**Previews**

**TLR Ignores Methylated RNA?**

CpG methylation of DNA silences TLR9-mediated innate immune recognition. In this issue of *Immunity*, Kariko et al. (2005) suggest that the innate immune recognition of RNA by TLR3, TLR7, or TLR8 is in fact controlled by modification of nucleotides, including methylation.

Nucleic acids such as DNA and RNA are essential elements of all living organisms. Evidence that has accumulated over the past few decades suggests that nucleic acids are normally sequestered but can be released from pathogens or damaged host cells and are then recognized by the innate immune system (Isaacs et al., 1963; Tokunaga et al., 1984). The recent discovery of Toll-like receptors (TLRs) and the extensive studies that followed have revealed that certain TLRs recognize nucleic acids derived from infectious organisms or damaged host cells, resulting in modulation of immune responses (Figure 1) (Akira and Takeda, 2004).

Although certain RNAs such as double-stranded (ds) RNA have long been known to be immunologically active, it is only recently that TLR-mediated recognition of RNA has been described. TLR3 recognizes dsRNA derived from virus or host mRNA (Alexopoulou et al., 2001; Kariko et al., 2004). TLR7 and TLR8 have recently been shown to recognize viral or synthetic single-stranded (ss) RNA (Heil et al., 2004; Diebold et al., 2004) or double-stranded, short-interfering RNA (siRNA) (Hornung et al., 2005). These findings have facilitated our understanding of innate immune recognition of RNA but have also left unsolved issues. In particular, we do not know exactly why a variety of self-RNA species, abundant in the host cells, are not continuously stimulating our immune system. Exactly what element(s) of RNA do TLR3, TLR7, or TLR8 recognize? In other words, are there any specific rules with respect to ligand structure or chemistry for elicitation of responses from these TLRs?

There are some clues in recent reports. An initial study indicated that the presence of GU- or U-rich sequences in virus-derived ssRNA might explain some of the differences between stimulatory and inert ssRNA (Diebold et al., 2004; Heil et al., 2004). Although this idea was attractive, a recent report showed that high GU content was not critical in the case of TLR7-mediated recognition of siRNA (Hornung et al., 2005). A plausible explanation was consequently offered: that normally abundant host ssRNAs are usually sequestered within intact cell membranes or immediately degraded by abundant RNases, thereby limiting their accessibility to the endosomal compartments in which TLR3, TLR7, or TLR8 reside. This spatial safety mechanism to prevent aberrant innate activation by RNA is broken by certain exceptional conditions such as a virus infection or tissue damage (Crozat and Beutler, 2004).

Other reports offered an alternative hypothesis, namely that there may still be a specific element in the RNA responsible for its immunomodulatory activity. Messenger RNAs derived from bacteria without poly(A) tails, but not from vertebrate mRNA with such sequences, displayed immunostimulatory activity on human myeloid dendritic cells (Koski et al., 2004). This could explain why bacterial, but not host, RNA is stimulatory. Similarly, unmethylated CpG motifs, more frequently observed in bacterial and certain viral DNA, may be mirrored in mRNA expressed by such pathogens. In fact, ssRNA with unmethylated CpG motifs was immunostimulatory, and its activity was abrogated if the 5' position of C was methylated, similar to that of CpG DNA, although the stimulatory effects were not simply mediated by TLR7 or TLR8 (Sugiyama et al., 2005). Interestingly, not only 5'-C methylation in CG dinucleotides but also 2'-O methylation of any nucleotides in ssRNA abrogated the immunostimulatory effects (Sugiyama et al., 2005). Many other modifications are currently known to be present in vertebrate RNAs as compared to bacterial RNAs, suggesting that these vertebrate-specific modifications, including methylation (see the web site http://medlib.med.utah.edu/RNAmods/), may be key to limiting their activity.

In this issue of *Immunity*, Kariko et al. (2005) extensively examined the effects of major RNA modifications on TLR-mediated innate immune activation and came up with surprising but intriguing results (Kariko et al., 2005). First, the authors observed that among natural RNA, lipofection of bacterial, but not mammalian, RNA (with an exception of mitochondrial RNA) activated human myeloid dendritic cells (DCs) to secrete TNFα. As noted above, mammalian RNA should have been active if the access of RNA to TLR-expressing endosomes is the safety mechanism keeping abundant self-RNA from mediating immune stimulation. Instead, these investigators demonstrated that modification of RNA, including methylation, is the major controller of RNA immunomodulatory activity. Synthetic ssRNAs of different sequences and lengths could activate TLR3-, TLR7-, and TLR8-expressing cells to secrete IL-8, an activity that was severely impaired when modified nucleosides such as N6-methyladenosine, 5'-methylcytidine, 2-thiouridine, or pseudouridine were introduced into the RNA. Because these modifications are observed more frequently in mammalian RNA than in RNA from bacteria, they may explain why synthetic unmodified RNA corresponding to host RNA sequences is immunostimulatory but natural host RNA is not. Overall, these new data strongly suggest that TLR3, TLR7, and TLR8 recognize “unmethylated RNA,” similar to TLR9 recognition of unmethylated CpG motifs in DNA.

This new study also extended our understanding of nucleic acid stimulation of TLRs in other ways. TLR3-
mediated RNA recognition was not altered by the presence of pseudouridine, which is known to stabilize RNA duplex formation. Conversely, modification of stimulatory RNA with N6-methyladenosine, which destabilizes RNA duplexes, abrogates TLR3-mediated immune activations. These results strongly support previous findings that TLR3 recognizes the double-stranded portion of RNA. Furthermore, N6-methyladenosine, found in many viral RNAs, also diminishes TLR7- and TLR8-mediated RNA recognition, implying that such modification is ideal for viral evasion of TLR-mediated immune responses, as the authors discuss. Finally, unmodified, but not modified, RNA potently activated in vitro generated or freshly isolated human DCs, leading to production of IL-12 and IFN-α as well as the enhanced expression of costimulatory molecules. These data will help guide development of therapeutic applications of RNA, because avoiding modification may enhance the potency of immunotherapy involving RNA-based vaccines or the use of RNA as an adjuvant. Conversely, appropriate nucleotide modifications could result in more efficient RNAi by dsRNA, including siRNA.

The new findings of Kariko et al. (2005) lead to two major conclusions about TLR-mediated RNA recognition. The first is that TLR3, TLR7, and TLR8 can recognize the same naturally isolated or synthetic RNA. The second is that the elements involved in TLR-mediated RNA responses are controlled by modifications such as methylation (Figure 1). Further confirmation and/or exploration of their findings in other experimental systems, such as in vivo studies with mutant mice altered in TLR and TLR signaling pathway, is still required to flesh out these new findings. It will be of interest to learn whether such modifications of RNA have an impact on TLR-independent innate recognition of RNA within cells through molecules such as RIG-I and MDA-5 and, of course, what the physiological relevance of their findings is to infectious diseases as well as to immunological disorders associated with RNA-induced immunomodulation.

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