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Review article

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Manipulation of the nucleotide pool in human, bacterial and plant immunity

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Abstract

The cell-autonomous innate immune system is responsible for sensing and mitigating viral infection at the level of individual cells. Many of the mechanisms used by the cell-autonomous innate immune system in eukaryotic cells are ancient and have evolutionary roots in bacterial systems that defend against phage infection. Studies from recent years have shown that modification of the free nucleotide pool is central to many of these conserved immune mechanisms. In this Review, we explain how immune pathways manipulate the available pool of nucleotides to deprive viruses of molecules essential for their replication, how immune proteins chemically modify nucleotides to generate immune signalling molecules, and how cell-autonomous innate immune mechanisms produce altered nucleotides that poison viral replication. We also discuss the mechanisms used by viruses to antagonize nucleotide-based immunity. Finally, we explore the evolutionary logic of using nucleotides as building blocks for immune responses.

Sections

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Introduction

Cell-autonomous innate immunity enables individual cells to sense and respond to viral infection before systemic immune responses have been activated[1,2]. Human cells encode a large number of sensors for viral invasion, including Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs), which can sense viral nucleic acids, proteins or other molecules as a signature of infection[3,4]. These sensors then activate signalling cascades whose typical outcome in human cells is the expression of multiple antiviral genes and, in some cases, the secretion of signalling factors such as interferons that can have effects on neighbouring cells[5]. Cell-autonomous innate immunity is a property shared across all domains of life[2].

Studies from recent years have shown that many key components of cellular immunity are conserved between bacteria, animals and plants[6,7]. Phylogenetic evidence suggests that these components first evolved in bacteria as anti-phage defence mechanisms and were then either inherited by early eukaryotes during the process of eukaryogenesis[7], or subsequently horizontally transferred from prokaryotes to eukaryotes[8,9]. Immune mechanisms that are conserved from bacteria to higher eukaryotes include the cyclic guanosine monophosphate (GMP)–adenosine monophosphate (AMP) synthase (cGAS)–stimulator of interferon genes (STING) pathway[10], RNA interference[11], inflammatory pathways such as pyroptosis[12], and many others[13].

With the recent expansion in our understanding of antiviral mechanisms in animals, plants and particularly bacteria, the free nucleotide pool is emerging as a central hub for viral mitigation and immune signalling. It has been shown that multiple immune pathways manipulate the free nucleotide pool to deprive viruses of molecules essential for their life cycle, use nucleotides as building blocks for immune signalling molecules, and modify nucleotides to form molecules that poison viral replication.

This Review article focuses on the role of chemical modification of free nucleotides in antiviral innate immunity across the tree of life. We discuss the diverse mechanisms by which immune pathways manipulate nucleotides and describe the various countermeasures that viruses have evolved to mitigate nucleotide-based immunity. Finally, we propose an evolutionary model to explain why nucleotides became a central axis of innate immunity that remained conserved from bacteria to plants and animals.

Manipulation of the nucleotide pool

Studies of both prokaryotic and eukaryotic immunity show that manipulating the cellular pool of free nucleotides is a successful strategy for inhibiting viral replication. Multiple pathways were found to specifically deplete a single type of nucleotide in response to viral infection, thus starving the virus of a molecule essential for its life cycle. Here, we detail how immune factors restrict viral replication by depleting deoxynucleotide triphosphates (dNTPs), adenosine triphosphate (ATP) or nicotinamide adenine dinucleotide (NAD^+).

Deoxynucleotide depletion

There are several cell-autonomous antiviral factors that are known to restrict viral replication by eliminating one of the available dNTPs, or all dNTPs, from the nucleotide pool. These include the sterile alpha motif and HD domain-containing protein 1 (SAMHD1) in human cells[14–16] and two families of antiviral enzymes in bacteria, comprising deoxycytidine triphosphate (dCTP) deaminases and deoxyguanosine triphosphate (dGTP) triphosphohydrolases (dGTPases)[17,18]. The principle of immunity in these cases is straightforward: in the absence of one or more dNTPs in the cell, the virus cannot successfully polymerize its genome. By contrast, host cells may be able to survive without requiring DNA replication until the virus is cleared by other means[19,20].

Human SAMHD1 restricts viral infection in non-dividing cells by depletion of all four dNTPs[14–16]. This is achieved by cleaving the triphosphate group from the deoxyribose-base moiety, thus converting dNTPs to deoxynucleosides that cannot be used for DNA replication[14–16] (Fig. 1a). SAMHD1-mediated dNTP depletion restricts DNA viruses and retroviruses that require dNTPs for their replication, and it has been particularly well characterized in the context of lentivirus infection; in cells expressing SAMHD1, dNTPs are scarce, which means that reverse transcription of the lentiviral genome becomes inefficient[14,15,21,22]. Some lentiviruses, such as HIV-2 and simian immunodeficiency virus, overcome SAMHD1-mediated restriction by encoding Vpx, a protein that specifically targets SAMHD1 for proteasomal degradation[23–26]. Multiple copies of Vpx are packaged in the virion and are released into the host cell after infection[27].

Bacteria encode dNTPases that show distant structural homology to SAMHD1 and have the same enzymatic activity of separating the triphosphate group from the deoxyribose base of the deoxynucleoside[28–30]. Some bacterial dNTPases have a strong preference for dGTP as their substrate[31–33], and a subset of these dGTPases have potent antiviral activity[18]. During phage infection, antiviral dGTPases in bacteria deplete the cell of dGTP, resulting in stalling of the DNA polymerase and inhibition of phage genome replication (Fig. 1a). Activation of antiviral dGTPases in *Escherichia coli* was suggested to occur in response to general transcription inhibition, which is often induced by phages as part of the host cell takeover process[18] (Fig. 1a). Some phages, including the coliphage T7, encode proteins that specifically bind and inhibit bacterial dGTPases[34,35].

Intriguingly, in addition to their roles in antiviral immunity, both SAMHD1 and bacterial dGTPases were shown to have a housekeeping role in regulating DNA repair. SAMHD1 is activated downstream of the DNA damage pathway[36] and localizes to DNA damage foci[37], wherein it promotes homologous recombination by recruiting the repair machinery to sites of double-stranded DNA (dsDNA) breaks[38]. Similarly, some bacterial dGTPases have been implicated in maintaining genomic stability and may participate in DNA repair processes[39]. As efficient DNA repair requires regulated amounts of dNTPs[40,41], it is logical that enzymes that regulate dNTP levels would participate in this process.

Another family of bacterial enzymes that confer resistance to phage infection are dCTP deaminases[17,18]. When a cell senses phage infection, these enzymes replace the amine residues of dCTP nucleotides with a carbonyl group, converting them into deoxyuridine triphosphates (dUTPs) and rendering the cell devoid of dCTP (Fig. 1a). dUTP is then rapidly converted into deoxyuridine monophosphate (dUMP), most probably by bacterial housekeeping enzymes[17,18]. Phage-mediated shutdown of cellular transcription was shown to trigger bacterial dCTP deaminase activity[17,18,42], similar to what was observed for the activity of bacterial antiviral dGTPases[18].

Manipulation of cellular ATP levels

Another category of immune factors that manipulate the nucleotide pool are those that alter ATP, which is essential for energy metabolism in all cell types. Various bacterial defence proteins contain an ATP nucleosidase domain that cleaves ATP and deoxyATP (dATP) molecules, separating the nucleobase from the sugar moiety[43] (Fig. 1b). The ATP nucleosidase domain is found in bacterial NLR-like proteins, cyclic-oligonucleotide-based anti-phage signalling systems (CBASS),

short prokaryotic Argonautes, defence-associated reverse transcriptase type 8 (DRT8), and Detocs[43]. In response to phage infection, these systems degrade ATP and dATP in infected cells. The reduction of ATP levels during the course of phage infection may cause cell death before the phage has completed its replication cycle, possibly by prematurely triggering the phage lysis machinery (the process by which

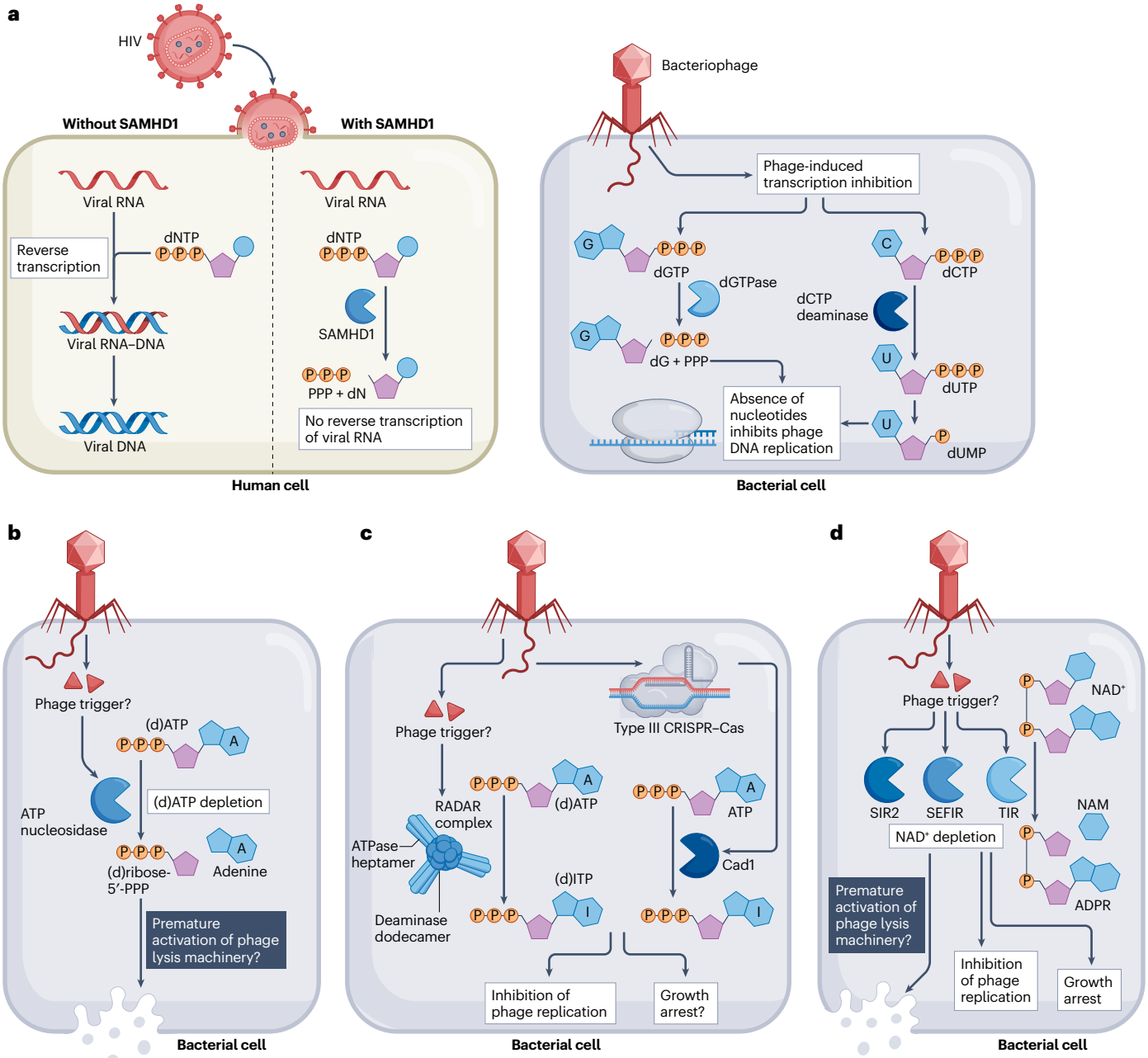


Fig. 1 | Nucleotide depletion as an antiviral strategy. 'Phage trigger' represents unknown phage components or phage-induced changes in cellular state. **a**, Deoxynucleotide triphosphate (dNTP) depletion. The human protein sterile alpha motif and HD domain-containing protein 1 (SAMHD1) cleaves triphosphate groups from dNTPs, thus depleting dNTPs and inhibiting reverse transcription of HIV RNA. In bacteria, deoxyguanosine triphosphate (dGTP) triphosphohydrolase (dGTPase) and deoxycytidine triphosphate (dCTP) deaminase enzymes deplete dGTP and dCTP, respectively, thereby preventing phage DNA replication. **b**, Degradation of adenosine triphosphate (ATP) and deoxyATP (dATP) by ATP nucleosidases renders the cell depleted of energy. In bacteria, ATP depletion is thought to also cause premature activation of the phage lysis machinery. **c**, The RADAR (restriction by an adenosine deaminase acting on RNA) defence system, and some versions of type III CRISPR–Cas systems, convert (d)ATP to (deoxy)inosine triphosphate (d)ITP upon sensing infection. ITP is thought to poison the nucleotide pool and lead to growth arrest and inhibition of phage replication. RADAR is composed of up to 12 RdrA ATPase heptamers docked to a central ball-shaped dodecamer structure formed by the deaminase RdrB. **d**, Defence systems with sirtuin (SIR2), SEFIR and Toll–interleukin receptor (TIR) effector domains degrade nicotinamide adenine dinucleotide (NAD^+) to ADP-ribose (ADPR) and nicotinamide (NAM) in response to phage infection. NAD^+ depletion inhibits phage replication and, in many cases, causes growth arrest or activates premature lysis of the infected cell. Cad1, CRISPR-associated adenosine deaminase 1; dUMP, deoxyuridine monophosphate.

phages induce the rupture of host cells to release newly synthesized viral particles)[43,44]. It is possible that ATP depletion has multiple effects on inhibiting phage propagation, serving both to deprive the phage of energy and nucleotide building blocks and to dysregulate its lysis[43].

ATP nucleosidase domains have also been observed in proteins from animals such as insects, corals and sponges, as well as in fungi, wherein they are frequently present in proteins suggested to be involved in innate immunity. For example, hundreds of NLR-like proteins in eukaryotes contain ATP nucleosidase domains at the amino terminus of the protein[43]. Other domains that are fused to ATP nucleosidase domains in eukaryotic proteins include STING, gasdermin and Death domains, which suggest that these proteins have immune functions[43]. An ATP nucleosidase domain in an NLR-like protein from the fungus *Hyaloscypha variabilis*, as well as a similar domain in an immune-like protein from the sponge *Amphimedon queenslandica*, was shown to have ATP and dATP cleavage activities, confirming that the specificity of this domain towards adenine nucleotides is conserved between bacterial and eukaryotic immune proteins[43].

Phylogenetic analyses suggest that the immune-related ATP nucleosidase domain evolved from the purine nucleoside phosphorylase (PNP) family of proteins[43]. PNP proteins have diverse housekeeping functions in nucleotide metabolism and recycling pathways in both prokaryotes and eukaryotes[45]. ATP nucleosidase sequences that are involved in host defence form a subclade within the larger phylogenetic tree of the PNP family; tree topology suggests that the immune function of ATP nucleosidases first emerged in bacteria and was later acquired by eukaryotes, possibly on multiple occasions[43].

The bacterial defence system RADAR (restriction by an adenosine deaminase acting on RNA) manipulates the ATP pool in a manner different from ATP nucleosidases (Fig. 1c). This system does not break apart the nucleotide but instead deaminates the adenine base, converting ATP and dATP into inosine triphosphate (ITP) and deoxyITP (dITP), respectively. The RADAR system consists of an ATPase protein (RdrA) and an adenosine deaminase (RdrB)[46]. Recent studies have shown that after infection, the adenosine deaminase of the RADAR complex generates large quantities of ITP and dITP, and that this process blocks viral replication. ATP and dATP are not eliminated from the cell during the course of RADAR activity[47], and thus it was hypothesized that the inosine derivatives somehow poison the nucleotide pool of the cell to inhibit phage replication[47,48].

Intriguingly, the RADAR system forms a giant supramolecular complex in vitro, whereby RdrB is assembled into a dodecameric ball-shaped structure with 12 surface-exposed deaminase active sites. RdrA forms heptameric rings, each fitting on top of one face of the deaminase dodecamer. In full occupancy of the complex, 12 RdrA heptamers can dock on the RdrB core, forming a 10-megadalton complex[47,48]. However, it remains unclear whether this complex forms in vivo and, if so, why such an elaborate complex might be necessary for the protective capacity of RADAR.

ATP-to-ITP conversion has also been described for a subset of type III CRISPR–Cas systems[49] (Fig. 1c). Like other CRISPR–Cas systems, type III systems recognize infection by base-pairing of the CRISPR guide RNA with the matching nucleic acid sequence of an invading phage[50]. In addition to recognition and cleavage of the target phage nucleic acid, type III CRISPR–Cas synthesizes a signalling molecule that activates other proteins in the system (discussed later in the section 'Production of cyclic oligoadenylates by type III CRISPR–Cas')[51,52]. One such protein is CRISPR-associated adenosine deaminase 1 (Cad1), which, once activated by the signalling molecule, converts ATP to ITP[49]. Although the exact mechanism by which the accumulation of ITP inhibits phage replication is unclear, it was shown to result in growth arrest of the bacterial cell, which eventually prevents phage propagation[49].

Conversion of ATP and dATP to (d)ITP has not been described in human cell-autonomous immunity, although mutations in adenosine deaminase enzymes that convert adenosine and deoxyadenosine into inosine and deoxyinosine have been implicated in immune disorders[53–55]. Adenosine deamination in the form of RNA and DNA editing has been identified as a defensive measure against viruses and transposons in animal cells[56].

NAD^+ depletion

Like ATP, nicotinamide adenine dinucleotide, in either oxidized (NAD^+) or reduced (NADH) forms, is an essential molecule for the energy balance of the cell[57]. This molecule comprises an adenine diphosphate (ADP) nucleotide covalently attached to a second ribose and a nicotinamide ring, and it functions as a cofactor in numerous essential metabolic processes, including oxidative phosphorylation[58], amino acid biosynthesis[59] and fatty acid biosynthesis[59].

NAD^+ depletion is a common outcome of multiple and diverse defence systems in bacteria (Fig. 1d). NAD^+ depletion can be achieved by several types of protein domains, including the sirtuin (SIR2) domain[60,61], the Toll–interleukin receptor (TIR) domain[62–66] and the SEFIR domain[67], which are NAD-specific hydrolases (NADases) that cleave NAD^+ into free nicotinamide and ADP-ribose (ADPR). SIR2, TIR and SEFIR NADase domains are present as effector modules in diverse bacterial immune systems, including CBASS, Thoeris and Pycsar (pyrimidine cyclase system for antiphage resistance), bacterial NLR-like proteins, prokaryotic Argonautes, SIR2–HerA (also known as Nezha), defence-associated sirtuin (DSR) systems, and retrons[46,61–71]. When SIR2-containing, TIR-containing or SEFIR-containing defence systems sense phage invasion, these domains become active, typically through oligomerization, and rapidly deplete NAD^+ from the infected cell. In many cases, NAD^+ depletion during phage infection was shown to result in premature death of the infected cell[60–66], possibly because depletion of cellular energy can prematurely activate the phage lysis machinery[44]. In support of this hypothesis, NAD^+ depletion in uninfected cells causes growth arrest but not cell death[60]. In some cases, depletion of NAD^+ was shown to protect against phages without causing growth arrest or cell death, and it was hypothesized that the temporary reduction of NAD^+ to low but not zero levels may be sufficient to interfere with the replication of some phages[60].

Defence systems that deplete NAD^+ are found in a substantial fraction of all sequenced bacteria[72]; correspondingly, phages have evolved multiple ways to evade this form of immunity. Some phages encode proteins that specifically bind NAD^+-depleting immune proteins and block their activation. One example is the DSR anti-defence protein 1 (DSAD1), a phage protein that inhibits the *Bacillus* SIR2-containing DSR2 protein[60,73]. Other phages have evolved biochemical pathways that reconstitute NAD^+ (ref. 72). The NAD reconstitution pathway 1 (NARP1) is one such pathway; it uses ADPR and nicotinamide, the direct degradation products of NAD^+, as substrates to re-build NAD^+. Adps, the first enzyme in the pathway, pyrophosphorylates ADPR to form ADPR-pyrophosphate, and Namat, the second enzyme, installs nicotinamide in place of the pyrophosphate, regenerating NAD^+. A second pathway, NARP2, reconstitutes NAD^+ through classical NAD^+ salvage enzymatic reactions. It was shown that NARP1 and NARP2 enable phages to counter various defence systems that involve NAD^+ depletion, including Thoeris, DSR1, DSR2 and SIR2–HerA[72].

Evidence for immune-related NAD^+ depletion in eukaryotic cells is scarce. Human TIR domains found in TLRs are not thought to have NADase activity; instead, they generally function as scaffolding domains for protein–protein interactions that transfer downstream immune signals[74]. The human TIR domain-containing protein SARM1 does have NADase activity and depletes NAD^+ in injured neurons as part of a regulated cell death pathway known as Wallerian degeneration[75–77]. Human SARM1 is not known to function in innate immunity but a *Caenorhabditis elegans* homologue of SARM1 was shown to be necessary for defence against some pathogens[78,79]. In some cases, the catalytic activity of TIR domains does not deplete NAD^+ but rather generates immune signalling molecules, a process that is described in detail in the next section.

Nucleotides as immune signalling molecules

A large and diverse set of immune pathways in animals, plants and bacteria use nucleotides as precursors for the formation of immune signalling molecules. These pathways typically comprise a sensor for pathogen infection, which subsequently produces a nucleotide-based signalling molecule. This molecule binds to and activates a second protein in the pathway, which then executes downstream immune functions. At least four such immune signalling pathways evolved in bacteria, and some of these have been evolutionarily inherited and integrated into the core innate immune systems of animals and plants. Various types of nucleotide signal are known to be produced by immune pathways, including cyclic oligonucleotides, linear non-canonically linked oligonucleotides, cyclic mononucleotides and derivatives of ADPR. In this section, we describe the main pathways of nucleotide-based signalling and their roles in animal, plant and bacterial immunity.

Production of cyclic oligonucleotides by cGAS and CBASS

The use of cyclic oligonucleotides as immune signalling molecules was first discovered in the cGAS–STING pathway in humans and mice[80,81] (Fig. 2a). In this pathway, viral or cellular dsDNA in the cytoplasm is sensed by cGAS[80]. Activated cGAS produces a signalling molecule comprising GMP and AMP linked in an asymmetrical cyclical configuration, whereby the phosphate of AMP binds the 3′OH of the GMP ribose, whereas the phosphate of GMP binds the 2′ OH of the AMP ribose[80–85]. The resulting molecule 2′3′-cyclic GMP–AMP (2′3′-cGAMP) binds the protein STING[81–84,86], which then activates a signalling cascade that culminates in the expression of interferons and other immune proteins[87,88]. 2′3′-cGAMP can also be transferred to neighbouring cells via gap junctions or by dedicated transporters, thus functioning as an intercellular immune signal[89–92].

Further studies have shown that cGAS is a member of a larger family of immune proteins, termed cGAS-like receptors (cGLRs)[93,94] (Fig. 2a). cGLRs are widely distributed in animals and are found in nearly all metazoan phyla[95]. For example, the *Drosophila melanogaster* cGLR1 protein, which has 25% sequence identity with human cGAS[95], was shown to recognize dsRNA as a signature of viral infection[93,94]. Beyond nucleic acid sensing, members of the cGLR family are hypothesized to recognize diverse pathogen-derived signals and have been expanded by gene duplication in multiple animal lineages. For example, the coral *Stylophora pistillata* encodes 42 cGLRs and 7 STING proteins, and the bivalve *Crassostrea virginica* has 225 cGLRs and 8 STING proteins, which could potentially enable differential immune responses to various stimuli[95].

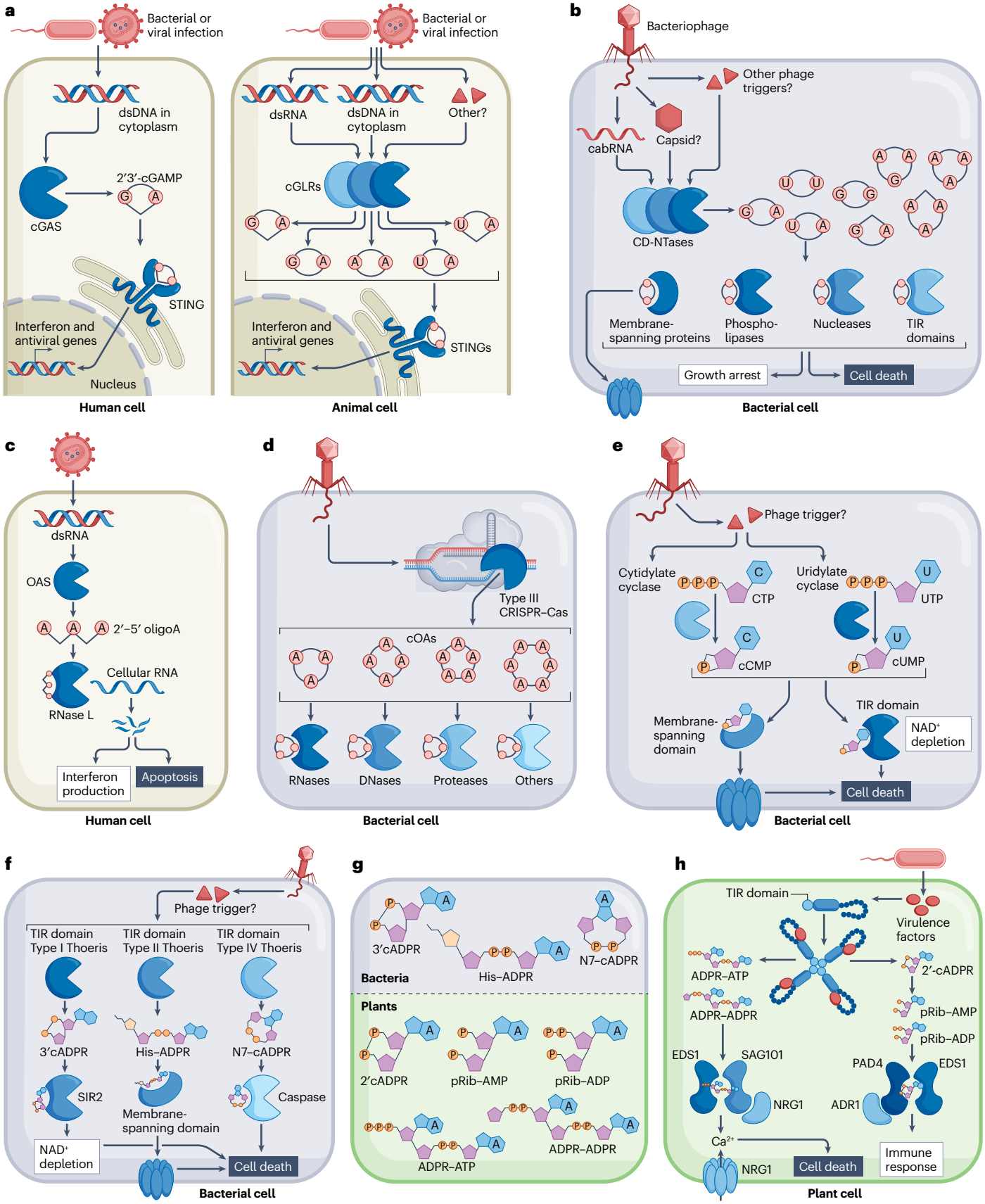
Although 2′3′-cGAMP is thought to be the major product of cGLRs[96], some cGLRs produce different types of cyclic-oligonucleotide signalling molecules that bind corresponding STING proteins[95,97] (Fig. 2a). For example, *D. melanogaster* cGLR1 produces 3′2′-cGAMP after sensing dsRNA, which activates STING-dependent antiviral immunity[93,94]. By contrast, some cGLRs from *S. pistillata* produce 3′3′-cyclic di-AMP (3′3′-cAA) and 3′3′-cyclic UMP–AMP (3′3′-cUA) in response to dsRNA, whereas oyster cGLRs sense dsDNA to synthesize 2′3′-cUA[95].

Recent studies have shown that the cGLR family of proteins, including human cGAS, originated from an ancient bacterial defence system known as CBASS[98,99] (Fig. 2b). CBASS systems are defined by the presence of a cGAS-like protein from the CD-NTase (cGAS/DncV-like nucleotidyltransferase) family[100,101], and – like the cGAS–STING pathway in animals – function in protection against viral (phage) infection[67,98,99]. In CBASS, the CD-NTase senses phage infection and then produces a cyclic-oligonucleotide signalling molecule that activates a CBASS effector protein. Unlike the cGAS–STING pathway, wherein activation of STING results in downstream immune signalling, activation of the CBASS effector protein usually (but not always[102]) causes death of the infected cell[98,103–108]. CBASS-mediated cell death occurs before the phage has assembled new viral particles within the infected cell and, thus, it clears infection by preventing viral spread. Numerous families of CBASS effectors have been documented, including phospholipases and membrane-spanning proteins that disrupt the cell membrane once activated[98,103,107], nucleases that indiscriminately cleave both phage and host DNA[104,106], TIR domain-containing proteins that deplete NAD^+ in response to infection[62,98], and others[67].

CBASS systems are abundant in prokaryotes and were detected in about 15% of all sequenced bacterial and archaeal genomes[67,99,109]. Diverse immune signals are produced by CD-NTases of CBASS systems (Fig. 2b). A large fraction produce 3′3′-cGAMP, a molecule similar but not identical to the molecule produced by human cGAS[100,110]. Other CD-NTases produce one or more of a large number of oligonucleotide signals, including the cyclic dinucleotides 3′3′-cUU, 3′3′-cUA, 3′3′-cGG and 3′2′-cGAMP, and the cyclic trinucleotides 3′3′3′-cAAG, 3′3′3′-cAAA and 2′3′3′-cAAA[62,63,99,104,106,108,111]. A recent preprint describes a subset of CBASS systems that produce the same signal as human cGAS, namely 2′3′-cGAMP[112].

CBASS effector proteins typically have a domain that specifically senses the nucleotide signal[67]. In a minority of CBASS, this receiver domain is a STING domain, which is thought to be the ancestor of eukaryotic STING proteins[62]. Other receiver domains include SAVED, which is found in about 30% of CBASS effectors[63,111,113]; CARF domains, which are also found in type III CRISPR–Cas systems[106,113]; a β-barrel signal-perceiving domain[103]; and others[67].

The most abundant CBASS configuration (type I systems) comprises only two genes, encoding the CD-NTase signal producer and the oligonucleotide-receiving effector[67]. However, a substantial fraction of CBASS systems encode additional ancillary genes that are possibly involved in regulation of the system[67,98]. Type II CBASS systems are associated with genes encoding proteins that are similar to eukaryotic ubiquitin-conjugating and ubiquitin-removing enzymes[98,107,114]. In some cases, it was shown that these enzymes can conjugate the CD-NTase to as-yet-unidentified target proteins, thereby enhancing its cyclase activity[115,116]. Conversely, other studies have shown that the ancillary proteins in type II CBASS conjugate the CD-NTase to membrane-associated phage shock protein A (PspA) to inhibit CD-NTase activity in the absence of phage infection[117]. In yet other cases, these ancillary proteins have been shown to generate chains of covalently linked CD-NTases, and this ‘poly-cGASylation’ enhances CD-NTase activity[118]. Other types of CBASS, including type III and type IV


a
Bacterial or viral infection
dsDNA in cytoplasm
2′3′-cGAMP
cGAS
STING
Interferon and antiviral genes
Nucleus
Human cell
Bacterial or viral infection
dsRNA
dsDNA in cytoplasm
Other?
cGLRs
Interferon and antiviral genes
STINGs
Animal cell
b
Bacteriophage
Other phage triggers?
cabRNA
Capsid?
CD-NTases
Membrane-spanning proteins
Phospho-lipases
Nucleases
TIR domains
Growth arrest
Cell death
Bacterial cell
c
dsRNA
OAS
2′–5′ oligoA
Cellular RNA
RNase L
Interferon production
Apoptosis
Human cell
d
Type III CRISPR–Cas
cOAs
RNases
DNases
Proteases
Others
Bacterial cell
e
Phage trigger?
Cytidylate cyclase
CTP
cCMP
Uridylate cyclase
UTP
cUMP
Membrane-spanning domain
TIR domain
NAD+ depletion
Cell death
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TIR domain Type I Thoeris
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TIR domain Type IV Thoeris
3′cADPR
His–ADPR
N7–cADPR
SIR2
Membrane-spanning domain
Caspase
NAD+ depletion
Cell death
Bacterial cell
g
3′cADPR
His–ADPR
N7–cADPR
Bacteria
Plants
2′cADPR
pRib–AMP
pRib–ADP
ADPR–ATP
ADPR–ADPR
h
TIR domain
Virulence factors
ADPR–ATP
ADPR–ADPR
2′-cADPR
pRib–AMP
pRib–ADP
EDS1
SAG101
NRG1
PAD4
EDS1
ADR1
Ca2+
NRG1
Cell death
Immune response
Plant cell

Fig. 2 | Nucleotides as immune signalling molecules. 'Phage trigger' represents unknown phage components or phage-induced changes in cellular state. In parts **a**–**d**, 2′–5′ linkages are represented by a triangular bond, whereas 3′–5′ linkages are represented by a rounded bond. **a**, The human cyclic GMP–AMP synthase (cGAS) and animal cGAS-like receptor (cGLR) proteins produce cyclic dinucleotides — such as cyclic GMP–AMP (cGAMP) — in response to foreign nucleic acid stimuli. These cyclic dinucleotides bind STING proteins and trigger a signalling cascade that culminates in the expression of interferons and other antiviral genes. ds, double-stranded. **b**, In the bacterial cyclic-oligonucleotide-based anti-phage signalling system (CBASS), proteins of the CD-NTase (cGAS/DncV-like nucleotidyltransferase) family produce cyclic dinucleotides or cyclic trinucleotides once they sense phage infection. These molecules bind and activate immune effector proteins that cause cell death or growth arrest. cabRNA, CBASS-activating bacteriophage RNA. **c**, 2′–5′-Oligoadenylate synthase (OAS) proteins in animals produce 2′–5′-linked linear oligoadenylate molecules (2–5A) when they recognize foreign (viral) RNA. 2–5A binds RNase L, which then indiscriminately cleaves cellular RNAs, resulting in apoptosis and the production of interferons. **d**, Type III CRISPR–Cas systems produce cyclic oligoadenylate (cOA) molecules that bind and activate various downstream effectors. **e**, Bacterial Pycsar (pyrimidine cyclase system for antiphage resistance) systems produce cyclic cytidine monophosphate (cCMP) and cyclic uridine monophosphate (cUMP) as immune signalling molecules, leading to cell death through depletion of nicotinamide adenine dinucleotide (NAD^+) or membrane disruption. **f**, In bacterial Thoeris systems, Toll–interleukin receptor (TIR) domain-containing proteins sense phage infection and produce various ADP-ribose (ADPR) isomers as immune signalling molecules. **g**, Various immune signalling molecules are produced by TIR domain-containing proteins in plants and bacteria. These signalling molecules are derived from processing of the precursor NAD^+. **h**, Plant TIR domain-containing proteins are activated by binding pathogen-derived virulence factors and then produce ADPR derivatives as immune signalling molecules. These molecules bind a complex comprising EDS1 and partner proteins (SAG101 or PAD4), which then recruit a second protein (NRG1 or ADR1) to trigger cell death or a downstream immune response. dsRNA, double-stranded RNA; pRib, phosphoribosyl.

systems, are associated with different sets of ancillary genes[67,108], the functions of which are unclear.

A large variety of phages are blocked by CBASS[98,103,119,120]. Although the phage-derived components that activate CBASS are largely unknown, CBASS activation has been characterized in several individual cases. For example, a CBASS from *Staphylococcus schleiferi* is activated by a conserved non-coding RNA expressed by some staphylococcal phages[121]. This RNA, termed CBASS-activating bacteriophage RNA (cabRNA), is encoded in the same locus that encodes phage terminase genes, but its role in the biology of the phage is currently unknown[121]. Multiple studies have shown that phages can evade CBASS defence by mutating their major capsid proteins[119–121], although whether the phage capsid directly activates CBASS is still unclear. Proteins in the phage virion assembly machinery have also been suggested to activate CBASS, including the phage prohead protease[120]. The presence of an allosteric site in a subset of CD-NTases that binds folate derivatives has led to the suggestion that these CD-NTases could be activated by altered folate metabolism in phage-infected cells[122–124], but direct evidence to support this model is lacking.

Production of linear oligoadenylates by OAS

The family of 2′–5′-oligoadenylate synthase (OAS) antiviral proteins that are encoded in the human genome function as sensors for viral dsRNA[125–127]. Once they recognize viral RNA, OAS proteins produce a linear chain of AMP molecules linked by 2′–5′ phosphodiester bonds (2–5A)[125,126,128] (Fig. 2c). These linear oligoadenylates then bind ribonuclease L (RNase L), activating it to non-specifically cleave all RNA in the cell[129–133]. RNA cleavage results in translation inhibition and apoptosis of the infected cell and can also induce interferon production[129,134–136]. Similarly to 2′3′-cGAMP in eukaryotes[89–92], 2–5A can be transferred between cells via gap junctions and dedicated transporters, triggering RNase L activity in neighbouring cells[137].

OAS proteins are widespread in eukaryotes[138] and are part of the same superfamily as cGAS, cGLR and CD-NTase proteins[101,138]. The logic of the OAS–RNase L pathway is similar to that of CBASS: recognition of infection leads to amplification of the signal via oligonucleotide signalling molecules, which activate an effector protein that causes cell death to prevent the spread of infection to nearby cells. Here, both structural and functional features of the defence system are conserved from bacteria to humans.

Production of cyclic oligoadenylates by type III CRISPR–Cas

A specific set of CRISPR–Cas systems, namely type III CRISPR–Cas, produce nucleotide signalling molecules in addition to their core function as RNA-guided nucleases[50,139] (Fig. 2d). The RNA–protein complex of type III CRISPR–Cas base-pairs with actively transcribing target phage RNA and cleaves it, as well as the DNA locus from which it is transcribed[140,141]. During this process, the Cas10 subunit of the RNA–protein complex produces the nucleotide signalling molecule cyclic oligoadenylate (cOA), which typically comprises a ring of multiple AMP molecules linked by 3′5′ bonds[51,52]. The size of the cOA molecules varies between versions of type III CRISPR–Cas and can consist of three to six AMPs[51,52]. The cOA molecules bind and activate a CRISPR-associated effector protein encoded by the CRISPR–Cas locus[51,52]. Effector proteins associated with type III CRISPR–Cas are typically composed of two domains: a receiver domain (usually CARF or SAVED) that senses the cOA signal, and another domain that causes growth arrest or cell death after cOA binding[142]. CRISPR-associated effectors in different bacteria vary and include RNases that indiscriminately cleave cellular and phage RNAs[143–145], DNases that cleave single-stranded DNA or dsDNA in a largely sequence-independent manner[104,146–150], proteases that activate a cascade of proteolytic events culminating in cleavage of a wide range of cellular proteins[151], proteins that disrupt the cell membrane[49,152,153], and others[142,154,155].

It was estimated that recognition of a single target RNA by the type III CRISPR–Cas complex triggers Cas10 to produce about 1,000 cOA molecules[156]. Once the target RNA is cleaved, Cas10 ceases to produce cOA molecules[140,157,158]. CARF and SAVED domains in effector proteins of type III CRISPR–Cas have been shown to slowly degrade the bound cOA so that in the absence of continued production of cOA molecules by the CRISPR–Cas complex, the toxic activity of the effector is eventually terminated[144,159–162]. Some type III systems also encode standalone enzymes known as ring nucleases that slowly degrade cOA molecules[163–166]. The ability of the system to regulate levels of the cOA signalling molecules explains the logic of type III CRISPR–Cas activity: recognition of a single transcribing phage locus causes temporary growth arrest, which slows down phage cycle progression until the infection is cleared by the CRISPR–Cas complex. Once the phage nucleic acids are cleaved, production of cOA signalling molecules stops, the remaining cOA molecules are slowly degraded, and cell growth can resume[161,167,168].

Whereas almost all type III CRISPR–Cas systems produce cOA signalling molecules, some notable exceptions have been described. Specifically, some type III CRISPR–Cas systems, including one from *Bacteroides fragilis*, have been shown to conjugate *S*-adenosyl methionine (SAM) to AMP via a phosphodiester bond, forming a SAM–AMP signalling molecule[152]. The effector protein in this system, known as CorA, binds SAM–AMP and inhibits cell growth, most probably via membrane disruption[152]. Phosphodiesterases and SAM–AMP lyases associated with CRISPR–Cas systems can degrade this signalling molecule to deactivate the system[152].

Production of cCMP and cUMP by Pycsar

Pycsar is another bacterial defence system that relies on nucleotide signalling. The domain composition of this system is similar to that of CBASS in that one protein recognizes infection and produces a signalling molecule and another protein senses the signalling molecule and exerts a toxic activity. Unlike CBASS, in which the signal-producing enzyme belongs to the CD-NTase family, the enzyme in Pycsar is a nucleotide cyclase that produces cyclic cytidine monophosphate (cCMP) or cyclic UMP (cUMP)[66] (Fig. 2e). During evolution, Pycsar probably borrowed domains from proteins involved in cAMP metabolism, as the Pycsar nucleotide cyclase is structurally similar to class III adenylate and guanylate cyclase proteins that are conserved in the housekeeping signalling systems of prokaryotes and eukaryotes[66]. In contrast to canonical adenylate cyclases, which have active-site residues that select for purine nucleotides, substitutions that occurred in the active site of Pycsar cyclases confer specificity for pyrimidine cyclization[66,169].

Pycsar effectors include TIR domain-containing NADases that deplete the cell of NAD^+ or membrane-spanning proteins that impair membrane integrity once activated by the nucleotide signal (Fig. 2e). In some cases, the Pycsar effector gene encodes an additional domain annotated as a 'cyclic nucleotide-binding domain' (Pfam accession PF00027), which is common in proteins that are regulated by cAMP and cGMP[170], providing further support for the evolutionary origin of Pycsar[66]. cCMP and cUMP have been detected in human cells and implicated in embryonic development[171,172] and cell death[173], but whether these molecules have a role in human biology remains unclear.

Production of ADPR derivatives by immune TIR domains

TIR domains are important components of TLRs and interleukin receptors in the human immune system[74]. When TLRs bind a pathogen-associated molecular pattern, their TIR domains oligomerize and activate downstream intracellular signalling[74,174]. After several decades of research in which TIRs were considered to have solely scaffolding roles, later studies have revealed that some TIR domains have catalytic activity[76]. Studies of these domains in the context of bacterial immunity[175,176] and plant immunity[177,178] suggested that catalytically active TIRs might produce signalling molecules involved in regulating immunity and cell death. These molecules were eventually characterized as derivatives of ADPR, and in recent years, there has been substantial progress in understanding their roles in bacterial and plant immunity[179,180].

The activity of TIR domains as signal producers in bacteria is best characterized in a family of defence systems called Thoeris[61,175]. In Thoeris defence systems, a protein with a TIR domain functions as the sensor for phage infection and, once infection is recognized, the TIR domain processes NAD^+ into an ADPR-containing signalling molecule (Fig. 2f). In type I Thoeris, the signalling molecule is 1′′–3′ glycocyclic ADPR (1′′–3′ gcADPR; abbreviated as 3′cADPR), a molecule in which the two ribose moieties in ADPR are linked[181]. TIR domains in type IV Thoeris also produce a variant of cyclic ADPR, but in this case, the ribose of ADPR is covalently attached to the N7 nitrogen atom of the adenine base, forming N7-cADPR[182]. In type II Thoeris, the signalling molecule is formed by conjugating ADPR to the amino acid histidine, generating a His–ADPR linear signalling molecule[183]. Although the identity of phage-derived factors that trigger signal production is unknown for most Thoeris systems, a type I Thoeris system from *Staphylococcus aureus* was shown in a recent preprint to be activated by a phage capsid protein during infection[184].

The mode of action of Thoeris systems is similar in principle to that of CBASS and Pycsar, whereby the immune signalling molecule binds an effector protein, activating it and leading to cell death[122]. Effectors of type I Thoeris contain a SLOG domain that binds 3′cADPR and a SIR2 domain that depletes NAD^+ from the cell once activated. In type II Thoeris, the effector contains a Macro domain that senses the His–ADPR signalling molecule, and membrane-spanning helices that compromise membrane integrity when the signal is bound[183]. Finally, the effector of type IV Thoeris systems is a caspase-like protease that indiscriminately cleaves cellular proteins when N7-cADPR is bound[182].

The identification of plant TIR domains with catalytic NADase activity led to the hypothesis that these TIRs might produce a signalling molecule involved in plant immunity[177,178]. The first signalling molecule characterized for a plant TIR was 1′′–2′ gcADPR (2′cADPR)[181], produced by the *Brachypodium distachyon* TIR domain-containing protein BdTIR[181,185,186] (Fig. 2g). This molecule is similar to bacterial 3′cADPR, but the two ribose moieties in 2′cADPR are linked by a 1′′–2′ O-glycosidic bond rather than the 1′′–3′ bond in the bacterial molecule. The same molecule (2′cADPR) was later found to be produced by a TIR domain-containing protein from rice, OsTIR[187]. Evidence from *Arabidopsis thaliana* suggests that 2′cADPR is further processed in these plant cells into phosphoribosyl adenosine monophosphate (pRib-AMP), in which the link between the two phosphates in 2′cADPR is hydrolysed[188]. The receiver complex for pRib-AMP is a heterodimer between the proteins EDS1 and PAD4, which recruits ADR1 to activate downstream immune signalling in *A. thaliana* and rice[187–190] (Fig. 2h). Crystal and cryogenic electron microscopy structures of active EDS1–PAD4 complexes also detected pRib-ADP molecules in the complex; one potential explanation for this is that pRib-AMP may be further phosphorylated to pRib-ADP after its production in the cell[188,189].

A second class of immune signalling molecules produced by plant TIR domain-containing proteins comprise ADPR conjugated to another nucleotide. Two such molecules were described: ADPR–ATP and ADPR–ADPR[191] (Fig. 2g). These molecules are sensed by a plant protein complex formed between EDS1 and SAG101 (refs. 191,192). After binding the signalling molecule, the EDS1–SAG101 complex recruits the protein NRG1 (refs. 191–194), which oligomerizes to form a membrane-spanning calcium channel, leading to Ca^{2+} influx that culminates in death of the infected plant cell[195,196] (Fig. 2h). Some plant TIR domains were also found to hydrolyse RNA and, probably, DNA molecules to produce 2′3′-cAMP and 2′3′-cGMP[197], but the role of these molecules in plant immunity is currently unclear.

Plant immune TIR domain-containing proteins are activated in response to various pathogen stimuli. The TIR domain-containing protein RPP1 in *A. thaliana* binds the ATR1 protein injected into plant cells by the mildew pathogen *Hyaloperonospora arabidopsidis*[198–200], and ROQ1 from *Nicotiana benthamiana* recognizes XopQ from the bacterial plant pathogen *Xanthomonas euvesicatoria*[201,202]. Binding of the virulence factor (ATR1 or XopQ) induces tetramerization and catalytic

activation of the TIR domains in RPP1 and ROQ1, triggering the production of an immune signal that eventually causes Ca^{2+} influx and death of the infected plant cell[199,203] (Fig. 2h). TIR-mediated signalling is a major axis of plant innate immunity; for example, *A. thaliana* encodes more than 100 TIR domain-containing proteins[204]. However, the pathogen-derived signals that activate the majority of these TIR domain-containing proteins are yet to be discovered.

Although the TIR domains in bacteria and plant immune systems synthesize various types of signalling molecule, there is a unifying principle in the production of almost all of these molecules. TIR domains are thought to produce signalling molecules in a multistep process[183,205]. First, the TIR processes NAD^+ by removing the nicotinamide ring from the connected ribose group. Then, the TIR transfers the ribose that was previously connected to the nicotinamide ring to another moiety to generate the signalling molecule. This moiety can be an atom within the same ADPR molecule, generating 2′cADPR or 3′cADPR[181,186] or N7-cADPR[182]; it can be the nucleotides ATP or ADPR to form ADPR–ATP or ADPR–ADPR[191], or it can be the amino acid histidine to generate His–ADPR[183]. The TIR-produced molecule can then be further processed to the final signalling molecule, for example, processing of 2′cADPR to pRib-AMP[188,189].

So far, TIR domain signalling through the production of small molecules has not been reported in animal immunity. In humans, most TIR domain-containing proteins lack catalytic activity, with the exception of SARM1, which was shown to deplete NAD^+ in nerve cells in response to axonal injury[76,77]. Intriguingly, although SARM1-mediated NAD^+ cleavage mostly produces ADPR and cADPR, it has also been shown to produce 2′cADPR and 3′cADPR as minor products[206]. Whether these molecules have a role in SARM1-mediated cell death remains unknown.

Viral strategies to overcome nucleotide signalling

Given the centrality and breadth of nucleotide-based immune signalling in all domains of life, it is not surprising that viruses have evolved a variety of mechanisms to evade or overcome these pathways, targeting every step of the process, including pathogen recognition, signal production, signal reception and effector activity (Fig. 3).

Viruses infecting both animal and bacterial cells encode enzymes that specifically target and degrade nucleotide-based immune signals, which effectively decouples virus sensing from immune effector activation (Fig. 3). For example, the widely distributed poxvirus immune nucleases (poxins) cleave 2′3′-cGAMP to prevent the activation of STING in eukaryotic cells[207,208] (Fig. 3a). In phages, the anti-CBASS

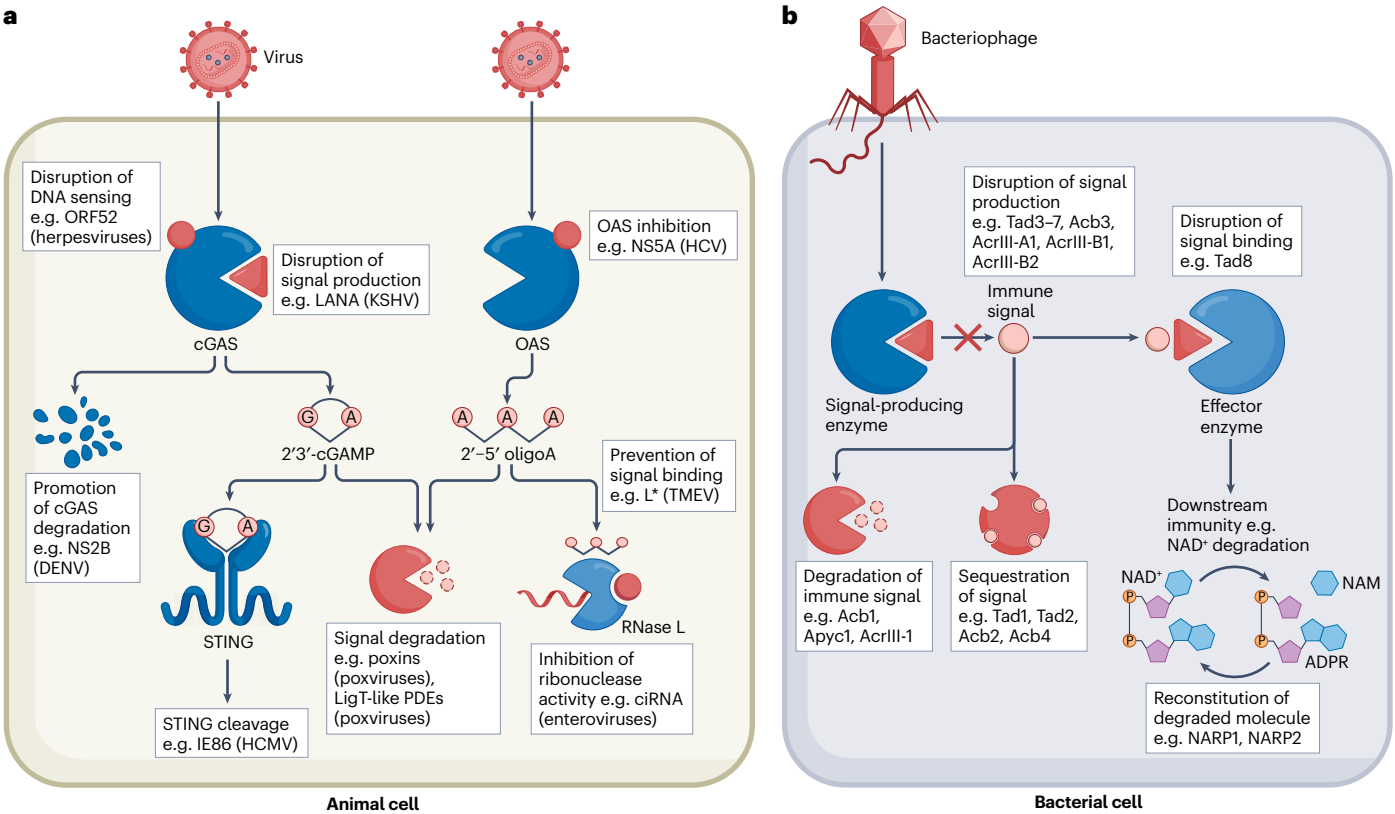

Fig. 3 | Viral evasion of nucleotide-based immune signalling. a, Viral evasion of the cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes (STING) and 2′–5′-oligoadenylate synthase (OAS) pathways in animal cells. Viral components can disrupt all stages of cGAS–STING and OAS pathways, including viral recognition, production and perception of immune signals, and downstream immune activation. **b**, Phage evasion of immune signalling in bacteria. Phages can inhibit bacterial immune signalling pathways at all stages, in a manner similar to viral evasion in animal cells, additionally including the sequestration of immune signals by 'sponge' proteins and reconstitution of the degraded molecule nicotinamide adenine dinucleotide (NAD^+) by NAD^+ reconstitution pathways (NARP). Acb, anti-CBASS; Acr, anti-CRISPR; ADPR, ADP-ribose; Apyc, anti-Pycsar; DENV, dengue virus; HCMV, human cytomegalovirus; HCV, hepatitis C virus; KSHV, Kaposi sarcoma-associated herpesvirus; LANA, latency-associated nuclear antigen; LigT-like PDEs, RNA ligase T-like phosphodiesterases; NAM, nicotinamide; ORF, open reading frame; Tad, Thoeris anti-defence; TMEV, Theiler's murine encephalomyelitis virus.

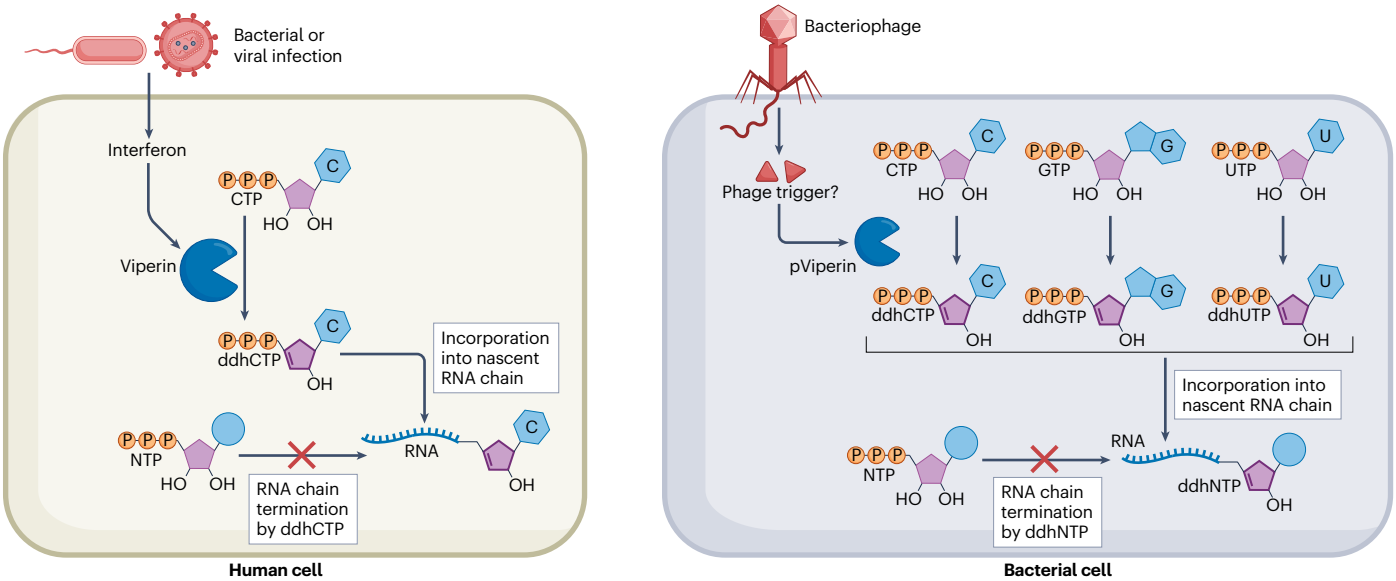

Fig. 4 | Viperins produce antiviral molecules. Human viperins, the expression of which is stimulated by interferons, produce 3′-deoxy-3′,4′-didehydro (ddh) CTP from cytidine triphosphate (CTP), whereas diverse prokaryotic viperins can produce ddhCTP, ddhGTP and ddhUTP from CTP, guanosine triphosphate (GTP) and uridine triphosphate (UTP), respectively. The missing 3′OH in these molecules prevents the addition of further nucleotides to the nascent RNA chain, resulting in chain termination during polymerization of viral RNA. pViperin, prokaryotic viperin.

protein 1 (Acb1) hydrolyses various CD-NTase-produced immune signals[209], including 3′3′-cGAMP, 3′3′-cUA and 3′3′3′-cAAA[209] (Fig. 3b). Acb1 is evolutionarily related to a family of RNA ligase T (LigT)-like phosphodiesterases (PDEs) encoded by viruses that infect eukaryotes[210,211] (Fig. 3a). These were shown to cleave signals including 2′3′-cGAMP, 3′3′-cGAMP and 3′2′-cGAMP via a mechanism equivalent to that of Acb1 (refs. 210,211), which implies that these LigT-like PDEs could function similarly to Acb1 against eukaryotic cGAS–STING products. Other LigT-like PDEs cleave the 2–5A molecules produced by OAS, thus evading OAS-based immunity[212–217]. Another example of a viral protein that degrades a bacterial immune signal is the anti-Pycsar protein 1 (Apyc1), which degrades cyclic mononucleotides to evade the Pycsar antiviral system[209]. Finally, phages were shown to carry nucleases that degrade cOA nucleotides produced by type III CRISPR–Cas, termed the anti-CRISPR (Acr) family AcrIII-1 (ref. 218) (Fig. 3b).

Another class of viral proteins that specifically target nucleotide-based immune signals are ‘sponge’ proteins (Fig. 3b). These proteins efficiently bind and sequester the signalling molecules before they can reach their target effector protein and elicit an immune response. Such sponge proteins were originally found encoded by phages that overcome the bacterial Thoeris system, and they were called Thoeris anti-defence 1 (Tad1) and Tad2 (refs. 181,183,219). It was later shown that Tad1 and Tad2 can also bind and sequester CBASS signals[220]. Tad1 forms a hexamer with six binding sites for the Thoeris signal 3′cADPR and CBASS-derived cyclic dinucleotides, and an additional two binding sites for CBASS-derived cyclic trinucleotides. Tad2 forms a tetramer with two binding sites for Thoeris-derived ADPR derivatives and two sites that can bind CBASS-derived cyclic dinucleotides[220]. The anti-CBASS protein Acb2 is another sponge protein that sequesters CD-NTase products to prevent the activation of cell death pathways[115,119]. Acb2 forms a hexamer with three binding sites for cyclic dinucleotides and two binding sites for cyclic trinucleotides[119,221], and it can also sequester trinucleotide cOA signals produced by some type III CRISPR–Cas systems[221]. A recent preprint reports that Acb4 is an additional viral sponge protein that binds CBASS-produced signalling molecules[222]. The sponge proteins Tad1, Tad2, Acb2 and Acb4 are widely distributed within phage genomes, and it was shown that different members of a given sponge protein family can differ in their binding preferences for immune signals[183,219].

Viral proteins can also interact with host immune proteins to prevent them from generating nucleotide-based immune signals. Many eukaryotic viruses carry proteins that inhibit cGAS activation (reviewed in ref. 223) (Fig. 3a). For example, ORF52 of several gammaherpesviruses binds to cGAS to disrupt its interaction with viral DNA, thereby preventing cGAMP production and subsequent activation of STING[224]. Similarly, the Kaposi sarcoma-associated herpesvirus protein latency-associated nuclear antigen (LANA) physically interacts with cGAS to prevent a downstream immune response[225]. The dengue virus NS2B protease promotes lysosomal degradation of cGAS[226], and other viral proteins, such as the human cytomegalovirus protein IE86, cause degradation of STING[227–229]. Viruses infecting animal cells can also inhibit the OAS–RNase L pathway through direct binding. Examples include the hepatitis C virus NS5A protein, which interacts with OAS to block signal production[230]; the L* accessory protein of Theiler’s murine encephalomyelitis virus, which blocks binding of the immune signal to RNase L[231,232]; non-coding RNAs in group C enteroviruses, termed ciRNAs, which competitively inhibit the endoribonuclease activity of RNase L[233,234]; and others[235]. In bacteria, six anti-Thoeris proteins (Tad3 to Tad8) and the anti-CBASS protein Acb3 have been shown to inhibit the respective anti-phage systems by physically binding to either the signal-generating enzyme or the signal-receiving effector protein, thereby blocking signal transduction to prevent activation of the system[236] (Fig. 3b). The anti-CRISPR proteins AcrIII-A1, AcrIII-B1 and AcrIII-B2 inhibit cyclic oligoadenylate

signalling in bacteria by binding and inhibiting the type III CRISPR–Cas RNA–protein complex[237–240].

Modified free nucleotides as virus replication inhibitors

Beyond their role as precursors for signalling molecules, modified free nucleotides can function as direct inhibitors of viral replication. Viperin (viral inhibitory protein, endoplasmic reticulum-associated interferon-inducible) is a human antiviral enzyme of the radical SAM family that modifies cytidine triphosphate (CTP) to form 3′-deoxy-3′,4′-didehydro (ddh)CTP[241] (Fig. 4, left). This modification removes the hydroxyl group from the 3′ carbon of the nucleotide, resulting in a modified nucleotide that forms a poison for replication of the viral nucleic acid. When the viral polymerase incorporates ddhCTP into the nascent RNA chain, the absence of a 3′OH group in the nucleotide prevents the addition of further nucleotides[241]. Viperin expression is strongly induced by interferons[242], and the chain-terminating capacity of its ddhCTP products protects human cells from many viruses, including flaviviruses[243]. Viperins were also suggested to possess additional antiviral traits unrelated to ddhCTP production[243].

Homologues of the human protein viperin were found in bacteria and archaea, and these were termed prokaryotic viperins (pVips) (Fig. 4, right). The pVip family is encoded by diverse prokaryotic species, and it was shown that different members of the family can produce different ddhNTPs. Whereas some pVips produce ddhCTP similarly to human viperin, other pVips modify UTP or GTP to produce ddhUTP or ddhGTP[244], and some pVips can produce multiple distinct ddhNTPs[244]. It was shown that pVips can block the infection cycle of phage T7 in *E. coli* and that their ddhNTP products inhibit chain elongation by the T7 RNA polymerase[244]. The production of ddhNTPs in bacterial and animal cells does not inhibit cellular growth[241,244], which suggests that host polymerases are not susceptible to the

Table 1 | Central antiviral immune pathways involving nucleotide manipulation

Protein domain activity	Examples	Taxonomy	Nucleotide involved
Nucleotide depletion			
dNTPase	SAMHD1	Eukaryotic	dNTP
	dGTPase	Prokaryotic	dGTP
dCTP deaminase	dCTP deaminase	Prokaryotic	dCTP
ATP nucleosidase	NLR-like proteins, CBASS, prokaryotic Argonautes, DRT8, Detocs	Prokaryotic, eukaryotic	(d)ATP
Adenosine deaminase	RADAR, type III CRISPR–Cas	Prokaryotic	(d)ATP
SIR2	Thoeris, SIR2–HerA (also known as Nezha), DSR systems, Kongming	Prokaryotic	NAD^+
TIR	CBASS, Pycsar, NLR-like proteins, prokaryotic Argonautes, retrons	Prokaryotic	NAD^+
SEFIR	SEFIR	Prokaryotic	NAD^+
Signalling molecules			
CD-NTase	cGAS–STING	Eukaryotic	2′3′-cGAMP
	cGLRs	Eukaryotic	2′3′-cGAMP, 3′2′-cGAMP, 3′3′-cAA, 3′3′-cUA, 2′3′-cUA
	CBASS	Prokaryotic	3′3′-cGAMP, 3′3′-cUU, 3′3′-cUA, 3′3′-cGG, 3′2′-cGAMP, 3′3′3′-cAAG, 3′3′3′-cAAA, 2′3′3′-cAAA, 2′3′-cGAMP
OAS	OAS–RNase L	Eukaryotic	2–5A
Cas10	Type III CRISPR–Cas	Prokaryotic	cOA, SAM–AMP
Nucleotide cyclase	Pycsar	Prokaryotic	cCMP, cUMP
TIR	Thoeris	Prokaryotic	3′cADPR, His–ADPR, N7-cADPR
	Plant TIRs	Eukaryotic	2′cADPR, pRib-AMP, pRib-ADP, ADPR–ATP, ADPR–ADPR, 2′3′-cAMP, 2′3′-cGMP
Adenosine deaminase	Kongming	Prokaryotic	dITP
Poisoning viral replication			
Viperin	Eukaryotic viperins	Eukaryotic	ddhCTP
	Prokaryotic viperins	Prokaryotic	ddhCTP, ddhUTP, ddhGTP

2–5A, 2′–5′-linked linear oligoadenylate; ADP, adenosine diphosphate; ADPR, ADP-ribose; AMP, adenosine monophosphate; cAA, cyclic di-AMP; cADPR, cyclic ADPR; CBASS, cyclic-oligonucleotide-based anti-phage signalling system; cGAMP, cyclic GMP–AMP; cGAS, cyclic GMP–AMP synthase; cGLR, cGAS-like receptor; cOA, cyclic oligoadenylate; cUA, cyclic UMP–AMP; dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; ddh, 3′-deoxy-3′,4′-didehydro; dGTP, deoxyguanosine triphosphate; dITP, deoxyinosine triphosphate; dNTP, deoxynucleotide triphosphate; DRT8, defence-associated reverse transcriptase type 8; DSR, defence-associated sirtuin; His, histidine; NAD^+, nicotinamide adenine dinucleotide; NLR, NOD-like receptor; OAS, 2′–5′-oligoadenylate synthase; pRib-ADP, phosphoribosyl ADP; pRib-AMP, phosphoribosyl AMP; Pycsar, pyrimidine cyclase system for antiphage resistance; RADAR, restriction by an adenosine deaminase acting on RNA; SAM, *S*-adenosyl methionine; SAMHD1, sterile alpha motif and HD domain-containing protein 1; SIR2, sirtuin; STING, stimulator of interferon genes; TIR, Toll–interleukin receptor.

incorporation of these nucleotides. Presumably, the rapid polymerization rate of some viral polymerases makes them more prone to incorporation of ddhNTPs.

Phylogenetic analyses suggest that eukaryotic viperins evolved from pVips, in particular those of Asgard archaea[9,245]. Viperins are thought to have been present in the last eukaryotic common ancestor[8] or even in the last universal common ancestor[9] but were differentially lost in eukaryotes, leading to a patchy distribution within eukaryotic clades[9]. For example, there are no viperins in land plants, although expression of prokaryotic and eukaryotic viperins in plants can restrict plant viruses[246].

Why nucleotides?

In recent years, it has become clear that the nucleotide pool has multiple major roles in immunity. The use of nucleotides as building blocks for immune signalling molecules has emerged multiple times in evolution, as has the strategy of selective depletion of nucleotides from the nucleotide pool to starve viruses of essential molecules (Table 1). As nucleotides are central for the replication of any virus, manipulation of the nucleotide pool is an effective form of defence. It is less clear why nucleotides, in particular, are used so frequently as intracellular immune signalling molecules, given that other molecules could also conceivably fulfil this purpose. A possible answer lies in the ancient nature of the virus–host arms race. Viruses are thought to have emerged shortly after the beginning of life, during the hypothesized 'RNA world' period[247,248]. At that time, nucleotides were the main substrate for biological innovation, and numerous nucleotide-modifying enzymatic activities evolved. The conservation and abundance of nucleotide-based immune signalling strategies in bacteria suggest that these signalling pathways also evolved early on and probably adopted some of the innovations in nucleotide-manipulating enzymatic activities for the purpose of antiviral immunity.

Another hypothesis suggests that the ancestral role of nucleotide-related immune proteins was nucleic acid repair after invasion by 'selfish' genetic elements such as transposable elements, integrative plasmids and prophages[249]. Byproducts of nucleic acid repair may have been adopted as early immune signals, leading to the eventual recruitment of nucleotide-processing enzymes into dedicated immune pathways[249].

The prevalence of nucleotides as signalling molecules could also be attributed to their biochemical properties. In human and animal cells, nucleotide-based immune signals such as 2′3′-cGAMP and 2–5A can be transferred from an infected cell to neighbouring cells via dedicated transporters and gap junctions, thereby triggering immune responses in these cells before they are infected[89–92,137]. This trait is beneficial in multicellular organisms, wherein the nucleotide signal triggers expression of antiviral genes, but not in unicellular organisms, wherein signalling directly activates cell death. Indeed, the spread of immune signals from infected bacteria to bystander cells would not be advantageous because the signal would cause the death of cells that had not been infected. Owing to their phosphate moieties, nucleotides are highly negatively charged and, thus, do not tend to passively diffuse across membrane barriers in an efficient manner. As a result, when a bacterial cell dies owing to the action of a system such as CBASS or Thoeris, the immune signals released from the cell do not induce death in nearby cells. This biochemical property of nucleotides may have influenced their early evolutionary selection as substrates for intracellular immune signalling molecules.

Conclusions and outlook

Despite many decades of research in molecular immunity, the centrality of nucleotides for immune purposes has only recently been recognized. Given this recent expansion of knowledge on nucleotide-centric immunity, it is probable that future studies will uncover new immune signalling molecules comprising modified nucleotides, or new immune pathways that manipulate the nucleotide pool in novel ways. Indeed, a recent study has reported a bacterial defence system called Kongming that produces dITP as an immune signalling molecule[250]. An adenosine deaminase protein within the Kongming system generates dITP in response to infection, and this modified nucleotide activates a Kongming-encoded SIR2-domain-containing protein to deplete NAD^+ from the cell[250]. We predict that additional pathways that use modified nucleotides as signalling molecules will be discovered in the future, in both bacteria and eukaryotes.

The variety of viral mechanisms that inhibit immune signalling leads to continuous evolutionary diversification of nucleotide-based immune signals. For example, the Tad2 protein from phage SPO1 efficiently inhibits type I Thoeris by binding and sequestering 3′cADPR[219], but it does not inhibit type II Thoeris because it cannot bind His–ADPR. This highlights how signal diversification in type II Thoeris can provide an advantage against viral inhibition. At the same time, some phages carry variants of Tad2 that bind His–ADPR and inhibit type II Thoeris[183], demonstrating the interplay between immune system diversification and phage adaptation. These interactions probably drive the ongoing diversification of both immune signals and their corresponding viral inhibitors. We, therefore, anticipate that future studies will document further diversification of immune signals for known defence systems such as Thoeris and CBASS.

Although it is already known that eukaryotes inherited some of their immune systems from bacterial defences, whether eukaryotic viruses similarly acquired anti-defence capacities from phages is currently unclear. A particularly intriguing piece of evidence for a shared mechanism of counter-defence was recently reported for viral LigT-like PDEs, which are used to cleave nucleotide-based immune signals in both phages and viruses infecting eukaryotes, suggesting a shared evolutionary origin[210–217]. It will be interesting to explore whether other mechanisms of viral counter-defence, for example, phage sponge proteins that sequester immune signals, are also shared between phages and eukaryotic viruses.

Research on nucleotide-based immunity has been progressing rapidly in recent years, especially in the context of bacterial defences. The molecular innovation at the heart of the virus–host arms race promises further exciting discoveries in this area.

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