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Evading innate immunity in nonviral mRNA delivery: don't shoot the messenger

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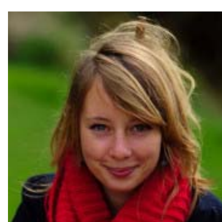
Teaser:

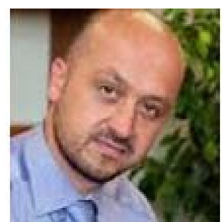
This review presents an overview of the immune-related hurdles that limit mRNA advance for non-immunotherapy related applications and suggest some promising methods to reduce this 'unwanted' innate immune response.

Author photographs and biographies

Joke Devoldere (1990) obtained her master's degree in pharmaceutical sciences at Ghent University (Belgium) in 2013. After graduating, she started her Ph.D. in the Ghent Research Group on Nanomedicines under the supervision of Prof. Katrien Remaut and Prof. Stefaan De Smedt and became a doctoral fellow of the Fund for Scientific Research-Flanders (FWO-Vlaanderen) in 2014. Her current research focuses on non-viral mRNA delivery: overcoming immunogenicity and the need for multiple administrations.



Heleen Dewitte (1987) obtained her master's degree in pharmaceutical sciences – drug development at Ghent University (Belgium) in 2010. In 2015, she obtained her PhD in Pharmaceutical and Medical Sciences at both Ghent University and the Vrije Universiteit Brussels under the joint promotorship of Prof. dr. Stefaan De Smedt and Prof. dr. Karine Breckpot. Her project lies on the interface between material science and immunology as it aims to develop novel micro- and nanomaterials for the delivery of antigen-coding mRNA to induce antitumor immunity. With her research, she won several awards, among which the "Therapeutic use of microbubbles" oral presentation award (Rotterdam, The Netherlands) and the Jan Feijen poster prize at the 13th European symposium on controlled drug delivery (Egmond aan Zee, The Netherlands).



Stefaan C. De Smedt (1967) graduated from Ghent University in 1995 and joined Janssen Research Foundation. He has been a post-doctoral fellow at the universities of Ghent and Utrecht. In 1999 he became Professor in Physical Pharmacy and Biopharmacy at Ghent University where he founded the Ghent Research Group on Nanomedicines. He served as dean of his Faculty from 2010 till 2014. He is a member of the Board of Directors of Ghent University. Since 2004 Stefaan serves as the European Associate Editor of the Journal of Controlled Release; In 2015 he became Editor of JCR. His research is at the interface between drug delivery, biophysics, material sciences and advanced optical imaging. Stefaan received the Scott Blair Biorheology Award, the Controlled Release Society Young Investigator Award 2006 and the APV Research Award 2010 for Outstanding Research Achievements in Pharmaceutical Sciences. Dr. Stefaan De Smedt holds patents on carriers for drug delivery and diagnostics. He is a scientific founder of Memobead Technologies, a spin-off from Ghent University, whose technology is currently under further development by Biocartis (Lausanne and Mechelen) and Mycartis (Gent).

Katrien Remaut (1978) graduated as Pharmacist at Ghent University (Belgium) in 2001. She then started research work in the Lab of General Biochemistry and Physical Pharmacy under guidance of Prof. De Smedt and Prof. Demeester. In 2007, she received the title of doctor in pharmaceutical sciences and continued research work as postdoctoral fellow of the Research Foundation Flanders. In 2009, Katrien joined the Directors Research Lab under guidance of Prof. Ian Mattaj at the European Molecular Biology Laboratory during 6 months. She received several scientific prizes among which the Prize of the Royal Academy of Medicine for Scientific Research in Pharmacy, period 2008 – 2011. In 2014, she was appointed tenure track professor at the Lab General Biochemistry and Physical Pharmacy, Faculty of Pharmaceutical Sciences.

Evading innate immunity in non-viral mRNA delivery: don't shoot the messenger

Abstract

In de field of non-viral gene therapy, *in vitro* transcribed (IVT) mRNA has emerged as a promising tool for the delivery of genetic information. Over the past few years it has become widely known the introduction of IVT mRNA into mammalian cells elicits an innate immune response which has favored mRNA use towards immunotherapeutic vaccination strategies. However, for non-immunotherapy related applications this intrinsic immune-stimulatory activity directly interferes with the aimed therapeutic outcome, as it can seriously compromise the expression of the desired protein. This review presents an overview of the immune-related obstacles that limit mRNA advance for non-immunotherapy related applications.

1 Introduction

Recent advances in the field of molecular biology have revolutionized mRNA as a therapeutic. The concept of nucleic acid based therapy emerged in 1990, when Wolff *et al*. reported successful expression of proteins into target organs by direct injection of either plasmid DNA (pDNA) or messenger RNA (mRNA) [1]. Although this pioneering study showed a similar potential of mRNA and pDNA to induce protein expression, it took another 10 years for *in vitro* transcribed (IVT) mRNA to compete with the success of DNA transfection. Initially the use of mRNA as a gene therapeutic was confronted with much skepticism due to its perceived instability and transient nature. However, recent research demonstrating the many advantages of mRNA over pDNA, brought about a new wave of interest into the use of IVT mRNA. A first convenience is that mRNA exerts its function in the cytoplasmic compartment. As a consequence, mRNA activity does not depend on nuclear envelope breakdown, which is a major disadvantage of pDNA transfection. In this regard, mRNA is an ideal candidate for protein expression in non-dividing cells, such as dendritic cells, which are otherwise hard to transfect [2]. Secondly, mRNA, unlike pDNA and viral vectors, lacks genomic integration and thus avoids potential insertional mutagenesis [3]. This provides mRNA with a substantial safety advantage for clinical practice. Thirdly, mRNA production is relatively easy and relatively low-priced, since there is no need to select and incorporate a specific promoter into the transfection construct [4]. Furthermore, since IVT mRNA is synthesized in a cell-free system, the production process, manufacturing material as well as the product quality can be easily standardized and controlled in good manufacturing process (GMP) conditions. GMP manufacturing of mRNA guaranties high batch-to-batch reproducibility and makes it easy to translate mRNA use from bench to bedside [5].

One of the applications in which induction of transient gene expression by mRNA transfection is of great interest is vaccination, in which transcripts encoding a certain antigen are administered directly *in vivo* or *ex vivo* via dendritic cell transfection in order to elicit antigen-

specific immune responses [6-9]. Besides the desired immune responses against the antigenic protein encoded by the mRNA, the mRNA itself is often the target of the immune system, making mRNA both the messenger and its own adjuvant. For immunotherapy, this intrinsic immune-stimulatory activity of mRNA is not a limiting factor, as it can increase the potency of the vaccine (as extensively reviewed elsewhere [3,5,10-14]). When extending the use of mRNA for applications outside this area, however, innate immune responses against mRNA can seriously compromise its delivery efficiency. To address these issues, this review aims to discuss the immune-related hurdles that need to be tackled to allow clinical application of IVT mRNA for non-immunotherapy related applications. We present a summary of the current knowledge of the signal pathways induced by mRNA transfection and suggest some promising methods to enhance mRNA expression by reducing this 'unwanted' innate immune response. Furthermore, we overview recent developments in the use of non-viral mRNA delivery for non-immunogenic purposes, such as protein-replacement therapies and regenerative medicine applications.

2 In vitro transcribed (IVT) mRNA

Interestingly, the production of functional mRNA by *in vitro* transcription was already reported in 1984 by Krieg *et al*. [15]. They synthesized mRNA using a phage RNA polymerase and a cloned cDNA template. Following this publication, a high number of technical refinements have been reported and kits for synthesis have been commercialized.

IVT mRNA is a single-stranded polynucleotide, structurally resembling naturally occurring eukaryotic mRNA. The sequence encoding the desired protein is called the open reading frame (ORF) and is located between two untranslated regions (UTRs). A 5' cap structure and a 3' poly(A) tail flank the mRNA at its extremities (Figure). Eukaryotic mRNA contains a 7-methylguanosine cap coupled to the mRNA via a 5'-5' triphosphate bridge (m^7GpppN). For efficient translation, binding of the 5'-cap to the eukaryote translation initiation factor 4E (eIF4E) is essential. Binding with the decapping enzymes (DCP1, DCP2, DCPS) on the other hand results in a loss of mRNA activity [16,17]. IVT mRNA can be capped either post-transcriptionally using recombinant capping enzymes [18] or during the *in vitro* transcription reaction by adding a synthetic cap analogue. The poly(A) tail, a long sequence of polyadenylate residues binds to the polyadenylate binding proteins (PABPs) leading to mRNA circularization, thereby increasing the affinity of eIF4E for the cap structure [19,20]. This synergistic interaction between the two termini of mRNA plays an important role in the stability of mRNA by limiting both decapping as well as 3' to 5' mRNA degradation.

Although IVT mRNA strongly resembles endogenous mRNA, it is still considered as foreign by the innate immune system. Over the past few years it has become known that the introduction of IVT mRNA into mammalian cells induces activation of several mechanisms of which the natural purpose is to identify and attack viral RNAs.

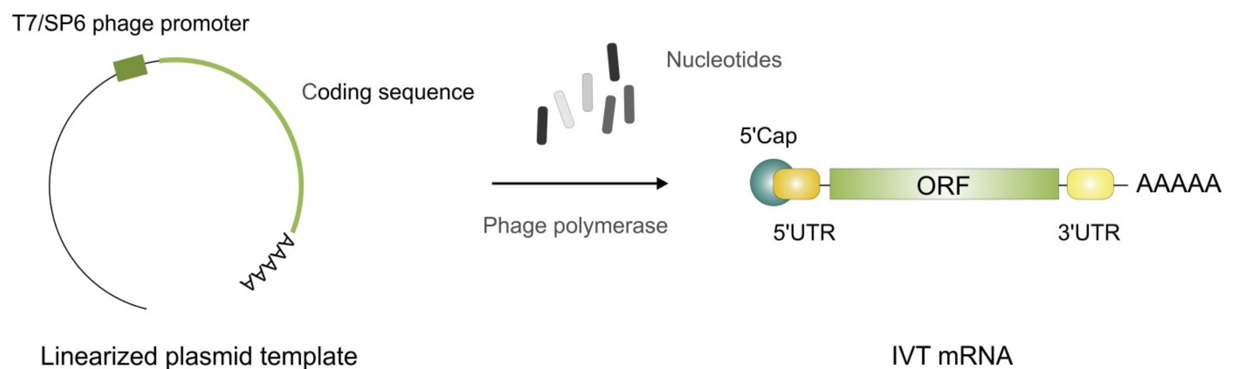


Figure 1 |In vitro transcription of mRNA. Capping of the mRNA can be done during the in vitro transcription reaction by addition of synthetic cap analogues or post-transcriptionally by means of recombinant capping enzymes. The poly(A) tail can be encoded in the template DNA or can be enzymatically added after *in vitro* transcription. Abbreviations: IVT, *in vitro* transcribed; ORF, open reading frame; UTR, untranslated region

3 The immune-stimulating activity of mRNA

3.1 Intracellular mRNA sensing pathways

Knowledge of the mechanisms recognizing and responding to viral intruders has furthered our understanding of the cytosolic sensors involved in innate immunity. These sensors have been shown to be activated mainly by viral nucleic acids, rather than viral proteins [21]. DNA, double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) found in viral genomes, as well as dsRNA-intermediates of viral replication are recognized by so-called pattern recognition receptors (PRRs) [22]. Stimulation of these PRRs activates a downstream cascade of signaling reactions, eventually inducing gene expression of pro-inflammatory cytokines and type I interferons (IFNs). By identifying the structural elements responsible for this activation, insight was gained into the immune-stimulatory activity of IVT mRNA.

Figure summarizes the main pathways involved in mRNA recognition. Two families of PRRs are thought to be responsible for the detection of IVT mRNA: the Toll-like receptors (TLRs) and the cytoplasmic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). TLRs, predominantly but not exclusively expressed in immune cells, are transmembrane receptors with leucine-rich repeats in the extracellular or intra-endosomal region and a signal-transduction or Toll/Interleukin (IL)-1 receptor (TIR) domain in the cytosolic region. Thirteen TLRs have so far been identified in humans and mice together [21,23,24]. Their location in the cell seems to correlate to the pathways by which their molecular ligands are processed [25]. Accordingly, the TLRs involved in the recognition of foreign mRNA – TLR3, TLR7 and TLR8 – are located in the endosomal compartment. As such, especially uridine-rich ssRNA was identified as a strong immune inducer, mainly via stimulation of TLR7 [26,27], whereas dsRNA rather activates TLR3 [28,29]. Generally, mRNA is considered ssRNA, causing the foreign IVT mRNA to be mostly recognized by the structurally homologous TLR7 and TLR8 receptors [26,30]. However, mRNA is also able to form secondary structures, such as hairpins, containing double

stranded sequences. These short segments interact with the dsRNA binding protein of the TLR3 signaling cascade, making mRNA a suitable ligand for TLR3 [28,29].

Following activation, PRRs transmit downstream signaling via specific adaptor molecules. For TLR7 and 8, the required adaptor is the Myeloid differentiation primary response gene 88 (MyD88). TLR3 transmits signals via TIR domain-containing adaptor inducing IFN-β (TRIF) [31]. The adaptor proteins MyD88 and TRIF initiate a signaling cascade that consists of a complex network of signaling molecules. These signaling networks cooperate, integrate and finally converge into the activation of several transcription factors, including nuclear factor- κB (NF-κB) and interferon regulatory factors (IRFs) 3 and 7 [32].

In addition to TLRs, IVT mRNA can be detected by RLRs, which are cytosolic RNA helicases. These sensors, mainly important in non-immune cells, include RIG-I, melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RIG-I has been long thought to specifically detect ssRNA bearing a '5-triphosphate (5'ppp) group [33,34]. Recent studies however, challenged this hypothesis and demonstrated that activation of RIG-I requires base pairing of the nucleoside carrying the 5'ppp. Evidence was provided that RIG-I is triggered by double-stranded, but not single-stranded, RNA containing 5'ppp [35,36]. In addition, Goubau *et al.* showed that also 5'-diphosphate (5'pp) dsRNA serves as an RIG-I ligand, thereby concluding that a minimal feature for RIG-I activation is a base-paired RNA with a free 5'pp [37]. Since endogenous RNA is processed and capped before entering the cytoplasm, its 5'ppp group is shielded from detection by RIG-I. IVT mRNA however, if co-transcriptionally capped, yields a significant fraction of uncapped single- and double-stranded molecules, which can trigger RIG-I signaling. The second RLR, MDA-5, is activated by cytoplasmic long dsRNA [38,39]. Recognition of RNA by RIG-I or MDA5 triggers an ATP-dependent change in the receptor conformation, which allows interaction with the mitochondrial adaptor molecule MAVS (also known as IPS-1). The obtained complex actuates several proteins to initiate downstream signaling, which similar to the activation of TLRs converges in the activation of several transcription factors. A third member of the RLRs is LGP2 (not depicted in Figure). LGP2 is much less explored and conflicting data have been published on its role in innate immune signaling. Although LGP2 was initially assumed to negatively regulate RLR-mediated signaling [40,41], more recent studies revealed a positive role for LGP2 in the regulation of type I IFN responses [42]. Nevertheless, experimental data of further studies are still controversial, with both overexpression and knockdown of LGP2 resulting in type I IFN production [43]. Whether LGP2 mediated signaling can be induced by IVT mRNA remains to be established.

It is clear from the above that both TLR and RLR sensors respond to mRNA stimulation by activation of transcription factors, such as NF-κB, IRF3 and IRF7. Both pathways converge in the activation of the IκB kinase (IKK) complex and the IKK-related kinases TANK-binding kinase 1 (TBK1) and IKKε. The IKK complex, which includes the kinases IKKα and IKKβ as well as the regulatory subunit IKKγ/NEMO, is responsible for the activation of NF-κB, whereas TBK1 and IKKε phosphorylate and activate IRF3 and IRF7 [31,44,45]. In unstimulated cells, NF-κB, IRF3

and IRF7 are located in the cytoplasm. Activation by the aforementioned kinases, however, causes them to translocate to the nucleus. Intranuclear, they bind to the type I IFN gene promoter, inducing expression of type I IFNs, in particular IFN-α and IFN-β. NF-κB additionally activates the expression of pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-12 (IL-12) [5,32].

As depicted in Figure , type I IFNs are secreted in the extracellular environment and bind to the transmembrane IFN receptor complex of the stimulated cell and adjacent cells. This receptor complex in turn induces a downstream transmission of signals through the so-called Janus kinase (JAK)-Signal transducer activator of transcription (STAT) pathway. The STAT proteins, STAT1 and STAT2, are phosphorylated by the Janus kinases JAK-1 and TYK-2, and bind a third factor, IRF-9 to form a transcription activator complex, the IFN-stimulated gene factor 3 (ISGF-3). Upon activation ISGF-3 translocates to the nucleus, where it initiates the transcription of more than 300 IFN-stimulated genes (ISGs). Of these ISGs, many encode for proteins that are components of the signaling pathways themselves, such as PRRs and transcription factors, thus providing an autocrine loop that amplifies IFN production [46]. However, several other ISGs encode for proteins which confer strong anti-viral activity, including dsRNA dependent protein kinase (PKR), 2'-5'-oligoadenylate synthetases (OASs) and RNA-specific adenosine deaminase (ADAR) [47]. It is interesting to point out that both type I IFNs as well as the pro-inflammatory cytokines, not only act in an autocrine fashion, but concurrently activate receptors in adjacent cells via paracrine secretion. As a result, upregulation of PRRs is induced in neighbouring cells, sensitizing them to subsequent exposure to nucleic acids [48,49].

Recently, Andries *et al.* demonstrated that another PRR family, the NOD-like receptors (NLRs) are also involved in the cytoplasmic recognition of IVT mRNA [50]. They demonstrated an upregulation of caspase-1 after non-viral carrier-mediated delivery of mRNA in respiratory cells. Caspase-1 is a zymogen, typically regulated by NLRs. The NLR most broadly associated with RNA sensing is NLRP3, which has been shown to respond to dsRNA [51]. NLRP3, also known as cryopyrin or Nalp3, forms a multiprotein complex with the adaptor protein ASC and caspase-1. This complex, called "the inflammasome", is responsible for the proteolytic maturation of the IL-1β and IL-18 cytokines. A recent study by Sabbah *et al*. has demonstrated that another member of the NLRs, NOD2, can also serve to detect ssRNA [52].

All these intracellular cascades have been shown to interact with each other in a complex network. It is this crosstalk together with the strength, timing and context of stimulation that determines the type and duration of immune responses. Besides this inter-pathway crosstalk, PRR-mediated signaling is regulated by polyubiquitination or deubiquitination of the involved proteins and can therefore be influenced by deubiquitinating enzymes [32].

3.2 Unwanted immune responses induced by mRNA recognition

As previously discussed, IVT mRNA-induced immune activation is considered beneficial for vaccination strategies as it can attribute to the desired cellular and humoral immune response. Especially the strong cytokine milieu that results from antigen-encoding IVT mRNA transfection is of particular interest, as this can boost dendritic cell maturation as well as T cell activation [53,54].

By contrast, for non-immunotherapy related applications this immune-stimulatory activity of IVT mRNA might be a major concern, as was shown in several mRNA-based reprogramming studies [48,49,55]. Signaling through the different PRR pathways forces the cells into an overall anti-viral state, affecting the efficiency of mRNA translation and causing RNA degradation. In this anti-RNA response, a key role is played by the ISG encoded proteins (Figure). To date, three anti-RNA pathways that shoot the messenger have been identified. These comprise the PKR, the OAS and the ADAR system.

PKR (also known as Eif2ak2) is a kinase that phosphorylates the α-subunit of the eukaryotic translation initiator factor 2 (eIF2α). Activation of PKR impairs eIF-2 activity, which results in an inhibition of general mRNA translation and thus stalls protein synthesis [56]. Besides this regulatory translational controlling function, PKR is also involved in various signaling pathways. Active PKR has been shown to provoke release of NF-κB from its inhibitory subunit, IκB, by stimulation of the IKK kinase complex, thereby activating the NF-κB transcription factor and promoting the expression of multiple genes [57]. Finally PKR also induces cellular apoptosis, which serves as a natural process of preventing further viral infection [56,58].

A second anti-RNA pathway involves the activation of OAS by dsRNA to produce of 2'-5'-oligoadenylates (2-5A) from ATP. These rare 2-5A oligomers have the capacity to induce the catalytical activity of the latent enzyme RNaseL, which causes cleavage of ssRNA, thus promoting RNA degradation [59]. In addition, the cleavage products can again bind and activate cytoplasmic PRRs, thus maintaining and amplifying the type I IFN loop [47,60].

Another ISG family that influences translation is the adenosine deaminases acting on RNA or ADARs. These genes encode for the ADAR enzyme, which catalyzes RNA editing through site-specific deamination of adenosine (A) to yield inosine (I). By inducing the formation of a weak I:U mismatch, ADARs are capable of destabilizing the RNA molecule. Moreover, conversion of A to I may alter the coding capacity of mRNA and thus the amino acid sequence of the encoded proteins [61,62]. Surprisingly, however, recent studies found that the absence of ADAR1, one of the three identified ADAR proteins, significantly increases IFN-mediated signaling, suggesting a role for ADAR1 as a suppressor of IFN responses [63,64]. Presumably, this negative feedback serves to prevent overreaction during viral infection. The mechanism by which ADAR1 impairs type I IFN response has not been thoroughly elucidated. One possibility is that ADAR1 edits the RNA in such a way that it no longer serves as an activator of innate immune signaling and loses its IFN inducing capacity [61,62]. Another feasible explanation is

that the RNA binding activity of ADAR1 is involved in the suppression of IFN signaling. Recently, Yang *et al.* demonstrated that ADAR1 binds dsRNA, thereby limiting cytosolic dsRNA sensing by RLRs [65]. In addition, ADAR1 suppresses activation of both PKR and IRF3, by a mechanism still to be resolved [66].

OAS as well as PKR and ADAR require IFN signaling for induction of their synthesis, but also call for dsRNA to initiate their activation. In this way all tree enzymes not only act as RNA-induced effectors but also serve as PRRs for the detection of dsRNA in the cytosol [47]. It is important to note that these are probably not the only ISGs that negatively influence IVT mRNA translation. Likely, additional IFN-induced proteins with similar roles exist, but await further investigation into their specific relevance.

The processes induced by these effectors not only hamper mRNA transfection, but also disfavor cell viability and can eventually result in apoptosis [67]. Besides type I IFNs, up-regulation of caspase-1 by NLR-mediated signaling too is detrimental to cells and causes programmed cell death [50]. This is probably one of the reasons why non-immunotherapeutic mRNA therapies are still in its infancy (as will be more thoroughly discussed in section 5).

4 Bypassing the intracellular innate immune system

Owing to the strong immune responses induced by mRNA transfection, the use of IVT mRNA has been mainly limited to therapeutic vaccination approaches. Over the past few years several strategies have been explored to decrease the immune-activating capacity of IVT mRNA in order to promote non-immunogenic applications, such as protein-replacement therapies and mRNA-based reprogramming methods. This review discusses three possible strategies to evade mRNA-induced immunity: i) optimization of delivery methods to shield the IVT mRNA and control its entry pathway into the cells, ii) modifications on the level of the mRNA template or the IVT mRNA molecule itself or iii) blocking key proteins involved in the intracellular recognition of IVT mRNA and its subsequent signaling cascades.

4.1 mRNA delivery methods

Most cell types show only limited cytoplasmic presence of IVT mRNA after spontaneous uptake of the naked transcript [68]. An exception to this are immature dendritic cells, which efficiently take up and accumulate mRNA by macropinocytosis [69]. By contrast, effective delivery of mRNA in other cell types requires alternative delivery methods. In addition to a facilitated uptake, most of these delivery methods have focused on the protection of mRNA against RNase degradation, thus increasing its extra- and intracellular stability. However, also the delivery route (endosomal vs. direct cytoplasmic entry) will determine which PRR the mRNA will encounter on its intracellular journey. Unfortunately, favoring particular delivery routes as a means to protect mRNA against PRR recognition has not been one of the main focus points so far.

Several strategies have been investigated to package the negatively charged mRNA into cationic carriers. These carriers condense the mRNA into positively charged complexes that interact with the negatively charged cell membrane, facilitating mRNA uptake [70,71]. Both viral and non-viral vectors have been investigated, with a better efficiency for the former but a higher safety and adjustability for the latter. Although knowledge of the cellular pathways involved in vector-mediated mRNA transfection expands by the day, their interaction with cellular components and the subsequent effects on cell function have been strongly overlooked so far. Evidence is emerging which indicates that most carriers exhibit an intrinsic immune-stimulating activity, inducing cell signaling cascades even without mRNA complexation [72].

One very clear example is the oldest and most widely used non-viral mRNA-carrier, protamine. Although this naturally occurring protein is demonstrated to protect the mRNA from degradation, mRNA:protamine complexes were shown to strongly induce innate immune response [73]. Scheel *et al*. indicate that protamine condensed mRNA stimulates the immune system through a MyD88-dependent pathway, suggesting that TLR7 and TLR8 are probably the receptors involved [74]. This immune activating capacity of protamine can be exploited for vaccination strategies, but seemed to inhibit the primary goal of mRNA delivery, i.e. expression of the encoded protein [73]. Other well-investigated mRNA carriers are cationic lipids and polymers. Spontaneous electrostatic interactions condense the mRNA into lipo- or polyplexes respectively [71]. Rejman *et al*. demonstrated that both lipid-based carriers, such as DOTAP/DOPE, and polymers, exemplified by poly-ethylene-imine (PEI) are capable of transfecting mRNA into cells with a higher and longer lasting protein expression found for liposomes than for polymers (the reason being currently unknown) [75]. As for the protamine-RNA complexes, also DOTAP and PEI RNA formulations were shown to be detected by TLR-7 and TLR-8 [26,30]. Furthermore, *Lonez et al.* conclude that multiple cationic lipids, such as stearylamine-liposomes and Lipofectamine, in itself activate intracellular immune pathways, independent of mRNA complexation, resulting in the induction of several pro-apoptotic and pro-inflammatory signaling molecules [76]. The activated immune profile will additionally depend on the particle size, as it has been shown that the immune system distinguishes nanometric and micrometric structures in order to adapt the response to viral or bacterial/fungal organisms [77]. Taken together, these examples show that when mRNA is formulated in particulate delivery systems, the immune-stimulatory effects of the resulting complex will be dictated by both the mRNA molecule, as well as by the nature of the carrier used.

Apart from packaging IVT mRNA into nanoparticles, enhanced uptake has also been achieved by physically disturbing the cell membrane. Methods like micro-injection, electroporation or sonoporation shuttle the mRNA directly in the cytosol and thus avoid detection by endosomal RNA receptors [78]. Whether or not mRNA delivery by one of these approaches is a suitable strategy to circumvent endosomal sensing of IVT mRNA remains to be elucidated. Nevertheless, studies in the field of cancer immunotherapy have demonstrated that neither

sono- nor electroporation results in strong activation of immune cells. In fact, both techniques require additional stimulation with adjuvants to induce therapeutically beneficial immune responses [79,80]. This might indicate that cytosolic PRRs are less immunogenic than endosomal TLRs. If so, cytosolic delivery might be the preferred route of administration for non-immunotherapy-related applications.

4.2 Modifying the mRNA

In the past few years, considerable efforts have been undertaken to increase the stability of the mRNA transcript by applying modifications to the plasmid template or to the mRNA molecule itself. As these modifications have been extensively reviewed elsewhere, we will only list these modifications that reduce IVT mRNA immunogenicity [5,11].

First of all, Koski *et al.*, provided evidence that enzymatic 3'-polyadenylation with a minimum of 150 adenosines lowers the immune stimulatory activity of synthetic mRNA [81]. Therefore, apart from increased stability, elongation of the poly(A) tail seems to be a good strategy to temper the immunogenic profile of IVT mRNA. To provide mRNA with a fixed poly(A) tail length, the adenosine residues are mostly encoded in the DNA template, as posttranscriptional polyadenylation yields mixtures of mRNAs with different poly(A) tail lengths.

A second strategy makes use of the observation that uncapped IVT mRNA bears a triphosphate group at the 5' end, which can be detected by the cytosolic RNA sensors RIG-I and PKR [37,82]. Therefore, shielding the 5'ppp with a synthetic cap analogue can evade immune activation. This can be achieved by addition of an anti-reverse cap analog (ARCA) during the *in vitro* transcription reaction or by means of posttranscriptional capping using recombinant capping enzymes. However, even with these methods it is impossible to accomplish a capping efficiency of 100% [83]. To further reduce the immunogenicity of the remaining uncapped mRNA, a phosphatase treatment can remove any resting triphosphates at the 5' end of the mRNA transcript [84]. Besides capping, also 2'-O-methylation at the penultimate nucleotide of the 5' end has been shown to prevent RIG-I binding and activation [84,85].

De-immunization of the mRNA construct can be further achieved by the incorporation of naturally occurring modified nucleosides. Kariko and colleagues demonstrated that activation of TLR3, TLR7 and TLR8 can be reduced or completely eliminated with RNA containing 5-methylcytidine (m5C), N6-methyladenosine (m6A), 5-methyluridine (m5U), pseudouridine or 2-thioruridine (s2U) [86]. Two of the modified nucleosides, S2U and pseudouridine, seem to reduce detection by RIG-I and PKR as well [33,87]. Additionally, in 2008, pseudouridine was shown to increase mRNA translation capacity, by improving the overall stability of mRNA and avoiding PKR recognition [88,89]. In the same vein, *Kormann et al.* indicated that replacement of 25% uridine and cytidine with s2U and m5C substantially reduced binding to PRRs and decreased innate immune activation, leading to an increased protein expression *in vitro* and *in vivo* [90].

Finally, purification of the IVT mRNA can further mitigate the immune stimulatory properties as was demonstrated by Kariko *et al*. by the removal of dsRNA contaminants through high-performance liquid chromatography purification [91].

4.3 Interfering with the signaling downstream pathways

Although a wide range of mRNA delivery techniques and modification strategies have been available for a while, activation of the innate immune cascades still remains a primary concern for mRNA transfections in non-immunogenic applications. In particular, repeated transfections seem to be problematic, as have been demonstrated when using mRNA for cellular reprogramming. The mRNA-triggered immune response seems to hypersensitize transfected cells, as well as neighboring cells to subsequent mRNA exposure, causing cell damage and eventually cell death [48,49]. In spite of this problem, repeated transfections are often required due to the transient nature of mRNA expression. The strong and detrimental immune responses against foreign mRNA originally serves to detect and prevent the spreading of RNA viruses in such a way that if needed, host cells are sacrificed to prevent further infection. RNA viruses, however, have developed a remarkable diversity of countermeasures to evade immune detection and down-regulate induced responses. Mimicking this viral immune-evasion could therefore be an interesting strategy to bypass the mRNA-triggered immune responses and increase the transfection efficiency of non-viral mRNA based gene delivery systems.

It is known that viruses inhibit innate immunity by avoiding or inhibiting specific immune-related proteins. Genetic analyses have revealed antagonistic activities against virtually all elements of the immune pathways. In this review, we aim to address some of these potential target points in order to bypass mRNA triggered innate immunity. Given the redundancy in possible interfering molecules, other examples than the ones listed here might also form a possible evading strategy. Since the innate immune response to mRNA is bimodal, evasion of the response can be divided in two aspects as well: prevention of the initial type I IFNs production (Figure) and inhibition of the auto- and paracrine effects of type I IFNs (Figure).

Prevention of type I IFN production

The most obvious approach to escape the negative effects of IFN induction is to intervene in their production. This can be achieved by (i) avoiding mRNA detection and/or (ii) intervene in the mRNA-induced signal transmission. A straightforward strategy to prevent mRNA-mediated IFN production would be to avoid detection in the first place. PRR mediated recognition of mRNA can be inhibited using small molecules, such as bafilomycin A1 and chloroquine, which can simply be added to the cell culture medium. Bafilomycin A1 acts as an endosomal TLR inhibitor by selectively blocking the vacuolar H^+-ATPase. As a result, bafilomycin increases the acidic endosomal pH, which is thought to be essential for the activation of TLR mediated signal transduction [92-94]. As for bafilomycin A1, the inhibitory acitivity of chloroquine has been generally ascribed to the inhibition of endosomal

acidification. However, Kuznik *et al.* recently demonstrated the effect of chloroquine on the endosomal pH to be negligible at concentrations required for TLR suppression. Instead, they proposed a direct interaction of chloroquine with nucleic acids, which causes a conformational change and makes the nucleic acid ligand unavailable for TLR recognition [94].

A second strategy to restrict IFN production is to intervene negatively in the mRNA-induced signal transmission. Over the past years, it has become evident that the activation of innate immune signaling involves ubiquitination of several immune-related proteins. A case in point is the ubiquitin-dependent activation of the RIG-I receptor required for the recruitment of MAVS and the subsequent signaling molecules. Ubiquitination also activates TRAF3 and TRAF6, which in turn activate, respectively, TBK1/IKKε and the IKK complex for subsequent phosphorylation of transcription factors. In addition, the IκB inhibitory protein depends on ubiquitination for its degradation and hence release of NF-κB. Considered together, administration of deubiquitinating enzymes could negatively regulate innate immunity [95]. Examples in this regard, include the deubiquitinating enzyme A (DUBA) and A20, which inhibit IRF3 and NF-κB activation respectively by deubiquitinating TRAF3 and TRAF6.

Alternatively, mRNA-induced signal transmission can be diminished by interfering with the PRR-adaptor molecule interaction. Pepinh-TRIF and Pepinh-MYD are two peptide inhibitors which contain specific domains of the adaptor molecules TRIF and MyD88. Administration of Pepinh-TRIF and Pepinh-MYD therefore competitive reduces interaction between these adaptormolecules and their respective TLRs [96,97]. Another technique to interrupt mRNA-induced signaling is through the administration of kinase inhibitors. Since the IKK complex and the IKK-related kinases, TBK1 and IKKε are responsible for the activation of NF-κB and IRF3/7 respectively, related inhibitors can minimize the ensuing IFN and cytokine production. BX795, a potent inhibitor of TBK1 and IKKε, has been shown to suppress the phosphorylation of IRF3, and thus activation of the IFN production [98]. In this regard, Awe *et al*. recently compared BX795 with an inhibitor of the IKK complex, BAY11, in their ability to increase mRNA-mediated protein expression by suppressing the innate immune response [99].

Besides inhibiting the adaptor molecules and the kinases evolved in innate immune signaling, the transcription factors itself can also be targeted. A variety of small molecule NF-κB antagonists are available, repressing cytokine and IFN expression. An example of this is dexamethasone, which is often used as a positive control for NF-κB inhibition [100]. Dexamethasone has been shown to counteract NF-κB activity in many cell types through upregulation of its cytoplasmic inhibitor IκB, thereby reducing the amount of NF-κB translocating to the nucleus [101-103]. Recently, Bhattacharryya *et al.* indicated this inhibition to be dependent on the type of TLR activated and the specific adaptor protein involved [104]. Another small molecule, phenylmethimazole (also known as C10) has been reported to block transcriptional activity of IRF3. Courreges *et al*. describe the molecular basis for this inhibition, which seems to be a prevention of dsRNA-induced IRF3 translocation and homodimerization

[105]. The observation that C10 blocks IRF3 transactivation is consistent with prior studies which demonstrate C10-mediated inhibition of the TLR3-regulated IRF3/IFN-β/STAT signal pathway [106,107]. In the same way, establishment of a cellular anti-RNA state can be prevented through inhibition of IRF7. By impairing the phosphorylation and nuclear translocation of IRF7, the ORF45 protein of Kaposi's sarcoma-associated herpesvirus blocks activation of type I IFN induction. Mechanistically, ORF45 acts as a decoy substrate for TBK1/IKKε and thus competitively inhibits IRF7 phosphorylation [108,109].

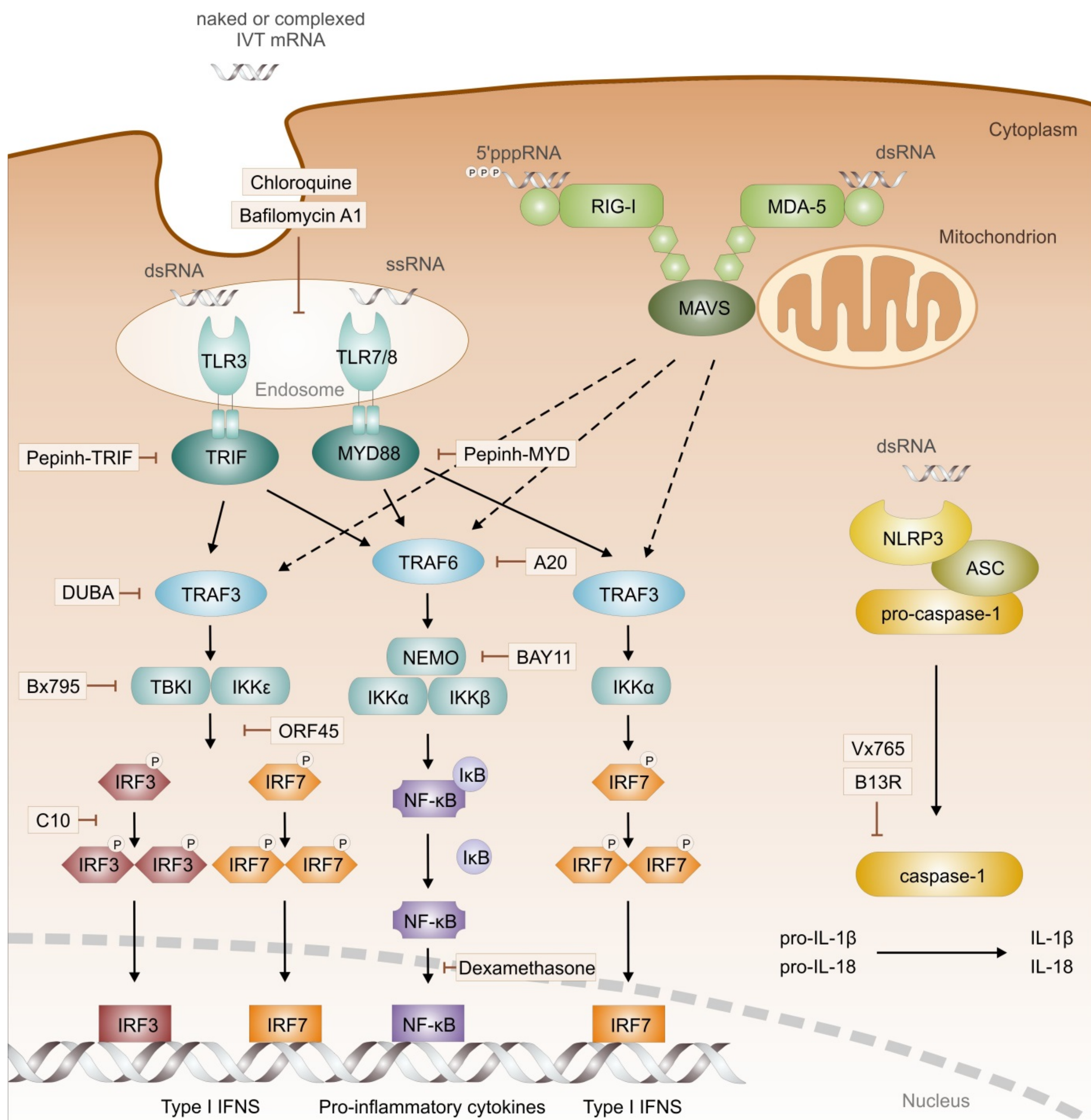


Figure 2 | Innate immune responses to intracellular delivery of IVT mRNA. Synthetic mRNA is recognized by several PRRs, including the endosomal TLR3 and TLR7/8 receptors and the cytoplasmic RIG-I, MDA-5 and NLRP3 sensors. Each PRR interacts with a specific adaptor molecule, which recruits the illustrated signaling molecules and activates downstream transcription factors IRF3, IRF7 and NF-κB. IRF3 and IRF7 regulate the expression of type I IFNs (IFNα and IFNβ), whereas NF-κB additionally controls the production of pro-inflammatory cytokines. Production of type I IFNs and can be inhibited at multiple levels: (i) minimize mRNA recognition through administration of PRR inhibitors, (ii) delivering deubiquitinating enzymes, (iii) inhibiting the adaptor molecules by means of peptide inhibitors, (iv) suppressing transcription factor

activation with kinase inhibitors, (v) counteracting transcription factor activity itself and (vi) applying caspase-1 inhibitors to prevent NLRP3-mediated cytokine production. Abbreviation**s:** 2-AP, 2-aminopurine; ADAR, RNA-specific adenosine deaminase; ASC, apoptosis-associated speck-like protein; dsRNA, double-stranded RNA; DUBA, deubiquitinating enzyme A; EIF2α, eukaryotic translation initiator factor 2; IFN, interferon; IFNAR, interferon-α/β receptor; IKK, IκB kinase; IL, interleukin; IRF, interferon regulatory factor; ISGF3, the IFN-stimulated gene factor 3; IVT, in vitro transcribed; JAK1, Janus kinase 1; MAVS, mitochondrial adaptor molecule; MDA-5, melanoma differentiation-associated protein 5; MyD88 Myeloid differentiation primary response gene 88; NEMO, NF-κB essential modulator; NF-κB, nuclear factor- κB; NLRP3, NOD-like receptor pyrin domain containing 3; OAS, 2'-5'-oligoadenylate synthetase; ORF, open reading frame; PKR, dsRNA dependent protein kinase; RIG-I, cytoplasmic retinoic acid-inducible gene I; ssRNA, single-stranded RNA; STAT, Signal transducer activator of transcription; TBK1, TANK-binding kinase 1; TLR, Toll-like receptor; TRAF, TNF receptor-associated factor; TRIF, TIR domain-containing adaptor inducing IFN-β; TYK2, tyrosine kinase 2; UTR, untranslated region

Inhibition of the IFN-induced effects

A second approach to quelling interferon-mediated immune activation is to inhibit the effects induced by IFN production. Again several options can be explored to accomplish this inhibition. The first is to block IFN transduction by inhibiting engagement with its receptor. IFN binding proteins or neutralizing antibodies compete with the cellular IFN receptor by capturing the secreted IFNs. As a consequence, they avert not only the autocrine IFN amplification loop, but also the induction of IFN-triggered signaling in neighbouring cells. The only IFN binding protein whose use has been extensively published is the Vaccinia Virus (VV) encoded B18R protein. B18R is a decoy receptor, specific for type I IFNs of various species that has been shown to increase cell viability during mRNA-based reprogramming protocols [55,110].

A second strategy to prevent IFN-induced effects is to inhibit IFN induced signal transduction. Proteins that interfere with the JAK/STAT signaling pathway will inhibit production of IFN effectors, such as PKR and OAS, but they will also suppress the upregulation of PRRs and transcription factors, thus reducing the second-wave IFN production. A commonly used JAK inhibitor is the small molecule ruxolitinib [111]. Ruxolitinib potently inhibits the phosphorylation of JAK1 and can therefore interrupt IFN-JAK-STAT signaling in mRNA-stimulated cells. In 2011, ruxolitinib was approved by the U.S. Food and Drug Administration for the treatment of myeolofibrosis, which underscores its potential use for clinical applications [112,113].

A third strategy to inhibit IFN response is to counter the action of the IFN-induced effectors. Accordingly, Gupta *et al*. recently discovered a specific, potent inhibitor of the human RNase L. Curcumin, a naturally occurring antioxidant, was shown to non-competitively inhibit RNase L, presumably by inducing a switch in the conformation of the enzyme, leading to a complete loss of its activity [114]. Likewise, PKR function can also be interrupted. For instance, Carroll *et al*. demonstrated inhibition of eIF2α activation by the Vaccinia virus protein, K3L. They revealed that K3L shows structural similarities to the eIF2α molecule and competes with eIF2α for its phosphorylation by PKR, thereby preventing inhibition of the protein synthesis

[115,116]. Another potent PKR inhibitor, which has been widely used for signaling analysis, is 2-aminopurine (2-AP). Attachment of 2-AP to the ATP-binding site of PKR prevents autophosphorylation of the protein kinase, thus inhibiting subsequent phosphorylation of eIF2α [117,118]. More recently, Jammi *et al.* identified an even stronger PKR inhibitor, known as C16. As for 2-aminopurine, this small molecule inhibits RNA-induced PKR autophosphorylation and rescues the PKR induced translation blockade [119,120]. Besides a direct inhibition of the IFN-induced effectors, some compounds prevent effector activation by sequestering dsRNA, as is described by Xiang *et al.* for the VV E3L protein. Since both PKR and 2-5A synthase require activation by dsRNA, sequestration of dsRNA by the E3L protein will hamper induction of both effectors [115]. Similarly, a cell-permeable peptide (PRI) containing a motif of the dsRNA binding domain of PKR has been reported to prevent PKR activation by sequestering of dsRNA molecules [121].

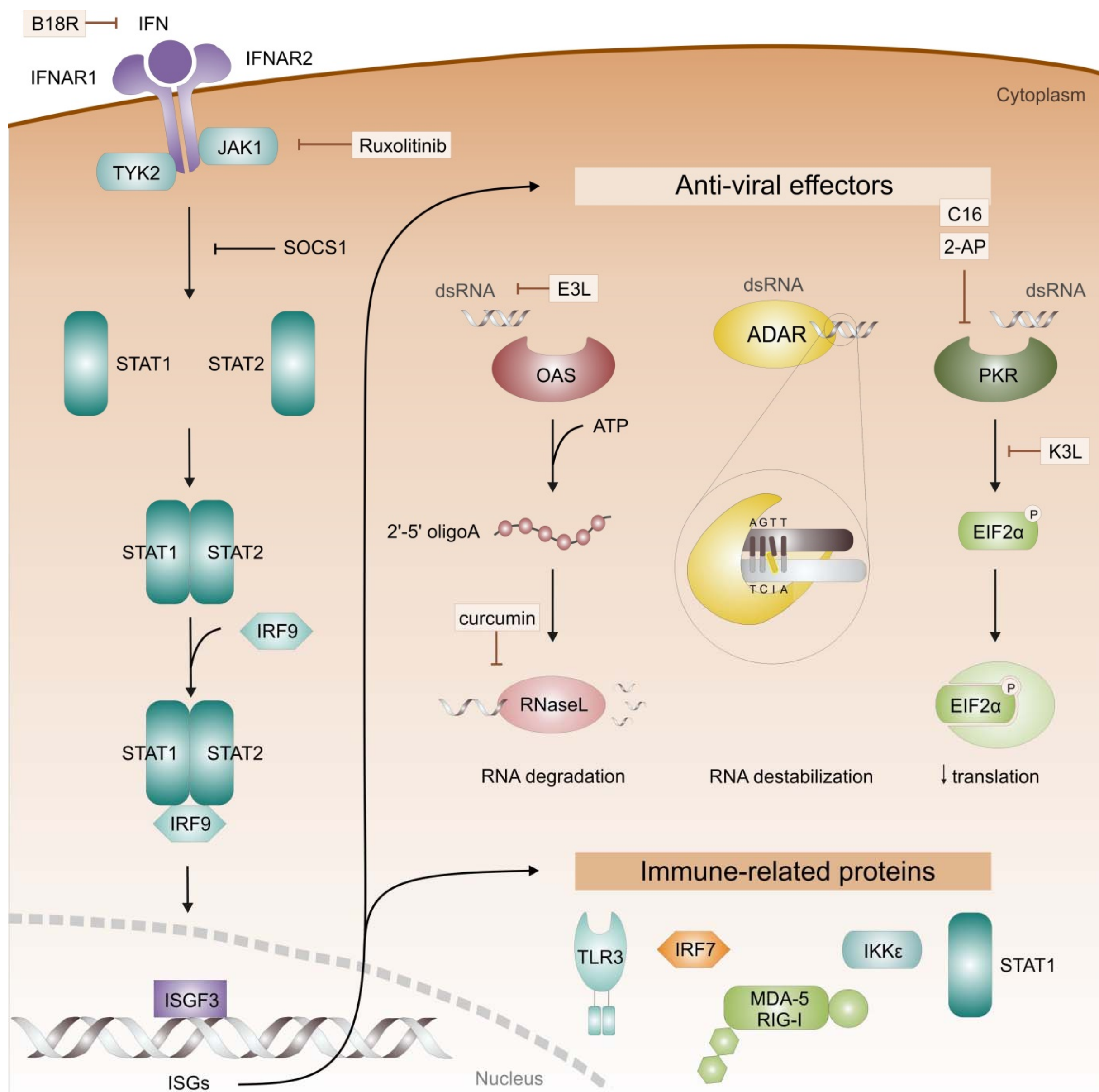


Figure 3 | IFN-mediated signaling. Following their production (cf. Figure) type I IFNs bind to autocrine or paracrine IFN receptor complexes, composed of IFNAR1 and IFNAR2. Recognition of IFNs stimulates the Jak kinases, Jak1 and Tyk2 to

phosphorylate Stat1 and Stat2, which form a transcription activator complex, ISGF3, together with IRF9. ISGF3 activates hundreds of ISGs, including genes encoding for anti-viral effectors OAS, PKR and ADAR. Overall, these create an anti-viral environment, enhancing RNA degradation, causing RNA destabilization and stalling RNA translation. Among the ISGs several genes encode for immune-related proteins, thereby initiating the transcription of a second wave of type I IFNs and amplifying the antiviral response. IFN-induced signaling can be avoided blocking different steps of the signaling cascade: (i) apply IFN-capturing proteins to prevent IFN-receptor binding, (ii) inhibit interferon-induced signaling by means of JAK/STAT inhibitors and (iii) administer molecules which minimize the antiviral action of IFN-induced proteins. Abbreviation**s:** 2-AP, 2-aminopurine; ADAR, RNA-specific adenosine deaminase; ASC, apoptosis-associated speck-like protein; dsRNA, double-stranded RNA; DUBA, deubiquitinating enzyme A; EIF2α, eukaryotic translation initiator factor 2; IFN, interferon; IFNAR, interferon-α/β receptor; IKK, IκB kinase; IL, interleukin; IRF, interferon regulatory factor; ISGF3, the IFN-stimulated gene factor 3; IVT, in vitro transcribed; JAK1, Janus kinase 1; MAVS, mitochondrial adaptor molecule; MDA-5, melanoma differentiation-associated protein 5; MyD88 Myeloid differentiation primary response gene 88; NEMO, NF-κB essential modulator; NF-κB, nuclear factor- κB; NLRP3, NOD-like receptor pyrin domain containing 3; OAS, 2'-5'-oligoadenylate synthetase; ORF, open reading frame; PKR, dsRNA dependent protein kinase; RIG-I, cytoplasmic retinoic acid-inducible gene I; ssRNA, single-stranded RNA; STAT, Signal transducer activator of transcription; TBK1, TANK-binding kinase 1; TLR, Toll-like receptor; TRAF, TNF receptor-associated factor; TRIF, TIR domain-containing adaptor inducing IFN-β; TYK2, tyrosine kinase 2; UTR, untranslated region

As is clear from the above, the intracellular immune responses are generated in cascades. Hence, proteins interfering at one level of a cascade will also influence distant signaling, leading to an even stronger immune-inhibition. In addition, one protein may inhibit different components of the immune signaling cascades. As such, *Xiang et al*. have demonstrated that besides dsRNA sequestration and direct inhibition of PKR, the E3L protein also prevents activation of IRF3, thereby not only blocking the second, but also the first wave of IFN production [115]. In the same way, 2-aminopurine was shown to impair nuclear translocation of phosphorylated IRF3, in addition to its inhibitory effect on PKR [118]. Another molecule which has recently been shown to target more than one element of the intracellular pathways is the anticancer drug, sunitinib. Although commonly known as an inhibitor of the vascular endothelial growth factor receptor (VEGF-R) and the platelet-derived growth factor receptor (PDGF-R), Jha *et al*. reported *in vivo* inhibition of both PKR and RNase L by sunitinib, due to a kinase homology between both effectors [122,123].

As discussed under 3.1, activation of PRRs can also result in the production of pro-inflammatory cytokines. These cytokines amplify the innate immune response to mRNA recognition and some of them negatively influence cell viability as they induce apoptosis. Cytokine-mediated signal transduction can be blocked in the same way as for IFN inhibition, i.e. restriction of cytokine production, prevention of receptor binding and inhibition of the cytokine-induced signaling pathways. To illustrate, production of IL-1 and IL-18 can be prevented by inhibition of their proteolytic maturation [124]. Since both cytokines require caspase-1 to activate their premature form, inhibitors of caspase-1 such as the VV B13R protein and the small molecule VX-765 prevent synthesis and secretion of both cytokines (Figure) [125]. As for IFNs, several viruses also secrete proteins that serve as decoy receptors to sequester extracellular cytokines and impede interaction with cellular cytokine receptors [126]. Furthermore, most cytokines are induced by activation of the NF-κB pathway. Therefore, the aforementioned NF-κB inhibitors will also decrease cytokine production.

It should be noted that instead of using classic small molecule inhibitors, every aspect of the IFN defense could also be targeted for inhibition by means of small interfering RNAs (siRNAs) [86,127,128] or short hairpin RNAs (shRNAs) [86,129,130]. siRNA and shRNA are short artificial dsRNA molecules used to silence gene expression via RNA interference by homology to the targeted gene. Although silencing by siRNA and shRNA has been initially considered sequence specific, Kariko *et al.* recently demonstrated suppression of nontargeted mRNA expression as well. In this paper evidence is provided that both siRNA and shRNA induce type I IFN signaling through TLR3 and activate sequence-independent inhibition of gene expression [131]. Therefore, their use to enhance non-viral mRNA transfection seems contradictory, as they trigger innate immunity per se. In this respect, the use of microRNAs could be considered as well. Since miRNAs have a natural role in regulating inflammatory responses, the chances at immune activation might be lower. In fact, Drews *et al.* observed absolutely no induction of a significant immune response when transfecting mouse fibroblasts with a mix of pluripotency-promoting miRNAs [48]. Nonetheless, a better understanding of the precise regulatory roles of miRNAs in innate immune signaling is needed in order to unravel their potential in manipulating the intracellular pathways.

Another more general strategy to neutralize the innate immune responses is the use of monoclonal antibodies targeting either signaling molecules or their receptors. Owing to the inefficient transport of monoclonal antibodies across cellular membranes, this method will mainly target extracellular elements of the innate immune system, such as the IFN receptor expressed on the surface of the cell or the circulating type I IFNs themselves. Inhibition of intracellular components of the immune pathways might be possible by integrating the antibody to the delivery vehicle.

Finally, we wish to stress that the list of potential immune-inhibitors is tremendously increasing and it is beyond the scope of this review to sum up all commercially available or virus-related inhibitors. Therefore, we have attempt to exemplify every possible strategy with at least one inhibitory molecule.

5 Current state of non-immunotherapy related mRNA applications

So far, cancer immunotherapy is the only field in which mRNA-based therapeutics are reaching clinical trials. Although mRNA has garnered broad interest for its utility in other medical indications, clinical translation has been hampered by its immunogenicity, limited stability and transient nature. The finding that the immune-stimulatory activity of RNA could be tempered by incorporation of modified nucleosides was crucial to extend the applicability of mRNA into other areas than immunotherapy [86].

Currently, the potential of IVT mRNA is being explored for a variety of applications, ranging from inherited or acquired metabolic disorders to regenerative medicine, all of which still

remain in preclinical stage. The first study in which IVT mRNA is used for the replacement of a deficient protein *in vivo*, was published in 1992. In this work, Jirikowski and colleagues demonstrated that direct injection of vasopressin-encoding mRNA in the hypothalamus of vasopressin-deficient rats led to the production of significant plasma levels of vasopressin and temporary reversed their diabetes insipidus [132]. For about a decade, this remained the only mRNA-based paper demonstrating the feasibility of using IVT mRNA to express therapeutic proteins *in vivo*. Advances in the optimization of IVT mRNA and the many conveniences coupled to its use, reinstituted mRNA as a possible method for protein replacement therapies. Ever since, a few studies have attempted *in vivo* mRNA administration targeting a variety of tissues (summarized in **Error! Reference source not found.**).

Table 1 | *In vivo* mRNA-based protein-replacement studies

Study	Application	mRNA	Frequency	Innate immune evasion		
				Delivery method	Modification	Signaling inhibitors
2003 Anderson et al. [133]	Reporter assay Hypoxic stress	Luc Hsp70	Single	Lipid-based	Non-modified mRNA	None
2008 Okumura et al. [134]	Melanoma	BAX	5 daily injection	Lipid-based	Non-modified mRNA	None
2011 Kormann et al. [90]	Congenital lung disease	SPB	Twice weekly aerosol	Aerosolisation of naked mRNA	m5C and s2U	None
2012 Kariko et al. [135]	Anaemia	EPO	Once weekly injection	Lipid/ polymer-based (TransIT®)	Pseudouridine	None
2013 Mays et al. [136]	Asthma	FOXP3	Single and repeated spraying (5 times)	Intratracheal high-pressure spraying of naked mRNA	m5C and s2U	None
2013 Zangi et al. [137]	Myocardial infarction	VEGFA	Single injection	Lipid-based (RNAiMAX®)	m5C and pseudouridine	B18R
2015 Baba et al. [138]	Olfactory nerve disfunctions	BDNF	Once daily	Polymer-based	m5C, 2sU and pseudouridine	None

Strikingly, although most studies prove their awareness of the immune-stimulatory activity of IVT mRNA by using modified mRNA, only Zangi *et al*. make use of an additional immune-inhibiting compound, B18R, however without stressing the function of this molecule [137]. Whether or not supplementation with immune-inhibiting molecules could enhance the level and duration of mRNA expression and thereby advance protein-replacement therapies, warrants further investigation. Furthermore, it is important to note that not all the

aforementioned molecules can evidently be used in an *in vivo* setting. Obviously, prior to clinical application, the toxicological profile of the selected therapeutic components should be determined diligently. As most signaling pathways are critical elements of cell physiology, supplementation with immune decreasing molecules should be further advanced with due caution.

Apart from protein-replacement applications, IVT mRNA has also been extensively used in the field of regenerative medicine for the reprogramming of cell fates. In 2007, Yamanaka and colleagues discovered that the expression of only 4 transcription factors could reverse the fate of human fibroblasts towards pluripotency [139]. From then on, researchers tried optimizing the transfection protocol in order to render a safe and stable generation of induced pluripotent stemcells (iPSCs). Yakubov *et al*. were the first to propose a mRNA-based approach as a solution to minimize genome integration as well as to increase reprogramming efficiency. In 2010, they demonstrated that lipid-based mRNA encoding four reprogramming factors could be used to induce expression of pluripotency markers in human fibroblasts [140]. Unfortunately, this study was limited by the absence of pluripotency verification tests, leading to the question whether these iPSCs were able to functionally differentiate into each of the three germ layers. In the same year, Warren *et al*. described mRNA-based reprogramming methodology that rendered iPSCs that met all the molecular and functional pluripotency requirements. In order to enhance the sustainability of the mRNA-mediated protein expression, the authors searched for approaches to reduce the immunogenic profile of IVT mRNA. To this end, modified IVT mRNA was used, which contained pseudouridine and m5C and was differed to a phosphatase treatment. In addition, the cell culture medium was supplemented with the soluble IFN inhibitor B18R to further mitigate innate immune responses [55]. This was in line with a previous protocol published by Angel *et al.*, which demonstrated that a combined knockdown of immune-related proteins with a siRNA cocktail rescues human fibroblasts from the innate immune response triggered by frequent non-modified mRNA transfection, and enables sustained, high-level expression of the encoded proteins. They also suggest that the use of small-molecule immunosuppressants either alone or in combination with siRNA might be a suitable strategy to increase the frequency of mRNA transfections, without compromising cell viability [49]. Since the onset of this initial approach, numerous refinements have been published, each claiming to reach a higher reprogramming efficiency (summarized in **Error! Reference source not found.**). Despite these achievements, cellular reprogramming still faces a lot of technical challenges and requires intensive optimization to become routinely applicable. Recently, Drews *et al*. attributed the lack of reproducibility to severe toxicity and cell death, still caused by activation of the innate immune response even by modified mRNA. In their assays supplementation with a variety of immunosuppressing compounds, including B18R, Pepinh-TRIF and Pepinh-MYD, did not down-regulate the immune response-related genes [48]. Similarly, Awe *et al*. reported that the reprogramming methodology of Warren and colleagues did not completely reduce the mRNA-induced innate immune responses in their experiments. They noticed a significant

degradation of their OCT4 encoding mRNA, which could not be prevented by B18R supplementation. Nevertheless, they suggest a different kind of small molecule-based inhibition of the innate immune response, namely the administration of BAY11. Being an inhibitor of the IKK complex, BAY11 diminishes the negative IFN-induced responses, such as decay of the encoding mRNA, thereby stabilizing mRNA expression [99].

Table 2 | mRNA-based reprogramming studies

Study	Frequency	mRNA	Innate immune evasion		
			Delivery method	Modification	Signaling inhibitors
2010 Yakubov et al. [140]	5 daily transfections	Oct4, Lin28, Sox2 and Nanog	Lipid-based (Lipofectamine)	Non-modified mRNA	None
2010 Angel et al. [49]	3 daily transfections	Oct4, Sox2, Klf4 and Utf1	Lipid-based (RNAiMAX)	Non-modified mRNA	SiRNA against IFNB1, Eif2ak2 (PKR) and STAT2
2010 Warren et al. [55]	17 daily transfections	Oct4, Sox2 Klf4, cMyc and Lin28	Lipid-based (RNAiMAX)	m5C and pseudouridine	B18R
2010 Plews et al. [141]	Single transfection	Oct4, Sox2 Klf4, cMyc LT	Electroporation	Non-modified mRNA	None
2012 Tavernier et al. [142]	3 consecutive transfections (day 1,3 and 6)	Oct4, Lin28 Sox2 and Nanog	Lipid-based (RNAiMAX)	Non-modified mRNA	None
2012 Drews et al. [48]	Single transfection	OCT4, Sox2, Klf4 and cMyc	Lipid-based (RNAiMAX)	Non-modified and m5C and pseudouridine modified mRNA	B18R Chloroquine TSA Pepinh-TRIF Pepinh-MYD
2012 Warren et al. [143]	9 daily transfections	M_3O, Sox2 Klf4, cMyc Lin28 and Nanog	Lipid-based (RNAiMAX)	m5C and pseudouridine	B18R
2012 Lee et al. [144]	Max. 17 daily transfections	Oct4, Sox2, Klf4 and cMyc	Non-specified	Modified mRNA (non-specified)	B18R shRNA against TLR3, TRIF and MyD88
2013 Awe et al. [99]	5 daily transfections	Klf4, cMyc, Oct4, Sox2 and Lin28	Lipid-based (RNAiMAX)	m5C and pseudouridine	B18R BX795 BAY11
2013 Mandal et al. [110]	14-16 daily transfections	Oct4, Sox2 Klf4, cMyc, Lin28 and NDG	Lipid-based (RNAiMAX or Stemfect RNA)	m5C and pseudouridine	B18R

2013 Yoshioka et al. [145]	Single transfection	1 single VEE RNA replicon,encoding Oct4, Sox2 Klf4, cMyc or GLIS1	Lipid-based (Lipofectamine)	Non-modified mRNA	B18R
2014 Krieg et al. [146]	5 daily transfections	Oct4, Sox2 Klf4 and cMyc	Lipid/polymer-based (TransIT®)	Mouse specific synthesized mRNA	None

Of note, for reprogramming strategies is not desirable to completely block the innate immune system, as a recent study of Lee *et al.* demonstrated a positive effect of TLR3 stimulation on the reprogramming efficiency. The authors discovered a striking difference in the gene expression profiles induced by viral delivery of reprogramming factors compared to other reprogramming methods, suggesting that viral vectors actively contribute to the reprogramming process. Functional studies indicated that the TLR3 pathway is required for efficient induction of pluripotency genes. Stimulation of TLR3 seems to affect the expression and/or distribution of epigenetic modifiers promoting an open chromatin configuration and thus nuclear reprogramming. Although these findings recommend stimulation of the innate immune system for efficient mRNA-based iPSC generation, the authors also note that the level of TLR3 should be balanced, as further stimulation can cause cell death [144].

Considering all of these data, it is clear that the innate immune response still represents the biggest hurdle for advancing non-immunotherapy applications. Especially when multiple mRNA transfections are required, IVT mRNA induces severe cytotoxicity, making repeated transfections over time almost impossible. The studies presented thus far provide the basis for further investigations into other immunosuppressing strategies. Use of other chemical compounds, as suggested in section 4.3, either alone or in combination, may allow frequent mRNA transfections and robust expression of the encoded protein.

6 Concluding remarks

IVT mRNA transfection is a versatile and promising tool for the delivery of genetic information. Unprecedented advances in controlling the stability of IVT mRNA have reestablished mRNA interest for a wide range of potential applications. However, the fact that IVT mRNA, despite its strong resemblance to naturally occurring mRNA, can be recognized by the innate immune system, presumably plays an important role in its applicability. For vaccination approaches, the inflammatory cytokine production resulting from mRNA-induced immune-stimulation might add to the effectiveness of the evoked immune response. For non-immunotherapy approaches, however, the story becomes quite different. In this review, we discussed a number of important considerations that should be taken into account when using IVT mRNA for non-immunogenic applications, such as protein-replacement therapy or cellular reprogramming.

Firstly, whether or not the induced innate immune response will affect the therapeutic outcome of the mRNA delivery will likely depend on the required mRNA application frequency, which in its turn is determined by the intended application. So far, mRNA-based reprogramming protocols require about 12 daily transfections, whereas transfection frequencies for long-term treatment of congenital diseases still remain to be elucidated.
Secondly, as soon as mRNA is delivered using a chemical or physical delivery method, the vehicle or technique will also play an undeniable role in the induction of innate immune responses. Besides influencing the mRNA uptake mechanism and as such favoring or avoiding contact with specific mRNA sensors, increasing evidence indicates that most RNA-carriers possess an intrinsic immune-stimulating activity, inducing cell signaling cascades independent of mRNA complexation.
Thirdly, over the years, considerable efforts have been made to unriddle mRNA recognition pathways and limit the immune-stimulatory activity of IVT mRNA. Besides the well-known modifications that can be made to the mRNA molecule itself, a number of potential immune-inhibitors have been identified and are currently under investigation. This review has focused on the different players involved in innate immunity signaling, all of which are potential targets to shut down to enhance the level and duration of mRNA expression. In this regard, it is worth mentioning a couple of side notes. For one thing, the inhibition of only one key molecule of a signaling pathway might be nullified as its function can be superseded by a connected pathway. Therefore, simultaneous inhibition on different levels of the mRNA recognition should be considered, as exemplified by several RNA based viruses (see section 4.3). In addition, evidence is emerging that the innate immune response might not be all bad for mRNA-based reprogramming purposes. Such observations prompt further investigation and will likely require fine-tuning the balance between immune suppression and immune stimulation. Furthermore, clearly not all combinations of immune-inhibitory strategies that are feasible in an *in vitro* cell culture setting (e.g. reprogramming of isolated stem cells) can be translated into the *in vivo* situation (e.g. *in situ* protein replacement). As most signaling pathways are involved in many other regulatory aspects of cells as well, care should be taken when one interferes with these critical elements, in order to avoid side effects. What is more, *in vivo* application of inhibitory molecules faces the same challenge as mRNA therapy per se: targeted delivery. So far, research on how to efficiently deliver molecules to the target cell type and avoid systemic exposure is still pending.

Moreover, it is highly likely that not all elements in the mRNA recognition pathways have been identified thus far. Since research into the cell type dependent reaction to intruding mRNA molecules is still in its infancy, there is bound to be limited insights available. Finally, we wish to stress that although these innate immune responses might appear to limit the use of mRNA for non-immunotherapy applications, as evidenced by the fact that clinical IVT mRNA-therapy is still very much in its infancy, these responses do not solely occur in response to mRNA. Long before the discussion of mRNA-induced immune triggering arose, we knew about TLR9 ligation of CpG-rich pDNA, also resulting in the secretion of type I IFN and IL-12 [147-149].

Although the use of mRNA has been extensively investigated over the past few years, non-immunotherapy-related *in vivo* applications are merely at the beginning of development. In this regard the use of small molecule immune-inhibitors might bring non-immunogenic mRNA strategies to a higher level. For protein replacement therapies in specific, substantial improvements will be required in the delivery of mRNA to efficiently target the desired cell type and ensure a duration of protein production that benefits patient compliance. Even though we still have a long way to go before mRNA can be used as an off-the-shelf drug further insight into the major hurdles compromising mRNA-based protein expression, as presented in this review, might provide new inspiration for the therapeutic development of mRNA.

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