RECONsidering sensing of cyclic di-nucleotides

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Summary

Detection of cyclic di-nucleotides (cdNs) by the STING pathway potently triggers type I interferon production and an anti-viral response. McFarland *et al.* now show that the mouse oxidoreductase RECON is a sensor for some bacterial cdNs and modulates innate signalling in a manner independent of STING to promote an anti-bacterial state.

Cyclic dinucleotides (cdNs) are bacterial second messengers that regulate many aspects of bacterial biology. For example, the intracellular bacterium *Listeria monocytogenes* produces cyclic di-AMP (c-di-AMP), which controls the bacterium's metabolism (Sureka et al., 2014). *L. monocytogenes* also secretes c-di-AMP into mammalian host cells, where it is detected by the innate immune system: c-di-AMP is an agonist for the pattern recognition receptor STING (STimulator of INterferon Genes) (Sauer et al., 2011; Woodward et al., 2010). Activation of STING triggers type I interferon (IFN) production, typically associated with viral infections. Experimental manipulation of c-di-AMP secretion by *L. monocytogenes* impacts bacterial growth in mouse models: reduced c-di-AMP secretion results in increased bacterial replication, and *vice versa* (Crimmins et al., 2008; Woodward et al., 2010). Surprisingly, however, the replication of *L. monocytogenes* is largely unchanged in mice with a non-functional *Sting* gene (Sauer et al., 2011). This observation indicates the existence of an additional sensor for c-di-AMP. In this issue of *Immunity,* McFarland *et al.* report that the mouse aldo-keto reductase family 1 member C13, which they rename RECON (REductase COntrolling NF-κB), acts as a receptor for c-di-AMP (McFarland et al., 2017). Binding of c-di-AMP to RECON inhibits its enzymatic activity, leading to increased activation of the pro-inflammatory transcription factor NF-κB and redirecting the cellular response towards an anti-bacterial rather than anti-viral state.

In addition to c-di-AMP, bacteria produce other cdNs including cyclic di-GMP (c-di-GMP) and cyclic GMP-AMP (3'3'-cGAMP). Much like c-di-AMP, these cdNs are STING agonists (Chen et al., 2016). Moreover, mammals are capable of synthesising cGAMP. The cytosolic DNA sensor cGAS is activated during infection with viruses or intracellular bacteria and generates an atypical 2'3'-mixed phosphodiester linkage cGAMP molecule (2'3'-cGAMP), which binds tightly to STING and thereby initiates an anti-viral state (Chen et al., 2016). To identify additional proteins that bind to cdNs, the authors employed a pull-down approach with immobilised c-di-AMP as bait and whole organ lysates from mice, followed by mass-spectrometry. The aldo-keto reductase RECON was

highly enriched in c-di-AMP pull-downs from liver lysates. Remarkably, the affinity of RECON for c-di-AMP was more than an order of magnitude higher than that of STING. Interestingly, McFarland *et al.* find that, in contrast to STING, the binding spectrum of RECON is confined to the bacterial cdNs c-di-AMP and 3'3'-cGAMP. As explained later, this degree of selectivity of RECON and STING for cdNs shapes the host's response to intracellular bacteria versus viruses.

Aldo-keto reductases are a family of enzymes that reduce the carbonyl groups of a variety of substrates and use NAD(P) as a cofactor (Penning, 2015). McFarland *et al.* find that binding to c-di-AMP and 3'3'-cGAMP inhibits RECON's enzymatic activity. In agreement with its binding spectrum, c-di-GMP and 2'3'-cGAMP do not modulate the enzymatic activity of RECON. To examine the molecular underpinnings of this inhibition, the authors solve the crystal structure of RECON complexed with c-di-AMP. Comparison of this structure with a previously solved structure of RECON with its cofactor NAD reveals that c-di-AMP not only competes with NAD for binding to the same pocket, but also induces a rearrangement of the catalytic site.

The authors next ask whether the inhibition of RECON activity by c-di-AMP has a role in the detection of *L. monocytogenes* by host cells. Upon infection of macrophages, a wide range of innate receptors detects *L. monocytogenes* (Figure 1). TLR2 and NOD1/2 recognise bacterial cell-wall components at the plasma membrane and in the cytosol, respectively (Boxx and Cheng, 2016). STING is both directly activated by c-di-AMP secreted by the bacterium (Sauer et al., 2011; Woodward et al., 2010) and by 2'3'-cGAMP synthesised by cGAS upon detection of bacterial DNA (Hansen et al., 2014). These signalling pathways activate the transcription factor NF-κB, which promotes the expression pro-inflammatory cytokines including TNF and IL-1β as well as bactericidal genes such as inducible nitric oxide synthase (NOS2). In addition, STING activates the transcription factor IRF3 and thereby triggers an anti-viral response characterised by type I IFN expression. McFarland *et al.* find that infection of murine macrophages with reduced expression of RECON *via* RNAi leads to increased induction of type I IFN, cytokines, chemokines, NOS2 and its

product, NO, while RECON overexpression has opposite effects. These observations suggest that RECON negatively regulates the sensing pathways responding to *L. monocytogenes* infection.

To examine the mechanisms underlying the inhibition of the innate response to *L. monocytogenes* by RECON, McFarland *et al.* compare type I IFN induction triggered by different cdNs. Ectopic expression of RECON strongly diminishes the IFN response in mouse macrophages after c-di-AMP stimulation, whereas the response to 2'3'-cGAMP – that is not bound by RECON – is largely unchanged. This suggests that RECON traps cdNs and thereby prevents activation of STING (Figure 1). This mechanism of cdN sequestration, however, does not account for the observation that depletion of RECON enhances induction of NF-κB target genes when macrophages are stimulated with purified TLR2 and NOD1 agonists. Moreover, the authors report that NOD1 stimulation and c-di-AMP synergistically induce NO production. Remarkably, this response was STING-independent at higher c-di-AMP doses. These observations lead the authors to postulate that RECON inhibits NF-κB signalling.

To investigate this in more detail, McFarland *et al.* use hepatocytes, which express high levels of RECON but not STING and provide a niche for *L. monocytogenes* replication *in vivo*. CRISPR/Cas9-mediated deletion of *Akr1c13* in the hepatocyte cell line TIB73 augments the activation of NF-κB target genes including *Nos2* in response to *L. monocytogenes* infection and purified TLR2 and NOD1 stimuli. In line with this observation, other markers of NF-κB activation such as NF-κB p65 nuclear translocation and IKKα phosphorylation were also enhanced in infected *Akr1c13*$^{-/-}$ cells. Interestingly, reconstitution of these cells with a catalytically inactive RECON mutant that retains c-di-AMP binding shows that enzymatic activity is required for blockade of NF-κB signalling. Taken together, Woodward and colleagues conclude that binding of c-di-AMP to RECON shifts the innate immune response to *L. monocytogenes* towards pro-inflammatory and away

from anti-viral gene expression *via* a dual mechanism involving reduced STING and enhanced NF-κB activation (Figure 1).

The discovery of RECON as a sensor of some bacterial cdNs is remarkable in a number of ways and poses several interesting questions. Firstly, it shows that the STING signalling axis, which may include DDX41 that binds to some cdNs (Parvatiyar et al., 2012), is not the only cdN sensing system. Secondly, RECON's enzymatic activity is inhibited by bacterial cdNs, demonstrating that microbial ligands of innate receptors can shape innate immunity not only as agonists that activate responses, but also as antagonists. An important question for future work pertains to the nature of the substrate modified by RECON, and how this molecule impacts NF-κB signalling in the context of different upstream stimuli. Thirdly, antiviral type I IFN responses upon infection with intracellular bacteria such as *L. monocytogenes* and *Mycobacterium tuberculosis* are associated with increased pathology (Boxx and Cheng, 2016). It is possible that bacterial c-di-AMP exacerbates this detrimental immune response by enhancing the activation of STING and tempting to speculate that RECON evolved to divert innate signalling away from a full-on anti-viral response. It can do so in two ways: by sequestering c-di-AMP from STING and by modulating NF-κB signalling. It is noteworthy that several allelic variants of the gene encoding STING have been found in human, and that some of these variants detect 2'3'-cGAMP derived from cGAS but not bacterial cdNs (Chen et al., 2016). This may reflect an adaptation to prevent anti-viral IFN responses during bacterial infection. Fourthly, it is interesting that the *AKR1C* gene family, to which RECON belongs, includes four genes in humans but is expanded in mice without clear 1-to-1 orthologues between species (Penning, 2015). Whether this reflects host-pathogen co-evolution and if a human *AKR1C* gene with similar functions as mouse RECON exists are important open questions. Finally, the relative contribution of the cdN sensors STING and RECON to immunity *in vivo* remains to be tested. RECON-deficient animals and mice expressing STING variants unable to bind bacterial cdNs will be important tools.

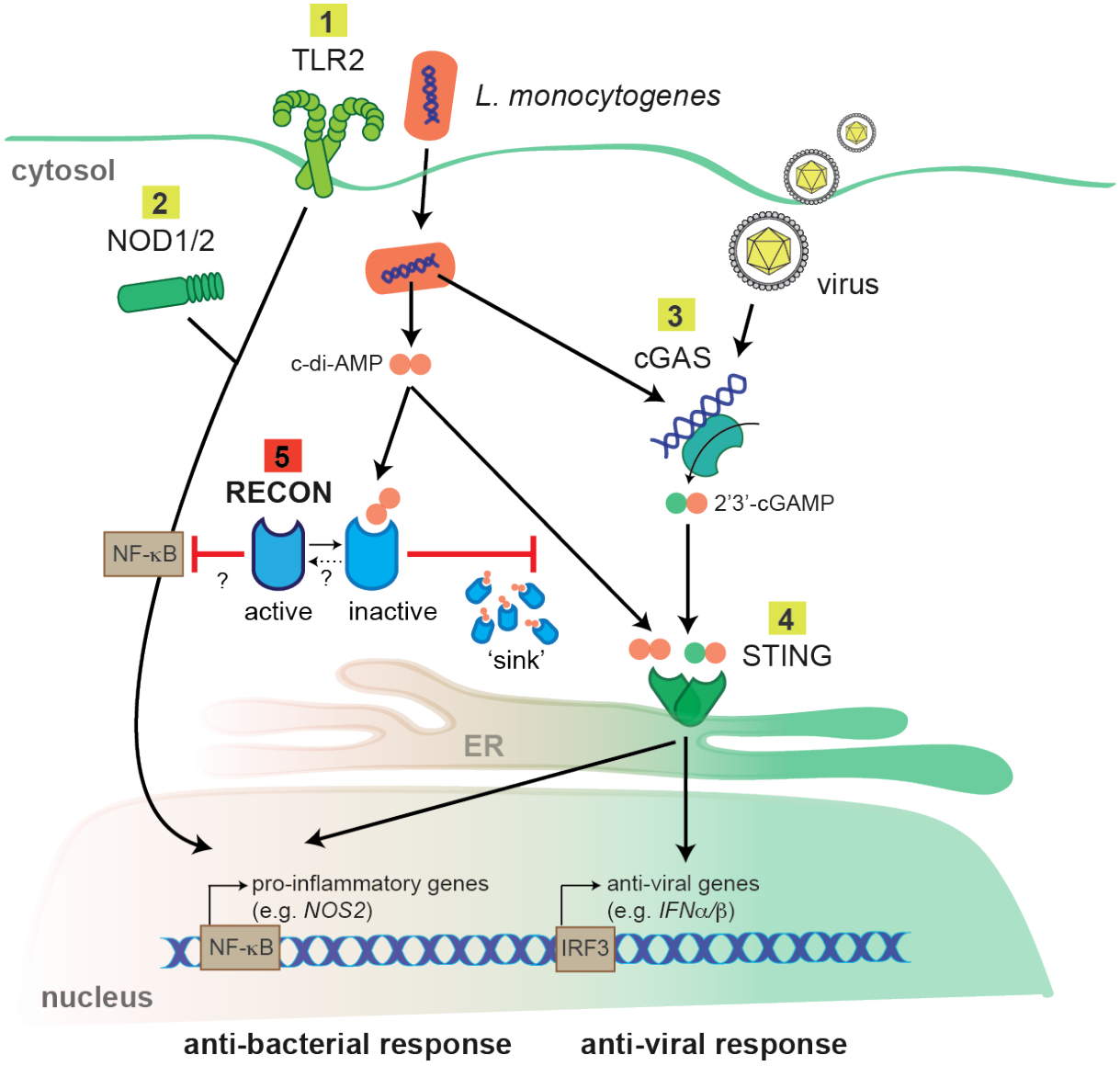

Figure 1. Binding of c-di-AMP to RECON potentiates the pro-inflammatory anti-bacterial response upon *Listeria monocytogenes* infection.

Innate immune sensors detecting *L. monocytogenes* infection are shown. These include TLR2 (1), NOD1/2 (2), cGAS (3) and STING (4). McFarland *et al.* report that RECON (5) recognizes c-di-AMP produced by *L. monocytogenes*. This dampens STING-dependent type I interferon induction by sequestration of c-di-AMP and also facilitates the induction of anti-bacterial genes *via* NF-κB.

References

Boxx, G.M., and Cheng, G. (2016). The Roles of Type I Interferon in Bacterial Infection. Cell host & microbe *19*, 760-769.

Chen, Q., Sun, L., and Chen, Z.J. (2016). Regulation and function of the cGAS-STING pathway of cytosolic DNA sensing. Nature immunology *17*, 1142-1149.

Crimmins, G.T., Herskovits, A.A., Rehder, K., Sivick, K.E., Lauer, P., Dubensky, T.W., Jr., and Portnoy, D.A. (2008). Listeria monocytogenes multidrug resistance transporters activate a cytosolic surveillance pathway of innate immunity. Proceedings of the National Academy of Sciences of the United States of America *105*, 10191-10196.

Hansen, K., Prabakaran, T., Laustsen, A., Jorgensen, S.E., Rahbaek, S.H., Jensen, S.B., Nielsen, R., Leber, J.H., Decker, T., Horan, K.A., *et al.* (2014). Listeria monocytogenes induces IFNbeta expression through an IFI16-, cGAS- and STING-dependent pathway. The EMBO journal *33*, 1654-1666.

McFarland, A.P., Luo, S., Ahmed-Qadri, F., Zuck, M., Thayer, E.F., Goo, Y.A., Hybiske, K., Tong, L., and Woodward, J.J. (2017). Sensing of bacterial cyclic dinucleotides by the oxidoreductase RECON promotes NF-κB activation and shapes a proinflammatory antibacterial state. Immunity.

Parvatiyar, K., Zhang, Z., Teles, R.M., Ouyang, S., Jiang, Y., Iyer, S.S., Zaver, S.A., Schenk, M., Zeng, S., Zhong, W., *et al.* (2012). The helicase DDX41 recognizes the bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate a type I interferon immune response. Nature immunology *13*, 1155-1161.

Penning, T.M. (2015). The aldo-keto reductases (AKRs): Overview. Chemico-biological interactions *234*, 236-246.

Sauer, J.D., Sotelo-Troha, K., von Moltke, J., Monroe, K.M., Rae, C.S., Brubaker, S.W., Hyodo, M., Hayakawa, Y., Woodward, J.J., Portnoy, D.A., and Vance, R.E. (2011). The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to Listeria monocytogenes and cyclic dinucleotides. Infection and immunity *79*, 688-694.

Sureka, K., Choi, P.H., Precit, M., Delince, M., Pensinger, D.A., Huynh, T.N., Jurado, A.R., Goo, Y.A., Sadilek, M., Iavarone, A.T., *et al.* (2014). The cyclic dinucleotide c-di-AMP is an allosteric regulator of metabolic enzyme function. Cell *158*, 1389-1401.

Woodward, J.J., Iavarone, A.T., and Portnoy, D.A. (2010). c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response. Science *328*, 1703-1705.