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RNA mediated toll-like receptor stimulation in health and disease

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Abbreviations: PBMCs, peripheral blood mononuclear cells; pDCs, plasmacytoid dendritic cells; DC, dendritic cells; CLRs, C-type lectin receptors; siRNA, small interfering RNAs; dsRNA, double stranded RNA; RNAi, RNA interference; bRNA, bacterial RNA; tRNA, transfer RNA; rRNA, ribosomal RNA; TLR, toll-like receptors; NLR, nucleotide-oligomerization domain protein (Nod)-like receptors; RIG-I, retinoic acid inducible gene I; Mda5, melanoma differentiation-associated gene-5; Nlrp, NLR family, pyrin domain containing; AIM2, absent in melanoma 2; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; MyD88, myeloid differentiation factor 88; IRAK, interleukin-1 receptor-associated kinase; TRAF, tumor necrosis factor (TNF) receptor associated factor; SLE, systemic lupus erythematosus; HIV, human immunodeficiency virus; TLR3-ECD, TLR3 ectodomain; IFNα, interferonalpha; IFN, interferon; IRF1, interferon regulatory factor-1; NFκB, nuclear factorκB; IL, interleukin; HMGB, high-mobility group box; ON, oligonucleotide; Bp, basepair; shRNA, short hairpin RNA; RISC, RNA-induced silencing complex

Besides their well-known functions in storage and translation of information, nucleic acids have emerged as a target of pattern recognition receptors that drive activation of innate immunity. Due to the paucity of building block monomers used in nucleic acids, discrimination of host and microbial nucleic acids as a means of self/foreign discrimination is a complicated task. Pattern recognition receptors rely on discrimination by sequence, structural features and spatial compartmentalization to differentiate microbial derived nucleic acids from host ones. Microbial nucleic acid detection is important for the sensing of infectious danger and initiating an immune response to microbial attack. Failures in the underlying recognitions systems can have severe consequences. Thus, inefficient recognition of microbial nucleic acids may increase susceptibility to infectious diseases. On the other hand, excessive immune responses as a result of failed self/foreign discrimination are associated with autoimmune diseases. This review gives a general overview over the underlying concepts of nucleic acid sensing by Toll-like receptors. Within this general framework, we focus on bacterial RNA and synthetic RNA oligomers.

Introduction

Although nucleic acids are mainly recognized as important for storage and translation of genetic information, cutting-edge research has continuously revealed new and exciting functions, including e.g., the myriad roles of regulatory noncoding RNAs, as well as the recognition by the immune system. Interestingly, some of those non-classical functions are interlinked: before the discovery and development of small interfering RNAs (siRNA),[1] the use of long double-stranded RNA (dsRNA) in attempts to elicit RNA interference (RNAi) in mammalian cells was unsuccessful, as a consequence of an interferon response by the innate immune system. This feature of the innate immune system has recently received immense attention, as its implications for health and disease became clear. For example, bifunctional RNA based therapeutics displaying immunostimulating and gene silencing activities alike[2] emerged as a new concept. At the heart of these developments is the recognition of nucleic acids in general and of RNA in particular, by receptors of the innate immune system, which are charged with the task to differentiate between RNA from the mammalian host ("self") and from invading pathogens ("non-self").

This review gives an overview of these highly interesting interactions. After an introduction to the topic of nucleic acids as pathogen-associated molecular patterns (PAMPs), we will focus on recognition of nucleic acids by Toll-like receptors (TLR). Particularly, this review will highlight recognition of bacterial and synthetic RNA because this topic has received less attention so far. However, we will also discuss recognition of bacterial DNA to support our ideas of three main principles by which the immune system differentiates self- and microbial-derived nucleic acids. We will finish with an analysis of the importance of nucleic acid recognition during infections as well as mislead activation in autoimmune diseases.

Innate Immune Sensing of Microbes Relies on Recognition of Conserved Patterns

With last year's Nobel Prize of Medicine being awarded to Jules A. Hoffmann, Bruce A. Beutler and Ralph M. Steinman for their

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discoveries in the field of innate immunity,[3-5] one of the most exciting developments in immunological research over the last two decades has now been honored. In fact, the rediscovery of innate immunity and its role for early defense of infections but also for inducing and shaping adaptive immunity has definitively changed our way of understanding of how the immune system works on the systemic level. Central to this field has been the seminal concept proposed by the late Charles Janeway in 1989.[6] He suggested that the co-stimulatory signal delivered by innate antigen presenting cells was inducible and regulated itself through the recognition of conserved microbial products. Thus, adaptive immunity was placed under the control of innate immunity.

Moreover, for the first time, a conceptual framework of how innate immunity is activated was proposed: Conserved microbial products, termed pathogen-associated molecular patterns (PAMPs), stimulate pattern recognition receptors (PRR), thus driving innate immunity's activation. Innate immunity can therefore function with a limited set of target structures, without the need to produce a large repertoire of different receptor specificities by somatic recombination and clonal selection. Since this seminal proposal of Janeway, the immunological community has made considerable progress in defining "pattern recognition" at the molecular basis.[7]

By now, pattern recognition receptors expressed by professional innate immune cells comprise several major groups:[8] Toll-like receptors (TLRs),[4,9-11] Nucleotide-oligomerization domain protein (Nod)-like receptors (NLRs),[12-14] C-type lectins (CLRs),[15] Retinoic acid inducible gene I (RIG-I) and Melanoma differentiation-associated gene-5 (Mda5),[16,17] the NLR family, pyrin domain containing proteins (Nlrp),[18] and Absent in melanoma 2 (AIM2) inflammasomes.[19-21]

For most of those receptors, microbial ligands have been identified that fit into the category of molecular patterns: they are expressed by a variety of different microbes and differ in their fine structure, but nevertheless are recognized by a common receptor. Additionally, molecules have been identified that are of self-origin but still drive pattern recognition receptors, especially under non-physiological conditions.[22] Conceptually, this fits to a guard theory[7,23] whereby innate immune receptors also survey integrity of cellular processes that are often targeted by pathogenic microbes.

Sensing of a microbial infection, once effected by a PRR, is relayed into a downstream cascade of signaling events reviewed in reference 24. The first line of relay factors, e.g., the central adaptor molecule Myeloid differentiation factor 88 (MyD88) as well as Interleukin-1 receptor associated kinases (IRAKs) and Tumor necrosis factor (TNF) receptor associated factors (TRAFs) are integral parts of innate immunity, while downstream elements funnel into more general pathways, such as e.g., nuclear factor κB (NFκB). Studies on signal transduction have led to the discovery of important interconnections among several signaling cascades, and provided insights into a complex signaling network (reviewed in ref. 25). Clearly, the recognition of the various PAMPs by PRRs of varying specificity for a given microbe is a highly orchestrated event. Its multiple facets may include degrees of redundancy[26] to ensure broad and unspecific recognition on one hand, as well as features aimed at high specificity such as cooperativity among PRRs.[27]

Furthermore, several recent studies on PRRs and relay proteins have promoted the notion that nucleic acid sensing by players of innate immunity significantly impacts adaptive immunity.[28-30] This connection extends to systemic lupus erythematosus (SLE), a prominent autoimmune disease involving the erroneous recognition of self-nucleic acids by the adaptive immune system.

General Aspects of Innate Immune Sensing of Microbial Nucleic Acids

Among the various PAMPs, nucleic acids stand out for several reasons, the most obvious being the chemical and structural resemblances between nucleic acids of the host ("self") and those of a potential pathogen ("non-self"). Consequently, faithful discrimination cannot exclusively rely on differences in the structure, and erroneous recognition occurs more readily than e.g., in the recognition of such distinctively bacterial molecules as flagellin or lipopolysaccharide. Correspondingly, many nucleic acid-sensing PRRs including e.g., TLRs3, 7–9, RIG-I, Mda5, AIM2 and DExD/H helicases, are known to also respond to nucleic acids of self-origin under pathological conditions.[31]

Nucleic acids of either provenance form very similar structures, including in particular the unsophisticated double-helix. Typical cellular RNA populations are mostly composed of ribosomal and transfer RNA, who's ubiquitously conserved three-dimensional structures account for 90% of total RNA. Hence, leverage for discrimination may be found in sequence elements or on the atomic level, i.e., in post-transcriptional modifications or differentially processed 5'- or 3'-extremities.

Indeed, from the published results modes of pattern discrimination emerge that may be classified according to the hierarchy of nucleic acid structure: (1) primary structure, including nucleotide composition, chemical modification and sequence, (2) higher order structures including elements of secondary and tertiary structure and (3) discrimination by localization. Those modes of recognition appear to be realized in each of the nucleic acid recognizing PRRs.

Here, we focus on recognition by TLRs and will only briefly touch other PRRs. We will start with a short introduction on individual TLRs that will highlight each of the three recognition principles, including also TLR9 as a DNA-sensing PRR. This will be followed by a more detailed analysis of bacterial and synthetic RNA by TLRs with respect to the named modes of self/foreign differentiation.

Structural Recognition of RNA through TLR3

TLR3 has first been recognized to be stimulated by double-stranded RNA, a molecular pattern that will occur during replication of RNA viruses.[32] TLR3 [33-35] as well as the other nucleic acids recognizing TLRs (TLR7,8,9) resides in the endosomes, thus being accessible to dsRNA taken up from extracellular (mode iii). Of the TLRs sensing nucleic acids, crystal structures are only available for the TLR3 ectodomain (TLR3-ECD). A

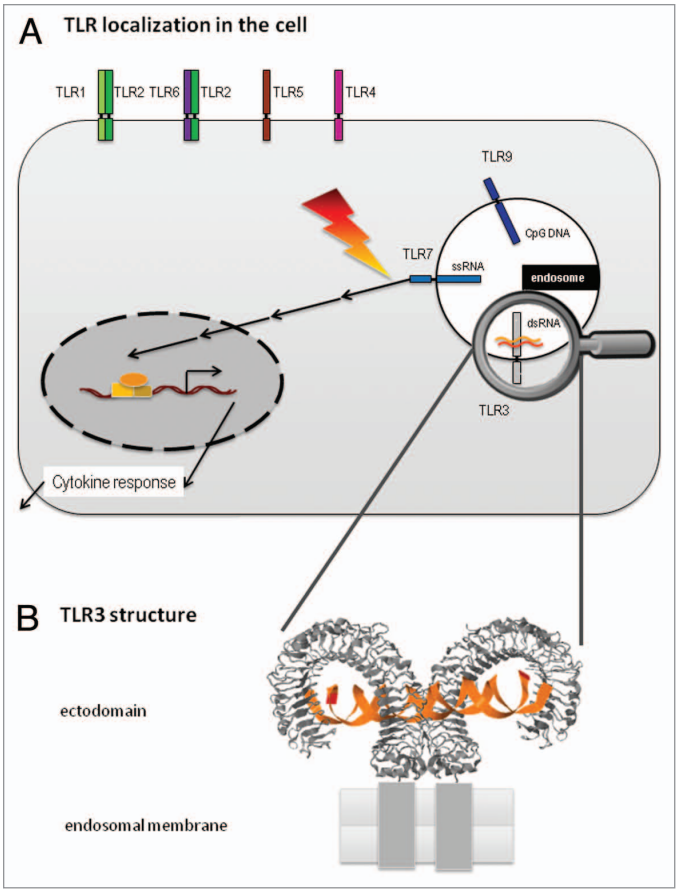

Figure 1. (A) Localization of TLRs. One set of TLRs is situated at the cell membrane. The set of TLRs which is responsible for sensing nucleic acids is located in the membrane of late endosomes/lysosomes. Upon activation by non-self nucleic acid, an inflammation signal is triggered through a signal cascade including MyD88, IRAK1/2/4, TRAF3/6, which ultimately leads to a cytokine response. (B) Horseshoe structure of TLR-ectodomain complexed to a 46mer dsRNA.[36]

structure of TLR3-ECD complexed to a 46 basepair (bp) dsRNA helix[36] reveals the typical horseshoe shape of the ectodomain. TLR3-ECD is dimerized (**Fig. 1**) for activation of the cytosolic signal transduction domain and ensuing signal transmission as a consequence of RNA binding. In its general shape, the TLR3 structure resembles X-ray based structures of TLR4,[37] and TLR1-TLR2.[38] TLR3-ECD binds dsRNA of at least 40 bps[36,39] at two sites located at opposite ends of the TLR3 horseshoe. An intermolecular contact between the two TLR3-ECD C-terminal domains coordinates and stabilizes the dimer. This critical length is above that of a typical helix occurring in normal cellular RNA such as e.g., tRNA, and in particular, miRNA and siRNA. Indeed, before the discovery of siRNA as duplexes of some 20 bps, attempts to induce RNAi in mammals with larger dsRNA was unsuccessful, presumably also as a consequence of TLR3 action.[1] Two RNA binding sites in each horseshoe function at pH below 6.5, which is a hallmark of the endosomal compartment,[36,40,41] likely due to protonation of histidine residues, whose positive charge then undergoes ionic interaction with the negatively charged RNA backbone.

Recognition of a ds helix by TLR3 is a perfect example of pattern recognition by secondary structure alone (mode ii, structural feature), which evidently occurs independently of any specific sequence motif. It involves recognition of relatively long stretches of dsRNA, a pattern that is common during RNA virus infection and replication but largely absent (at least within the endosome where TLR3 is expressed) in normal cell physiology. Indeed, recognition of dsRNA by TLR3 has been shown to be sequence independent.[32] Many studies involve the use of poly I:C as a surrogate for natural dsRNA in the stimulation of for TLR3. Although certainly helpful, such results need to be interpreted with some caution as different commercially available poly I:C compounds exert somewhat differential effects, [e.g., interleukin (IL)-12p70 induction in DCs], probably dependent on differences in molecular weight and the fact that it is not a physiologically or even naturally occurring substance. Also, stimulation of additional receptors including cytosolic Mda5 and RIG-I has been observed.[42] Of note, mammalian mRNA, i.e., self-RNA has also been postulated to be a TLR-3 ligand,[43] which might signal the presence of necrotic debris from neighboring cells. Possibly, structured elements, especially in the UTRs of mRNA might form secondary structures that could induce TLR-3 signaling.

Sensing of RNA through TLR7

TLR7 and TLR8, the latter of unclear functional competence in mice, have first been identified to be activated by single-stranded RNA (ssRNA) and were suggested to participate in virus recognition, e.g., influenza virus.[44,45] TLR7 (as well as TLR3, 8, 9, 13) is expressed intracellularly within endosomes,[35] a compartment where RNA can be sensed during e.g., uptake of viral particles[45] but which has only sporadic contact with host-derived RNA, e.g., after endocytic uptake of debris from necrotic tissue.[46,47]

Specifically, TLR7 exerts an important function to trigger IFNα release from plasmacytoid dendritic cells (pDCs).[44,45] Self/foreign discrimination involves a certain sequence dependency, and nucleotide modifications modulate recognition:[48-50] TLR7 is activated by single stranded RNA ONs with an apparent preference for G and U rich sequences.[44,45,51] Although length dependence of single stranded RNA ONs has been controversially discussed, it appears that efficient activation requires a length of at least 21 nucleotides.[44,52,53] This discussion is complicated by the fact that contradictory studies were often conducted with various read-out systems on ONs of different sequence, composition and modification state.

It has been suggested that the nucleotide preference might be related to a similar nucleotide composition of viral sequences in certain RNA viruses, including e.g., Influenza A and human immunodeficiency virus.[44,54,55] Synthetic RNA ONs encoding such sequences stimulate sequence-dependent cytokine responses via TLR7 and TLR8. It has therefore been speculated that these GU rich sequences have evolved as a PAMP of invariant nucleotide composition for the recognition of viruses.[44,54]

However, TLR7 is also a major player in the recognition of bacterial RNA: in pDCs bacterial RNA stimulated TLR7 thus leading to secretion of Interferonα (IFNα) [56] but in myeloid DCs

TLR7 was dispensable. In contrast to the findings of TLR7 independency of bRNA immunostimulation in myeloid DCs, another group showed that IFNβ production in response to phagosomal but not cytosolic bacteria used TLR7. Immunostimulation was achieved through lysosomal recognition of bacterial RNA and activated TLR7, MyD88 and interferon regulatory factor-1 (IRF1).[57] It might be that induction of IFNα occurs through a different pathway as compared with typical NFκB dependent innate immune genes. The latter report suggested that bacterial mRNA was specifically active whereas others showed that rRNA (rRNA) is equally potent.[56]

By conjecture of the known TLR horseshoe structures, it may be assumed hat TLR7, 8 form a similar double horseshoe structure upon activation.[58] However, the binding modes of their ligands are still ill understood. While typical approaches to the definition of key features have focused on either two of the parameters length, sequence, modification state or secondary structure, the solution, as in the case of TLR3, is of three-dimensional nature and therefore most likely must include three-dimensional features of the RNA ligand. Our current understanding of TLR7 ligands thus cannot be consolidated by a simple model such as dsRNA, as shown in the TLR3 structure.[36] In simplified presentations, TLR7 is often portrayed as the ssRNA-sensing TLR with a structural specificity that is seemingly complementary to the dsRNA-sensing TLR3. However higher structural features are also of importance, since TLR7 was recently shown to recognized bacterial tRNAs.[49,50]

Even more puzzling than the recognition of such divergent structures as ssRNA and tRNA, but not of dsRNA, is the existence of a number of of small molecular compounds that activate TLR7, TLR8 or both.[59-61] Among these small molecules, Imiquimod and Resiquimod are shown in **Figure 2** because of their clinical relevance. Both may be viewed as nucleoside analogs, although it is unclear if the recognition mode by TLR7 bears any resemblance to RNA recognition. More striking however, is the TLR recognition of several guanosine derivatives including Loxoribine, while, guanosine itself shows no interaction.[61] While it is out of the scope of this review to elucidate structure-function relationships of such compounds in depth, it is duly noted that the efficiency of such small molecules implies a mode of action that appears fundamentally different from that of RNA ON recognition by TLRs. Yet, the recognition of guanosine derivatives provokes a comparison with the guanosine-rich RNA ONs mentioned above.[52] In the absence of any conclusive in-depth study, we speculate that small molecules as well as ssRNA might aggregate to form higher ordered structures.[62] Beyond the discussed implications from the structure of Loxoribine, this hypothesis is based on the fact that many highly active ssRNA ONs do not span a distance comparable to the 46mer in the TLR3 structure. It has indeed been suggested, that TLR7 activating RNA ONs that are presumed single stranded, actually form secondary structures,[48,63] possibly including hybridization of several RNA ONs to a larger complex, which then might be able to bridge the TLR subunits for dimerization. This concept has also been discussed based on stimulation of TLR7/8 using phosphorothioate RNA ONs.[63]

Although there is so far no experimental structural basis for recognition by TLR7, the recognition of tRNA structures by TLR7 [49,50] strongly suggests an involvement of larger structures.

DNA Sensing by TLR9

Although TLR9 itself is known for DNA recognition, it is discussed here as a relative of the RNA-sensing TLRs. Indeed many clues in the studies of TLRs 3 and 7 have been taken from pioneering work on TLR9. TLR9 has been shown to recognize hypomethylated bacterial DNA that is rich in CG dinucleotides.[64] Whereas hypomethylated CG dinucleotides are frequent in bacterial DNA, CG motifs are suppressed in eukaryotic DNA and furthermore are highly methylated.[65] As bacterial DNA mediated TLR9 stimulation can be mimicked by synthetic CpG ONs[66] and is abolished by GC inversion, this receptor provides a clear example for a (albeit minimal) sequence-dependent pattern recognition (mode i). However, a recent report claims that CG sequence dependency can be observed mainly when using synthetic, phosphorothioate modified DNA ONs but might be less important for natural backbones.[67] Despite the bias of CG sequences in bacterial vs. mammalian genomes, this dinucleotide element alone contains too little information for efficient discrimination. Indeed, further control is achieved through recognition of cytosine methylation which acts as a negative determinant, i.e., its presence in mammalian DNA prevents triggering of TLR9.

In addition to these structural features (mode i), the endosomal expression of TLR9[68] adds an additional level of control (compartmentalization, mode iii). Interestingly, forced expression of TLR9 at the cellular surface promotes recognition of self-DNA, thus abolishing self/foreign discrimination.[69] Also, increasing the amount of self-DNA either by interference with lysosomal DNA degradation in DNase I knockouts[70,71] or through increased delivery by anti-DNA antibodies,[72,73] results in increased stimulation through self-derived DNA. Using a combined approach of mutational analysis and homology modeling, it was suggested that TLR9 recognizes bacterial DNA in a manner similar to TLR3 with two binding sites in the extracellular domain that possibly interact through charges with the nucleic acid.[74] TLR9 has to be cleaved in the extracellular domain[75,76] which appears to be necessary to bind the double stranded helix in a curvature-dependent manner.[77]

Excursion: cytoplasmic nucleic acid recognition also relies on sequence, spatial and structural features. Besides endosomal TLRs a variety of cytoplasmic PRRs rely on similar principles for the recognition of foreign nucleic acids, adapted to the parameters of a different cellular compartment. Although we focus on TLRs, this paragraph will give an abbreviated overview to avoid the impression that sensing is taking place exclusively in the endosome. Interestingly, cytoplasmic PRRs rely on similar structural recognition principles, although they differ in fine details. For example RIG-I and Mda5 are cytosolic receptors that recognize viral RNA. The mode of pattern recognition is best characterized for RIG-I as crystal structures with and without RNA are now reported in reference 78 and 79. RIG-I has first been shown to interact with uncapped, 5'-tri-phosphorylated RNA,

a pattern that would be produced during viral replication processes but not in eukaryotic RNA biology as capping and some nucleotide modifications occur during eukaryotic posttranscriptional RNA processing in the nucleus, before export of mRNAs into the cytosol.[80] Yet, double-stranded RNA longer than 100 bp can stimulate RIG-I without the need for 5'-tri-phosphate ends.[81] The crystal structure of RIG-I with RNA shows that ligand-free RIG-I has an open conformation in which the signaling domain is sequestered, but closes upon dsRNA binding. Blunt end 5'ppp-dsRNA is bound by the helicase and the C-terminal domain thus enabling signal induction and inducing further cooperative RIG-I binding on dsRNA. Thus, RIG-I combines the sensing of 5'-trisphosphates and dsRNA with compartmentalization.

Cytosolic DNA recognition also relies on localization as a major discriminatory principle, because lack of cytosolic DNA is typical of normal cell physiology. AIM2 was reported to recognize cytosolic DNA and induce maturation of IL-1β and IL-18 through a new type of inflammasome.[19-21,82] IFI16 is a cytoplasmic receptor, which was recently identified as being also stimulated by intracellular DNA.[83] AT-rich stem loop DNA motifs in the genome of plasmodia are recognized by still another unknown DNA receptor.[84]

Aspects of Endosomal Recognition of Nucleic Acids

As has been outlined in the separate presentations of the nucleic acid receptors TLR3, 7 and 9, all these PRRs recognize their ligands in intracellular endolysosomes, thus adding a layer of control to pattern recognition by means of compartmentalization.[27] The similarities among these TLRs, which are probably rooted in their evolutionary relationship, do not only include recognition modes, but extend to signaling cascades and intracellular trafficking.[85] It is unclear how many components of these networks are yet to be discovered, but probably the present picture is still incomplete.

The recognition of nucleic acids itself may actually be aided by accessory proteins, as suggested by the fact that high-mobility group box (HMGB) proteins 1, 2 and 3 were found to bind to immunogenic nucleic acids and to contribute to activation of TLR3, 7, 9 by their cognate ligands. HMGBs increased nucleic acid uptake, thus acting as universal sentinels for nucleic acids.[86] Moreover, HMGB1 was suggested to affect DNA curvature thus contributing to DNA-TLR9 interaction.[77]

An interesting aspect of intracellular trafficking was discovered in a mutagenesis screen, which identified a mutant named 3d[85] that abolished immunostimulation by the three endosomal TLRs. The underlying mutation was found to reside in Unc93b1, a conserved protein of the endoplasmic reticulum that turned out to be crucial for delivering TLR3, 7 and 9 to the endosome[87] (as well as TLR13 in mice). The Unc93b1 mutant D34A upregulated ligand-induced trafficking of TLR7 but downregulated delivery of TLR9, from which it was inferred that wild-type Unc93b1 might have evolved to naturally bias responses to nucleic acids toward DNA- but against RNA-sensing.[88] Importantly, Unc93b1 was found to be involved in resistance to infections with *Toxoplasma gondii*[89] and streptococci;[90] human

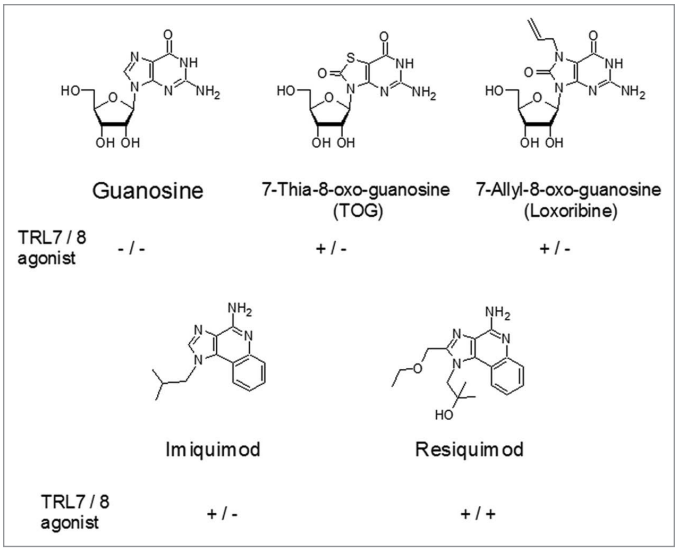

Figure 2. Small molecule purine agonists of TLR7 and/or TLR8. The respective names are indicted under the chemical structures and triggering of TLR7 and/or TLR8 [61] is indicated below the name.

Unc93b1 deficiency resulted in susceptibility to Herpes simplex virus encephalitis.[91]

Evolutionary relations between TLR7, 8 and 9 are also observed when modeling the extracellular receptor domains.[58] All nucleic acid recognizing TLRs are cleaved by proteolysis in a stepwise manner,[92,93] however it remains controversial to what extent this cleavage represents simple degradation, or a processing step that contributes to recognition.[74-76]

Co-receptor usage might be an additional way to shape nucleic acid responses. CD14, a well-known co-receptor for TLR4 in lipopolysaccharide (LPS) recognition recently was shown to be involved in TLR7 and TLR9 recognition as well.[94] CD14 was shown to contribute to nucleic acid uptake as well as promoting endosomal TLR activation in response to vesicular stomatitis virus.

Posttranscriptional Processing and Modifications that Discriminate Self from Foreign RNA

Microbial foreign RNA comes from many sources and in various compositions, therefore distinction by biased nucleotide composition alone is insufficient. However, bacterial and eukaryotic RNA processing differs significantly and thus offers leverage for efficient identification of prokaryotic RNA. Features that may be used to identify eukaryotic RNA include the cap structure of mRNA (which will also pass inspection by cytoplasmic RIG-I as discussed above), polyadenylation and post-transcriptional nucleotide modification, the latter being generally more pronounced in eukaryotic RNA. Of note, since viral RNA is processed in eukaryotic cells, its recognition by these features may be less efficient than that of bacterial RNA (bRNA).

An effect of polyadenlation was discovered by comparison of IL-12 secretion from human monocyte-derived DCs, which was high upon stimulation with bRNA, but low with eukaryotic

RNA.[95] In-vitro transcribed mRNA that lacked a poly(A) tail was also immunostimulatory and this property was lost by enzymatic 3'-polyadenylation. DCs activated by bRNA to secrete high amounts of IL-12 in turn induced T-helper cell differentiation to the T-helper-1 subset which was discussed to be a biological meaningful shift to defend against intracellular microbes.

A fundamental work also based on the comparison of eukaryotic RNA vs. bRNA was published in 2005.[46] Here, activation of TLR3, 7 and 8 was specifically addressed and found to be inefficient with mammalian cytosolic RNA, but efficient with bRNA and, most interestingly, with mitochondrial RNA. Of note, the presence of stimulatory mitochondrial RNA in eukaryotic cells is a further example of the contribution of compartmentalization in RNA sensing. As mitochondria are presumed descendents from bacterial symbionts, mitochondrial transcription and translation carry distinct bacterial traits, which also extend to RNA processing and thus mitochondrial RNA. This includes a distinctly lower content of modified nucleotides, which was subsequently revealed to be a major contribution to the observed effect.

It was observed that in vitro transcribed RNA containing randomly incorporated m^6A or s^2U lacked TLR3 stimulation and that m^6A, m^5C, m^5U, s^2U and Ψ modifications suppressed activation of TLR7 and 8 (see **Fig. 3** for structures). Primary DCs on the other hand, could be stimulated by m^6A and m^5C modified RNA and were inhibited when Ψ was introduced in parallel. In further studies, these modifications were also shown to improve translation of the respective mRNA by diminishing activation of cytosolic components of innate immunity.[97,98]

This concept of a "relative hypomodification" of bRNA vs. eukaryotic RNA as a basis for pattern recognition by TLRs was subsequently taken up in many studies, from which 2'-O-methylation of the ribose emerged as a recurrent feature of eukaryotic nature, which efficiently prevents triggering of TLR response. Several studies based on synthetic 2'-O-methylated RNA observed suppressing effects for TLR7 in human as well as murine plasmacytoid DCs and monocytes.[48,99,100] Those recent findings fit well to old observations that within dsRNA-homopolymers 2'-O-methylation (poly I:Cm, poly A:Um) induced less type I IFN.[101] 2'-O-methylation is naturally occurring at significantly higher abundance in eukaryotic as compared with prokaryotic (and mitochondrial) RNA. This assessment is valid for various RNA species including mRNA, rRNA, tRNA and several other noncoding RNAs.[102-104]

Eukaryotes (and archaea) have evolved a sophisticated system that allows variable targeting of 2'-O-methylation by guide RNAs, most of which direct abundant modification to rRNA. By virtue of their abundance, rRNA, which actually presents 80–90% of a cell's RNA population, and tRNA (tRNA, ~10%) should in principle account for most of the RNA mediated TLR stimulation, and the frequent occurrence of 2'-O-methylated residues in rRNA may have a function in TLR mediated RNA sensing. However, since rRNA exists mostly in ribosomes and is thus tightly covered by proteins, tRNA is probably the most abundant free RNA species being sampled in endosomes.

Two recent studies show that most bacterial tRNA isoacceptor species as well as all unmodified in vitro transcribed tRNAs elicit a strong interferon response from pDCs via TLR7.[49,50] In contrast, mammalian tRNA isoacceptor species and certain bacterial tRNAs were non-stimulatory. A detailed analysis identified 2'-O-methylated guanosines at two different positions, G_m18 and G_m34, as efficient in suppressing a TLR7 response. Of note, G_m18 is present in certain bacteria and tRNAs containing G_m18 act as TLR7 antagonists, suppressing a response to other bacterial tRNA molecules that would otherwise elicit a response. More antagonistic modifications remain to be identified in mammalian tRNAs,[49] which, by containing up to 25% modified nucleotides, are the most heavily modified RNAs known so far.[105]

In summary, the recent dynamic development of this field has identified post-transcriptional processing and modification features as a leverage for the definition of PAMPs in RNA. This has major implications for potential therapeutic applications, including RNAi based strategies, as well as vaccination strategies[106] that employ mRNA.[107] Both, avoiding recognition or deliberate activation of innate immunity may be desirable, depending on the therapeutic strategy. For either case, the use of naturally occurring or non-natural synthetic modifications is being actively investigated.[97,98,107]

Modulation of TLR Activation by Synthetic RNA

Following the analysis of principles that allow discrimination of natural, microbial RNA from host RNA we will now center on synthetic RNA in a therapeutic perspective. Aside from mRNA based vaccination,[106] synthetic RNA is being develop with three main features: (1) antisense, ribozyme or RNAi based gene silencing,[108,109] or activation,[110,111] (2) target recognition of aptamer structures[109] and (3) deliberate immunostimulation or avoidance thereof.[2]

Therapeutic antisense approaches have been in development for decades, and unwanted activation of the immune system is a long standing problem. Academia and industry alike have responded with the development of ever new building blocks (known as phosphoramidites) for incorporation into synthetic oligonucleotides by solid phase synthesis.[112] In addition to modulation of immunostimulation, desirable properties of artificial modifications include resistance against nucleolytic degradation and optimized base-pairing which in turn should result in maximized antisense or RNAi effects. Phosphoramidite building blocks corresponding to chemical alterations of almost every atom of RNA nucleosides have been explored,[112] and may be roughly divided into modifications of the nucleobase and modifications of the ribose-phosphate backbone; some examples are given in **Figure 3**. This concerns in particular the 2'-OH position[113] that, for one, marks the border between RNA and DNA, second, is critical for resistance against nucleases and third, appears to be an important feature in TLR7 recognition, as is evident from the role of 2'-O-methlyation. Modern oligonucleotide therapeutics typically contain a mix of modified nucleotides, some of which are naturally occurring, e.g., 2'-O-methlyated nucleosides, and some of which are clearly artificial, such as 2'-fluorinated nucleosides, which defy definitions of DNA and RNA. Phosphorothioates (**Fig. 3**) present a very special case: originally conceived as a

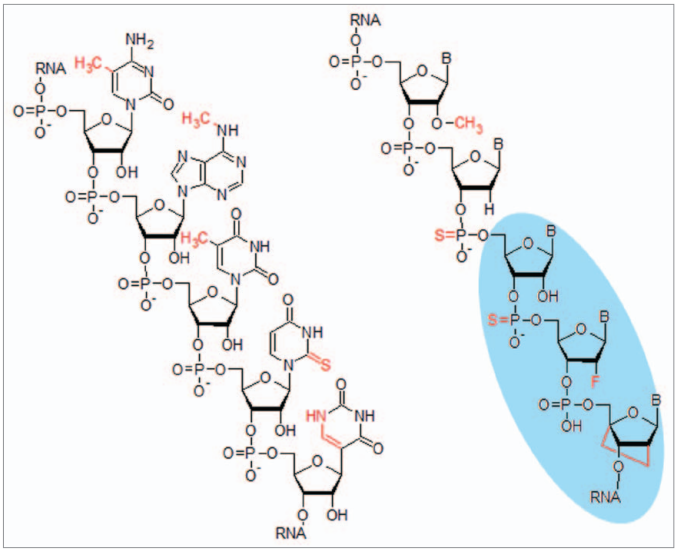

Figure 3. Selected chemical modifications (red) at the nucleobase shown on the left strand include the naturally occurring m^5C, m^6A, m^5U, s^2U and Ψ, (see ref. 96 for nomenclature). Modifications at the ribose shown on the right strand include the naturally occurring 2′-O-Me and desoxyphosphorothioate, as well as the synthetic ribophosphorothioate, 2′-fluoro and locked nucleic acid (LNA) modifications highlighted in blue, which have not (yet) been discovered in nature.

human invention by Eckstein, DNA phosphorothioates were recently revealed to be naturally occurring.[114-116] The phosphorothioate modification in both DNA and RNA enhances stability, improves cellular uptake both via active transport[117] and by increased membrane permeability, and importantly, increases activation of the immune system.[118]

Oligonucleotides for targeted TLR stimulation. Many nucleic acid preparations elicit an ill-defined response of the innate immune system because of pleiotropic effects: for example, synthetic poly I:C can be used to stimulate TLR3, however this is not specific as cytosolic Mda5 and RIG-I might be triggered as well.[119] Because of differential expression of the various TLRs in different professional immune cells,[120] activation of multiple PRRs may seriously complicate clinical development. Development of ONs with sequence elements or chemical modifications that allow selective targeting of a given TLR or immune cell is therefore of high interest. Early implementations of the idea to use ONs for deliberate activation of the innate immune system were targeting TLR9 because its recognition of CpG DNA ONs was best understood among the TLRs early on.[66,121,122] The emergence of ligand profiles for TLR3 [32] and TLR7,[44,45] went hand in hand with the development of motifs for selective TLR targeting: such motifs include 5'-GUC CUU CAA-3',[53] 5'-UGU GU-3',[123] as well as a 5'-CUG AAU U-3' motif, which stimulated TLR7 and pDCs when contained in duplex-forming ONs.[53] Forsbach et al. report sequences that specifically trigger a specific set of cytokines via TLR8, while avoiding the type I IFNs response that is typical of TLR7-mediated pDC activation.[124] Along the same line, it was shown that by manipulation of molecular structure and mode of delivery, either selective TLR7 stimulation and IFNα secretion from pDC, or TLR8 stimulation and IL12p70 secretion from monocytes can be achieved.[125]

Phosphorothioate modifications in the backbone as well as cationic lipid formulation were shown to be effective to use RNA as DC stimulus.[126] Intra-tumor injection of stabilized single stranded RNA could be used to trigger anti-tumor immunity and thus opens the field for the development of synthetic TLR7 agonists.[127] Potential therapeutic fields include adjuvant activity for new vaccines[106] as well as treatment of viral infections, cancer and allergies.[128,129] In another approach RNA segments were coupled through their 3' ends resulting in enhanced nuclease resistance and increased immunostimulation of TLR8 without lipid carriers.[130] Substitution with 7-deazaguanosine for guanosine also resulted in an immunomodulatory compound with increased stimulation of TLR7 and 8. Another RNA ON that additionally contained a CpG motif was reported which induced IL-12 in peripheral blood mononuclear cells (PBMCs) without special delivery agents when phosphorothioate modified.[131] Overcoming the need for cellular delivery might be achieved by presence of a poly(G) motif that leads to higher order structures and increased nucleic acid uptake.[118,132]

Immune activation by siRNA. Based on the hallmark observation that RNA interference can be induced in mammalian cells with 21-nucleotide duplexes, which avoid the typical powerful immune response of longer dsRNA,[1] siRNA has become a universal molecular tool and potential therapeutic drug.[108] An early report claiming that siRNA does not induce type I IFN[133] has been convincingly contradicted in several studies.[134-137] One such case reports an IFN response to shRNA vectors.[134] In a genome wide expression analysis, 21 bp siRNAs induced a considerable number of genes of the IFN response.[135] This response involved the dsRNA-activated serine-threonine kinase PKR. However, as found by analysis of cells deficient in IFN signaling, the gene silencing effect was independent of an IFN response, showing that both functions—gene knockdown and immunostimulation—rely on pathways that are clearly different.[135]

Careful recent analysis strongly suggests that immune response to siRNA in a TLR3, 7 or 8 dependent manner is sequence-dependent, thus reconciling several of the above reports.[53,138,139] Sequence specific TLR7 activation depended upon delivery by cationic lipoplexes and endosomal maturation.[53,139] There is recurrent emergence of GU-rich or U-rich regions which are particularly efficient in TLR7 stimulation[53,123,140] by "entire" siRNAs, i.e., duplexes composed of sense and antisense strand. These nucleotide combinations appear to be particularly effective in ss siRNA components (sense or antisense),[48,139] possibly because they have a higher propensity for forming secondary structures through mismatched G:U basepairs.[141]

Another report claimed that siRNA and short hairpin RNA (shRNA) induced IFNα and TNFα secretion in professional innate immune cells. The secretion could be inhibited by blocking Toll/Interleukin-1 receptor domain-containing adaptor inducing IFNβ (TRIF) or interferon regulatory factor 3 signaling and could be increased by TLR3 overexpression, thus identifying sequence independent TLR3 activation as an additional mode of action.[138] Understanding the molecular details and requirements

of RNA/TLR stimulation will in turn allow the development of more specific siRNAs that avoid immunostimulation, as well as to predict the potential of any given RNA sequence in terms of innate immune activation.[142]

Besides stimulation of endosomal TLRs, it was shown that 27-mer siRNAs with blunt ends but not with 2-base 3' overhangs triggered cytoplasmic RIG-I to induce type I IFN[143] thus providing an explanation why endogenous miRNAs with a Dicer signature avoid immunorecognition. Importantly, as many cell lines have impaired IFN response pathways, use of primary cells will be necessary to assess the stimulatory potential of synthetic RNAs. An independent study confirmed that the expression of 90% of genes that were regulated by the TLR7 ligand R848 (**Fig. 3**) was also modified by ss siRNA. However, TLR-independent genes were activated as well confirming the existence of multiple siRNA recognition systems.[144]

While activation of the innate immune system is a serious concern in the development of siRNA therapeutics, where it must typically be avoided as an aspect of siRNA toxicity,[112,113] alternative strategies include immunostimulation as a beneficial therapeutic feature, e.g., in antiviral or anticancer strategies.[2,145-148]

Using Modifications to Decrease Immunostimulation by siRNA

In contrast to the deliberate immunostimulation mentioned above, avoidance of an interferon response is a necessity in most therapeutic applications of RNA. Beyond sequence optimization, chemical modification is the method of choice, which, though occasionally cumbersome, allows optimization of other parameters as well, including nuclease resistance etc. First generation siRNAs in clinical trials typical contain a mix of phosphorothioates, 2'-O methyl- and 2'-desoxynucleotide modifications.[108,149] The advantages of 2'-O methylation include immunosilencing, increased nuclease resistance, low cost, and the fact that its degradation products occur naturally in the cell. Indeed, siRNAs containing 2'-O-methyl modifications at every other nucleotide,[150] are now in clinical trials.[151] The 2'-hydroxyl group does not play a critical role at most positions in siRNA[135] and thus is an attractive candidate to dissect immunostimulation from RNAi. Indeed, substitution of 2'-hydroxyl uridines with either 2'-fluorouridines, 2'-deoxyuridines (dU) or 2'-O-methyluridines (U_m) decreased immune activation of siRNAs within PBMCs.[140] Among those modifications, 2'-O-methylation not only abolished immunostimulation (in PBMCs including pDCs) but also suppressed activity of an unmodified, stimulatory strand, thus acting as an antagonist. U_m-containing ONs inhibited stimulation by unmodified siRNAs at nanomolar concentrations. Using global gene expression analysis, U_m-siRNA abrogated nearly all 270 genes that were induced by ss siRNA in human monocytes. Thus, U_m acts as dominant negative, suppressing modification and indeed, it also suppressed TLR7/8 stimulation in PBMCs by bRNA.[100] In contrast, dU lacked immunostimulation but did not act in a suppressive manner, an observation also confirmed by others.[48] Likewise, incorporation of 2'-O-methyl residues into siRNA was sufficient to suppress immunostimulation even if the modification was not incorporated into the immunostimulatory strand, confirming a role as antagonistic modification.[99] Of note in this report the nucleoside modification did not alter RNA interference. In contrast, others observed that 2'-O-methyl modifications also decreased efficacy of gene knockdown.[48] It was reported that modifications have to be restricted to certain positions in a siRNA[152] thus possibly explaining the slightly varying findings.

In general, modifications in the middle of the siRNA duplex are not well tolerated, as this part is important for activity of the RNA induced silencing complex (RISC),[153] an important fact in attempts to use modifications to decrease siRNA side effects. Also, incorporation of such modifications in the passenger strand might be recommended as this should have less effect on RNAi.[154]

Incorporation of dU or dT into siRNA allowed for dissection of immunostimulation and RNA interference.[48] Whereas IFNα secretion from PBMCs was decreased (or abolished at concentration necessary for RNA interference), gene knockdown was as efficient as with unmodified siRNA. For maximal effects, both strands of the siRNA had to be modified as those modifications behaved as "silent" but not "suppressive" modifications. Interestingly, 2'-deoxyriboses with nucleobases other than uridine were not effective, arguing for a specific recognition of uridine by TLR7. Also, neither 5-methylcytidine nor 7-deazaguanosine, both modifications affecting the surface of the major groove of a duplex, modified IFNα secretion. Abrogation of immunostimulation by incorporation of U_m or G_m into one strand of the siRNA duplex was confirmed to be effective and such modified siRNA was efficient and specific in systemic gene knockdown.[144,155] From those results it was suggested to use alternating 2'-O-ribose methylation as a general strategy to generate siRNA.[150] In contrast to suppressive effects of A_m, U_m and G_m, we observed that C_m was not sufficient to suppress immunostimulation[48] arguing for a lack of recognition of cytidine residues.

RNA Immunostimulation in Infections

The role of nucleic acid recognizing TLRs in infections is largely defined by pDCs, for which a large body of evidence[156] documents the requirement for TLR9 to respond to DNA viruses[157-159] and for TLR7 mediated type I IFN induction in response to RNA viruses, including influenza virus, respiratory syncytial virus, Sendai virus or vesicular stomatitis virus.[45,55] MyD88 dependent but TLR9 independent recognition of murine cytomegalovirus might indicate that further receptors exist.[160]

TLR-related innate immunity affects induction of adaptive immunity[29] and therefore defects in pattern recognition receptors can affect both limbs of the immune system. Some valuable insight comes from recent reports on human patients with genetic deficiencies of components of the innate immune system, which frequently result in predispositions to bacterial or viral infections. For example, children with a dominant-negative allele of TLR3 were found to be susceptible to Herpes simplex-virus-1 encephalitis.[161] Patients suffering from primary deficiency of IRAK4, an important signaling molecule for most TLRs, display a loss of PBMC responsiveness to TLR ligands including

R848 (also known as Resiquimod, a TLR 7, 8 ligand displayed in **Fig. 2**) and CpG ON (TLR9 ligand).[162,163] As an apparent consequence, patients were prone to invasive pneumococcal diseases in young childhood and half of them died. With increasing age the infection susceptibility vanished. IFN responses to most viruses tested were not severely affected and no specific viral infections in IRAK-4 deficient patients have been reported arguing for a redundant role of IFN inducing TLRs, especially the nucleic acid receptors, in viral defense.

Genetic predispositions to viral infections have been reported for autosomal recessive deficiency in Unc93b1, a protein necessary for trafficking of endosomal, nucleic acid detecting TLRs[164,165]. This resulted in impaired antiviral IFN responses and Herpes simplex virus-1 encephalitis in children.[91] Unc93b1 defect that abrogates TLR3, 7 and 9 functions was also reported to decrease resistance to infection with the parasite *Toxoplasma gondii*.[89] Furthermore sensing of group B streptococci by macrophages and cellular activation was dependent on bacterial ssRNA and involved MyD88 and Unc93b1, although the established RNA sensors TLR3 and TLR7 were dispensable.[90] Further studies in a mouse model of group A streptococci-induced lethal subcutaneous cellulitis analyzed type I IFN induction of macrophages and pDCs.[166] Type I IFN induction in macrophages was dependent on endosomal recognition of streptococcal DNA. In contrast myeloid DCs recognized streptococcal RNA in a MyD88 but TLR7 independent manner. The results suggest that nucleic acid detection is important for defense of *Streptococcus pyogenes* which evades immune recognition by other TLRs.

A role of TLR7 in signaling toward fungal infections was reported for IFNβ induction in DCs in response to Candida infection. IFNβ induction was dependent on TLR7, MyD88 and IRF1 and occurred after completed phagocytosis.[167] A similar report also showed that IFNβ induction in myeloid DCs to Candida infection required MyD88 and partly TLR7 or TLR9.[168] Moreover it was shown that Candida DNA as well as RNA is recognized. Type I IFN induction was necessary to control fungal growth. Although yeast RNA has been shown to bear nucleoside modifications similar to other eukaryotes and was not overly stimulatory in vitro,[56] it is possible that enrichment within the endolysosome during severe infections overcomes classical self/foreign discrimination principles. This would be compatible with a model in which intrinsic structural features (nucleoside modifications) in cooperation with spatial control (endolysosome) would account for overall discrimination of self and foreign nucleic acids.

Manipulating the Course of Natural Infections through RNA-Mediated TLR Stimulation

As nucleic acid recognizing TLRs contribute to resistance against virus infections it was speculated that TLR7 agonists might be used to increase anti-viral responses especially when chronic infections are established. In this line it was shown that TLR7 stimulation results in increased anti-Hepatitis C (HCV) immunity by means involving IFN dependent as well as independent effects.[169] In a cell culture system HCV levels were reduced by treatment with a TLR7 agonist. As TLR7 is expressed in hepatocytes it was speculated that TLR7 stimulation could be used to reinforce HCV immunity.

Usage of antagonists for nucleic acid sensing TLRs might be a strategy to avoid overshooting reactions toward such pathogens for which nucleic acid detection is of importance during natural immune responses. It was reported that cerebral malaria in an experimental model with *Plasmodium berghei* is caused by excessive immunostimulation with TLR9 and possibly other nucleic acid recognizing TLRs.[170] Using E6446, a synthetic antagonist composed of benzoxazole with two-sided pyrrolidine rings that blocks TLR9 and TLR8 at higher concentrations, severe symptoms of cerebral malaria could be successfully inhibited.

Mislead Self RNA Recognition and Autoimmune Diseases

Whereas under healthy, physiological conditions discrimination of self- and foreign-derived nucleic acids is achieved through sequence and structure as well as spatial control (outlined above), recent publications indicate that self-tolerance of nucleic acids can be broken in certain pathologies. Thus, recognition of self-nucleic acids through TLRs is not entirely prevented. An important finding was first published for recognition of self-DNA by TLR9. B cells prone to produce IgG autoantibodies known as rheumatoid factors were shown to be stimulated by dual engagement of the antigen receptor as well as TLR9.[72] Chromatin-antibody immune complexes activated autoreactive B cells by triggering an endosomal TLR. Increased uptake and delivery of self-DNA within those chromatin complexes contributed to the breakdown of TLR-mediated self/foreign discrimination. Similarly, dsDNA-specific antibodies triggered autoreactive B cells through antigen receptor/TLR9 coengagement.[171] A two-stage model for autoimmunity in systemic lupus erythematosus was proposed that discriminates TLR-independent and TLR-dependent processes.[172] TLR-independent dendritic cell uptake of self-derived cell debris is followed by amplification through TLR recognition of nucleic acids in respective complexes. Both phases are dependent on type I IFN. These observation fit well to an experimental observation which shows that ectopic expression of TLR9 at the cell surface results in reactivity with mammalian DNA, emphasizing the need for control by localization.[69] In turn, conditions in which spatial control is overcome, e.g., increased occurrence and uptake of self-DNA, defects in vesicle trafficking or membrane integrity, autoimmunity might develop through unphysiological triggering of TLRs in conjunction with self-antigen presentation.

Similar to the findings of synergistic activation of the B-cell receptor and TLR9 it was subsequently shown that this paradigm also holds true for RNA autoantigens. RNA containing immune complexes triggered the B-cell receptor together with TLR7.[173] This response was markedly enhanced by IFNα thus resembling a situation of disease progression in patients with autoimmune systemic lupus erythematosus. Autoimmune-prone mice lacking the nucleic acid response through MyD88 deficiency had reduced chromatin autoantibody titers. Of note, pDC activation and IFNα secretion can occur with self-nucleic acids through

increased endolysosomal delivery, thus overcoming spatial control. It was shown that nucleic acid/immunoglobulin complexes can be taken up into pDCs through the low-affinity Fc receptor (FcγRIIA). Internalization is followed by TLR9 activation overcoming the physiological spatial control.[174] Another way to deliver self-DNA is by interaction with the antimicrobial peptide LL37.[175] LL37 increased delivery of aggregated DNA into the early endosome where it triggered TLR9. Thus, LL37 converts self-DNA to stimulatory DNA and, interestingly, LL37 plays a role in pDC activation in psoriasis.[176] It can be speculated that similar findings hold true for RNA delivery and TLR7 stimulation.

It has also been shown that high mobility group box protein 1 is a general nucleic acid sensor that delivers nucleic acids into TLR-bearing vesicles and synergistically activates the cell through its receptor for advanced glycation end products (RAGE).[69] HMGB1 is a nuclear-DNA binding protein that can be released by dying cells and interacts with aggregated DNA. Later, it was shown that HMGB1, 2, 3 act as general nucleic acid sensors including DNA and RNA and facilitate stimulation of TLR3, 7, 9 by their cognate ligands.[86] HMGB knockout mice were defective in DNA or RNA induced type I IFN secretion and proinflammatory cytokine induction. Thus, HMGBs could be promiscuous nucleic acid sensors that act together with specific TLRs to mediate self/foreign discrimination.

Similar to findings for DNA it was shown that self-RNA rich in uridine and guanosine and RNA in small nuclear ribonucleoprotein can induce type I IFN in plasmacytoid DCs in a TLR7 dependent manner.[177] Thus, nucleic acid recognizing TLRs might play a role in pathological activation of plasmacytoid DCs and B-lymphocytes to produce autoantibodies against DNA and RNA as observed in systemic lupus erythematosus.[178] In this line TLR7 gene duplication has been found to promote autoimmune diseases, plasmacytoid DC activation, type I IFN production and autoantibody formation in mice.[179,180] In turn, TLR7 deficiency results in decreased susceptibility for autoimmune diseases with lowered serum autoantibody levels.[181,182]

Fc gamma receptor independent, but strictly TLR7 dependent induction of autoimmunity was reported in a model with 2,6,10,14-tetramethylpentadecane induced lupus-like disease with immune complex nephritis and autoantibodies to DNA and ribonucleoproteins.[183] TLR7 triggered type I IFN was also shown to upregulate MHC class I expression and induction of autoimmune T-cell responses in a model of pancreatic lymphocytic choriomeningitis virus glycoprotein expression.[184] Autoreactive cytotoxic T cells were only activated when an additional TLR stimulus was provided.

TLR3 contribution to autoimmunity has been reported for autoimmune liver damage and rheumatoid arthritis.[185,186] Thus, incubation of fibroblasts that bear TLR3 with necrotic synovial fluid cells from rheumatoid arthritis patients resulted in increased chemokine and cytokine secretion. Interestingly, Unc93b1 signaling (implicating TLR3, 7, 8, 9 nucleic acid sensing) was necessary to remove developing autoreactive B cells and patients with Unc93b1 deficiency had defective central and peripheral B-cell tolerance with accumulation of autoreactive mature B cells.[187]

Glucocorticoids are used to treat autoimmune diseases and glucocorticoids decrease proinflammatory NFκB signaling. However, glucocorticoids lose activity in long-term treatment. Recently, it was shown that pDCs triggered through self-nucleic acids via TLR7 or TLR9 induced NFκB for cell survival. NFκB activation in pDCs however was not sensitive to glucocorticoids and in turn pDCs produced IFNα was not decreased.[188] TLR7/9 antagonists might therefore be of use to decrease pDC activation and to spare glucocorticoid. In a model of collagen-induced arthritis reduction of TLR7 expression by means of lentiviral transfer resulted in decreased inflammation and increased clinical outcome.[189]

The common topic in all reports that claim breakdown of self-tolerance for nucleic acid recognizing TLRs is that the underlying patterns that mediate self/foreign discrimination are not entirely specific for microbes. Perhaps this is the reason why spatial restriction occurs for TLR3, 7, 8, 9. This allows for an additional level of control that under healthy conditions excludes self-reactivity.

Closing Remarks

Recognition of nucleic acids should be, as such, a simple task on the molecular level, because of the few building blocks present. However, discrimination of nucleic acids originating from the host organism against that of an invading pathogen is even more daunting, probably for the very same reasons. As we have outlined here, mammals have evolved multiple sophisticated sensors in different cellular compartments. More discrimination principles unravel as newer and more realistic assays are being developed. In all likelihood, more receptors for nucleic acid recognition await detection and after completion of this task, new challenges appear. Not only do all parameters for recognition and discrimination have to be clarified, if therapeutic use is to emerge from this research; a much more challenging task may be to define the interplay between the various receptors, whose signaling, in its sum, must be what alarms our body to an imminent infection. Although single receptors may make dominating contributions in the detection of e.g., cytosolic viral RNA, the variety of different receptors and the use of common molecular relays in the signaling pathways strongly suggests the possibility of cooperative effects in pathogen sensing, where the full-fledged alarm mode might be most efficiently stimulated upon detection of multiple PAMPs by different receptors.

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