OLIGONUCLEOTIDES
Volume 19, Number 2, 2009
© Mary Ann Liebert, Inc.
DOI: 10.1089/oli.2009.0180

REVIEW

siRNA and Innate Immunity

Marjorie Robbins, Adam Judge, and Ian MacLachlan

Canonical small interfering RNA (siRNA) duplexes are potent activators of the mammalian innate immune system. The induction of innate immunity by siRNA is dependent on siRNA structure and sequence, method of delivery, and cell type. Synthetic siRNA in delivery vehicles that facilitate cellular uptake can induce high levels of inflammatory cytokines and interferons after systemic administration in mammals and in primary human blood cell cultures. This activation is predominantly mediated by immune cells, normally via a Toll-like receptor (TLR) pathway. The siRNA sequence dependency of these pathways varies with the type and location of the TLR involved. Alternatively nonimmune cell activation may also occur, typically resulting from siRNA interaction with cytoplasmic RNA sensors such as RIG1. As immune activation by siRNA-based drugs represents an undesirable side effect due to the considerable toxicities associated with excessive cytokine release in humans, understanding and abrogating this activity will be a critical component in the development of safe and effective therapeutics. This review describes the intracellular mechanisms of innate immune activation by siRNA, the design of appropriate sequences and chemical modification approaches, and suitable experimental methods for studying their effects, with a view toward reducing siRNA-mediated off-target effects.

Introduction

Innate immune activation via short interfering ribonucleic acid (siRNA) represents a significant undesirable side effect *in vivo* due to the toxicities associated with excessive cytokine release and associated inflammatory syndromes, particularly evident in humans. Despite the allure of harnessing such immune activation for *in vivo* antiangiogenic (Kleinman et al., 2008), anticancer (Poeck et al., 2008), or anti-infective therapies, the particularly exquisite sensitivity of humans to cytokine-mediated toxicities urges caution (Judge and Maclachlan, 2008). Furthermore, an understanding of how to recognize and minimize innate immune effects will be helpful to researchers as they attempt to identify the extent to which specific RNAi is occurring in their models.

Systemic administration of synthetically manufactured siRNA duplexes can activate the innate immune response, inducing high levels of inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), and interferons (IFNs), particularly IFN-alpha (IFNα) (Hornung et al., 2005; Judge et al., 2005). Effective intracellular delivery of siRNA in human peripheral blood mononuclear cells (PBMC) induces significant cytokine release, including high-level IFNα production from plasmacytoid dendritic cells (pDC) *in vitro* (Hornung et al., 2005; Judge et al., 2005). When activated, the innate immune response protects the host from potentially pathogenic infectious agents or other foreign bodies. Innate immunity to siRNA is either Toll-like receptor (TLR)-mediated or non-TLR-mediated. The RNA-sensing TLRs (TLR3, 7, and 8) (Fig. 1) are predominantly located inside the cell and engage nucleic acids released from invading pathogens as they are degraded within the endosomal/lysosomal compartment. TLR3 is also located on the surface of certain cell types (Fig. 1). Non-TLR-mediated innate responses are triggered by siRNA binding to proteins such as retinoic acid inducible gene 1 (RIG1) within the cytoplasm (Fig. 1). If siRNAs are delivered and presented appropriately, they have the capacity to simultaneously activate both TLR-dependent and -independent immune responses.

Although TLRs are activated early in the response to infection they are also important throughout the infection process, linking innate to adaptive immunity. Immunostimulatory siRNAs induce cytokines via innate immune responses but also potentiate the production of antibodies to components of the delivery vehicle (adaptive immunity) (Judge and Maclachlan, 2008). Antibodies to the delivery vehicle are considered a negative result of immune activation via siRNA, since they have the potential

Tekmira Pharmaceuticals Corp., Burnaby, British Columbia, Canada.

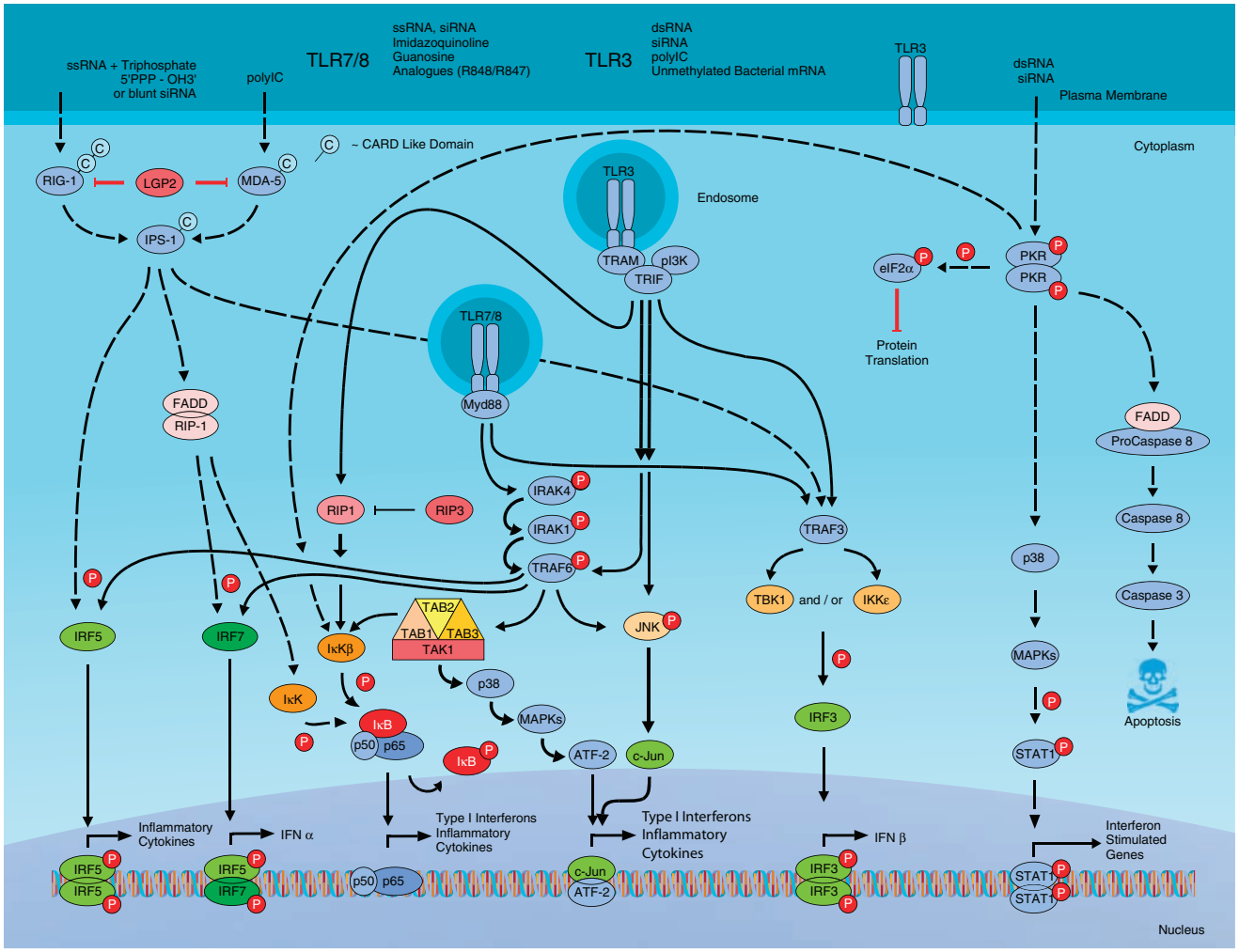

FIG. 1. Immune recognition of siRNA and dsRNA by TLR3/7/8, RIG1/MDA5, and PKR. Signaling through the endosomally located TLR7 and TLR8 occurs via the adaptor protein MyD88, which in turn forms a complex with IRAK-1, IRAK-4, and TRAF6. This results in the activation and nuclear translocation of NF-κB (p50 and p65 subunits) and activation of ATF2-c-Jun transcription factor (leading to expression of inflammatory cytokines) and the IRF7 transcription factor (leading to expression of IFNα subtypes). Endosomal TLR3 signaling occurs through the TRIF adaptor leading to activation of IRF3 and IFNβ expression. TLR3 signaling also activates NF-κB and ATF2-c-Jun causing inflammatory cytokine production. Upon binding to blunt-ended dsRNA or triphosphate-ssRNA, the cytoplasmic protein RIG1 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 trans-phosphorylation 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.

to enhance the clearance and impede the efficiency of subsequent treatments of the siRNA drug.

TLR-Dependent Response to siRNA

Thirteen TLR receptors, TLR1–13, have been identified in mouse and humans. Three TLRs, TLR7 (Heil et al., 2004; Hornung et al., 2005; Gantier et al., 2008), TLR8 (Heil et al., 2004; Forsbach et al., 2008), and TLR3 (Karikó et al., 2004; Kleinman et al., 2008), have been shown to be activated by siRNA. TLR7/8 ligands include siRNA and their constituent RNA oligonucleotides, while TLR3 recognizes only duplex siRNA. Signaling via these intracellular TLR requires endosomal acidification and maturation. This process (and thus TLR-dependent immune activation) can therefore be inhibited by chloroquine and related buffering agents that prevent effective acidification of the endosome (Diebold et al., 2004; Judge et al., 2005).

TLR7 and TLR8

Although TLR8 is phylogenetically related to TLR7 and is activated by structurally similar ligands, the cellular expression patterns of these two receptors are distinct. TLR7 is

constitutively expressed by pDC and B cells and its expression can be up-regulated upon activation of certain other immune cell types. TLR8 is constitutively expressed by myeloid DC (mDC), monocytes, and macrophages in humans (Zarember and Godowski, 2002). It is the cellular expression patterns, rather than the nature of the TLRs themselves, that is likely responsible for the differing cytokine profiles evoked by TLR7 and TLR8 (predominantly pDC-derived IFNα for TLR7 and mDC-derived proinflammatory cytokines for TLR8) (Gorden et al., 2005). Murine TLR8 does not respond to conventional TLR7/8 ligands and, until recently (Gorden et al., 2006), was considered to be nonfunctional in mice. These interspecies variations are an important consideration when comparing RNA-mediated immune activation in murine and human model systems.

TLR7 has been shown to recognize ssRNA viruses (such as vesicular stomatitis virus or influenza), as well as polyuridine and synthetic ssRNA rich in guanosine and uridine (such as RNA40, derived from a GU-rich segment of HIV RNA) (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004). In humans, TLR8 recognizes similar U-rich or GU-rich ssRNA and is also likely activated by RNA viruses (Heil et al., 2004). More recently, it has been suggested that RNA ligands for TLR7 are defined simply by the presence of a ribose sugar backbone and multiple uridine groups in close proximity (Diebold et al., 2006). TLR7 (and likely TLR8) activation is highly dependent on the nucleotide sequence of the RNA, while TLR3 responses to dsRNA are less sequence-dependent. Since the majority of overt innate immune activation by siRNA *in vivo* (ie, systemic cytokine induction) is mediated through TLR7/8 in immune cells, RNA oligonucleotide duplexes can range from being highly potent immune activators to having no apparent immunostimulatory capacity. Perhaps not surprisingly, the same is true for their constitutive oligonucleotide single strands (Hornung et al., 2005; Judge et al., 2005; Sioud, 2005).

The precise nature of the RNA motifs recognized by TLR7/8 remains obscure. We originally 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 immunostimulatory capacity of the duplex, whereas introduction of the 5′-UGU-3′ motif into an siRNA duplex had the opposite effect. By avoiding GU-rich sequences, we have shown that it is possible to select functional siRNA duplexes with inherently low immunostimulatory capacity (Judge et al., 2005). However, it is clear that the 5′-UGU-3′ motif is not the only RNA sequence recognized by TLR7/8 (Diebold et al., 2004; Hornung et al., 2005; Judge et al., 2005; Sioud, 2005; Diebold et al., 2006). This is illustrated by the fact that the large majority of unmodified siRNA sequences demonstrate at least some level of immunostimulatory activity, suggesting that TLR7/8 likely recognize a simple pattern of ribonucleotides occurring at high frequency in conventionally designed synthetic siRNA. This hypothesis has recently been validated by Diebold et al. (2006) who demonstrated that the presence of uridine and a ribose sugar backbone (two hallmarks that distinguish RNA from DNA) are both necessary and sufficient for TLR7 recognition, and that short ssRNA only require several uridines in close proximity to render them effective TLR7 agonists. These findings, consistent with our own observations relating to siRNA duplexes, prompt a redefining of what may be regarded as "basic stimulatory motifs" within RNA. The sequence-dependent nature of the TLR-mediated immune response has significant consequences in the development of siRNA technologies, highlighting the critical requirement for selecting appropriate experimental controls. Genuine RNAi effects must be distinguished from nonspecific effects induced by active and control siRNA sequences with distinct immunostimulatory profiles (Robbins et al., 2008).

Synthetic siRNAs and shRNAs are immunostimulatory when delivered into immune cells, triggering IFNα production from pDCs and inflammatory cytokine production from mDCs (Hornung et al., 2005; Judge et al., 2005; Sioud, 2005). The magnitude of this innate immune response is governed by the siRNA sequence, and in mice is primarily mediated via TLR7 activation (Hornung et al., 2005; Judge et al., 2005). The striking similarities between the responses in mouse and human systems (eg, IFNα induction from pDC, chloroquine sensitivity, and RNA sequence specificity) imply that siRNA activates TLR7 in human immune cells, and is also likely to be a ligand for human TLR8 (Judge et al., 2005). Using TLR7 or TLR8 expressing HEK-293 cells and an NF-κB reporter, Forsbach et al. (2008) showed that a GU-rich RNA oligo with specificity for both TLR7 and TLR8 indeed activated NF-κB in both cell lines. An AU-rich RNA oligo with specificity only for TLR8 activated NF-κB in the TLR8 cell line alone. These data suggest that GU-rich motifs in siRNA will activate both TLR7/8, while AU-rich motifs in siRNA will preferentially activate TLR8.

TLR7/8-mediated recognition and concomitant immune stimulation by siRNA can be inhibited by chemical modification, notably by introduction of 2′-*O*-methyl (2′OMe)-modified nucleotides (Judge et al., 2006). Methylation of the ribose sugar backbone at this site appears to require no positional context to inhibit TLR7/8 activation. As an extension to these studies, we have found that 2′OMe-modified RNA acts as an antagonist to TLR7-mediated immune activation by both ssRNA and small molecule TLR7 agonists (Robbins et al., 2007).

TLR7 and TLR8 signal exclusively through the adaptor protein MyD88 (Fig. 1) (Akira and Takeda, 2004; Takeda and Akira, 2005; Kawai and Akira, 2006). MyD88 contains a C-terminal domain with considerable homology to both TLRs and the IL-1 receptor, a so-called cytoplasmic Toll/interleukin-1R (TIR) domain. MyD88 also contains a region of significant homology to the N-terminal region of TNF-R1 and Fas, a so-called "death domain." MyD88 associates with the TIR domains of TLR7 and TLR8 (Takeda and Akira, 2005) and upon activation, forms a signaling complex with the intermediate molecules IRAK-1, IRAK-4, and TRAF6. The subsequent signaling events and nuclear translocation of NF-κB lead to activation of transcription factors such as IRF5 and IRF7, responsible for up-regulating expression of IFNα and inflammatory cytokines (Akira and Takeda, 2004; Kawai and Akira, 2006). This process occurs rapidly in immune cells that are directly responsive to TLR7 and TLR8 ligands such as pDC and B cells, due to their constitutive expression of the TLRs together with key signaling components including IRF7. The outcome of TLR7/8 engagement has been shown to depend on the spatiotemporal regulation of the MyD88-IRF7 pathway (Asselin-Paturel and Trinchieri, 2005; Honda et al., 2005). Production of inflammatory cytokines, such as TNFα, appears to be promoted when TLR7 engagement and signaling occur in the lysosomal compartment, whereas

production of IFNα occurs when the signals are propagated within early endosomes The balance between these two responses is dictated by the rate of trafficking through the endo/lysosomal compartment, and is influenced by both the structure of the nucleic acid and its mechanism of uptake.

TLR3

Toll-like receptor-3 has a unique gene structure among the TLRs and belongs to its own subfamily distinct from TLR7 and TLR8 (Takeda et al., 2003). At the cellular level, TLR3 expression in human leukocytes is largely restricted to mature mDCs (Muzio et al., 2000) while in mice TLR3 is found in a wider variety of immune cell types (Applequist et al., 2002). Recently, TLR3 expression on the cell surface has been noted in primary human endothelial cells such as lung, aorta, dermis, choroidal, and umbilical vein (Kleinman et al., 2008). TLR3 can also be expressed in a number of other cell types including endothelial, epithelial, and fibroblast lines.

Alexopoulou et al. identified dsRNA as a TLR3 agonist by examining the response of macrophages, splenocytes, or bone-marrow-derived DCs in TLR3 knockout mice to polyinosinic–polycytidylic acid (polyIC), a long synthetic dsRNA homolog (Alexopoulou et al., 2001). Endothelial cell surface TLR3 is also activated by exogenous siRNA of at least 21 nt in length *in vivo*, leading to the production of cytokines such as IFNγ and IL-12 (Kleinman et al., 2008). It is assumed that formulated siRNA, once delivered to cells and thus located intracellularly, for example in the endosome or cytoplasm, is less likely to activate cell surface TLR3. However, formal testing of this hypothesis is complicated by the need to demonstrate the intracellular location of TLR3 in the cells under study. While Kleinman et al. reported that both unformulated (naked) siRNA and siRNA–cholesterol conjugates caused reduction of choroidal neovascularization in TLR3 mice (but not in TLR3–/– mice), it is unclear whether this effect is mediated by activation of cell surface TLR3, intracellular TLR3, or some combination of both. Clearly, further exploration of the mechanism by which siRNA-mediated TLR3 activation may contribute to off-target effects is required.

As well as being evolutionarily distinct from TLR7/8, engagement of TLR3 by dsRNA activates distinct intracellular signaling pathways (Fig. 1). Unlike the other nucleic acid sensing TLRs that signal through MyD88, TLR3 signals through the TRIF adaptor protein (Yamamoto et al., 2003). TRIF signals for the downstream production of IFNβ and IFNα4 via TANK-binding kinase-1 (TBK1), mediating activation of the transcription factor IRF3 (Peters et al., 2002; Yamamoto et al., 2002). A positive feedback mechanism occurs in TLR3 signaling in which the production of IFNβ and IFNα4 signals back to the cell causing the up-regulation of IRF7 and other IFN-inducible genes. This leads to the production of additional IFNα subtypes and amplification of the response (Kawai and Akira, 2006). TLR3 activation through TRIF also activates RIP1 and TRAF6, leading to the downstream activation of NF-κB, ATF, and c-Jun transcription factors and the induction of inflammatory cytokines such as IL-6 and TNFα (Kawai and Akira, 2006).

Cytoplasmic RNA Sensors (non-TLR)

In addition to the TLR-based systems, mammalian cells have evolved a number of TLR-independent mechanisms that recognize cytoplasmic RNA species and are hallmarks of viral infection and replication. These non-TLR-mediated responses to cytoplasmic RNA, including siRNA, are often induced via the dsRNA-binding protein kinase (PKR) or RIG1 proteins (Fig. 1). RIG1 is a cytoplasmic RNA helicase that has recently been shown to play an important role in antiviral defense (Yoneyama et al., 2004; Kato et al., 2005; Kato et al., 2006). RIG1 acts as a sensor of viral RNA via binding and activation by either single-stranded or double-stranded RNA containing uncapped 5′-triphosphates, a characteristic of some viral RNAs (Yoneyama et al., 2004; Hornung et al., 2006; Marques et al., 2006; Pichlmair et al., 2006). These structural features of RIG1 ligands are also found in certain siRNA constructs, including blunt-ended and *in vitro* transcribed siRNA. RIG1 is also IFN-inducible and therefore its expression is rapidly up-regulated in an autocrine or paracrine manner, following initiation of an interferon response.

RIG1 is a DexD/H-box-containing RNA helicase that contains two caspase recruitment domains (CARD) near its N-terminus. Upon dsRNA binding at the C-terminal helicase domain, RIG1 signals for the activation of IRF3 and NF-κB, leading to the production of IFNβ and inflammatory mediators, respectively (Fig. 1) (Yoneyama et al., 2005; Hiscott et al., 2006). RIG1 signaling is transmitted through an adaptor protein variously named IFNβ promoter stimulator 1 (IPS-1; [Kawai et al., 2005]), mitochondrial antiviral signaling (MAVS; [Seth et al., 2005]), virus-induced signaling adaptor (VISA; [Xu et al., 2005]), or Cardif [Meylan et al., 2005]. The adaptor fas-associated protein with death domain (FADD) is also required for dsRNA signaling via RIG1, functioning downstream of the IPS-1 adaptor and upstream of IRF7 for the production of Type I IFN (Balachandran et al., 2007).

Until the identification of TLRs and RIG1, another protein, PKR, was considered to be the primary sensor of viral dsRNA. PKR is an IFN-inducible serine–threonine kinase expressed in most mammalian cells (Meurs et al., 1990; Clemens and Elia, 1997). PKR is activated by N-terminal binding to long dsRNA, causing homodimer formation and autophosphorylation on serine and threonine residues. dsRNA-activated PKR catalyzes the phosphorylation of proteins such as eIF2α and IκB, leading to the general inhibition of translation in addition to activation of NF-κB (Fig. 1) (Meurs et al., 1990; Williams, 2001). Several earlier studies ascribed the siRNA-related up-regulation of interferon-inducible genes to a PKR-mediated effect (eg, [Sledz et al., 2003]); however given our current understanding, it is now believed that RIG1 activation may be responsible for many of these early observations (Kim et al., 2004; Hornung et al., 2006). It is therefore unclear whether canonical siRNA can induce functional activation of the PKR response. PKR can *interact* with dsRNA as short as 11 bp, independent of nucleotide sequence, although PKR *activation* is thought to require >30 bp of contiguous dsRNA (Manche et al., 1992). Recently, higher sensitivity kinase assays have facilitated the observation that synthetic siRNAs, either blunt or 21-mer duplex containing canonical 3′overhangs, are able to mediate some level of PKR activation (Marques et al., 2006; Puthenveetil et al., 2006; Zhang et al., 2006); however, it is unclear from these *in vitro* studies whether this level of PKR activation leads to NF-κB activation or translational inhibition.

Unlike TLR-mediated immune recognition, the recognition of RNA by the cytoplasmic receptors RIG1 and PKR

is considered to be sequence-independent. However, RIG1 and PKR have evolved to recognize certain other physical and structural characteristics of RNA. Following the initial discovery that 21-mer siRNA can initiate RNAi in mammalian cells without activation of the PKR-mediated interferon response (Elbashir et al., 2001; Gitlin et al., 2002), several studies subsequently reported that interferon induction from cell lines may still occur under certain experimental conditions (Bridge et al., 2003; Sledz et al., 2003; Kim et al., 2004). Kim et al. demonstrated that phage polymerase-transcribed siRNA activated interferon responses when transfecting nonhematopoietic cell lines, due to the presence of uncapped 5′-triphosphate groups that characterize such RNA (Kim et al., 2004). This observation likely explains earlier reports of IFN induction in RNAi experiments that also utilized nonsynthetic siRNA (Sledz et al., 2003). The causal molecular mechanisms for these effects have now been elucidated. It appears that RNA containing uncapped 5′-triphosphate groups binds to and activates RIG1, resulting in potent IFN responses from RIG1-expressing cells (Hornung et al., 2006; Pichlmair et al., 2006). Uncapped 5′-triphosphate groups on RNA therefore represent a pathogen-associated molecular pattern that helps distinguish virally derived from host RNA. For siRNA constructs synthesized using phage polymerase, the induction of an interferon response through RIG1 activation needs to be considered, even when working in cell lines that do not express TLR7/8. In this regard, Kim et al. provide a methodology for engineering T7 siRNA synthesis that removes the initiating 5′-triphosphate, thereby alleviating interferon induction by this class of siRNA (Kim et al., 2004).

Further studies have highlighted additional structural features of chemically synthesized siRNA that can activate the RIG1-mediated interferon pathway (Marques et al., 2006). Asymmetrical siRNA possessing a standard 3′ overhang at one end and a blunt 5′-antisense end have become more widely used in an attempt to improve RNAi potency (Soutschek et al., 2004; Kim et al., 2005). Marques et al. (2006) have subsequently demonstrated that blunt-ended RNA, including siRNA, can also bind to and activate RIG1. Again, this effect appears to be independent of the RNA sequence but is enhanced as the length of the oligonuceotide increases from 21 to 27 base pairs, and is reduced if the blunt end is converted to an overhang. This study also reported the interesting observation that certain batches of conventional 21-mer siRNA with 3′ overhangs could activate IFN responses equivalent to those induced by blunt-ended or 5′-triphosphate siRNA. This appears to be associated with siRNA batches of poor quality or purity, presumably containing uncharacterized RNA products capable of activating RIG1 or one of the other cytoplasmic RNA receptors. Such variability between siRNA batches and suppliers means individual researchers should determine whether or not their particular siRNA preparations are capable of activating an interferon response, even when working with cell lines *in vitro*. Unfortunately, it would appear insufficient to rely solely on the generalized principles described in the literature to make this assessment.

Nonhematological cell lines do not typically express TLR7/8 and therefore do not manifest the robust innate immune response to siRNA that is readily observed both *in vivo* and in primary blood cell cultures. However, unlike RNA-sensing TLR, cytoplasmic RNA receptors are expressed in a wider variety of mammalian cell types, providing the cells with an autonomous antiviral defense mechanism. As structural features of certain transcribed and synthetic siRNA have been shown to activate the RIG1 pathway, it is not surprising that cell lines that naturally express high levels of RIG1, such as the lung fibroblast MRC5, or glioblastoma T98G, mount strong interferon responses when transfected with blunt-ended siRNA (Marques et al., 2006). In our experience, blunt-ended siRNA can also induce interferon responses (albeit more weakly), in a variety of commonly used human cell lines of diverse lineage. The magnitude of the response is presumably dictated by the differential expression levels of RIG1 or equivalent RNA receptors.

A given siRNA duplex may contain multiple structural, physical, and sequence-related features that are recognized by the various immune receptors. As such, a particular siRNA may be able to simultaneously activate both TLR and non-TLR pathways, depending on its structure and sequence and which cell types are transfected. For example, an unmodified, blunt-ended siRNA such as the 21/23-mer ApoB1 duplex can be a potent activator of both TLR7/8 as it traffics through the endosomal compartment (Judge et al., 2006) and RIG1 when it is released into the cell cytoplasm ([Marques et al., 2006] and Adam Judge, Marjorie Robbins, 2007 unpublished data). Each pathway results in the induction of type I interferons and may contribute to the toxic and off-target effects observed with these unmodified siRNA. It is important therefore that the ability of a chosen siRNA to activate any one of multiple pathways is assessed.

Cell culture conditions can also directly affect the sensitivity of cells to siRNA-mediated activation. For example, PKR, RIG1, and a number of TLRs including TLR3 and TLR7 are themselves induced by interferons, while critical components of these signaling pathways can also be up-regulated by 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. In addition, the ability of siRNA to activate an immune response is influenced by a number of other factors including the chemical structure and the mode of intracellular delivery, which must be taken into account. Each of these is discussed in more detail below.

Cellular Uptake

Due to the poor cellular uptake of naked siRNA, effective intracellular delivery requires some means of enablement (Kleinman et al., 2008). For this reason, the use of delivery vehicles to present siRNA to the cytoplasmic RNAi machinery is essential for most researchers. The most widely used vehicles for this purpose are based on positively charged (cationic) agents that complex with, or encapsulate, the negatively charged nucleic acid. These include cationic lipid formulations (eg, Lipofectamine) and cationic polymers (eg, polyethylenimine [PEI]). Both form positively charged complexes with siRNA and facilitate intracellular delivery through electrostatic interactions with the membrane of the target cell. Importantly, these delivery vehicles are typically taken up into cells by endocytosis, and are concentrated in an endosomal compartment prior to releasing siRNA into the cytoplasm. These are the very cellular compartments where the RNA-sensing pattern recognition receptors

are localized. It is worth noting that immunologists have exploited this feature of cationic lipids and polycations to potentiate the immunostimulatory effects of RNA and DNA for many years. It should therefore come as no surprise that similar effects are also observed with siRNA.

Naked siRNA molecules do not typically activate an immune response in blood cells either *in vitro* or *in vivo* (Heidel et al., 2004; Judge et al., 2005). The relationship between siRNA delivery and immune stimulation reflects a number of factors: (1) The relative efficiency of intracellular uptake; (2) enhanced bioavailability and exposure to innate immune cells *in vivo*; (3) protection of the siRNA from rapid nuclease degradation; and (4) altered physical characteristics of the siRNA complex resulting in more efficient engagement of immune receptors. The nature of the delivery vehicle utilized in a given experiment can have a dramatic effect on both the quality and magnitude of the immune response to siRNA. For example, human blood cells stimulated with siRNA encapsulated in stable nucleic acid lipid particles (SNALP) or complexed with PEI exhibit a response dominated by IFNα secretion. In contrast Lipofectamine and polylysine, which form larger, more heterogeneous complexes with siRNA, tend to elicit a predominantly inflammatory cytokine biased response from the same human blood cell population (Judge et al., 2005).

We have found that a number of parameters affect how delivery vehicles potentiate immune stimulation by a particular siRNA duplex. These include the chemical composition of the delivery vehicle as well as physical properties such as the size and charge ratio of the formulated material. *In vivo*, additional variables also need to be considered including the pharmacokinetics and biodistribution of the formulated siRNA, as well as the route of administration. We have noted that differences in these parameters can result in the magnitude of the immune response changing by greater than 100-fold. In our experience to date however, we have found that siRNA duplexes with inherent immunostimulatory activity will 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 (SNALP).

Chemical Modifications

Stabilization of synthetic siRNA against rapid nuclease degradation is often regarded as a prerequisite for *in vivo* and therapeutic applications. This is achieved using chemistries previously developed to achieve the stabilization of ribozymes or antisense oligonucleotide drugs (Manoharan, 2004). These include chemical modifications to the 2′-OH group in the ribose sugar backbone such as 2′-*O*-methyl (2′OMe) and 2′-fluoro (2′F) substitutions, and are incorporated as 2′-modified nucleotides during oligonucleotide synthesis. A number of reports have shown that siRNA containing 2′OMe, 2′F, 2′-deoxy (2′-H), or "locked nucleic acid" (LNA) modifications may retain functional RNAi activity, indicating that these chemistries can be compatible with the RNAi machinery (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). However, these modifications to siRNA appear to be tolerated only in certain ill-defined positional or sequence-related contexts as, in many cases, their introduction can have a negative impact on RNAi activity (Chiu and Rana, 2003; Czauderna et al., 2003; Elmen et al., 2005; Hornung et al., 2005; Prakash et al., 2005).

The use of appropriate delivery vehicles that confer nuclease protection to the encapsulated or complexed siRNA can obviate the need to use siRNA stabilization chemistries as a means of protection from nuclease degradation. However these same chemistries, in particular the use of 2′-OMe nucleotides, have also been revealed to prevent recognition of the siRNA by the innate immune system (Judge et al., 2006; Cekaite et al., 2007). This additional benefit can have the striking result of rendering siRNA immunologically inert, without needing to modify the basic RNA sequence, and so avoiding this particular potential for loss of RNAi activity.

One chemical modification strategy to generate siRNA suitable for use as naked molecules involves the systematic substitution of all ribonucleotides with 2′-F, 2′-OMe, or 2′-deoxy nucleotides based on the pattern of purines and pyrimidines in the given sequence (Chiu and Rana, 2003; Morrissey, Blanchard, et al., 2005; Morrissey, Lockridge, et al., 2005). We have found that these siNA molecules (so-called as they contain no native ribose sugar) exhibit only minimal immunostimulatory activity compared to the native siRNA sequence when administered to mice in lipidic formulations (Morrissey, Lockridge, et al., 2005). This is perhaps unsurprising given the fact that the ribose sugar is a definitive characteristic of RNA and therefore likely plays an integral role in the recognition of RNA by nucleic acid sensing receptors. This has recently been directly demonstrated in the case of TLR7-mediated recognition of RNA (Diebold et al., 2006). From the initial studies in which multiple stabilization chemistries were utilized in the siNA duplex, it was not possible to determine which chemical modifications in particular were responsible for abrogating immune stimulation. Furthermore, the application of this chemical modification strategy frequently causes significant inhibition of RNAi activity, and calls for a stochastic screening approach to select modified duplexes that retain gene silencing activity (Chiu and Rana, 2003).

We have found that the selective incorporation of as few as two 2′OMe guanosine or uridine residues in the sense strand of highly immunostimulatory siRNA molecules is sufficient to abrogate siRNA-mediated interferon and inflammatory cytokine induction in human PBMC and in mice *in vivo* (Judge et al., 2006). This most likely reflects profound inhibition of the TLR7/8-mediated pathway that dominates the response to siRNA in immunological settings. Strikingly, the degree of 2′OMe modification required to exert this profound anti-inflammatory effect on the RNA represents ~5% of the native 2′-OH positions in the siRNA duplex and no other modifications are required. This can be achieved using either 2′OMe-uridine, -guanosine, or -adenosine residues in any combination (surprisingly, the incorporation of 2′OMe-cytidines by themselves has significantly less effect in dampening immune stimulation [Karikó et al., 2005; Judge et al., 2006]). Coupled with a suitable delivery vehicle, these minimally modified siRNAs are able to mediate potent RNAi effects *in vitro* and *in vivo* without induction of a detectable cytokine response (Judge et al., 2006).

Recently, we have revised our modification strategy to suggest that researchers consider selective modification of both strands of the siRNA to eliminate subtle residual immune stimulation that can still manifest as antibody production *in vivo* (Judge et al., 2009). The incorporation of 2′OMe nucleotides in the antisense strand of an siRNA duplex, however, can impact the gene silencing activity of a given siRNA sequence (Czauderna et al., 2003; Prakash et al., 2005; Judge et al., 2006). Therefore, testing of the modified duplex should be conducted alongside the native duplex to confirm RNAi potency has been retained. We have found that 2′OMe modification of <20% of the total nucleotides is generally well tolerated ([Judge et al., 2006] and Judge et al. (2009). Since our original description of this chemical modification strategy (Judge et al., 2006), two studies examining the mechanism of RNAi have shown that the sense strand of an siRNA duplex is typically cleaved as a precursor to efficient assembly of the RISC complex (Matranga et al., 2005; Leuschner et al., 2006). This process follows the same rules established for the siRNA-guided cleavage of a target mRNA, resulting in RISC-directed cleavage between positions 9 and 10 of the sense strand. Leuschner et al. showed that chemical modification of the nucleotide at position 9 of the sense strand (or introduction of a phosphorothioate linker between nucleotides 9 and 10) could inhibit sense strand cleavage and subsequently reduce the efficiency of RISC assembly and RNAi activity (Leuschner et al., 2006). Similarly, we have found that the introduction of 2′OMe nucleotides at position 9 in the sense strand can reduce RNAi activity, even in the absence of any chemical modifications to the antisense strand of the siRNA duplex (Adam Judge, 2006; unpublished observations). Although this negative effect is not absolute and does not appear to affect all duplexes, the chemical modification of this particular sense strand nucleotide is best avoided when designing siRNA to have minimal immunostimulatory activity.

It has been suggested that human TLR may be preferentially activated by pathogen-derived RNA based on their lack of modified nucleosides that typically occur at much higher frequency in most mammalian RNA (Karikó et al., 2005). The introduction of a variety of naturally occurring modified nucleosides, including 2′OMe nucleosides, into RNA was shown to inhibit TLR activation and immune stimulation (Karikó et al., 2005). Findings with 2′OMe-modified siRNA are consistent with this report, particularly with respect to the low degree of modification required to abrogate RNA-mediated immune stimulation and the differential effects of modifying uridine versus cytidine residues on the activation of primary immune cells. This suggests that 2′OMe-modified siRNA may avoid recognition by the innate immune system due to an evolutionary mechanism by which immune receptors distinguish pathogen-derived RNA from host cell RNA.

Chemical modification of siRNA can also block their interaction with RIG1 in the cell cytoplasm, thereby inhibiting interferon responses that are induced via this pathway. Marques et al. reduced but did not eliminate this immune recognition by the incorporation of two DNA nucleotides into the blunt end of the siRNA (Marques et al., 2006). We have confirmed these observations in our own unpublished studies; however, DNA-modified blunt-ended siRNAs are still fully capable of eliciting potent inflammatory responses through TLR7/8-mediated immune stimulation. Initial reports describing RIG1 activation by 5′-triphosphate RNA indicated that global substitution of uridines with 2′OMe-uridine in these transcribed RNA profoundly inhibited interferon induction in monocyte cultures (Hornung et al., 2006). This implies that 2′OMe nucleotides are also able to inhibit the recognition of stimulatory RNA by RIG1. We and others have found that 2′OMe modification to blunt-ended siRNA not only inhibits TLR7/8-mediated immune activation, but also abrogates the interferon response in RIG1-sensitive cell lines such as T98G and MRC5 (Adam Judge, Marjorie Robbins, 2007 unpublished data; [Collingwood et al., 2008; Zamanian-Daryoush et al., 2008]).

It is unclear how the introduction of 2′OMe nucleotides prevents recognition of the duplex RNA by the immune system, as the exact nature of the physical interaction between TLR7/8 and RNA is unknown. This is also true for other defined TLR7 and TLR8 ligands such as the commonly used guanosine analogs (Lee et al., 2003). Further work is therefore required to confirm the precise mechanism before the molecular basis underlying the inhibitory effects of 2′OMe modification can be fully elucidated. However, it is interesting to note the ability of 2′OMe RNA to inhibit immune stimulation by small molecule TLR7 agonists, suggesting some form of TLR7 antagonism (Robbins et al., 2007).

A number of other stabilization chemistries are routinely used in synthetic siRNA design that may also influence immune recognition and RNAi potency. Locked nucleic acids (LNA) that contain a 2′-O, 4′-C methylene bridge in the sugar ring have been shown to partially reduce the immunostimulatory activity of an siRNA (Hornung et al., 2005), while siRNAs containing inverted deoxy abasic end caps retain immunostimulatory activity (Morrissey, Lockridge, et al., 2005). No evidence of a trans-inhibitory effect similar to that observed with 2′OMe modifications (whereby 2′OMe-modified RNA annealed to unmodified immunostimulatory RNA generates a nonimmunostimulatory duplex [Judge et al., 2006; Robbins et al., 2007]) has been observed with LNA-modified duplexes. As we have described, a complex combination of 2′-F, 2′-H, and 2′-OMe chemistries can generate a fully modified siNA duplex with minimal immunostimulatory activity (Morrissey, D.V., Lockridge et al., 2005). The introduction of 2′-F-modified nucleotides into RNA 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 modified nucleotides. As such, the use of 2′-F and 2′-H chemistries alone tends to have unpredictable effects on the immunostimulatory activity of the modified siRNA (Adam Judge, Ian MacLachlan, 2005 unpublished observations). Taken as a whole, these observations suggest that immune stimulation by siRNA is particularly sensitive to inhibition by 2′OMe modifications versus other established stabilization chemistries.

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 siRNA or siRNA-targeting genes that are not present in the mammalian genome, such as green fluorescent protein (GFP) or firefly luciferase (Luc), were once considered adequate controls. Since the inherent immunostimulatory activity of siRNA is

sequence-dependent, a certain amount of screening may be required to ensure that negative control siRNAs are as immunostimulatory as their active counterparts (eg, Geisbert et al., 2006). A preferred approach would be to utilize chemically modified siRNA with minimal residual immunostimulatory activity for both active and control duplexes and to confirm this within the context of the disease model in question. This may help avoid the misinterpretation of preclinical results (see Robbins et al., 2008).

Measuring Immunostimulation

As the nature of the cytokine response to siRNA can differ according to timing, cell type, delivery vehicle, route of administration, and so on, the absence of immune stimulation at a single time point for a single cytokine does not necessarily indicate the absence of immune stimulation in general. Antibodies and ELISA kits are widely available for the determination of IFNα, IFNβ, and a great variety of inflammatory cytokines. For *in vivo* studies, testing for both IFNα and suitable inflammatory cytokines (including IL-6 and TNFα) in the plasma of treated animals is recommended to monitor for systemic activation of the innate immune response. A similar panel of cytokines can also be used to test for stimulation of primary immune cell cultures *in vitro*. For cell lines, the detection of IFNβ, IL-6, or the chemokine IL-8 in the supernatant of treated cells can be used as a measure of siRNA-mediated activation through the RIG1/MDA5/PKR pathways. However, the secretion of these cytokines is cell-line-dependent and cannot be used in isolation to determine whether these cytoplasmic RNA receptor pathways have been engaged.

Measurement of the immune response at an appropriate time after siRNA treatment is critical in order to make a valid assessment. Systemic administration of immunostimulatory siRNA formulations to mice can lead to detectable elevations in serum cytokines within 1–2 hours, with the timing dependent on the type of delivery vehicle and the cytokine being assessed. It is important to note however that elevations in serum cytokines are transient and typically resolve within 12–24 hours after initial treatment. This is true even for highly stimulatory siRNA that can induce ng/mL quantities of cytokines in the blood. By way of example, intravenous administration of lipidic (SNALP) formulations containing immune stimulatory siRNA leads to elevations in serum IL-6 by 2 hours, with a peak between 4 and 6 hours and resolution by 16 hours after a single treatment. IFNα follows a similar pattern but with a 2-hour delay in peak serum levels. It should again be emphasized however that these time frames are entirely dependent on the nature of the delivery vehicle. It is therefore important that researchers define this parameter within their own models and select an appropriate time point to capture this aspect of the innate immune response. The assessment of cytokine responses from *in vitro* cell cultures is less time-sensitive, since the secreted cytokines tend to accumulate in the tissue culture supernatant. Therefore, the measurement of cytokines at a single time point 16–24 hours after siRNA treatment is often adequate for detection of a response. However, this does not reflect the dynamics of the cytokine response *per se*; researchers who choose to monitor cytokine induction through mRNA analysis also need to define the most appropriate time point for sample collection (eg, Marques et al., 2006).

Although the measurement of secreted cytokines is both an efficient and meaningful readout of innate immune stimulation, it is not necessarily the most sensitive method to assess the response. Great care should therefore be taken when interpreting negative cytokine data; the absence of proof is not proof of absence. Within our laboratory we have utilized 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 than plasma cytokines (Robbins et al., 2008) (Judge et al., 2009). We have measured strong IFIT1 mRNA induction in both liver and spleen of siRNA-treated mice in the absence of any detectable plasma IFNα protein or related immunotoxicity. This likely reflects low-level, local IFN induction that does not manifest as a systemic cytokine response. Alternatively, direct induction of IFIT1 via activation of IRF3 in the absence of type I IFN protein has also been reported (Geiss et al., 2001). Although the immunostimulatory activity that can be detected by IFIT1 mRNA analysis is not sufficient to cause overt symptoms of toxicity in mice, it is clearly associated with off-target effects in gene expression patterns. It will be up to individual researchers to demonstrate whether or not such low-grade responses to siRNA are of sufficient magnitude to mediate nonspecific antiviral or antitumoral effects in preclinical models.

Off-Target Effects

Although it is now well known that synthetically manufactured siRNA duplexes can induce high levels of inflammatory cytokines and Type I interferons following systemic administration in mammals (Hornung et al., 2005; Judge et al., 2005), very little of the literature is devoted to determining the extent to which these effects may contribute to the "efficacy" associated with the use of siRNA. siRNAs have been reported as efficacious in several *in vivo* virus infection models, including influenza A (Ge et al., 2004; Tompkins et al., 2004), herpes simplex virus (HSV) (Palliser et al., 2006), respiratory syncitial virus (RSV) (Bitko et al., 2005), parainfluenza (PIV) (Bitko et al., 2005), and hepatitis B virus (HBV) (Morrissey, Lockridge, et al., 2005) infections 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). Activation of innate immunity and production of cytokines such as type I interferon are known to inhibit viral infection. For example, polyI:C in complex with poly-l-lysine and methylcellulose (polyICLC), either alone or encapsulated in liposomes, protects mice from otherwise lethal Influenza A infection (Saravolac et al., 2001). In spite of the obvious interplay between immune stimulation and antiviral activity, at the time of writing, only one published study has compared chemically modified to unmodified (immunostimulatory) siRNA (Morrissey, Lockridge, et al., 2005). Here researchers found that nonspecific reduction of Hepatitis B viral titer (HBV) *does* occur *in vivo* when using unmodified, immunostimulatory control siRNA. Chemical modification of both control and active siRNAs, in a manner that abrogates the siRNAs' immunostimulatory activity, prevents the nonspecific efficacy associated with the control while maintaining specific antiviral efficacy of the active siRNA (Morrissey, Lockridge, et al., 2005 and see Robbins et al., 2008). This serves to highlight not only the potential

contribution of immune stimulation to antiviral efficacy but also one of the strategies by which one may abrogate the effect.

The potential for immune stimulation to manifest as nonspecific "efficacy" is not confined to antiviral applications. In oncology, an area of intense interest to investigators working in RNAi, the immunostimulatory effects of nucleic acids have been intentionally harnessed for therapeutic benefit (Foss, 2002; Krieg, 2006; Brannon-Peppas et al., 2007). Furthermore, several interferons are approved for use as drugs in humans and IFNα in particular is used to treat many types of cancer. In spite of the well-known antitumor effects associated with stimulation of the innate immune system, attempts to characterize the extent to which siRNA-mediated immune stimulation contributes to antitumor efficacy are in the minority (Santel et al., 2006). Interestingly, siRNA-mediated immunostimulation has recently been harnessed for therapy of melanoma (Poeck et al., 2008). Unfortunately, Poeck et al. do not determine the extent to which RNAi is responsible for the antitumor effect in their model *in vivo* by using modified siRNA as a negative control for immunostimulation. Again, investigators should be encouraged to determine the extent to which RNAi-mediated effects occur in their models in the absence of immunostimulation.

One mechanism by which IFN may contribute to antitumor effects involves the antiangiogenic activity of IFNα and IFNβ (Folkman and Ingber, 1992). IFN inhibits the ability of endothelial cells to form new blood capillaries, with IFN acting specifically to suppress VEGF expression at the mRNA level (von Marschall et al., 2003; Wu et al., 2005). This highlights another area where the interpretation of preclinical results may be confounded by the immunostimulatory activity of active siRNA. The VEGF pathway is an attractive target for other applications, in addition to oncology. Macular degeneration, an eye disease characterized by dysregulated macular capillary growth, has been targeted using siRNA directed against VEGF. Given that unintended immune stimulation and concomitant IFN production have the potential to down-regulate both VEGF protein and mRNA, confirmation that VEGF knockdown is mediated by RNAi and not via an interferon response induced by the active siRNA is fundamental to the interpretation of experiments targeting VEGF (see [Kleinman et al., 2008; Robbins et al., 2008]). Recent work by Kleinman et al. suggests that siRNA-mediated immune stimulation via TLR3 has the potential to manifest as antiangiogensis in the absence of bona fide RNAi.

Toxicology

In mammals, the recognition of canonical siRNA duplexes as a foreign entity by the TLR system acts as a trigger for the rapid release of inflammatory mediators as part of the host's natural defense against pathogens. The use of delivery systems to confer the required drug-like properties to siRNA promotes the immune response (Hornung et al., 2005; Judge et al., 2005), while "naked" siRNA, in the absence of a delivery system, are thought not to stimulate TLR7/8 (Heidel et al., 2004) (naked [nonformulated] siRNA duplexes of 21 bp or longer have been reported to activate cell surface TLR3 on endothelial cells, causing nonspecific antiangiogenic effects in models of choroidal neovascularization [Kleinman et al., 2008]). TLR7 and TLR8 expression allow innate immune cells to respond directly to siRNA. pDC respond by producing large amounts of IFNα that activates a generalized antiviral state in the body that includes the maturation of antigen-presenting cells (APCs) that can initiate potent T-cell responses (Ito et al., 2001). Soluble factors that are released by pDC in this initial phase of the reaction, including IFNα, also recruit and activate other immune cells to amplify the inflammatory response. This is brought about in part by the up-regulation of TLR7 on monocytes, macrophages, NK cells, and neutrophils, enabling these cells to respond to siRNA (Kadowaki et al., 2001; Hornung et al., 2002). IFNα signaling can also induce up-regulation of the enzymes cyclooxygenase-2 (COX-2) (Yeo et al., 2003) and nitric oxide synthase (iNOS) (Sharara et al., 1997), resulting in the biosynthesis of eicosanoids and nitric oxide, respectively. In this manner, immunostimulatory siRNA can trigger an inflammatory cascade culminating in the high-level production of an array of cytokines, plus other vasoactive and inflammatory mediators. In our experience, these inflammatory reactions can also exacerbate any underlying chemical toxicities associated with the siRNA or delivery vehicle.

Although beneficial in host defense, overstimulation of TLR-mediated pathways is pathological. Treatment of humans with the TLR ligands 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 flu-like symptoms: fever, chills, rigors, and hypotension. Onset of symptoms typically occurs within hours of 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. Reasons for this remain unclear, but it may reflect interspecies differences in either TLR signaling or differential sensitivity to the ensuing inflammatory response. Although the clinical syndrome induced by these agents is multifactorial, reflecting the complexity of the inflammatory cascade, many of the symptoms can be directly attributable to the action of cytokines. Evidence to support this comes from the clinical experience with recombinant cytokines as therapeutics, such as IFNα, interleukin-1β (IL-1β), and TNFα, where dose-limiting toxicities typically include fever, chills, rigors, and hypotension.

In our experience, preclinical toxicology in mice has proven to be nonpredictive for the severe immunotoxicities observed in human subjects after the intravenous administration of immunostimulatory plasmid DNA (Adam Judge, Ian MacLachlan, 2004; unpublished data). However, the discovery that these symptoms of toxicity in humans correlated with significant increases in blood cytokines, and that the response to the DNA-based drug could be replicated *in vitro* using TLR9-expressing human blood cells, provides us a better understanding with which to extrapolate the effects of immune stimulation by siRNA-based drugs in preclinical models.

Although rat and mouse models successfully reproduce several aspects of the cytokine response and associated toxicities 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 hypotension). Neither do they predict the low doses at which immunotoxicities manifest in humans

(<0.003 mg plasmid DNA/kg body weight). By our understanding, no preclinical model has been described for a DNA-based therapeutic that is predictive of the human response to low-dose, systemic administration of lipid-formulated immunostimulatory DNA. However, the preclinical toxicities associated with DNA-based drugs are typically ascribed to TLR9-mediated immune stimulation, and this can be partly modeled in a human system using primary immune cell cultures containing TLR9-expressing pDC. It is proposed therefore that the etiologies of the toxicities observed in preclinical models and in humans are fundamentally similar; the difference between species lies in the degree of sensitivity to the DNA and its immunostimulatory effects. In this respect, we find no reason to believe that the clinical effects of a TLR7/8-mediated immune response to an immune stimulatory siRNA-based drug would be fundamentally different from that of an immune stimulatory DNA-based drug.

Conclusion

Due to the multitude of cell types and mechanisms through which siRNA can interact with the mammalian immune system, the potential to activate off-target immune-mediated effects is a consideration for any researcher utilizing RNAi technologies. The extent to which these immunological pathways may be activated is dependent upon 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 where dose-limiting immunotoxicities may manifest at subtherapeutic siRNA doses. Inflammatory responses are also a relevant concern for local routes of drug administration, due to 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 utilized *in vivo*. Antibody responses against the drug conjugate or carrier can severely impair multidose treatment regimes due to rapid drug clearance, loss of tissue targeting, and their potential to trigger hypersensitive reactions in patients when reexposed to the drug.

Fortunately, relatively simple ways have been identified to engineer siRNA that avoid recognition by TLRs and other nucleic acid sensing receptors. In particular, this can be achieved by introducing chemically modified nucleotides into the siRNA duplex in a manner that does not significantly impact RNAi activity. These chemistries are commonly used in RNA synthesis and are readily available to researchers through conventional suppliers. The progress made in this aspect of siRNA design illustrates how chemical modification strategies can overcome many, if not all, of the challenges associated with the immunostimulatory potential of siRNA. We are confident that through the judicious application of well-informed siRNA design and the use of increasingly effective delivery systems, demonstrations of systemic RNAi in human subjects will soon be realized.

References

AKIRA, S., and TAKEDA, K. (2004). Toll-like receptor signalling. Nat. Rev. Immunol. **4**, 499–511.

ALEXOPOULOU, L., CZOPIK-HOLT, A., MEDZHITOV, R., and FLAVELL, R. (2001). Recognition of double-stranded RNA and activation of NF-KB by toll-like receptor 3. Nature. **413**, 732–738.

ALLERSON, C.R., SIOUFI, N., JARRES, R., PRAKASH, T.P., NAIK, N., BERDEJA, A., WANDERS, L., GRIFFEY, R.H., SWAYZE, E.E., and BHAT, B. (2005). Fully 2′-modified oligonucleotide duplexes with improved *in vitro* potency and stability compared to unmodified small interfering RNA. J. Med. Chem. **48**, 901–904.

APPLEQUIST, S.E., WALLIN, R.P., and LJUNGGREN, H.G. (2002). Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines. Int. Immunol. **14**, 1065–1074.

ASSELIN-PATUREL, C., and TRINCHIERI, G. (2005). Production of type I interferons: plasmacytoid dendritic cells and beyond. J. Exp. Med. **202**, 461–465.

BALACHANDRAN, S., VENKATARAMAN, T., FISHER, P.B., and BARBER, G.N. (2007). Fas-associated death domain-containing protein-mediated antiviral innate immune signaling involves the regulation of Irf7. J. Immunol. **178**, 2429–2439.

BITKO, V., MUSIYENKO, A., SHULYAYEVA, O., and BARIK, S. (2005). Inhibition of respiratory viruses by nasally administered siRNA. Nat. Med. **11**, 50–55.

BRANNON-PEPPAS, L., GHOSN, B., ROY, K., and CORNETTA, K. (2007). Encapsulation of nucleic acids and opportunities for cancer treatment. Pharm. Res. **24**, 618–627.

BRIDGE, A.J., PEBERNARD, S., DUCRAUX, A., NICOULAZ, A.-L., and IGGO, R. (2003). Induction of an interferon response by RNAi vectors in mammalian cells. Nat. Genet. **34**, 263–264.

CEKAITE, L., FURSET, G., HOVIG, E., and SIOUD, M. (2007). Gene Expression Analysis in Blood Cells in Response to Unmodified and 2′-Modified siRNAs Reveals TLR-dependent and Independent Effects. J. Mol. Biol. **365**, 90–108.

CHIU, Y., and RANA, T. (2003). siRNA function in RNAi: A chemical modification analysis. RNA. **9**, 1034–1048.

CLEMENS, M.J., and ELIA, A. (1997). The double-stranded RNA-dependent protein kinase PKR: structure and function. J. Interferon Cytokine Res. **17**, 503–524.

COLLINGWOOD, M.A., ROSE, S.D., HUANG, L., HILLIER, C., AMARZGUIOUI, M., WIIGER, M.T., SOIFER, H.S., ROSSI, J.J., and BEHLKE, M.A. (2008). Chemical modification patterns compatible with high potency dicer-substrate small interfering RNAs. Oligonucleotides. **18**, 187–200.

CZAUDERNA, F., FECHTNER, M., DAMES, S., AYGÜN, H., KLIPPEL, A., PRONK, G.J., GIESE, K., and KAUFMANN, J. (2003). Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. Nucleic Acids Res. **31**, 2705–2716.

DER, S.D., ZHOU, A., WILLIAMS, B.R., and SILVERMAN, R.H. (1998). Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc. Natl. Acad. Sci. USA. **95**, 15623–15628.

DIEBOLD, S.S., KAISHO, T., HEMMI, H., AKIRA, S., and REISE SOUSA, C. (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science. **303**, 1529–1531.

DIEBOLD, S.S., MASSACRIER, C., AKIRA, S., PATUREL, C., MOREL, Y., and REIS e SOUSA, C. (2006). Nucleic acid agonists for Toll-like receptor 7 are defined by the presence of uridine ribonucleotides. Eur. J. Immunol. **36**, 3256–3267.

ELBASHIR, S.M., HARBORTH, J., LENDECKEL, W., YALCIN, A., WEBER, K., and TUSCHL, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. **411**, 494–498.

ELMEN, J., THONBERG, H., LJUNGBERG, K., FRIEDEN, M., WESTERGAARD, M., XU, Y., WAHREN, B., LIANG, Z., ØRUM, H., KOCH, T., and WAHLESTEDT, C. (2005). Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. Nucleic Acids Res. **33**, 439–447.

FOLKMAN, J., and INGBER, D. (1992). Inhibition of angiogenesis. Semin. Cancer Biol. **3**, 89–96.

FORSBACH, A., NEMORIN, J.G., MONTINO, C., MÜLLER, C., SAMULOWITZ, U., VICARI, A.P., JURK, M., MUTWIRI, G.K., KRIEG, A.M., LIPFORD, G.B., and VOLLMER, J. (2008). Identification of RNA sequence motifs stimulating sequence-specific TLR8-dependent immune responses. J. Immunol. **180**, 3729–3738.

FOSS, F.M. (2002). Immunologic mechanisms of antitumor activity. Semin. Oncol. **29**, 5–11.

GANTIER, M.P., TONG, S., BEHLKE, M.A., XU, D., PHIPPS, S., FOSTER, P.S., and WILLIAMS, B.R. (2008). TLR7 is involved in sequence-specific sensing of single-stranded RNAs in human macrophages. J. Immunol. **180**, 2117–2124.

GE, Q., FILIP, L., BAI, A., NGUYEN, T., EISEN, H.N., and CHEN, J. (2004). Inhibition of influenza virus production in virus-infected mice by RNA interference. Proc. Natl. Acad. Sci. USA. **101**, 8676–8681.

GEISBERT, T.W., HENSLEY, L.E., KAGAN, E., YU, E.Z., GEISBERT, J.B., DADDARIO-DICAPRIO, K., FRITZ, E.A., JAHRLING, P.B., MCCLINTOCK, K., PHELPS, J.R., LEE, A.C., JUDGE, A., JEFFS, L.B., and MACLACHLAN, I. (2006). Postexposure protection of guinea pigs against a lethal ebola virus challenge by RNA interference. J. Infect. Dis. **193**, 1650–1657.

GEISS, G., JIN, G., GUO, J., BUMGARNER, R., KATZE, M.G., and SEN, G.C. (2001). A comprehensive view of regulation of gene expression by double-stranded RNA-mediated cell signaling. J. Biol. Chem. **276**, 30178–30182.

GITLIN, L., KARELSKY, S., and ANDINO, R. (2002). Short interfering RNA confers intracellular antiviral immunity in human cells. Nature. **418**, 430–434.

GORDEN, K.B., GORSKI, K.S., GIBSON, S.J., KEDL, R.M., KIEPER, W.C., QIU, X., TOMAI, M.A., ALKAN, S.S., and VASILAKOS, J.P. (2005). Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. J. Immunol. **174**, 1259–1268.

GORDEN, K.K., QIU, X.X., BINSFELD, C.C., VASILAKOS, J.P., and ALKAN, S.S. (2006). Cutting Edge: activation of murine TLR8 by a combination of imidazoquinoline immune response modifiers and PolyT oligodeoxynucleotides. J. Immunol. **177**, 6584–6587.

HEIDEL, J.D., HU, S., LIU, X.F., TRICHE, T.J., and DAVIS, M.E. (2004). Lack of interferon response in animals to naked siRNAs. Nat. Biotechnol. **22**, 1579–1582.

HEIL, F., HEMMI, H., HOCHREIN, H., AMPENBERGER, F., KIRSCHNING, C., AKIRA, S., LIPFORD, G., WAGNER, H., and BAUER, S. (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science. **303**, 1526–1529.

HISCOTT, J., LIN, R., NAKHAEI, P., and PAZ, S. (2006). MasterCARD: a priceless link to innate immunity. Trends Mol. Med. **12**, 53–56.

HONDA, K., OHBA, Y., YANAI, H., NEGISHI, H., MIZUTANI, T., TAKAOKA, A., TAYA, C., and TANIGUCHI, T. (2005). Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. Nature. **434**, 1035–1040.

HORNUNG, V., ELLEGAST, J., KIM, S., BRZÓZKA, K., JUNG, A., KATO, H., POECK, H., AKIRA, S., CONZELMANN, K.K., SCHLEE, M., ENDRES, S., and HARTMANN, G. (2006). 5′-Triphosphate RNA is the ligand for RIG-I. Science. **314**, 994–997.

HORNUNG, V., GUENTHNER-BILLER, M., BOURQUIN, C., ABLASSER, A., SCHLEE, M., UEMATSU, S., NORONHA, A., MANOHARAN, M., AKIRA, S., de FOUGEROLLES, A., ENDRES, S., and HARTMANN, G. (2005). Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat. Med. **11**, 263–270.

HORNUNG, V., ROTHENFUSSER, S., BRITSCH, S., KRUG, A., JAHRSDÖRFER, B., GIESE, T., ENDRES, S., and HARTMANN, G. (2002). Quantitative expression of Toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunol. **168**, 4531–4537.

ITO, T., AMAKAWA, R., INABA, M., IKEHARA, S., INABA, K., and FUKUHARA, S. (2001). Differential regulation of human blood dendritic cell subsets by IFNs. J. Immunol. **166**, 2961–2969.

JUDGE, A.D., BOLA, G., LEE, A.C., and MACLACHLAN, I. (2006). Design of noninflammatory synthetic siRNA mediating potent gene silencing *in vivo*. Mol. Ther. **13**, 494–505.

JUDGE, A., and MACLACHLAN, I. (2008). Overcoming the innate immune response to small interfering RNA. Hum. Gene Ther. **19**, 111–124.

JUDGE, A.D., SOOD, V., SHAW, J.R., FANG, D., MCCLINTOCK, K., and MACLACHLAN, I. (2005). Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat. Biotechnol. **23**, 457–462.

JUDGE, A.D., ROBBINS, M., TAVAKOLI, I., LEVI, J., HU, L., FRONDA, A., AMBEGIA, E., MCCLINTOCK, K., MACLACHLAN, I. (2009). Confirming the RNAi-mediated mechanism of action of siRNA-based therapeutics in mice. J. Clin. Invest. 119, 661–73.

KADOWAKI, N., HO, S., ANTONENKO, S., MALEFYT, R.W., KASTELEIN, R.A., BAZAN, F., and LIU, Y.J. (2001). Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J. Exp. Med. **194**, 863–869.

KARIKÓ, K., BHUYAN, P., CAPODICI, J., and WEISSMAN, D. (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.

KARIKÓ, K., BUCKSTEIN, M., NI, H., and WEISSMAN, D. (2005). Suppression of RNA recognition by Toll-like receptors: The impact of nucleoside modification and the evolutionary origin of RNA. Immunity. **23**, 165–175.

KATO, H., SATO, S., YONEYAMA, M., YAMAMOTO, M., UEMATSU, S., MATSUI, K., TSUJIMURA, T., TAKEDA, K., FUJITA, T., TAKEUCHI, O., and AKIRA, S. (2005). Cell type-specific involvement of RIG-I in antiviral response. Immunity. **23**, 19–28.

KATO, H., TAKEUCHI, O., SATO, S., YONEYAMA, M., YAMAMOTO, M., MATSUI, K., UEMATSU, S., JUNG, A., KAWAI, T., ISHII, K.J., YAMAGUCHI, O., OTSU, K., TSUJIMURA, T., KOH, C.S., REIS E SOUSA, C., MATSUURA, Y., FUJITA, T., and AKIRA, S. (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature. **441**, 101–105.

KAWAI, T., and AKIRA, S. (2006). Innate immune recognition of viral infection. Nat. Immunol. **7**, 131–137.

KAWAI, T., TAKAHASHI, K., SATO, S., COBAN, C., KUMAR, H., KATO, H., ISHII, K.J., TAKEUCHI, O., and AKIRA, S. (2005). IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat. Immunol. **6**, 981–988.

KIM, D.H., BEHLKE, M.A., ROSE, S.D., CHANG, M.S., CHOI, S., and ROSSI, J.J. (2005). Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. Nat. Biotechnol. **23**, 222–226.

KIM, D-H, LONGO, M., HAN, Y., LUNDBERG, P., CANTIN, E., and ROSSI, J.J. (2004). Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase. Nat. Biotechnol. **22**, 321–325.

KLEINMAN, M.E., YAMADA, K., TAKEDA, A., CHANDRASEKARAN, V., NOZAKI, M., BAFFI, J.Z., ALBUQUERQUE, R.J., YAMASAKI, S., ITAYA, M., PAN, Y., APPUKUTTAN, B., GIBBS, D., YANG, Z., KARIKÓ, K., AMBATI,

B.K., WILGUS, T.A., DIPIETRO, L.A., SAKURAI, E., ZHANG, K., SMITH, J.R., TAYLOR, E.W., and AMBATI, J. (2008). Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature. **452**, 591–597.

KRIEG, A.M. (2006). Therapeutic potential of Toll-like receptor 9 activation. Nat. Rev. **5**, 471–484.

LAYZER, J., MCCAFFREY, A., TANNER, A., HUANG, Z., KAY, M., and SULLENGER, B. (2004). *In vivo* activity of nuclease resistant siRNAs. RNA. **10**, 766–771.

LEE, J., CHUANG, T.H., REDECKE, V., SHE, L., PITHA, P.M., CARSON, D.A., RAZ, E., and COTTAM, H.B. (2003). Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: Activation of Toll-like receptor 7. Proc. Natl. Acad. Sci. USA. **100**, 6646–6651.

LEUSCHNER, P.J., AMERES, S.L., KUENG, S., and MARTINEZ, J. (2006). Cleavage of the siRNA passenger strand during RISC assembly in human cells. EMBO Rep. **7**, 314–320.

LI, B.J., TANG, Q., CHENG, D., QIN, C., XIE, F.Y., WEI, Q., XU, J., LIU, Y., ZHENG, B.J., WOODLE, M.C., ZHONG, N., and LU, P.Y. (2005). Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. Nat. Med. **11**, 944–951.

LUND, J.M., ALEXOPOULOU, L., SATO, A., KAROW, M., ADAMS, N.C., GALE, N.W., IWASAKI, A., and FLAVELL, R.A. (2004). Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc. Natl. Acad. Sci. USA. **101**, 5598–5603.

LYNN, M., ROSSIGNOL, D.P., WHEELER, J.L., KAO, R.J., PERDOMO, C.A., NOVECK, R., VARGAS, R., D'ANGELO, T., GOTZKOWSKY, S., and MCMAHON, F.G. (2003). Blocking of responses to endotoxin by E5564 in healthy volunteers with experimental endotoxemia. J. Infect. Dis. **187**, 631–639.

MANCHE, L., GREEN, S.R., SCHMEDT, C., and MATHEWS, M.B. (1992). Interactions between double-stranded RNA regulators and the protein kinase DAI. Mol. Cell. Biol. **12**, 5238–5248.

MANOHARAN, M. (2004). RNA interference and chemically modified small interfering RNAs. Curr. Opin. Chem. Biol. **8**, 570–579.

MARQUES, J.T., DEVOSSE, T., WANG, D., ZAMANIAN-DARYOUSH, M., SERBINOWSKI, P., HARTMANN, R., FUJITA, T., BEHLKE, M.A., and WILLIAMS, B.R. (2006). A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. Nat. Biotechnol. **24**, 559–565.

MATRANGA, C., TOMARI, Y., SHIN, C., BARTEL, D.P., and ZAMORE, P.D. (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell. **123**, 607–620.

MEURS, E., CHONG, K., GALABRU, J., THOMAS, N.S., KERR, I.M., WILLIAMS, B.R., and HOVANESSIAN, A.G. (1990). Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell. **62**, 379–390.

MEYLAN, E., CURRAN, J., HOFMANN, K., MORADPOUR, D., BINDER, M., BARTENSCHLAGER, R., and TSCHOPP, J. (2005). Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature. **437**, 1167–1172.

MICHIE, H.R., MANOGUE, K.R., SPRIGGS, D.R., REVHAUG, A., O'DWYER, S., DINARELLO, C.A., CERAMI, A., WOLFF, S.M., and WILMORE, D.W. (1988). Detection of circulating tumor necrosis factor after endotoxin administration. N. Engl. J. Med. **318**, 1481–1486.

MORRISSEY, D.V., BLANCHARD, K., SHAW, L., JENSEN, K., LOCKRIDGE, J.A., DICKINSON, B., MCSWIGGEN, J.A., VARGEESE, C., BOWMAN, K., SHAFFER, C.S., POLISKY, B.A., and ZINNEN, S. (2005). Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus infection. Hepatology. **41**, 1349–1356.

MORRISSEY, D.V., LOCKRIDGE, J.A., SHAW, L., BLANCHARD, K., JENSEN, K., BREEN, W., HARTSOUGH, K., MACHEMER, L., RADKA, S., JADHAV, V., VAISH, N., ZINNEN, S., VARGEESE, C., BOWMAN, K., SHAFFER, C.S., JEFFS, L.B., JUDGE, A., MACLACHLAN, I., and POLISKY, B. (2005). Potent and persistent *in vivo* anti-HBV activity of chemically modified siRNA's. Nat. Biotechnol. **23**, 1002–1007.

MUZIO, M., BOSISIO, D., POLENTARUTTI, N., D'AMICO, G., STOPPACCIARO, A., MANCINELLI, R., VAN'T VEER, C., PENTON-ROL, G., RUCO, L.P., ALLAVENA, P., and MANTOVANI, A. (2000). Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J. Immunol. **164**, 5998–6004.

PALLISER, D., CHOWDHURY, D., WANG, Q.Y., LEE, S.J., BRONSON, R.T., KNIPE, D.M., and LIEBERMAN, J. (2006). An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. Nature. **439**, 89–94.

PETERS, K.L., SMITH, H.L., STARK, G.R., and SEN, G.C. (2002). IRF-3-dependent, NFkappa B- and JNK-independent activation of the 561 and IFN-beta genes in response to double-stranded RNA. Proc. Natl. Acad. Sci. USA. **99**, 6322–6327.

PICHLMAIR, A., SCHULZ, O., TAN, C.P., NÄSLUND, T.I., LILJESTRÖM, P., WEBER, F., and REIS e SOUSA, C. (2006). RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. Science. **314**, 997–1001.

POECK, H., BESCH, R., MAIHOEFER, C., RENN, M., TORMO, D., MORSKAYA, S.S., KIRSCHNEK, S., GAFFAL, E., LANDSBERG, J., HELLMUTH, J., SCHMIDT, A., ANZ, D., BSCHEIDER, M., SCHWERD, T., BERKING, C., BOURQUIN, C., KALINKE, U., KREMMER, E., KATO, H., AKIRA, S., MEYERS, R., HÄCKER, G., NEUENHAHN, M., BUSCH, D., RULAND, J., ROTHENFUSSER, S., PRINZ, M., HORNUNG, V., ENDRES, S., TÜTING, T., and HARTMANN, G. (2008). 5′-triphosphate-siRNA: turning gene silencing and Rig-I activation against melanoma. Nat. Med. **14**, 1256–1263.

PRAKASH, T., ALLERSON, C.R., DANDE, P., VICKERS, T.A., SIOUFI, N., JARRES, R., BAKER, B.F., SWAYZE, E.E., GRIFFEY, R.H., and BHAT, B. (2005). Positional effect of chemical modifications on short interference RNA activity in mammalian cells. J. Med. Chem. **48**, 4247–4253.

PUTHENVEETIL, S., WHITBY, L., REN, J., KELNAR, K., KREBS, J.F., and BEAL, P.A. (2006). Controlling activation of the RNA-dependent protein kinase by siRNAs using site-specific chemical modification. Nucleic Acids Res. **34**, 4900–4911.

ROBBINS, M., JUDGE, A., AMBEGIA, E., CHOI, C., YAWORSKI, E., PALMER, L., MCCLINTOCK, K., and MACLACHLAN, I. (2008). Misinterpreting the therapeutic effects of siRNA caused by immune stimulation. Hum. Gene Ther. [Epub Ahead of print].

ROBBINS, M., JUDGE, A., LIANG, L., MCCLINTOCK, K., YAWORSKI, E., and MACLACHLAN, I. (2007). 2′-O-methyl-modified RNAs Act as TLR7 Antagonists. Mol. Ther. **15**(9), 1663–1669.

SANTEL, A., ALEKU, M., KEIL, O., ENDRUSCHAT, J., ESCHE, V., DURIEUX, B., LÖFFLER, K., FECHTNER, M., RÖHL, T., FISCH, G., DAMES, S., ARNOLD, W., GIESE, K., KLIPPEL, A., and KAUFMANN, J. (2006). RNA interference in the mouse vascular endothelium by systemic administration of siRNA-lipoplexes for cancer therapy. Gene Ther. **13**, 1360–1370.

SARAVOLAC, E.G., SABUDA, D., CRIST, C., BLASETTI, K., SCHNELL, G., YANG, H., KENDE, M., LEVY, H.B., and WONG, J.P. (2001). Immunoprophylactic strategies against respiratory influenza virus infection. Vaccine. **19**, 2227–2232.

SETH, R.B., SUN, L., EA, C.K., and CHEN, Z.J. (2005). Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell. **122**, 669–682.

SHARARA, A.I., PERKINS, D.J., MISUKONIS, M.A., CHAN, S.U., DOMINITZ, J.A., and WEINBERG, J.B. (1997). Interferon (IFN)-alpha activation of human blood mononuclear cells *in vitro* and *in vivo* for nitric oxide synthase (NOS) type 2 mRNA and protein expression: Possible relationship of induced NOS2 to the anti-hepatitis C effects of IFN-alpha *in vivo*. J. Exp. Med. **186**, 1495–1502.

SIOUD, M. (2005). Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNA

is sequence dependent and requires endosomal localization. J. Mol. Biol. **348**, 1079–1090.

SLEDZ, C.A., HOLKO, M., de VEER, M.J., SILVERMAN, R.H., and WILLIAMS, B.R. (2003). Activation of the interferon system by short-interfering RNAs. Nat. Cell. Biol. **5**, 834–839.

SOUTSCHEK, J., AKINC, A., BRAMLAGE, B., CHARISSE, K., CONSTIEN, R., DONOGHUE, M., ELBASHIR, S., GEICK, A., HADWIGER, P., HARBORTH, J., JOHN, M., KESAVAN, V., LAVINE, G., PANDEY, R.K., RACIE, T., RAJEEV, K.G., RÖHL, I., TOUDJARSKA, I., WANG, G., WUSCHKO, S., BUMCROT, D., KOTELIANSKY, V., LIMMER, S., MANOHARAN, M., and VORNLOCHER, H.P. (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature. **432**, 173–178.

STEVENSON, H.C., ABRAMS, P.G., SCHOENBERGER, C.S., SMALLEY, R.B., HERBERMAN, R.B., and FOON, K.A. (1985). A phase I evaluation of poly(I,C)-LC in cancer patients. J. Biol. Response Mod. **4**, 650–655.

TAKEDA, K., and AKIRA, S. (2005). Toll-like receptors in innate immunity. Int. Immunol. **17**, 1–14.

TAKEDA, K., KAISHO, T., and AKIRA, S. (2003). Toll-like Receptors. Ann. Rev. Immunol. **21**, 335–376.

TOMPKINS, S.M., LO, C.Y., TUMPEY, T.M., and EPSTEIN, S.L. (2004). Protection against lethal influenza virus challenge by RNA interference *in vivo*. Proc. Natl. Acad. Sci. USA. **101**, 8682–8686.

VON MARSCHALL, Z., SCHOLZ, A., CRAMER, T., SCHÄFER, G., SCHIRNER, M., OBERG, K., WIEDENMANN, B., HÖCKER, M., and ROSEWICZ, S. (2003). Effects of interferon alpha on vascular endothelial growth factor gene transcription and tumor angiogenesis. J. Natl Cancer Inst. **95**, 437–448.

WILLIAMS, B.R. (2001). Signal integration via PKR. Sci. STKE **2001**, RE2.

WU, W.Z., SUN, H.C., SHEN, Y.F., CHEN, J., WANG, L., TANG, Z.Y., ILIAKIS, G., and LIU, K.D. (2005). Interferon alpha 2a down-regulates VEGF expression through PI3 kinase and MAP kinase signaling pathways. J. Cancer Res. Clin. Oncol. **131**, 169–178.

XU, L.G., WANG, Y.Y., HAN, K.J., LI, L.Y., ZHAI, Z., and SHU, H.B. (2005). VISA is an adapter protein required for virus-triggered IFN-beta signaling. Mol. Cell. **19**, 727–740.

YAMAMOTO, M., SATO, S., HEMMI, H., HOSHINO, K., KAISHO, T., SANJO, H., TAKEUCHI, O., SUGIYAMA, M., OKABE, M., TAKEDA, K., and AKIRA, S. (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science. **301**, 640–643.

YAMAMOTO, M., SATO, S., MORI, K., HOSHINO, K., TAKEUCHI, O., TAKEDA, K., and AKIRA, S. (2002). Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J. Immunol. **169**, 6668–6672.

YEO, S.J., YOON, J.G., and YI, A.K. (2003). Myeloid differentiation factor 88-dependent post-transcriptional regulation of cyclooxygenase-2 expression by CpG DNA: Tumor necrosis factor-alpha receptor-associated factor 6, a diverging point in the Toll-like receptor 9-signaling. J. Biol. Chem. **278**, 40590–40600.

YONEYAMA, M., KIKUCHI, M., MATSUMOTO, K., IMAIZUMI, T., MIYAGISHI, M., TAIRA, K., FOY, E., LOO, Y.M., GALE, M., JR., AKIRA, S., YONEHARA, S., KATO, A., and FUJITA, T. (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J. Immunol. **175**, 2851–2858.

YONEYAMA, M., KIKUCHI, M., NATSUKAWA, T., SHINOBU, N., IMAIZUMI, T., MIYAGISHI, M., TAIRA, K., AKIRA, S., and FUJITA, T. (2004). The RNA helicase RIG-1 has an essential function in double-stranded RNA induced innate antiviral responses. Nat. Immunol. **5**, 730–737.

ZAMANIAN-DARYOUSH, M., MARQUES, J.T., GANTIER, M.P., BEHLKE, M.A., JOHN, M., RAYMAN, P., FINKE, J., and WILLIAMS, B.R. (2008). Determinants of cytokine induction by small interfering RNA in human peripheral blood mononuclear cells. J. Interferon Cytokine Res. **28**, 221–233.

ZAREMBER, K.A., and GODOWSKI, P.J. (2002). Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J. Immunol. **168**, 554–561.

ZHANG, Z., WEINSCHENK, T., GUO, K., and SCHLUESENER, H.J. (2006). siRNA binding proteins of microglial cells: PKR is an unanticipated ligand. J. Cell. Biochem. **97**, 1217–1229.

Address reprint requests to:
Dr. Ian MacLachlan
Chief Scientific Officer
Tekmira Pharmaceuticals Corp.
100-8900 Glenlyon Parkway
Burnaby, B.C. V5J 5J8
Canada

E-mail: IMaclachlan@tekmirapharm.com

Received for publication January 9, 2009; accepted after revision March 11, 2009.