Review

Innate recognition of non-self nucleic acids

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Published: 10 March 2008

Genome Biology 2008, **9**:211 (doi:10.1186/gb-2008-9-3-211)

The electronic version of this article is the complete one and can be found online at http://genomebiology.com/2008/9/3/211

Abstract

The immune system has evolved a plethora of innate receptors that detect microbial DNA and RNA, including Toll-like receptors in the endosomal compartment and RIG-I-like receptors and Nod-like receptors in the cytosol. Here we discuss the recognition of and responses to non-self nucleic acids via these receptors as well as their involvement in autoimmune diseases.

The function of the immune system is to protect the organism from invading pathogens. To avoid collateral damage to the body's own tissues, it must be able to distinguish infectious non-self entities from self tissues. Antigen-specific lymphocytes - T cells and B cells - recognize pathogens through T-cell receptors and immunoglobulins, respectively, which are generated by somatic gene rearrangement. But although these antigen-specific receptors allow the recognition of a vast number of different molecules, they have no intrinsic ability to distinguish non-self from self. Instead, it is believed that signals delivered through the so-called pattern recognition receptors of the innate immune system are fundamental in recognizing infectious non-self entities, thus preparing the body for the initiation of a full antigen-specific immune response that targets invading pathogens but not self tissues [1]. The receptors utilized by the innate immune system recognize microbial components, known as pathogen-associated molecular patterns, that are essential for the survival of the microorganism and are therefore difficult for it to alter. Different receptors interact with different pathogen molecules, and show distinct expression patterns, activate specific signaling pathways and lead to distinct anti-pathogen responses [2,3]. The molecules recognized include, for example, components of bacterial and fungal cell walls, flagellar proteins and viral surface proteins - molecules that are unique to the pathogen and not found in the host. Another major group of pathogen molecules specifically recognized by innate immune receptors comprises microbial DNA and RNA. Because nucleic acids are present in all organisms, the host has evolved specialized mechanisms for recognizing non-self nucleic acids while maintaining tolerance (non-responsiveness) to self nucleic acids. In this article, we will review several systems of pattern recognition receptors involved in the recognition of non-self nucleic acids, including the Toll-like receptors (TLRs), the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and the Nod-like receptors (NLRs) (Table 1). Mechanisms for recognizing non-self nucleic acids are not fail-safe, however, and under abnormal conditions recognition of self DNA and RNA occurs, leading to the development of autoimmunity. This is discussed in the last section of this review.

TLRs mediate recognition of microbial nucleic acids in the endosomal compartment

Some innate immune receptors, including the TLRs and the NLRs, recognize pathogen components via leucine-rich repeats (LRRs) in the receptor. Of these, the TLRs are the best studied. TLRs elicit cellular responses by signaling through their cytoplasmic Toll-interleukin-1 receptor (TIR) domain, which recruits TIR-containing adaptors. These adaptors, which include MyD88, TRIF/TICAM-1, TRAM and TIRAP/Mal, mediate intracellular events that lead to the expression of antimicrobial and inflammatory genes [2,4]. TLRs can be classified into two groups on the basis of their subcellular localization. TLR1, 2, 4, 5 and 6 are all present at the plasma membrane and recognize pathogen components present in the extracellular milieu. The second

Table 1

Major pattern recognition receptors involved in the recognition of non-self nucleic acids

Receptor family	Location	Ligand	Receptor	Adaptor
Toll-like receptors (TLRs)	Endosomes	CpG DNA, abnormal DNA	TLR9	MyD88
		ssRNA	TLR7/TLR8	MyD88
		dsRNA	TLR3	TRIF
RIG-I-like receptors (RLRs)	Cytosol	5'-triphosphate ssRNA	RIG-I	IPS-1
		dsRNA	MDA5	IPS-1
		dsRNA	LGP2	IPS-1
		dsDNA	DAI	Unknown
Nod-like receptors (NLRs)	Cytosol	Bacterial and viral RNA	Cryopyrin	ASC

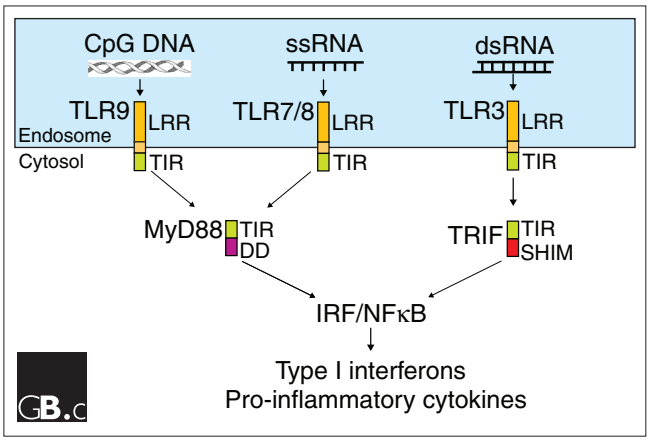

Figure 1
Recognition of microbial RNA and DNA by endosomal Toll-like receptors (TLRs). TLR9, TLR7 (and TLR8), and TLR3 recognize CpG DNA, single-stranded RNA (ssRNA), and double-stranded RNA (dsRNA), respectively. TLR9 and TLR7/8 signal through a Toll-interleukin-1 receptor- (TIR-) containing adaptor molecule MyD88, whereas TLR3 signals exclusively through a different adaptor, TRIF. MyD88 and TRIF induce the expression of genes for type I interferons and pro-inflammatory cytokines by activating transcription factors of the IRF and NFκB families. DD, death domain; IRF, interferon response factor; LRR, leucine-rich repeat; NF, nuclear factor; SHIM, RIP homotypic interaction motif.

group includes TLR3, 7, 8 and 9, which localize to intracellular compartments such as endosomes. All these intracellular TLRs share the ability to sense viral and bacterial nucleic acids (Figure 1), which they gain access to when microbial DNA and RNA are released following the degradation of endocytosed microbial particles in late endosomes or lysosomes. As abnormal recognition of self DNA and RNA is associated with autoimmune diseases, the endosomal localization of nucleic acid-specific TLRs is important in preventing their contact with self nucleic acids [5].

TLR9 was the first TLR identified to interact with nucleic acids, and its classical ligand is CpG DNA, an immunostimulatory DNA composed of unmethylated CpG dinucleotides with particular flanking sequences [6]. The CpG motif is abundant in bacterial genomes as well as in the DNA of viruses such as herpes simplex virus 1 (HSV-1), HSV-2 and murine cytomegalovirus (MCMV), allowing these pathogens to be recognized by TLR9. In contrast, in mammalian genomes the CpG motif occurs much less frequently and is highly methylated, which does not activate innate immunity. CpG DNA induces a conformational change in TLR9 that is required for its activation [7]. In addition to CpG DNA, recent findings indicate that oligodeoxyribonucleotides containing no CpG motifs but with a phosphorothioate backbone, or modified nucleotides with a bicyclic heterobase can activate the innate immune system in a TLR9-dependent manner. Therefore, TLR9 might have evolved to recognize not only unmethylated CpG motifs as a conserved molecular pattern in pathogen DNA but also abnormal composition, structure or chemical features in any kind of DNA [8].

TLR9 is highly expressed in dendritic cells, the 'professional' antigen-presenting cells that link innate and adaptive immune responses, and in other immune system cells such as B cells. TLR9 expression patterns are different in humans and mice. In mice, TLR9 is expressed broadly in the different subsets of dendritic cells, whereas in humans it is exclusively expressed by plasmacytoid dendritic cells (pDCs), a subtype characterized by the ability to secrete high levels of type I interferons in response to viral infection. In pDCs, TLR9 acts as a sensor of viral infection, which leads to the transcription of type I interferons, particularly interferon-α, through the MyD88-interferon response factor 7 (IRF7) signaling pathway. In other cells, such as conventional dendritic cells (cDCs) and macrophages, TLR9 ligands are poor at inducing type I interferons but they can induce inflammatory cytokines through the MyD88-IRF1 pathway [8]. TLR9-deficient mice show increased susceptibility to MCMV infection but not to

local infection by HSV-1 [9-11], indicating a specific role for TLR9 in viral sensing and antiviral responses.

Double-stranded RNA (dsRNA), along with its synthetic analog polyinosinedeoxycytidylic acid (poly I:C) is a potent inducer of the type I interferons and is recognized by TLR3 [12]. Double-stranded RNA can be generated during viral infection as a replication intermediate for single-stranded RNA (ssRNA) viruses or as a byproduct of symmetrical transcription in DNA viruses. TLR3 is expressed in cDCs, which avidly phagocytose dying cells, and in a variety of epithelial cells, including airway, uterine and intestinal epithelial cells that function as efficient barriers to infection. Expression of TLR3 in these cells is also rapidly induced by treatment with poly I:C or type I interferons. TLR3 signals exclusively through the TLR adaptor TRIF, leading to the IRF3-dependent induction of type I interferons. As dsRNA is a universal viral product, TLR3 was originally thought to play a key role in antiviral immunity. However, the induction of type I interferons and the maturation of dendritic cells after viral infection occur in a TLR3-independent manner [13], and TLR3-deficient mice respond normally to infection with many viruses, including MCMV, vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV) and reovirus [14-16]. Indeed, TLR3-deficient mice are more resistant than normal to lethal West Nile virus infection. In this infection, a strong inflammatory response mediated by TLR3 in peripheral tissues results in the disruption of the blood-brain barrier, facilitating entry of the virus into the brain, suggesting that the interaction of TLR3 with West Nile virus actually facilitates the infection [17]. A role for TLR3 in exacerbating immunopathology has also been found in infection with influenza virus and Punta Toro virus [18,19]. *In vivo*, therefore, TLR3 plays an important role in evoking inflammatory responses in response to RNA virus infection - a response that can be detrimental to the host - rather than in the production of type I interferons. In addition to detecting viral dsRNA, TLR3 can also be activated by dsRNA from the helminth parasite *Schistosoma* in dendritic cells [20].

The TLR7 and TLR8 genes are very similar in sequence to each other and are both located on the X chromosome. Mouse TLR7 and human TLR8 recognize synthetic antiviral imidazoquinolines (such as R848 and Imiquimod), certain guanine nucleotide analogs (for example, loxoribine), and uridine-rich or uridine/guanosine-rich ssRNA of both viral and host origin [21-23]. Although both TLR7 and TLR8 are expressed in mice, mouse TLR8 appears to be nonfunctional. TLR7 and TLR8 are present in the endosomal membranes of pDCs, indicating that access to ssRNA may be a key factor for activation of these cells via these receptors. Like TLR9, TLR7 and TLR8 signal exclusively through MyD88 to induce an interferon response. TLR7-deficient pDCs are defective in interferon-α production after stimulation with influenza virus [22], and TLR7-deficient mice show increased sensitivity to VSV [23], indicating a role for TLR7 in antiviral defense *in vivo*.

Cytosolic sensors of nucleic acids are ubiquitous triggers of interferon responses

In 1963, two groups, including one led by the discoverer of interferon, Alick Isaacs, reported that DNA and RNA derived from pathogens or host cells were able to activate chicken or mouse fibroblasts to produce interferon [24,25]. TLRs specific for nucleic acids are expressed only in a subset of immune-system cells, whereas almost all nucleated cells can produce type I interferons in response to viral infection, and so TLRs are unlikely to mediate the interferon response in fibroblasts. Indeed, fibroblasts lacking the key TLR adaptors MyD88 and TRIF are still capable of inducing type I interferons after viral infection, indicating that TLRs are not required for viral detection in these cells [26]. Recent studies indicate that the ubiquitous interferon response to immunostimulatory nucleic acids is mediated by cytosolic RNA-binding proteins - the RLR family and by cytosolic DNA sensors that include the recently identified protein DAI (DNA-dependent activator of interferon-regulatory factors) (Figure 2).

RIG-I and melanoma differentiation-associated gene 5 (MDA5)/Helicard are DExD/H box RNA helicases that have recently been implicated in the regulation of interferon gene expression following sensing of viral RNA in the cytosol. Both of these proteins contain caspase recruitment and activation domains (CARDs) and detect RNA viruses and synthetic poly I:C [27-29]. The critical determinant in RIG-I stimulation by RNA is the presence of triphosphates at the 5' end of ssRNA [30,31]. Host 5'-triphosphate ssRNA exist in the nucleus but not the cytoplasm; host ssRNA in the cytoplasm are normally capped or processed. The agonist for MDA5 remains uncharacterized, although MDA5 is known to respond to dsRNA. A third member of the RLR family, the protein LGP2, shares homology with RIG-I and MDA5 in the helicase domain and can bind dsRNA. However, LGP2 lacks a CARD domain and has been proposed to act as a negative regulator of RIG-I or MDA5 signaling [32,33].

Upon RNA recognition, RIG-I and MDA5 signal through the adaptor molecule interferon-β promoter stimulator 1 (IPS-1, also known as MAVS, Cardif and VISA) to downstream signaling proteins that include the adaptor Fas-associated death domain-containing protein (FADD), and the death-domain-containing kinase RIP1, TANK-binding kinase 1 (TBK1) and the inducible I-kappaB kinase (IKK-*i*), leading to transcription of the interferon genes [2,34]. Experiments with mice deficient in RIG-I or MDA5 indicate that the two helicases are critical for host antiviral responses and distinguish different viruses [35,36]. RIG-I is essential for the production of interferon in response to RNA viruses such as paramyxoviruses, influenza virus and Japanese encephalitis

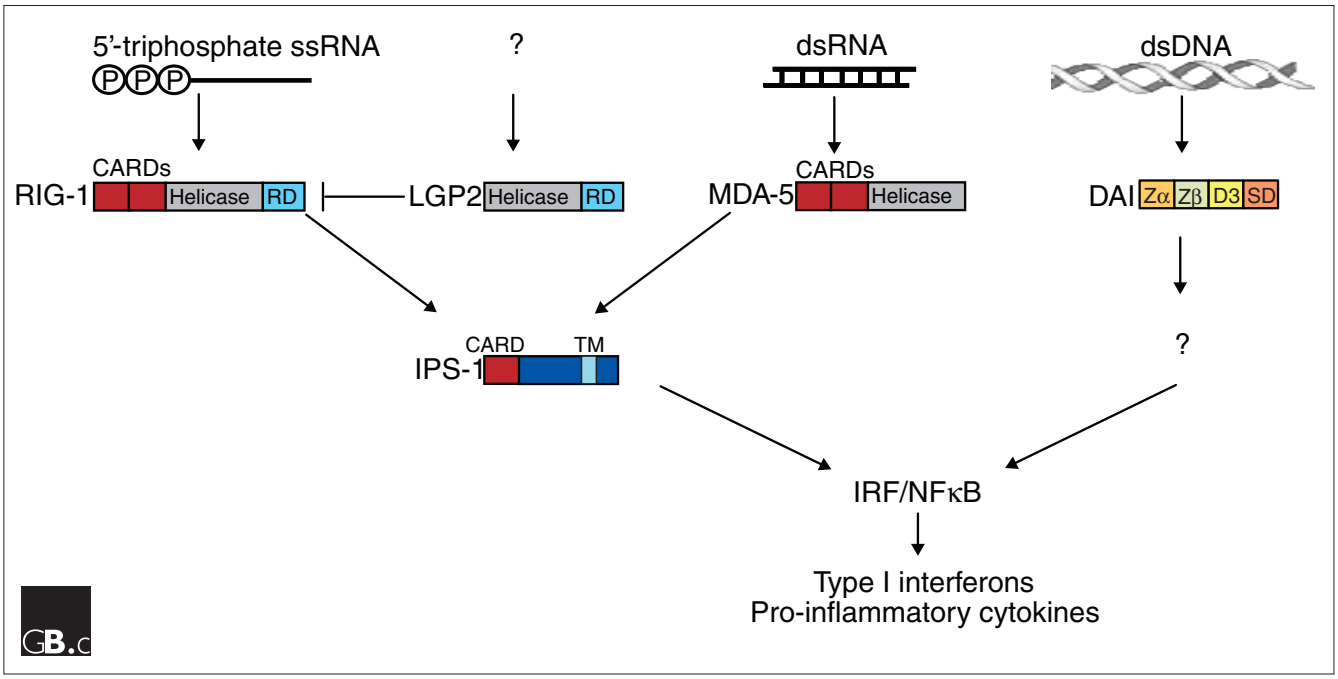

Figure 2
Recognition of microbial RNA and DNA by cytoplasmic pattern recognition receptors. RIG-I and MDA5 recognize 5'-triphosphate ssRNA and dsRNA from RNA viruses and trigger signaling cascades via a CARD-containing adaptor molecule, IPS-1. IPS-1 induces the expression of genes for type I interferons and pro-inflammatory cytokines by activating transcription factors of the IRF and NFκB families. The role of LGP2 in RNA virus recognition is unclear and LGP2 has been proposed to inhibit RIG-I activity. DAI recognizes dsDNA and induces gene expression through an unknown adaptor molecule. Modular structures of the receptor proteins are shown with the abbreviations of the domains as follows: CARD, caspase activating recruitment domains; helicase, RNA-binding domain; RD, repressor domain; TM, transmembrane domain; Zα and Zβ, Z-DNA binding domain α and β; D3, tentative name for an additional DNA-binding region; SD, signaling domain.

virus, whereas MDA5 is critical for picornavirus detection. Furthermore, compared with control mice, RIG-I$^{-/-}$ and MDA5 $^{-/-}$ mice are highly susceptible to infection with the respective RNA viruses [35,36].

A pathway analogous to that stimulated by RIG-I and MDA5 has been suggested to signal an innate immune response to DNA in the cytosol. HSV-1 elicits type I interferon production via both TLR9-independent and -dependent pathways [37]. Similarly, cytosolic DNA has been shown to be an interferon-activating ligand independent of TLRs in intracellular infection with the bacterium *Listeria monocytogenes* [38]. Cytosolic DNA sensing does not require RIG-I or MDA5 [39], suggesting the involvement of a new factor. The first cytosolic DNA sensor identified was the interferon-inducible protein DAI, which can activate IRF3 and induce an interferon response in cells [40]. Binding of dsDNA to DAI enhances the protein's association with the transcription factor IRF3 and the kinase TBK1. Overexpression of DAI in mouse fibroblasts selectively enhances DNA-mediated induction of type I interferons and of other genes involved in innate immunity. Thus, DAI may be central to detection of the DNA of viruses, bacteria, fungi and parasites that enter host cells. Generation and analysis of mice deficient in DAI should define its function more clearly. Inhibition of DAI synthesis by small interfering RNAs reduced interferon production after DNA stimulation but did not abolish it [40], suggesting that additional cytosolic DNA sensors might exist.

The induction of non-transcriptional responses to nucleic acids

A common feature of the recognition of non-self nucleic acids by TLRs, RLRs and DAI is their ability to induce robust activation of genes for interferons and/or pro-inflammatory cytokines, which endow these receptors with a central role in innate immunity. Various other mechanisms that recognize non-self nucleic acids do not result in gene activation. One important post-transcriptional pathway for the regulation of innate immunity is mediated by the intracellular NLRs [41,42]. These cytosolic pattern recognition receptors induce the activation of the protease caspase-1 through the assembly of large protein complexes called inflammasomes. One of the NLRs, cryopyrin/Nalp3, mediates caspase-1 activation and the processing and secretion of the cytokines interleukin 1β (IL-1β) and IL-18 in response to bacterial and viral RNA, poly I:C and the imidazoquinolines R837 and

R848 as well as to viral infection [43]. Whether cryopyrin/Nalp3 directly recognizes RNA and imidazoquinoline is unclear, because no direct association has been reported. NLRX1, a mitochondrially localized NLR, plays a negative role in antiviral immunity by antagonizing the interaction between RIG-I and IPS-1, suggesting that NLRX1 functions as a modulator of the responses to pathogen components rather than as a receptor that regulates antiviral innate immunity [44].

Several enzymes whose activities depend on the presence of dsRNA are also involved in innate immunity. One mechanism involves two enzymes, protein kinase R (PKR) and general control nonderepressible-2 (GCN-2), which phosphorylate the α subunit of translation initiation factor 2, leading to the downregulation of protein translation, and therefore of virus replication, within infected cells [45]. In PKR-deficient mice, antiviral responses are reduced when dsRNA or interferon are given along with the virus as coactivators. However, PKR deficiency does not affect the induction of type I interferons by dsRNA and viruses, suggesting that PKR is acting as an effector of interferon action rather than in the induction of interferon synthesis. Another protein that is stimulated by dsRNA is 2'-5' oligoadenylate synthetase, an enzyme that synthesizes short oligoadenylates that in turn activate the endoribonuclease RNase L. Upon activation, RNase L promotes the cleavage of both cellular and viral RNAs. As with PKR, the analysis of RNase L-deficient mice revealed the requirement for this enzyme in interferon-dependent antiviral actions [46]. Although PKR and RNase L become activated by dsRNA and are implicated in antiviral immunity, they are mainly effectors of interferon action and are dispensable for interferon production, and generally are not defined as pattern recognition receptors.

Sensing of self nucleic acids can result in autoimmunity

Although all the defense mechanisms described above are designed for the recognition of microbial nucleic acids, they can in some circumstances lead to the recognition of host DNA and RNA and the development of autoimmunity [47]. TLRs specific for nucleic acids are normally localized to the endosomal compartment, which may be the safeguard against contact with self DNA [5]. Unfortunately for the host, endogenous RNA and DNA are also able to activate TLR7 and TLR9 if they enter the endosomal compartment. A rich potential source of self RNA and DNA are the remains of host cells that have died via necrosis or apoptosis. Normally such apoptotic debris appears to be cleared rapidly by macrophages, which in humans do not express TLR7 or TLR9. But if there is a delay in apoptotic clearance, or if there are autoantibodies that interact with the antigens exposed on the apoptotic cells, this material can be misdirected to pDCs and B cells, where the nucleic acids can activate TLR7 and/or TLR9, resulting in the secretion of type I interferons by pDCs and the differentiation of B cells into plasma cells. Such a mechanism is likely to be the cause of pDC secretion of type I interferons in systemic lupus erythematosus (SLE) patients, in which increased serum concentrations of interferon-α correlate with disease activity and probably contribute to disease pathogenesis. In the mouse model of SLE, the genetic locus Y chromosome-linked autoimmune accelerator (*Yaa*) acts as a disease accelerator, promoting SLE in genetically susceptible mouse strains. Recent genetic studies revealed that *Yaa* is a duplication and translocation of the TLR7 gene from the X chromosome onto the Y chromosome, increasing the dosage of the TLR7 gene in the cell [48,49]. Further studies showed that the gene dosage of TLR7 is directly related to the risk of autoimmunity [50].

TLR-independent mechanisms for the recognition of self nucleic acids also contribute to autoimmunity. Deoxyribonuclease II (DNase II) in macrophages cleaves the DNA of engulfed apoptotic cells and of engulfed nuclei that have been expelled from erythroid precursor cells. In mice deficient in DNase II, genomic DNA cannot be degraded and accumulates in the macrophage phagosome, leading to TLR-independent, interferon-mediated autoimmune pathology [51]. Whether DAI or other cytosolic DNA sensors contribute to such autoimmunity awaits further investigation.

The discovery of pattern recognition receptors has thus revolutionized our understanding of innate immunity, explaining why and how multiple and diverse infectious agents are recognized by a limited number of innate immune receptors that trigger antimicrobial responses [1]. We are beginning to appreciate how a variety of such receptors at distinct cellular localizations recognize non-self nucleic acids derived from infectious microorganisms and initiate a proper immune response against them, while at the same time maintaining tolerance to self DNA and RNA to prevent development of autoimmunity. How such a delicate balance is established is not well understood and it probably involves both the receptors themselves and the way host nucleic acids are handled in the cell. For example, sequestration of certain TLRs in the endosomal compartments and packaging of mammalian DNA into high-order chromatin structure may both prevent the accidental activation of innate responses to self nucleic acids. It will also be important to dissect the molecular mechanisms and structural basis of the interactions of these receptors with their ligands, a task that should become easier following the recent elucidation of the structures of several TLRs [52-55]. Finally, the mechanisms underlying the sensing of cytosolic DNA by DAI and other possible factors have not yet been established and await further investigation. Eventually, research on innate recognition of non-self nucleic acids is likely to be translated into the development of new strategies for the prevention and therapy of infectious and autoimmune diseases.

Acknowledgements

We thank Fran Manzo for assistance with editing and Thirumala-Devi Kanneganti for critical reading of the manuscript. HC acknowledges support from the Arthritis Foundation and NIH (K01AR053573). RAF is an investigator of the Howard Hughes Medical Institute.

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