbit, rat and with lesser affinity mouse type I IFNs [146,148–151]. Several reprogramming studies have used this inhibitor as an adjuvant to non-amplifying and self-amplifying mRNA delivery, where it was either administered as the soluble protein in the cell culture medium or expressed *in situ* by co-transfecting B18R mRNA with mRNA encoding for the protein of interest (POI). Results indicated a positive effect on cell viability and high protein expression, which is useful for the repetitive transfections often required during cell reprogramming [152–156]. Furthermore, B18R was also useful

as adjuvant therapy to modified mRNA delivery during these reprogramming studies [152,153,156]. Although modified mRNA is known to efficiently reduce innate immune responses, it has been opted that B18R is used to neutralize remaining type I IFNs [155,157,158]. Besides its use for reprogramming purposes, B18R has also been explored in several non-reprogramming studies, where it has demonstrated to decrease type I IFN production and increase mRNA translation [159–161].

It should be noted that the beneficial effect of B18R on cell viability could not be replicated by both Gomez *et al.* and Minnaert *et al.* [159,160]. However, this could be explained by the fact that these studies did not involve multiple mRNA transfections in contrast to the cell reprogramming studies. As Michel *et al.* showed, the increase in cell viability caused by B18R was only generated starting from the 5th transfection [162]. Furthermore, conflicting results regarding the effect of B18R on protein expression and immune response suppression have been reported [149]. The research group of Byrne and Adjaye was not able to increase reprogramming transcription factor expression or to reduce immune activation in fibroblast cells in the presence of B18R [143,163,164]. Also Poleganov *et al.* observed no enhanced protein expression or reduced IFN-β production upon addition of B18R mRNA or B18R protein [155]. Again, Michel *et al.* only observed an increase in protein expression upon 2 consecutive mRNA transfections co-delivered with B18R mRNA but not with the B18R protein [162]. Taken together, it appears that the effect of B18R on both cell viability and protein expression is dependent on the transfection frequency and duration, the cell type, and the POI.

Besides B18R, two other VV proteins, E3 and K3, have been tested to block innate immune response activation during mRNA transfections, although their precise mode of action remains elusive. The 25 kDa protein E3 is suggested to act primarily by dsRNA binding, thereby preventing activation of anti-viral state pathways, and direct PKR inhibition which hampers homodimerization (**Figure 2**) [146,165–167]. Moreover, it was recently suggested by Mehta *et al.* that E3 would prevent apoptosis upon viral infection by stimulation of apoptosis inhibitor F1 expression. The latter is allegedly mediated by PKR inhibition as described before [168]. K3, a 10.5 kDa protein with 28% homology to eIF2α, is suggested to act as a competitive substrate for PKR (**Figure 2**) [146,165–167,169].

Poleganov *et al.* delivered non-modified mRNA encoding for the immune evasion proteins B18R, E3 and K3 together with non-modified mRNA encoding for different reprogramming factors, to Human Foreskin Fibroblast (HFF) cells. The authors observed a three-fold increase in protein expression mediated by E3 and K3 mRNA delivery, which further increased to 4.6-fold upon the additional delivery of B18R mRNA, despite the fact that B18R mRNA alone remained without effect. Consequently, the authors measured an 80% decrease in IFN-β secretion due to E3 and K3, which even further reduced upon addition of B18R mRNA or the B18R protein. Additionally, the addition of the immune evasion proteins also prevented cell death upon multiple mRNA transfections [155]. Beissert *et al.* performed a similar follow-up study where mRNA encoding the same three immune evasion proteins was delivered, as an adjuvant therapy for saRNA transfection in HFF cells. They observed that E3 inhibited PKR very efficiently, but K3 inhibition of eIF2α phosphorylation was less pronounced. B18R efficiently neutralized IFNs but only showed, similar to K3, a moderate effect on protein expression. However, when all three VV proteins were combined, both B18R and K3 did provide an additive effect to E3, which was confirmed *in vivo* [165]. Finally, Liu and colleagues delivered modified E3, K3 and B18R encoding mRNA as a pretreatment before transfection of HFF and HepG2 cells with unmodified mRNA. All three VV proteins increased protein expression, with E3 being the most potent inhibitor, without inducing cell toxicity. Furthermore, a reduced IFN-β production was seen in both cell types upon E3 mRNA delivery, albeit for B18R and K3 this effect seemed cell-type dependent [167].

Taken together, the different VV proteins described above all show potential as innate immune inhibitors to enhance treatment efficiency both in terms of cell viability or protein expression. Future studies to elucidate the molecular mechanisms involved and to determine *in vivo* effects more in detail will provide insight about their future clinical potential.

4.3.1.2. Influenza A virus proteins: NS1 and PB1-F2

The influenza A virus (IAV) belongs to the Orthomyxoviridae virus family and is a single-stranded RNA virus, consisting of eight RNA segments. Similar to VVs, several mechanisms have been developed by the IAVs to evade the innate immune response, including the expression of proteins, which might inhibit triggers and signaling pathways of the innate immunity. Below, we will discuss two noteworthy non-structural IAV proteins and their influence on innate immunity [170,171].

The non-structural protein 1 (NS1) is encoded by the eighth mRNA segment of the influenza A virus and consists of both an RNA binding domain and an effector domain. Innate immune signaling is prevented through several mechanisms depending on the virus strain of origin including inhibition of IRF3, direct PKR inhibition and OAS inhibition by dsRNA binding (**Figure 2**). Moreover, upon viral infection, NS1 proteins encoded by some virus strains can also inhibit the cleavage and polyadenylation factor 30 (CPSF30) function, which interferes with pre-mRNA processing in the nucleus. Hence, the transport to the cytoplasm is inhibited, which therefore hampers the expression of many genes, such as ISGs. However, several additional immune response suppression mechanisms mediated by NS1 still require further study [167,170,172–174]. Phua *et al.* co-delivered unmodified mRNA encoding for six different NS1 proteins, derived from different virus strains and subtypes, together with unmodified luciferase mRNA in fibroblasts and observed an increase in luciferase protein expression by all NS1 proteins. Although IFN reduction was similar for all NS1 proteins, NS1-TX91 and NS1-VN showed a higher protein expression in comparison to the others, which was attributed to their CPSF30 inhibiting activity. Furthermore, the authors could increase the protein expression of modified mRNA upon addition of modified NS1-TX91 mRNA. *In vivo*, however, the effects were not as outspoken as compared to *in vitro* settings [175]. In a later study, the same research group delivered eGFP encoding unmodified mRNA together with NS1-HK encoding unmodified mRNA to several cell types and noticed enhanced eGFP expression in BJ fibroblasts, HepG2, RAW 264.7 and pMEF cells, accompanied by a reduced IFN production. Furthermore, cell viability increased upon NS1 mRNA delivery, both after single and multiple mRNA transfections. However, when testing several NS1 proteins, originating from different virus strains, this was only observed for the NS1 proteins that did not inhibit CPSF30. Of note, these effects appeared to be cell type- and dose-dependent, so further in-depth research is required [172]. Recently, Liu *et al.* pretreated HepG2 and BJ Fibroblasts with modified mRNA encoding NS1 and the three VV proteins E3, K3 and B18R before transfection with unmodified luciferase mRNA. The authors showed a superior behavior for NS1 and no synergistic effect of the VV immune evasion proteins could be noticed, explained by a possible overlap in function between E3 and NS1 [167]. Although more studies are needed to further elucidate the immune evasion mechanisms of NS1 and to determine efficacy and safety *in vivo*, the abovementioned results show great potential for this protein to enhance mRNA translation.

A second potentially interesting non-structural IAV protein is PB1-F2, which has been linked to several effects such as type I IFN response suppression. However, most of the effects seem to be virus strain dependent. Although Le Goffic *et al.* described an increase in type I IFN activity mediated by PB1-F2, several more recent studies have contradicted this observation [174,176–178]. A type I IFN antagonist activity could be observed for both the PB1-F2 wild type (WT) protein and the mutated PB1-F2 N66S protein, containing a serine instead of an arginine residue at position 66 [174,177,178]. The molecular mechanisms underlying this type I IFN inhibitory effect are still not fully elucidated but would be situated in the RIG-I pathway, where association between RIG-I and MAVS is suggested to be hampered, which would mainly interfere with the IRF3 function (**Figure 2**) [174,177,178]. Although more in-depth mechanistic studies are needed, it might be worthwhile investigating the potential of PB1-F2, both in its WT and mutated form, to enhance IVT mRNA translation and protein expression.

4.3.1.3. MERS-CoV ORF4a and PIV-5 V self-amplifying mRNA inhibitors

The studies described above demonstrate that applying two different mRNAs, i.e. encoding the protein of interest on the one hand and the immune inhibitor on the other, has shown good potential to reduce IVT mRNA-induced innate immune responses. However, the fact that cells have to receive both mRNA formulations remains a difficulty. Recently, Blakney *et al.* provided an interesting alternative to overcome this hurdle. They established saRNA constructs containing coding regions for both the POI and an innate immune inhibitor. An *in vitro* screen of several different inhibitors encoded by the saRNA showed the superiority of the MERS-CoV ORF4a and PIV-5 V protein in different human cell lines. The MERS-CoV ORF4a protein binds to dsRNA, which hampers PACT activation of the RIG-I and MDA-5 receptors, while the PIV-5 V protein binds to MDA-5 directly. As such, these proteins could possibly interfere with IFN production. The mechanism of increased protein expression was studied in MRC5 cells and they indeed found a decrease of NF-κB and IRF3 in the early stages upon transfection. The *in vitro* results were however not confirmed *in vivo*, as neither of the proteins led to a substantial and significant increase in protein expression in both BALB/c and C57BL/6 mice. In contrast, both proteins were able to increase the percentage of transfected cells in human skin explants [4]. Nevertheless, this innate immune evasion strategy remains interesting to explore. However, future studies should include more in-depth research on the delivery system dependency, anti-vector immunity, possible combination of inhibitors and effects in humans [4].

4.3.2.Non-viral immune evasion mechanisms

Besides the use of viral immune evasions proteins, many other non-viral molecules have been tested for their potential to reduce immune responses and increase protein expression, of which we will provide a selection below.

4.3.2.1. RNA Interference-mediated immune suppression

Small interfering RNAs (siRNAs) are short dsRNA sequences that can be designed to silence any gene in a sequence specific manner [10]. The research group of Angel *et al.* combined mRNA for cell reprogramming with an siRNA cocktail targeting different immune-related molecules and found that knockdown of IFN-β, PKR and STAT2 completely recovered cell viability upon multiple mRNA transfections [179]. These studies highlight the complexity of the immune responses and the fact that inhibiting multiple factors might be needed to evade immune responses efficiently [24]. Furthermore, Lee *et al.* showed the use of short hairpin RNAs (shRNAs) to inhibit the function of TLR3, TRIF and MYD88. Although the authors performed the experiments to elucidate the role of these receptor and transcription factors in pluripotency induction, this mechanism could also be explored to suppress immune-component activity beneficial for treatment efficiency [180]. At last, the use of micro RNAs (miRNAs), small non-coding RNA sequences, could also be explored. Zhang *et al.* demonstrated the potential of miR-29c to protect the deubiquitinating enzyme A20. The latter is involved in the suppression of NF-κB and IRF signaling pathways, thereby inhibiting the innate immune response during IAV infections. Results showed that miR-29c was capable to protect A20 mRNA against RISC-degradation by acting as an RNA decoy. Moreover, A20 protein expression was enhanced leading to reduced pro-inflammatory cytokine expression [171]. Although this study does not involve IVT mRNA transfection, the function of miRNAs in reducing synthetic mRNA-related innate immune responses might be worthwhile investigating. Yet, it is important to consider that siRNA, shRNA and miRNA molecules might also activate the immune system, which possibly counteracts their effects. However, chemical modification of these compounds can possibly overcome this problem [181–183].

4.3.2.2. Small molecule inhibitors

Several small molecules have been identified that can inhibit key components of the innate immune pathways, thereby possibly reducing IFN production. However, Liu *et al.* tested 15 different small molecular compounds and found that none of them enhanced mRNA transfection efficiency in human

fibroblasts [144]. Also Drews *et al.* tested several small molecules to suppress innate immune responses in HFF cells, but neither chloroquine, Pepinh-TRIF, Pepinh-MYD88, Trichostatin A or the VV protein B18R induced a considerable reduction of the innate immune response as measured by expression of innate immune associated genes [143]. On the other hand, Awe *et al.* showed that BAY11, a IKK complex inhibitor, was more potent than the TBK1 and IKKε inhibitor BX795 to increase protein expression and modified mRNA stability, mediated by reduction of NF-κB expression [163].

Also the integrated stress response inhibitor (ISRIB), a molecule capable of enhancing translation by improving eIF2B complex formation, demonstrated to enhance protein expression in the early transfection stages (0-6h) upon luciferase mRNA delivery [184,185]. The authors did notice, however, that the corticosteroid dexamethasone was more capable of increasing translation efficiency in the intermediate to late stages upon transfection. When both components were delivered together a two-phase increase in protein expression was observed. When dexamethasone was used for further *in vivo* studies, it improved luciferase expression after intravenous delivery of dexamethasone-palmitate loaded mRNA lipoplexes, possibly attributed to its NF-κB inhibiting activity [185]. A very recent study of Zhong *et al.* tested another corticosteroid, clobetasol propionate, which was able to reduce type I IFN response and significantly enhanced expression upon intradermal electroporation of a self-amplifying mRNA vaccine against the Zika virus [142].

Taken together, the use of viral and non-viral innate immune inhibitors is an interesting strategy to consider during the development of mRNA-based therapeutics for different applications. The inhibitors can be used as an adjuvant to self-amplifying mRNA, modified and unmodified conventional mRNA. Moreover, they can provide an added value when multiple mRNA transfections are required, for instance with cellular reprogramming. Finally, inhibitors can be delivered as soluble proteins or as mRNA constructs, possibly co-encapsulated with the therapeutic mRNA in a nanoparticle carrier. However, which inhibitor should be selected and how it should be used is cell type and therapy dependent.

5. mRNA vaccines

Vaccines can prevent against a variety of infectious diseases, by priming the immune system which is then able to recognize and destroy the pathogen upon future encounter. Most of the current vaccines are based on attenuated or inactivated pathogens, or recombinant produced antigens. Over the recent years, many researchers have recognized the advantages of conventional and self-amplifying mRNA-based vaccines for both infectious diseases and cancer treatment [3,5,21,23,35,186,187]. First, the mRNA sequence can be easily and rapidly adapted to express the antigen of choice, which is very convenient for personalized treatment with cancer vaccines or to provide quick responses against emerging infectious diseases [16,17,21,23,35,36]. Second, depending on the mRNA vaccine design and immunogenicity profile, mRNA vaccines have shown great versatility to treat different disease indications, ranging from infectious diseases, cancer treatment, and even autoimmune diseases [188–190]. At last, mRNA does not require penetration into the nucleus to perform its action, making it useful for transfection of slow or non-dividing cells, such as dendritic cells (DCs) [35]. Despite this plethora of advantages, some attention points remain. As mRNA is a labile molecule, *in vitro* and *in vivo* stability should be improved by for example optimizing the mRNA backbone structure, the delivery route and especially by formulating the mRNA into nanoparticles. Several different administration routes and delivery carriers have been explored to improve the *in vivo* stability and to promote efficient intracellular delivery [17,21,30,191–194]. Another key challenge in mRNA vaccine development is to find the optimal balance between the mRNA-mediated innate immune stimulation and antigen expression, which should be fine-tuned depending on the specific therapeutic application. This interesting topic has been also discussed in several other reviews [16,17,34,192,195].

As outlined in Section 3, type I IFNs and many other proteins, encoded by ISGs are produced upon the cellular entrance of synthetic mRNA by activation of PRR signaling pathways [10,16,34,37,38]. On the one hand, type I IFN signaling induced by synthetic mRNA plays a key role in the immune activation of both innate and adaptive immune cells, mimicking the immune events upon a natural infection with viruses. On the other hand, the type I IFN reaction can inhibit the translation of mRNA and could therefore also work detrimental for the vaccine efficiency. Moreover, the inflammatory effects of type I IFNs can be a culprit for the safety of mRNA vaccines, as they play - in all likelihood - an important role in the reactogenicity of mRNA vaccines [17,18,21,34–36,196]. Therefore, the key towards a successful mRNA vaccine might be a well-balanced innate immune response, while avoiding excessive innate immune triggering in order to sustain the protein expression needed to reach the antigenic threshold for T cell and B cell activation. However, it is not completely understood how to hit this sweet spot. This challenge becomes somewhat more complicated, since the carrier system, such as LNPs, might not only contribute to the mRNA delivery efficiency, but can also possess intrinsic innate immune effects that can influence and maybe even overrule the innate immune activity of mRNA vaccines [11,197,198]. Moreover, the mRNA vaccine design may have to be tailored to the specific therapeutic application and the associated desired adaptive immune response. Indeed, differential kinetics and amount of mRNA encoded antigen expression and type I IFNs might be required for the generation of antibody producing B cells, helper $CD4^+$ T cells or cytotoxic $CD8^+$ T cells [199–202]. A more in-depth overview about the role of this self-adjuvant effect on the efficacy of mRNA vaccines and the use of other immune adjuvants, will be provided below. After all, as mRNA vaccination has truly gained more attention since the recent COVID-19 pandemic, this subject is more than ever important to consider.

5.1. Type I IFN-mediated effects: beneficial or detrimental for mRNA vaccines?

A first requirement for sufficient mRNA vaccine efficiency is that the antigen expression needs to reach the antigenic threshold for T cell and B cell activation [202–204]. After the mRNA translation process, preferentially taken place in antigen presenting cells (APCs), produced antigens can, depending on their signaling properties, accumulate in the cytosolic compartment, be secreted into the extracellular matrix environment, or be exposed at the cellular surface as a membrane protein antigen. The latter two transport mechanisms make the antigenic proteins accessible for B cell recognition [205,206]. Secreted antigens can also be recycled into APCs, thereby entering the major histocompatibility complex (MHC) II pathway to be presented to $CD4^+$ T cells [207,208]. Produced antigens in the cytosol can be directly processed into antigenic peptides, followed by their presentation in MHC-I complexes to $CD8^+$ T cells [17,34,209]. Besides this antigen signal, other stimulatory signals are needed in order to activate adaptive immune cells [210]. This includes their interactions with co-stimulatory molecules (e.g. CD40, CD80 and CD86) and pro-inflammatory cytokines, as illustrated in **Figure 4**. The type I IFN reaction, induced by mRNA vaccines, may trigger several stimulatory effects on both innate and adaptive immune cells that can contribute to the vaccine efficacy. This self-adjuvant effect of mRNA vaccines promotes the activation of APCs, as type I IFN signaling leads to the upregulation of co-stimulatory molecules, stimulates antigen presentation pathways, and activates the production of pro-inflammatory cytokines and chemokines. Moreover, type I IFNs can directly act as a third cytokine signal for T cell activation. However, at the same time, type I IFNs can interfere with the mRNA translation process, lowering the antigen availability, and therefore in some cases might negatively impact the vaccine outcome [17,34,35,201,211–215].

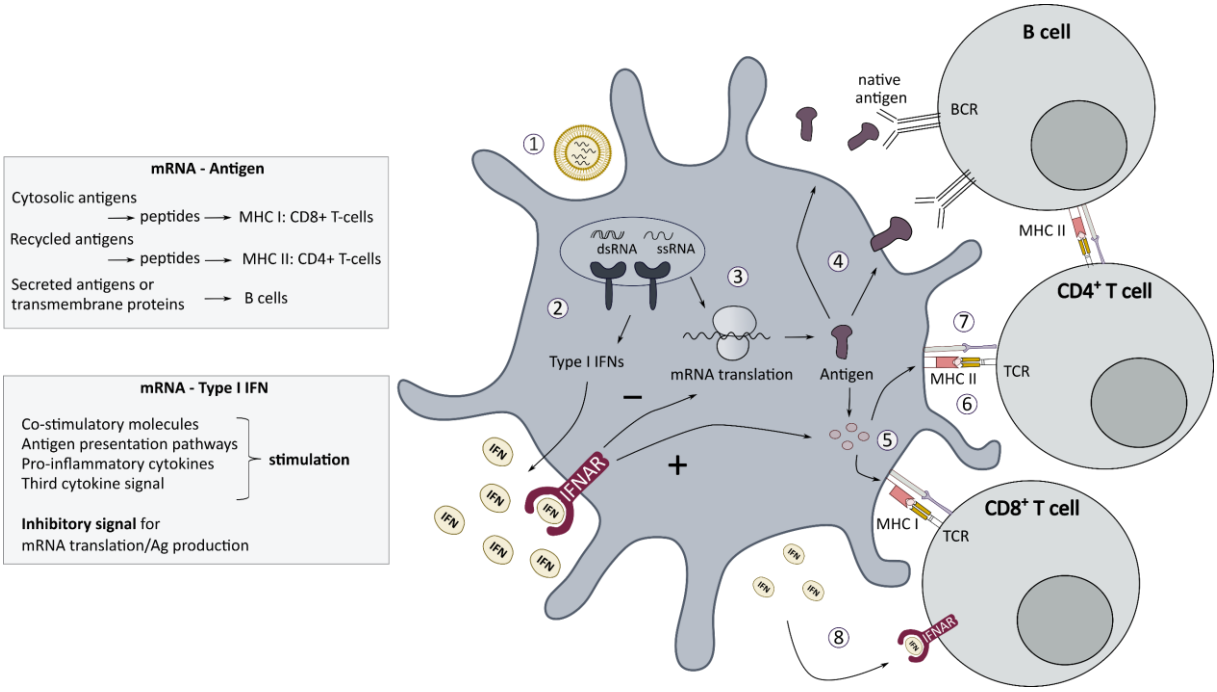


Figure 4. From mRNA delivery to immunity activation. Upon mRNA delivery to APCs (1), mRNA sensing by PRRs leads to the production of type I IFNs, ISGs and pro-inflammatory cytokines (2). A crucial step is that mRNA is efficiently translated resulting in the production and accumulation of antigen proteins in the cytosol (3). Subsequently, antigen proteins can be secreted in the extracellular environment or presented as a transmembrane protein to B cells, depending on their signaling properties (4). Moreover, antigen processing to peptides occurs by several pathways (5), which are presented via the MHC-I and MHC-II molecules to $CD8^+$ and $CD4^+$ T cells, respectively (6). Interaction of T cells with co-stimulatory molecules on the APC surface serves as a second necessary signal for T cell activation (7). All of these activation signals are boosted by type I IFNs. Moreover, type I IFNs, present in the extracellular environment, can interact with their receptor on the T cell surface, thereby acting as a direct third activation signal (8). Abbreviations: BCR, B cell receptor; TCR, T cell receptor

In the context of the development of mRNA vaccines for COVID-19, the focus was laid on the generation of neutralizing antibody responses against the SARS-COV-2 Spike glycoprotein [216]. At the end of last year, two COVID-19 mRNA vaccines, BNT162b2 and mRNA-1273, were granted authorization for emergency use. These COVID-19 mRNA vaccines are composed of HPLC-purified, m1Ψ-containing mRNA formulated in LNPs to allow their delivery upon intramuscular administration [216]. By mitigating the type I IFN activity, *e.g.* using modified uridines and extensive purifications methods, more durable protein expression and thus prolonged antigen availability can be achieved [216]. In general, a prolonged mRNA expression and continuous presentation of antigens via MHC-II molecules is expected to promote the induction of helper $CD4^+$ T cells and to elicit Germinal Center (GC) reactions. In these GCs, formed in secondary lymphoid tissues such as in the spleen and lymph nodes, B cells undergo cell divisions, affinity maturation and isotype switching. Eventually, this will determine the quality and magnitude of the antibody response [202]. Lederer *et al.* demonstrated that intramuscular immunization with a SARS-CoV-2 (m1Ψ) mRNA-LNP vaccine in mice induced robust spike-specific GC B cells and follicular $CD4^+$ T cell responses [217]. Moreover, a recent study report provided first clinical evidence that vaccination with the SARS-CoV-2 mRNA vaccine, BNT162b2, in humans induces a robust and persistent GC B cell response in the draining axillary lymph nodes of all participants after boosting [218].

In contrast, the use of “immunogenic” unmodified mRNA has been the preferred choice in the design of mRNA vaccines for cancer vaccination, where the main focus is to elicit cytotoxic $CD8^+$ T cell responses to eliminate tumor cells [216]. In light of this, several conventional mRNA vaccination

studies for cancer immunotherapy highlighted the role of type I IFNs to elicit cytotoxic T cells and therefore their influence on vaccine efficiency. Additionally, it is important to remark that type I IFNs have also shown to possess direct antitumor effects, exerted via two mechanisms: inhibition of tumor cell growth and angiogenesis [205]. Kranz *et al.* illustrated that intravenous injection of RNA-lipoplexes induced IFN-α secretion leading to strong $CD8^+$ T cell responses. When an IFNAR blocking antibody was administered, the T cell effector response dropped and antitumor immunity was decreased in contrast to mice not treated with IFNAR antibodies [219]. Furthermore, Broos and colleagues observed a decrease in mRNA expression upon intravenous administration of mRNA-RNAiMAX complexes, due to type I IFN signaling. However, the tumor regression and cytotoxic T cell response was considerably lower in IFNAR deficient mice, corroborating the role of type I IFN secretion. Of note, it is important to mention that both intramuscular and subcutaneous injection did not lead to the desired $CD8^+$ T cell expansion and activity [220]. The latter suggests an important role for the administration route on the final outcome. Additionally, the carrier system needs to be optimized according to the route of administration, as different anatomical and physiological barriers for successful mRNA delivery will be encountered. For instance, as also shown by Hassett *et al.*, mRNA lipoplexes composed with the cationic lipid DOTAP fail to obtain protein expression when these particles are intramuscularly injected [221]. However, these particles are effective to induce $CD8^+$ T cells upon intravenous administration, by successfully targeting and transfecting APCs in the spleen [196,219].

The impact of type I IFN signaling on the vaccine outcome was shown to be different for saRNA vaccines. The antigen expression kinetics from saRNA differs from conventional mRNA, as it is preceded by the translation of the replicase complex and as it is more intense and long lasting [3,20]. Also, due to structural and possible productional differences between mRNA and saRNA, their innate immune-stimulating activity might not be equally strong. Hence, the intensity and timing of the elicited innate immune response and the final outcome of type I IFNs on vaccine efficiency might differ as well. Pepini and colleagues revealed that intramuscular injection of a saRNA-based vaccine induced an early and robust type I IFN and ISG response in mice, which negatively impacted initial antigen expression. In IFNAR knockout mice, reporter antigen expression was significantly higher and sustained upon intramuscular LNP-mediated saRNA delivery. Moreover, a saRNA vaccine encoding respiratory syncytial virus (RSV) F protein was shown to be more potent in IFNAR knockout compared to wild-type mice, as illustrated by an increase in $CD8^+$ T cell responses and in total IgG anti-RSV F antibody titer [213]. Additionally, Zhong *et al.* reported on the negative effect of type I IFNs on saRNA vaccine efficiency. They showed that intradermal electroporation of their saRNA vaccine encoding the pre-membrane and envelope (prM-E) glycoproteins of Zika virus (ZIKV) elicited higher ZIKV E specific $CD8^+$ T cell responses, antibody titers and seroconversion rates in IFNAR1 knockout mice in comparison to wild-type mice [222].

It should be noted that the different experimental conditions used in the many, sometimes contradictory, studies available make it difficult to compare results with one another. It has however been suggested that the amount of type I IFN secretion as well as the timing of type I IFN signaling are crucial for the final outcome [16,34,208,212,220,223,224]. Depending on whether the type I IFNs act before or after T cell priming, the effects can be detrimental or beneficial respectively, as discussed by De Beuckelaer *et al.* [34]. T-cell activation followed by or simultaneous with type I IFN signaling stimulates clonal proliferation and differentiation. In contrast, when the opposite order of events occurs, proliferation might be inhibited and T cell apoptosis can take place. An explanation for this complex mechanism was found in the STAT signaling pathway. When T cells are activated by antigen recognition, the transcription factor STAT4 is activated. Subsequent IFNAR stimulation can re-activate STAT4, thereby inducing proliferation and blocking apoptosis. Although, if IFNAR stimulation occurs before T cell priming, the STAT1 protein can be upregulated resulting in apoptosis and inhibition of proliferation [34,215,224]. In fact, both the administration route and carrier can determine the order of signaling and the effect of type I IFNs on $CD8^+$ T cell responses, making it important aspects of the vaccine formulation [16,34,225].

5.2. Adjuvant therapies

All things considered, both antigen expression and innate immune stimulation are crucial for the vaccine efficiency [16,17,220]. The self-adjuvant effect of mRNA therapeutics is one of the aspects that contributed to the increasing interest in conventional and self-amplifying mRNA-based vaccines. However, type I IFN production can come at a cost of both non-amplifying and self-amplifying mRNA translation, which can work detrimental for the vaccine efficiency. Currently, researchers continuously aim to develop optimal mRNA vaccine formulations where the antigen is adequately expressed and innate immune responses are contributing to the final outcome without hampering the safety. The question however remains, if only the application of the mRNA modifications is enough to reach this goal. Besides adaption of the mRNA structure as extensively described in this review and the optimization of delivery vehicles, many researchers have focused on the use of other immune-stimulatory compounds. Especially, in the field of cancer immunotherapy, this could be of interest to increase $CD8^+$ T cell response [216]. We provide some examples below but for an extensive list of the explored options, we refer to [16,17,21,34].

As mentioned above, the use of modified uridines can improve the intracellular mRNA stability and translation efficiency by reduction of PRR signaling, but can also come at a cost of T cell priming and functioning [19,34,226]. Therefore, Verbeke *et al.* explored the use of the TLR4-agonist monophosphoryl lipid A (MPLA), as an adjuvant to nucleoside-modified mRNA-loaded liposomes. Results indicated that type I IFN signaling was extremely reduced using nucleoside-modified mRNA, while $CD8^+$ T cell responses were maintained if MPLA was incorporated in the modified mRNA liposomes [17,196]. A later study by the same researchers, revealed the potential of intravenously injected mRNA Galsomes, liposomes containing nucleoside-modified mRNA encoding the antigen of interest and α-galactosylceramide, a glycolipid to promote anti-tumor immunity. The authors observed a more potent $CD8^+$ T cell response compared to liposomes containing immunogenic unmodified mRNA or modified mRNA alone, resulting in substantial tumor regression [227]. Furthermore, CureVac has developed the RNActive® vaccine formulation, which consists of naked sequence-engineered mRNA supplemented with mRNA-protamine complexes to induce immune responses upon TLR7 activation. In order to benefit from both considerable antigen expression and immune stimulation, an appropriate ratio between free mRNA and mRNA-protamine complexes should be maintained [17,21,34,228]. Another vaccine adjuvant formulation, TriMix, contains a mixture of three different mRNA molecules encoding CDL40, CD70 and constitutive active TLR4 (caTRL4) that boost DC activation [17,34,35]. TriMix and tumor antigen mRNA co-electroporated in DCs progressed as a treatment strategy for melanoma [229]. Recently, a phase II clinical trial was completed where patients received TriMixDC-MEL together with the checkpoint inhibitor ipilimumab, to block negative feedback signals in the generated immune response, long term follow-up results indicate an overall survival of 28% after 5 years and a clear correlation between the clinical outcome and the tumor-antigen-specific $CD8^+$ T cell responses was observed [230,231]. Furthermore, this adjuvant therapy co-delivered with HIV immunogen sequence (HTI)-encoding mRNA was explored as treatment option for HIV patients. Promising results were gathered upon intranodal administration during preclinical studies and the formulation entered phase I and phase II clinical trials. However, the latter was terminated due to insufficient immunity induction compared to placebo [232–234]. At last, a very recent and interesting study by Tse *et al.* evaluated a mutated version of the stimulator of interferon genes ($STING^{V155M}$) as an adjuvant for mRNA-based cancer immunotherapy. Mice were vaccinated by intramuscular injection of LNPs containing mRNA encoding the antigen of choice together with mRNA encoding the adjuvant. They observed a strong $CD8^+$ T cell response and increased vaccine efficiency in terms of tumor growth and survival. It is however remarkable that the adjuvant effect is linked to the production of type I IFNs, which has been considered detrimental for antigen expression when generated as part of the self-adjuvant effect of the antigen-encoding-mRNA. However, as discussed above, the authors acknowledge the timing and amount of type I IFN production as critical factors for the fate of $CD8^+$ T

cell responses. To address this issue, several mass ratios of antigen/adjuvant were tested for their potential to induce $CD8^+$ T cell activation [224]. Future studies, focused especially on the influence of adjuvant therapies on the mRNA expression kinetics, would be useful.

Taken together, mRNA vaccine development is all about achieving optimal contribution of the different elements involved. Researchers agree that several aspects such as administration route, mRNA design and formulation, cell type, and translation from animals to humans will define which pathways and effects are triggered [17,34,192,196]. Different options to optimize vaccine efficiency have been explored. The question however remains if optimal vaccine efficiency can be obtained solely by fine-tuning of the mRNA molecule or if there is room for improvement with other immune adjuvant therapies. Moreover, an interesting but often neglected aspect to take into account is the adjuvant effect of the delivery vehicle itself. However, with the very recent approval of two mRNA vaccine formulations to control the COVID-19 pandemic, we believe a new era of mRNA vaccine development has started. The ongoing research by many scientists over the world will surely increase our knowledge which can possibly improve the progress of mRNA therapeutics in general.

6. Conclusions

During the last decade, important investments have been made in the research and development of mRNA- and saRNA-based therapeutics. Up till now, most preclinical and clinical reports on mRNA therapeutics focus on its potential for vaccination purposes against infectious diseases and cancer, but the IVT mRNA platform is assumed to be versatile and promising for other applications as well, including protein replacement therapy, gene editing, and cell fate determination and reprogramming. Although the applications are very diverse, some common hurdles should be taken into account in order to fully exploit their potential as therapeutics. In this review, we focus on one of these hurdles and discuss in depth the immune-stimulatory properties of IVT mRNA and saRNA.

It is important to realize that the therapeutic outcome of mRNA- and saRNA-based therapies can either benefit or suffer from RNA-induced immune stimulation depending on the treatment application. For non-immunotherapy applications, we can unambiguously state that mRNA-induced immune stimulation should be avoided. Moreover, the induced innate immune response is highly unfavorable for frequent mRNA administration, which might be required for protein replacement therapies and mRNA-based cell reprogramming protocols. In contrast, for vaccination approaches, the situation is more complex and not fully elucidated yet. Here, it will be crucial but challenging to find a balance between the negative impact of the induced type I IFNs on antigen expression and RNA amplification on the one hand, and the potential of type I IFNs to instigate B and T cell responses on the other making the mRNA to become its own adjuvant. Moreover, conflicting reports regarding the influence of type I IFNs on CD8+ T cell responses further increase complexity.

Consequently, a variety of strategies have been explored to control the innate immune response elicited by mRNA- and saRNA-based therapeutics and more in-depth research is still ongoing. The vast majority of studies on this topic investigate mRNA-based therapeutics, whereas only a few solely focus on reducing the innate immunogenicity of saRNA. To us this is not surprising because up till now, except for some saRNA-mediated cell reprogramming studies, the use of saRNA was restricted to vaccination approaches in which the self-adjuvant properties of saRNA were considered as an important benefit for vaccine potency. However, nowadays, it is generally realized that controlling the innate immune response to saRNA is of vital importance to obtain effective and safe saRNA-based vaccines in the end, and therefore researchers started looking into strategies to modulate the innate immune response to saRNA. Furthermore, if saRNA-based therapeutics would be explored in the future for other non-immunotherapy approaches, it will be even more crucial to reduce its immune-stimulating activity.

Currently, the methods that have been applied to limit the immune-stimulatory activity of IVT mRNA and saRNA can be divided into three main categories. Firstly, every element of the mRNA or saRNA backbone itself can be altered, which is a very convenient strategy to optimize its stability and to evade innate immune stimulation directly or indirectly. In this regard, the most frequently applied and successful approach is the incorporation of modified nucleosides. However, caution should be taken because conflicting studies report that incorporation of modified nucleosides did not impact or even negatively impacted protein expression. Once again, this highlights the complexity and shows that variations in delivery system, administration route, target cell and mRNA properties may also affect the evoked innate immune response. Secondly, there is no doubt that proper production and purification of the mRNA or saRNA are essential requirements to obtain a safe and effective end product, free of immunogenic byproducts such as short abortive RNA transcripts and dsRNAs. Thirdly, both conventional and self-replicating mRNA delivery can be combined with innate immune inhibitors to quell its immune-stimulating activity. This approach is an attractive alternative to other rather time consuming and expensive approaches described above. In addition, to retain saRNA functionality, neither nucleoside modification nor sequence alteration is tolerated, which increases the interest in the use of innate immune inhibitors. Both for mRNA and saRNA, promising results were obtained already in multiple studies with a variety of inhibitors, highlighting their potential and the need for further investigation. However, it is important to realize that the choice of inhibitor is also depending on the target cell type and the treatment application, which complicates the identification of innate immune inhibitors that might be applied universally for mRNA- and saRNA-based therapeutics.

It is clear that the mRNA- or saRNA-mediated innate immune response is a complex interplay of many factors, like the mRNA properties, target cell, the delivery vehicle and the administration route, which define the fate of the mRNA. The immune-stimulating activity of the carrier itself is often overlooked and future research on this topic would be of interest. Since many different players are involved in the innate immunity signaling pathway itself, it is not surprising that reducing the innate immune-stimulating activity of mRNA and saRNA is not straightforward and none of the methods described in this review can be regarded as the golden standard to achieve this goal. Moreover, a combination of different strategies will be typically required to modulate the innate immunogenicity of mRNA and saRNA according to the desired application. In conclusion, although numerous efforts have been made already with regard to innate immune sensing of mRNA and saRNA, there are still knowledge gaps requiring further research and head-to-head comparisons between mRNA platforms. We believe that progress in this field will be made soon, as the recent marketing of the mRNA-based vaccines developed by Pfizer/BioNTech and Moderna is an ultimate boost for the research and development of numerous other mRNA- and saRNA-based therapeutics.

7. Declarations of interest

Declarations of interest: none

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