



# Identification of U11snRNA as an endogenous agonist of TLR7-mediated immune pathogenesis

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The activation of innate immune receptors by pathogen-associated molecular patterns (PAMPs) is central to host defense against infections. On the other hand, these receptors are also activated by immunogenic damage-associated molecular patterns (DAMPs), typically released from dying cells, and the activation can evoke chronic inflammatory or autoimmune disorders. One of the best known receptors involved in the immune pathogenesis is Toll-like receptor 7 (TLR7), which recognizes RNA with single-stranded structure. However, the causative DAMP RNA(s) in the pathogenesis has yet to be identified. Here, we first developed a chemical compound, termed KN69, that suppresses autoimmunity in several established mouse models. A subsequent search for KN69-binding partners led to the identification of U11 small nuclear RNA (U11snRNA) as a candidate DAMP RNA involved in TLR7-induced autoimmunity. We then showed that U11snRNA robustly activated the TLR7 pathway *in vitro* and induced arthritis disease *in vivo*. We also found a correlation between high serum level of U11snRNA and autoimmune diseases in human subjects and established mouse models. Finally, by revealing the structural basis for U11snRNA's ability to activate TLR7, we developed more potent TLR7 agonists and TLR7 antagonists, which may offer new therapeutic approaches for autoimmunity or other immune-driven diseases. Thus, our study has revealed a hitherto unknown immune function of U11snRNA, providing insight into TLR7-mediated autoimmunity and its potential for further therapeutic applications.

U11snRNA | TLR7 | autoimmune diseases | DAMPs | type I IFN

Activated B and T lymphocytes with autoreactivity against a diverse array of self-derived molecules are central mediators of autoimmune disease pathophysiology. However, an essential role of innate immune responses in the initiation and maintenance of autoimmune disorders has increasingly come into focus. For example, the exuberant production of type I interferons (IFNs) and proinflammatory cytokines is linked to autoimmunity by either an inappropriate triggering of innate immune receptors or genetic mutations in the receptor pathways that govern the production of those cytokines (1–3). In general, when these cytokines are produced *en masse* and/or chronically, they contribute to a “break” in immune tolerance and to triggering the differentiation and expansion of self-reactive pathogenic T and B cells (1–3).

Among the innate immune receptors, genetic and functional data have implicated a causative role for nucleic acid-sensing receptors. Typically, RNA-sensing Toll-like receptor 7 (TLR7) and DNA-sensing TLR9 have been most extensively studied for their involvement in autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (1–6). In

this context, growing evidence indicates that, in addition to pathogen-derived nucleic acids, self-derived nucleic acids also activate those innate receptors, thereby contributing to autoimmunity (1–3, 7–10).

TLR7 has been well-described for its role in the detection of virus-derived single-stranded RNAs (ssRNAs) or other RNAs that contain ssRNA structures (1–3). In the context of autoimmunity, the chronic and/or excessive exposure of self-derived RNA to TLR7 is thought to be critical in driving disease (1–3). Deficiency

## Significance

Immunogenic damage-associated molecular patterns (DAMPs), typically released from dying cells, can evoke chronic inflammatory or autoimmune disorders via their activation of innate immune receptors. Since an association of RNA-sensing Toll-like receptor 7 (TLR7) signaling with autoimmunity is well-documented, identification of a DAMP(s) that triggers TLR7 is critical to understanding the disease pathogenesis. By generating the synthetic compound KN69 that inhibits autoimmunity in mouse models, we identified U11 small nuclear RNA (U11snRNA) as a target of KN69 and strong activator of TLR7. We found a correlation between high serum level of U11snRNA and autoimmune diseases in human subjects and mouse models. Finally, we generated TLR7 agonists and TLR7 antagonists. Our study provides therapeutic insight into autoimmunity and other diseases.

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in the *Tlr7* gene results in reduced autoimmune symptoms in mouse disease models (2, 4–6). Conversely, amplified expression of TLR7 in mice bearing the Y chromosome-linked autoimmune acceleration (*Yaa*) gene allele shows accelerated development of antibody to RNA-containing self-antigen and incidence of lupus nephritis (4–6).

In humans, associations between TLR7 polymorphisms and the prevalence of SLE have been demonstrated in both Asian and Caucasian populations, although this was not confirmed in other ethnicities (11). SLE is found far more often in women and the *Tlr7* gene, located on X chromosomes, escapes X-chromosome inactivation in distinct populations of immune cells, such as B cells, plasmacytoid dendritic cells (pDCs), and monocytes (12). In this regard, cell-intrinsic activation of TLR7 appears to directly contribute to the functional responses of B cells in SLE (13–15). In addition, it is also reported that *Tlr7* copy number is a risk factor for the onset of juvenile SLE (16).

A hallmark of TLR7 activation is the robust production of type I IFNs (1–3), and an IFN-inducible transcriptional signature has been observed in the peripheral blood of patients across several autoimmune diseases (17, 18). In fact, the inappropriate stimulation of the type I IFN response by an abnormal accumulation of an endogenous RNA ligand(s) would activate TLR7 to cause autoinflammatory and autoimmune disorders by type I IFNs, and hence they are termed type I interferonopathies (19).

Due to the importance of TLR7 in disease pathogenesis, there is considerable interest in identifying a culprit endogenous ligand(s). In this context, the U1 small nuclear RNA (U1snRNA) component of U1 small nuclear ribonucleoprotein (U1snRNP) immune complexes has been proposed as a candidate since U1snRNA and synthetic oligoribonucleotides derived from its stem-loop regions can induce type I IFN and proinflammatory cytokines in pDCs (7). Of note, type I IFN signaling in B cells is critical for isotype switching to the pathogenic IgG2a isotype (20). Thus, when dead cells are not appropriately cleared by phagocytic cells, autoreactive B cells can become activated by self-derived RNAs released by the dead cells, which may also contribute to the development of autoimmune diseases. However, the causative role of U1snRNA and other endogenous RNAs in autoimmune diseases has not been rigorously demonstrated.

In the present study, we first generated a chemical compound, termed KN69, that relieves disease burden in mouse models of RA and SLE. Using KN69, we next identified U1small nuclear RNA (U11snRNA) as a KN69-binding RNA and TLR7 agonistic ligand. U11snRNA is a noncoding RNA critical component for the minor spliceosome protein complex, which is also involved in alternative splicing (21). We show that U11snRNA activates TLR7 more robustly than reference agonist RNAs, such as polyuridine (polyU) and U1snRNA, in pDCs and other immune cells, and provide a structure–activity relationship unique for U11snRNA. We also offer evidence for a pathogenic role of U11snRNA in a mouse model of arthritis and find significant elevation of U11snRNA in the sera of RA and SLE patients. Finally, we describe the development of a U11snRNA-derived TLR7 agonist and antagonist. Our study in toto places U11snRNA as a potential causative agent of autoimmune disease and demonstrates a proof-of-concept approach by which a U11snRNA-based agonist and antagonist, respectively, controls TLR7-driven protective and pathogenic immune responses.

## Results

**Development of the Immunosuppressive Compound KN69.** We first screened a low–molecular-mass compound library to identify those compounds that impeded nucleic acid-mediated immune responses. One of the “hit” compounds was modified through synthetic chemistry to develop a compound that suppresses the responses more strongly than the predecessor. As the result of this approach, a compound, termed KN69 (2-[(1-benzylpiperidin-4-yl)amino]-

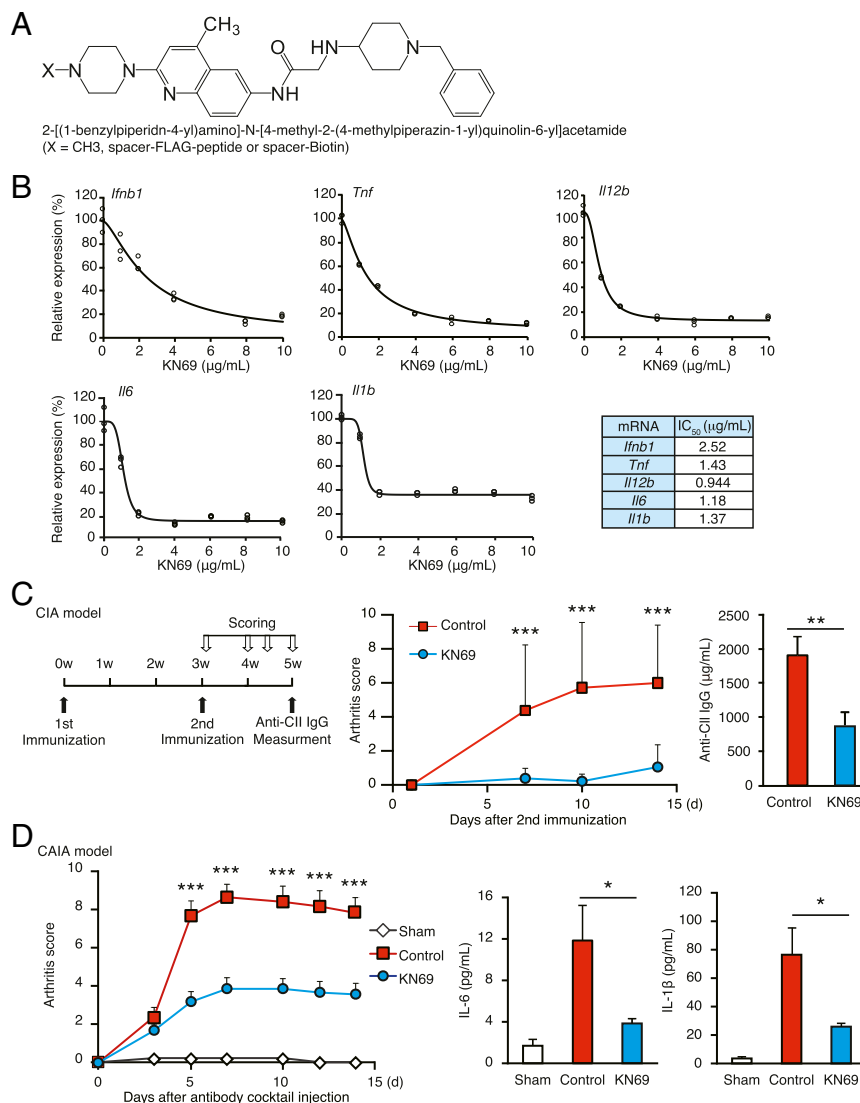
*N*-[4-methyl-2-(4-methylpiperazin-1-yl)quinolin-6-yl]acetamide) (Fig. 1A), was identified that strongly and selectively inhibited ssRNA-mediated immune responses in vitro (Fig. 1B and *SI Appendix*, Fig. S1A–C). Notably, KN69 also suppressed development of pathogenic features in several autoimmune models including collagen-induced arthritis (CIA), anticollagen antibody-induced arthritis (CAIA), and graft-versus-host disease that mimics SLE in mouse (Fig. 1C and D and *SI Appendix*, Fig. S1D). Considering the suppressive effect of KN69 on ssRNA-mediated immune response (Fig. 1B and *SI Appendix*, Fig. S1A–C), we then suspected KN69 interferes with the activation of an RNA-sensing receptor signaling pathway(s) in vivo.

**Screening of the KN69 Target(s).** To identify an endogenous target(s) of KN69, we generated a FLAG-tagged KN69 to perform pull-down assays with whole-cell lysates of Raji cells which express RNA-sensing receptors including TLR7 (22). KN69-associated molecules were then examined by proteome analysis with a nano-LC-MS system (23). This analysis found that KN69 bound to RNA-binding proteins but not to any of the known RNA-sensing receptors or related proteins (Fig. 2A and *SI Appendix*, Fig. S2A). Consistently, the coprecipitated KN69-binding nucleic acids were sensitive to enzymatic treatment by RNase A but not DNase I, indicating that the nucleic acids are RNAs with a single-stranded structure(s) (Fig. 2B).

We then examined whether KN69 directly binds to nucleic acids by surface plasmon resonance (SPR) analysis and found its direct interaction with ssRNA, but not double-stranded RNA (dsRNA), ssDNA, or dsDNA (Fig. 2C). These data indicate that KN69 directly and selectively binds to endogenous ssRNAs to inhibit the development of autoimmunity. The details of the selective KN69 binding to ssRNA remain unknown. We further characterized KN69-binding RNA by comprehensive RNA-sequencing (RNA-seq) analysis. As shown in Fig. 2D, 9 RNA sequences, including snRNAs and ribosomal RNAs (rRNAs), were identified as KN69-binding RNA in the absence of protein (see also *SI Appendix*, Fig. S2B). In fact, these comprised a majority (more than 97%) of the total mapped reads (Fig. 2E).

**Association of U11snRNA with Autoimmunity.** To further identify a KN69-targeted RNA(s) involved in autoimmunity, we sought to measure relative amounts of the above RNA species (Fig. 2E) in the sera from BXSB/MpJ-*Yaa* (BXSB.*Yaa*) mice, a model of SLE, and from mice with CAIA after disease development. We found notable elevation of U11snRNA levels observed in the sera of these mice (Fig. 3A and B). On the other hand, levels of other KN69-binding RNAs, such as U1snRNA and 28S rRNA, remained unchanged, indicating the selective correlation between the serum U11snRNA levels and disease development in these models. In the case of the BXSB.*Yaa* mice, the serum U11snRNA levels were significantly increased by age (*SI Appendix*, Fig. S3A), whereas no such increase was found for U1snRNA and 28S rRNA. In addition, a strong correlation was also seen between serum U11snRNA levels and renal IFN signature, a hallmark of the pathogenesis of SLE and other autoimmune diseases (Fig. 3C) (17, 18). Consistent with this, serum U11snRNA levels also correlated with kidney injury marker 1 (KIM-1), a biomarker of kidney injury (Fig. 3D). In contrast, no such correlation was observed between these and U1snRNA or 28S rRNA (Fig. 3D and *SI Appendix*, Fig. S3B).

Levels of the KN69-binding RNAs were also measured in the sera of human RA and SLE patients. Interestingly, a statistically significant elevation of U11snRNA, but not other RNAs examined, was observed in the sera from RA patients as compared with healthy donors (Fig. 3E and *SI Appendix*, Fig. S3C). A significant correlation was also seen between serum levels of U11snRNA and pathological status of patients such as disease score and serum rheumatoid factors (Fig. 3F). Furthermore, U11snRNA levels



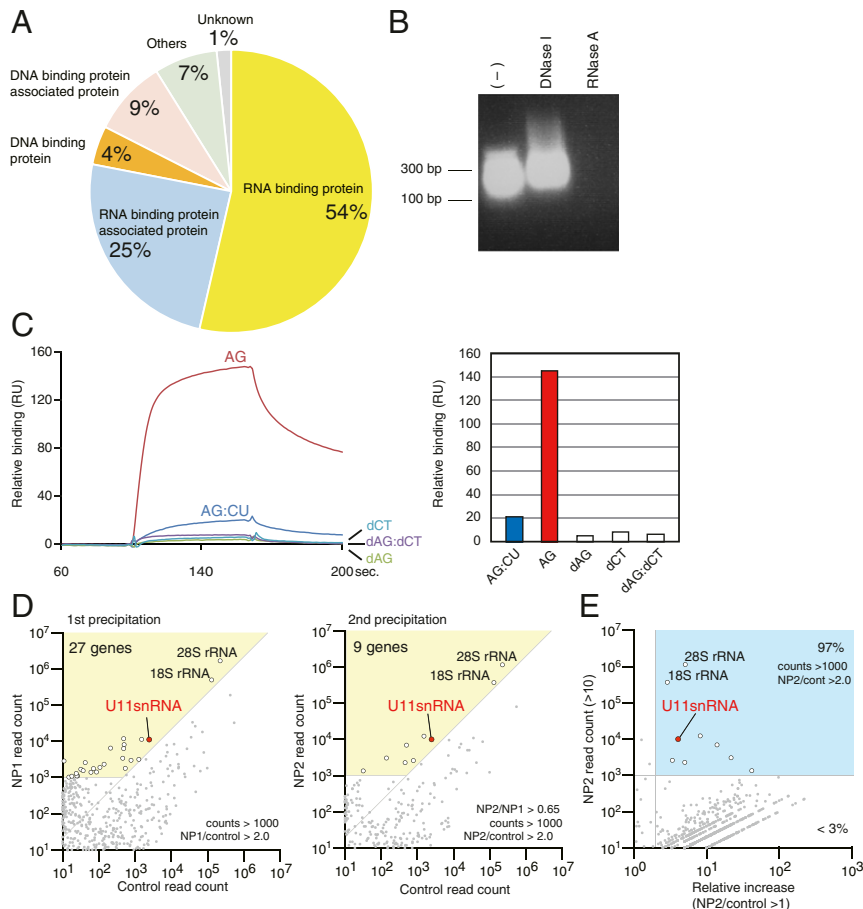
**Fig. 1.** Suppression of innate immune response and autoimmunity by KN69 (X, CH<sub>3</sub>) and its derivatives (X, spacer-FLAG peptide or spacer-biotin). (B) Inhibition of TLR7-mediated induction of *Ifnb1*, *Tnf*, *Il12b*, *Il6*, and *Il1b* mRNA by KN69. Splenocytes were stimulated with polyU (2 µg/mL) for 4 h in the presence of the indicated concentrations of KN69 and then cytokine mRNA levels were measured by qRT-PCR analysis. Results are shown as relative expression values to KN69-untreated cells. A summary of estimated IC<sub>50</sub> values for KN69 inhibition of individual genes is shown (Bottom Right). (C) Therapeutic effect of KN69 in a model of collagen-induced arthritis. Schematic view of the experimental procedure (Left), arthritis score measure by time (Middle), and serum concentration of anti-collagen II (anti-CII) IgG on day 14 after the second immunization (Right). Control, *n* = 20; KN69, *n* = 20. \*\**P* < 0.01, \*\*\**P* < 0.001. (D) Therapeutic effect of KN69 on the anti-collagen antibody-induced arthritis mice model. Arthritis score (Left) and serum cytokine production on day 14 are shown (Right). Sham, *n* = 6; control, *n* = 10; KN69, *n* = 10. \**P* < 0.05, \*\*\**P* < 0.001.

were increased in the synovial fluid of the RA patients as compared with osteoarthritis (OA) patients (Fig. 3G). Consistently, significant elevation of serum U11snRNA levels and their correlation to pathological status (SLE activity measure; SLAM) were also observed with SLE patients (Fig. 3H and I). These results, which corroborate KN69-mediated suppression of disease development in the mouse models, lend support to the notion that U11snRNA may also contribute to human autoimmunity.

**Immunogenic and Pathogenic Potential of U11snRNA.** We next examined the potential of U11snRNA to activate innate immune responses. We first compared the immunogenicity of full-length U11snRNA with other RNAs, including U1snRNA, which has also been implicated in autoimmunity (7). Interestingly, U11snRNA induced type I IFN gene expression in splenocytes far more strongly than U1snRNA or other endogenous RNAs, each of

which was complexed with the cationic liposome-forming compound DOTAP (Fig. 4A) (24). Since the gene induction by U11snRNA was abolished in splenocytes from TLR7-deficient, but not TLR9-deficient, mice (Fig. 4B and SI Appendix, Fig. S44), we concluded that U11snRNA is indeed a bona fide TLR7 agonist. Consistent with the cell type-specific TLR7 signaling pathways (1–3), U11snRNA activated robust type I IFN gene expression in pDCs and proinflammatory cytokine genes in conventional pDCs (cDCs) (SI Appendix, Fig. S4B). Perhaps expectedly, the U11snRNA-induced gene expression was inhibited by KN69 in all cell types tested (Fig. 4C and D).

We next examined the pathogenic potential of U11snRNA to induce joint inflammation (17, 18). As shown in Fig. 4E, intraarticular U11snRNA injection caused arthritis, and it was suppressed when KN69 was coinjected with U11snRNA. On the other hand, a comparable amount of total RNA isolated



**Fig. 2.** Comprehensive analysis of KN69-interacting molecules and identification of U11snRNA. (A) Classification of KN69-binding proteins shown in *SI Appendix, Fig. S2A*. KN69-binding proteins were classified into the 6 indicated groups and the frequency of classified proteins is shown in the pie graph. (B) Electrophoresis analysis of the KN69-binding nucleic acids treated with DNase I (200 U/mL) or RNase A (1 mg/mL) for 1 h. (C) SPR analysis for the binding of KN69 to ssRNA [dsRNA poly(AG:CU), AG:CU; poly(AG), AG; ssDNA poly(dAG), dAG; poly(dCT), dCT; dsDNA poly(dAG:dCT), dAG:dCT]. Sensorgrams are shown (*Left*) with time (s) and relative binding units (RU). Maximum RUs are shown (*Right*). (D) RNA-seq analysis of KN69-binding RNA. Read count data for RNAs precipitated by first nucleic acid precipitation in the presence of proteins (NP1; *Left*) or second nucleic acid precipitation in the absence of proteins (NP2; *Right*) are shown. Dots in the yellow-colored area indicate significantly enriched RNAs after the NP1 procedure (*Left*) or NP2 procedure (*Right*) as compared with genes from control RNA from Raji cells. (E) Analysis of RNA-seq data of RNAs precipitated by NP2. Relative increase of read counts after the NP2 procedure was calculated as compared with those in control RNA, and shown with NP2 read count data. The blue-colored area includes the genes whose read counts met the criteria noted.

from HEK293T cells was far less effective in inducing the pathogenesis (*SI Appendix, Fig. S4C*). Although further rigorous examination will be required, these observations in toto support the idea that U11snRNA is one, if not the only, culprit that provokes the pathogenesis of TLR7-mediated autoimmune diseases.

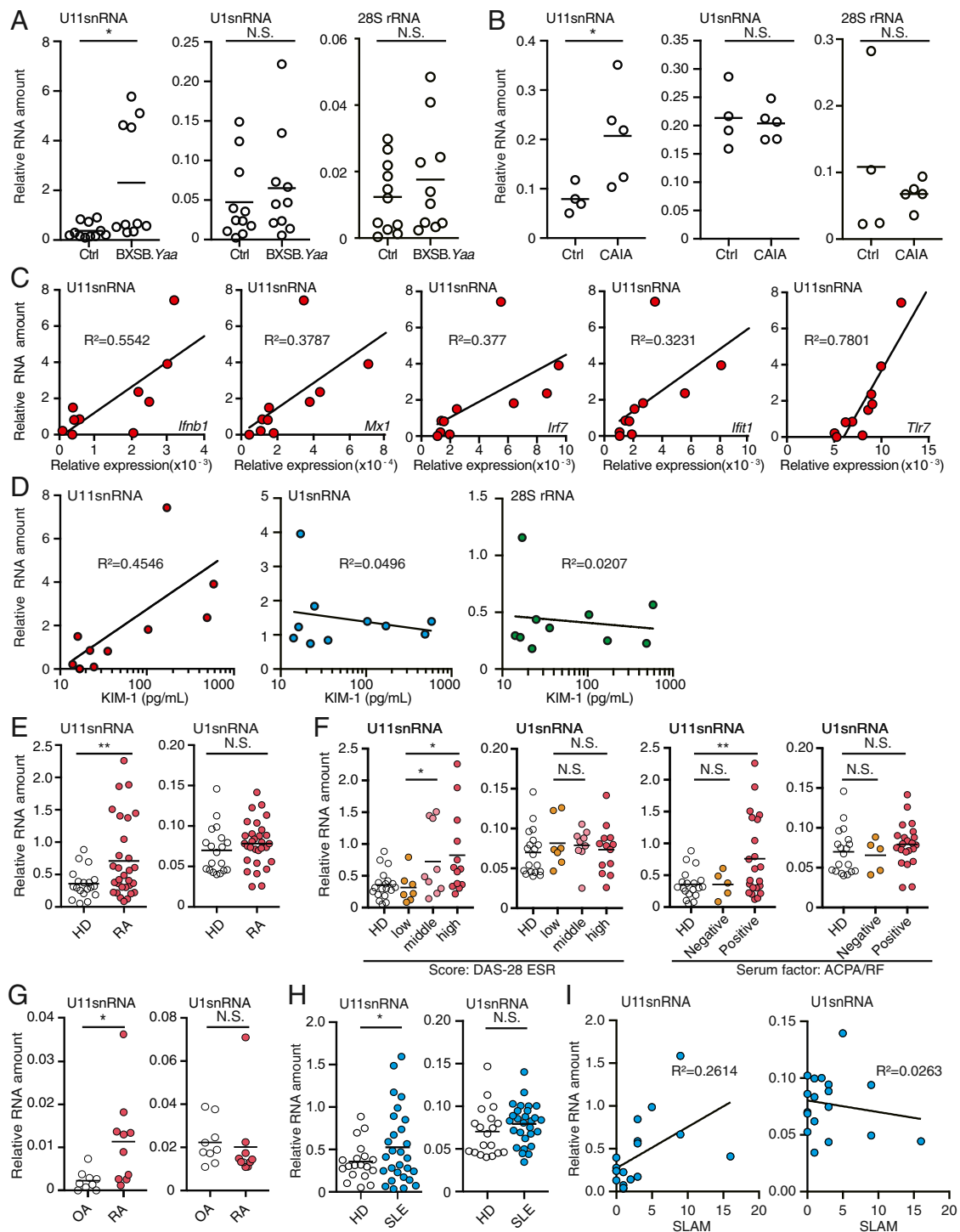
#### Possible Mechanism of U11snRNA Delivery for Immune Pathogenesis.

How does U11snRNA activate TLR7-expressing cells in vivo? Conventional wisdom is that intranuclear and intracellular RNAs are released into the extracellular environment upon cell death, such as necrosis (25). TLR7 is located in the endosomal compartment, necessitating delivery of RNA across the cell membrane of nonphagocytic cells. Cationic antimicrobial peptides, such as human cathelicidin LL-37, released by cells under inflammatory conditions have a high affinity for nucleic acids and can shuttle nucleic acids across membranes (26). In fact, the contribution of LL-37 to autoimmunity and type I IFN induction has been reported (27). LL-37 in complex with self-DNA potently stimulates type I IFN production via activation of TLR9 in pDCs, which may drive autoimmunity in psoriasis (28).

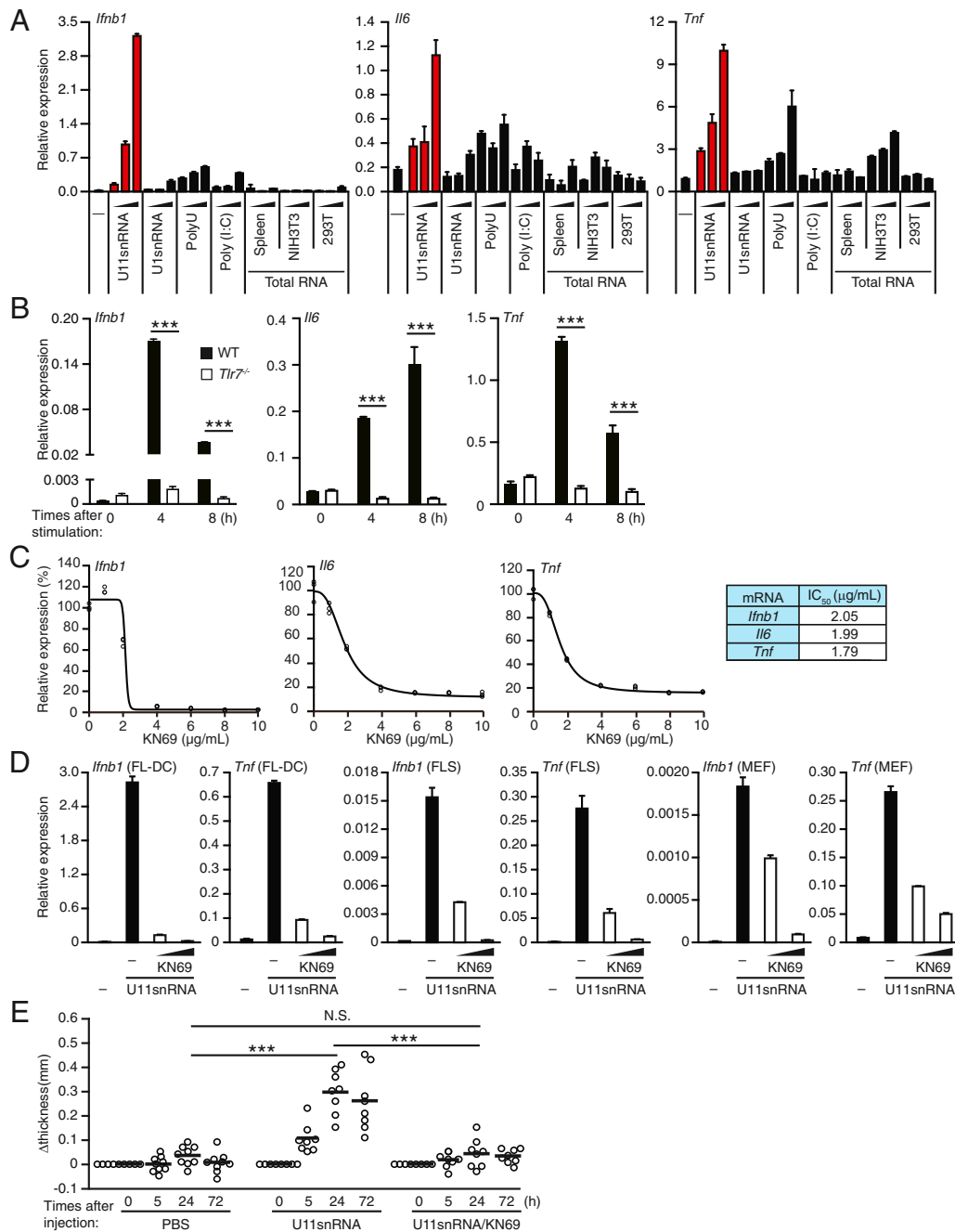
We therefore hypothesized that LL-37 also forms a complex with U11snRNA, acting as a shuttle across membranes for the delivery of U11snRNA. Indeed, we observed a complex formation of mouse U11snRNA, the sequence of which is almost identical to its human counterpart (29), in the presence of LL-37 (*SI Appendix, Fig. S4D*). Further, this complex induced IFN- $\beta$  and other cytokine mRNAs in splenocytes without DOTAP, whereas other RNAs tested were again inert in the gene induction even in the presence of LL-37 (*SI Appendix, Fig. S4E*). These results suggest that LL-37 is one of the mediators of U11snRNA, permitting its delivery to TLR7 in the endosomal compartment (*Discussion*).

#### Designing TLR7 Agonists and Antagonists Based on the Structure of U11snRNA.

To identify the region(s) of U11snRNA responsible for the activation of TLR7, KN69-binding, U-rich oligonucleotides within U11snRNA were synthesized along with the KN69-binding regions of 18S and 28S rRNAs (*SI Appendix, Fig. S5A*). These oligonucleotides were then examined for their potential to induce type I IFN gene expression. We found that a short U11snRNA fragment that contains a putative Smith (Sm) protein-binding region (the Sm site spans from 87 to 96 nt from its 5' region) (*SI*



**Fig. 3.** Relationship between U11snRNA and manifestation of autoimmune diseases. (A) qRT-PCR analysis of serum U11snRNA, U1snRNA, and 28S rRNA from BXSB/MpJ-Yaa (BXSB.Yaa) and control BXSB/MpJ-Yaa<sup>+</sup> (Ctrl) mice. Around 24-wk-old mice were used. \**P* < 0.05; N.S., not significant. (B) qRT-PCR analysis of serum U11snRNA, U1snRNA, and 28S rRNA from anti-CAIA or control BALB/c (Ctrl) mice. \**P* < 0.05. (C) Correlation analysis between relative amount of serum U11snRNA and expression of IFN signature genes in kidney from various aged BXSB.Yaa mice; 7- to 20-wk-old mice were used. (D) Correlation analysis between relative RNA amount of the indicated RNA and kidney injury marker (KIM-1) in serum from various aged BXSB.Yaa mice; 7- to 20-wk-old mice were used. (E) qRT-PCR analysis of the indicated RNA amount in the serum of rheumatoid arthritis patients or healthy donors (HD). \*\**P* < 0.01. (F) Relative RNA levels of serum U11snRNA or U1snRNA in HD or various pathological statuses of RA patients. (F, Left) Disease activity score 28 erythrocyte sedimentation rate (DAS-28 ESR). (F, Right) Serum rheumatoid factors (ACPA, anti-citrullinated protein/peptide antibody; RF, rheumatoid factor): low, RA patients with a DAS-28 score lower than 3.2; middle, RA patients with a DAS-28 score between 3.2 and 5.1; high, RA patients with a DAS-28 score higher than 5.1; negative, both ACPA- and RF-negative RA patients; positive, ACPA- and/or RA-positive patients. \**P* < 0.05, \*\**P* < 0.01. (G) qRT-PCR analysis of the indicated RNA levels in the synovial fluids from RA or osteoarthritis patients. \**P* < 0.05. (H) qRT-PCR analysis of the indicated genes in the serum from SLE patients or HD. These samples and samples in E were simultaneously examined, and results are separately shown to be understood easily. \**P* < 0.05. (I) Correlation analysis between relative serum amount of the indicated RNA and pathological score of SLE (SLAM).

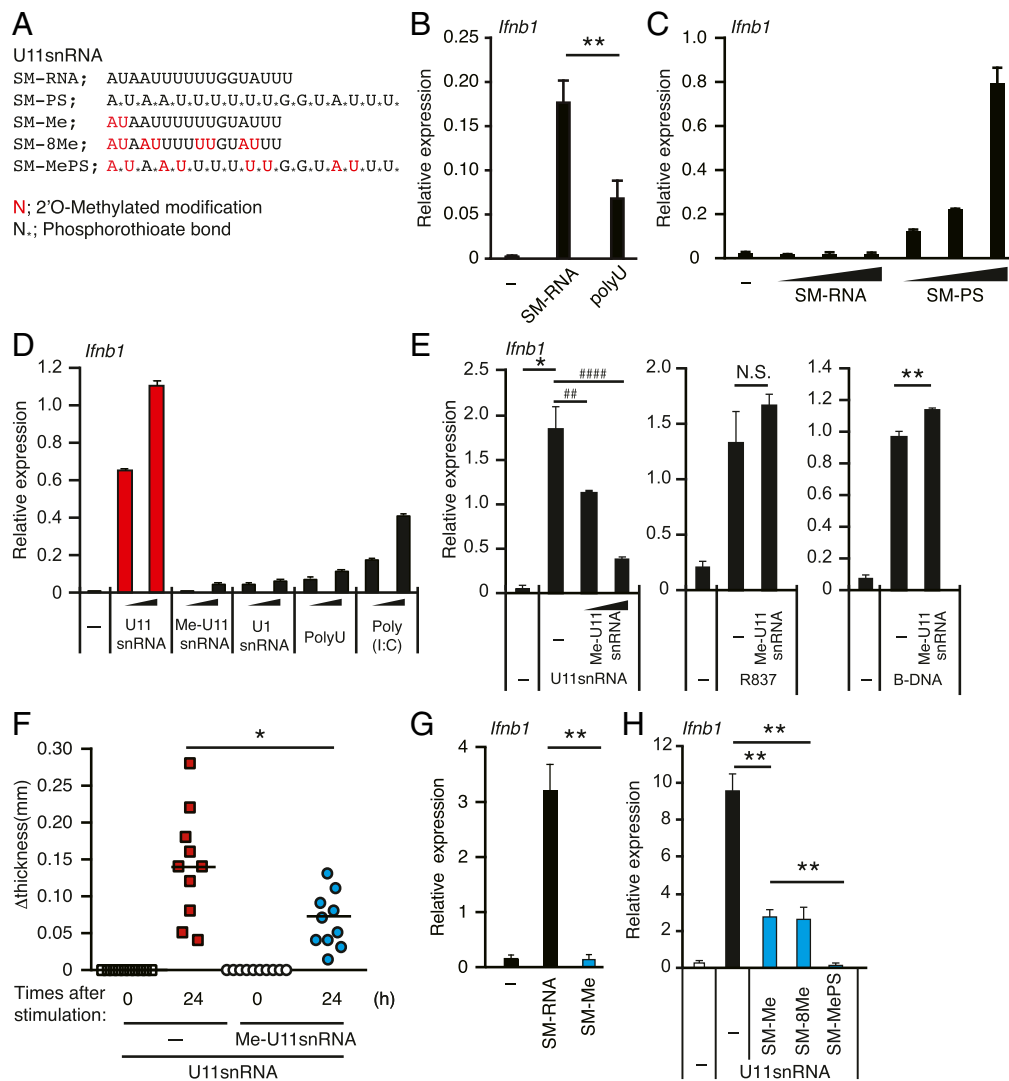


**Fig. 4.** Induction of cytokine genes by U11snRNA and other RNAs. (A) qRT-PCR analysis of *Ifnb1*, *Il6*, and *Tnf* mRNA in splenocytes after stimulation by the indicated RNA (0.25, 0.5, or 1.0 μg/mL) for 4 h. (B) qRT-PCR analysis of the indicated cytokine mRNA in wild-type (WT) or TLR7-deficient (*Tlr7*<sup>-/-</sup>) splenocytes stimulated with U11snRNA (2 μg/mL) for 4 h. \*\*\**P* < 0.001. (C) qRT-PCR analysis of the indicated cytokine mRNA induction in splenocytes stimulated with U11snRNA (2 μg/mL) for 4 h in the presence of KN69. IC<sub>50</sub> values of KN69 for the induction of individual genes are listed (Right). (D) qRT-PCR analysis of *Ifnb1* and *Tnf* mRNA in the indicated types of cells stimulated with U11snRNA (5 μg/mL) for 4 h in the presence of KN69 (4 or 8 μg/mL). FL-DCs, bone marrow-derived dendritic cells cultured in the presence of Flt-3 ligand; FLS, fibroblast-like synoviocytes; MEF, mouse embryonic fibroblasts. (E) U11snRNA (0.96 μg per joint) was intraarticularly injected into BALB/c mice with or without KN69 (0.96 μg per joint). Knee joint swelling was examined at 0, 5, 24, and 72 h (Right). \*\*\**P* < 0.001; N.S., not significant.

Appendix, Fig. S5A) (30) showed the strongest activity in inducing type I IFN gene expression (SI Appendix, Fig. S5B). This Sm region of U11snRNA, termed SM-RNA, is U-rich and also contains 2 G residues (SI Appendix, Fig. S5A); therefore, it fulfills the structural elements required for TLR7 activation (31). Further, according to the proposed secondary structure of U11snRNA, the region is single-stranded (SI Appendix, Fig. S5C) (30). In fact, short oligonucleotides based on the sequence of the SM site (SM-

RNA) also induced expression of IFN-β mRNAs to a level higher than that induced by polyU (Fig. 5 A and B). On the other hand, the oligonucleotides derived from 18S and 28S rRNAs showed very weak, if any, activity (SI Appendix, Fig. S5B).

On the basis of these findings, we sought to design a more potent agonist of TLR7, which would be useful for the enhancement of prophylactic and/or therapeutic immune responses. Since phosphorothioate (PS) modification in RNA or DNA enhances



**Fig. 5.** Rationally designed TLR7 agonists and antagonists. (A) Sequences of RNA oligonucleotides used in this study. SM-RNA, 85 to 101 nt of human U11snRNA. Others, modified derivatives of SM-RNA. 2'-O-methylated nucleotides are shown in red. A period denotes those with a phosphorothioate backbone. (B) qRT-PCR analysis of *Ifnb1* mRNA in FL-DCs stimulated with the indicated RNA (2.0  $\mu$ g/mL) for 4 h.  $**P < 0.01$ . (C) qRT-PCR analysis of *Ifnb1* mRNA in splenocytes after stimulation with different concentrations of RNA (20, 40, or 100  $\mu$ g/mL; without DOTAP) for 4 h. (D) qRT-PCR analysis of *Ifnb1* mRNA in splenocytes stimulated with the indicated RNAs (0.5 or 1  $\mu$ g/mL) for 4 h. (E) qRT-PCR analysis of *Ifnb1* mRNA in splenocytes stimulated with U11snRNA (2  $\mu$ g/mL), R837 (1  $\mu$ g/mL), or B-DNA (10  $\mu$ g/mL) in the presence or absence of 2'-O-methylated U11snRNA (Me-U11snRNA; 2 or 4  $\mu$ g/mL) for 4 h.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ . (F) U11snRNA (0.81  $\mu$ g per joint) was intraarticularly injected into female BALB/c mice with or without Me-U11snRNA (0.81  $\mu$ g per joint). Knee joint swelling was examined 24 h after injection.  $*P < 0.05$ . (G) Relative gene expression for *Ifnb1* mRNA as assessed by qRT-PCR in splenocytes stimulated with the indicated RNA oligonucleotide (2  $\mu$ g/mL) for 4 h.  $**P < 0.01$ . (H) qRT-PCR analysis of *Ifnb1* mRNA in splenocytes stimulated with U11snRNA (2  $\mu$ g/mL) for 4 h in combination with or without the indicated 2'-O-methylated residue-containing SM-RNAs (4  $\mu$ g/mL).  $**P < 0.01$ .

their immunogenic potential (32, 33), we introduced PS linkages into the sugar backbone of SM-RNA to create a new oligo RNA, termed SM-PS (Fig. 5A). As shown in Fig. 5C, SM-PS was much more potent in the induction of IFN- $\beta$  mRNA than SM-RNA in splenocytes.

We next evaluated the structure-to-activity relationship of U11snRNA. Native RNAs typically undergo modifications which are critical for their structure and function (34), and these modifications suppress the potential of RNA to activate the immune system (35). In fact, 2'-O-methylation of RNA interferes with TLR7 activation (35–37). In this context, it is interesting that, unlike other KN69 target RNAs, U11snRNA is unmethylated (29, 38–40). Thus, we examined the effect of 2'-O-methylation on the U11snRNA-mediated gene induction by introducing 2'-O-methyl residues within U11snRNA (Me-U11snRNA) (Materials and

Methods). Interestingly, methylation of only 3 nt was sufficient to lose its immunogenicity and, instead, become a competitive inhibitor of U11snRNA for TLR7 activation (Fig. 5D and E). Further, Me-U11snRNA showed significantly lower activity to induce arthritis than U11snRNA (SI Appendix, Fig. S4C) and inhibited the induction of U11snRNA-mediated joint inflammation (Fig. 5F), suggesting Me-U11snRNA functions as an antagonist for disease pathogenesis.

We also introduced 2'-O-methylation at the 5'-terminal AU residues of otherwise immunostimulatory SM-RNA (SM-Me) (Fig. 5A). Expectedly, SM-Me also lost its activity to induce the type I IFN gene (Fig. 5G) and, instead, strongly inhibited TLR7 activation by unmodified U11snRNA (Fig. 5H). These observations are consistent with the idea that 2'-O-methylated RNA can bind to TLR7 with a higher affinity than its unmodified counterpart,

thereby outcompeting those stimulatory RNA sequences for TLR7 binding (37). Finally, we modified SM-Me by introducing 2'-O-methylation and PS linkage in SM-Me to increase antagonism (Fig. 5A). Although the resulting 2'-O-methylated SM-RNA (SM-8Me) did not show any increase of antagonism, further PS-modified SM-8Me (SM-MePS) suppressed U11snRNA-mediated gene induction much more strongly than SM-Me (Fig. 5H), indicating that SM-MePS functions as a strong TLR7 antagonist.

## Discussion

It has become clear that “selfness” is no guarantee of tolerance; DAMPs represent a potential danger signal when released into the extracellular matrix by activating innate receptors (41). The activation of TLR7 by endogenous RNA has long been implicated in the development of autoimmunity, yet the culprit RNA(s) that triggers these responses has still remained unclear (1–6). In the present study, we first developed a compound, KN69, that suppresses autoimmunity in several mouse models (Fig. 1 C and D and *SI Appendix, Fig. S1D*). A subsequent search of KN69-binding partners revealed RNA, but not DNA or protein. Among these, U11snRNA was found to be most active to evoke innate immune response via the TLR7 pathway.

In fact, U11snRNA contains sequences, particularly in the SM region, which fulfill a known criterion as a TLR7 agonist ligand, namely a U-rich sequence together with a G residue(s) (*SI Appendix, Fig. S5A*). Further, we also showed that the lack of 2'-O-methylation, where a methyl group is added to the 2' hydroxyl of the ribose moiety of a nucleoside, is an important structural feature for the agonistic activity of U11snRNA (Fig. 5D). In fact, other KN69 target RNAs that contain this modification are less potent TLR7 agonists (*SI Appendix, Fig. S5B*). Consistent with this, introduction of 2'-O-methylation converted U11snRNA or its derivative SM site RNA (SM-Me) from a TLR7 agonist to an antagonist (Fig. 5 E, G, and H). Thus, these results provide a rationale for the selective TLR7 activation by U11snRNA.

U11snRNA activated immune cells and nonimmune cells, and induced arthritis in vivo, features suppressed by KN69 (Fig. 4 A and C–E). On the other hand, U1snRNA, an endogenous RNA implicated in triggering inflammation or autoimmunity (7, 42–44), was far less immunogenic by comparison (Fig. 4A). Although it is difficult to reconcile our data and those of previous studies (42–44), we can offer the following possible explanation. First, the natural U1snRNA contains 2'-O-methylated residues, which reduce RNA's immunogenicity (Fig. 5D). Previous studies used unmethylated synthetic RNA (42–44). Second, the in vitro transcribed RNA in a previous study contained a 5'-triphosphate structure that can activate another innate receptor, RIG-I (45). We speculate 2'-O-methylation modifications present in many abundant endogenous RNAs have evolved to suppress undesirable immune activation by these RNAs. The reason U11snRNA is unmodified is currently unknown, but we speculate that the unmodified structure may be important for its original function in the mRNA splicing machinery.

We also adduced evidence that U11snRNA has pathogenic function in the development of joint arthritis in mice, which is suppressed by KN69 or U11snRNA-derived antagonist RNA (Figs. 4E and 5F). To further validate the in vivo role of U11snRNA, we attempted to generate *Rnu11* gene knockout mice; however, perhaps expectedly from its original, essential role in pre-mRNA splicing (21), even heterozygous mice were not viable (*SI Appendix, Fig. S6*).

Although further work is required to better understand U11snRNA's role in autoimmunity, our results show that 1) U11snRNA is the main target of KN69, which suppresses autoimmune symptoms in mouse models, 2) U11snRNA strongly activates TLR7, which is involved in autoimmunity, and 3) there is a correlation between U11snRNA levels in the sera of RA and

SLE patients and disease burden. In total, these data suggest that the U11snRNA is a long-sought culprit DAMP RNA involved in the development of autoimmunity. Clearly, further work will be required to rigorously examine whether there will be an additional endogenous RNA(s) involved in the pathology at present. It remains to be clarified how U11snRNA is delivered to TLR7. Our results suggest the involvement of an antimicrobial peptide, namely LL-37, in the delivery of U11snRNA; however, the mouse ortholog cathelicidin-related antimicrobial peptide (CRAMP) may be inactive for the RNA delivery (46). In view of previous reports that indicate a role of antimicrobial peptides in nucleic acid-mediated pathogenesis (27), we infer that LL-37 and/or another peptide(s) may cooperate in the delivery of extracellular U11snRNA to TLR7 (*SI Appendix, Fig. S4 D and E*).

It is interesting that the serum U11snRNA levels are selectively increased in the sera of autoimmune model mice and patients (Fig. 3). Although the mechanism(s) underlying this phenomenon is currently unknown, it is possible that the RNA is complexed with other molecules, for example LL-37, upon cell death, rendering the RNA resistant to degradation. Alternatively, one may envisage the presence of an unknown mechanism(s) by which this RNA is selectively released by the cells. Clearly, further study is required to clarify this point.

Finally, the generation of a U11snRNA-derived TLR7 agonist and antagonist may provide new clues for their therapeutic use. Namely, SM-PS may be useful for the development of an effective adjuvant or to boost robust antitumor immunity (47), while SM-MePS will be effective for the treatment of TLR7-mediated autoimmunity.

## Materials and Methods

**Mice.** *Tlr7*<sup>-/-</sup> and *Tlr9*<sup>-/-</sup> mice described previously (48, 49) were kindly provided by S. Akira, Osaka University, Osaka, Japan. C57BL/6 and BALB/c mice were purchased from CLEA Japan. DBA/1, DBA/2, and B6D2F1 (BDF1) mice were obtained from Charles River Laboratories Japan. BXSb/MpJ-Yaa and BXSb/MpJ-Yaa+ mice were purchased from Japan SLC. CAG-Cre transgenic mice were obtained from The Jackson Laboratory. All animal experiments were done in accordance with guidelines of the University of Tokyo and RIKEN Kobe Branch.

**Human Samples.** We recruited RA patients, SLE patients, OA patients, and self-reported healthy donors. Individuals under 20 y of age or with active infection were excluded. RA patients fulfilled the 1987 revised American College of Rheumatology (ACR) criteria or the 2010 ACR/European League against Rheumatism classification criteria, and disease activity was assessed based on the disease activity score of 28 joints (DAS-28 ESR). SLE patients fulfilled the 1997 ACR criteria for SLE, and the disease activity was assessed according to the systemic lupus activity measure (SLAM) score. OA patients fulfilled the clinical ACR criteria. All donors provided written informed consent, and the use of human peripheral blood, synovial fluid samples, and clinical data were approved by the Ethical Committee of the University of Tokyo Hospital (nos. 10154 and G3582). The methods were carried out in accordance with the approved guidelines.

**Data Availability.** Detailed information for reagents, cells, plasmids, and experimental procedures is provided in *SI Appendix*. RNA-sequencing data were deposited in the DNA Data Bank of Japan (BioProject ID code PRJDB8733).

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