Review Article

Molecular Basis for the Immunostimulatory Potency of Small Interfering RNAs

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Small interfering RNAs (siRNAs) represent a new class of antigen agents, which has emerged as a powerful tool for functional genomics and might serve as a potent therapeutic approach. However, several studies have showed that they could trigger several bystander effects, including immune activation and inhibition of unintended target genes. Although activation of innate immunity by siRNAs might be beneficial for therapy in some instances, uncontrolled activation can be toxic, and is therefore a major challenging problem. Interestingly, replacement of uridines in siRNA sequences with their 2′-modified counterparts abrogated siRNA bystander effects. Here we highlight these important findings that are expected to facilitate the rational design of siRNAs that avoid the induction of bystander effects.

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INTRODUCTION

A variety of well-documented approaches have been developed for inhibiting gene expression. These include the use of antisense RNAs, oligodeoxynucleotides (ODNs), ribozymes, and RNA interference (RNAi) [1–3]. Among these approaches, RNAi-mediated mRNA degradation is emerging as the most highly effective antigen strategies [2, 3]. Although the same phenomenon was described in transgenic plants in the late 1980s [4], only in 1998 Fire and colleagues have demonstrated that ds RNA introduced into Caenorhabditis elegans can silence the expression of homologous target gene by directing degradation of its mRNA [5]. During RNAi, the precursor double-stranded (ds) RNA is processed by the Dicer endonuclease into short 21–24 nucleotides ds siRNAs containing 2-base 3′-overhangs. Subsequently, the siRNA duplexes are then incorporated into a multiprotein complex, the RNA-induced silencing complex (RISC), which mediates the degradation of mRNAs with sequence homologous to the siRNA. Recent studies have showed that nucleotides 2–8 of the siRNA antisense strand form a seed sequence that directs target mRNA recognition [6]. Some of the protein components of RISC have been identified and assigned functions. Argonaute 2 (Ago2) is the RNA endonuclease that cleaves target mRNA [7].

Despite the demonstration of RNAi in plants and worms, researchers have been pessimistic about using RNAi in mammalian cells due to the induction of the interferon (INF) pathway by long ds RNAs (> 30 nucleotides), leading to nonspecific inhibition of protein synthesis and degradation of mRNAs [8]. However, Tuschl and colleagues found that siRNA duplexes mimicking the siRNAs generated by Dicer cleavage in lower organisms could trigger RNAi without inducing the IFN pathway, which is observed with long double-stranded RNA in most cell types [9]. This discovery has paved the way for the study of gene function in mammalian cells. In contrast to invertebrates, mammalian cells do not have transitive RNAi because of the absence of RNA-dependent RNA polymerase [2, 3]. Consequently, the number of siRNA molecules introduced into a cell limits gene silencing induced by synthetic siRNAs. To circumvent this potential problem, several groups have developed expression vectors that direct the synthesis of RNA duplexes or short hairpin RNAs (shRNAs) in mammalian cells, leading to sustained production of siRNAs [3]. One approach for silencing relies on the expression of shRNAs under the control of H1 or U6 pol III promoter, which is active in most mammalian cell types.

During the last 5 years, siRNA-mediated gene knockdown has become a standard method for studying gene function and drug target validation. However, despite the success that has been achieved, some serious concerns such as the activation of innate immunity, inhibition of unintended target mRNAs, and potential interference with regulatory functions...
of endogenous microRNAs need to be resolved prior therapeu-
tic applications in humans [2, 10–12].

**CELLULAR RESPONSE TO siRNAs**

The gradual maturation of RNAi technology from the labo-
atory to the clinic involves several major challenges, many
of which still need to be resolved. These include delivery to
target cells, intracellular stability, and specificity [2, 3]. It
should be noted that the introduction of synthetic siRNAs
into mammalian cells in culture is relatively simple by trans-
fection reagents [9]. siRNAs can be chemically synthesized,
or produced by in vitro transcription or by digestion of long
dsRNAs by recombinant RNase III or Dicer [2, 3]. Most of
the techniques that have been used for antisense delivery can
be applied to synthetic siRNAs and to DNA constructs en-
gineered to express shRNAs. These include electroporation,
cell microinjection, and lipophilic transfection [13].

Although siRNAs were initially thought to be small
enough to avoid the activation of the IFN pathway [9], re-
cent studies showed they could activate innate immunity in
mammalian cells [10, 14–16]. In this respect, Sledz and col-
leagues reported that siRNA could activate PKR, and the ef-
fects were sequence independent and do not occur with the
sense or the antisense RNA used to prepare the siRNA du-
plexes [15]. In contrast, Kariko and colleagues found that
siRNA could activate cytokine and interferon production via
TLR3, a receptor known to bind viral ds RNAs [16]. TLR3 is
mainly expressed on the cell surface. However, we and oth-
ers recently have demonstrated that PKR and TLR-3 do not
represent the major mechanism by which chemically syn-
thesized siRNA activate innate immunity [17–19]. In addi-
tion, internalization of ds siRNAs or ss siRNAs is required
for immune activation. Indeed, inhibitors of endosomal mat-
uration/acidification like bafilomycin A1, a drug that inhibit
endosomal H⁺-proton pumps, blocked immune activation
[17], indicating the involvement of endosomal TLR7 and
TLR8. Consistent with the role of endosomal TLRs in siRNA
signaling, TLR7 knockout mice did not mount immune acti-
vation in response to siRNAs [18]. Taken together, the avail-
able data indicate that immune cells such as monocytes and
dendritic cells recognize ds siRNAs and ss siRNAs through
TLR7 and TLR8, leading to the production of inflammatory
cytokines and type I interferons [20]. We also found that ss
siRNAs are more effective than ds siRNA in triggering TLR7
and TLR8 responses [17, 21]. In addition, we have found
that human bone marrow hematopoietic CD34⁺ progeni-
tor cells express TLR7 and TLR8. Interestingly, incubation of
these cells with either immunostimulatory synthetic siRNAs
or R848, a specific ligand for TLR7 and TLR8, induced their
differentiation into the myeloid lineage (Sioud et al in prepa-
ration). Thus, the interaction of viral RNAs with CD34⁺ pro-
genitor cells may increase the pool of innate immune cells.

As mentioned above mammalian cells respond to siR-
NAs, provided they are delivered to the endosomes. Notably,
the immune system uses a set of germ line encoded recep-
tors called pattern recognition receptors (PRRs) to recognize
common microbial structures known as pathogen associated
molecular patterns [22]. Whereas several TLRs are expressed
in the cell surface, TLR3, TLR7, TLR8, and TLR9, traffic be-
tween the endoplasmic reticulum and intracellular compart-
ment such as the endosomes and the lysosomes [22]. An ob-
vious function of these trafficking pathways is to scan for
viral and/or bacterial nucleic acids, thus playing a central
role in innate antiviral responses. Therefore siRNAs inter-
nalized via endocytosis are more likely to activate endoso-
mal TLRs. It should be noted that cytoplasmic delivery of
immunostimulatoty synthetic siRNAs or shRNA did not in-
duce immune response in human blood cells [17, 21], sug-
gesting that they are not recognized by cytosolic sensors of
ds RNAs. In this respect, a recent study has showed that en-
dogenously expressed shRNAs are not immunostimulatory
in human cells [23]. Interestingly, Williams and colleagues
demonstrated that the presence of 2-base 3'–overhangs in
synthetic siRNAs or in Dicer processed shRNAs blocks the
activation of RIG-1, a major cytosolic sensor for viral ds
RNAs [24].

**STRUCTURE FEATURES THAT ACTIVATED TLR7 AND TLR8**

Initial experiments indicate that some types of secondary
structures and/or specific nucleotides are responsible for the
activation of NF-κB signaling pathway by siRNAs in adherent
PBMC, an enriched monocyte population [10]. Monocytes
are circulating peripheral blood cells that can be differenti-
ated by cytokines into macrophages of different phenotypes
as well as into dendritic cells. As mentioned above, siRNA ef-
fects are sequence dependent and can occur with ds siRNAs
and ss siRNAs [20]. Thus, what is the nature of IFN-inducing
motif present in one sequence but absent in another? Al-
though GU dinucleotides were found to trigger TLR7 and
TLR8 activation [25], their absolute requirement in siRNA
activation of innate immunity is still not clear [21]. Judge
and colleagues identified one RNA motif and its immunos-
timulatory effect seems to depend on the GU content [19].
However, Hornung and colleagues identified a second RNA
motif that is recognized by TLR7 in the context of siRNA du-
plexes and the activity does not depend on GU content [18].
It is worth noting that several siRNA sequences without GU
nucleotides activated the immune system [21, 26]. Thus, it is
likely that in addition to GU dinucleotides other characteris-
tics such as RNA structure, base position, and base composi-
tion of the siRNA flanking sequences may be involved.

**2'-RIBOSE MODIFICATIONS OF URIDINES BLOCKS IMMUNE ACTIVATION**

The identification of the immunostimulatory sequence that
can activate innate immunity will allow the design of siRNAs
to minimize activation or to increase the immune response
for combating infections and tumor cells. Considering the
simplicity of the immunostimulatory motifs and their high
frequency in human RNAs, it is desirable to find strategies
that evade immune recognition of siRNAs. At least two ba-
sic strategies can be used to block immune recognition of
siRNA. One is to use delivery agents that avoid the delivery and/or retention of siRNA within the endosomes. The other is to apply chemical modifications. Regarding the first strategy, Song and colleagues described an antibody-based delivery strategy that can result in gene silencing without immune activation [27]. The second strategy relies in the use of modified nucleotides, which are essential to protect siRNAs from nuclease degradation and ameliorate their pharmacokinetic parameters in vivo [13]. However, the chemical modification that blocks immune activation must be chosen carefully so as not to inhibit siRNA silencing activity. Thus, finding the appropriate chemical modifications for inhibiting siRNA immune activation will be important for exploring their therapeutic applications. In this respect, replacement of the 2′-hydroxyl uridines with either 2′-fluoro, 2′-deoxy, or 2′-O-methyl uridines abrogated immune recognition of siRNAs by TLRs [21]. Thus, endosomal TLRs can distinguish between modified and unmodified RNAs (Figure 1). In order to distinguish between self and nonself RNAs, the immune system may use both endosomal compartmentalization and RNA modification strategies [20]. Collectively, these recent findings offer the possibility of choosing the appropriate modifications that evade immune activation without reducing siRNA-silencing potency. Of note, siRNA with either 2′-fluoro uridines or 2′-deoxy uridines maintained silencing activity [26, 28].

Another potential source of toxicity is the destruction of cellular mRNAs that share partial homology to the siRNA sequences. Indeed, recent studies demonstrated that both siRNAs and microRNAs could interact with undesired target mRNAs via base pairing of only few nucleotides, leading to inhibition of gene expression [12, 29]. Interestingly, we have found that chemical modifications of siRNAs not only evade immune activation but also reduce the ss siRNAs and ds siRNA “off-target effects” [30]. Although the evading mechanism is not known, it is probable that the interaction of ss siRNAs or ds siRNAs with unintended cellular mRNAs is affected by chemical modifications.

**IMMUNOSTIMULATORY siRNAs AS VACCINE ADJUVANTS**

It is generally accepted that initiation of a specific immune response requires activation of innate immunity resulting in a proinflammatory response. The produced cytokines and chemokines assist in activating and directing the adaptive immune responses. Therefore, a vaccine has to induce a proinflammatory response to be effective. As discussed above, siRNA sequences containing immunostimulatory motifs induced interferons, chemokines, proinflammatory cytokines, monocyte differentiation, and dendritic cell maturation [20]. Activated DCs produce high levels of IL-12, INF-α, and proinflammatory cytokines such as IL-6 and TNF-α. IFN-α triggers not only innate immune defense such as the activation of NK cells, but also adaptive Th-1 responses, which are important for killing tumor cells and virus infected cells [20]. Engagement of endosomal TLR7 and/or TLR8 with ss siRNAs causes activation of at least three key transcription factors, NF-κB, IFN regulatory factor (IRF)-3, and IRF-7, which are important immune responses [30]. Previous studies have shown that the activation of TLR9 can improve both cell-mediated and humoral responses to antigens [31]. Notably, endosomal TLR9 recognize unmethylated 2′-deoxyribo (cytidine-phosphate-guanosine) CpG motifs that are more commonly found in bacterial and viral genomes [32]. Most, but not all, CpG dinucleotides are methylated in the genomic DNA of vertebrates. Although much remains to be revealed regarding the adjuvant potency of RNA oligonucleotides, the reported data indicate that ss siRNA- and ds siRNAs-containing immunostimulatory RNA motifs might improve cancer and viral vaccines.

**CONCLUDING REMARKS**

As with any new agent, issues of delivery and specificity are major obstacles before siRNAs can be used in patients. Similar to antisense oligonucleotides, certain synthetic siRNAs activated innate immunity via TLRs, in particular TLR7 and TLR8. Therefore, there is a need to examine
the immunostimulatory effects of any potential therapeutic siRNA in human immune cells prior to clinical applications. The recent findings clearly show that the 2′-hydroxyl uridines are absolutely essential for TLR7 and/or TLR8 recognition and signaling. Indeed, replacement of uridines with 2′-fluoro, 2′-deoxy, or 2′-O-methyl modified counterparts abrogated immune activation by ss siRNA and siRNA duplexes. Alternatively, the presence of 2′-modified uridines might protect siRNAs for being sensed by the immune system. Also, most of the other bystander effects not related to immune activation were also inhibited by chemical modifications. These findings will enable the rational design of siRNAs that avoid the induction of bystander effects.

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