Adjuvants, the Elephant in the Room for RNA Vaccines

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Abstract Adjuvants are crucial components of vaccines. Nevertheless, they are frequently considered as mere "excipients", and their mode of action is often poorly understood. Although the attractiveness of mRNA as an immunogen has been recognized already more than thirty years ago, it wasn't until the current COVID-19 crisis that its full potential was shown. From a fringe approach, it has now become a leading technology in vaccine development which will no doubt result in a tremendous boost in both prophylactic and therapeutic vaccination settings. The issue of finding the right adjuvant is especially relevant for mRNA-based vaccines, as mRNA itself is a strong activator of innate immune responses which represents a double-edged sword. Moreover, given the high sensitivity of RNA to ambient RNases, and to improve delivery efficiency, in recent years, a lot of effort has been invested in developing ways to package the mRNA in so-called nanoparticle formulations. Currently approved mRNA-based vaccines are all formulated in lipid nanoparticles, but many other approaches are being explored, each of which will result in a different type of

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immune stimulation. In this chapter, we want to provide an overview of the potential adjuvant effect of different types of nanoparticles and implications for vaccine development.

Keywords Adjuvant · RNA vaccines · Innate immunity · Lipid nanoparticles

Abbreviations

AP-1	Activator protein-1
ASC	Apoptosis-associated Speck-like protein containing caspase activation and recruitment domains
CARDS	Caspase activation and recruitment domains
CTL	Cytotoxic T lymphocyte
DCs	Dendritic cells
DDX	Dead-box helicase
DHX	DExH-box helicase
dsRNA	Double-stranded RNA
eIF2α	Eukaryote initiation factor 2α
IFN	Interferon
IFNAR	IFN-α/β receptor
IIPs	Innate inhibiting proteins
IL	Interleukin
IP-10	Interferon-induced protein-10
IRF	IFN regulatory factor
ISRE	Interferon-stimulated response element
IVT	In vitro transcribed
JAK-STAT	Janus kinase—signal transducer and activator of transcription
LGP2	Laboratory of genetics and physiology 2
LNP	Lipid nanoparticle
m1ψ	N1-methylpseudouridine
m^5C	5-Methylcytidine
m^6A	6-Methyladenosine
MAPK	Mitogen activation protein kinase
MAVS	Mitochondrial antiviral signalling protein
MCP-1	Monocyte chemoattractant protein
MDA5	Melanoma differentiation-associated protein 5
MERS-CoV-ORF4a	Middle East respiratory syndrome coronavirus
MPLA	Monophosphoryl lipid A
MyD88	Myeloid differentiation primary response protein 88
NF-κβ	Nuclear factor κ-light-chain enhancer of activated B cells
NLRP3	NOD-/leucine-rich repeat- and pyrin domain-containing protein 3
NOD	Nucleotide-binding oligomerization domain

NSP	Non-structural proteins
OAS	2′-5′Oligoadenylate synthase
PAMP	Pathogen-associated molecular pattern
PKR	Protein kinase R
PRR	Pattern recognition receptors
RIG-I	Retinoic acid-inducible gene-I
RLR	RIG-I-like receptors
S2U	2-Thio-uridine
sa-RNA	Self-amplifying RNA
SARS-CoV	Severe acute respiratory syndrome coronavirus 2
ssRNA	Single-stranded RNA
STING	Stimulator of IFN genes
Tfh	Follicular helper T cells
TIR	Toll-IL-1-receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
TRIF	TIR-domain-containing adaptor-inducing IFN-β
UTP	Uridine-5′-triphosphate
ψ	Pseudouridine

1 Introduction

The first in vivo application of mRNA dates back to 1990, when expression of in vitro transcribed (IVT) mRNA after intramuscular injection in mice has been described (Wolff et al. 1990). Shortly after, IVT mRNA was shown to induce an immune response, as an injection in the tongue muscle elicited antigen-specific antibodies (Conry et al. 1995). In the meantime, a liposome formulation of IVT mRNA injected subcutaneously was proven to induce virus-specific cytotoxic T lymphocytes (CTLs) in several mouse models (Martinon et al. 1993). Already 30 years ago, this work paved the way for the development of mRNA-based COVID-19 vaccines. Nevertheless, many hurdles still needed to be cleared for routine development of mRNA vaccines. One of the problems that arose was the fact that the innate immune system was activated after injection of mRNA, which led to reduced translational levels. In addition, mRNA is not as stable as its double-stranded nucleic acid counterpart DNA, which meant it had to be packaged in order to be delivered efficiently in vivo. In this chapter, we will discuss the improvement of mRNA vaccines, the next-generation mRNA vaccines, the self-amplifying RNA (sa-RNA) and the adjuvant mechanism of mRNA and of the packaging nanoparticles.

2 mRNA as a Natural Adjuvant

IVT mRNA is produced from a template plasmid DNA by a phage polymerase, usually T7 or SP6 (Konarska and Sharp 1989). However, this process is not without errors; during the in vitro production of mRNA, the phage polymerase generates promoter- and run-off transcript-dependent and -independent double-stranded RNA (dsRNA) contaminants (Gholamalipour et al. 2018; Mu et al. 2018) which can activate the innate immune system in several ways, conferring efficient adjuvant properties to the IVT mRNA. For nucleic acid sensing in general, we refer to a recent review (Bartok and Hartmann 2020). In this chapter, we will mainly discuss the receptors involved in the recognition of IVT mRNA and the nanoparticles it is formulated in.

In addition to classic, non-replicating mRNA, the next-generation IVT mRNA is the so-called sa-RNA and has the capacity to replicate itself via the viral replicase complex, which serves as an in situ translation machinery and enables prolonged antigen production (Ljungberg and Liljeström 2014). This type of mRNA is derived from single-stranded RNA (ssRNA) viruses, e.g. alphaviruses (Sindbis virus, Venezuelan Equine Encephalitis virus, among others) and encodes not only the antigen of interest (which substitutes for the viral structural proteins) but also four non-structural proteins (NSP1-4) and a subgenomic promoter (Maruggi et al. 2019). The main benefit of sa-RNA is that it allows for a prolonged antigen exposure. For instance, sa-RNA encoding for luciferase packaged in a lipid nanoparticle was injected intramuscularly, and bioluminescence was detected for up to 63 days in vivo (Geall et al. 2012), compared to only 8 days for non-replicating mRNA (Pardi et al. 2015). Furthermore, sa-RNA was shown to induce broad and potent immunity after delivery in a lipid nanoparticle (LNP) (Geall et al. 2012). The production process of IVT mRNA and sa-RNA is similar, leading to the same by-products which give rise to immune activation. In addition, during the self-replicating process, dsRNA is inevitably formed resulting in RNA recognition by RNA sensors (Pepini et al. 2017), making both forms of mRNA natural adjuvants.

2.1 Endosomal RNA Recognition

After engulfment of the IVT mRNA or LNPs, the mRNA ends up in the endosome where it comes into contact with several toll-like receptors (TLRs) (Fig. 1, part 1). TLRs are pattern recognition receptors (PRRs), which play a crucial role in innate immunity as they are responsible for the initial detection of the so-called pathogen-associated molecular patterns (PAMPs), in this case ssRNA and dsRNA. TLR3 recognizes dsRNA and signals through toll-interleukin(IL)-1-receptor-domain-containing adaptor-inducing interferon (IFN)-β (TRIF) (Oshiumi et al. 2003), and TLR7 and 8 are triggered by ssRNA and signal through myeloid differentiation factor 88 (MyD88) (Diebold et al. 2004; Heil et al. 2004). In addition, two other endosomal TLRs recognize RNA: TLR10 was only recently discovered to respond to dsRNA (Lee et al.

2018), and TLR13 recognizes a specific sequence within bacterial ribosomal RNA (Hidmark et al. 2012); the latter two are only detected in lower vertebrates and rodents and will not be further discussed in this chapter.

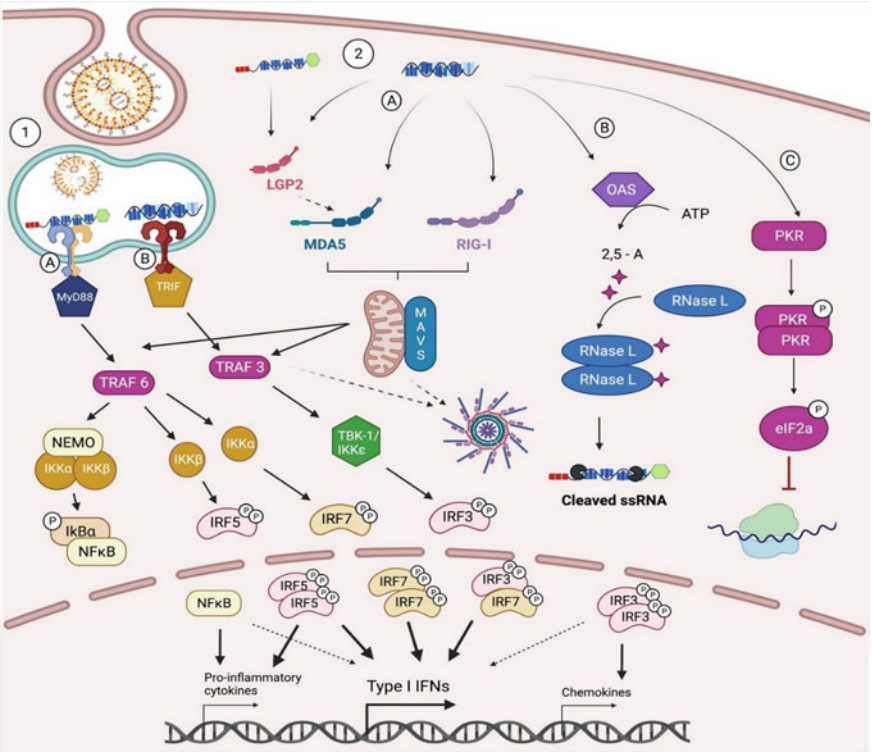

Fig. 1 Mechanisms of innate immune sensing of IVT mRNA. mRNA-containing lipid nanoparticles are taken up by bystander cells via endocytosis (1). In the endosomes, ssRNA (A) and dsRNA (B) are recognized by toll-like receptors (TLR)7/8 and TRL3, and these receptors in turn activate MyD88 and TLR3 in Toll-interleukin-1 domain-containing adapter-inducing interferon-β (TRIF) respectively. Eventually, this results in the stimulation of tumour necrosis factor receptor-associated factor (TRAF) 3 and 6 resulting in the activation of transcription factors such as nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) and interferon (IFN) regulatory factors (IRF) 3, 5 and 7. Subsequently, this gives rise to the transcription of genes encoding for inflammatory cytokines, type I IFNs and chemokines. In the cytosol (2), dsRNA and ssRNA are recognized by the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) laboratory of genetics and physiology 2 (LGP2), melanoma differentiation-association protein 5 (MDA5) and RIG-I (A). LGP2 cannot by itself activate MAVS but has a role as facilitator for other RLRs. MAVS in turn results in the activation of TRAF3 and TRAF6 and may as well have an influence on inflammasome activation. Upon recognition of dsRNA by oligoadenylate synthase (OAS), OAS is phosphorylated and recruits RNase L resulting in RNA degradation (B). In addition, dsRNA is recognized by protein kinase R (PKR) (C), which leads to phosphorylation of eukaryote initiation factor 2a, leading to the abrogation of translation. (Created with Biorender.com)

Downstream TRIF signalling results in the activation of several transcription factors. Via tumour necrosis factor (TNF) receptor-associated factors (TRAF)3, it results in phosphorylation and translocation of IFN regulatory factor (IRF) 3. In addition, the mitogen activation protein kinases (MAPKs), stimulating activator protein (AP)-1 translocation and nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) activation are the result of TRIF signalling. Lastly, TRAF3 can also trigger the assembly of the inflammasome by the complexation of nucleotide-binding oligomerization domain (NOD)-, leucine-rich repeat- and pyrin domain-containing protein (NLRP)3, the adaptor protein, apoptosis-associated speck-like protein containing caspase activation and recruitment domains (CARD) (ASC) and caspase-1 (Kelley et al. 2019). MyD88 signalling mainly results in activation of NF-κB, MAPK, IRF5 and 7. TRIF and MyD88 activation therefore results in the secretion of similar inflammatory cytokines (such as TNF-α and IL-6), chemokines CXCL-8 (IL-8) and more importantly type I IFNs (Bartok and Hartmann 2020). The signalling cascades starting with TRIF or MyD88 therefore share some signalling proteins but can also work supplementary. For instance, MyD88 signalling results in the downstream production of pro-IL-1β. Simultaneously, TRAF3 will allow inflammasome assembly, resulting in active caspase-1 which can subsequently cleave the pro-IL-1β precursor in active IL-1β.

The first mechanism for IVT mRNA recognition was discovered in 2004, where it was shown that IVT mRNA could stimulate TLR7 in mice and led to the production of IFN-α among other cytokines (Diebold et al. 2004). In the same issue, TLR7 and TLR8 were revealed as the human receptors for ssRNA (Heil et al. 2004). These receptors are found on the endosomal membrane, unsurprisingly, the site where the mRNA is located after engulfment inside the cell. TLR3, which is known for binding to dsRNA, also plays an important role in the recognition of mRNA, as dsRNA is a by-product of IVT mRNA. Thus, early work showed that IVT mRNA (the whole mixture) acts as a ligand for TLR3, 7 and 8 resulting in maturation and release of cytokines by primary monocyte-derived dendritic cells (DCs) after lipofection (Karikó et al. 2005). These findings were recently confirmed when it was shown that modified mRNA gives rise to MyD88 dependent activation of the type I IFN pathway (Nelson et al. 2020).

2.2 Cytoplasmic RNA Sensors

In order for mRNA to be efficiently translated, it needs to be released from the endosome into the cytoplasm to reach the ribosomes. This release occurs passively but is much more efficient when the mRNA is packaged in LNPs and other types of packaging materials such as polymers or cell-penetrating peptides. Therefore, when mRNA is released into the cytosol, it encounters a new set of RNA sensors (Fig. 1, Part 2). One group of sensors is the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) which include three different family members: RIG-I, the melanoma

differentiation associated protein 5 (MDA5) and the laboratory of genetics and physiology 2 (LGP2) protein. All RLRs contain a central helicase domain and a carboxy-terminal regulatory domain. These two domains join forces into recognizing RNAs. Additionally, RIG-I and MDA5 contain two amino terminal CARDs and are able to interact with the IFN-β promoter stimulator protein adaptor protein, better known as mitochondrial antiviral-signalling protein (MAVS), and induce downstream signal transduction pathways (Onomoto et al. 2021). In the presence of dsRNA or ssRNA with 5′ phosphate, conformational changes lead to an exposed CARD domain. Subsequently, in the presence of ATP, the CARD domain will interact with the adaptor protein MAVS, leading to type I IFN transcription (Onomoto et al. 2021). LGP2 lacks the CARD domain, making it an atypical RLR, but although it cannot interact with MAVS, it is able to recognize and bind to dsRNA as well as ssRNA (Takahasi et al. 2009). In case of recognition of viral RNA, LGP2 acts as a facilitator for RIG-I and MDA5 (Duic et al. 2020). Nevertheless, its role in IVT mRNA sensing is uncertain and LGP2 will therefore not be further discussed.

The RLRs are part of a much larger family called the DEAD/DExH-box RNA helicases (DDX/DHX). This group entails many understudied RNA helicases which may aid in cytosolic RNA sensing by for instance acting as a co-receptor for MDA5 or RIG-I or by enhancing RIG-I signalling (Bartok and Hartmann 2020). Some members of the family have been described to even directly activate the inflammasome. However, the role of DDX/DHX proteins in IVT mRNA sensing requires thorough investigation and will not be discussed in detail in this chapter.

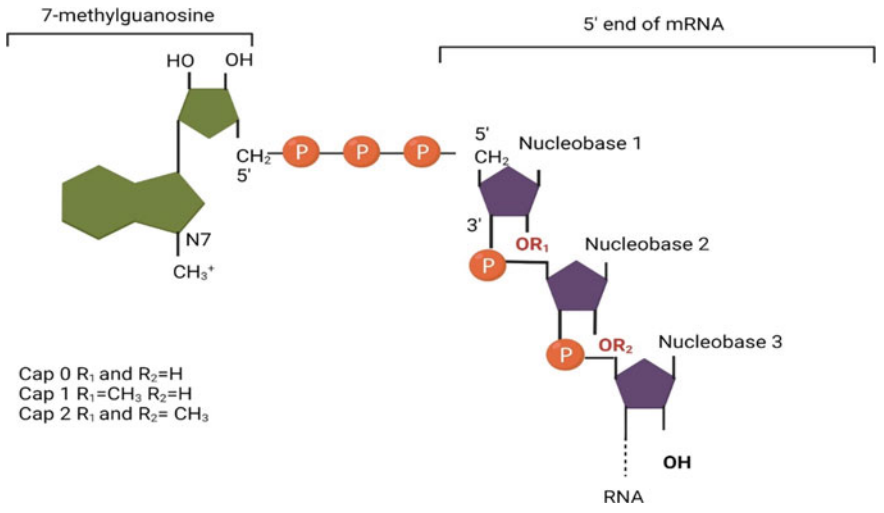

Fig. 2 Structures of cap 0, cap 1 and cap 2. The difference between these capping systems lies in the addition of a methylation group at the 2′ position of the 5′ penultimate and antepenultimate of the transcript. In some cases, the addition of a methyl group in these positions enhances the translation efficiency in vivo compared to the corresponding cap 0-mRNA (Created with Biorender.com)

Downstream signalling of RIG-I and MDA5 results in activation of MAVS (Brisse and Ly 2019). It has been shown that the ATPase activity of MDA5 and RIG-I helicases differ in a length-dependent manner: RIG-I is efficient in recognizing short poly I:C molecules, while MDA5 is only activated by long stretches of poly I:C (the cut-off being around 300 bp) (Yoneyama et al. 2005; Kato et al. 2008). RIG-I is activated by dsRNA over 18 bp but requires extra motif such as 5′ triphosphate, among others (Hornung et al. 2006). For RNA which is not poly I:C, the motifs recognized by MDA5 are still not well defined (Hartmann 2017).

To avoid recognition of RIG-I, a synthetic cap analogue was introduced to the 5′ end of IVT mRNA. To date, there are three different capping systems that can be used in IVT mRNA: cap0 (m7G(5′)pppN1pN2p), cap1 (m7G(5′)pppN1mpNp) or cap2 (m7G(5′)pppN1mpN2mp). As depicted in Fig. 2, the main difference of these capping systems lies in the methylation status of the 2′ position of the 5′ penultimate and antepenultimate nucleoside (Zhong et al. 2018). Importantly, capping mRNA with a cap 0 structure reduced RIG-I activation while cap 1 or cap 2 completely abrogated RIG-I recognition of IVT mRNA and subsequent type IFN induction (Schuberth-Wagner et al. 2015).

Downstream of MAVS, IRF3 is phosphorylated which leads to subsequent dimerization and translocation to the nucleus where they induce type I IFN genes (Brisse and Ly 2019). While the RLRs mainly give rise to the activation of the type I IFN response, other cytoplasmic RNA sensors such as 2′-5′ oligoadenylate synthase (OAS) and protein kinase R (PKR) have a more direct effect. Once OAS is activated, it in turn stimulates RNase L, which will be responsible for the cleavage of RNA. PKR on the other hand will phosphorylate eukaryote initiation factor 2α (eIF2α), resulting in an abrogation of translation in the cell. PKR and OAS where shown to be activated by IVT mRNA, leading to not only the induction of type I IFNs but also, and more importantly, to the cleavage of mRNA and the abrogation of translation (Nallagatla and Bevilacqua 2008; Anderson et al. 2010, 2011).

For vaccination purposes, this type I IFN induction is highly beneficial, stimulating B- as well as T-cell responses (McNab et al. 2015). However, type I IFNs are also association with cell death induction and due to the antiviral state of immune cells, translation is abrogated, and mRNA is actively degraded by RNases. Luckily, a solution for this conundrum came from the field of gene therapy.

3 Type I IFNs: The Double-Edged Sword

Type I IFNs play a key role in initiating and sustaining a solid T- and B-cell-mediated immune response in the context of infection or cancer (McNab et al. 2015). Type I IFNs result in maturation of DCs (Santini et al. 2000; Breckpot et al. 2005), attraction of cytotoxic $CD8^+$ T cells to the tumour environment (Fuertes et al. 2011) and increased MHCI expression (Hofbauer et al. 2001), leading to enhanced antigen presentation towards $CD8^+$ T cells. Besides maturation, type I IFNs also stimulate DCs to promote isotype switching in B cells and enhance humoral immunity and

memory response (Le Bon et al. 2001). The mechanism might be related to the fact that type I IFNs stimulate follicular helper T cell (Tfh) differentiation which can subsequently aid in the differentiation of B cells during germinal centre formation in the lymph nodes (Ray et al. 2014). Therefore, the use of type I IFNs as an adjuvant or an immune stimulator was assessed in cancer research. For instance, systemic administration of type I IFN in breast cancer mouse models resulted in a decrease in tumour progression and metastasis to the bone and prolonged metastasis free survival via NK-cell anti-tumour function (Slaney et al. 2013; Rautela et al. 2015). In the context of melanoma, type I IFNs are used in the clinic after resection as an adjuvant therapy, to prevent relapse and formation of metastasis (Mocellin et al. 2013). Therefore, in theory, the type I IFNs, driven by the natural adjuvant properties of mRNA, should be at the basis of a strong immune response. Unfortunately, the reality is not black and white.

During investigations into the potential use of IVT mRNA as a gene therapy platform, one big problem occurred: the type I IFN activity not only reduced translation of the IVT mRNA but also gave rise to substantial cytotoxicity resulting in cell death (Andries et al. 2013). The reduced translation is the result of the type I IFNs binding to their receptor, a dimer of IFN-α/β receptor (IFNAR)1 and 2 (from here onwards called IFNAR). After signalling through the Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway, the IFN-stimulated genes are transcribed, including PKR and OAS. Not only the presence of nucleic acids but also type I IFNs can induce cell death (both immunologically silent and proinflammatory), with as a primary goal limiting the propagation of (RNA) viruses (Bartok and Hartmann 2020). Therefore, a crucial element is to avoid elements in the IVT mRNA-resembling viral nucleic acids. In the context of sa-RNA, type I IFN responses inhibit the amplification of the RNA replicon resulting in a loss of the efficiency of this vaccine platform. It has been reported that sa-RNA elicits an inflammatory response within a few hours by the upregulation of IFN-stimulated genes. In the absence of type I IFN signalling, RNA vaccine potency was shown to be improved (Pepini et al. 2017). Undoubtedly, different strategies need to be designed to balance out this "ying and yang" effect.

3.1 Nucleoside Modifications

By studying naturally occurring forms of RNA, the presence of several nucleoside variants in various types of mammalian and bacterial RNA were identified. Firstly, it was discovered that monocyte-derived DCs treated with bacterial RNA produced high amounts of TNF-α. This high induction of TNF-α was not seen upon transfection with mammalian RNA except for mitochondrial RNA. The induction of TNF-α was inversely correlated to the extent of nucleoside modification found in the type of RNA. Thorough investigation led to the realization that specific modification of the RNA molecules (6-methyl adenosine (m^6A), pseudouridine (ψ) and 5-methylcytidine (m^5C) reduced the production of TNF-α, IFN-α and other cytokines,

presumably through a reduced activation of the endosomal TLRs (Karikó et al. 2005). Subsequently, it was shown that nucleoside modification in combination with adding a capping structure on the IVT mRNA significantly reduced type I IFN production through a RIG-I-dependent process (Hornung et al. 2006; Karikó et al. 2008). In addition, reduced activation of PKR was observed when IVT mRNA was generated with modified nucleosides, yet whether there was reduced binding to PKR or not has so far not been clearly established (Nallagatla and Bevilacqua 2008; Anderson et al. 2010). Moreover, IVT mRNA fully substituted by ψ did not only lead to a reduced activation of OAS1 but was also resistant to cleavage by RNase L (Anderson et al. 2011). Recently, it was confirmed that 1-methyl-pseudouridine (m1ψ)-containing RNA led to a reduced activation of TLR7/8 (Nelson et al. 2020). In conclusion, the use of modified nucleosides, in particular ψ or m1ψ, results in increased levels of translation of the IVT mRNA. The chemical modifications also significantly reduce the induction of type I IFNs (Table 1). However, basal immune activation can still be detected.

3.2 Techniques for dsRNA Removal

As mentioned earlier, phage polymerases are prone to errors, resulting in dsRNA contaminants in the IVT mRNA-generated mixture. By removing these contaminants with HPLC purification, pure IVT mRNA was obtained and no type I IFN induction was observed anymore in DCs after transfection (Karikó et al. 2011). However, this technique is expensive, and approximately 50% of the mRNA is lost during the process. Therefore, other strategies were developed to further "clean-up" the IVT mRNA.

At the level of T7 polymerase transcription, there are several ways to prevent the polymerase from making these errors: reducing $MgCl_2$ concentration in the reaction mixture (Mu et al. 2018), creating an optimal dNTP ratio (Nelson et al. 2020) or using a heat-stable T7 RNA polymerase (Wu et al. 2020). Adding modified nucleosides might also have an influence on the dsRNA content, however there is still some conflicting information depending on the detection method of the dsRNA and the purification method used (phenol:chloroform and native gel electrophoresis (Mu et al. 2018) versus oligo-dT purification and dsRNA ELISA (Nelson et al. 2020). At the level of the purification process itself, progress has also been made, as it was shown that passing the IVT mRNA over a cellulose column could filter out dsRNA with a 70 to 80% recovery of the mRNA (Baiersdörfer et al. 2019). As a result, lithium chloride or oligo-dT-enriched mRNA after dsRNA removal gave rise to higher translation efficiencies and is "immunosilent", meaning that type I IFNs were no longer produced in vitro or in vivo (Baiersdörfer et al. 2019; Nelson et al. 2020).

For sa-RNA, the removal of dsRNA is considered to be less helpful since dsRNA intermediates are produced continually during the self-amplification cycle. These dsRNA intermediates may cause a translational shutdown due to their recognition

Table 1 Overview of innate immune stimulation by IVT mRNA

	Mechanism of action	Solution to reduce activation	Remarks	References
ssRNA	TLR7		Polyuridine—viral ssRNA and IVT mRNA similar IFNα response Mouse ex vivo/in vitro	Diebold et al. (2004)
Adenosine, uridine, cytidine	TLR 3	m^6A s2U		Karikó et al. (2005)
	TLR 7 and 8	m^6A, m^5C, m^5U, s2U, ψ		
5′-triphosphate RNA	RIG-I	5′-cap structure s2U, ψ, 2′-O-methylated UTP	MDA5 does not recognize this 5′-triphosphate RNA	Hornung et al. (2006)
Uridine	Not RIG-I	ψ better than m^5C, m^5U and s2U		Karikó et al. (2008)
Uridine	PKR	Ψ, m^6A, m^5C, s2U, s4U, 2′-dU, I^5U abrogated PKR activation	No significant effect on binding	Nallagatla and Bevilacqua (2008)
Cytidine, uridine and adenosine	PKR	Ψ, m^6A and m^5C decreased PKR activation	Reduced binding to PKR	Anderson et al. (2010)
Uridine	OAS1 RNase L	Ψ	Less OAS1 activation Less efficiently cleaved by RNase L	Anderson et al. (2011)
dsRNA	MDA5	m^5C, ψ, m1ψ result in reduced dsRNA formation and MDA5 stimulation Reducing $MgCl_2$ in reaction T7	MDA5 forms filaments together with dsRNA	Mu et al. (2018)
Uridine	TLR7/8 signalling	m1ψ		Nelson et al. (2020)
dsRNA impurities	Cytoplasmic sensors RIG-I/MDA5	dsRNA reduction by custom NTP ratio	Signalling via MAVS	

TLR—toll-like receptor; IVT—in vitro transcribed; IFN—interferon; MDA5—melanoma differentiation-associated protein; RIG-I—retinoic acid-inducible gene I; PKR—protein kinase R; OAS—oligoadenylate cyclase; s2/4U—2/4- thio-uridine; m^5C—5-methylcytidine; m^5U—5-methyluridine; i^5U—5-iodineuridine; ψ—pseudouridine; UTP—uridine 5′-triphosphate; m1ψ—N1-methylpseudouridine; m^6A -6-methyladenosine; dU—deoxy uridine

by the cytoplasmic RNA sensors (Pepini et al. 2017). Different strategies based on the escape mechanism of different viruses have been explored to overcome this hurdle. One of the most appealing strategies to dampen the type I IFN and to escape innate sensing is the use of innate inhibiting proteins (IIPs). For instance, the vaccinia virus IIPs E3, K3 and B18 each play a specific role in counteracting the host's antiviral response. E3 and K3 inhibit PKR and B18 disrupts the type I IFN signalling pathway by acting as a decoy receptor and thus preventing the interaction between extracellular IFN and IFNAR. As for influenza proteins, the non-structural protein 1 of the influenza A virus shows a multifunctional role as it inhibits the immune related proteins PKR, OAS, IRF3 and NF-κβ. Co-transfection of these three IIPs improved substantially the translational capacity of sa-RNA compared to the transfection of sa-RNA only (Beissert et al. 2017). As this strategy requires the administration of two different mRNA formulations (one encoding the protein of interest and one encoding the IIPs), the co-localization of both mRNAs in the same cell is an important prerequisite to overcome innate immune sensing. However, this cannot be guaranteed. More recently, a proof-of-concept study reported the use of a sa-RNA construct encoding regions for both the protein of interest and the IIPs. In this study, a plethora of IIPs derived from different viruses were screened in vitro, based on their targets in the type I IFN pathway and on their effect on protein expression and immunogenicity. One of the most promising IIPs that was identified from the screening was the accessory protein ORF 4a of MERS-CoV and the V protein of parainfluenza type 5, which showed superiority in different human cell lines. It has been reported that the ORF 4a protein of MERS-CoV has the highest potential to counteract innate immune sensing, as it is able to inhibit IFN production and IFN stimulated response element (ISRE) promoter element signalling pathways. PIV-5 on the other hand binds to MDA5 directly and inhibits its activity. These proteins could therefore dampen the IFN production (Blakney et al. 2020). Besides the use of viral IIPs, many other non-viral molecules have also been explored for their potential to reduce antiviral responses and have also been elaborately reviewed (Minnaert et al. 2021). One of the most recent attempts to quench the type I IFN response is the use of corticosteroids, a class of anti-inflammatory drugs, in combination with IIPs and cellulose-based mRNA purification. Among the corticosteroids, it was shown that clobetasol propionate, especially when applied topically, enhanced the translation of sa-RNA against Zika virus upon intradermal electroporation and reduced type I IFN responses. Although this approach might be beneficial in the context of gene therapy, it should be avoided in vaccination context as clobetasol propionate prevents the formation of antibodies against sa-RNA encoded antigens (Zhong et al. 2021).

Undoubtedly, immunosilent mRNA is of great importance in the context of gene therapy. However, for vaccination against cancer or infectious diseases, well-balanced amounts of type I IFNs could contribute to improving the vaccine. To investigate this, the role of IFNAR was assessed.

3.3 Role of Type I IFN Receptor

As stated before, systemic type I IFN activation by PRRs facilitates the adaptive immune response and induces DC activation. However, type I IFN activation is also associated with reduced translation of the IVT mRNA. To study this delicate balance in more detail, IFNAR knock-out mouse models were used in mRNA vaccination studies. After intravenous injection of mRNA-lipoplexes encoding various tumour antigens, the IFN-α induced was shown to be critical for an efficient $CD8^+$ T-cell-mediated anti-tumour response (Kranz et al. 2016). Similar results were obtained and noticed that even though translation improved in IFNAR knock-out mice, antigen-specific lysis by CTLs was decreased in these knock-out mice (Broos et al. 2016). To further complicate matters, conflicting observations were made when mRNA was injected via different routes, i.e. intradermally and subcutaneously, not only did translation improve in IFNAR knock-out mice, the antigen-specific CTL-mediated response was also higher (Pollard et al. 2013; De Beuckelaer et al. 2016; Udhayakumar et al. 2017). For sa-RNA vaccines, the limited data available shows that the type I IFN response also impeded the subsequent immune response upon intradermal electroporation (Zhong et al. 2019).

It was suggested that the discrepancy in these results might be due to differences in timing, rather than dosing. It was shown that after intravenous injection, mRNA is immediately translated and presented in the spleen by plasmacytoid DCs to T cells. The plasmacytoid DCs simultaneously produce type I IFNs, providing the "second signal", needed for efficient proliferation and activation of T cells. On the contrary, after intradermal or subcutaneous injection the translation lags behind and the DCs have to migrate from the skin to the lymph nodes to stimulate the T cells. By this time, the DCs are already producing type I IFNs, leading to the "second signal" without a first signal, resulting in T-cell apoptosis (De Beuckelaer et al. 2017). However, this hypothesis does not take into account that the type I IFNs will likely dilute systemically and may not be present at such high levels in the lymph nodes. On the other hand, the hypothesis is partly supported by the fact that the expression of IFNAR is primarily important on $CD4^+$ T cells and not $CD11c^+$ DCs to support the CTL response (Van Hoecke et al. 2020). In summary, two solutions remain to counter these effects: either decreasing the type I IFN response or speeding up the translation in the lymph nodes. It was shown that modifying the IVT mRNA but not removing the dsRNA contaminants partly reduces the type I IFN response (Nelson et al. 2020). In addition, by packing the mRNA in nanoparticles smaller than 200 nm, the bulk part was shown to migrate to the lymph nodes after injection, leading to fast and local translation (Manolova et al. 2008). For self-replicating mRNA, it has also been shown that packaging in LNPs (<100 nm) leads to expression in the lymph nodes (Huysmans et al. 2019). However, the impact on the immune reaction elicited has so far not been investigated.

When looking to the COVID-19 mRNA vaccines, there is a large difference in the efficacy of on the one hand the CureVac vaccine (CVnCoV) and on the other hand the vaccines produced by Moderna (mRNA-1273, Spikevax) and BioNTech

(BNT162b2, COMIRNATY). CVnCoV does not use modified nucleosides and therefore presumably dose reduction is required to avoid cytotoxicity due to excessive type I IFN induction, resulting in lower efficacy (Kremsner et al. 2021). mRNA-1273 and BNT162b2 use IVT mRNA fully substituted by m1ψ and obtain very high efficacies after a second dose (Baden et al. 2021; Sahin et al. 2021). While Moderna uses a purification technique of the mRNA via oligo-dT capture (Corbett et al. 2020) and dsRNA removal by cellulose chromatography (Baiersdörfer et al. 2019; Laczkó et al. 2020), BioNTech remains vague in its description and uses magnetic particle purification (Vogel et al. 2021) in addition to an undisclosed method of dsRNA removal (EMA 2021). Complete removal of dsRNA accordingly would imply that the resulting mRNA is immunologically silent, which is in contradiction to the result obtained by the clinical trials (Baden et al. 2021; Sahin et al. 2021). However, the mRNA encoding the full-length SARS-CoV-2 spike protein is over 3 kb long, meaning that there is plenty of space for the formation of secondary structures which might still be able to trigger both endosomal and cytoplasmic sensors.

4 mRNA Delivery Systems

In order to address the elephant in the room, we have to answer the following question: if the COVID-19 vaccines use immunosilent mRNA, why are they so immunogenic? The answer could perhaps in part be found in the nanoparticles used to package the mRNA. However, we only begin to understand the mechanisms behind the immune activation capacity of nanoparticles in general and lipid-based nanoparticles in particular. The current state of the art about how different intracellular pathways are activated by cationic liposomes was summarized, and they conclude that empty cationic liposomes are able to activate MAPK and result in NF-κB-dependent and -independent release of inflammatory cytokines, chemokines and co-stimulatory molecules in vitro. In addition, generation of ROS and Ca^{2+} influx also contribute to this mechanism as well as the activation of apoptotic cascade induction and inflammasome activation, leading to release of IL-1β (Lonez et al. 2012). Subsequent research with cationic lipopolyamines confirms activation of MAPK, NF-κB and NLRP3, giving rise to TNF-α, IL-6 and IL-1β in in vitro models (Li et al. 2018; Zhang et al. 2021). In most of these studies, type I IFNs were not assessed, but their induction cannot be excluded because a mild induction of type I IFNs exist for both liposomes and liposome-formulated mRNA. However, the trigger for all these inflammatory pathways still depends on the nature of the liposome including characteristics such as size, charge and composition. In addition, in these studies only one-component cationic liposomes are assessed, while in reality, the field has shifted from the cationic nanoparticles, which are often associated with systemic and cellular toxicity (Kedmi et al. 2010; Rietwyk and Peer 2017), to neutral nanoparticles by using ionizable lipids. These lipids have a neutral charge at physiological pH, reducing cell death, but become positively charged when the pH decreases (e.g. in the endo-lysosomal environment), ensuring endosomal escape of the mRNA cargo. Moreover, LNPs are

not made up of one single component but used in combination with helper lipids, PEGylated lipids and cholesterol. Only a limited amount of data is available on the immune stimulatory capacity of these lipid nanoparticles, without the presence of mRNA.

While for gene therapy purposes, LNPs were made less and less immunogenic; it was sought to identify LNPs which are safe yet provide a potent stimulus for the immune system. After screening a large number ($n = 1080$) of cationic ionizable lipids, they found that lipids containing cyclic amino head groups stimulate the stimulator of IFN genes (STING) pathway (Miao et al. 2019). In this way, type I IFNs are induced even when using modified mRNA. Another approach is adding well-defined adjuvants to the LNPs. For example, addition of monophosphoryl lipid A (MPLA) reversed the effect of immunosilent mRNA and induced higher levels of IL-6, IFN-γ and monocyte chemoattractant protein (MCP)-1/CCL-2 after 12 h compared to the LNPs with unmodified mRNA or LNPs with modified mRNA without MPLA (Verbeke et al. 2017). The combination of lipid nanoparticles with α-galactosylceramide packaging (polymer-)mRNA on the other hand led to the engagement of invariant NKT cells (Guevara et al. 2019; Verbeke et al. 2019). Improved invariant NKT cell engagement led to increased secretion of IFN-γ, IL-4, IL-12p70, IL-6 and TNF-α (Verbeke et al. 2019) and thus improved subsequent cellular immunity.

Specifically for the COVID-19 vaccines, previous work with similar formulations for other infectious diseases mainly showed the induction of Tfh and subsequent germinal centre B-cell responses (Pardi et al. 2018, 2019; Laczkó et al. 2020). However, recent work illustrates the inflammatory potential of the LNPs used in the vaccines against SARS-CoV-2. After intradermal injection in C57BL/6 mice, gene analysis in skin explants showed activation of RIG-I, NOD-like receptor and TLR signalling resulting in production of inflammatory cytokines and chemokines among which IL-1β, IL-6 and IFN-γ induced protein (IP)-10/CXCL-10 (Ndeupen et al. 2021). Even though the empty LNPs were administered via the skin and not via the muscle, it still shows the inflammatory potential of the COVID-19 vaccines. However, it still needs to be explored in more detail what type of inflammatory reaction, activating which components of the immune system, is associated with the most robust adaptive immune response.

In summary, although research into the immune stimulatory capacity of nanoparticles is incomplete, there are several indications that they might have a bigger influence than previously thought. Nevertheless, there is still insufficient information available about the immune response elicited by the different components of the LNPs themselves.

5 Conclusion

For the safe and reliable production of mRNA vaccines, it is advisable to purify the IVT and test for the absence of dsRNA contaminants (as is done for BNT162b2 and

mRNA-1273). In addition, the use of modified nucleosides for RNA synthesis has now been established as superior to the use of unmodified mRNA. Still, even when using modified, purified, dsRNA-free mRNA, secondary structures within the mRNA could still trigger the innate immune system. Especially for long IVT mRNA strands, e.g. the spike protein of SARS-CoV-2, base-pairing leads to loop formation and results in the formation of a complex secondary structure. The dsRNA components within this secondary structure or other motifs could still trigger innate immune sensors such as RLRs. Although this has not been discussed in depth in this chapter, many other RNA helicases (especially members of the DEAD/DExH-box helicase family, e.g. DDX1 or DHX9, DHX15) have been discovered and we have only started to scratch the surface with regard to their involvement in innate immune reactions. For future mRNA vaccine design, we therefore need to pinpoint which sequences or secondary structures within the IVT mRNA are related to an efficient adaptive immune response and which receptors play a role in their recognition. A more targeted modulation of either of these components should lead to more potent vaccines.

In the future, it will be interesting to develop sa-RNA, as they allow for longer antigen translation and are already effective at lower dosages. This means that with the same amount of sa-RNA, more vaccines can be made than for IVT mRNA. In addition, sa-RNA intrinsically represents a long IVT mRNA strand due to the incorporation of non-structural proteins and other sequences. Furthermore, during the replication cycle loop formation occurs, resulting in dsRNA fragments, which leads to type I IFN induction. When purified, sa-RNAs are useful tools in IVT mRNA vaccination. Interestingly, modern in silico techniques enable the design of potent sa-RNA vectors yielding both high translation and immunity by balancing type I IFN effects. For sa-RNA, self-adjuvanting activity is generally considered beneficial, but side-by-side comparisons between modified and non-modified sa-RNA are still lacking. Moreover, the clinical trials that have been performed so far, used unmodified nucleosides. The use of corticosteroids, especially when topically administered, has been suggested to improve translation efficiency for sa-RNA. However, this approach is less applicable in the context of vaccination as it completely abrogates both cellular and humoral responses (Zhong et al. 2021; Minnaert et al. 2021). Nevertheless, progress has been made in the field of IIPs, showing their potential to enhance the translation efficiency of sa-RNAs.

Not only the mRNA but also the LNP has the capacity to stimulate the immune system, as the COVID-19 vaccines have showed us that the LNPs alone can be inflammatory. However, the mechanism leading to the adaptive immune response and the correlation with the inflammatory response, still has to be determined. Yet, it is possible to determine the mechanism underlying the induction of an efficient adaptive immune response and nanoparticles could be designed to stimulate particularly $CD8^+$ T cells or B cells. In order to move forward, it is very important to take a systematic approach, assessing every pathway that might be induced after vaccination, from known molecules such as MDA5 and RIG-I to less well-known molecules such as for instance non-RLR RNA helicases. Research should also not stop once a product is on the market, since often not every aspect of the mechanism behind the adjuvant activity has been fully elucidated, especially when taking into account the rapid approval of

COVID-19 mRNA vaccines. By starting to understand how today's mRNA vaccines stimulate the immune system, we can design a new generation of even better vaccines for the future.

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