ELSEVIER

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

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

Joke Devoldere, Heleen Dewitte, Stefaan C. De Smedt and Katrien Remaut

Laboratory for General Biochemistry and Physical Pharmacy, Department of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, B-9000 Ghent, Belgium

In the field of nonviral 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 that the introduction of IVT mRNA into mammalian cells elicits an innate immune response that has favored mRNA use toward immunotherapeutic vaccination strategies. However, for non-immunotherapy-related applications this intrinsic immune-stimulatory activity directly interferes with the aimed therapeutic outcome, because 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.

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 ten 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 owing 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 nondividing 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, because there is no need to select and incorporate a specific promoter into the transfection construct [4]. Furthermore, because IVT mRNA

Corresponding author:. Remaut, K. (Katrien.Remaut@UGent.be)

Joke Devoldere obtained her master's degree in pharmaceutical sciences at Ghent University (Belgium) in 2013. After graduating, she started her PhD 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 nonviral mRNA delivery: overcoming immunogenicity and the need for multiple administrations.

Heleen Dewitte 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 Ghent University and the Vrije Universiteit Brussels under the joint supervision of Prof Stefaan De Smedt and Prof Karine Breckpot. Her project lies at the interface between material science and immunology, aiming to develop novel micro- and nano-materials for the delivery of antigen-coding mRNA to induce antitumor immunity. With her research, she has won several awards, including 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 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 2010–2014. He is a member of the Board of Directors of Ghent University. Since 2004, Stefaan has served as the European Associate Editor of the Journal of Controlled Release; and in 2015 he also 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, and its technology is currently under further development by Biocartis (Lausanne and Mechelen) and Mycartis (Gent).

Katrien Remaut graduated as a pharmacist at Ghent University (Belgium) in 2001. She then started research work in the Laboratory of General Biochemistry and Physical Pharmacy under the 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 Laboratory under the guidance of Professor Ian Mattaj at the European Molecular Biology Laboratory for six months. She received several scientific prizes among which features the Prize of the Royal Academy of Medicine for Scientific Research in Pharmacy 2008–2011. In 2014, she was appointed tenure track professor at the Laboratory of General Biochemistry and Physical Pharmacy, Faculty of Pharmaceutical Sciences.

1359-6446/
http://dx.doi.org/10.1016/j.drudis.2015.07.009

is synthesized in a cell-free system, the production process, manufacturing material and 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 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 the messenger and its own adjuvant. For immunotherapy, this intrinsic immune-stimulatory activity of mRNA is not a limiting factor, because it can increase the potency of the vaccine, 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 nonviral mRNA delivery for non-immunogenic purposes, such as protein-replacement therapies and regenerative medicine applications.

IVT mRNA

Interestingly, the production of functional mRNA by *in vitro* transcription has already been reported in 1984 by Krieg and Melton [15]. They synthesized mRNA using a phage RNA polymerase and a cloned cDNA template. Following this publication, a high number of technical refinements were 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 (Fig. 1). 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 (eIF)4E is essential. Binding with the decapping enzymes (DCP1, DCP2, DCPS) by contrast 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 analog. 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 part in the stability of mRNA by limiting decapping as well as 3′–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.

The immune-stimulating activity of mRNA

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 proinflammatory 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 2 summarizes the main pathways involved in mRNA recognition. Two families of PRRs are thought to be responsible for

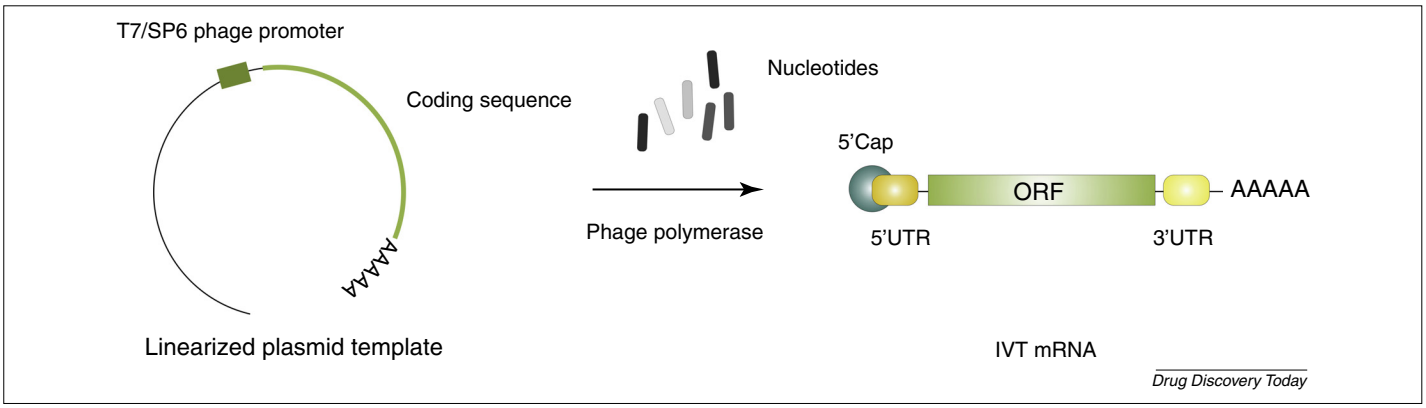

FIGURE 1

In vitro transcription of mRNA. Capping of the mRNA can be done during the *in vitro* transcription reaction by addition of synthetic cap analogs 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.

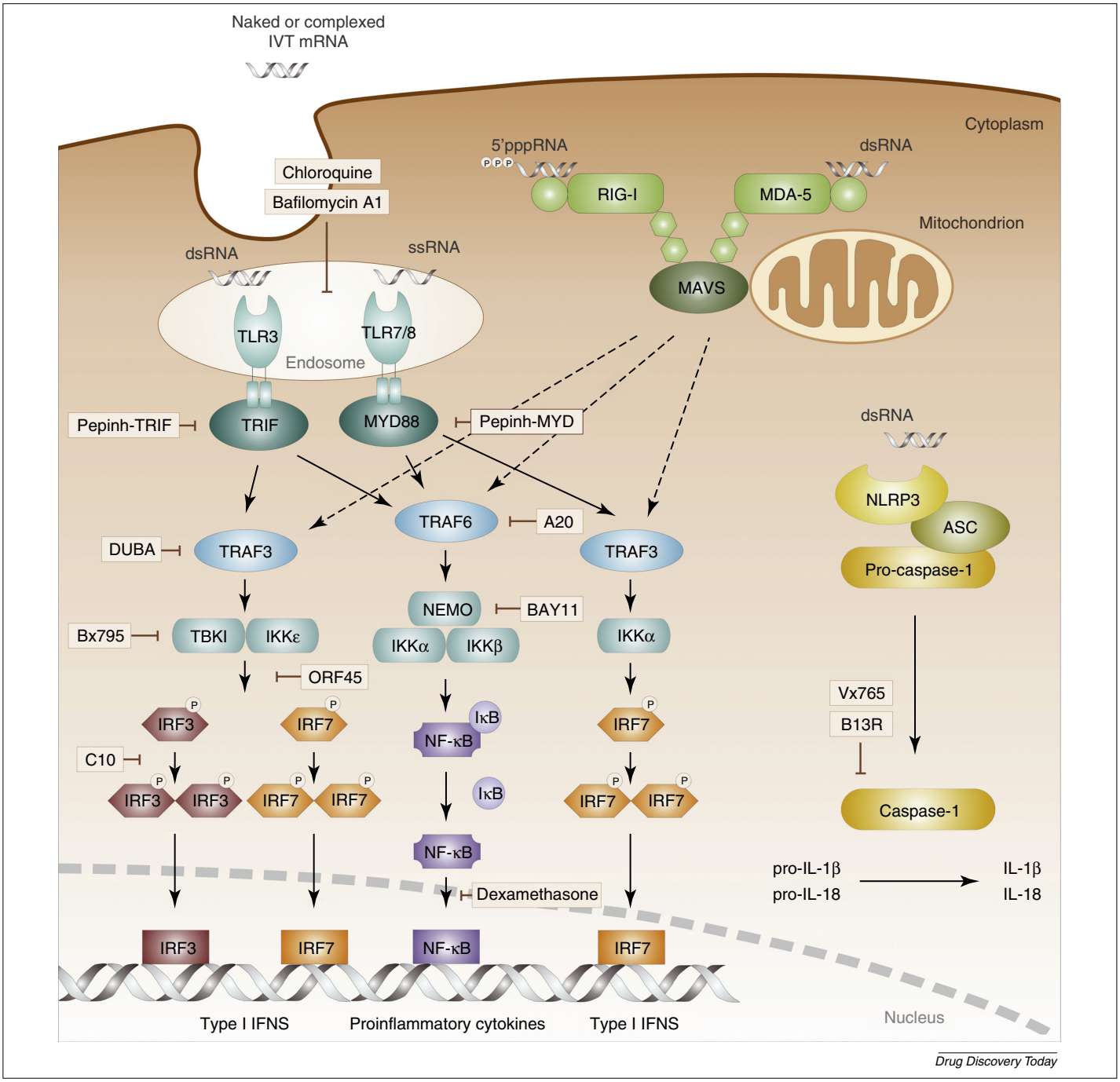

FIGURE 2

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 proinflammatory cytokines. Production of type I IFNs and can be inhibited at multiple levels: (i) minimizing 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. Abbreviations: 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.

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 intraendosomal 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 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 TLR8 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 factor (IRF)3 and IRF7 [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 specifically to detect ssRNA bearing a 5′-triphosphate (5′ppp) group [33,34]. Recent studies, however, have challenged this hypothesis and demonstrated that activation of RIG-I requires base-pairing of the nucleoside carrying the 5′ppp. Evidence was therefore 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 5′-diphosphate (5′pp) dsRNA also serves as a RIG-I ligand, thereby concluding that a minimal feature for RIG-I activation is a base-paired RNA with a free 5′pp [37]. Because 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 that can trigger RIG-I signaling. The second RLR, MDA5, 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 that, 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 Fig. 2). LGP2 is much less explored and conflicting data have been published on its role in innate immune signaling. Although LGP2 was initially assumed to regulate RLR-mediated signaling negatively [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 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 that 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 proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-6 and IL-12 [5,32].

As depicted in Fig. 3, 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: 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 that confer strong antiviral 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 type I IFNs as well as the proinflammatory 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 neighboring 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), is also involved in the cytoplasmic recognition of IVT mRNA [50]. They demonstrated an upregulation of caspase-1 after nonviral 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

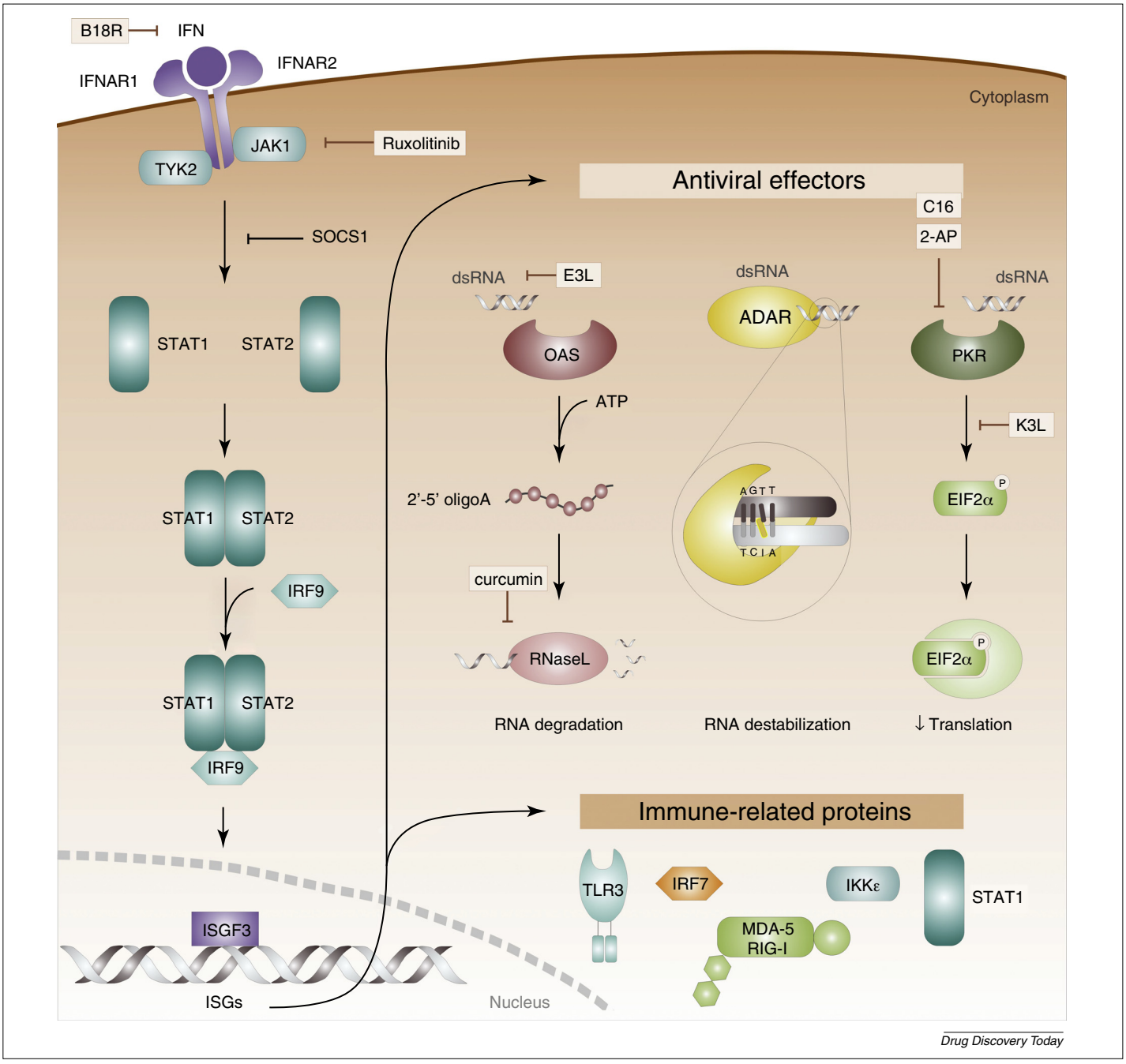

FIGURE 3

IFN-mediated signaling. Following their production (Fig. 2) type I IFNs bind to autocrine or paracrine IFN receptor complexes comprising 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 antiviral effectors OAS, PKR and ADAR. Overall, these create an antiviral 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 by 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 that minimize the antiviral action of IFN-induced proteins. Abbreviations: 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.

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 poly-ubiquitination or deubiquitination of the involved proteins and can therefore be influenced by deubiquitinating enzymes [32].

Unwanted immune responses induced by mRNA recognition

As previously discussed, IVT mRNA-induced immune activation is considered beneficial for vaccination strategies because it can attribute to the desired cellular and humoral immune response. The strong cytokine milieu that results from antigen-encoding IVT mRNA transfection is of particular interest, because 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 antiviral 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 (Fig. 3). To date, three anti-RNA pathways that shoot the messenger have been identified. These comprise the PKR, the OAS and the ADAR systems.

PKR (also known as Eif2ak2) is a kinase that phosphorylates the α-subunit of eIF2α. Activation of PKR impairs eIF2 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 for 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 catalytic activity of the latent enzyme RNase L, 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 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 can 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 PKR and IRF3, by a mechanism still to be resolved [66].

OASs 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 three enzyme types 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. It is probable that 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, upregulation of caspase-1 by NLR-mediated signaling is also detrimental to cells and causes programmed cell death [50]. This is probably one of the reasons why non-immunotherapeutic mRNA therapies are still in their infancy (as will be more thoroughly discussed below).

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 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; and (iii) blocking key proteins involved in the intracellular recognition of IVT mRNA and its subsequent signaling cascades.

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 intra-cellular stability. However, the delivery route (endosomal versus direct cytoplasmic entry) will also 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]. Viral and nonviral 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 that 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 nonviral mRNA carrier, protamine. Although this naturally occurring protein is demonstrated to protect the mRNA from degradation, mRNA–protamine complexes were shown to induce the innate immune response strongly [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 poly-plexes, respectively [71]. Rejman *et al*. demonstrated that 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, DOTAP and PEI RNA formulations were also shown to be detected by TLR7 and TLR8 [26,30]. Furthermore, Lonez *et al*. concluded that multiple cationic lipids, such as stearylamine-liposomes and Lipofectamine®, activate intracellular immune pathways, independent of mRNA complexation, resulting in the induction of several proapoptotic and proinflammatory signaling molecules [76]. The activated immune profile will additionally depend on the particle size, because it has been shown that the immune system distinguishes nanometric and micrometric structures to adapt the response to viral or bacterial and 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 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 microinjection, electroporation or sonoporation shuttle the mRNA directly in the cytosol and thus avoid detection by endosomal RNA receptors [78]. Whether mRNA delivery using 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 electro-poration result 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.

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. Because 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, because post-transcriptional 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 analog 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 post-transcriptional capping using recombinant capping enzymes. However, even with these methods it is impossible to accomplish a capping efficiency of 100% [83]. To reduce the immunogenicity of the remaining uncapped mRNA further, a phosphatase treatment can remove all resting triphosphates at the 5′ end of the mRNA transcript [84]. Besides capping, 2′-*O*-methylation at the penultimate nucleotide of the 5′ end has also 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), *N*6-methyladenosine (m6A), 5-methyluridine (m5U), pseudouridine or 2-thiouridine (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].

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 has 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 because of the transient nature of mRNA expression. The strong and detrimental immune responses against foreign mRNA originally serve 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 downregulate 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 nonviral 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 to bypass mRNA-triggered innate immunity. Given the redundancy in possible interfering molecules, other examples to those listed here might also form a possible evading strategy. Because 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 IFN production (Fig. 2) and inhibition of the auto- and para-crine effects of type I IFNs (Fig. 3).

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) intervening 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 with bafilomycin A1, the inhibitory activity 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 TBK1/IKKε and the IKK complex, respectively, 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 that contain specific domains of the adaptor molecules TRIF and MyD88. Administration of Pepinh-TRIF and Pepinh-MYD therefore reduces interaction between these adaptor molecules and their respective TLRs [96,97]. Another technique to interrupt mRNA-induced signaling is through the administration of kinase inhibitors. Because 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 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 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, Bhattacharyya *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 [149]. The observation that C10 blocks IRF3 transactivation is consistent with previous studies that demonstrate C10-mediated inhibition of the TLR3-regulated IRF3/IFN-β/STAT signal pathway [105,106]. 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 herpes virus blocks activation of type I IFN induction. Mechanistically, ORF45 acts as a decoy substrate for TBK1/IKKε and thus competitively inhibits IRF7 phosphorylation [107,108].

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 neighboring cells. The only IFN-binding protein that has been extensively published regarding its use is the vaccinia virus (VV)-encoded B18R

protein. B18R is a decoy receptor, specific for type I IFNs of various species, and has been shown to increase cell viability during mRNA-based reprogramming protocols [55,109].

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 second-wave IFN production. A commonly used JAK inhibitor is the small-molecule ruxolitinib [110]. 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 FDA for the treatment of myelofibrosis, which underscores its potential use for clinical applications [111,112].

A third strategy to inhibit IFN response is to counter the action of the IFN-induced effectors. Accordingly, Gupta and Rath recently discovered a specific, potent inhibitor of the human RNase L. Curcumin, a naturally occurring antioxidant, was shown to inhibit RNase L noncompetitively, presumably by inducing a switch in the conformation of the enzyme, leading to complete loss of its activity [113]. Likewise, PKR function can also be interrupted. For instance, Carroll *et al*. demonstrated inhibition of eIF2α activation by the VV 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 [114,115]. 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 [116,117]. More recently, Jammi *et al*. identified an even stronger PKR inhibitor, known as C16. As for 2-AP, this small molecule inhibits RNA-induced PKR autophosphorylation and rescues the PKR-induced translation blockade [118,119]. 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. Because PKR and 2-5A synthase require activation by dsRNA, sequestration of dsRNA by the E3L protein will hamper induction of both effectors [114]. 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 dsRNA molecules [120].

Clearly, 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 can 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 [114]. In the same way, 2-AP was shown to impair nuclear translocation of phosphorylated IRF3, in addition to its inhibitory effect on PKR [117]. Another molecule that 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 vascular endothelial growth factor receptor (VEGF-R) and platelet-derived growth factor receptor (PDGF-R), Jha *et al*. reported *in vivo* inhibition of PKR and RNase L by sunitinib, as a result of a kinase homology between both effectors [121,122].

As discussed above, activation of PRRs can also result in the production of proinflammatory 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 [123]. Because 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 [124] (Fig. 2). As for IFNs, several viruses also secrete proteins that serve as decoy receptors to sequester extracellular cytokines and impede interaction with cellular cytokine receptors [125]. 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) [86,126,127] or short hairpin RNAs (shRNAs) [86,128,129]. 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 shows siRNA and shRNA induce type I IFN signaling through TLR3 and activate sequence-independent inhibition of gene expression [130]. Therefore their use to enhance nonviral mRNA transfection seems contradictory, because they trigger innate immunity. In this respect, the use of microRNAs could be considered as well. Because 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]. Nevertheless, a better understanding of the precise regulatory roles of miRNAs in innate immune signaling is needed 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 rapidly increasing and it is beyond the scope of this review to sum up all commercially available or virus-related inhibitors. Therefore, we have attempted to exemplify every possible strategy with at least one inhibitory molecule.

Current state of non-immunotherapy-related mRNA applications

So far, cancer immunotherapy is the only field in which mRNA-based therapeutics have reached 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 at the 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 temporarily reversed their diabetes insipidus [131]. 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 Table 1).

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, but without stressing the function of this molecule [132]. 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, before clinical application, the toxicological profile of the selected therapeutic components should be determined diligently. Because most signaling pathways are crucial elements of cell physiology, supplementation with immune-decreasing molecules should be further advanced with caution.

Apart from protein-replacement applications, IVT mRNA has also been extensively used in the field of regenerative medicine for the reprogramming of cell fate. In 2007, Yamanaka and colleagues discovered that the expression of only four transcription factors could reverse the fate of human fibroblasts toward pluripotency [133]. From then on, researchers tried optimizing the transfection protocol to render a safe and stable generation of induced pluripotent stemcells (iPSCs). Yakubov *et al*. were the first to propose an 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 [134]. Unfortunately, this study was limited by the absence of pluripotency verification tests, leading to the question regarding whether these iPSCs were able to differentiate functionally 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. 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 mitigate innate immune responses further [55]. This was in line with a previous protocol published by Angel and Yanik, which demonstrated that a combined knockdown of immune-related proteins with an siRNA cocktail rescues human fibroblasts from the innate immune response triggered by frequent nonmodified 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,

TABLE 1

***In vivo* mRNA-based protein-replacement studies**

Refs	Application	mRNA	Frequency	Innate immune evasion		
				Delivery method	Modification	Signaling inhibitors
[139]	Reporter assay Hypoxic stress	Luc Hsp70	Single	Lipid-based	Nonmodified mRNA	None
[140]	Melanoma	BAX	Five daily injections	Lipid-based	Nonmodified mRNA	None
[90]	Congenital lung disease	SPB	Twice weekly aerosol	Aerosolization of naked mRNA	m5C and s2U	None
[141]	Anemia	EPO	Once weekly injection	Lipid/polymer-based (TransIT®)	Pseudouridine	None
[142]	Asthma	FOXP3	Single and repeated spraying (five times)	Intratracheal high-pressure spraying of naked mRNA	m5C and s2U	None
[132]	Myocardial infarction	VEGFA	Single injection	Lipid-based (RNAiMAX®)	m5C and pseudouridine	B18R
[143]	Olfactory nerve dysfunctions	BDNF	Once daily	Polymer-based	m5C, 2sU and pseudouridine	None

TABLE 2

mRNA-based reprogramming studies

Refs	Frequency	mRNA	Innate immune evasion		
			Delivery method	Modification	Signaling inhibitors
[134]	Five daily transfections	Oct4, Lin28, Sox2 and Nanog	Lipid-based (Lipofectamine®)	Nonmodified mRNA	None
[49]	Three daily transfections	Oct4, Sox2, Klf4 and Utf1	Lipid-based (RNAiMAX™)	Nonmodified mRNA	siRNA against IFNB1, Eif2ak2 (PKR) and STAT2
[55]	Seventeen daily transfections	Oct4, Sox2 Klf4, cMyc and Lin28	Lipid-based (RNAiMAX™)	m5C and pseudouridine	B18R
[144]	Single transfection	Oct4, Sox2 Klf4, cMyc LT	Electroporation	Nonmodified mRNA	None
[145]	Three consecutive transfections (day 1,3 and 6)	Oct4, Lin28 Sox2 and Nanog	Lipid-based (RNAiMAX™)	Nonmodified mRNA	None
[48]	Single transfection	OCT4, Sox2, Klf4 and cMyc	Lipid-based (RNAiMAX™)	Nonmodified and m5C- and pseudouridine-modified mRNA	B18R Chloroquine TSA Pepinh-TRIF Pepinh-MYD
[146]	Nine daily transfections	M_3O, Sox2 Klf4, cMyc Lin28 and Nanog	Lipid-based (RNAiMAX™)	m5C and pseudouridine	B18R
[135]	Max. 17 daily transfections	Oct4, Sox2, Klf4 and cMyc	Nonspecified	Modified mRNA (nonspecified)	B18R shRNA against TLR3, TRIF and MyD88
[99]	Five daily transfections	Klf4, cMyc, Oct4, Sox2 and Lin28	Lipid-based (RNAiMAX™)	m5C and pseudouridine	B18R BX795 BAY11
[109]	Fourteen to sixteen daily transfections	Oct4, Sox2 Klf4, cMyc, Lin28 and NDG	Lipid-based (RNAiMAX™ or Stemfect™ RNA)	m5C and pseudouridine	B18R
[147]	Single transfection	One single VEE RNA replicon, encoding Oct4, Sox2, Klf4, cMyc or GLIS1	Lipid-based (Lipofectamine®)	Nonmodified mRNA	B18R
[148]	Five daily transfections	Oct4, Sox2 Klf4 and cMyc	Lipid/polymer-based (TransIT®)	Mouse-specific synthesized mRNA	None

each claiming to reach a higher reprogramming efficiency (summarized in Table 2). 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 downregulate 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].

Of note, for reprogramming strategies it is not desirable to block the innate immune system completely – a recent study by 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 with 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, because further stimulation can cause cell death [135].

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 above, either alone or in combination, could allow frequent mRNA transfections and robust expression of the encoded protein.

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 re-established 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 part 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 is different. In this review, we have 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 probably 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 part 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 understand 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 because 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. In addition, evidence is emerging that suggests the innate immune response might not be all bad for mRNA-based reprogramming purposes. Such observations prompt further investigation and will probably 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). Because most signaling pathways are involved in many other regulatory aspects of cells as well, care should be taken when interfering with these crucial elements to avoid side effects. What is more, *in vivo* application of inhibitory molecules faces the same challenge as mRNA therapy: targeted delivery. So far, research on how to deliver molecules efficiently to the target cell type and avoid systemic exposure is still pending.

It is highly likely that not all elements in the mRNA recognition pathways have been identified thus far. Because research into the cell-type-dependent reaction to intruding mRNA molecules is still in its infancy, there are 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 [136–138].

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 specifically, substantial improvements will be required in the delivery of mRNA to target the desired cell type efficiently and ensure 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.

Acknowledgement

Joke Devoldere is a doctoral fellow of the Research Foundation-Flanders, Belgium (FWO-Vlaanderen).

References

1 Wolff, J.A. *et al.* (1990) Direct gene transfer into mouse muscle *in vivo*. *Science* 247, 1465 1468

2 Leonhardt, C. *et al.* (2014) Single-cell mRNA transfection studies: delivery, kinetics and statistics by numbers. *Nanomedicine* 10, 679 688

3 Pascolo, S. (2008) Vaccination with messenger RNA (mRNA). *Handb. Exp. Pharmacol.* 183, 221 235

4 Tavernier, G. *et al.* (2011) mRNA as gene therapeutic: how to control protein expression. *J. Control. Release* 150, 238 247

5 Sahin, U. *et al.* (2014) mRNA-based therapeutics developing a new class of drugs. *Nat. Rev. Drug Discov.* 13, 759 780
6 Conry, R.M. *et al.* (1995) Characterization of a messenger RNA polynucleotide vaccine vector. *Cancer Res.* 55, 1397 1400
7 Martinon, F. *et al.* (1993) Induction of virus-specific cytotoxic T lymphocytes *in vivo* by liposome-entrapped mRNA. *Eur. J. Immunol.* 23, 1719 1722
8 Tang, D.C. *et al.* (1992) Genetic immunization is a simple method for eliciting an immune response. *Nature* 356, 152 154
9 Hoerr, I. *et al.* (2000) In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies. *Eur. J. Immunol.* 30, 1 7
10 Kallen, K.J. and Thess, A. (2014) A development that may evolve into a revolution in medicine: mRNA as the basis for novel, nucleotide-based vaccines and drugs. *Ther. Adv. Vaccines* 2, 10 31
11 Van Lint, S. *et al.* (2015) The ReNAissanCe of mRNA-based cancer therapy. *Expert Rev. Vaccines* 14, 235 251
12 Pollard, C. *et al.* (2013) Challenges and advances towards the rational design of mRNA vaccines. *Trends Mol. Med.* 19, 705 713
13 Kreiter, S. *et al.* (2011) Tumor vaccination using messenger RNA: prospects of a future therapy. *Curr. Opin. Immunol.* 23, 399 406
14 Pascolo, S. (2015) The messenger's great message for vaccination. *Expert Rev. Vaccines* 14, 153 156
15 Krieg, P.A. and Melton, D.A. (1984) Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs. *Nucleic Acids Res.* 12, 7057 7070
16 Li, Y. and Kiledjian, M. (2010) Regulation of mRNA decapping. *Wiley Interdiscip. Rev. RNA* 1, 253 265
17 Coller, J. and Parker, R. (2004) Eukaryotic mRNA decapping. *Annu. Rev. Biochem.* 73, 861 890
18 Martin, S.A. *et al.* (1975) Purification of mRNA guanylyltransferase and mRNA (guanine-7-) methyltransferase from vaccinia virions. *J. Biol. Chem.* 250, 9322 9329
19 Wells, S.E. *et al.* (1998) Circularization of mRNA by eukaryotic translation initiation factors. *Mol. Cell* 2, 135 140
20 Gallie, D.R. (1998) A tale of two termini: a functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. *Gene* 216, 1 11
21 Akira, S. *et al.* (2006) Pathogen recognition and innate immunity. *Cell* 124, 783 801
22 Kawai, T. and Akira, S. (2006) Innate immune recognition of viral infection. *Nat. Immunol.* 7, 131 137
23 Akira, S. *et al.* (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* 2, 675 680
24 Kumar, H. *et al.* (2009) Toll-like receptors and innate immunity. *Biochem. Biophys. Res. Commun.* 388, 621 625
25 Yamamoto, A. *et al.* (2009) Current prospects for mRNA gene delivery. *Eur. J. Pharm. Biopharm.* 71, 484 489
26 Heil, F. *et al.* (2004) Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 303, 1526 1529
27 Diebold, S.S. *et al.* (2006) Nucleic acid agonists for Toll-like receptor 7 are defined by the presence of uridine ribonucleotides. *Eur. J. Immunol.* 36, 3256 3267
28 Alexopoulou, L. *et al.* (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413, 732 738
29 Kariko, K. *et al.* (2004) mRNA is an endogenous ligand for Toll-like receptor 3. *J. Biol. Chem.* 279, 12542 12550
30 Diebold, S.S. *et al.* (2004) Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303, 1529 1531
31 Akira, S. and Takeda, K. (2004) Toll-like receptor signalling. *Nat. Rev. Immunol.* 4, 499 511
32 Yoneyama, M. and Fujita, T. (2010) Recognition of viral nucleic acids in innate immunity. *Rev. Med. Virol.* 20, 4 22
33 Hornung, V. *et al.* (2006) 5′-Triphosphate RNA is the ligand for RIG-I. *Science* 314, 994 997
34 Pichlmair, A. *et al.* (2006) RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science* 314, 997 1001
35 Schlee, M. *et al.* (2009) Recognition of 5′ triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity* 31, 25 34
36 Schmidt, A. *et al.* (2009) 5′-Triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proc. Natl. Acad. Sci. U. S. A.* 106, 12067 12072
37 Goubau, D. *et al.* (2014) Antiviral immunity via RIG-I-mediated recognition of RNA bearing 5′-diphosphates. *Nature* 514, 372 375
38 Pichlmair, A. *et al.* (2009) Activation of MDA5 requires higher-order RNA structures generated during virus infection. *J. Virol.* 83, 10761 10769
39 Zust, R. *et al.* (2011) Ribose 2′-*O*-methylation provides a molecular signature for the distinction of self and non-self mRNA dependent on the RNA sensor Mda5. *Nat. Immunol.* 12, 137 143
40 Rothenfusser, S. *et al.* (2005) The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *J. Immunol.* 175, 5260 5268
41 Komuro, A. and Horvath, C.M. (2006) RNA- and virus-independent inhibition of antiviral signaling by RNA helicase LGP2. *J. Virol.* 80, 12332 12342
42 Satoh, T. *et al.* (2010) LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. *Proc. Natl. Acad. Sci. U. S. A.* 107, 1512 1517
43 Liniger, M. *et al.* (2012) Chicken cells sense influenza A virus infection through MDA5 and CARDIF signaling involving LGP2. *J. Virol.* 86, 705 717
44 Ikeda, F. *et al.* (2007) Involvement of the ubiquitin-like domain of TBK1/IKK-i kinases in regulation of IFN-inducible genes. *EMBO J.* 26, 3451 3462
45 Israel, A. (2010) The IKK complex, a central regulator of NF-kappaB activation. *Cold Spring Harb. Perspect. Biol.* 2, a000158
46 Sen, G.C. and Sarkar, S.N. (2007) The interferon-stimulated genes: targets of direct signaling by interferons, double-stranded RNA, and viruses. *Cur. Top. Microbiol. Immunol.* 316, 233 250
47 Sadler, A.J. and Williams, B.R. (2008) Interferon-inducible antiviral effectors. *Nat. Rev. Immunol.* 8, 559 568
48 Drews, K. *et al.* (2012) The cytotoxic and immunogenic hurdles associated with non-viral mRNA-mediated reprogramming of human fibroblasts. *Biomaterials* 33, 4059 4068
49 Angel, M. and Yanik, M. (2010) Innate immune suppression enables frequent transfection with RNA encoding reprogramming proteins. *PLoS ONE* 5, e11756
50 Andries, O. *et al.* (2013) Innate immune response and programmed cell death following carrier-mediated delivery of unmodified mRNA to respiratory cells. *J. Control. Release* 167, 157 166
51 Kanneganti, T.D. *et al.* (2006) Critical role for cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J. Biol. Chem.* 281, 36560 36568
52 Sabbah, A. *et al.* (2009) Activation of innate immune antiviral responses by Nod2. *Nat. Immunol.* 10, 1073 1080
53 Heufler, C. *et al.* (1996) Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. *Eur. J. Immunol.* 26, 659 668
54 Banchereau, J. and Steinman, R.M. (1998) Dendritic cells and the control of immunity. *Nature* 392, 245 252
55 Warren, L. *et al.* (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* 7, 618 630
56 Garcia, M.A. *et al.* (2007) The dsRNA protein kinase PKR: virus and cell control. *Biochimie* 89, 799 811
57 Kumar, A. *et al.* (1994) Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B. *Proc. Natl. Acad. Sci. U. S. A.* 91, 6288 6292
58 Balachandran, S. *et al.* (2000) Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. *Immunity* 13, 129 141
59 Silverman, R.H. (2007) Viral encounters with 2′,5′-oligoadenylate synthetase and RNase L during the interferon antiviral response. *J. Virol.* 81, 12720 12729
60 Borden, E.C. *et al.* (2007) Interferons at age 50: past, current and future impact on biomedicine. *Nat. Rev. Drug Discov.* 6, 975 990
61 George, C.X. *et al.* (2014) An RNA editor, adenosine deaminase acting on double-stranded RNA (ADAR1). *J. Interferon Cytokine Res.* 34, 437 446
62 Samuel, C.E. (2012) ADARs: viruses and innate immunity. *Curr. Top. Microbiol. Immunol.* 353, 163 195
63 Hartner, J.C. *et al.* (2009) ADAR1 is essential for the maintenance of hematopoiesis and suppression of interferon signaling. *Nat. Immunol.* 10, 109 115
64 Qiu, W. *et al.* (2013) ADAR1 is essential for intestinal homeostasis and stem cell maintenance. *Cell Death Dis.* 4, e599
65 Yang, S. *et al.* (2014) Adenosine deaminase acting on RNA 1 limits RIG-I RNA detection and suppresses IFN production responding to viral and endogenous RNAs. *J. Immunol.* 193, 3436 3445
66 Pfaller, C.K. *et al.* (2011) Protein kinase PKR and RNA adenosine deaminase ADAR1: new roles for old players as modulators of the interferon response. *Curr. Opin. Immunol.* 23, 573 582
67 Pichlmair, A. and Sousa, C.R.E. (2007) Innate recognition of viruses. *Immunity* 27, 370 383
68 Lorenz, C. *et al.* (2011) Protein expression from exogenous mRNA: uptake by receptor-mediated endocytosis and trafficking via the lysosomal pathway. *RNA Biol.* 8, 627 636

69 Diken, M. *et al.* (2011) Selective uptake of naked vaccine RNA by dendritic cells is driven by macropinocytosis and abrogated upon DC maturation. *Gene Ther.* 18, 702 708

70 Pathak, A. *et al.* (2009) Recent trends in non-viral vector-mediated gene delivery. *Biotechnology J.* 4, 1559 1572

71 Tros de Ilarduya, C. *et al.* (2010) Gene delivery by lipoplexes and polyplexes. *Eur. J. Pharm. Sci.* 40, 159 170

72 Dewitte, H. *et al.* (2014) Nanoparticle design to induce tumor immunity and challenge the suppressive tumor microenvironment. *Nano Today* 9, 743 758

73 Fotin-Mleczek, M. *et al.* (2011) Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity. *J. Immunother.* 34, 1 15

74 Scheel, B. *et al.* (2005) Toll-like receptor-dependent activation of several human blood cell types by protamine-condensed mRNA. *Eur. J. Immunol.* 35, 1557 1566

75 Rejman, J. *et al.* (2010) mRNA transfection of cervical carcinoma and mesenchymal stem cells mediated by cationic carriers. *J. Control. Release* 147, 385 391

76 Lonez, C. *et al.* (2012) Cationic lipids activate intracellular signaling pathways. *Adv. Drug Deliv. Rev.* 64, 1749 1758

77 Rettig, L. *et al.* (2010) Particle size and activation threshold: a new dimension of danger signaling. *Blood* 115, 4533 4541

78 Wang, W. *et al.* (2013) Non-viral gene delivery methods. *Curr. Pharm. Biotechnol.* 14, 46 60

79 Dewitte, H. *et al.* (2014) The potential of antigen and TriMix sonoporation using mRNA-loaded microbubbles for ultrasound-triggered cancer immunotherapy. *J. Control. Release* 194, 28 36

80 Bonehill, A. *et al.* (2008) Enhancing the T-cell stimulatory capacity of human dendritic cells by co-electroporation with CD40L, CD70 and constitutively active TLR4 encoding mRNA. *Mol. Ther.* 16, 1170 1180

81 Koski, G.K. *et al.* (2004) Cutting edge: innate immune system discriminates between RNA containing bacterial versus eukaryotic structural features that prime for high-level IL-12 secretion by dendritic cells. *J. Immunol.* 172, 3989 3993

82 Nallagatla, S.R. *et al.* (2007) 5′-Triphosphate-dependent activation of PKR by RNAs with short stem-loops. *Science* 318, 1455 1458

83 Grudzien-Nogalska, E. *et al.* (2007) Phosphorothioate cap analogs stabilize mRNA and increase translational efficiency in mammalian cells. *RNA* 13, 1745 1755

84 Pardi, N. *et al.* (2013) *In vitro* transcription of long RNA containing modified nucleosides. *Methods Mol. Biol.* 969, 29 42

85 Wang, Y.L. *et al.* (2010) Structural and functional insights into 5′-ppp RNA pattern recognition by the innate immune receptor RIG-I. *Nat. Struct. Mol. Biol.* 17, 719 781

86 Kariko, K. *et al.* (2005) Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* 23, 165 175

87 Nallagatla, S.R. and Bevilacqua, P.C. (2008) Nucleoside modifications modulate activation of the protein kinase PKR in an RNA structure-specific manner. *RNA* 14, 1201 1213

88 Kariko, K. *et al.* (2008) Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Mol. Ther.* 16, 1833 1840

89 Anderson, B.R. *et al.* (2010) Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation. *Nucleic Acids Res.* 38, 5884 5892

90 Kormann, M.S. *et al.* (2011) Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. *Nat. Biotechnol.* 29, 154 157

91 Kariko, K. *et al.* (2011) Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. *Nucleic Acids Res.* 39, e142

92 Hacker, H. *et al.* (1998) CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* 17, 6230 6240

93 He, X. *et al.* (2013) Recognition of pathogen-associated nucleic acids by endosomal nucleic acid-sensing toll-like receptors. *Acta Biochim. Biophys. Sin.* 45, 241 258

94 Kuznik, A. *et al.* (2011) Mechanism of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines. *J. Immunol.* 186, 4794 4804

95 Rajsbaum, R. and Garcia-Sastre, A. (2013) Viral evasion mechanisms of early antiviral responses involving regulation of ubiquitin pathways. *Trends Microbiol.* 21, 421 429

96 Loiarro, M. *et al.* (2005) Peptide-mediated interference of TIR domain dimerization in MyD88 inhibits interleukin-1-dependent activation of NF-{kappa}B. *J. Biol. Chem.* 280, 15809 15814

97 Toshchakov, V.U. *et al.* (2005) Differential involvement of BB loops of toll-IL-1 resistance (TIR) domain-containing adapter proteins in TLR4- versus TLR2-mediated signal transduction. *J. Immunol.* 175, 494 500

98 Clark, K. *et al.* (2009) Use of the pharmacological inhibitor BX795 to study the regulation and physiological roles of TBK1 and IkappaB kinase epsilon: a distinct upstream kinase mediates Ser-172 phosphorylation and activation. *J. Biol. Chem.* 284, 14136 14146

99 Awe, J.P. *et al.* (2013) BAY11 enhances OCT4 synthetic mRNA expression in adult human skin cells. *Stem Cell Res. Ther.* 4, 15

100 Rowland, T.L. *et al.* (2001) Differential effect of thalidomide and dexamethasone on the transcription factor NF-kappa B. *Int. Immunopharmacol.* 1, 49 61

101 Auphan, N. *et al.* (1995) Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science* 270, 286 290

102 Scheinman, R.I. *et al.* (1995) Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science* 270, 283 286

103 Chauhan, D. *et al.* (2002) Identification of genes regulated by dexamethasone in multiple myeloma cells using oligonucleotide arrays. *Oncogene* 21, 1346 1358

104 Bhattacharyya, S. *et al.* (2010) TAK1 targeting by glucocorticoids determines JNK and IkappaB regulation in Toll-like receptor-stimulated macrophages. *Blood* 115, 1921 1931

105 Harii, N. *et al.* (2005) Thyrocytes express a functional toll-like receptor 3: overexpression can be induced by viral infection and reversed by phenylmethimazole and is associated with Hashimoto's autoimmune thyroiditis. *Mol. Endocrinol.* 19, 1231 1250

106 Schwartz, A.L. *et al.* (2009) Phenylmethimazole decreases Toll-like receptor 3 and noncanonical Wnt5a expression in pancreatic cancer and melanoma together with tumor cell growth and migration. *Clin. Cancer Res.* 15, 4114 4122

107 Zhu, F.X. *et al.* (2002) A Kaposi's sarcoma-associated herpesviral protein inhibits virus-mediated induction of type I interferon by blocking IRF-7 phosphorylation and nuclear accumulation. *Proc. Natl. Acad. Sci. U. S. A.* 99, 5573 5578

108 Zhu, F.X. *et al.* (2010) Antagonism of host antiviral responses by Kaposi's sarcoma-associated herpesvirus tegument protein ORF45. *PLoS ONE* 5, e10573

109 Mandal, P.K. and Rossi, D.J. (2013) Reprogramming human fibroblasts to pluripotency using modified mRNA. *Nat. Protoc.* 8, 568 582

110 Kambara, H. *et al.* (2014) Negative regulation of the interferon response by an interferon-induced long non-coding RNA. *Nucleic Acids Res.* 42, 10668 10680

111 Quintas-Cardama, A. *et al.* (2010) Preclinical characterization of the selective JAK1/2 inhibitor INCB018424: therapeutic implications for the treatment of myeloproliferative neoplasms. *Blood* 115, 3109 3117

112 Quintas-Cardama, A. and Verstovsek, S. (2013) Molecular pathways: Jak/STAT pathway: mutations, inhibitors, and resistance. *Clin. Cancer Res.* 19, 1933 1940

113 Gupta, A. and Rath, P.C. (2014) Curcumin, a natural antioxidant, acts as a noncompetitive inhibitor of human RNase L in presence of its cofactor 2-5A *in vitro*. *Biomed. Res. Int.* 2014, 817024

114 Xiang, Y. *et al.* (2002) Blockade of interferon induction and action by the E3L double-stranded RNA binding proteins of vaccinia virus. *J. Virol.* 76, 5251 5259

115 Carroll, K. *et al.* (1993) Recombinant vaccinia virus K3L gene product prevents activation of double-stranded RNA-dependent, initiation factor 2 alpha-specific protein kinase. *J. Biol. Chem.* 268, 12837 12842

116 Hu, Y. and Conway, T.W. (1993) 2-Aminopurine inhibits the double-stranded RNA-dependent protein kinase both *in vitro* and *in vivo*. *J. Interferon Res.* 13, 323 328

117 Sugiyama, T. *et al.* (2012) Mechanism of inhibition of lipopolysaccharide-induced interferon-beta production by 2-aminopurine. *Mol. Immunol.* 52, 299 304

118 Jammi, N.V. *et al.* (2003) Small molecule inhibitors of the RNA-dependent protein kinase. *Biochem. Biophys. Res. Commun.* 308, 50 57

119 Ingrand, S. *et al.* (2007) The oxindole/imidazole derivative C16 reduces *in vivo* brain PKR activation. *FEBS Lett.* 581, 4473 4478

120 Nekhai, S. *et al.* (2000) A cell-permeable peptide inhibits activation of PKR and enhances cell proliferation. *Peptides* 21, 1449 1456

121 Jha, B.K. *et al.* (2011) Inhibition of RNase L and RNA-dependent protein kinase (PKR) by sunitinib impairs antiviral innate immunity. *J. Biol. Chem.* 286, 26319 26326

122 Jha, B.K. *et al.* (2013) Suppression of antiviral innate immunity by sunitinib enhances oncolytic virotherapy. *Mol. Ther.* 21, 1749 1757

123 Smith, G.L. *et al.* (2013) Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity. *J. Gen. Virol.* 94, 2367 2392

124 Kettle, S. *et al.* (1997) Vaccinia virus serpin B13R (SPI-2) inhibits interleukin-1beta-converting enzyme and protects virus-infected cells from TNF- and Fas-mediated apoptosis, but does not prevent IL-1beta-induced fever. *J. Gen. Virol.* 78, 677 685

125 Mantovani, A. *et al.* (2001) Decoy receptors: a strategy to regulate inflammatory cytokines and chemokines. *Trends Immunol.* 22, 328 336

126 Myskiw, C. *et al.* (2009) Vaccinia virus E3 suppresses expression of diverse cytokines through inhibition of the PKR, NF-kappaB, and IRF3 pathways. *J. Virol.* 83, 6757 6768

127 Yoneyama, M. *et al.* (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* 5, 730 737
128 Allen, I.C. *et al.* (2009) The NLRP3 inflammasome mediates *in vivo* innate immunity to influenza A virus through recognition of viral RNA. *Immunity* 30, 556 565
129 Schmolke, M. *et al.* (2014) RIG-I detects mRNA of intracellular *Salmonella enterica* serovar Typhimurium during bacterial infection. *MBio* 5, e01006 e01014
130 Kariko, K. *et al.* (2004) Small interfering RNAs mediate sequence-independent gene suppression and induce immune activation by signaling through Toll-like receptor 3. *J. Immunol.* 172, 6545 6549
131 Jirikowski, G.F. *et al.* (1992) Reversal of diabetes insipidus in Brattleboro rats: intrahypothalamic injection of vasopressin mRNA. *Science* 255, 996 998
132 Zangi, L. *et al.* (2013) Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. *Nat. Biotechnol.* 31, 898 907
133 Takahashi, K. *et al.* (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861 872
134 Yakubov, E. *et al.* (2010) Reprogramming of human fibroblasts to pluripotent stem cells using mRNA of four transcription factors. *Biochem. Biophys. Res. Commun.* 394, 189 193
135 Lee, J. *et al.* (2012) Activation of innate immunity is required for efficient nuclear reprogramming. *Cell* 151, 547 558
136 Krieg, A.M. *et al.* (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374, 546 549
137 Halpern, M.D. *et al.* (1996) Bacterial DNA induces murine interferon-gamma production by stimulation of interleukin-12 and tumor necrosis factor-alpha. *Cell Immunol.* 167, 72 78
138 Ballas, Z.K. *et al.* (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* 157, 1840 1845
139 Anderson, D.M. *et al.* (2003) Stability of mRNA/cationic lipid lipoplexes in human and rat cerebrospinal fluid: methods and evidence for nonviral mRNA gene delivery to the central nervous system. *Hum. Gene Ther.* 14, 191 202
140 Okumura, K. *et al.* (2008) Bax mRNA therapy using cationic liposomes for human malignant melanoma. *J. Gene Med.* 10, 910 917
141 Kariko, K. *et al.* (2012) Increased erythropoiesis in mice injected with submicrogram quantities of pseudouridine-containing mRNA encoding erythropoietin. *Mol. Ther.* 20, 948 953
142 Mays, L.E. *et al.* (2013) Modified Foxp3 mRNA protects against asthma through an IL-10-dependent mechanism. *J. Clin. Invest.* 123, 1216 1228
143 Baba, M. *et al.* (2015) Treatment of neurological disorders by introducing mRNA *in vivo* using polyplex nanomicelles. *J. Control. Release* 201, 41 48
144 Plews, J.R. *et al.* (2010) Activation of pluripotency genes in human fibroblast cells by a novel mRNA based approach. *PLoS ONE* 5, e14397
145 Tavernier, G. *et al.* (2012) Activation of pluripotency-associated genes in mouse embryonic fibroblasts by non-viral transfection with *in vitro*-derived mRNAs encoding Oct4, Sox2, Klf4 and cMyc. *Biomaterials* 33, 412 417
146 Warren, L. *et al.* (2012) Feeder-free derivation of human induced pluripotent stem cells with messenger RNA. *Sci. Rep.* 2, 657
147 Yoshioka, N. *et al.* (2013) Efficient generation of human iPSCs by a synthetic self-replicative RNA. *Cell Stem Cell* 13, 246 254
148 El-Sayed, A.K. *et al.* (2014) Pluripotent state induction in mouse embryonic fibroblast using mRNAs of reprogramming factors. *Int. J. Mol. Sci.* 15, 21840 21864
149 Courreges, M.C. *et al.* (2012) Phenylmethimazole blocks dsRNA-induced IRF3 nuclear translocation and homodimerization. *Molecules* 7, 12356 12377