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BRIEF REVIEWS

Nucleic Acid-Sensing TLRs as Modifiers of Autoimmunity¹Jonathan A. Deane and Silvia Bolland²

The immune system requires precise regulation of activating and inhibitory signals so that it can mount effective responses against pathogens while ensuring tolerance to self-components. Some of the most potent activation signals are triggered by innate immune molecules, particularly those in the TLR family. Recent studies have shown that engagement of TLRs plays a significant role in both innate and adaptive immunity. This review focuses on the ways that TLR function might contribute to the etiology of lupus-like syndromes in the context of an autoimmune-prone environment. By considering the sources, localization, and expression of both nucleic acids and the molecules that bind them, we discuss several ways that innate immunity can play a role in the development of systemic autoimmunity. The Journal of Immunology, 2006, 177: 6573–6578.

Innate¹² immunity involves the activation of host responses triggered by the recognition of repeating sequences generally restricted to infectious organisms such as viruses, bacteria, and intracellular parasites (1–3). Recent findings in the study of TLR activation seem to imply that many of these patterns, normally of microbial origin, cannot be strictly recognized separately from what is commonly called “self.” Innate receptors TLR3, TLR7, TLR8, and TLR9, for example, recognize nucleic acid types that are normally found in bacteria and viruses (3), as do the RNA helicases melanoma differentiation-associated gene 5 and retinoic acid-inducible gene I (4). Because these types of nucleic acids can also be found endogenous in a mammalian cell, the question of why these nucleic acids are not normally recognized by the innate immune system to promote autoimmunity remains unresolved. It was initially hypothesized that unique aspects of sequence or nucleoside modifications allowed for host/pathogen discrimination (5–7), but there is increasing evidence that such differences are insufficient to explain how responses to self-nucleic acids are normally suppressed in healthy individuals (7, 8). This review highlights recent findings that show that in some instances, autoimmunity is indeed driven by aberrant innate immune responses to DNA and RNA. We discuss how synergy between adaptive and innate immune responses can lead to autoimmunity, including

aspects such as colocalization between receptor and ligand, the impact that methylation and other modifications have on the receptors' sensitivity to nucleic acids, and gene dosage of these receptors. Overall, studies characterizing the role of innate immunity in autoimmune-prone settings provide insightful clues that may shed light on gender bias, influence of environmental factors, and the stochastic pathogenesis that is often seen in the development of autoimmunity.

TLRs: the usual suspects for autoimmune responses

TLRs were first characterized in humans in 1998 (9), and initial functional studies focused on their importance in responding to pathogens such as Gram-negative bacteria (10–12). Among the ligands identified so far are DNA, RNA, LPS and related molecules, flagellar proteins, lipopeptides, zymosan, profilin-like molecules, and the malarial pigment hemozoin (3). Details on specific ligands and unique signaling pathways used by individual TLRs are discussed elsewhere (3). For the purposes of this review, it is important to highlight the four nucleic acid-sensing TLRs: TLR3 shows specificity for poly(I:C) compounds and dsRNA, while TLRs 7 and 8 recognize imidazoquinolones and ssRNA, and TLR9 binds dsDNA. While TLR3 can be on the cell surface or in the endosome, TLRs 7, 8, and 9 are thought to be restricted to the endosome. The list is likely to grow as progress continues in this field.

In terms of the general signal transduction pathways activated downstream of TLRs, the key functional outcome of TLR ligation is the production of an inflammatory response through transcription factors such as NF- κ B (1). While some TLRs seem to rely mainly on MyD88 to produce cytokines like IL-6 and TNF- α , others also recruit adaptor molecules and transcription factors such as Toll/IL-1R-domain-containing adaptor-inducing IFN- β , TNFR-associated factor 6, IL-1R-associated kinase-1, and IFN regulatory factor-7 to potently stimulate the transcription of IFN- α (3, 13–15). This last cytokine is especially interesting to those studying autoimmune systemic lupus erythematosus because its expression has been correlated to disease severity in some studies (16). Of the TLRs, the DNA- and RNA-binding members have been shown to stimulate the production of large amounts of IFN- α , mostly derived from plasmacytoid dendritic cells (14, 15). Thus, the fact that some

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TLRs recognize nucleic acids, combined with their inflammatory properties, makes them good candidates for molecules that could potentiate autoimmunity because Abs to nucleic acids are key indicators of systemic inflammatory syndromes such as lupus and scleroderma (17, 18). These facts suggest that the overlap of specificity between autoantibodies and TLRs can be more than a coincidence, as we will discuss below.

Nucleic acid-sensing TLRs provide a key costimulatory signal for autoreactive B cells

A key finding that brought the logical link between the TLRs that recognize nucleic acids and the production of Abs that recognize nucleic acids involved studies of AM14 transgenic mice, whose B cells express Abs specific for self-IgG, also known as rheumatoid factor (19). In this study, antinucleosomal IgG was mixed with cell lysates to stimulate B cell proliferation in transgenic B cells. Furthermore, this stimulation was DNase sensitive and MyD88 dependent, and TLR9 inhibitory oligonucleotides could abrogate the response of the autoreactive B cells. These observations provided the first report of a synergistic effect between a DNA-binding TLR and a BCR that binds and internalizes DNA-including complexes. Further support of the role of DNA as a stimulus of autoreactive B cells was shown by Fields et al. (20), using transgenic 3H9 B cells, which have a DNA-specific BCR. With this system, CpG-mediated proliferation was also sensitive to TLR-inhibiting oligonucleotides, supporting the idea that combined signals through TLRs and the BCR provide a means to activate autoreactive B cells. The importance of DNA as an autoimmune stimulus was also advocated in mice lacking DNase I, which developed a lupus-like syndrome (21).

Several groups have tested the involvement of TLR9 in the activation of autoreactive B cells *in vivo* by crossing TLR9-knockout (ko)³ mice to a number of models of lupus disease and have reported varied and sometimes conflicting results. First, Christensen et al. (22) used the *Mrl/lpr* lupus model to show that mice deficient in TLR9 specifically lack anti-DNA Abs while retaining other autoantibody specificities. Even with the absence of anti-DNA Abs, kidney disease was not prevented in these mice. Wu and Peng (23), using the same *Mrl/lpr* model crossed to TLR9-ko, have reported a protective effect of TLR9 in autoimmunity, since their *Mrl/lpr* mice developed more severe glomerulonephritis and increased anti-DNA Abs when deficient in TLR9. Lartigue et al. (24) found that, in the absence of TLR9, B6.*lpr* mice do not produce anti-nucleosome Abs but do produce other autoantibody specificities. Glomerulonephritis was also enhanced in the absence of TLR9 in this model, as well as in the mutated phospholipase C γ model reported by Yu et al. (24, 25). The study by Lartigue et al. (24) also reported a switch in specificities toward nucleolar autoantibodies. Ehlers et al. (26) have used the Fc γ RIIB-deficient lupus model to show that mice that lack TLR9 do not switch to IgG2a and IgG2b pathogenic autoantibodies, and glomerulonephritis was prevented in the 56R/3H9 anti-DNA Tg model (22, 26). It was initially thought that the difference in the two studies on *Mrl/lpr* mice is the number of backcrosses that were performed, since effects from the *Mrl/B6/129* mixed background can be profound. Indeed, more recent work by Christensen et al. (27) has confirmed that lethality is exacerbated in fully backcrossed

Mrl/lpr mice lacking TLR9. On the other hand, they show that TLR7-deficient *Mrl/lpr* mice have blocks in anti-RNA autoantibody responses and disease severity. In agreement with the importance of TLR7, Berland et al. (28) have found that TLR7-deficient mice bearing an anti-RNA BCR knock-in allele are protected from a lupus like syndrome.

Overall, the majority of studies have shown that the absence of TLR9 in murine models of lupus results in a change in autoantibody specificities and that this effect not only does not eliminate the inflammatory pathology in the kidney, but in fact in many cases TLR9 deficiency seems to enhance this pathology. In the context of a genetic background that allows for a systemic loss of tolerance, TLR9 seems to be a factor in shaping autoantibody specificities, perhaps by allowing activation or expansion of B cells with DNA-related specificities. Meanwhile, TLR9's role in the pathogenesis of inflammatory disease observed in lupus models seems to be more debatable at this point, while TLR7 may play a more dominant role in promoting lupus-like syndromes (29).

Because most of these studies are based on mouse models using transgenic receptors, it is still an open question whether these results will be applicable to human disease. So far, human studies have shown that sera from lupus patients can stimulate IFN- α production in a TLR7/8-dependent manner (30). Also, IFN- α found in blood from pediatric lupus patients and implicated in the pathogenesis of the disease was able to mature monocytes into fully differentiated dendritic cells capable of Ag presentation (31).

Avoiding mixed messages: why foreign nucleic acids stimulate TLRs

An immediate question that comes to mind when one considers the fact that there are nucleic acid-sensing molecules in the immune system is why these proteins fail to respond to the large amount of host nucleic acids present in a tolerant individual. Some explanations have focused on unique aspects of sequence differences in viral and bacterial nucleic acids that could allow the TLRs that bind them to distinguish them from mammalian sequences. In the case of TLR9, studies using AM14 B cells showed that hypomethylated CpG DNA sequences were critical for optimal stimulation to occur (6), which was in agreement with initial findings showing defective responses to CpG DNA in TLR9-deficient mice (32). Extensive sequence analysis of stimulatory RNA has found multiple modifications in sequence content, 2' modifications, and differences in poly(A) length to be important for providing pathogen-specific stimulation of RNA-sensing TLRs (33–37). More recently, the significance of methylation has been expanded from CpG DNA motifs to the case of RNA (5). The work by Kariko et al. (5) suggests that hypomethylated RNA contributes the maximum immunogenic signal to TLRs 3, 7, and 8. As a whole, there is strong evidence that TLR sensitivity to nucleic acids is dependent upon species-specific aspects of the DNA and RNA that they recognize.

Conversely, suppressive oligonucleotides have been studied for the ability of particular sequences and modifications to block inflammatory responses (38). Because of their stability, many investigators have focused on phosphorothioate-modified oligonucleotides, and they have shown promise in being therapeutically effective for some mouse models of lupus (39, 40). Extensive work with different inhibitory sequences has led to the isolation of sequences that selectively inhibit only TLR7,

³ Abbreviations used in this paper: ko, knockout; RNP, ribonucleoprotein.

only TLR9, or both (8). These TLR-specific reagents will likely be useful to determine contributions of each receptor in different systems, as well as in therapies aimed at specific pathways. Because studies of suppressive oligos involve comparing effectiveness of different oligonucleotides, they also underscore the idea that particular sequences and chemical modifications are critical for providing selective recognition of TLRs.

Even though microbial nucleic acids are primary natural agonists for TLRs, they are not the only existing ligands that can engage these receptors. For instance, the aforementioned studies of AM14 B cell activation with chromatin complexes used activatory DNA that originated from mammalian cell lysates (8, 19, 41). In those experiments, there were no microbial nucleic acids to account for the activation that was observed. Moreover, microbial and mammalian DNAs are not easily differentiated by sequence motifs. It is known that mammalian genomes do contain hypomethylated CpG motifs on sections referred to as CpG islands (42). Why these do not seem to normally stimulate TLRs and lead to inflammation is a matter of open debate. One interesting idea is that mammalian genomes contain suppressive sequences that function in a manner similar to inhibitory oligonucleotides, such that positive signals from endogenous CpG islands and stimulatory RNA sequences are overruled by suppressive nucleic acids from the host genome. It has been shown, for example, that repetitive elements in mammalian telomeres suppress bacterial DNA-induced activation (43). Regardless, such objections have led some to seek other mechanisms to supplement what has been discovered through sequence and modification analyses on which the next section focuses.

Location, location, location: are TLRs and self nucleic acids kept apart?

Another way that self nucleic acids may fail to activate nucleic acid sensing molecules in a healthy individual is through their sequestration from their receptors. Recent work, particularly from Medzhitov and colleagues, has served to emphasize that colocalization of Ags and TLRs may be the key for optimal activation of the immune system. Initial studies showed that phagocytosed Ags do not enter the appropriate intracellular location in the absence of TLR ligands, suggesting that phagosomal maturation is critically regulated by TLR signaling (44). A newer report went on to show that unless the TLR ligand LPS is directly conjugated to the Ag, efficient processing and presentation of that Ag does not take place (45). In those experiments, the protein Ag and LPS were added individually but simultaneously, and similar maturation was not observed. Thus, it seems that cells of the immune system are not generically activated by TLRs to process and present just any protein Ag that is taken up. Instead, colocalization of the TLR signal and the Ag might be the crucial factor. Another study from the same group focused on the recognition of cytosolic DNA, showing that while wild-type TLR9 was not potently activated by host DNA, a chimeric TLR9 that was improperly localized in the cytosol was responsive to host DNA (46). Taken together, these studies suggest that, in addition to sequence modifications, TLRs and their ligands are generally sequestered, and this is a key means to avoid recognition of self nucleic acids.

While studies of the importance of TLR ligand localization answer some important questions, they also raise new questions about how pathogens and the host are distinguished. First, since phagocytosis of apoptotic cells occurs through the endosome,

how is it that TLRs 7–9 are not stimulated in the tolerant host? Second, as has been mentioned before, TLRs have served to modulate the adaptive immune response, and this has been taken advantage of in a therapeutic manner because most adjuvants contain TLR ligands. However, in those systems, the vaccination target is not physically conjugated to the adjuvant, and yet the adjuvant is able to stimulate an immune response. Does this imply that adjuvants have the capacity to bring the TLR ligands that they contain together with the Ags used in vaccination? If so, how might this occur? Overall, studies on unique patterns and in pathogenic nucleic acids and the importance of localization have clarified how it is that a tolerant host normally fails to respond to its own nucleic acids. Fig. 1 illustrates how a strict requirement for nucleic acid composition and localization may prevent the activation of TLRs and thus maintain tolerance.

Altered gene dosage and TLRs

Studies of human genome polymorphisms underscore the idea that aberrant gene expression due to duplications and deletions may be widespread throughout a genetically heterogeneous population like our own (47). Such alterations may increase or decrease the amount of protein normally maintained at optimal levels in a healthy individual. For example, gene dosage differences in NFAT-regulating proteins have been strongly implicated in causing the developmental defects seen in Down's Syndrome (48). Arron et al. (48) found that a 1.5-fold increase in NFAT-regulating molecules due to chromosome 21 trisomy was sufficient to account for altered cranial development and behavior seen in mouse models of Down's Syndrome. In the field of autoimmunity, a similar train of thought has linked *FcγR3* gene dosage to predisposition to glomerulonephritis (49). Furthermore, a link between *TLR* gene dosage and autoimmunity has recently been found in the Y chromosome autoimmune accelerator (*Yaa*) mouse (50). In this system, our group found that an extra copy of *TLR7* was duplicated onto the Y chromosome of the *Yaa* allele, accelerating autoimmunity in the *FcγRIIB*-ko mouse model by making B cells hyperresponsive to TLR7 ligands in vitro and in vivo (51). A similar

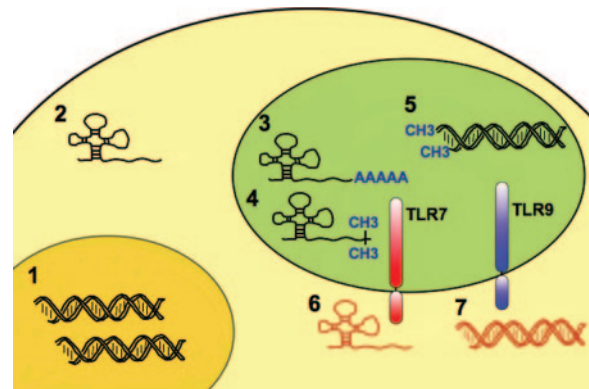


FIGURE 1. Proper regulation of nucleic acid-sensing TLRs involves active and passive mechanisms. Sequestration of self-DNA (1) and RNA (2) away from the endosome, shown in green, prevents activation of TLRs. Increased poly(A) length in mRNA (3) and methylation of both RNA (4) and DNA (5) provide a sequence and modification-specific means to activate TLRs. Suppressive sequences in host RNA (6) or DNA (7) may also actively repress activation of TLRs. For purposes of simplicity, TLR8 is not drawn, although it may act in a similar manner as TLR7.

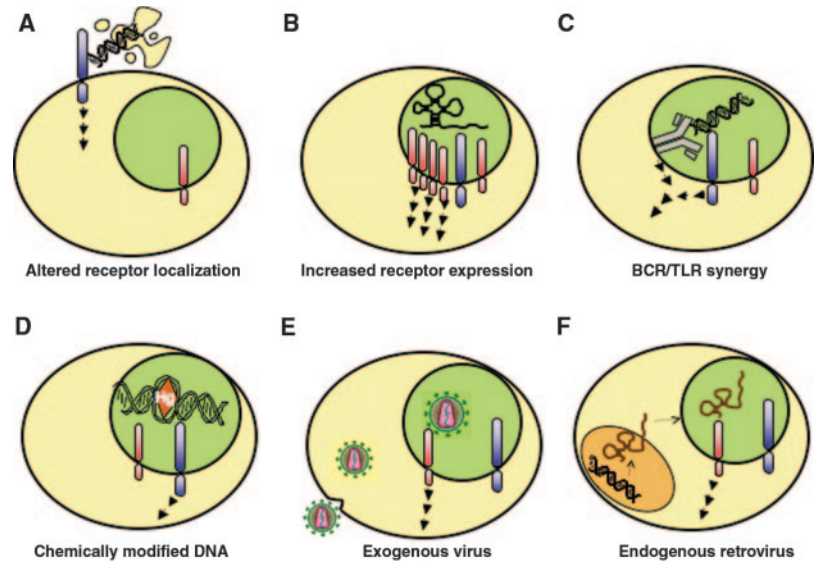


FIGURE 2. Multiple pathways that may break tolerance through the activation of nucleic acid-sensing TLRs. Improper localization of TLRs away from the endosome (A), increased TLR expression (B), immune activation to induce the colocalization of stimulatory nucleic acids and TLRs (C), cationic metal induced alteration of nucleic acid structure (D), and exogenous (E) or endogenous (F) viral nucleic acids may serve to stimulate the nucleic acid-sensing TLRs and promote autoimmunity.

hyperresponsiveness to TLR7 ligands was also seen in dendritic cells from *Yaa* mice (J. A. Deane, unpublished observations). In addition to showing a quantitative increase in response, the *Yaa* allele is able to qualitatively influence the nature of the autoantibody response so that instead of anti-chromatin autoantibody response, *Yaa* mice show an anti-nucleolar pattern (52), which is indicative of reactivity to RNA and/or ribonucleoproteins (RNPs). These results nicely complement those obtained by in vitro-stimulating B cells with TLR7 agonists, as RNase-treated lysates were shown to ablate proliferation in AM14 B cells that were stimulated with anti-RNA Abs (41). On a more molecular level, the same study found that AM14 B cells isolated from mice lacking TLR7 were unable to respond to these mixtures of anti-RNA IgG and RNA. Additionally, several studies have shown that endogenous mammalian ligands of TLR7 such as small RNPs are capable of stimulating B cells and plasmacytoid dendritic cells (8, 30, 53).

Thus, the *Yaa* system shows that by doubling the *TLR7* gene dosage, B cells secreting Abs specific to RNA are selected, and because of this dysregulation, the lupus-like disease seen in the $Fc\gamma RIIB$ -ko mouse model is exacerbated. These findings were also supported by another recent study by Subramanian et al. (54), which also reported the *TLR7* gene duplication in *Yaa* mice and used autoantigen arrays to show a bias for RNPs and RNA-associated autoantigens. Subramanian et al. (54) also considered the contribution of T follicular helper cell (T_{FH}) development as another factor involved in accelerating autoimmunity. Indeed, previous studies have shown that in the absence of TLR signaling, T-dependent Ab production is decreased substantially (55). It is probable that both dendritic cell and B cell Ag presentation are enhanced upon signaling through TLRs, which could drive Th cell development and activation. TLRs may also influence autoimmune responses in a T cell intrinsic fashion, as suggested by the fact that direct stimulation of T cells through TLRs can occur (56, 57). In a similar manner, our own studies of the *Yaa* mouse showed through mixed bone marrow chimera studies that the nucleolar autoantibody specificity is intrinsic to the *Yaa* B cells, demonstrating that increased expression of TLR7 in B cells preferentially selects those cells with RNA specificities (51). It is interesting to note that an increase in nucleolar specificities was also observed

in some lupus mouse models crossed to the TLR9-ko, perhaps suggestive of increased engagement of TLR7 in the absence of TLR9 in those cases (24, 25). While the precise mechanisms that are critical for this phenomenon need to be elucidated, the *Yaa* mouse model undoubtedly shows that a 2-fold increase in TLR gene dosage can dramatically impact an autoimmune pathology. These results could be restricted to the case of the C57BL/6 strain of mice, but it is also possible that they ultimately provide clues as to the type of changes in TLR function that would be expected to modify pathologies in humans.

Apart from alterations in gene dosage, signals via the BCR or cytokine receptors may control the level of TLR7 and TLR9 expression in human naive B cells and mouse follicular B cells, thus overcoming this important checkpoint and increasing responsiveness to their ligands (58–61). This implies that improper regulation of TLR protein levels could also impact autoimmunity in a manner similar to what was seen in gene dosage studies. Further studies will determine whether checkpoints in *TLR* gene regulation are indeed impaired in some cases.

Implications

Even in the absence of polymorphisms in *TLR* gene dosage, two other phenomena related to lupus have intriguing characteristics that may link systemic autoimmunity to improper TLR expression and function. First, given the approximate female:male ratio of 9:1 seen in lupus patients (62), it is possible that some of this skewing is due to improper inactivation of the X-linked TLRs. Given recent studies highlighting sections of the X chromosome that fail to be completely activated (63, 64), it is exciting to hypothesize that some instances of lupus are due to dysregulated X inactivation of *TLR7/8* alleles. Interestingly, it has been reported very recently that TLR7 ligands induce higher IFN- α production in females (65).

Another remarkable phenomenon seen in lupus patients is the lack of complete genetic penetrance. For example, among identical twins, the observed correlation of lupus is roughly between 25 and 50% (66, 67). This has led many to consider what environmental cues are important for lupus pathogenesis (68, 69). As a result, it is possible that viral infections may serve to exacerbate lupus through TLR stimulation. While molecular mimicry has long been linked to autoimmune responses such as

rheumatic carditis (70), where a streptococcal protein has some homology to a host protein, it may also be the case that viral nucleic acids may provide a “nonspecific” activation of TLRs that serves to potentiate autoimmune responses. There are, however, some studies that challenge the hypothesis linking infection to autoimmunity (reviewed in Ref. 71). A second potential origin of microbe-derived nucleic acids that can potentially stimulate may stimulate TLRs and play a role in autoimmunity could be genomically encoded endogenous viruses. Some of them have been shown to be transcribed as a result of immune cell activation, and they could possibly provide T cell help due to superantigen stimulation (72, 73). An additional source of TLR-activating molecules could be chemical modification of nucleic acids, for example, by heavy metal incorporation. While the phenomenon of metal-induced autoimmunity is well known (74), the mechanisms behind it have been debated. One possibility is that these metals serve to alter the structure of host nucleic acids to make them recognizable to nucleic acid binding TLRs. Alternatively, given the fact that many view the synergy between autoreactive BCRs and TLRs to be mainly dependent upon localization of the nucleic acids with the TLR that recognizes it, it is possible that the differences seen in the development of lupus between genetically related individuals are due to very rare colocalization events of host nucleic acids with endosomes that contain TLRs. Whether there are active mechanisms to sequester and inhibit such an event remains to be determined. Fig. 2 shows six of the above described scenarios where TLRs are activated in a cell to induce autoimmunity. By investigating all of these possibilities, we may clarify our understanding of lupus and tolerance in general, which may lead to better treatments and/or prevention strategies.

Summary

The past several years have seen much progress in the understanding of how nucleic acid-sensing TLRs may drive autoreactive immune responses at the molecular, cellular, and organism levels. The growing interest in suppressive oligonucleotides that are specific to one or more TLRs will likely be a key means to apply these findings from bench to the bedside. While less is known about it, TLR3 should also be considered as a key molecule that may potentiate autoimmunity, as one report has suggested (75). Additionally, the consideration of innate immune responses as key molecules involved in autoimmunity may extend beyond the TLR field. For example, the RNA-sensing helicases RIG-I and MDA5 have been characterized recently and implicated in antiviral inflammatory responses in a TLR-independent manner (4, 76). The possibility that they may also potentiate the development of autoimmune disease is likely to be the subject of active investigation in the years to come. Last, while many studies have focused on lupus, other autoimmune syndromes such as scleroderma, Sjogren’s syndrome, and rheumatoid arthritis could be tied to an improperly regulated innate immune response. Time, and more research, will tell.

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