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Review

Overcoming the Innate Immune Response to Small Interfering RNA

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ABSTRACT

Many types of nucleic acid, including canonical small interfering RNA (siRNA) duplexes, are potent activators of the mammalian innate immune system. Synthetic siRNA duplexes can induce high levels of inflammatory cytokines and type I interferons, in particular interferon-α, after systemic administration in mammals and in primary human blood cell cultures. These responses are greatly potentiated by the use of delivery vehicles that facilitate cellular uptake of the siRNA. Although the immunomodulatory effects of nucleic acids may be harnessed therapeutically, for example, in oncology and allergy applications, in many cases immune activation represents a significant undesirable side effect due to the toxicities associated with excessive cytokine release and associated inflammatory syndromes. The potential for siRNA-based drugs to be rendered immunogenic is also a cause for concern because the establishment of an antibody response may severely compromise both safety and efficacy. Clearly, there are significant implications both for the development of siRNA-based drugs and in the interpretation of gene-silencing effects elicited by siRNA. This review provides the background information required to anticipate, manage, and abrogate the immunological effects of siRNA and will assist the reader in the successful *in vivo* application of siRNA-based drugs.

INTRODUCTION

The introduction of long double-stranded RNA (dsRNA) into mammalian cells typically leads to the global degradation of mRNA, inhibition of protein synthesis, and activation of the interferon system. These processes are thought to reflect activation of the cells' autonomous antiviral response (Samuel, 2001). This response is based on the recognition of cytoplasmic dsRNA as a signature of viral infection and is mediated by the activation of dsRNA-binding receptors such as PKR (dsRNA-binding protein kinase; Saunders and Barber, 2003) and the RNA helicases RIG-I (retinoic acid-inducible gene-I) and MDA-5 (melanoma differentiation-associated protein-5) (Yoneyama *et al*., 2004; Kato *et al*., 2005, 2006). A consequence of this nearly ubiquitous defense mechanism is that it precludes the use of long dsRNA Dicer substrates when studying RNA interference (RNAi) in mammalian cell lines, because of the myriad of off-target effects associated with activation of the innate antiviral response. However, this barrier has been overcome by the demonstration that short (21-mer) small interfering RNA (siRNA) duplexes are the active product of dsRNA cleavage by Dicer and can mediate RNAi in mammalian cell lines without activating the interferon response associated with transfection of longer dsRNA (Elbashir *et al*., 2001). The seminal early works describing RNAi in mammalian cells inadvertently helped foster the widely held belief that siRNA molecules were immunologically inert. This, despite several reports that later showed that chemically synthesized and virally transcribed siRNA are capable of activating components of the interferon response in certain *in vitro* culture systems (Bridge *et al*., 2003; Sledz *et al*., 2003; Kim *et al*., 2004). Given the early interest in whether or not siRNA could induce an interferon response, it is remarkable that the potential for siRNA to activate mammalian immune cells has, until more recently, been largely ignored. This is particularly surprising when one considers that the immune stimulation associated with antisense DNA oligonucleotides was found to be responsible for the bulk of the off-target effects associated with this then novel class of gene-

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silencing agent. Research into the immune-mediated effects of antisense molecules subsequently helped to define CpG sequence motifs as the primary immunostimulatory element in DNA (Krieg, 2002). An understanding of these phenomena has led to the careful reconsideration of the antisense literature and an improved understanding of the mechanism of action underlying various "antisense" drugs.

In many ways mirroring the early history of antisense technology, we and several other groups have shown that siRNA has the inherent capacity to activate potent immune responses *in vivo* or when delivered to responsive cell types *in vitro* (Hornung *et al*., 2005; Judge *et al*., 2005; Sioud, 2005). Immune stimulation and the induction of interferons by siRNA provide not only the potential for strong nonspecific effects with respect to target gene expression and function, but the ensuing inflammatory response can also result in significant toxicities when siRNAs are administered *in vivo*. In this review, we outline some of the factors that may influence the immunostimulatory properties of siRNA and highlight the need for these to be taken into consideration when designing and interpreting results from siRNA experiments. Finally, we describe ways to mitigate these unwanted side effects, in particular by including chemical modifications to the siRNA that can completely abrogate their immunostimulatory activity.

MECHANISMS OF siRNA-MEDIATED IMMUNE STIMULATION

Innate immune responses can be triggered through a variety of so-called pattern recognition receptors, including Toll-like receptors (TLRs), that are activated by molecules typically associated with viral, bacterial, or fungal pathogens (Takeda *et al*., 2003; Kawai and Akira, 2006; Schlee *et al*., 2007). The TLR-dependent arm of the innate immune response is regulated by a number of TLR family members, each having a specialized function in recognizing particular pathogen-associated molecules. These include cell wall components, lipopolysaccharides (LPSs), flagellae, and nucleic acids derived from bacteria and viruses. An important feature of all pattern recognition receptors is that the ability to engage their target ligands is determined by the cellular location of the receptor. For example, the nucleic acid-sensing TLRs are typically located intracellularly and engage nucleic acids released from invading pathogens as they are degraded within the endosomal/lysosomal compartment. The innate immune system can also be activated by nucleic acids through TLR-independent mechanisms composed of a disparate series of cytoplasmic receptors including PKR, and the RNA helicase enzymes RIG-I and MDA-5. These RNA sensors are situated within the cytoplasm of the cell, an ideal location to monitor for the presence of certain RNA species that are hallmarks of viral infection and replication. As we shall describe, siRNAs have the capacity to activate both TLR-dependent and -independent immune responses on the basis of two fundamental principles: (1) siRNA transfection into cells exposes it to the endosomal and cytoplasmic RNA-sensing receptors of the innate immune system; and (2) siRNA is an exogenous nucleic acid that can possess the same molecular signatures as viral RNA.

It is now clear that specialized cells of the mammalian innate immune system are highly responsive to stimulation by native siRNA sequences (Hornung *et al*., 2005; Judge *et al*., 2005; Sioud, 2005). Synthetic siRNA duplexes or hairpin RNA can elicit the secretion of interferon (IFN)-α and inflammatory cytokines from both murine and human blood cells at levels comparable to those induced by immunostimulatory CpG oligodeoxynucleotides (ODNs). This robust *in vitro* response is recapitulated when formulated siRNAs are administered to mice, bringing about the characteristic inflammatory reaction caused by activation of the innate immune response. This appears to be mediated primarily through the activation of TLR7 and/or TLR8 (Hornung *et al*., 2005; Judge *et al*., 2005), both of which have been shown to recognize short RNA species in the endosomal compartment (Diebold *et al*., 2004; Heil *et al*., 2004). siRNAs have been reported to activate cellular responses *in vitro* through the dsRNA receptor TLR3 (Karikó *et al*., 2004), although it is unclear at present whether this pathway contributes significantly to the immune response to siRNA *in vivo* (Hornung *et al*., 2005; Schlee *et al*., 2006). Certain siRNA constructs can also stimulate an IFN response by activation of the RNA helicase RIG-I (Kim *et al*., 2004; Marques *et al*., 2006). These various pathways of siRNA-mediated immune activation are summarized in Fig. 1.

FACTORS INFLUENCING IMMUNE RECOGNITION OF siRNA

Responsive cell types

Immune responsiveness to siRNA is cell type dependent because of the selective expression pattern of TLRs and the RNA helicases. This heterogeneity in receptor expression has undoubtedly been a contributing factor in the numerous conflicting reports on the immunobiology of siRNA. The reactivity of immune cells to siRNA is contingent on their pattern of TLR expression, in particular, the functional expression of TLR7 and/or TLR8. Plasmacytoid dendritic cells (pDCs) that constitutively express TLR7 in both mice and humans are directly activated by siRNA to secrete high levels of IFN-α (Hornung *et al*., 2005; Judge *et al*., 2005). In contrast, human myeloid lineage cells including monocytes and myeloid DCs preferentially express TLR8 and respond to stimulatory RNA by secreting inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF) rather than IFN-α (Gorden *et al*., 2005; Judge *et al*., 2005). The induction of IFN-α by pDCs acts to prime other immune cells such as monocytes, DCs, natural killer (NK) cells, and B cells to become more responsive to RNA-mediated activation through upregulation of TLR7/8 and other ancillary signaling components (Liu, 2005). B cells have a TLR expression profile similar to that of pDCs and are therefore activated by the same TLR9 and TLR7 ligands including RNA (Bekeredjian-Ding *et al*., 2005; Peng, 2005). As we discuss later, TLR7 signaling in B cells potentiates their differentiation into antibody-secreting plasma cells, and this has direct implications for the potential of drug formulations containing stimulatory siRNA to be immunogenic.

The majority of cell lines do not express functional TLR7 or TLR8 and are therefore unable to recapitulate the robust innate

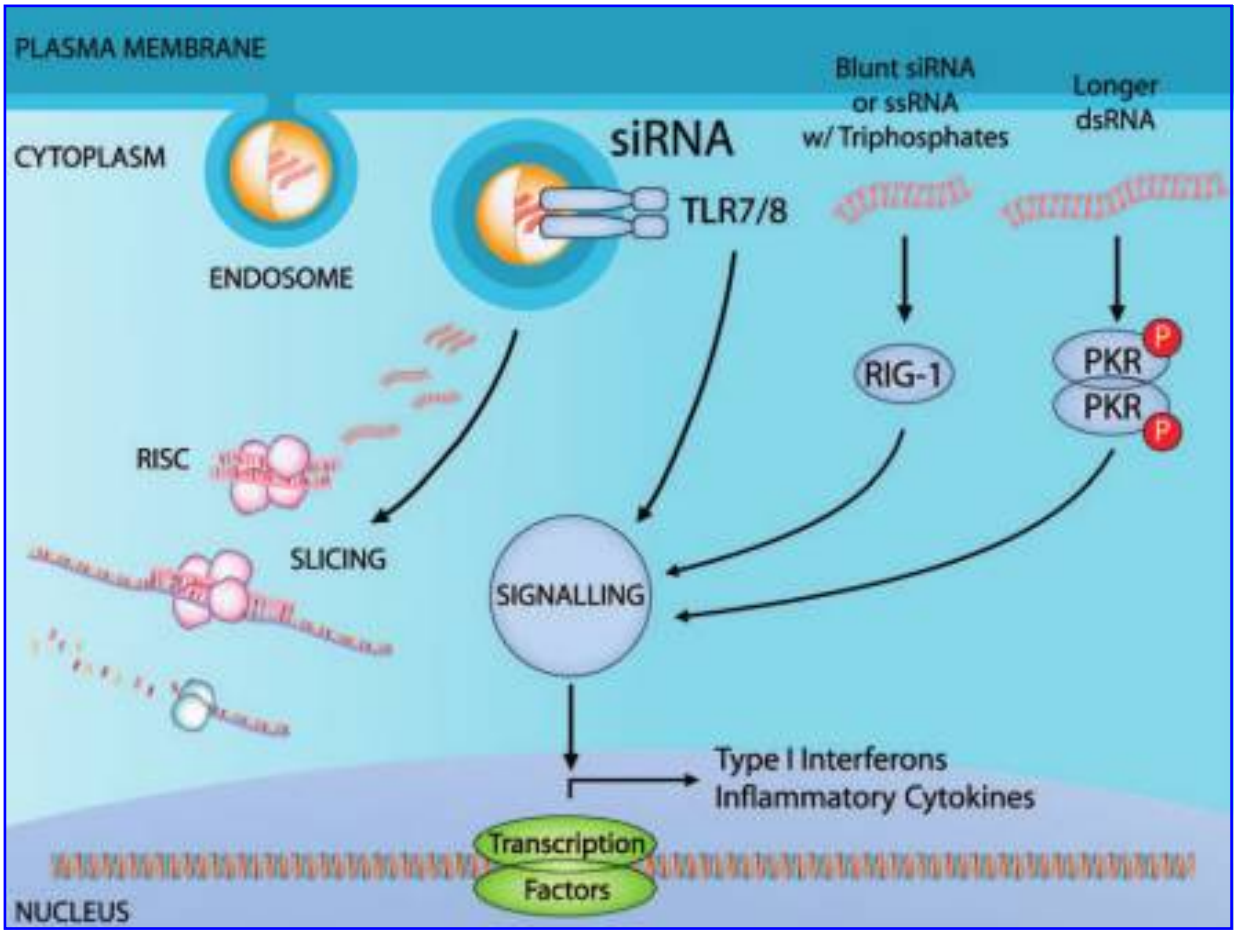

FIG. 1. Immune recognition of siRNA and dsRNA by TLR7/8, RIG-I, and PKR. Signaling through endosomally located TLR7 and TLR8 occurs via the adaptor protein MyD88, which in turn forms a complex with IRAK-1, IRAK-4, and TRAF6. This results in the activation and nuclear translocation of NF-κB and activation of ATF2-c-Jun transcription factor (leading to expression of inflammatory cytokines) and the IRF-7 transcription factor (leading to expression of IFN-α subtypes). On binding to blunt-ended dsRNA or triphosphate-ssRNA, the cytoplasmic protein RIG-I signals through the IPS-1 protein adaptor and TRAF6-activating IRF5 (leading to inflammatory cytokine production) and TRAF3-activating IRF3 and IRF7 (leading to type I IFN production). Binding of long dsRNA to PKR causes its dimerization and transphosphorylation, leading to the phosphorylation of proteins such as eIF2α and IκB. These in turn cause a general inhibition of translation and nuclear translocation of NF-κB, respectively. Phosphorylated PKR also activates p38 MAPK and STAT1, culminating in the transcription of interferon-stimulated genes. *Abbreviations*: dsRNA, double-stranded RNA; eIF2α, elongation initiation factor-2α; IκB, inhibitor of NF-κB; IPS-1, interferon-β promoter stimulator-1; IRAK, interleukin-1 receptor-associated kinase; IRF, interferon regulatory factor; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation factor-88; NF-κB, nuclear factor-κB; P, phosphate; PKR, dsRNA-binding protein kinase; RIG-I, retinoic acid-inducible gene-I; RISC, RNA-induced silencing complex; siRNA, small interfering RNA; ssRNA, single-stranded RNA; STAT1, signal transducer and activator of transcription-1; TLR, Toll-like receptor; TRAF, TNFR (tumor necrosis factor receptor)-associated factor.

immune response to siRNA. Cytoplasmic RNA receptors, however, are expressed in a wider variety of mammalian cell types. Cell lines that naturally express RIG-I mount strong interferon responses to siRNAs that contain particular structural features recognized by this receptor (Kim *et al*., 2004; Marques *et al*., 2006). As a practical matter it should also be noted that cell culture conditions can directly affect the sensitivity of cells to siRNA-mediated immune activation. For example, critical components of both the TLR-dependent and -independent pathways are themselves induced by interferons and other physiological factors such as cellular stress. Therefore, the potential exists for many cell types to be primed, either intentionally or inadvertently, to respond to siRNA, depending on the experimental conditions.

Delivery vehicles

The use of delivery vehicles to enable synthetic siRNA uptake and presentation to the cytoplasmic RNAi machinery is seen as essential by researchers conducting both *in vitro* and *in vivo* studies. Most vehicles used for this purpose are based on positively charged (cationic) agents that complex with, or en-

capsulate, the negatively charged nucleic acid. These include cationic lipid formulations (e.g., Lipofectamine) and cationic polymers such as polyethylenimine (PEI) that form positively charged siRNA complexes that facilitate intracellular delivery through electrostatic interactions with the membrane of the target cell. A common feature of such systems is that they are taken up by endocytosis and concentrated in the endosomal compartment before releasing siRNA into the cytoplasm. An inadvertent consequence of this process is the efficient delivery of siRNA into the very cellular compartments where the RNA-sensing receptors of the innate immune system are located. It is worth noting that immunologists have exploited this feature of cationic lipids and polycations to potentiate the immunostimulatory effects of nucleic acids for many years. It should therefore come as no surprise that similar effects are also observed on effective delivery of siRNA.

Immune stimulation by siRNA may be potentiated by delivery vehicles because of a number of factors: (1) efficient uptake into intracellular compartments, (2) protection of siRNA from nuclease degradation, thereby enhancing exposure to immune cells, and (3) enhanced engagement or cross-linking of immune receptors by siRNA when complexed with its carrier. The nature of the delivery vehicle can have a dramatic effect on both the quality and magnitude of the immune response to siRNA. For example, our group has shown that human blood cells stimulated with siRNA encapsulated in stable nucleic acid–lipid particles (SNALPs) or complexed with PEI induce a response that is dominated by IFN-α secretion. In contrast, Lipofectamine and polylysine, which form larger, more heterogeneous siRNA complexes, tend to elicit a predominantly inflammatory cytokine response from the same human blood cell cultures (Judge *et al*., 2005). The nature of the response is affected by numerous parameters including the size, charge ratio, and chemical composition of the delivery vehicle as well as its pharmacokinetics, biodistribution, and route of administration. We have noted that the magnitude of cytokine induction by a particular siRNA can differ by greater than two orders of magnitude in mice depending on the delivery vehicle. In our experience, however, we have found that siRNA duplexes with inherent immunostimulatory activity invariably activate some measure of immune stimulation when tested appropriately in responsive immune cell types, irrespective of the delivery system employed. These include the typical commercial transfection reagents as well as our own proprietary lipid delivery technologies.

siRNA sequence

Individual siRNA duplexes vary considerably in their capacity to activate the TLR-mediated immune response, because of inherent differences in their nucleotide sequence (Hornung *et al*., 2005; Judge *et al*., 2005; Sioud, 2005). Unlike the defined CpG DNA motifs that characterize TLR9 agonists, the precise nature of the RNA sequences recognized by TLR7/8 remains obscure. We have described siRNA duplexes with GU-rich sequences as being highly immunostimulatory and identified 5′-UGU-3′ motifs within particular siRNAs that apparently confer this activity (Judge *et al*., 2005). Substitution of the uridine groups in this motif significantly reduced the immunostimulatory activity of the duplex whereas introduction of the 5′-UGU-3′ motif into an siRNA duplex had the predicted effect of increasing activity. On the basis of these observations, we have been able to select functional siRNA sequences that lack GU-rich regions and have inherently low immunostimulatory capacity (Judge *et al*., 2005). However, it is clear from our own work and that of others that the 5′-UGU-3′ motif is one of many recognized by TLR7/8 (Diebold *et al*., 2004; Hornung *et al*., 2005; Judge *et al*., 2005; Sioud, 2005; Diebold *et al*., 2006). This is supported by the observation that the large majority of native siRNA sequences possess at least some immunostimulatory activity, suggesting that TLR7/8 recognizes a simple pattern of ribonucleotides occurring at high frequency in conventionally designed synthetic siRNA. This hypothesis has been validated by Diebold and coworkers, who demonstrated that a ribose sugar backbone and multiple uridine residues in close proximity (two hallmarks that define RNA from DNA) are all that is necessary for TLR7 recognition of single-stranded RNA (ssRNA) (Diebold *et al*., 2006). These findings go some way to refining the basic concept of what may be regarded as a stimulatory "motif" within RNA and are consistent with our own observations relating to siRNA duplexes.

siRNA structure

Unlike the TLR7/8-mediated response, the recognition of RNA by the cytoplasmic receptors RIG-I, MDA-5, and PKR is considered to be sequence independent. However, these receptors have evolved to recognize certain other physical and structural characteristics of RNA that are thought to be representative of viral infection. One trigger for this response appears to be viral or phage-transcribed RNA that naturally contains uncapped 5′-triphosphate groups. Kim and coworkers found that transcribed 5′-triphosphate siRNA activated an interferon response when transfected into nonhematopoietic cell lines (Kim *et al*., 2004). It has subsequently been reported that 5′-triphosphate RNA binds to and activates RIG-I, leading to potent IFN responses from RIG-I-expressing cells (Hornung *et al*., 2006; Pichlmair *et al*., 2006). Blunt-ended dsRNA may also cause RIG-I activation (Marques *et al*., 2006). This becomes a relevant concern as asymmetrical siRNAs containing a blunt 5′-antisense end have become more widely used in an attempt to improve potency (Soutschek *et al*., 2004; Kim *et al*., 2005; Rose *et al*., 2005). The binding and activation of RIG-I by blunt-ended siRNA increase as the duplex length is increased from 21 to 27 base pairs and is inhibited by the introduction of overhangs that reduce RIG-I helicase activity (Marques *et al*., 2006). On the basis of a small number of unique RNA duplexes examined, this effect appears to be sequence independent, although testing of a wider panel of siRNA is required to determine whether nucleotide sequence plays a role in either RIG-I binding or its ability to unwind dsRNA. Another important finding from RIG-I studies is the ability of certain batches of canonical 21-mer siRNAs to induce significant IFN responses from RIG-I-sensitive cell lines. This appears to correlate with siRNA batches of poor quality or purity that are thought to contain uncharacterized RNA impurities capable of activating RIG-I or one of the other cytoplasmic RNA receptors (Marques *et al*., 2006). Unfortunately, such variability places the onus on individual investigators to determine whether their particular siRNA preparations are capable of activating an interferon response in their model system.

It should be appreciated that a given siRNA duplex may activate multiple immune receptor pathways depending on the various structural, physical, and sequence-related features. For example, an unmodified, blunt-ended siRNA can be a potent activator of both TLR7/8 as it trafficks through the endosomal compartment (Hornung *et al*., 2005; Judge *et al*., 2006a) and RIG-I when it is released into the cell cytoplasm (Marques *et al*., 2006; and A. Judge, unpublished data). Each pathway results in the induction of type I interferons and may contribute to toxic and off-target effects. It is important to determine the ability of a chosen siRNA to activate any one of these multiple pathways.

CONSEQUENCES OF ACTIVATING THE INNATE IMMUNE RESPONSE

False-positive efficacy

Activation of innate immunity and the production of IFNs have well characterized effects in modulating viral replication, tumor growth, angiogenesis and immunological processes such as inflammation. An extra degree of caution is advised when operating in these areas to avoid the potential misattribution of therapeutic effects caused by immune stimulation. To confirm that efficacy is mediated by RNAi, it is critical for researchers to appreciate the capacity for siRNA to activate immune responses and to fully characterize the immunostimulatory potential of both active and control siRNA sequences. An especially problematic area is that of infectious diseases. The antiviral efficacy of siRNA has been reported in numerous virus infection models, including influenza A (Ge *et al*., 2004; Tompkins *et al*., 2004), herpes simplex virus (HSV) (Palliser *et al*., 2006), respiratory syncytial virus (RSV) (Bitko *et al*., 2005), parainfluenza (PIV) (Bitko *et al*., 2005), and hepatitis B virus (HBV) (Morrissey *et al*., 2005b) in mice, Ebola virus infection in guinea pigs (Geisbert *et al*., 2006), and severe acute respiratory syndrome (SARS) in both monkeys and mice (Li *et al*., 2005). In spite of the obvious interplay between immune stimulation and antiviral activity, surprisingly few RNAi studies of this nature have adequately examined or controlled for immune stimulation. One study to date that directly compared immunostimulatory versus nonimmunostimulatory siRNAs (Morrissey *et al*., 2005b) has found a differential effect of nontargeting control siRNA on the reduction of HBV titers that was indicative of nonspecific immune-mediated viral inhibition.

Another problematic area is that of oncology. The immunostimulatory effects of nucleic acids have been intentionally harnessed as a cancer therapy for many years (Foss, 2002; Krieg, 2006; Brannon-Peppas *et al*., 2007). Activation of the immune response by siRNA can promote nonspecific tumor inhibition by several mechanisms involving either general adjuvant effects that enhance the host's cellular response or through the direct antitumor activity of cytokines such as the IFNs and TNF. Some of these mechanisms, such as NK cell activity and innate cytokine induction, are active even in the immunodeficient mouse strains commonly used for human tumor modeling. However, once again reports that adequately describe the extent to which siRNA-mediated immune stimulation contributes to antitumor efficacy are rare. An illustration of how IFN induction by siRNA may confound the interpretation of results is found in the antiangiogenic activity of IFN-α and -β (Folkman and Ingber, 1992). IFN inhibits the ability of endothelial cells to form new blood capillaries, acting specifically to suppress vascular endothelial growth factor (VEGF) expression at the mRNA level (von Marschall *et al*., 2003; Wu *et al*., 2005). The VEGF pathway is an attractive target for RNAi in oncology (Schiffelers *et al*., 2004; Song *et al*., 2005; Behlke, 2006) as well as in other indications such as macular degeneration, an eye disease characterized by dysregulated macular capillary growth (Reich *et al*., 2003). Given that unintended immune stimulation and concomitant IFN production have the potential to downregulate both VEGF protein and mRNA, confirmation that VEGF knockdown is mediated by RNAi and not via an interferon response induced by siRNA is fundamental to the interpretation of experiments targeting VEGF or other components of the angiogenesis pathways.

The use of appropriate controls should facilitate the straightforward interpretation of siRNA studies. However, there is currently little consensus as to what constitutes the "best" control for a given siRNA when considering immune-mediated effects. Noncoding "scrambled" siRNAs or siRNAs targeting nonmammalian genes such as green fluorescent protein (GFP) were once considered adequate controls; however, every native siRNA sequence has a distinct immunostimulatory profile and therefore a differential capacity to induce off-target effects. It is curious that a common GFP siRNA sequence selected for use as a negative control by many groups developing therapeutic siRNA strategies (e.g., Novina *et al*., 2002; Reich *et al*., 2003; Song *et al*., 2003, 2005; Flynn *et al*., 2004; Ge *et al*., 2004; Tompkins *et al*., 2004; Thomas *et al*., 2005; Palliser *et al*., 2006) has unusually low immunostimulatory activity whereas the active siRNAs used by these same groups are immunostimulatory when evaluated on either human peripheral blood mononuclear cells (PBMCs) or murine bone marrow-derived Flt3 ligand (Flt3L) DCs, or *in vivo* in mice (unpublished data). When previously published influenza efficacy studies (Ge *et al*., 2004) are repeated with chemically modified, nonstimulatory active siRNA or, alternatively, immunostimulatory negative controls, the results clearly suggest that a differential induction of IFN between control and active siRNAs has affected the results and interpretation of this and a number of other published *in vivo* knockdown studies (Judge and MacLachlan, 2008). Because the inherent immunostimulatory activity of siRNAs is sequence dependent, a certain amount of screening may be required to ensure that negative control siRNAs are as immunostimulatory as their active counterparts (e.g., Geisbert *et al*., 2006). A preferred approach would be to use chemically modified siRNAs with minimal residual immunostimulatory activity for both active and control duplexes and to confirm the immunobiology of selected siRNA within the context of the disease model in question. This approach would help minimize the potential for false-positive results and help avoid the misinterpretation of preclinical RNAi studies.

Acute toxicities

Although beneficial in host defense, overstimulation of TLR-mediated pathways is pathological. Treatment of humans with

TLR ligands such as LPS (TLR4) (Michie *et al*., 1988; Lynn *et al*., 2003) or poly(I:C) (TLR3) (Stevenson *et al*., 1985) results in an inflammatory response syndrome characterized by flulike symptoms, fever, chills, rigors, and hypotension. Onset of symptoms typically occurs several hours after administration, distinguishing them from immediate hypersensitivity or infusion reactions. This response can be replicated in a number of animal models including rodents, although the doses required to induce symptoms can vary over orders of magnitude according to the species. The clinical syndrome induced by TLR ligands is multifactorial, reflecting the complexity of the inflammatory cascade; however, many of the symptoms can be directly attributed to the action of cytokines (Thomson and Lotze, 2003). Informed by the well-characterized responses to TLR ligands and our current understanding of the biology underlying siRNA-mediated immune stimulation, we propose the following model describing the mechanism of immunotoxicity after systemic administration of immunostimulatory RNA (summarized in Fig. 2):

1. Endocytosis of siRNA carriers by pDCs and other immune cells expressing TLR7/8 causes activation and the secretion of IFN-α and cytokines including IL-6 (Hornung *et al*., 2005; Judge *et al*., 2005).
2. IFN-α acts on surrounding immune cells including monocytes, macrophages, and NK cells to upregulate TLR7/8, making them responsive to the siRNA and amplifying the production of inflammatory cytokines, including IL-6, IL-1β, and TNF, together with proinflammatory chemokines (Mohty *et al*., 2003; Hart *et al*., 2005; Liu, 2005; Bekeredjian-Ding *et al*., 2006).
3. Systemic cytokine release induces symptoms of fever, rigors, and chills and also acts directly on the liver to induce the acute-phase reaction that elevates serum proteins associated with host defense such as complement, pentraxins, and coagulation factors.
4. IL-1β, TNF, and other vasoactive molecules activate vascular endothelium, upregulating adhesion molecules, triggering margination and pavementing of blood cells that manifests as transient lymphopenia, and thrombocytopenia. Increasing vascular permeability may result in the development of hypotension, exacerbating the extravasation of blood cells into surrounding tissues.
5. Cyclo-oxygenase (COX)-2 and nitric oxide synthase enzyme pathways are upregulated either directly through TLR signaling or indirectly through the action of cytokines (Sharara *et al*., 1997; Yeo *et al*., 2003), resulting in eicosanoid and nitric oxide production. These mediators contribute to the symptoms of toxicity, including pain.
6. In the most severe cases, dysregulation of the protective negative feedback mechanisms allows continued amplification of the systemic inflammatory response, worsening hypotension, vascular leak, and intravascular coagulation that ultimately leads to multiorgan failure.

Although the severity of any inflammatory reaction to siRNA will depend on the nature of the siRNA, delivery vehicle, and route of administration, we believe that the risks for serious adverse effects manifesting in the clinic are real. Our preclinical experience indicates that these inflammatory reactions can also exacerbate any underlying chemical toxicities associated with the siRNA or delivery vehicle.

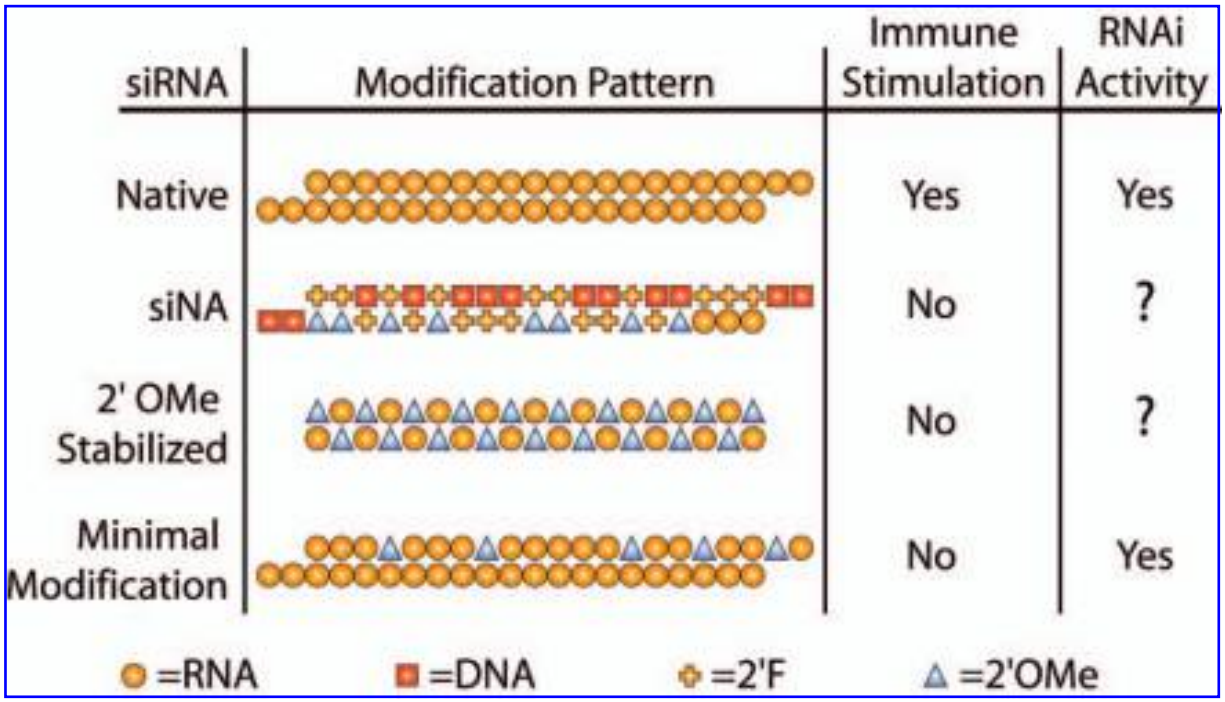

FIG. 2. Chemical modification of siRNA. Chemical modifications have been applied to siRNA in an effort either to confer stability from nuclease-mediated degradation or to abrogate the inherent immunostimulatory properties of canonical siRNA duplexes. The various approaches are described in text.

Species differences

At the time of this writing, siRNA-based therapeutics have yet to be systemically administered to humans. Therefore, no direct clinical precedent exists with which to compare the toxicities observed in preclinical studies. It should be noted that the relative sensitivities of different species to TLR-mediated immune responses are considered idiosyncratic and notoriously difficult to predict. The best defined of these is the response to LPS; humans and chimpanzees are exquisitely sensitive to the toxic effects of LPS whereas baboons and other primates are relatively resistant (Smirnova *et al*., 2000). A similar spectrum of sensitivities to LPS is seen within rodents. With respect to immunostimulatory nucleic acids, dramatic differences also exist between species, with humans being exquisitely sensitive to the toxic effects of poly(I:C), for example (Stevenson *et al*., 1985). In our experience, preclinical toxicology in mice and rats has proven to be nonpredictive for the dose-limiting immunotoxicities observed in human subjects after the intravenous administration of immunostimulatory plasmid DNA (A. Judge and I. MacLachlan, unpublished data).

Although rat and mouse models successfully reproduce the TLR-mediated cytokine response identified in human subjects receiving lipid-encapsulated plasmid DNA, neither model replicates the full extent of the toxicities observed in humans, most notably the development of fever, rigors, and hypotension, or predict the low doses at which clinical immunotoxicities manifest (less than 0.003 mg of plasmid DNA per kilogram body weight). By our understanding, no preclinical model has been described for a DNA-based therapeutic that is predictive of this human response, including several reports in nonhuman primates (Tsuboniwa *et al*., 2001; Webb *et al*., 2001; Quezada *et al*., 2002). However, the discovery that these symptoms of toxicity in humans correlated with significant increases in blood IFN-α, and that this could be replicated *in vitro* with TLR9-expressing human blood cells, provides us with a better understanding with which to extrapolate the toxic effects of immune stimulation by siRNA-based drugs in preclinical models into the clinical setting.

Interspecies differences in sensitivity to the toxic effects of TLR ligands may be due to several reasons. First, TLRs from different species can be preferentially activated by different immunostimulatory motifs or structures within the respective ligands (Krieg, 2002). Second, the range of cell types expressing particular TLRs, and therefore responsive to stimulation, can differ between species; and third, species differ in their direct sensitivity to the toxic effects of cytokines and inflammatory mediators. Critical differences in the sensitivity of human subjects to the toxic effects of immune stimulation have been highlighted in an ill-fated phase 1 study of a CD28 agonist monoclonal antibody designed to activate T cell responses. Life-threatening shocklike symptoms developed rapidly in healthy volunteers after the first administration of the immunostimulatory monoclonal antibodies. These symptoms were subsequently attributed to "on-target" effects of the CD28 agonist causing excessive cytokine release, leading to a severe shocklike syndrome (Suntharalingam *et al*., 2006). Most strikingly, these effects occurred at a low dose and were apparently unpredicted by the extensive preclinical testing leading up to this first human trial.

Taken together, it is believed that the identification of a preclinical model capable of predicting the clinical symptoms that may result from siRNA-mediated immune stimulation will require a stochastic approach to screening species. To the best of our knowledge, no published studies have highlighted such a model. Using the precautionary principle, we believe it prudent to minimize the potential risk for adverse clinical reactions by using siRNA with minimal immunostimulatory activity, particularly when considering systemic routes of administration.

Multiple dosing and toxicities associated with drug immunogenicity

The potential for a drug or its excipient to be immunogenic is a serious concern because the establishment of an antibody response can severely compromise both the safety and efficacy of a drug. The strong adjuvant effects of nucleic acids have been widely used in preclinical immunization protocols and vaccine development to promote antibody responses (Klinman, 2004; Krieg, 2006). Similarly, it has been shown that the encapsulation of CpG DNA and plasmid DNA within delivery vehicles can potentiate antibody responses against components of the carrier, even after low-dose, single administration (Boeckler *et al*., 1999; Li *et al*., 2001; Semple *et al*., 2005; Judge *et al*., 2006b). We have found that loading immunostimulatory siRNA into lipidic vehicles can render an otherwise inert carrier immunogenic (Judge *et al*., 2006b; and A. Judge, unpublished data). Surprisingly, this is evidenced by the induction of both IgM and IgG antibodies against the polyethylene glycol (PEG) component of the vehicle and is driven by the activation of B cells that have taken up the stimulatory siRNA. In this example, the immunostimulatory activity of siRNA is thought to potentiate antibody responses against the PEG–lipids both by direct B cell stimulation through TLR7 (Peng, 2005) and through the concurrent induction of cytokines that support activated B cell growth and differentiation (Jego *et al*., 2003). A similar TLR7-mediated mechanism has been proposed for the induction of autoantibodies by RNA complexes in the autoimmune disease systemic lupus erythematosus (SLE) (Lau *et al*., 2005; Vollmer *et al*., 2005; Christensen *et al*., 2006; Pisitkun *et al*., 2006; Subramanian *et al*., 2006).

The immune response against delivery vehicles containing siRNA can be surprisingly robust, generating sufficient titers of serum antibodies in mice to mediate accelerated blood clearance, a loss of disease site targeting, and acute hypersensitive reactions on subsequent readministration (Judge *et al*., 2006b; and A. Judge, unpublished data). PEG is generally regarded as nonimmunogenic yet significant antibody responses against PEGylated vehicles can be raised by the encapsulation of immunostimulatory siRNA. It is expected that attachment of inherently more immunogenic components such as targeting ligands, peptides, or antibodies to delivery systems carrying stimulatory siRNA will likely exacerbate this problem (Phillips and Dahman, 1995; Harding *et al*., 1997; Li *et al*., 2002). Perhaps not surprisingly, native (immunostimulatory) siRNAs delivered in human transferrin-conjugated PEG-modified cyclodextrin-based carriers have been shown to result in the production of anti-human transferrin antibodies in nonhuman primates (Heidel *et al*., 2007). It remains to be seen whether

active targeting technologies can be made to be compatible with the delivery of immunostimulatory siRNA.

MEASUREMENT OF siRNA-MEDIATED IMMUNE RESPONSE

The nature and extent of the immune response to siRNA are dictated by a multitude of variables that differ with each experimental model and methodology employed to affect RNA interference. It is therefore essential that researchers conduct the appropriate studies that are able to monitor for the induction of an immune response in their particular experimental setting. The measurement of systemic cytokine release into the blood of treated animals is a routine assay that can confirm significant activation of the innate immune response. To make this a valid assessment, however, it is critical to realize that (1) siRNA formulations can differentially induce either IFNs and/or inflammatory cytokines such as TNF; (2) the kinetics of this response vary depending on the model; and (3) detectable increases in plasma cytokine levels are typically short-lived. Therefore, analysis must be performed on multiple cytokines over a 1- to 12-hr time course in order to conclude an absence of a systemic cytokine response to siRNA. Such an assessment should also be supported by cytokine release assays from primary immune cell cultures *in vitro*.

Although the measurement of secreted cytokines is a meaningful readout of innate immune stimulation, it is not necessarily the most sensitive method by which to assess the response. Great care should therefore be taken when interpreting negative cytokine data. Within our laboratory we have used IFN-inducible IFIT1 (p56) mRNA, the most strongly induced mRNA in response to type I IFN (Der *et al*., 1998) or dsRNA (Geiss *et al*., 2001), as a more sensitive measure of immune stimulation. Strong IFIT1 mRNA induction in both liver and spleen can be observed in siRNA-treated mice in the absence of detectable plasma IFN-α protein (unpublished data). This likely reflects local IFN induction that does not manifest as a systemic cytokine response. It will be up to individual researchers to demonstrate the presence or absence of local IFN induction and whether or not such low-grade responses to siRNA are of sufficient magnitude to mediate nonspecific antiviral or antitumoral effects in preclinical models.

OVERCOMING IMMUNE STIMULATION BY siRNA

Although ssRNA is generally regarded as the ligand for TLR7 and TLR8, it is clear that immune stimulation by duplexed siRNA is not mediated by unincorporated oligonucleotide strands that typically contaminate siRNA preparations (Hornung *et al*., 2005; Judge *et al*., 2005). This is an important consideration as it implies that immune activation by siRNA cannot be overcome by improved manufacture or downstream purification of the annealed duplex. Alternative ways of overcoming this barrier to the development of siRNA therapeutics are therefore required. Drug-related side effects that involve adverse immune responses are often managed by premedication with immunosuppressive glucocorticoids such as dexamethasone or with nonsteroidal antiinflammatory drugs (NSAIDs). This approach may be useful within the context of siRNA because the immunostimulatory properties of siRNA are expected to be sensitive to inhibition by glucocorticoids via their molecular effects on key components of TLR signaling pathways (Moynagh, 2003). Glucocorticoids may also be of benefit in symptom management because of their ability to block cytokine receptor signaling and the biosynthesis of both eicosanoids and nitric oxide (Bianchi *et al*., 2000; Moynagh, 2003). In human studies, it has been shown that glucocorticoid administration is a potent suppressor of the IFN-α response elicited by the intravenous administration of lipid-encapsulated plasmid DNA, helping ameliorate the severe immunotoxicities that can manifest in human subjects (A. Judge and I. MacLachlan, unpublished data).

A far more preferable alternative to managing the toxicities associated with the use of immunostimulatory siRNA is to avoid inducing them altogether. On the basis of the finding that immune activation by siRNA is sequence dependent, it is possible to design active siRNA with negligible immunostimulatory activity by selecting sequences that lack immunostimulatory sequence motifs (Judge *et al*., 2005). Although this strategy has proven successful, it significantly limits the number of novel siRNA sequences that can be designed against a given target and requires a certain degree of screening because of the relatively ill-defined nature of immunostimulatory RNA sequence motifs. Clearly, a more robust approach is required.

Chemical modification

The chemical modification of siRNA to provide nuclease resistance and suitable *in vivo* pharmacology has often been regarded as a prerequisite for *in vivo* use (Czauderna *et al*., 2003; Soutschek *et al*., 2004; Morrissey *et al*., 2005a). However, the development of delivery vehicles that protect siRNA from nuclease degradation has bypassed the absolute requirement that siRNA be chemically stabilized. This has allowed researchers to use native RNA sequences for both *in vitro* and *in vivo* RNAi applications, exposing them to the inherent risks of unintended immune stimulation. Chemical modification to abrogate immune stimulation often relies on stabilization chemistries that were previously developed for ribozyme and antisense oligonucleotide drugs, including nucleotides modified with 2′-OH substitutions such as 2′-*O*-methyl (2′-OMe) and 2′-fluoro (2′-F) (Manoharan, 2004). Although some of these stabilization chemistries, when applied judiciously, can be compatible with the RNAi machinery of cells (Chiu and Rana, 2003; Czauderna *et al*., 2003; Layzer *et al*., 2004; Allerson *et al*., 2005; Elmen *et al*., 2005; Hornung *et al*., 2005; Prakash *et al*., 2005) they appear to be tolerated only in certain ill-defined positional or sequence-related contexts as their introduction into a siRNA duplex can have unpredictable negative effects on RNAi activity (Chiu and Rana, 2003; Czauderna *et al*., 2003; Elmen *et al*., 2005; Hornung *et al*., 2005; Prakash *et al*., 2005; Judge *et al*., 2006a). Our preclinical work developing siRNA for therapeutic use has revealed that some common chemical modifications originally developed to confer nuclease resistance can prevent the recognition of modified siRNA by the innate immune system (Morrissey *et al*., 2005b; Judge *et al*., 2006a). This has the

striking result of rendering appropriately designed, fully active siRNA incapable of activating a deleterious immune response and offering a relatively simple design-based solution to the problems associated with immune stimulation.

One chemical modification strategy used to generate siRNA suitable for use as naked molecules involves substituting greater than 90% of the ribonucleotides with 2′-F, 2′-OMe, and 2′-deoxy nucleotides according to the pattern of purines and pyrimidines in a given sequence (Chiu and Rana, 2003; Morrissey *et al*., 2005a,b). We have found that lipidic formulations of many of these so-called siNA molecules induce minimal immune activation when compared with the native siRNA (Fig. 3). Furthermore, these siNA formulations demonstrated improved tolerability and reduced off-target effects in a mouse model of hepatitis B infection (Morrissey *et al*., 2005b). Although dramatic, the loss of immunostimulatory activity in siNA is perhaps unsurprising given the fact that the native ribose sugar backbone, a requirement for RNA-mediated TLR7 activation (Diebold *et al*., 2006), is completely replaced in these molecules. Although there are examples in which the siNA design strategy has been successfully employed, it should be noted that this extensive chemical modification approach frequently results in the significant loss of RNAi activity when applied to native siRNA sequences (Chiu and Rana, 2003; and A. Judge and I. MacLachlan, unpublished observations). This means that a stochastic screening approach of siNA is required to identify duplexes that have retained gene-silencing activity.

Through our work focusing on the design of nonimmunostimulatory siRNA, we have identified 2′-OMe nucleotides as being particularly effective in preventing the recognition of siRNA by the innate immune system (Judge *et al*., 2006a).

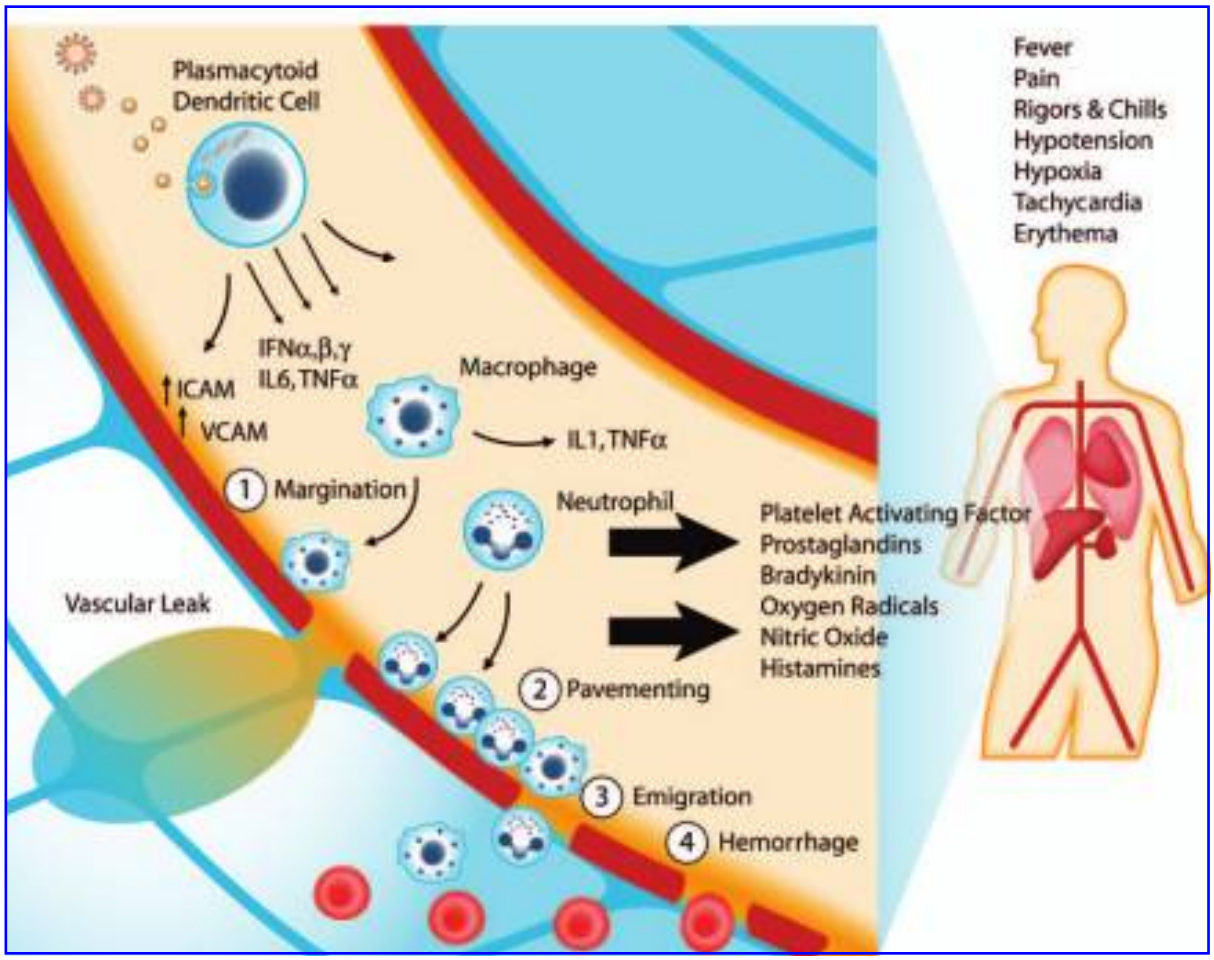

FIG. 3. Downstream effects of innate immune stimulation. IFN-α produced by pDCs acts on surrounding monocytes and macrophages, causing the production of high levels of inflammatory cytokines including IL-6, IL-1β, and TNF-α. Systemic release of IFN-α, IL-6, and IL-1β induces symptoms of fever, rigors, and chills. Cytokines released into the blood cause activation of vascular endothelial cells, resulting in a cascade of effects on blood cells including (1) margination, (2) pavementing, (3) emigration, and (4) hemorrhage, exacerbating lymphopenia and thrombocytopenia. Activation of cells by siRNA, either directly or via the action of cytokines, can upregulate COX-2 and nitric oxide synthase enzyme pathways, resulting in eicosanoid (prostaglandins, etc.) and nitric oxide production, contributing to the symptoms of toxicity including pain. IL-1β and TNF-α acting on vascular endothelium result in the development of hypotension. *Abbreviations*: ICAM, intercellular adhesion molecule; VCAM vascular cell adhesion molecule.

These findings are supported by the work of other groups studying the immunostimulatory effects of RNA (Karikó *et al*., 2005; Cekaite *et al*., 2007). The selective incorporation of 2′-OMe nucleotides into highly immunostimulatory siRNA molecules is sufficient to abrogate induction of the innate immune response and associated toxicities in mice (Judge *et al*., 2006a). Importantly for drug development, we have also observed a similar lack of immune stimulation by 2′-OMe-modified siRNA in both nonhuman primates (Zimmermann *et al*., 2006) and primary human immune cell cultures (Judge *et al*., 2006a), indicating that the inhibitory mechanism is preserved across species. This most likely reflects the profound inhibition or avoidance of TLR7/8 activation by siRNA (Cekaite *et al*., 2007; Robbins *et al*., 2007). Strikingly, inhibition of the immune response requires as few as 2 of 42 native ribonucleotides in a canonical siRNA duplex to be substituted with 2′-OMe nucleotides and requires no other chemical modifications to the siRNA. This effect is mediated by 2′-OMe-uridine, -guanosine, or -adenosine residues, either alone or in combination whereas the incorporation of 2′-OMe-cytidines alone into RNA is significantly less effective in dampening immune stimulation (Karikó *et al*., 2005; Judge *et al*., 2006a). The molecular mechanism underlying this phenomenon remains unclear. Although in some ways analogous to TLR9 activation being inhibited by the direct methylation of CpG dinucleotides within immunostimulatory DNA, the 2′-OMe modification reflects methylation of the ribose sugar backbone rather than the pyrimidine base and there appears to be no strict sequence or positional requirement within RNA for 2′-OMe nucleotides to inhibit TLR7/8 activation. It has been reported that 2′-OMe RNA interacts with TLR7 without signaling (Cekaite *et al*., 2007) and can antagonize TLR7-mediated immune activation by both RNA and small-molecule TLR7 agonists (Robbins *et al*., 2007).

Active siRNA containing more extensive chemical modifications can be generated if nuclease resistance is the primary objective (reviewed in Manoharan, 2004). Several of these published chemical modification strategies have historically used 2′-OMe nucleotides, either alone or in combination with other stabilization chemistries (Czauderna *et al*., 2003; Soutschek *et al*., 2004; Morrissey *et al*., 2005a) and we would anticipate that some of these modified siRNA would avoid significant activation of the immune response. We have confirmed this in two examples (Morrissey *et al*., 2005b; Zimmermann *et al*., 2006).

Because the indiscriminate incorporation of 2′-OMe nucleotides into siRNA can negatively impact RNAi (Czauderna *et al*., 2003; Prakash *et al*., 2005; Judge *et al*., 2006a) it is important to test the modified siRNA alongside the native duplex to confirm the retention of gene-silencing activity. Because only a small number of 2′-OMe nucleotides is required to abrogate the immune response to siRNA, we have adopted a more selective modification approach in which 2′-OMe-uridine or -guanosine residues are preferentially incorporated into the passenger (sense) strand of the duplex to help minimize the potential for attenuating RNAi (Judge *et al*., 2006a). Since our initial report, two studies examining the mechanism of RNAi have shown that the passenger strand undergoes guide strand-directed cleavage between nucleotide positions 9 and 10 as a precursor to efficient assembly of the RNA-induced silencing complex (RISC) (Matranga *et al*., 2005; Leuschner *et al*., 2006). Chemical modification of the nucleotide at position 9 of the passenger strand can inhibit this processing and reduce the efficiency of RISC assembly and resulting RNAi activity (Leuschner *et al*., 2006). Consistent with this observation, we have found that 2′-OMe nucleotides at position 9 in the passenger strand reduce the RNAi activity of certain siRNA duplexes, even in the absence of modifications to the guide strand (A. Judge, unpublished observations). With this in mind, our selective approach to chemical modification has proven to be a reliable method for generating active siRNAs that, in conjunction with a suitable delivery vehicle, are able to mediate RNAi in animals without activating the innate immune response. 2′-OMe nucleotides are a standard chemistry available from many of the RNA suppliers at little or no additional cost over conventional ribonucleotides. Researchers who use synthetic siRNA in their studies should have no trouble adopting this approach to the design of nonimmunostimulatory siRNA.

A number of other siRNA stabilization chemistries influence immune recognition. Locked nucleic acids (LNAs) that contain a 2′-O, 4′-C methylene bridge in the sugar ring have been shown to partially reduce the immunostimulatory activity of siRNA but can also attenuate RNAi activity (Hornung *et al*., 2005). LNA modifications do not appear to display the transinhibitory effect observed with 2′-OMe modifications, whereby 2′-OMe RNA annealed to an immunostimulatory RNA generates a nonimmunostimulatory duplex (Judge *et al*., 2006a; Robbins *et al*., 2007). As we have described, siRNAs containing inverted deoxy abasic end caps remain immunostimulatory whereas some fully modified siNAs with a combination of 2′-F, 2′-H, and 2′-OMe chemistries have minimal immunostimulatory activity (Morrissey *et al*., 2005b). The incorporation of 2′-F-modified nucleotides alone has been reported to reduce immune stimulation (Cekaite *et al*., 2007) although we have found that the inhibitory effects of this chemistry appear to depend on the siRNA sequence and/or the position and extent of the 2′-F-modified nucleotides. As such, we believe the effects of 2′-F and 2′-H nucleotides on the immunostimulatory activity of siRNA are more difficult to predict (A. Judge and I. MacLachlan, unpublished observations). Taken as a whole, these observations suggest that immune stimulation by siRNA can be modulated by a number of different chemical modifications although it appears particularly sensitive to inhibition by the inclusion of 2′-OMe nucleotides versus other well-characterized stabilization chemistries.

Because siRNA may initiate innate immune responses via both TLR-dependent and independent mechanisms it is important that any strategy to prevent immune stimulation be able to inhibit each of these pathways. For siRNA constructs with blunt ends or those synthesized using phage polymerase, the potential for RIG-I activation needs to be considered, even when working in cell lines that do not typically express TLR7/8. In this regard, methodologies have been described that alleviate interferon induction by these classes of siRNA. For transcribed siRNA constructs, T7 siRNA synthesis can be engineered to remove initiating 5′-triphosphates (Kim *et al*., 2004). For long blunt-ended siRNA, it has been found that the original design of incorporating DNA nucleotides into the blunt end to help direct Dicer cleavage (Rose *et al*., 2005) also has the effect of inhibiting RIG-I activation (Marques *et al*., 2006). We have observed similar effects with DNA-modified blunt-ended siRNA in cell lines; however, these siRNAs are still fully capable of

eliciting potent inflammatory responses through TLR7/8-mediated immune stimulation in primary immune cells. RIG-I activation and interferon induction by 5′-triphosphate RNA in monocyte cultures was profoundly inhibited by global substitution of 2′-OMe-uridines into transcribed RNA (Hornung *et al*., 2006), suggesting that 2′-OMe modifications to siRNA can inhibit both TLR7/8 and RIG-I-mediated responses. We have observed similar inhibitory effects with selective 2′-OMe modification of blunt-ended siRNA, generating active duplexes with no immunostimulatory activity in either primary immune cells or RIG-I-sensitive cell lines (A. Judge and I. MacLachlan, unpublished data). It would appear therefore that the incorporation of 2′-OMe nucleotides into immunostimulatory siRNA is able to circumvent both the TLR-dependent and -independent mechanisms primarily responsible for initiating immune responses against these RNA species.

FUTURE DIRECTIONS

The multitude of cell types and mechanisms through which siRNA can interact with the mammalian immune system means that the potential to activate off-target immune-mediated effects is a genuine concern for any researcher using RNAi technologies. The extent to which these immunological pathways may be activated is dependent on the sequence, structure, and quality of the siRNA together with the type of delivery system employed to transfect the siRNA into cells. Therefore, the onus falls on the individual researcher to determine whether or not a particular siRNA formulation activates an immune response within their experimental models. Appropriate study design is required to accurately make this assessment and needs to be conducted on each of the active and control siRNA sequences under consideration.

The implications of activating an immune response for siRNA-based therapeutics run significantly beyond the much discussed "off-target" effects on gene expression associated with the induction of an interferon response. Activation of TLR-mediated immune responses can lead to significant immunotoxicities caused by the release of proinflammatory cytokines and interferons. These are of particular concern for systemic RNAi applications, in which dose-limiting immunotoxicities may manifest at subtherapeutic siRNA doses. Inflammatory responses are also a relevant concern for local routes of drug administration because of injection site reactions and the potential for these to spread systemically. Even in the absence of overt inflammatory reactions, low-grade stimulation of the immune system by siRNA significantly increases the likelihood of an antibody response developing against components of any siRNA delivery vehicle that is used *in vivo*. Antibody responses against the drug conjugate or carrier can severely impair multidose treatment regimens due to rapid drug clearance, loss of tissue targeting, and their potential to trigger hypersensitive reactions in patients when reexposed to the drug.

Fortunately, chemical modification of the native siRNA duplex provides a reproducible method to profoundly inhibit the immune response elicited by siRNA administration. This can alleviate many, if not all, of the problems surrounding immunotoxicity, immunogenicity, and off-target gene effects. Design rules for chemically modified siRNA that successfully abrogate immune stimulation while retaining RNAi activity have been described and no doubt these principles will be further refined. Such progress overcomes one more barrier to the safe and effective development of siRNA-based therapeutics.

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