Silencing or Stimulation? siRNA Delivery and the Immune System

Kathryn A. Whitehead,[1] James E. Dahlman,[2] Robert S. Langer,[1,2,3] and Daniel G. Anderson[1,2,3]

[1]The David H. Koch Institute for Integrated Cancer Research, [2]Harvard-MIT Division of Health Sciences and Technology, and [3]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142; email: kawhite@mit.edu, jdahlman@mit.edu, rlanger@mit.edu, dgander@mit.edu

Annu. Rev. Chem. Biomol. Eng. 2011. 2:77–96

First published online as a Review in Advance on February 14, 2011

The *Annual Review of Chemical and Biomolecular Engineering* is online at chembioeng.annualreviews.org

This article's doi: 10.1146/annurev-chembioeng-061010-114133

1947-5438/11/0715-0077$20.00

Keywords

isRNA, immunostimulatory RNA, bifunctional siRNA, TLR7, 2′ modification

Abstract

Since its inception more than a decade ago, the field of short interfering RNA (siRNA) therapeutics has demonstrated potential in the treatment of a wide variety of diseases. The power behind RNA interference (RNAi) therapy lies in its ability to specifically silence target genes of interest. As more biological data have become available, it has become evident that, in addition to mediating RNAi, siRNA molecules have the potential to potently induce the innate immune system. One of the significant challenges facing the field today is the differentiation between therapeutic effects caused by target-specific, RNAi-mediated gene silencing and those caused by nonspecific stimulation of the innate immune system. Unless appropriate experimental measures are taken to control for RNA-induced immunostimulation, genetic manipulation can be confused with immune activation. This review attempts to provide an accessible background in siRNA-relevant immunology and to highlight the ways in which siRNA can be engineered to avoid or provoke an innate immune response.

INTRODUCTION

RNA interference (RNAi): an evolutionarily conserved cellular mechanism by which the presence of double-stranded RNA promotes the sequence-specific silencing of complementary mRNA

In recent years, excitement surrounding the discovery of RNA interference (RNAi) has mounted as researchers have taken advantage of this endogenous cellular mechanism to address a broad range of diseases using specially designed short interfering RNAs (siRNAs). RNAi therapeutics have the potential to treat a variety of diseases by inducing the specific and reversible loss of expression of target genes. For example, RNAi has the potential to treat cancer by silencing genes that promote uncontrolled cell proliferation (1). High cholesterol potentially can be kept in check by using RNAi to silence a gene that promotes low-density lipoprotein retention (2). Using such gene knockdown approaches, researchers have been able to address a variety of disease states in rodent and nonhuman primate models, including hepatitis B virus (3), Huntington's disease (4), hypercholesterolemia (2), and Ebola virus (5, 6). More recently, siRNA delivery to solid tumors has been reported in humans (7).

On the cellular level, RNAi is an endogenous mechanism by which sequence-specific siRNA induces gene silencing through the targeting and cleavage of complementary messenger RNA (mRNA) within the cytoplasm of the cell (8, 9) (**Figure 1**). RNAi is triggered by the presence of double-stranded RNA (dsRNA), which is cleaved by the intracellular enzyme Dicer into 21–base pair fragments of siRNA (10). The siRNA is loaded into a protein complex called the RNA-induced silencing complex (RISC), which unwinds the siRNA, retaining the antisense strand (11).

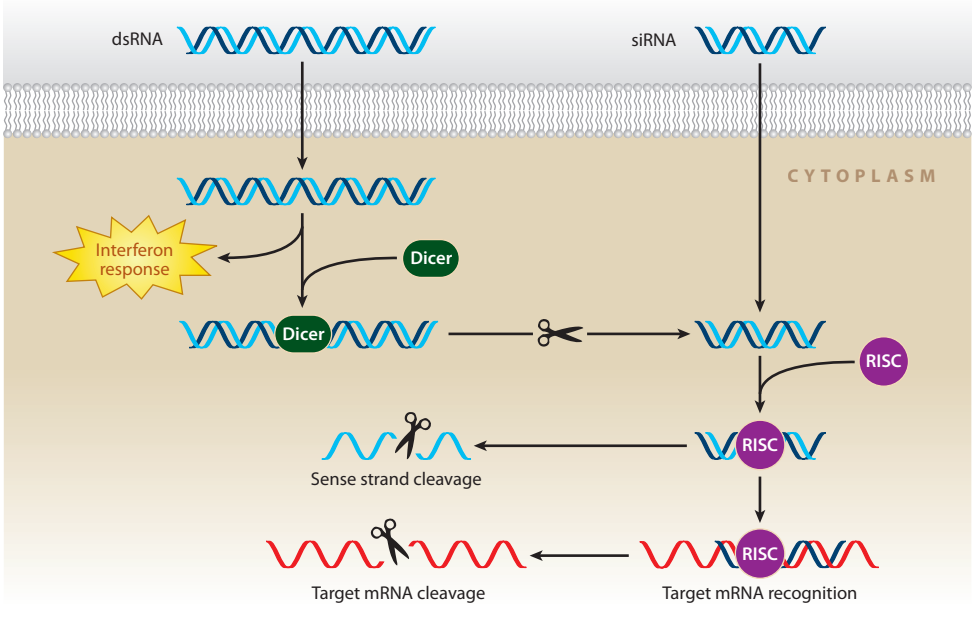

Figure 1

RNA interference leads to gene silencing at the messenger RNA (mRNA) level. When long double-stranded RNA (dsRNA) is introduced into the cytoplasm, it is cleaved into small interfering RNA (siRNA) by the enzyme Dicer (*left*). Alternatively, synthetic siRNA can be introduced directly into the cell (*right*). The siRNA is then incorporated into the RNA-induced silencing complex (RISC), which unwinds the siRNA duplex and mediates the cleavage of the sense strand. The activated RISC-siRNA complex binds to and degrades mRNA that is complementary to the antisense siRNA strand. This results in silencing of the target gene and any proteins that it may encode.

RISC then seeks out and cleaves any mRNA that is complementary to the antisense strand, thus silencing the target gene and preventing protein production.

Unfortunately, dsRNA, the natural trigger of RNAi, can be a potent activator of an innate immune interferon (IFN) response when longer than 30 base pairs (12). This characteristic seemingly limits any therapeutic advantage offered by the RNAi pathway. RNAi likely developed evolutionarily as a natural defense mechanism against viruses, given that long dsRNA is a hallmark of viral infection (13). Fortunately, it is possible to introduce into the cell synthetically designed siRNA that circumvents Dicer mechanics as well as the immunostimulation associated with long dsRNA (9) (**Figure 1**).

After several early reports suggested that siRNA delivery did not result in a dsRNA-induced innate immune response (9, 14, 15), siRNA became widely regarded as nonimmunostimulatory. Although many reports began to emerge on the therapeutic applicability of RNAi, far fewer studies attempted to examine the effect of siRNA delivery on the immune system. However, we now know that siRNA can provoke the innate immune system (16–18). At its most basic level, siRNA delivery involves the introduction of foreign material into a robust biological environment and therefore has the potential to sound the immunological alarm.

Acknowledgment and control of the immunostimulatory potential of siRNA are important aspects of siRNA therapeutic development. This review describes the basic functioning of the innate immune system as well as the ways in which the immune system recognizes and reacts to siRNA. It is our hope that the information contained herein will enable readers to design their own siRNA delivery studies to account for immune stimulation as well as to evaluate critically the immunological aspects of the siRNA delivery literature. Before we discuss engineering issues that affect immunostimulatory properties of siRNA, we must first understand some of the basic principles of how the innate immune system operates.

short interfering RNA (siRNA): small fragments of RNA, approximately 21 base pairs in length, that are able to independently induce both RNA interference and immune stimulation

RISC: RNA-induced silencing complex

IFN: interferon

Innate immune system: the branch of the immune system that provides a fast-acting, temporary, nonspecific response to foreign materials

THE RNA-RELATED IMMUNE SYSTEM

Over time, competition between ancient pathogens and our evolutionary ancestors has led to a complex network of defensive cells and molecules that we call the immune system. This system has equipped us with an arsenal of defense mechanisms that seek out and destroy nonself molecules, including bacteria, fungi, and viruses. The mammalian immune system has evolved to recognize siRNA as a signature of viral infection, and as such, siRNA is capable of inducing a potent and potentially dangerous innate immune response. The following section highlights aspects of the immune system most relevant to siRNA design and its subsequent therapeutic application.

The Innate Immune System Responds to siRNA

The human immune system is divided into two separate branches: the innate immune system and the adaptive immune system. The innate immune system is generally the first defense against infection and responds to pathogens in a generic fashion (19). The innate immune response is acute; it acts swiftly and potently for a short window of time without conferring any long-term or protective immunity to the host. In contrast, the adaptive immune system, which developed later in the evolutionary process, comprises highly specialized B and T cells that are trained to react to very specific portions of pathogenic molecules (20). On one hand, upon recognizing a pathogen, B cells respond by triggering the production of antibodies specific to that pathogen. T cells, on the other hand, are responsible for the destruction of pathogens and infected host cells as well as the recruitment of other immune cells. Both B and T cells differentiate into long-lived memory cells, which confer immunological memory to the host body long after the infection has passed.

IL: interleukin

TNF: tumor necrosis factor

pattern recognition receptors (PRRs): a diverse group of receptors present throughout the cell that recognize and respond to specific pathogenic components of foreign molecules.

Toll-like receptors (TLRs): a class of pattern recognition receptors that play an active role in RNA recognition by the innate immune system

The process of recognizing and responding to nonself nucleic acids such as siRNA is governed almost exclusively by the innate immune system. Innate immune responses are typically divided into two categories: acute inflammatory responses and antiviral responses. The inflammatory response is characterized by an induction of small signaling molecules called cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12) and tumor necrosis factor α (TNF-α). Interleukins promote the development and differentiation of B, T, and natural killer (NK) cells, thus linking the innate and adaptive immune systems (21). TNF-α acts to promote inflammation, induce apoptosis, and inhibit viral replication (22). Together, these proinflammatory cytokines stimulate phagocytosis, the process by which macrophages internalize and destroy invading pathogens (23).

The antiviral response, however, is marked by the release of a group of cytokines called Type I IFNs, a class of proteins that includes IFN-α and IFN-β, upregulate the expression of more than 100 antiviral genes, leading to an antiviral state (24, 25). Antiviral genes perform many regulatory functions, notably inducing the proliferation of NK cells and memory T cells. Both inflammatory and antiviral responses are capable of destroying pathogens and eliciting adaptive immune responses. Phagocytotic cells such as macrophages, neutrophils, dendritic cells (DCs), and NK cells quickly differentiate into cells whose primary focus is microbe destruction. For instance, antiviral responses are characterized by the rapid release of IFN-α from a subset of DCs called plasmacytoid DCs (pDCs). If this response does not eliminate the pathogen, DCs activate the adaptive immune system to assist in the overall immune response (26).

The innate immune system generates both inflammatory and antiviral responses through the stimulation of pathogen or pattern recognition receptors (PRRs). These receptors recognize distinct pathogenic patterns that are not present on self-cells (26–28) and can distinguish between viruses, fungi, bacteria, and other pathogens. Importantly for siRNA delivery, the innate immune system has evolved to include multiple PRRs that recognize different aspects of RNA structure, providing a redundancy that makes immunostimulation difficult to escape. These varied PRRs are depicted in **Figure 2**. The following sections discuss the PRRs that are responsible for the detection of and response to siRNA.

Toll-Like Receptors Recognize RNA

Toll-like receptors (TLRs) are a class of PRRs that recognize structurally conserved regions of foreign pathogens. The name reflects a structural similarity shared with a *Drosophila* (fruit fly) protein encoded by the gene *Toll* (29). Each member of the TLR family is responsible for the detection of particular pathogen-associated molecules. For example, some TLRs recognize bacterial components, such as flagellin, whereas others recognize fungal molecules, such as β-glucan. To date, 10 functional TLRs (TLRs 1–10) have been identified in humans, and 12 functional TLRs (TLRs 1–9 and TLRs 11–13) have been discovered in mice (30). Toll-like receptors with relevance to siRNA delivery include TLR3, which recognizes dsRNA, and TLR7 and TLR8, which recognize single-stranded RNA (ssRNA).

TLR3 responds to dsRNA, which is a typical characteristic of viral replication found in lysed or apoptotic virally infected cells (31). Studies have indicated that the horseshoe-like structure of TLR3 facilitates dsRNA recognition (32, 33). Interactions between TLR3 and dsRNA were originally reported in 2001 when TLR3-deficient mice exhibited reduced immune responses to dsRNA viruses (34). Since then, it has been demonstrated that TLR3 also recognizes dsRNA products derived from the replication of single-stranded (ssRNA) viruses (35). Because siRNA has a double-stranded structure, TLR3 has also been shown to respond to siRNA (36, 37).

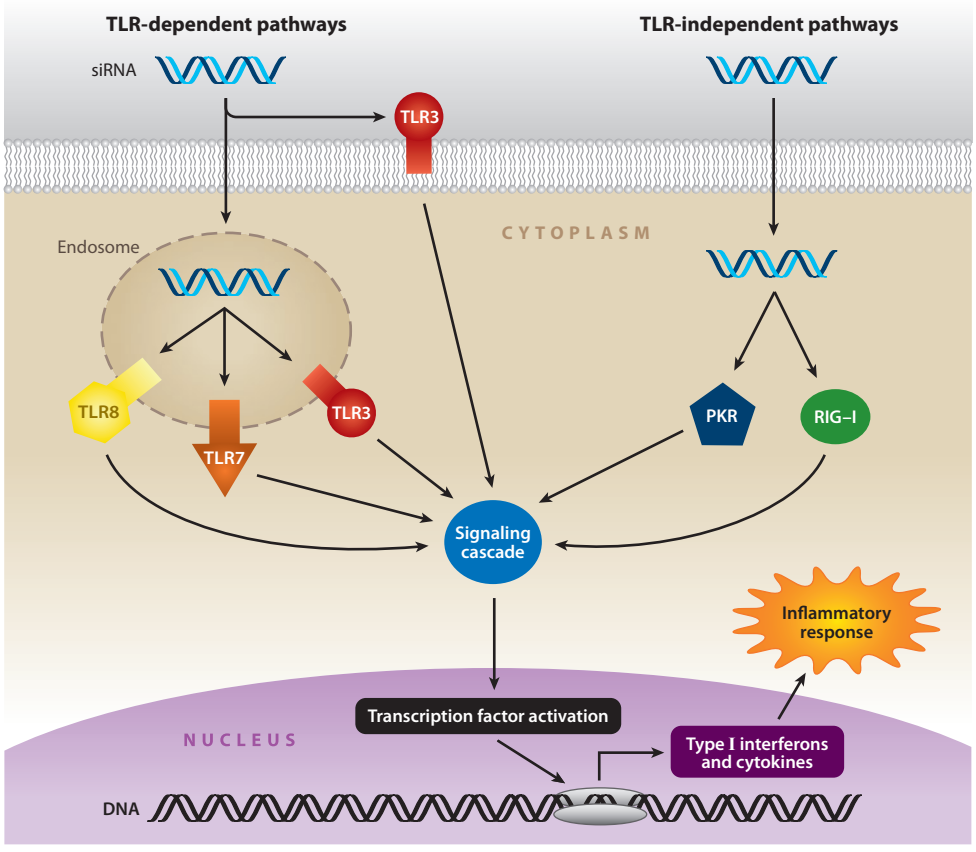

Figure 2

Short interfering RNA (siRNA) provokes immune stimulation via multiple pathogen recognition receptors. Toll-like receptor 3 (TLR3) (*red*) exists both on the cell surface and in subcellular compartments of select cells. TLR7 (*orange*) and TLR8 (*yellow*) exist solely in the endosomes and lysosomes of specialized immune cells and recognize siRNA in a sequence-dependent manner. PKR, a dsRNA-dependent protein kinase, and retinoic acid-inducible gene 1 protein (RIG-I) are present in the cytoplasm of certain cell types and can detect and react to siRNA in a sequence-independent fashion. When activated, each receptor sets off a unique immune signaling cascade that causes increased transcription of mRNA encoding for Type I interferons and inflammatory cytokines.

In humans, TLR3 is expressed in endosomes and on the cell surface of select cell populations. Specifically, TLR3 is found in the endosomes of mature dendritic cells (mDCs), fibroblasts, and epithelial cells (38, 39) as well as on the surface of fibroblasts and epithelial cells. Importantly, TLR3 expression is heterogeneous across different cell lines and across different species. Unlike humans, mice express TLR3 in macrophages, dendritic cells, B cells, T cells, and neutrophils (40). TLR3 activation sets off an immunostimulatory cascade that results in the increased production of IFN-α and IFN-β.

TLR7 is an important pattern recognition receptor that responds to ssRNA in a sequence-specific manner. It is located exclusively in the intracellular vesicles, including endosomes, lysosomes, and the endoplasmic reticulum of pDCs and B cells (31, 41). Originally discovered in 2002, TLR7 initially was shown to respond to particular antiviral and antitumor compounds (42).

Two years later, Diebold, Heil, and coworkers (43, 44) demonstrated that ssRNAs trigger TLR7. TLR7 also reacts to ssRNA viruses (45). siRNAs, which are composed of two strands of ssRNA, were subsequently shown to elicit a TLR7-mediated response in a sequence-dependent manner (18, 46, 47). When activated in the endosome, TLR7 initiates a signal cascade that results in the upregulation of interferon molecules IFN-α and IFN-β (48). When activated in the lysosome, TLR7 induces the production of inflammatory cytokines TNF-α and IL-12 (49). In B cells, TLR7 activation primes the adaptive immune system by initiating the differentiation of B cells into antibody-secreting plasma cells (41).

PKR: a double-stranded RNA-dependent protein kinase

RIG-I: retinoic acid–inducible gene 1 protein

TLR8 is another PRR that recognizes ssRNA, and it shares several similarities with TLR7. It, too, responds to ssRNA in a sequence-dependent fashion and is expressed only within intracellular vesicles. Like TLR7, TLR8 has been shown to respond to GU-rich motifs (43). However, unlike TLR7, it has also been shown to respond to AU-rich motifs (50). In addition, TLR8 appears in only myeloid lineage cells, which include monocytes, macrophages, and mDCs. Interestingly, although TLR8 has been shown to play an active role in the human immune system, its activity appears to be present, but reduced, in mice (27, 51). Downstream, TLR8 activation results in the upregulation of the same inflammatory molecules as the TLR7 response, although the relative expression of these molecules differs (52). Additionally, TLR8 activation induces the production of IL-1 and IL-6, which amplify the immune response (52, 53). Interestingly, siRNA is not the only nucleic acid capable of inducing an innate immune response, as DNA is recognized by TLR9 (see sidebar, DNA Can Activate the Innate Immune System).

Cytoplasmic Receptors Recognize RNA

Toll-like receptors are not the only class of PRRs that recognize and respond to siRNA. Although TLRs guard the endosomal compartments against pathogenic infection, several additional proteins mediate immune response to siRNA in the cytoplasm (**Figure 2**). These include a dsRNA-dependent protein kinase called PKR (54) as well as the RNA helicase protein retinoic acid–inducible gene 1 protein (RIG-I) (31, 55). Each of these recognizes and responds to sequence-independent structural characteristics of RNA.

PKR is a protein kinase expressed in the cytoplasm of most mammalian cells that responds to dsRNA (56), although the specific features of siRNA that it recognizes remain unclear. PKR can

DNA CAN ACTIVATE THE INNATE IMMUNE SYSTEM

RNA is not the only oligonucleotide capable of activating the innate immune system when introduced into the body. It has been shown that DNA containing particular sequences of nucleotides, called CpG motifs, stimulate a proinflammatory response upon recognition by TLR9 (115–117). CpG is shorthand for cytosine-phosphate-guanine, meaning that the two nucleotides are neighboring units on a linear DNA strand. TLR9 is similar to TLR7, as it appears in the subcellular compartments of plasmacytoid dendritic cells and causes a strong interferon response when stimulated (103). As it was discovered earlier, immunostimulatory DNA technology is more developed than the corresponding siRNA technology and is moving toward application in a clinical setting. At the time of this writing, Coley Pharmaceutical Group, together with GlaxoSmithKline (GSK), is conducting a Phase III clinical trial for the treatment of non-small cell lung cancer. Specifically, in conjunction with GSK's immunotherapeutic drug, the trial is using CpG-DNA as a TLR9-antagonizing adjuvant to induce an enhanced immune response to lung cancer cells. Such efforts in the field of immunostimulatory DNA highlight the therapeutic potential of immune-activating siRNA once a greater understanding of its biology is in place.

react to as little as 11 base pairs of dsRNA in a nonsequence-specific fashion (57). PKR activation leads to the inhibition of protein translation as well as to an interferon response (56, 58). Both traditional siRNA and blunt siRNA have been shown to induce moderate levels of PKR activation (57, 59, 60). Whereas each strand of traditional siRNA contains a two–base pair 3′ overhang, blunt siRNA has no overhangs (**Figure 3*a***).

RIG-I recognizes uncapped 5′-triphosphate structures of dsRNA or ssRNA, as the lack of a cap is a trademark of viral RNA sequences (13, 55, 61, 62). **Figure 3*b*** depicts the backbone of native, capped RNA. Blunt-ended siRNA can also generate a RIG-I-mediated immune response, independent of siRNA sequence (59). This is a concern for synthetic siRNAs transcribed in vitro

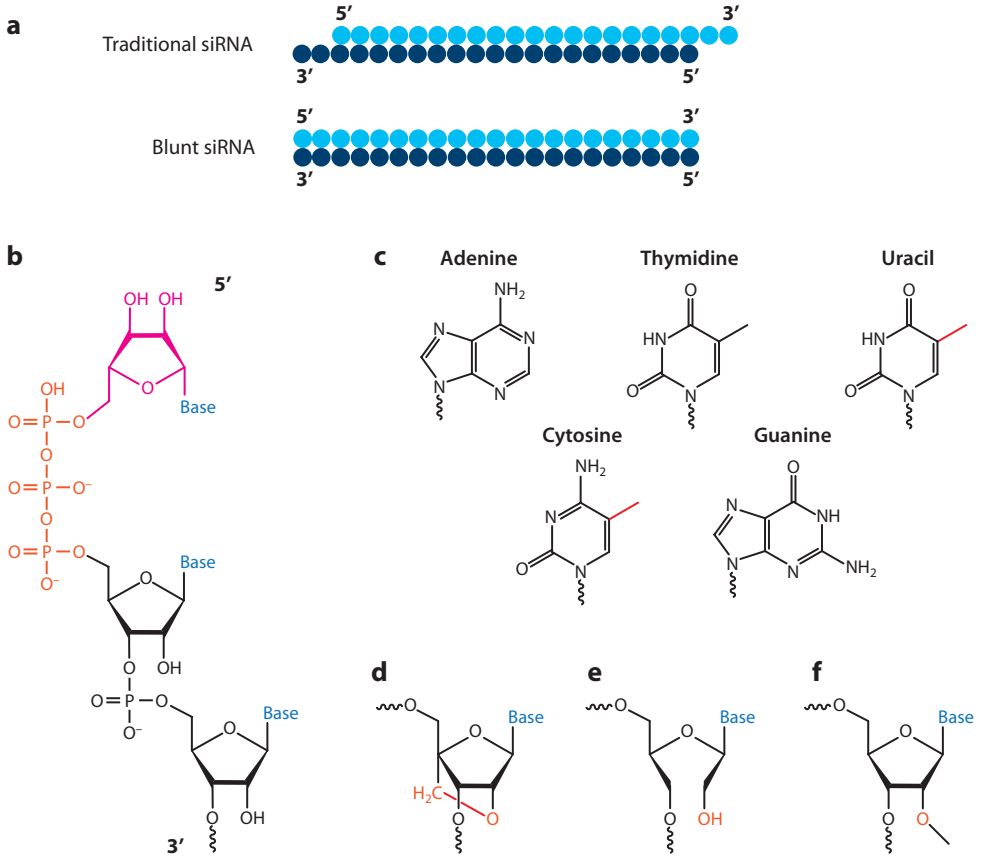

Figure 3

The chemical structure of small interfering RNA (siRNA) affects immune stimulation. (*a*) Traditional siRNA duplexes contain two–base pair 3′ overhangs on each strand. Blunt siRNA has no overhangs. (*b*) At the 5′ end, native duplexed RNA contains a triphosphate group (*orange*) capped with a methyl-guanosine nucleotide (*magenta*). Uncapped siRNA, with only a triphosphate at the 5′ end, causes immunostimulation via retinoic acid–inducible gene 1 protein (RIG-I). (*c*) Oligonucleotide bases (*black*) include adenine, thymidine, cytosine, guanine, and uracil. A nucleobase modification, in particular addition of a 5-methyl group (*red*), is shown for cytosine and uracil. (*d*) A locked nucleic acid modification inserts a 2′-O, 4′-C methylene bridge into the ribose ring of the nucleotide. (*e*) Unlocked nucleic acids are modified to remove the 2′-3′ carbon bond in the sugar ring. (*f*) The utilitarian 2′-*O*-methyl modification can mitigate siRNA-induced immunostimulation while retaining RNA interference potency.

Table 1 Pattern recognition receptors (PRRs) uniquely recognize and respond to RNA

PRR	Ligand	Cell line expression	Subcellular location	Primary response
TLR3	siRNA dsRNA	Epithelial cells Fibroblasts	Cell surface	IFN-α IFN-β IFN-γ
		Epithelial cells Fibroblasts mDCs	Endosome	IFN-α IFN-β IFN-γ
TLR7	siRNA ssRNA	pDCs	Endosome	IFN-α IFN-β
			Lysosome	TNF-α IL-12
		B cells		B cell differentiation
TLR8	siRNA ssRNA	Monocytes Macrophages mDCs	Endosome	IFN-α IFN-β
			Lysosome	TNF-α IL-1 IL-6 IL-12
PKR	Long dsRNA	Most mammalian cells	Cytoplasm	IFN-α IFN-β Inhibition of protein translation
RIG-I	Uncapped 5′-triphosphate RNA and blunt RNA	Fibroblasts mDCs	Cytoplasm	IFN-α IFN-β

Abbreviations: TLR, Toll-like receptor; siRNA, small interfering RNA; dsRNA, double-stranded RNA; IFN, interferon; mDCs, mature dendritic cells; ssRNA, single-stranded RNA; pDCs, plasmacytoid dendritic cells; TNF-α, tumor necrosis factor α; IL, interleukin; PKR, double-stranded RNA-dependent protein kinase; RIG-I, retinoic acid–inducible gene 1 protein.

or by phage polymerases, which often develop blunt ends (63). RIG-I is expressed in fibroblasts and mDCs (64) and, once activated, initiates an immunological cascade that generates a strong interferon response (25, 59).

In summary, the mammalian immune system has been trained to recognize siRNA as a foreign molecule that is likely a sign of viral infection and to respond robustly. As such, these oligonucleotides are capable of eliciting a potent anti-inflammatory response via a diverse group of pattern recognition receptors existing in varied cell lines and varied cellular locations. **Table 1** summarizes the heterogeneous PRR expression patterns in different cell types within the mammalian system. TLR3 can interact with siRNA on the cell surface, TLRs 3, 7, and 8 recognize siRNA in subcellular compartments, and PKR and RIG-I respond to siRNA in the cytoplasm. Each PRR, depending on its location, induces a unique immunological response to an siRNA target; together the PRRs result in a dynamic and robust anti-inflammatory response to siRNA delivery. Finally, PRR expression changes over time as environmental conditions vary (65). Because the immunological response to siRNA delivery is so multifaceted, the immunostimulatory potential of siRNA must be considered during the design and execution of siRNA studies.

FACTORS INFLUENCING siRNA-MEDIATED IMMUNE ACTIVATION

The diverse repertoire of PRRs in mammalian systems recognizes and reacts to many different features of siRNA. The following sections describe the ways in which siRNA and its associated delivery vehicle can be engineered either to provoke or to evade the innate immune system.

siRNA Sequence Influences Immunostimulation

The innate immune system employs a variety of strategies to ensure the detection of and response to pathogenic signatures. Whereas cytoplasmic RNA receptors such as PKR and RIG-I recognize RNA regardless of its sequence, TLR7 (and TLR8 in humans) recognize ssRNA in a sequence-specific manner (18, 43, 44, 46). Specifically, Judge and colleagues (18) found that the presence of a 5′-UGU-3′ sequence within RNA results in a potent TLR7-mediated interferon response. More generally, siRNAs rich in GU-rich motifs tended to provoke more immunostimulatory activity, whereas a decrease in the presence of uridine residues had the opposite effect. In a separate study, Hornung and coworkers (46) identified 5′-GUCCUUCAA-3′ as an immunostimulatory motif capable of inducing cytokine production independent of the number of GU nucleosides. This motif lost immunostimulatory activity in TLR7 knockout mice, suggesting that TLR7 plays a critical role in mounting an immune response to such sequences. However, these are only two of the many specific RNA sequences that the TLR7/8 arm of the innate immune system has been found to recognize (18, 44, 46, 47, 66).

Interestingly, it has been determined that the only molecular characteristics that are necessary and sufficient to stimulate a TLR7/8-mediated immune response are the presence of a ribose sugar backbone and multiple uridine residues in close proximity to one another (66). These are the two defining attributes that distinguish RNA from DNA. As such, all unmodified ssRNA molecules have the potential to invoke immunostimulatory activity to some extent, with the magnitude of the response determined by the presence of specific sequences within the strand. The substitution of certain residues within an RNA molecule has been shown to diminish the proinflammatory response. In particular, substituting guanosine with adenosine resulted in reduced TNF-α and IL-6 production in blood cells (peripheral blood mononuclear cells), whereas substituting uridine with adenosine decreased IFN-α production in plasmacytoid dendritic cells (43, 67).

siRNA Structure Influences Immunostimulation

In addition to the specific nucleotide sequence, the chemical structure of the siRNA duplex can also influence the degree of innate immune response. RIG-I, for example, has been shown to bind to ssRNA or dsRNA containing uncapped 5′-triphosphate groups (**Figure 3*b***), which results in an interferon-mediated immune response (61–63). Uncapped RNA is a sign of viral infection and subsequently induces inflammatory action. Additionally, Marques and colleagues (59) have reported that blunt-ended dsRNA can provoke immunostimulatory activity through recognition by RIG-I. Such activity is reduced upon incorporation of 3′ overhangs onto either or both RNA strands, as RIG-I loses its ability to unwind and bind to the RNA. This study suggests a structural basis for self versus nonself RNA discrimination. Although self microRNAs present in the cytoplasm have been processed by Dicer to include 3′ overhangs, viral dsRNAs typically possess blunt ends (59).

Nucleotide structure also has a profound effect on innate immune system activation. Researchers originally explored the idea of making chemical modifications to the 2′-OH group on the ribose ring of the RNA backbone as a way of decreasing the susceptibility of RNA to

endonuclease degradation. While exploring alternative 2′ chemistries, it was discovered that such changes can reduce or even eliminate the innate immune response.

2′-*O*-methyl (2′-*O*-Me) modification: this modification of the 2′ position of the sugar ring is one of the most effective at mitigating RNA-induced immunostimulation while retaining RNAi potency

Locked nucleic acids (LNAs; **Figure 3*d***) are modified from traditional nucleic acid structure through the introduction of a 2′-*O*, 4′-C methylene bridge in the ribose ring. When inserted into an RNA strand, such a modification can reduce immune recognition and response (68–70) but can also compromise RNAi potency depending on point of insertion (46). Reduced immunostimulation occurs only when both strands of the siRNA are modified with LNAs (71, 72).

In contrast, unlocked nucleic acids (UNAs) have been explored more recently as an alternative approach to modifying siRNA duplex stability and off-targeting effects (**Figure 3*e***). UNAs are missing the bond between the 2′ and 3′ carbon positions on the sugar ring of the nucleotide, which permits increased molecular flexibility (73). Initial studies have established guidelines on which positions on the antisense strand are the best choices for introducing UNAs to inhibit off-targeting effects while retaining RNAi potency (74, 75). Although the immunostimulatory properties of UNAs have yet to be examined, the modification is a distinctly nonviral characteristic and thus has the potential to reduce immune system activation.

Various laboratories have demonstrated the ability of alternative 2′ modifications to modulate the innate immune response, including 2′-F, 2′-H (i.e., 2′-deoxy) and 2′-*O*-methyl (2′-*O*-Me) chemistries (3, 47, 76). The extent of immune inhibitory activity of 2′-F modifications is dependent upon the position of insertion into the siRNA sequence as well as the number of nucleotide substitutions within the duplex (49). 2′-H modifications mimic DNA structure and offer the ability to evade immune recognition while keeping normal TLR7/8 function intact (77). Cekaite and coworkers (77) demonstrated that 2′-H modification of either uridine or thymidine residues contained within the RNA strand can reduce unwanted off-target effects of RNAi. More recently, it has also been shown that modifying an siRNA duplex with a combination of DNA analogs, 2′-F-modified RNAs, and LNAs can lend enhanced silencing efficacy while reducing immunostimulatory properties in human blood cells (78).

Relative to other types of nucleotide modification, 2′-*O*-Me modifications (**Figure 3*f***) act as potent inhibitors of RNA-induced immune stimulation without diminishing RNAi potency. Modification of the 2′ sugar position with an *O*-methyl group successfully inhibits TLR7/8-mediated recognition of siRNA by the immune system as well as eliminates the RIG-I-mediated immunostimulatory effects of uncapped 5′-triphosphate siRNAs (18, 61, 77, 79). Judge and colleagues (71) found that, when performed on a limited number of residues within the sense strand of siRNA, 2′-*O*-Me modification was capable of abolishing immunostimulation without adversely inhibiting RNAi ability. In fact, they determined that modifying as few as two guanosine or uridine residues within an RNA molecule effectively extinguished TLR7/8-mediated immune activity in both human blood cells and in mice (71). More recently, it has been suggested that judiciously inserting 2′-*O*-Me modifications into both strands of the siRNA duplex may be the best approach to minimize immunostimulatory effects, although antisense strand modifications need to be performed with care to avoid hindering RNAi activity (1).

Interestingly, RNAs containing 2′-*O*-Me modifications have been identified as TLR7 antagonists (72, 80). In other words, the presence of 2′-*O*-Me-modified RNA effectively shuts off TLR7 activity, causing significant reductions in inflammatory cytokine production even when TLR7 is further provoked with immunostimulatory ligands. It has been speculated that such antagonistic activity may be part of the cell's self versus nonself recognition system. Self-RNAs often contain 2′ modifications on the sugar ring. When TLR7 detects what it perceives as self-RNA, it may turn itself off to prevent an autoimmune reaction to other self-ligands in the vicinity. Such a finding could have important implications for the development of therapeutics for autoimmune diseases.

Some guidance is available regarding which nucleotide positions in an siRNA sequence are best suited for 2′ modifications. Jackson and colleagues (81) found that the introduction of a 2′-*O*-Me substitution at nucleotide position 2 of the antisense strand reduced the off-target silencing of mRNA transcripts that shared partial complementarity to the antisense strand. Interestingly, modifications at nucleotide position 9 on the sense strand have been shown to interfere with RISC assembly and subsequent sense strand cleavage (82), and can therefore reduce RNAi efficiency (49). In general, the positions of modifications within a specific RNA duplex need to be chosen carefully to avoid reducing the potency of the RNAi process (46, 68, 76, 83).

Nucleobase structure has also been manipulated in an attempt to reduce proinflammatory effects caused by siRNA delivery. On the basis of evidence that methylation of the nucleotides of immunostimulatory DNA abrogates immune recognition, Karikó et al. (84) introduced methyl groups onto several different types of nucleobases, including 5-methyl-cytidine and 5-methyl-uridine, which are shown in **Figure 3*c***. Their results indicate that such nucleobase modifications can decrease an siRNA-provoked immune response, although questions remain as to whether or not base modification will interfere with base pairing and ultimate RNAi ability.

siRNA Delivery Vehicles Influence Immunostimulation

Because siRNA is large (~13 kDa) and has a negatively charged backbone, its ability to readily cross lipophilic cellular membranes is decreased. As such, a delivery vehicle is usually required for effective delivery in both cell culture and animals. In vivo, a delivery vehicle also protects siRNA from various threats to exogenously introduced therapeutics, including phagocytosis and enzymatic degradation. Through the inclusion of different surface moieties, a delivery vehicle can extend circulation time and/or potentially target specific cell types within the body through ligand-receptor interactions. Interestingly, naked siRNA, although relatively inefficient at inducing gene silencing, does not necessarily cause innate immune activation (85). In contrast, the use of siRNA delivery material can have a substantial effect on siRNA-promoted immunostimulation, and the choice of delivery vehicle can account for immune responses varying up to two orders of magnitude (49, 86, 87). Delivery vehicles can act through a variety of mechanisms, escorting siRNA molecules across the membrane in unique ways and into differing subcellular compartments en route to the cytoplasm. Therefore, depending on the path of the delivery package, RNA will be exposed to differing numbers and types of pattern recognition receptors.

For example, cationic materials have been a popular choice of delivery vector, as they readily condense RNA because of electrostatic attraction. Most are believed to enable intracellular delivery by mediating endosomal uptake into the cell and subsequent endosomal escape into the cytoplasm, where the siRNA can interact with the RNAi machinery (88). When coupled to a cationic delivery material, siRNA is guided through several subcellular locations, enabling it to interact with TLRs in the endosomal compartments of immune cells as well as with RIG-I and PKR, which are present in the cytoplasm of most cell types. Therefore, this type of vehicle exposes its cargo to many PRRs, rendering the siRNA more prone to innate immune recognition and response than a delivery vehicle that avoided trafficking through the endosomal and lysosomal compartments. For example, a lipid-like siRNA delivery material, C12-200, has been shown to gain cellular entry through pinocytosis in vitro (89). Because pinocytosis avoids the endosomal/lysosomal pathway, the C12-200 delivery mechanism may potentially limit TLR-induced immunostimulatory action.

The size, charge, and in vivo biodistribution of a delivery nanoparticle can also alter both the intensity of an immune response as well as the resulting cytokine expression profile. In 2005, Judge and coworkers (18) compared and contrasted immune responses to the delivery of siRNA to human blood cells using various delivery materials. They found that although stable nucleic acid

lipid particle (SNALP)-encapsulated and polyethylenimine-complexed siRNA caused an IFN-α immune response, the use of polylysine, which forms larger delivery particles, resulted in an inflammatory response dominated by cytokines, including IL-6 and TNF-α (18). It has also been demonstrated that using different formulation techniques can increase significantly the immunostimulatory properties of the lipidoid delivery vehicle ND98-5 (90).

EXPERIMENTAL APPROACHES TO SILENCING AND STIMULATION

Today, researchers can design experiments in a way that decouples gene silencing effects from immune stimulation effects. This is especially important in light of the ability of immunostimulatory RNA to lend therapeutic benefit.

siRNA-Induced Immune Stimulation Can Be Monitored and Controlled

This section describes careful designs for siRNA delivery studies that should provide convincing evidence of RNAi-induced efficacy and/or immunostimulation-induced efficacy. Information regarding the proinflammatory nature of a particular siRNA and its delivery vehicle is of utmost importance when evaluating potential for further clinical development.

Following siRNA delivery, it is possible to measure cytokine production by collecting and evaluating cell supernatant (in vitro experiments) or animal serum (in vivo experiments) (91). RIG-I and PKR-mediated immune stimulation can be preliminarily assessed in certain (but not all) cell types by screening for the presence of IFN-β, IL-6, and IL-8. To assess TLR-mediated immunostimulatory action, transfection must occur in a primary immune cell culture. Presence of IFN-α, IL-6, and TNF-α in vitro is suggestive of TLR-induced innate immune activation, whereas the upregulation of IFN-α, IL-6, and TNF-α in animal sera indicates systemic inflammatory action in vivo (49).

Cytokine levels increase transiently upon siRNA-induced immune activation, and the timing of maximum inflammatory levels can vary significantly depending on the animal model, siRNA delivery vehicle, and cytokine in question. Most cytokine levels elevate, peak, and disappear anywhere from 1 to 24 h after the initial dosing of siRNA (49). Given the variability in cytokine expression profiles, Robbins and colleagues (49) recommend that sample collection take place at multiple time points to ensure the best chance of detecting inflammatory action. Results should be interpreted with caution, keeping in mind that performing an exhaustive study of cytokine activation is difficult.

When in need of a more sensitive assay to probe for evidence of immunostimulation, mRNA levels of a gene called *IFIT1* can be quantified in transfected cells using the quantitative polymerase chain reaction (qPCR). *IFIT1* expression occurs upstream of IFN expression, and therefore, upregulation of *IFIT1* can be observed prior to increases in IFN production (93). Using such an approach, it is possible to detect low levels of immunostimulatory action through increases in IFIT1 mRNA levels without detection of IFN-α in serum (1).

Many siRNA delivery studies attempt to use control siRNAs to evaluate any nonspecific effects that may occur in addition to the intended RNAi. The issue of nonspecific immune stimulation is of particular relevance in the treatment of illnesses that benefit from an innate immune response, such as viral infections or cancer. The demonstration that control siRNAs cannot achieve the same therapeutic effects as target-specific siRNAs is often meant to instill confidence that sequence-specific RNAi is taking place and that immune stimulation is not factoring into positive outcomes. Unfortunately, it is now known that siRNA-induced immune stimulation is sequence dependent (18, 46, 47). Therefore, it cannot be expected that any two siRNAs (e.g., the experimental and control siRNAs) will elicit the same immunological response.

Control siRNAs need to be selected carefully to avoid misinterpreted results. A popular control siRNA encoding a nonmammalian gene, green fluorescent protein, has recently been shown to have an unusually low immunostimulatory profile (94). The use of such a control has the potential to mislead investigators into believing that the positive effects of siRNA therapy are due solely to target-specific silencing rather than activation of the innate immune system. Any control siRNAs used in comparative analysis need to be chosen carefully to ensure that the control siRNA induces the same level of immunostimulatory activity as the therapeutic siRNA in question.

VEGF: vascular endothelial growth factor

One of the most useful approaches when performing siRNA delivery studies in vivo involves the use of chemical modifications that minimize the immunostimulatory potential of the siRNA delivery complex. This may include the use of strategically placed 2′-*O*-Me modifications and/or substitution of uridine residues with 2′-deoxythymidine residues. Trial and error is often required in the design of a nonimmunostimulatory siRNA sequence that retains RNAi potency.

Using modified target siRNAs in conjunction with modified control siRNAs would allow the differentiation between sequence-specific versus off-target RNAi effects while minimizing the influence of innate immune activity. Results obtained with modified versus unmodified RNA can be compared should there be an interest in provoking the immune system for the treatment of diseases that benefit from proinflammatory action. This experimental approach will assist both investigators and the scientific community in more fully appreciating the complicated, multifaceted effects of siRNA on a living organism.

Immunostimulatory RNA Can Have Therapeutic Activity

Because certain classes of diseases can potentially benefit from inflammatory action, siRNA studies must be designed carefully to differentiate between target-specific, RNAi-mediated therapeutic effects and nonspecific immunostimulatory therapeutic effects. For example, extensive demonstrations have shown that organisms ward off viral infections with greater speed and efficiency when the innate immune system is stimulated (95, 96).

For example, Morrissey and coworkers (3) designed a study to differentiate between RNAi and immune-induced therapeutic outcomes in a hepatitis B virus (HBV) mouse model (3). Upon finding that unmodified anti-HBV siRNAs stimulated production of IFN-α, IL-6, and TNF-α, chemically modified siRNAs were designed that abrogated immune effects. Both modified and unmodified anti-HBV similarly reduced HBV titers in mice at the same dose, indicating that the siRNA-mediated anti-HBV outcome was a sequence-specific effect of RNAi.

Another consequence of innate immune stimulation that can be exploited for therapeutic purposes is antiangiogenic activity. Vascular endothelial growth factor (VEGF) and its receptor, VEGFR, have been indicated as key proteins in stimulating the proliferation of blood vessels (97–99). Because interferons act specifically to suppress VEGF expression at the mRNA level (100), the expression of either IFN-α or IFN-β may result in reduced angiogenesis (101). Accordingly, numerous investigations have explored the application of immunostimulatory nucleic acids, both DNA (102, 103) and RNA (104, 105), in cancer therapy.

Angiogenesis also has implications in the treatment of age-related macular degeneration (AMD), an eye disease caused by excessive vascularization of the retina that can lead to blindness. Some of the most advanced siRNA clinical trials involve presumed RNAi-induced silencing of the VEGF or VEGFR genes. Recently, it has been demonstrated that the inhibition of vascularization is an siRNA class effect, that is, any siRNA 21 nucleotides or longer of any sequence is capable of achieving a therapeutic outcome in AMD models (106). Interestingly, these nonspecific effects were found to occur without the detection of off-target effects or IFN-α or IFN-β, suggesting that TLR7/8 immune stimulation was not mediating antiangiogenic activity. Upon

further analysis, it was found that siRNAs can interact with TLR3 present on the surface of human choroidal endothelial cells, which causes a release of IFN-γ and IL-12 and subsequent reductions in neovascularization (106).

Silencing and Immunostimulatory siRNA Properties Can Be Used Synergistically

Although induction of the innate immune system often is viewed as an unwanted side effect of siRNA delivery, in certain diseases, such as cancer or viral infection, it may be desirable to stimulate inflammatory action. One study in particular, carried out by Gantier and colleagues (107), aimed to identify sequence-based modifications that would allow for the design of bifunctional siRNAs with both proinflammatory and specific silencing activities, and with potentially increased therapeutic benefits. It was found that the introduction of a nonpairing uridine bulge in the passenger strand robustly increased immunostimulatory activity in human immune cells while rendering no effect on the silencing efficiency of the siRNA. A separate examination, in addition to confirming the immunostimulatory nature of motifs heavy in uridine, determined that lower hybridization strength between the two RNA strands yields a more immunostimulatory product (108).

Several studies have set out to take advantage of both the gene-silencing and immune-stimulating abilities of siRNA synergistically to achieve a therapeutic effect (80, 109, 110). In one example, Poeck and coworkers (105) induced substantial apoptosis of metastatic melanoma lung tumors in a mouse model using uncapped 5′-triphosphate siRNA targeting the *Bcl2* gene. The 5′-triphosphate ends characteristic of viral RNA are recognized by RIG-I in the cytosol (61, 62), triggering a strong IFN response. This immunostimulatory response was combined with an siRNA targeting *Bcl2*, an oncogene that promotes tumor proliferation (111, 112). Comparison of the results of delivering uncapped 5′-triphosphate siRNA with capped siRNA showed that both *Bcl2* silencing and RIG-I-mediated immunostimulation were needed to achieve maximal tumor suppression (105). Chemically modified siRNAs were not used in this study, so the precise contribution of *Bcl2* silencing relative to RIG-I activation was not ascertainable. It will be interesting to determine the relative contributions of immunostimulation to silencing in future studies.

FUTURE OUTLOOK

The real power behind RNAi therapy lies in its ability to potently and specifically silence target genes and their associated proteins. The introduction of nonself siRNA into the cell, however, can provoke a potentially robust innate response. Given the myriad of cell types that respond to RNA and pattern recognition receptors acting through multiple mechanisms in varied cellular locations, significant potential exists for siRNA to induce immune-based off-target effects. Such immunostimulation depends on a wide variety of factors, including the sequence and structure of RNA, delivery vehicle, target cell type, and animal model being examined. Therefore, each study must be individually crafted to properly control and account for siRNA-mediated innate immune activation.

In a clinical setting, the consequences of overstimulating the innate immune system can be dire. A robust proinflammatory response can induce chills, fever, and hypotension in patients (113). In the unfortunate event of a cytokine storm, patients are at risk of nausea, hypotension, vasodilation, depletion of white blood cells, prolonged cardiovascular shock, acute respiratory distress syndrome, and even death (114). Given these potential complications, the acknowledgment

and control of immunostimulatory action is of utmost importance in moving siRNA into higher-order animal models and clinical trials.

The good news is that a wealth of information regarding methods to alleviate siRNA-induced immunostimulatory effects has been uncovered in recent years. It is relatively straightforward to engineer siRNAs to include nucleotide modifications that reduce or eliminate TLR-mediated immune effects without negatively impacting gene silencing ability. Particularly useful to this end are 2′ nucleotide modifications, which are available to researchers through typical laboratory resource suppliers. Although such modified RNAs come at an increased cost to researchers, they offer the scientific community the much-needed ability to differentiate between the therapeutic effects of RNAi-induced gene silencing and immunostimulatory action. Moving forward, careful consideration of RNA-mediated innate immune stimulation will ensure the full potentiation of siRNA-based therapeutics and the broadest application of RNAi in the clinic.

SUMMARY POINTS

1. RNA interference is a cellular mechanism that mediates potent gene-specific silencing. Short interfering RNAs (siRNAs) have the potential to be used therapeutically to silence genes involved in the pathogenesis of a wide variety of diseases, including viral infection, hereditary disorders, and cancer.
2. Exogenous siRNA introduced into the body has the potential to activate the innate immune system through interaction with pattern recognition receptors (PRRs).
3. PRRs capable of recognizing and responding to siRNA include Toll-like receptor 3 (TLR3), which recognizes double-stranded RNA, and TLR7 and 8, which recognize single-stranded RNA. The cytoplasmic PRR proteins PKR and RIG-I are also able to mount an immune response against siRNA.
4. The nucleotide sequence of siRNA affects its immunostimulatory properties. For example, motifs high in uridine content trigger a TLR7-mediated immune response.
5. The chemical structure of siRNA affects its immunostimulatory properties. Modifications to the 2′ position on the ribose ring of the RNA backbone can substantially reduce innate immune activation while retaining RNAi potency.
6. The delivery vehicle used to facilitate siRNA transfection can affect the innate immune response by altering the delivery pathway and the PRRs that interact with the siRNA in different cellular locations.
7. Delivery experiments for siRNA need to be designed carefully to differentiate between therapeutic effects caused by gene silencing versus those caused by immunostimulation. The easiest way to ensure reduced immunostimulatory properties is to introduce 2′ modifications into the siRNA duplex.
8. Awareness of and control for siRNA-induced immune activation will ensure the most rapid development of siRNA therapeutics in the clinic.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The authors would like to thank C. Levins, A. Vegas, and J. Matthews for their insight and feedback during the preparation of this manuscript.

LITERATURE CITED

1. Judge AD, Robbins M, Tavakoli I, Levi J, Hu L, et al. 2009. Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. *J. Clin. Investig.* 119:661–73
2. Frank-Kamenetsky M, Grefhorst A, Anderson NN, Racie TS, Bramlage B, et al. 2008. Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates. *Proc. Natl. Acad. Sci. USA* 105:11915–20
3. Morrissey DV, Lockridge JA, Shaw L, Blanchard K, Jensen K, et al. 2005. Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nat. Biotechnol.* 23:1002–7
4. DiFiglia M, Sena-Esteves M, Chase K, Sapp E, Pfister E, et al. 2007. Therapeutic silencing of mutant huntingtin with siRNA attenuates striatal and cortical neuropathology and behavioral deficits. *Proc. Natl. Acad. Sci. USA* 104:17204–9
5. Geisbert TW, Lee ACH, Robbins M, Geisbert JB, Honko AN, et al. 2010. Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. *Lancet* 375:1896–905
6. Geisbert TW, Hensley LE, Kagan E, Yu EZ, Geisbert JB, et al. 2006. Postexposure protection of guinea pigs against a lethal Ebola virus challenge is conferred by RNA interference. *J. Infect. Dis.* 193:1650–57
7. Davis ME, Zuckerman JE, Choi CHJ, Seligson D, Tolcher A, et al. 2010. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature* 464:1067–70
8. **Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391:806–11**
9. **Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411:494–98**
10. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409:363–66
11. Ameres SL, Martinez J, Schroeder R. 2007. Molecular basis for target RNA recognition and cleavage by human RISC. *Cell* 130:101–12
12. Kleinschmidt WJ, Ellis LF, Van Fbank RM, Murphy EB. 1968. Interferon stimulation by a double stranded RNA of a mycophage in statolon preparations. *Nature* 220:167–68
13. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, et al. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441:101–5
14. Xia H, Mao Q, Paulson HL, Davidson BL. 2002. siRNA-mediated gene silencing in vitro and in vivo. *Nat. Biotechnol.* 20:1006–10
15. Leirdal M, Sioud M. 2002. Gene silencing in mammalian cells by preformed small RNA duplexes. *Biochem. Biophys. Res. Commun.* 295:744–48
16. **Bridge AJ, Pebernard S, Ducraux A, Nicoulaz A-L, Iggo R. 2003. Induction of an interferon response by RNAi vectors in mammalian cells. *Nat. Genet.* 34:263–64**
17. Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BRG. 2003. Activation of the interferon system by short-interfering RNAs. *Nat. Cell Biol.* 5:834–39
18. Judge AD, Sood V, Shaw JR, Fang D, McClintock K, MacLachlan I. 2005. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat. Biotechnol.* 23:457–62
19. Hoffmann JA, Kafatos FC, Janeway C, Ezekowitz RAB. 1999. Phylogenetic perspectives in innate immunity. *Science* 284:1313–18
20. Iwasaki A, Medzhitov R. 2010. Regulation of adaptive immunity by the innate immune system. *Science* 327:291–95
21. Feve B, Bastard J-P. 2009. The role of interleukins in insulin resistance and type 2 diabetes mellitus. *Nat. Rev. Endocrinol.* 5:305–11

8. The landmark study that identified and described the phenomenon of RNA interference.

9. The first study to determine that exogenous siRNA is capable of sequence-specific gene silencing in mammalian cells.

16. The first study to demonstrate that siRNA is capable of inducing an interferon response in mammalian cells.

22. Locksley RM, Killeen N, Lenardo MJ. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 104:487–501
23. Stuart LM, Ezekowitz RA. 2008. Phagocytosis and comparative innate immunity: learning on the fly. *Nat. Rev. Immunol.* 8:131–41
24. Liu Y-J. 2005. IPC: Professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu. Rev. Immunol.* 23:275–306
25. Garcia-Sastre A, Biron CA. 2006. Type 1 interferons and the virus-host relationship: a lesson in detente. *Science* 312:879–82
26. Janeway C Jr, Medzhitov R. 2002. Innate immune recognition. *Annu. Rev. Immunol.* 20:197–216
27. Akira S, Uematsu S, Takeuchi O. 2006. Pathogen recognition and innate immunity. *Cell* 124:783–801
28. Takeuchi O, Akira S. 2010. Pattern recognition receptors and inflammation. *Cell* 140:805–20
29. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. 1998. A family of human receptors structurally related to *Drosophila* Toll. *Proc. Natl. Acad. Sci. USA* 95:588–93
30. Akira S, Takeda K, Kaisho T. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* 2:675–80
31. Kawai T, Akira S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* 11:373–84
32. Bell J, Askins J, Hall P, Davies D, Segal D. 2006. The dsRNA binding site of human Toll-like receptor 3. *Proc. Natl. Acad. Sci. USA* 103:8792–97
33. Choe J, Kelker MS, Wilson IA. 2005. Crystal structure of human Toll-like receptor 3 (TLR3) ectodomain. *Science* 309:581–85
34. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature* 413:732–38
35. Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. 2004. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat. Med.* 10:1366–73
36. Kleinman ME, Yamada K, Takeda A, Chandrasekaran V, Nozaki M, et al. 2008. Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. *Nature* 452:591–97
37. Cho WG, Albuquerque RJC, Kleinman ME, Tarallo V, Greco A, et al. 2009. Small interfering RNA-induced TLR3 activation inhibits blood and lymphatic vessel growth. *Proc. Natl. Acad. Sci. USA* 106:7137–42
38. Kawai T, Akira S. 2008. Toll-like receptor and RIG-1-like receptor signaling. *Ann. N. Y. Acad. Sci.* 1143:1–20
39. Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, et al. 2003. Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J. Immunol.* 171:3154–62
40. Applequist SE, Wallin RPA, Ljunggren HG. 2002. Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines. *Int. Immunol.* 14:1065–74
41. Bekeredjian-Ding IB, Wagner M, Hornung V, Giese T, Schnurr M, et al. 2005. Plasmacytoid dendritic cells control TLR7 sensitivity of naive B cells via type I IFN. *J. Immunol.* 174:4043–50
42. Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, et al. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat. Immunol.* 3:196–200
43. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, et al. 2004. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. *Science* 303:1526–29
44. Diebold SS, Kaisho T, Hemmi H, Akira S, Sousa CRE. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303:1529–31
45. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, et al. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc. Natl. Acad. Sci. USA* 101:5598–603
46. Hornung V, Guenthner-Biller M, Bourquin C, Ablasser A, Schlee M, et al. 2005. Sequence-specific potent induction of IFN-α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat. Med.* 11:263–70
47. Sioud M. 2005. Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. *J. Mol. Biol.* 348:1079–90

46. The first evidence that siRNA induces a sequence-specific immune response mediated by TLR7.

48. Lee J, Chuang T-H, Redecke V, She L, Pitha PM, et al. 2003. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7. *Proc. Natl. Acad. Sci. USA* 100:6646–51
49. Robbins M, Judge A, MacLachlan I. 2009. siRNA and innate immunity. *Oligonucleotides* 19:89–102
50. Forsbach A, Nemorin J, Montino C, Mueller C, Samulowitz U, et al. 2008. Identification of RNA sequence motifs stimulating sequence-specific TLR8-dependent immune responses. *J. Immunol.* 180:3729–38
51. Gorden KKB, Qiu XX, Binsfeld CCA, Vasilakos JP, Alkan SS. 2006. Cutting edge: activation of murine TLR8 by a combination of imidazoquinoline immune response modifiers and polyT oligodeoxynucleotides. *J. Immunol.* 177:6584–87
52. Gorden KB, Gorski KS, Gibson SJ, Kedl RM, Kieper WC, et al. 2005. Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. *J. Immunol.* 174:1259–68
53. Zarember KA, Godowski PJ. 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.* 168:554–61
54. Clemens MJ, Elia A. 1997. The double-stranded RNA-dependent protein kinase PKR: structure and function. *J. Interferon Cytokine Res.* 17:503–24
55. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, et al. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* 5:730–37
56. Meurs E, Chong K, Galabru J, Thomas NSB, Kerr IM, et al. 1990. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. *Cell* 62:379–90
57. Zhang Z, Weinschenk T, Guo K, Schluesener HJ. 2006. siRNA binding proteins of microglial cells: PKR is an unanticipated ligand. *J. Cell. Biochem.* 97:1217–29
58. Williams BRG. 2001. Signal integration via PKR. *Sci. STKE* 2001:re2
59. Marques JT, Devosse T, Wang D, Zamanian-Daryoush M, Serbinowski P, et al. 2006. A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. *Nat. Biotechnol.* 24:559–65
60. Puthenveetil S, Whitby L, Ren J, Kelnar K, Krebs JF, Beal PA. 2006. Controlling activation of the RNA-dependent protein kinase by siRNAs using site-specific chemical modification. *Nucl. Acids Res.* 34:4900–11
61. Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, et al. 2006. 5′-Triphosphate RNA is the ligand for RIG-I. *Science* 314:994–97
62. Pichlmair A, Schulz O, Tan CP, Naslund TI, Liljestrom P, et al. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science* 314:997–1001
63. Kim D-H, Longo M, Han Y, Lundberg P, Cantin E, Rossi JJ. 2004. Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase. *Nat. Biotechnol.* 22:321–25
64. Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, et al. 2005. Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 23:19–28
65. Akira S, Uematsu S, Takeuchi O. 2006. Pathogen recognition and innate immunity. *Cell* 124:783–801
66. Diebold S, Massacrier C, Akira S, Paturel C, Morel Y, Reis e Sousa C. 2006. Nucleic acid agonists for Toll-like receptor 7 are defined by the presence of uridine ribonucleotides. *Eur. J. Immunol.* 36:3256–67
67. Sioud M. 2006. Single-stranded small interfering RNA are more immunostimulatory than their double-stranded counterparts: a central role for 2′-hydroxyl uridines in immune responses. *Eur. J. Immunol.* 36:1222–30
68. Elmén J, Thonberg H, Ljungberg K, Frieden M, Westergaard M, et al. 2005. Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. *Nucl. Acids Res.* 33:439–47
69. Dahlgren C, Wahlestedt C, Thonberg H. 2006. No induction of anti-viral responses in human cell lines HeLa and MCF-7 when transfecting with siRNA or siLNA. *Biochem. Biophys. Res. Commun.* 341:1211–17
70. Fluiter K, Mook OR, Baas F. 2009. The therapeutic potential of LNA-modified siRNAs: reduction of off-target effects by chemical modification of the siRNA sequence. *Methods Mol. Biol.* 487:189–203
71. Judge AD, Bola G, Lee ACH, MacLachlan I. 2006. Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo. *Mol. Ther.* 13:494–505

59. Demonstration that the end structure of siRNA duplexes, independent of sequence, can cause innate immune stimulation.

72. Robbins M, Judge A, Liang L, McClintock K, Yaworski E, MacLachlan I. 2007. 2′-O-methyl-modified RNAs act as TLR7 antagonists. *Mol. Ther.* 15:1663–69
73. Langkjær N, Pasternak A, Wengel J. 2009. UNA (unlocked nucleic acid): a flexible RNA mimic that allows engineering of nucleic acid duplex stability. *Bioorg. Med. Chem.* 17:5420–25
74. Bramsen JB, Pakula MM, Hansen TB, Bus C, Langkjaer N, et al. 2010. A screen of chemical modifications identifies position-specific modification by UNA to most potently reduce siRNA off-target effects. *Nucl. Acids Res.* 38:5761–73
75. Kenski DM, Cooper AJ, Li JJ, Willingham AT, Haringsma HJ, et al. 2010. Analysis of acyclic nucleoside modifications in siRNAs finds sensitivity at position 1 that is restored by 5′-terminal phosphorylation both in vitro and in vivo. *Nucl. Acids Res.* 38:660–71
76. Chiu Y-L, Rana TM. 2003. siRNA function in RNAi: a chemical modification analysis. *RNA* 9:1034–48
77. Cekaite L, Furset G, Hovig E, 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
78. Deleavey GF, Watts JK, Alain T, Robert F, Kalota A, et al. 2010. Synergistic effects between analogs of DNA and RNA improve the potency of siRNA-mediated gene silencing. *Nucl. Acids Res.* 38:4547–57
79. Hamm S, Latz E, Hangel D, Müller T, Yu P, et al. 2010. Alternating 2′-O-ribose methylation is a universal approach for generating non-stimulatory siRNA by acting as TLR7 antagonist. *Immunobiology* 215:559–69
80. Furset G, Sioud M. 2007. Design of bifunctional siRNAs: combining immunostimulation and gene-silencing in one single siRNA molecule. *Biochem. Biophys. Res. Commun.* 352:642–49
81. Jackson AL, Burchard J, Leake D, Reynolds A, Schelter J, et al. 2006. Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. *RNA* 12:1197–205
82. Leuschner PJF, Ameres SL, Kueng S, Martinez J. 2006. Cleavage of the siRNA passenger strand during RISC assembly in human cells. *EMBO Rep.* 7:314–20
83. Czauderna F, Fechtner M, Dames S, Aygun H, Klippel A, et al. 2003. Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. *Nucl. Acids Res.* 31:2705–16
84. Karikó K, Buckstein M, Ni H, Weissman D. 2005. Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* 23:165–75
85. Heidel JD, Hu S, Liu XF, Triche TJ, Davis ME. 2004. Lack of interferon response in animals to naked siRNAs. *Nat. Biotechnol.* 22:1579–82
86. Kornek M, Lukacs-Kornek V, Limmer A, Raskopf E, Becker U, et al. 2008. 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP)-formulated, immune-stimulatory vascular endothelial growth factor A small interfering RNA (siRNA) increases antitumoral efficacy in murine orthotopic hepatocellular carcinoma with liver fibrosis. *Mol. Med.* 14:365–73
87. Cubillos-Ruiz JR, Engle X, Scarlett UK, Martinez D, Barber A, et al. 2009. Polyethylenimine-based siRNA nanocomplexes reprogram tumor-associated dendritic cells via TLR5 to elicit therapeutic antitumor immunity. *J. Clin. Investig.* 119:2231–44
88. Akinc A, Thomas M, Klibanov AM, Langer R. 2005. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J. Gene Med.* 7:657–63
89. Love KT, Mahon KP, Levins CG, Whitehead KA, Querbes W, et al. 2010. Lipid-like materials for low-dose, in vivo gene silencing. *Proc. Natl. Acad. Sci. USA* 107:1864–69
90. Nguyen DN, Chen SCY, Lu J, Goldberg M, Kim P, et al. 2009. Drug delivery-mediated control of RNA immunostimulation. *Mol. Ther.* 17:1555–62
91. Nguyen DN, Kim P, Martínez-Sobrido L, Beitzel B, García-Sastre A, et al. 2009. A novel high-throughput cell-based method for integrated quantification of type I interferons and in vitro screening of immunostimulatory RNA drug delivery. *Biotechnol. Bioeng.* 103:664–75
92. Deleted in proof
93. Der SD, Zhou A, Williams BRG, Silverman RH. 1998. Identification of genes differentially regulated by interferon α, β, or γ using oligonucleotide arrays. *Proc. Natl. Acad. Sci. USA* 95:15623–28
94. Robbins M, Judge A, Ambegia E, Choi C, Yaworski E, et al. 2008. Misinterpreting the therapeutic effects of small interfering RNA caused by immune stimulation. *Hum. Gene Ther.* 19:991–99

95. Ko S-Y, Ko H-J, Chang W-S, Park S-H, Kweon M-N, Kang C-Y. 2005. α-Galactosylceramide can act as a nasal vaccine adjuvant inducing protective immune responses against viral infection and tumor. *J. Immunol.* 175:3309–17
96. Lindqvist M, Persson J, Thorn K, Harandi AM. 2009. The mucosal adjuvant effect of α-galactosylceramide for induction of protective immunity to sexually transmitted viral infection. *J. Immunol.* 182:6435–43
97. Ferrara N, Henzel WJ. 1989. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 161:851–58
98. Folkman J, Klagsbrun M. 1987. Angiogenic factors. *Science* 235:442–47
99. Plate KH, Breier G, Weich HA, Risau W. 1992. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature* 359:845–48
100. von Marschall Z, Scholz A, Cramer T, Schafer G, Schirner M, et al. 2003. Effects of interferon alpha on vascular endothelial growth factor gene transcription and tumor angiogenesis. *J. Natl. Cancer Inst.* 95:437–48
101. Folkman J, Ingber D. 1992. Inhibition of angiogenesis. *Semin. Cancer Biol.* 3:89–96
102. Brannon-Peppas L, Ghosn B, Roy K, Cornetta K. 2007. Encapsulation of nucleic acids and opportunities for cancer treatment. *Pharm. Res.* 24:618–27
103. Krieg AM. 2006. Therapeutic potential of Toll-like receptor 9 activation. *Nat. Rev. Drug Discov.* 5:471–84
104. Pries R, Wulff S, Kesselring R, Börngen K, Xie L, Wollenberg B. 2008. Up-regulation of NK cell function against head and neck cancer in response to ss-isRNA requires TLR7. *Int. J. Oncol.* 33:993–1000
105. Poeck H, Besch R, Maihoefer C, Renn M, Tormo D, et al. 2008. 5′-Triphosphate-siRNA: turning gene silencing and Rig-I activation against melanoma. *Nat. Med.* 14:1256–63
106. Kleinman ME, Yamada K, Takeda A, Chandrasekaran V, Nozaki M, et al. 2008. Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. *Nature* 452:591–97
107. Gantier MP, Tong S, Behlke MA, Irving AT, Lappas M, et al. 2010. Rational design of immunostimulatory siRNAs. *Mol. Ther.* 18:785–95
108. Goodchild A, Nopper N, King A, Doan T, Tanudji M, et al. 2009. Sequence determinants of innate immune activation by short interfering RNAs. *BMC Immunol.* 10:40
109. Kortylewski M, Swiderski P, Herrmann A, Wang L, Kowolik C, et al. 2009. In vivo delivery of siRNA to immune cells by conjugation to a TLR9 agonist enhances antitumor immune responses. *Nat. Biotechnol.* 27:925–32
110. Iversen PO, Sorensen DR, Sioud M. 2010. A combined immunostimulatory and immunoinhibitory short interference RNA reduces hypercoagulability in a rat model of acute promyelocytic leukaemia. *Thromb. Haemost.* 104:350–54
111. Vaux DL, Cory S, Adams JM. 1988. *Bcl-2* gene promotes haemopoietic cell survival and cooperates with c-*myc* to immortalize pre-B cells. *Nature* 335:440–42
112. McDonnell TJ, Deane N, Platt FM, Nunez G, Jaeger U, et al. 1989. *bcl-2*-Immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* 57:79–88
113. Michie HR, Manogue KR, Spriggs DR, Revhaug A, O'Dwyer S, et al. 1988. Detection of circulating tumor necrosis factor after endotoxin administration. *N. Engl. J. Med.* 318:1481–86
114. Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, et al. 2006. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N. Engl. J. Med.* 355:1018–28
115. Krieg AM. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546–49
116. Hartmann G, Weiner GJ, Krieg AM. 1999. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc. Natl. Acad. Sci. USA* 96:9305–10
117. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, et al. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740–45

106. Demonstration that the antiangiogenic effects seen upon siRNA delivery to the eye are caused by nonsequence-specific activation of a TLR3-mediated inflammatory response.

Annual Review of Chemical and Biomolecular Engineering

Volume 2, 2011

Contents