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Authors

Lind, Nicholas A
Rael, Victoria E
Pestal, Kathleen
et al.

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Regulation of the nucleic acid-sensing Toll-like receptors

Nicholas A. Lind[1,3], Victoria E. Rael[1,3], Kathleen Pestal[1,3], Bo Liu[1,2] and Gregory M. Barton[1] ✉

Abstract | Many of the ligands for Toll-like receptors (TLRs) are unique to microorganisms, such that receptor activation unequivocally indicates the presence of something foreign. However, a subset of TLRs recognizes nucleic acids, which are present in both the host and foreign microorganisms. This specificity enables broad recognition by virtue of the ubiquity of nucleic acids but also introduces the possibility of self-recognition and autoinflammatory or autoimmune disease. Defining the regulatory mechanisms required to ensure proper discrimination between foreign and self-nucleic acids by TLRs is an area of intense research. Progress over the past decade has revealed a complex array of regulatory mechanisms that ensure maintenance of this delicate balance. These regulatory mechanisms can be divided into a conceptual framework with four categories: compartmentalization, ligand availability, receptor expression and signal transduction. In this Review, we discuss our current understanding of each of these layers of regulation.

CpG motifs
Short single-stranded oligodeoxynucleotides containing unmethylated CpG motifs function as Toll-like receptor 9 (TLR9) agonists. Different classes of CpG oligodeoxynucleotide are given letter designations (for example, CpG-A, CpG-B and CpG-C) based on the distinct responses they elicit.

[1]*Division of Immunology and Pathogenesis, Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, USA.*

[2]*Present address: CAS Key Laboratory of Molecular Virology and Immunology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China.*

[3]*These authors contributed equally: Nicholas A. Lind, Victoria E. Rael, Kathleen Pestal.*

✉*e-mail: barton@berkeley.edu*

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Toll-like receptors (TLRs) are a family of innate immune receptors whose activation is crucial for the induction of innate and adaptive immune responses. Expression of TLRs in antigen-presenting cells links the recognition of pathogens both to the induction of innate immune effector mechanisms that limit pathogen replication and to the initiation of adaptive immunity[1]. TLRs recognize conserved microbial features shared by broad pathogen classes, which enables a limited set of receptors to recognize the tremendous diversity of microorganisms potentially encountered by the host. Five mammalian TLRs can be activated by nucleic acid ligands (referred to here as NA-sensing TLRs): TLR3 recognizes double-stranded RNA; TLR7, TLR8 and TLR13 recognize fragments of single-stranded RNA with distinct sequence preferences; and TLR9 recognizes single-stranded DNA containing unmethylated CpG motifs. NA-sensing TLRs are particularly relevant for the detection of viruses because viruses generally lack other common, invariant features that are suitable for innate immune recognition. However, NA-sensing TLRs can also detect nucleic acids from other pathogen classes, and each of these receptors has been implicated in the host response to diverse pathogens (TABLE 1).

Targeting nucleic acids greatly expands the breadth of microorganisms that can be recognized by TLRs but comes with the trade-off of potentially sensing self-nucleic acids. Indeed, improper activation of NA-sensing TLRs by self-nucleic acids has been linked to several autoimmune and autoinflammatory disorders, including systemic lupus erythematosus (SLE) and psoriasis[2–8]. One possible strategy for limiting such adverse outcomes is recognition of specific features that distinguish foreign nucleic acids from self-nucleic acids. However, although ligand preferences based on sequence or chemical modifications do reduce the likelihood of TLR responses to self-nucleic acids, discrimination between foreign and self-nucleic acids is not based solely on these differences[9,10]. NA-sensing TLRs also rely on mechanisms that reduce the likelihood that they will encounter self-nucleic acids and/or dampen the response when self-nucleic acids are nevertheless detected. These mechanisms collectively set a precisely tuned threshold for receptor activation: too low a threshold would result in sensing of self-nucleic acids and autoimmunity, whereas too high a threshold would hinder defence against the very pathogens that the NA-sensing TLRs aim to detect (FIG. 1). Recent research has shown that multiple mechanisms function together to determine this threshold for a given TLR. The picture emerging from these studies is becoming quite complex, as each NA-sensing TLR is subject to distinct modes of regulation, suggesting that the 'solution' to the problem of self versus non-self discrimination may be different for each TLR. Such receptor-specific regulation probably explains the differences in the relative contributions of different NA-sensing TLRs to autoimmune diseases. For example, inappropriate activation of TLR7 by self-nucleic acid is much more consequential than TLR9 activation in animal models of SLE, even though both TLR7 and TLR9 can contribute to pathology[11–13]. Although the field has taken initial steps towards identifying the molecular

Table 1 | **Key examples of pathogen recognition by nucleic acid-sensing Toll-like receptors**

Receptor	Ligand specificity	Class of pathogen recognized	Examples
TLR3	dsRNA	dsRNA viruses	Reovirus
		ssRNA viruses	Respiratory syncytial virus, hepatitis C virus
		DNA viruses	HSV-1, HSV-2, vaccinia virus
		Retroviruses	HIV-1
		Bacteria	Lactic acid-producing bacteria
		Protozoa	*Neospora caninum*
TLR7 and TLR8	ssRNA and RNA breakdown products	ssRNA viruses	Influenza A virus, SARS-CoV
		Retroviruses	HIV-1
		Bacteria	Group B streptococcus, *Borrelia burgdorferi*
		Fungi	*Candida* spp.
		Protozoa	*Leishmania major*
TLR9	ssDNA (containing CpG motifs)	DNA viruses	HSV-1, HSV-2, HPV, adenovirus
		Bacteria	*Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Mycobacterium tuberculosis*
		Fungi	*Aspergillus fumigatus*, *Candida* spp.
		Protozoa	*Plasmodium falciparum*, *Leishmania major*
TLR13 (mice)	ssRNA	Bacteria	*Staphylococcus aureus*, *Streptococcus pneumoniae*
		ssRNA viruses	Vesicular stomatitis virus

ds, double-stranded; HPV, human papillomavirus; HSV, herpes simplex virus; SARS-CoV, severe acute respiratory syndrome coronavirus; ss, single-stranded; TLR, Toll-like receptor.

basis of this specialized regulation, substantial additional work is needed to determine how such closely related receptors can make different contributions to disease outcomes.

With these questions in mind, here, we review our understanding of the mechanisms controlling activation of, and signalling by, NA-sensing TLRs. We believe that this discussion is timely, as several strategies to modulate NA-sensing TLR responses, both positively and negatively, are being pursued therapeutically (BOX 1). We discuss the four categories of regulatory mechanism that influence NA-sensing TLR responses: compartmentalization, ligand availability, receptor expression and signal transduction.

Compartmentalization

Activation of all NA-sensing TLRs is restricted to endosomes, and this intracellular compartmentalization is crucial for both their function and their regulation (FIG. 2). NA-sensing TLRs encounter pathogen-derived nucleic acids when microorganisms are internalized and degraded, either through endocytosis or phagocytosis[14,15]. This mode of recognition enables cells to detect pathogens without being infected, which reduces the likelihood that pathogens can inhibit TLR-mediated induction of immunity. By contrast, cytosolic sensors of nucleic acids can generally detect pathogen ligands only when cells are directly infected, which makes them more prone to interference from pathogen evasion strategies.

Localization of NA-sensing TLRs to endosomes also achieves a crucial regulatory function by sequestering these receptors away from self-nucleic acids. The importance of this sequestration was first demonstrated by the finding that certain types of immune cell that are normally unresponsive to self-nucleic acids can be activated if these ligands are efficiently delivered to endosomes[7,16]. This concept is further illustrated by studies in which NA-sensing TLRs have been mislocalized to the plasma membrane, which increases their access to extracellular nucleic acids[17]. Mice engineered to express mislocalized TLR9 have fatal systemic inflammation and anaemia[18,19]. These examples illustrate the importance of mechanisms that limit the activation of NA-sensing TLRs to endosomes. In the following sections, we discuss our current understanding of how such compartmentalization is achieved.

Receptor trafficking. The NA-sensing TLRs are translated at the endoplasmic reticulum and trafficked via the classical secretory pathway to endosomes[14]. Trafficking itself is a regulatory mechanism, as the number of functional TLRs in endosomes and lysosomes influences the receptor activation threshold. It is beyond the scope of this Review to cover all aspects of the trafficking of NA-sensing TLRs, which have been reviewed in detail elsewhere (for example, REFS[14,15]), but we highlight the key players and the latest developments.

All NA-sensing TLRs require the 12-pass transmembrane protein UNC93B1 to exit the endoplasmic reticulum and traffic to endosomes[20–22]. UNC93B1 stays associated with NA-sensing TLRs during their trafficking, and it is now clear that UNC93B1 also mediates regulatory functions after exit from the endoplasmic reticulum[21,23–25]. For example, the association of UNC93B1 with NA-sensing TLRs is essential for their stability in and beyond the endoplasmic reticulum[23]. Non-functional alleles of UNC93B1 that abolish interaction with NA-sensing TLRs result in reduced receptor stability, failure of export from the endoplasmic reticulum and loss of function[22,23,26]. These findings have clinical relevance, as humans with loss-of-function mutations in UNC93B1 are non-responsive to ligands for NA-sensing TLRs and show increased susceptibility to certain viruses[27]. Missense mutations in UNC93B1 can alter the trafficking of NA-sensing TLRs, leading to marked functional consequences. For example, the D34A mutation in UNC93B1 leads to preferential export of TLR7 from the endoplasmic reticulum at the expense of TLR9, which presumably increases the amount of TLR7 within endosomes[28]. This increase in the level of 'functional' TLR7 is sufficient to trigger lethal inflammation in mice[29]. That a single point mutation in this crucial chaperone is sufficient to disrupt the distribution of NA-sensing TLRs underscores the carefully tuned and interconnected nature of NA-sensing TLR regulation.

Although the NA-sensing TLRs all localize to endosomes, the trafficking routes they use, as well as the nature of the compartments where they ultimately reside, are surprisingly diverse. The process of dissecting

Systemic lupus erythematosus
(SLE). A chronic autoimmune disease characterized by the production of antinuclear autoantibodies and often associated with production of type I interferon. The pathology of SLE can affect joints, skin, brain, lungs, kidneys and blood vessels.

Psoriasis
A chronic autoimmune disease characterized by inflammation of the skin, leading to raised patches of dry and scaly skin.

Cytosolic sensors of nucleic acids
Innate immune sensors of infection that detect nucleic acids and reside in the cytosol. Examples include the cGAS–STING pathway and AIM2, which detect DNA, and RIG-I and MDA5, which detect RNA.

the mechanisms responsible for this diversity remains in its infancy, but some insight has been gained from recent studies. S100A9, a calcium-binding and zinc-binding protein, has been identified as a regulator of TLR3 compartmentalization but is not required for the trafficking of other TLRs. The S100A9–TLR3 interaction is crucial for the distribution of TLR3 in late endosomes, and accordingly, S100A9 deficiency in mice leads to a reduced response to the TLR3 agonist polyinosinic:polycytidylic acid (polyI:C)[30]. The mechanism by which S100A9 specifically controls TLR3 trafficking to late endosomes remains unclear. There is also evidence that TLR7 and TLR9 are subject to distinct trafficking regulation. To reach endosomes, TLR9 must first traffic to the plasma membrane, where it is then internalized into endosomes by adaptor protein complex 2 (AP-2)-mediated endocytosis[21]. By contrast, TLR7 does not require this AP-2-mediated step but instead can interact with AP-4, which suggests that TLR7 may traffic directly from the Golgi to endosomes[21]. For each of these examples, the biological relevance of such differential trafficking remains unclear, and further studies are needed to dissect the importance of such regulation.

There are clear examples of functional specialization owing to the regulated trafficking of NA-sensing TLRs, which illustrate the potential of this line of research. In plasmacytoid dendritic cells (pDCs) (BOX 2), the localization of TLR7 and TLR9 is further controlled by AP-3, which moves these TLRs into a specialized type of lysosome-related organelle from which TLR signalling leads to the transcription of type I interferon (IFN) genes[31,32]. Trafficking of TLR9 to this specialized endosome also requires phosphatidylinositol 3-phosphate 5-kinase[33]. Interestingly, there is growing evidence that the machinery controlling trafficking of TLR7 and TLR9 to this compartment is distinct. For example, TLR7-driven IFNα production by pDCs requires a cellular redistribution of TLR7-containing lysosomes mediated by linkage to microtubules[34]. The GTPase ARL8B is required for this redistribution of TLR7, but TLR9-induced IFNα production occurs independently of this mechanism[34]. pDCs also use a process known as LC3-associated phagocytosis for the formation of the TLR9–IFN signalling cascade in response to DNA-containing immune complexes[35]. The distinct modes by which each NA-sensing TLR populates the endosomal network suggest a granular level of sorting with potentially profound functional consequences. The lack of definitive molecular markers for some of these specialized endosomal organelles and the challenge of isolating such compartments for biochemical analyses have slowed progress in this field.

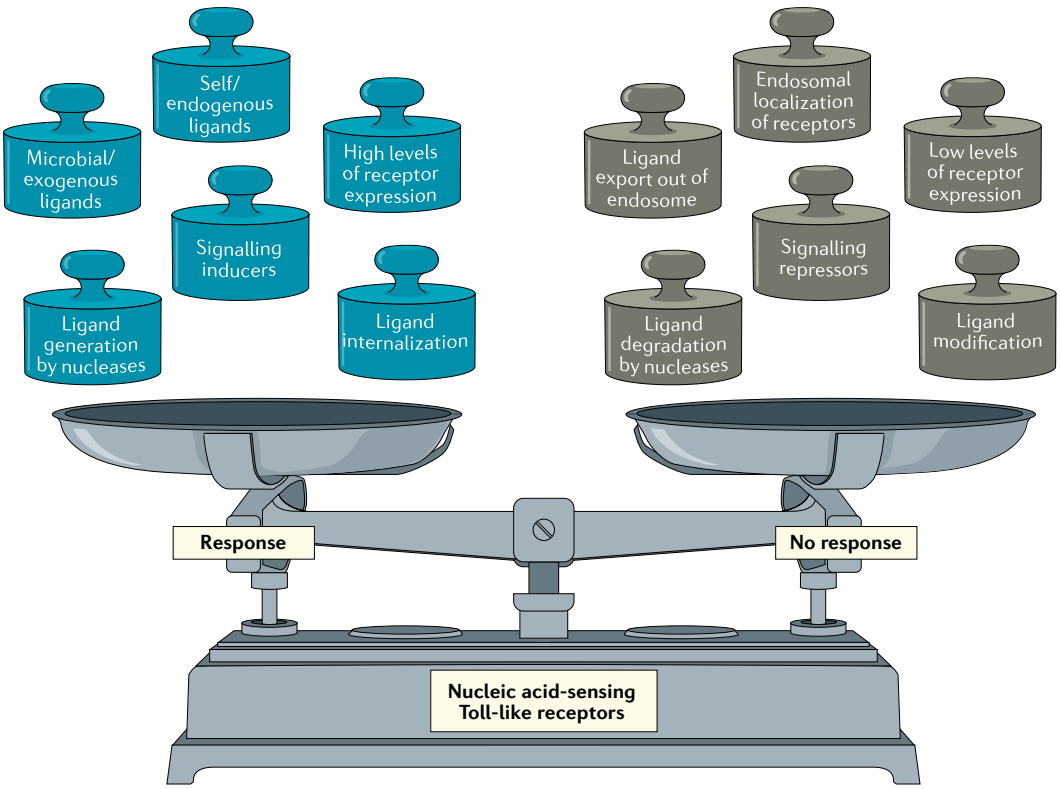


Fig. 1 | **The activation of nucleic acid-sensing Toll-like receptors is finely balanced by a complex array of regulatory mechanisms.** The nucleic acid-sensing Toll-like receptors (NA-sensing TLRs) are capable of sensing a wide variety of potential pathogens, but this breadth of recognition comes at the potential cost of autoimmunity induced by host nucleic acids. Activation of these receptors must therefore be carefully balanced, such that they remain inactive under homeostatic conditions but are still sensitive to increases in endosomal levels of nucleic acids that could indicate the presence of microorganisms. Each weight on the left-hand side of the scale represents a known input that drives the activation of NA-sensing TLRs, whereas the weights on the right-hand side represent known restraints on TLR activation.

Box 1 | **Nucleic acid-sensing Toll-like receptors as therapeutic targets**

Therapeutics that modulate nucleic acid-sensing Toll-like receptor (NA-sensing TLR) responses are being pursued in several contexts (reviewed extensively in REF.[117]). Because the ligands for these receptors are relatively small and easy to synthesize, progress in generating synthetic agonists or inhibitors has been rapid relative to other TLRs.

Numerous clinical trials are testing the efficacy of agonists for TLR3, TLR7, TLR8 and TLR9 in contexts where increased activation of innate and adaptive immune responses should be beneficial. These trials can be broadly grouped into two categories: adjuvants for vaccines targeting infectious disease and therapies aimed at boosting immune responses against cancer. The TLR9 agonist CpG 1018 is FDA-approved as an adjuvant in the hepatitis B vaccine HEPLISAV-B[118], and several additional TLR agonist compounds are being tested in vaccines against viral pathogens[117]. Two potent vaccines for coronavirus disease 19 (COVID-19), developed by Moderna and Pfizer/BioNTech, are mRNA-based vaccines that probably trigger an immune response at least in part through stimulation of NA-sensing TLRs. By contrast, the use of NA-sensing TLR agonists to boost anticancer immune responses has been met with only limited success so far. Currently, the only FDA-approved TLR agonist for treatment of cancer is imiquimod, an agonist for TLR7 and for the NLRP3 inflammasome, which is used to treat basal cell carcinoma, but many agonists for TLR3, TLR7, TLR8 and TLR9 are being tested for efficacy against various types of cancer. Most of these trials involve combining TLR agonists with therapeutics that block one or more immune checkpoints.

Antagonists of TLR7, TLR8 and TLR9 are being pursued to treat autoimmune disorders, such as systemic lupus erythematosus and psoriasis, in which activation of these TLRs has been implicated in disease pathology or progression. Unfortunately, although antagonists for TLR7 and TLR9 produced promising results in preclinical models, clinical trials focused on systemic lupus erythematosus have failed to meet primary end points[117]. Trials focused on psoriasis have been more promising. The modest success of these therapeutics probably reflects the complex aetiology of these diseases and underscores the importance of increasing our understanding of the mechanisms that control TLR regulation and function.

Receptor processing. The compartmentalized activation of NA-sensing TLRs is reinforced by a requirement that their ectodomains undergo proteolytic processing before they can respond to ligands[36,37]. Specific proteases have been implicated in ectodomain processing[36–40], but it is likely that many enzymes can mediate this required step. Although uncleaved TLRs can still bind to ligands, it is clear that processing is a prerequisite for stabilization of receptor dimers and activation[36,41]. Proteolytic processing occurs most efficiently in the acidic environment of endosomes and lysosomes. As a result, the NA-sensing TLRs are inactive in the endoplasmic reticulum and during trafficking, which further reduces the chance of stimulation by self-nucleic acids (FIG. 2). Human TLR7 and TLR8 can also be cleaved at a neutral pH by furin-like proprotein convertases, which suggests that they may become functional before reaching the acidic environment of endolysosomes[42,43]. The consequences of this potential 'early' activation of TLR7 and TLR8 in humans, if any, have not been determined.

Ligand availability

The compartmentalization of NA-sensing TLRs as a strategy to achieve self versus non-self discrimination relies on complementary mechanisms that control ligand availability within endosomes. Several factors influence the amount of nucleic acids within endosomes and determine whether those nucleic acids are capable of activating TLRs. Nucleases can reduce the availability of self-nucleic acids (FIG. 2), but ligand digestion as a means of negative regulation of the NA-sensing TLRs is nuanced because nucleic acids can be self or microbial. Furthermore, the ligands for some NA-sensing TLRs are very small RNA fragments (BOX 3), so degradation of self-nucleic acids may not always prevent recognition and may even facilitate it. Similarly, the digestion of microbial nucleic acids can also promote recognition by generating molecules that are capable of activating TLRs.

Although our current understanding makes it challenging to place the mechanisms affecting ligand availability into a unified conceptual framework, we find it useful to group such mechanisms into four general categories: nucleic acid digestion that reduces the concentration of activating ligands, internalization of ligands, nucleic acid digestion that generates activating ligands, and physical sequestration of ligands out of the endosome. Together, these four categories of regulation establish the levels of available ligands within endosomes, which contributes to the balance between sensing potential pathogens and avoiding recognition of self-nucleic acids (FIG. 1).

Digestion of nucleic acids to limit ligand availability. In recent years, investigators have identified multiple nucleases, the absence of which leads to sensing of self-nucleic acids as well as the onset of autoimmunity in both mice and humans. DNase I-like 3 (DNASE1L3), a secreted DNA endonuclease, has a positively charged carboxy-terminal peptide that enables it to access apoptotic cell microparticles and digest the DNA within[44]. Mutations in this nuclease lead to a form of paediatric SLE in humans[45], and mice lacking DNASE1L3 develop autoimmunity that is driven synergistically by TLR7 and TLR9 (REFS[44,46]). The contribution of TLR7, an RNA sensor, is unexpected and could be owing to the reported capacity of TLR7 to respond also to deoxyguanosine[47] or to the functional competition between TLR7 and TLR9 that has been previously described[28,29,48].

Deficiencies in other nucleases cause severe autoinflammation that manifests earlier in life. Phospholipase D3 (PLD3) and PLD4 were recently identified as membrane-anchored 5′ exonucleases that degrade TLR9 ligands within endolysosomes[2]. Mice lacking either enzyme develop TLR9-dependent and IFNγ-dependent inflammatory disease; mice lacking both PLD3 and PLD4 develop severe disease that is fatal early in life[2], similar to the disease that develops when mice express mislocalized TLR9, the activation of which is no longer restricted to endosomes[19]. Mice lacking DNase II, another endolysosomal endonuclease, also develop severe disease in utero[49]. In contrast to the TLR9-dependent disease in PLD3-deficient and PLD4-deficient mice, the embryonic lethality of DNase II-deficient mice can be attributed to activation of the cGAS–STING pathway upon endosomal rupture and can be rescued by eliminating type I IFN signalling[49–51]. However, in these rescued mice, the accumulation of self-DNA in endosomes leads to TLR-dependent autoantibody production, arthritis and splenomegaly, and deficiency in UNC93B1 reduces this residual disease[52–54].

In addition to these examples, it is likely that other nucleases are also involved in preventing the induction

Apoptotic cell microparticles
Small membrane-coated particles released from apoptotic cells that contain genomic DNA and chromatin, as well as RNA.

cGAS–STING pathway
An innate immune sensing pathway that detects the presence of cytosolic double-stranded DNA and triggers the transcription of type I interferon and other genes involved in the host response.

Neutrophil extracellular traps
Web-like structures of DNA released into the extracellular space by neutrophils. Neutrophil extracellular traps can trap microorganisms and prevent their dissemination, but in some contexts, they can be a source of self-nucleic acids that drive autoimmune diseases, such as systemic lupus erythematosus and psoriasis.

of NA-sensing TLR-dependent autoimmunity. An obvious candidate is another secreted DNase, DNase I; mice and humans with DNase I deficiency have symptoms of SLE but this disease has not yet been linked directly to aberrant TLR activation[55,56].

The examples discussed above involve enzymes that metabolize DNA but not RNA. Does an analogous group of RNases digest potential RNA ligands for TLR3, TLR7, TLR8 and TLR13? The RNase A family is one possible candidate, and overexpression of RNase A proteins reduces disease in a TLR7-dependent model of autoimmunity[57]. However, existing single knockouts of RNase A family members do not cause autoinflammation or autoimmunity[58,59]. Functional redundancy between family members may mask phenotypes in these contexts. Studies to overcome this limitation, perhaps by knocking out multiple RNases at the same time, have not been carried out to our knowledge. Alternatively, it is possible that the RNA-sensing TLRs are more reliant on alternative modalities of regulation, such as the physical sequestration mechanisms described below.

The distinct timelines of disease triggered by deletion of the genes that encode these nucleases are also compelling. In contrast to autoimmune diseases triggered by TLR7 and TLR8, TLR9-dependent autoinflammation develops in utero or extremely early in life. It is possible that TLR9 is simply expressed at an earlier time in development than TLR7 or TLR8 and therefore is capable of driving disease earlier. Alternatively, this observation may hint that early development is particularly sensitive to the loss of enzymes that are responsible for degrading DNA ligands rather than RNA ligands. The rapid tissue development and remodelling early in life, accompanied by increased apoptosis, could explain why digestion of potential TLR9 ligands is particularly crucial at this time point.

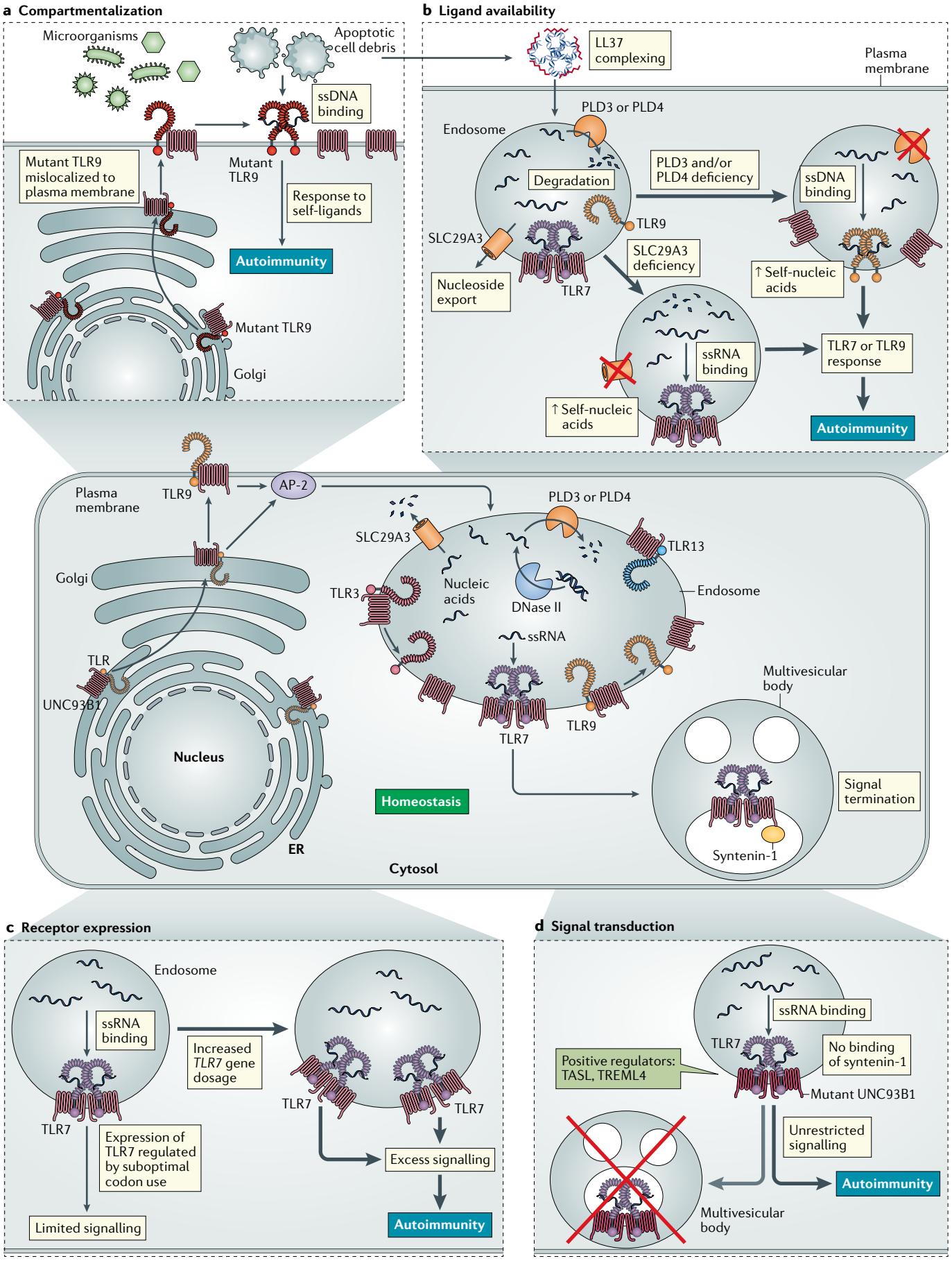
Internalization of ligands. Another factor that influences the ligand availability for activation of NA-sensing TLRs is the extent to which ligands are internalized by cells. The uptake of microorganisms varies across cell types; macrophages and dendritic cells are highly phagocytic, whereas certain other cell types are most likely to internalize microorganisms only when specific receptor–ligand interactions occur. For example, B cells are generally poor at acquiring antigen but will readily internalize antigens bound by their B cell receptor.

Receptor-mediated uptake of ligands also affects responses to self-nucleic acids. The first demonstration of this principle was made in the context of B cells expressing a B cell receptor specific for self-immunoglobulin. These B cells internalize immune complexes containing self-DNA or self-RNA, leading to synergistic activation of B cells through TLR9 or TLR7 and the B cell receptor[16,60]. This mechanism effectively 'breaks' the receptor compartmentalization achieved through localization of NA-sensing TLRs to endosomes. A similar uptake of immune complexes containing self-nucleic acids can occur via engagement of Fc receptors on dendritic cells[61,62]. Other receptors, such as the receptor for advanced glycosylation end products (RAGE)[63–65], have also been implicated in uptake and delivery of nucleic acids to NA-sensing TLRs.

Another mechanism that facilitates cellular uptake of and aberrant responses to extracellular self-nucleic acids is the formation of complexes between self-nucleic acids and certain self-proteins. The clearest example of this concept is the association of self-DNA and self-RNA with the antimicrobial peptide LL37 (also known as CAMP)[7,66]. This interaction condenses the nucleic acids, promoting uptake into endosomes and reducing degradation by nucleases. LL37-complexed self-RNA and self-DNA potently stimulate TLR7 and TLR9 in pDCs, leading to type I IFN production. This mechanism is particularly relevant to the recognition of neutrophil extracellular traps. Neutrophils express high levels of LL37, and neutrophil extracellular traps seem to be a major source of self-nucleic acids in the context of autoimmune diseases such as SLE and psoriasis[67–69]. Additional proteins, such as HMGB1, have also been shown to bind to self-nucleic acids and protect them from digestion by nucleases[64,70].

Processing of nucleic acids to generate ligands. The degradative environment of the endosome has an important role in breaking down microorganisms to expose their nucleic acids, but there is accumulating evidence that recognition of nucleic acids in some cases requires further processing by nucleases to generate ligands that can activate TLRs. The lysosomal endoribonucleases RNase T2 and RNase 2 function upstream of human TLR8-dependent RNA recognition, cleaving before and after uridine residues to generate TLR8 ligands from the RNA of certain pathogens[71,72]. DNase II, described above as a nuclease that is required to prevent responses to self-DNA, is also required to generate TLR9 ligands in some contexts[53,54,73]. DNase II-deficient cells, also lacking the type I IFN receptor to prevent lethality, fail to respond to CpG-A, which assembles into large oligomers, whereas responses to less complex CpG-B ligands are unaffected[73]. Similar analyses have revealed that other sources of DNA, such as *Escherichia coli* genomic DNA and even self-DNA, require processing by DNase II before TLR9 recognition[53,54]. Thus, DNase II provides an example of the nuanced nature of ligand processing as it functions to prevent the accumulation of DNA in some contexts while at the same time generating ligands that can stimulate TLR9.

Whether stimulation of the other NA-sensing TLRs requires ligand digestion by specific nucleases remains an open question. TLR13 recognizes bacterial ribosomal RNA sequences that are embedded within the ribosome and therefore are not readily accessible[74,75]. This specificity implies that some level of ligand processing must take place for TLR13 to be activated by bacterial ribosomal RNA. However, a specific nuclease that is required for this processing has not yet been discovered. This example, as well as the digestion of pathogen-derived RNA by RNase T2 and RNase 2 described above, highlights a prime area for future research — to determine how ligands derived from pathogens, rather than synthetic ligands, are processed and made available to the NA-sensing TLRs.


a Compartmentalization
Microorganisms
Apoptotic cell debris
ssDNA binding
Mutant TLR9 mislocalized to plasma membrane
Mutant TLR9
Response to self-ligands
Autoimmunity
Mutant TLR9
Golgi
b Ligand availability
LL37 complexing
Plasma membrane
PLD3 or PLD4
Endosome
Degradation
PLD3 and/or PLD4 deficiency
ssDNA binding
TLR9
SLC29A3
SLC29A3 deficiency
Nucleoside export
TLR7
↑ Self-nucleic acids
ssRNA binding
TLR7 or TLR9 response
↑ Self-nucleic acids
Autoimmunity
Plasma membrane
TLR9
AP-2
PLD3 or PLD4
SLC29A3
TLR13
Golgi
TLR3
Nucleic acids
DNase II
Endosome
ssRNA
TLR
UNC93B1
Nucleus
TLR7
TLR9
Multivesicular body
Homeostasis
Signal termination
Syntenin-1
ER
Cytosol
c Receptor expression
Endosome
ssRNA binding
TLR7
Increased TLR7 gene dosage
TLR7
TLR7
Expression of TLR7 regulated by suboptimal codon use
Excess signalling
Limited signalling
Autoimmunity
d Signal transduction
ssRNA binding
TLR7
No binding of syntenin-1
Positive regulators: TASL, TREML4
Mutant UNC93B1
Unrestricted signalling
Autoimmunity
Multivesicular body

◀ Fig. 2 | **The four categories of regulatory mechanisms for nucleic acid-sensing Toll-like receptors.** At homeostasis (centre panel), multiple regulatory mechanisms — compartmentalization, ligand availability, receptor expression and signal transduction — function collectively to limit the responses of nucleic acid-sensing Toll-like receptors (NA-sensing TLRs) to self-nucleic acids, while preserving responses to microbial nucleic acids. All NA-sensing TLRs require the transmembrane protein UNC93B1 to exit the endoplasmic reticulum (ER), where they are translated, and traffic via the classical secretory pathway to endosomes. To reach an endosome, TLR9 must first traffic to the plasma membrane, where it is internalized by adaptor protein complex 2 (AP-2)-mediated endocytosis. UNC93B1 also mediates regulatory functions after exit from the ER. For example, TLR3 and TLR9 are released from UNC93B1 in the endosome, whereas TLR7 remains associated. This subjects TLR7 to additional regulation through binding of syntenin-1 to UNC93B1, which leads to sorting of the UNC93B1–TLR7 complex to multivesicular bodies and termination of signalling. The fate of UNC93B1–TLR13 complexes in endosomes is currently not known. The distinct stoichiometry of dimeric UNC93B1–TLR7 complexes, compared with monomeric UNC93B1–TLR3 complexes, is also depicted. Breakdown of these regulatory mechanisms has been linked to the induction of autoimmunity and/or autoinflammation. For each category of regulatory mechanism, representative examples of how that regulation can break down are indicated. Detailed discussions of additional examples are provided in the main text. **a** | Compartmentalization. Mislocalization or defective compartmentalization of mutant TLR9 enables recognition of extracellular self-DNA. **b** | Ligand availability. Association of self-DNA and self-RNA with the antimicrobial peptide LL37 promotes their uptake into endosomes and reduces degradation by nucleases. Loss of the membrane-anchored 5′ exonucleases phospholipase D3 (PLD3) and PLD4 or of the nucleoside exporter SLC29A3 increases the availability of self-nucleic acids within endosomes and triggers activation of TLR9 or TLR7, respectively. **c** | Receptor expression. Overexpression of TLR7 increases the number of receptors in endosomes, which enables responses to otherwise non-stimulatory levels of self-RNA. **d** | Signal transduction. Loss of syntenin-1 binding to mutant UNC93B1 disrupts the sorting of UNC93B1–TLR7 complexes into multivesicular bodies, which is required to restrict TLR7 responses to self-RNA. Positive regulators of NA-sensing TLRs, such as TASL and TREML4, can enhance downstream signalling. ssDNA, single-stranded DNA; ssRNA, single-stranded RNA.

Ligand sequestration. TLR7 and TLR8 can recognize extremely short fragments of RNA and, in some cases, even single nucleosides[47,76–78] (BOX 3), so digestion of nucleic acids does not completely solve the problem of potential recognition of self-nucleic acids. In other words, nucleases may not be able to digest RNA ligands to the extent that they can no longer be recognized by TLR7 and TLR8. An additional mechanism that reduces the concentration of RNA in endosomes is the transport of ligands out of the endosomal lumen and into the cytosol. One clear example of this principle comes from recent preprint data of mice lacking SLC29A3, a member of the solute carrier family that functions to maintain nucleoside homeostasis. These mice accumulate endosomal nucleosides and develop disease with many of the hallmarks observed in other models of TLR7 dysregulation[79]. SLC29A3-deficient mice and humans have other abnormalities, most likely related to altered nucleoside homeostasis[80–83], but much of the autoimmune pathology in mice is rescued by TLR7 deficiency[79]. This example further illustrates how fundamental homeostatic mechanisms contribute to the carefully balanced recognition system of endosomal TLRs.

A second example of ligand sequestration involves the export of double-stranded RNA from endosomes. SIDT1 and SIDT2, which are the mammalian orthologues of the SID-1 double-stranded RNA transporter in *Caenorhabditis elegans*, are present in the endosome and facilitate transport of double-stranded RNA into the cytoplasm[84,85]. Mice deficient in SIDT2 have enhanced TLR3-mediated signalling, which indicates that physical sequestration of double-stranded RNA by SIDT2 can help to dampen TLR3 responses[84]. However, the enhanced TLR3 signalling in this model does not result in overt disease, which may indicate that accumulation of endosomal double-stranded RNA self-ligands is not problematic at steady state.

An open question is whether similar transporters exist to remove ligands for TLR9 or TLR13 from the endosome. Ligand digestion may be more important for regulating these NA-sensing TLRs, as they generally recognize longer nucleic acids than TLR7 or TLR8. Finally, it is unclear whether these transporters interfere with the ability of NA-sensing TLRs to recognize foreign nucleic acids by also reducing the availability of microbial ligands. There may be an additional, undiscovered layer of regulation that prevents microbial nucleic acids from being transported out of the endosome and thus avoiding recognition. It is also possible that microbial nucleic acids are occasionally transported from the endosome but that this is a worthwhile trade-off to prevent the induction of autoimmunity. Alternatively, the presence of pathogen-derived nucleic acids may increase overall ligand concentration enough to overcome the effect of export by transporters.

Receptor expression

The previous sections describe how NA-sensing TLRs localize to intracellular compartments separate from the majority of extracellular nucleic acids and how ligand digestion and sequestration reduce the concentration of self-nucleic acids that do reach the endosome. Collectively, these two categories of regulation operate to reduce the likelihood that a given NA-sensing TLR will encounter self-nucleic acids. Another component of this system is regulation of TLR expression, which determines the number of receptors in a given cell and, in combination with trafficking regulation, the number of receptors in the endosome available for ligand recognition. Maintaining low levels of TLR expression makes sense conceptually, as the concentrations of both ligand and receptor within the endosome determine the activation threshold for each TLR (FIG. 1). Increased levels of either will favour TLR activation and the induction of aberrant responses to self-nucleic acids. However, if expression of TLRs is too low, then responses to microbial ligands become compromised.

Relatively little is known about the molecular mechanisms that regulate expression of the NA-sensing TLRs. Most TLR transcripts contain a high frequency of suboptimal codons, which limits the number of NA-sensing TLRs present in the endosome[86,87]. For TLR7, this suboptimal codon usage regulates expression by altering the rates of translation and transcription and by modulating RNA stability[21,87]. TLR9 is an outlier among the TLRs, with super-optimal codon usage[86,87]. The fact that TLR9 does not require this additional limitation on receptor levels imposed by suboptimal codon usage aligns with the observation, discussed below, that TLR9 overexpression does not induce autoimmunity.

Despite the lack of information regarding the underlying mechanisms that control TLR expression, there is no

Suboptimal codons
Codons that are used at a lower than expected frequency in a given genome. Such codons often result in less efficient translation, which is thought to be owing, at least in part, to the limited availability of corresponding transfer RNAs.

doubt that maintaining low levels of certain NA-sensing TLRs is necessary to avoid autoimmunity. In mice and humans, increasing gene dosage of *TLR7* and/or *TLR8*, both located on the X chromosome, can lead to immune pathology[3–5,88–95]. It has been suggested that failed X-inactivation can lead to increased expression of TLR7 and TLR8 in individuals with more than one X chromosome[93], which could contribute to observed sex differences in susceptibility to certain autoimmune diseases. The sensitivity to overexpression of TLR7 and TLR8 is in contrast to studies of TLR9 overexpression in mice, for which there is no major pathology observed even with two additional copies of a *Tlr9* transgene expressed in every cell[96].

There is currently no satisfactory explanation for the disparate effects of overexpression of TLR7 and/or TLR8 versus TLR9, but several possibilities merit discussion. The ligands that are recognized by each receptor might have a crucial role — TLR7 and TLR8 can be stimulated by small fragments of RNA, whereas TLR9 requires longer strands of CpG DNA to initiate signalling (BOX 3). There could simply be a higher concentration of these simple RNA ligands than of DNA in endosomes. Alternatively, endosomal DNA levels might be tightly controlled by the above-discussed DNases, whereas the sequestration strategies used to reduce endosomal RNA levels are less efficient. It is also possible that there are more diverse intracellular sources of TLR7 and TLR8 ligands, including RNA from endogenous retroviruses and retroelements. Finally, there is the possibility that undiscovered regulatory mechanisms provide additional protection specifically against TLR9-induced autoimmunity.

Cell-type-specific expression and/or specialization of the NA-sensing TLRs also require further consideration. In this Review, we have largely assumed that regulation of the NA-sensing TLRs is similar across cell types, yet there are clear examples of different cell types with distinct patterns of TLR expression and unique modes of regulation (BOX 2). These differences are illustrated by the distinct mechanisms of disease associated with dysregulation of TLR7 and TLR8 versus TLR9. In the former case, disease is driven by type I IFN and pDCs, whereas in the latter case, it is driven by IFNγ and natural killer cells[2,5,11,19,97,98]. How differences in TLR expression (and potentially signalling) lead to such distinct disease manifestations is relatively understudied, and future work will first require the development of tools that enable dissection of TLR signalling and regulation in cell types that are often rare or difficult to isolate.

Finally, a discussion of the expression of NA-sensing TLRs is not complete without noting the presence of marked differences between species. There is substantial variation between mice and humans, particularly with respect to the single-stranded RNA sensors. TLR13 senses bacterial and viral single-stranded RNA in mice but is not encoded in the human genome[74,75,99] (TABLE 1). Instead, human TLR8 seems to have many of the same sensing functions as mouse TLR13 (REF. [100]). TLR8 itself is also the source of a major difference between mice and humans, in that TLR8 was originally thought to be non-functional in mice owing to its inability to respond to known ligands of human TLR8 (REF. [101]). Although there have been subsequent reports that mouse TLR8 does respond to certain ligands, its relative role in the hierarchy of single-stranded RNA-sensing TLRs is still an open question. There is also evidence of variation in cell-type-specific expression of the NA-sensing TLRs between mice and humans[102]. Expression of TLR3 and TLR9 is generally more limited in humans than in mice. For example, TLR9 expression is restricted to pDCs and B cells in humans but it is expressed much more broadly in mice[103,104]. The evolutionary history of the NA-sensing TLRs is a topic primed for further dissection, and species-specific expression patterns will probably provide further clues to the function and regulation of the NA-sensing TLRs.

Signal transduction

The above regulatory mechanisms, which ensure the proper balance of ligand availability and receptor expression in endosomes, are complemented by mechanisms that regulate signal transduction after activation of the NA-sensing TLRs. Properly modulated signalling functions as an extra layer of protection against inappropriate activation, terminates signalling after activation and ensures the appropriate functional outcome of signalling for different cell types (BOX 2). The general molecular players in TLR signalling are not covered in detail here,

Box 2 | Cell-type-specific regulation of nucleic acid-sensing Toll-like receptors

Expression levels of the nucleic acid-sensing Toll-like receptors (NA-sensing TLRs) can differ between cell types, and signalling and regulatory differences specific to a given cell type can further tailor the output of TLR activation.

Plasmacytoid dendritic cells (pDCs) are the prototypical example of specialized regulation of NA-sensing TLRs. In response to stimulation through TLR7 or TLR9, pDCs produce type I and type III interferons (IFNs), in contrast to other cell types that mainly produce nuclear factor-κB-dependent cytokines in response to TLR7 or TLR9 ligands[119]. This specialized signalling in pDCs probably ensures that antiviral IFNs are produced during viral infections because pDCs need not be infected to detect viral nucleic acids; by contrast, cytosolic sensing pathways for nucleic acids are often directly antagonized by viral proteins expressed in infected cells. Relevant and crucial to their ability to produce type I and type III IFNs is the expression of members of the IFN response factor (IRF) family by pDCs[120,121], as well as several other specialized signalling mechanisms[122–124].

There is also evidence of specialized signalling by NA-sensing TLRs in B cells. TLR signalling can lead to B cell proliferation and antibody production, and certain unique aspects of signal transduction in B cells have been identified as being crucial for these responses. Responses to TLR9 ligands require the adaptor protein DOCK8, which links TLR9 activation to activation of the transcription factor STAT3 (REF. [125]). B cell responses are impaired in patients with mutations in *DOCK8* (REFS [125,126]). Another example has been revealed by studies of diffuse large B cell lymphoma. A subset of these tumours depends on mutations in the TLR adaptor MYD88, which drives a TLR9-dependent proliferative and survival signal[127]. Whether these signalling features apply to other NA-sensing TLRs in B cells remains to be seen, as does the extent to which they operate in untransformed cells.

Certain cell types limit responses to nucleic acids by lacking expression of NA-sensing TLRs. To prevent aberrant responses to self-nucleic acids within engulfed cells, tissue-resident macrophages do not express TLR9 and also repress signalling from endosomal TLRs, while maintaining signalling from extracellular TLRs[96,128]. These mechanisms probably prevent autoinflammation or autoimmunity driven by a cell type with particularly high levels of exposure to endogenous ligands. Similarly, TLR7 and TLR9 are not expressed in intestinal epithelial cells, perhaps preventing aberrant immune responses in the ligand-rich environment of the gut microbiota[129].

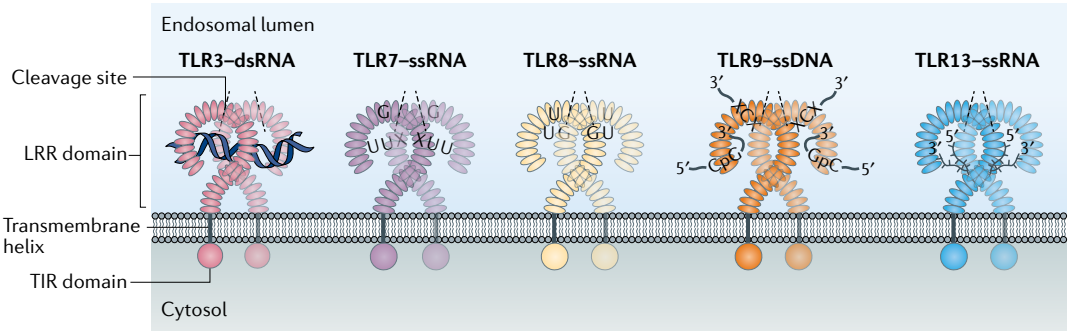
Box 3 | **Structural features of ligand recognition by nucleic acid-sensing Toll-like receptors**

The nucleic acid-sensing Toll-like receptors (NA-sensing TLRs) have common structural features, including an extracellular leucine-rich-repeat (LRR) domain that binds ligand, a transmembrane helix and a cytosolic Toll/IL-1 receptor (TIR) domain (see the figure). Ligand binding induces dimerization of the TLR and activation of downstream signalling pathways.

Ligands for TLR3 have the longest length requirement of the NA-sensing TLRs, with a minimum of 40 bp of double-stranded RNA (dsRNA) being necessary for activation. The dsRNA segment spans the two ligand-binding sites that are located on opposite ends of each TLR3 ectodomain[130,131]. By contrast, activation of TLR7 can occur with simple single-stranded RNA (ssRNA) ligands that require only one guanosine nucleoside at the first ligand-binding site together with a minimum of a ssRNA trimer containing a uridine dimer (UUX) at the second ligand-binding site[78,132]. Ligand binding by TLR8 is quite similar to TLR7, but with a preference for binding uridine in the first binding site and UG dimers in the second binding site[77,133].

CpG motifs within a DNA segment are necessary for activation of TLR9 (REF.[41]), but the motifs that induce maximum stimulation differ between mice and humans[134,135]. A second binding site in TLR9 for 5′-XCX DNAs cooperatively promotes receptor dimerization, together with CpG DNA. This second site has similarities with the nucleoside-binding pockets in TLR7 and TLR8, which highlights a common feature of ligand recognition by NA-sensing TLRs. Binding of the two sites in TLR9 can be achieved by a single DNA molecule, with spacing of at least 10 bp between the 5′-XCX and CpG motifs, or by two distinct DNA segments[136].

Unlike the other NA-sensing TLRs, ligand recognition by TLR13 is sequence specific. The 13-nucleotide ssRNA ligand for TLR13 (5′-XCGGAAAGACCXX-3′) has been identified in a conserved region of bacterial 23S ribosomal RNA and similar ssRNA sequences in viruses[74,75,99,137].



and we refer the reader to other excellent reviews on that topic[1,105]. Instead, we focus on the regulatory mechanisms that are imposed selectively on the NA-sensing TLRs, which are not as well understood.

There are relatively few examples of signalling regulation that are specific for the NA-sensing TLRs, although recent studies have identified several key positive regulators. The newly identified protein TASL, encoded by the SLE-associated gene *CXorf21* (also known as *TASL*), interacts with the endolysosomal transporter SLC15A4 to facilitate activation of IRF5, another SLE-associated protein[106,107], and the transcription of inflammatory genes in response to stimulation of TLR7, TLR8 and TLR9, but not TLR2 (REFS[108–110]). This work illustrates how genetic variation in positive regulators of NA-sensing TLR signalling can also influence responses to self-nucleic acids, presumably by lowering the threshold for activation that leads to a biologically meaningful transcriptional response. TREML4, another positive regulator of TLR7, TLR9 and TLR13 signalling, promotes MYD88 recruitment to the TLR and STAT1 phosphorylation and activation of transcription[111]. TREML4 deficiency in mice results in impaired production of IFNβ and the chemokine CXCL10 in response to TLR7 activation but does not affect TLR9-mediated activation of the IFN pathway[111]. Whereas TASL and TREML4 positively impact the signalling of multiple NA-sensing TLRs, there is one reported example of a signalling regulator that might be specific for TLR9. CD82, a transmembrane protein, positively regulates TLR9 signalling by promoting myddosome assembly[112,113]. In each of these cases, much more work is needed to understand the precise mechanisms by which these proteins control the signalling of NA-sensing TLRs. Whether these signalling modulators are specific to individual NA-sensing TLRs or whether they are generally involved in signalling pathways originating from the endosome also remains an open question.

UNC93B1, which was discussed above as a chaperone that facilitates transport of the NA-sensing TLRs to the endosome, also regulates signalling by the NA-sensing TLRs. Recent work has shown that UNC93B1 has distinct mechanisms of regulatory control over TLR3, TLR7 and TLR9 (REFS[24,25]). Although UNC93B1 controls the trafficking of all three TLRs, upon reaching the endosome, TLR3 and TLR9 are released from UNC93B1, whereas TLR7 remains associated[25]. This continued association with UNC93B1 allows for more nuanced control of TLR7 signalling. Upon TLR7 stimulation, UNC93B1 recruits syntenin-1 to the UNC93B1–TLR7 complex, leading to the sorting of the complex into multivesicular bodies and ultimately terminating signalling[24]. Mutations in UNC93B1 that prevent binding of syntenin-1 induce TLR7 hyperresponsiveness and severe TLR7-dependent autoimmune disease in mice. Notably, a similar mutation in the carboxy-terminal tail of UNC93B1 was recently identified by a genome-wide association study as the causative genetic variant in dogs with a form of cutaneous lupus erythematosus[114]. Thus, UNC93B1, through

Myddosome
A multiprotein signalling complex consisting of the adaptor protein MYD88 and members of the IL-1 receptor-associated kinase (IRAK) family that assembles upon activation of all Toll-like receptors (TLRs), except for TLR3.

Syntenin-1
A PDZ domain-containing adaptor protein that has been implicated in the biogenesis of exosomes and that supports intraluminal budding and delivery of cargo into intraluminal vesicles.

Multivesicular bodies
Specialized organelles within the endolysosomal network that are characterized by the presence of vesicles within their lumen. These intraluminal vesicles form by budding from the limiting membrane into the lumen. Sorting of cargo into multivesicular bodies is a common mechanism by which receptor signalling is terminated.

its association with syntenin-1, can tune TLR7 signalling and promote signal termination to prevent autoimmunity. Because TLR9 and TLR3 are released from UNC93B1 in the endosome, these receptors are not regulated by this mechanism. Whether distinct UNC93B1-mediated mechanisms restrict activation of these TLRs remains to be determined.

Many additional questions remain regarding UNC93B1-mediated regulation of the NA-sensing TLRs. How does a single protein differentially traffic and regulate multiple NA-sensing TLRs, as well as TLR5, TLR11 and TLR12, which also require UNC93B1 for proper localization? Recent structures of UNC93B1 bound to TLR3 or TLR7 have shown that these TLRs interact with similar regions of UNC93B1; however, the stoichiometry differs between the UNC93B1–TLR3 complex and the UNC93B1–TLR7 complex, indicating the existence of distinct interaction surfaces that may impact TLR function[115]. Comparisons of additional structures of UNC93B1 bound to each of the TLRs will certainly be an important aspect of future studies focused on defining new regulatory mechanisms. It remains unclear whether UNC93B1 exerts specific regulation of individual NA-sensing TLRs beyond the recently described syntenin-1 pathway. It is also possible that UNC93B1-mediated regulation of NA-sensing TLRs differs across cell types.

Conclusions and future challenges

The NA-sensing TLRs facilitate the recognition of a diverse array of potential pathogens but must be tightly regulated to avoid improper responses to self-nucleic acids. We have discussed four general categories of mechanisms that influence responses by NA-sensing TLRs and establish the balance that enables discrimination between self and foreign nucleic acids. The field has made substantial progress defining these mechanisms over the past decade (FIG. 2), but numerous open questions remain, which we have noted throughout this piece.

There is increasing evidence that each NA-sensing TLR is subject to individualized regulation at multiple levels. Trafficking patterns vary significantly between the NA-sensing TLRs, despite the fact that they all depend on UNC93B1. Modulation of ligand availability is similarly nuanced, with proper regulation of the RNA-sensing TLRs dependent on ligand transporters, whereas the level of TLR9 ligands is regulated by nucleases. These distinct regulatory mechanisms probably determine the contribution of specific TLRs to certain disease states, such as the opposing roles played by TLR7 and TLR9 in experimental models of autoimmunity[11–13], yet the logic behind such differences is still poorly understood. Future discoveries will no doubt provide additional pieces to the puzzle and will probably reveal that the regulatory nuances we currently perceive as unnecessarily complex are in fact rooted in functional differences between each of the NA-sensing TLRs. It is also possible that conceptual commonalities will emerge. For example, endosomal transporters for TLR9 and TLR13 ligands, or RNases involved in the removal of potential TLR7 ligands, may be identified.

The extent to which NA-sensing TLR responses are specialized in different immune cell types is also of great interest. We have described one example in which increased signalling of NA-sensing TLRs is wired to facilitate type I IFN production in pDCs, and another in which TLR9 expression and signalling are reduced in tissue-resident macrophages so that they can safely clear apoptotic cells (BOX 2). On the basis of these examples, it seems likely that the function of NA-sensing TLRs is also specialized in other immune and non-immune cell types. Some evidence already exists for such specialization in B cells (BOX 2). Further progress in this area will require better tools to track receptor expression and function in specific cell types, particularly for cells that are rare and/or difficult to access.

The challenge for the next decade is to unravel the mechanisms that influence each of the four regulatory categories that we have laid out here (compartmentalization, ligand availability, receptor expression and signal transduction) for each NA-sensing TLR. Ideally, these studies will incorporate the recognition of microbial ligands, as differences in the processing and recognition of nucleic acids in the context of microorganisms may be missed with the use of purely synthetic ligands[116]. The insights gained in the years ahead will undoubtedly reveal new therapeutic approaches for enhancing and inhibiting the activation of these key innate immune receptors (BOX 1).

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