Sensing the hybrid—a novel PAMP for TLR9

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The host immune response is initiated by pattern recognition receptors (PRR) when triggered by specific ligands known as pathogen-associated molecular patterns (PAMP). Toll-like receptor 9 is highly expressed in plasmacytoid dendritic cells and is well described as a PRR for CpG DNA which induce the production of cytokines and type I interferons. In this issue of The EMBO Journal, Rigby *et al* identify RNA–DNA hybrids as a novel PAMP specifically recognized by TLR9 in dendritic cells.

See also: **RE Rigby *et al*** (March 2014)

The ability of the organism to re-establish homeostasis after infections relies on the immune system and its capacity to detect, oppress, and eliminate infection. The innate immune system uses germ line-encoded pathogen recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMP) or danger-associated molecular patterns (DAMPs) which initiate the immune response. Triggering of a PRR leads to secretion of cytokines and interferons (IFNs), which activate the immune response against virus infections, but may also lead to autoimmune disease and immunopathology if inadequately regulated (Schenten & Medzhitov, 2011). Several PRRs that recognize nucleic acids are known to survey different cellular compartments and are therefore often thought of as endosomal or cytoplasmic PRRs. Equally important to the cellular location of the PRR–PAMP interaction is the specific molecular structure of a nucleic acid species being recognized. It is well established to some molecular detail how RNA is

detected by cytoplasmic RIG-I-like receptors (RLRs) or by endosomal TLRs (Kawai & Akira, 2008). Cytoplasmic localization of DNA is also a potent stimulator of innate immune reactions and involves DNA binding by cGAS and stimulation of a pathway dependent on the signaling molecule STING, eventually leading to IFN expression (Paludan & Bowie, 2013). The role of other proposed DNA sensors, such as IFI16, in this pathway represents an outstanding question in this field (Unterholzner *et al*, 2010). The 3′-prime repair exonuclease 1 (TREX1), which degrades reverse-transcribed products from endogenous retroviruses and also regulates the immune response to retrovirus infections, represents an important negative regulator of STING-dependent signaling. In humans, genetic deficiency for Trex1 results in the autoimmune disorder, Aicardi–Goutières syndrome (AGS) and Trex1-deficient mice also develop autoimmune disorders (Crow & Rehwinkel, 2009). Interestingly, AGS has also been linked to mutations in three different subunits of the RNase H enzyme, which is responsible for digestion of RNA in RNA–DNA hybrids, thus suggesting that accumulation of RNA–DNA hybrids in RNase H-deficient patients triggers inflammatory responses (Crow & Rehwinkel, 2009). However, the RNA–DNA hybrid has not been characterized as a PAMP, and no PRR detecting this molecular signature has been described.

TLR9 is well established as a PRR for endosomal DNA and is known to recognize unmethylated CpG motifs on thioester backbone, and the 2′-deoxyribose backbone on natural DNA (Haas *et al*, 2008; Yasuda *et al*, 2009). In the present issue of *The EMBO Journal*, Rigby *et al* identify the RNA–DNA

hybrid as a novel PAMP which induce cytokine and IFN responses when detected by TLR9 in mouse dendritic cells (DCs) (Rigby *et al*, 2014) (Fig 1). The newly discovered PAMP opens up new possibilities for TLR9 as an important PRR in retroviral infections as well as in the onset of autoimmune diseases such as AGS, psoriasis, and Systemic lupus erythematosus (Crow & Rehwinkel, 2009).

With the fundamental hypothesis that RNA–DNA hybrids constitute an additional class of immunostimulatory nucleic acids species, Rigby *et al* establish a method to obtain highly purified synthetic RNA–DNA hybrid (R:D60) which contains no significant amount of unannealed single-stranded (ss) RNA or ssDNA. The authors target R:D60 for intracellular delivery by lipofection and show that R:D60 induce inflammatory cytokines and IFN α responses in conventional DCs and plasmacytoid (p) DCs derived from Fms-like tyrosine kinase 3-ligand-differentiated bone marrow-derived DCs. Use of cells from knockout mice shows that TLR9 and MyD88 but not TLR7 or RLRs are essential to the response to R:D60. Hereafter, it is carefully excluded that the response is due to contamination from the purification process and shown that neither unannealed ssRNA nor ssDNA are able to induce TLR9-dependent immune responses. Importantly, the authors show that the RNA–DNA duplex remains intact following delivery to the endolysosomal compartment and report a high affinity of the ectodomain of TLR9 for RNA–DNA hybrid exceeding the affinity for the conventional DNA ligand. The study is concluded with experimental evidence that RNA–DNA hybrids do accumulate in

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Figure 1. Nucleic acid recognition by TLRs and cytosolic sensors.

Endogenous or exogenous nucleic acid structures can be detected by PRRs in the cytoplasm and endosomes to activate signaling stimulating expression of type I IFNs and inflammatory cytokines. The article by Rigby *et al* identifies the RNA:DNA hybrid as a PAMP for TLR9.

cytoplasmic and endosomal fractions for B3T3 fibroblast-like cells infected with the retrovirus Moloney murine leukemia virus suggesting a potential role for TLR9 in the recognition of retrovirus infection. With this work, Rigby *et al* are first to firmly establish RNA–DNA hybrids as PAMPs and identify TLR9 as its PRR in DC and hereby raise the important question whether TLR9 may be a physiologically relevant PRR in the defense against retrovirus infections (Mackelprang *et al*, 2014). The key questions raised by the work presented here include whether human TLR9 shares the dual PAMP specificity now evident for the murine receptor, which would allow for TLR9 to play a novel role in inflammatory disease. The newly identified PAMP may originate from retrovirus infections or from reverse transcription of endogenous retroviral elements, which have been suggested to accumulate in RNase H-deficient AGS patients (Crow & Rehwinkel, 2009). Future studies need to reveal how the RNA–DNA hybrid reaches the endosome

possibly by autophagy or through uptake via an endosomal route (Lee *et al*, 2007). In addition, it is key to now understand whether the RNA–DNA PAMP actually accumulates in endosomal compartments in cells expressing TLR9, such as pDCs, and whether this stimulates innate immune responses. In addition to endogenous and exogenous retroviruses, the RNA–DNA hybrid may also accumulate during infections with hepatitis B virus, thus representing another globally important human disease where the new knowledge may become of importance (Summers & Mason, 1982). The discovery of the RNA–DNA hybrid as a novel class of immunostimulatory nucleic acids also opens up the possibility of the existence of a cytoplasmic receptor for this PAMP. Further work is needed to clarify these issues.

All together, the work by Rigby *et al* establishes the RNA–DNA hybrid as a *bona fide* PAMP for TLR9 and opens up new questions regarding its role in host defense and inflammatory diseases.

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