Table 1
Profiles of reviewed clinical studies that compared vaccines with and without adjuvants (numbers indicate references).

Vaccine type		Adjuvant			
		Aluminum	AS03	MF59	Others[a]
Pandemic	Whole virion	9, 21, 22	Nil	Nil	Nil
	Subunit/split	12–15	10, 11, 22	16–19	20, 24
	Recombinant	23	Nil	Nil	Nil
Seasonal	Subunit	Nil	Nil	25–27	Nil

[a] One study used Matrix M™ [20] and the other used Inulin [24].

Immunogenicity was reviewed by the increase in geometric mean of the antibody titer (GMT), vaccinee ratio of seropositivity, and ratio of seroconversion. The antibody titer was measured by either hemagglutinin inhibition assays or microneutralizing assays. The safety profile was reviewed as the frequency of vaccine-related adverse reactions, comprising local reactions of pain, induration, erythema, etc., and systemic reactions of fever, malaise, headache, etc. Since the trial designs differed, especially in doses, schedules, subject backgrounds, and details of the definitions of immunogenicity, inter-trial comparisons were not reasonable, but the authors gained the impression that adjuvanted vaccines caused more frequent adverse reactions, regardless of the adjuvant used. The severity of the adverse events was slight or moderate, and no serious adverse events were reported, indicating that these influenza vaccines adjuvanted with aluminum salts, MF59 or AS03 are tolerable.

Seven studies on aluminum adjuvanted vaccines included various types of whole virion vaccines [9,21,22], subunit/split vaccines [12–15] and recombinant vaccines [23]. They satisfied the European Medical Agency's criteria for assessment of influenza vaccine [28,29], no matter which type of vaccine were used. For example in the two doses whole-virus H5N1 vaccine study, GMT increase on 21days after the second administration was between 2.7 and 5.2 when Aluminum adjuvant was added, and was between 3.2 and 5.9 without adjuvant [9].

On the other hand, compared with studies on vaccine with other adjuvants (AS03 [10,11,22], MF59 [16–19,25–27] and others [20,24]) the trends for the adjuvant effects on the vaccine immunogenicity differed among the adjuvants, in that aluminum showed lower adjuvanticity than MF59, AS03, or other adjuvants, irrespective of the dose of aluminum (300–1000 μg/dose) or the form of aluminum (hydroxide or phosphate). One study with two doses split vaccine (7.5 μg HA per dose) adjuvanted with MF59 showed 406.9 of GMT on 21days after the second administration, while non-adjuvanted vaccine showed 156.6 [19]. Higher adjuvanticity of MF59 than aluminum salts has also been shown in a trial on hepatitis B virus vaccines [30], etc.

The protective efficacy of influenza vaccines is mostly assessed by the clinical occurrence of confirmed influenza or influenza-like illness. Direct comparisons between MF59 adjuvanted and non-adjuvanted trivalent subunit influenza vaccines showed that adjuvanted vaccines exhibited higher effectiveness in both young children in Canada [27] and elderly people in Italy [31]. In the former study where influenza illness was confirmed by means of real-time polymerase-chain-reaction in nasopharyngeal aspirates or swabs, the effectiveness of the adjuvanted vaccine was shown by decreased influenza occurrence by 75%; 13 cases among 1937 adjuvanted vaccine group presented influenza illness whereas 50 cases of 1772 non-adjuvanted vaccine group showed influenza illness [27]. In the latter study in elderly people, the protective efficacy of the adjuvanted vaccine appeared to be less, since the odds ratio for developing influenza-like illness with the non-adjuvanted vaccine (versus adjuvanted vaccine) was 1.52, while the odds ratio for non-vaccinated people (versus vaccinated) was 2.16 [31].

From these experiences, it can be said that adjuvants in subunit influenza vaccines enhance the immunogenicity except for aluminum salts, but their adjuvanticity may need more improvement to prevent clinical influenza illness sufficiently.

3. Whole virion vaccines: vaccines with "unintended adjuvant"?

While subunit/split vaccines contain virus surface proteins as the vaccine antigens, whole virion vaccines are made of whole influenza virus particles that have been inactivated, typically by formaldehyde treatment. Therefore, these vaccines are composed of not only surface proteins, such as neuraminidase and hemagglutinin (for type A and type B, as the most commonly used vaccine antigens) or hemagglutinin esterase (for type C), but also matrix proteins and genomic RNA.

A review of three whole virion vaccines suggested that they were effective even though they were without aluminum adjuvants, and one of them was more effective than the aluminum-adjuvanted whole virion vaccine [9]. Superior immunogenicity of a whole virion influenza vaccine has been demonstrated in several Toll-like receptor (TLR) 7-knockout mouse experiments, which suggested it was dependent on TLR7 signaling [32,33]. Sialo-suger chains of host bind to influenza viruses but TLR7 specifically recognizes RNA of pathogens. These studies suggest that remaining RNA of influenza virus in the whole virion vaccine might unintentionally function as an adjuvant through TLR7 signaling. It is an interesting concept that a whole virion vaccine product might contain a "built-in adjuvant" when we call aluminum salts, MF59, or AS03 are artificially added as adjuvants. However, its generalization to other single-stranded RNA virus vaccines is controversial, since TLR7 and TLR8 polymorphisms did not affect the measles vaccine antibody response [34] and a transcriptional analysis of human blood cells found similar results for a vaccine against yellow fever and poly ICLC, the specific ligand of TLR3 [35].

4. Mechanisms of influenza vaccine adjuvants

The differences in the mechanisms of aluminum and other adjuvants are not yet fully understood, but they are commonly known to induce mild inflammation with immune cell recruitment at the injection site and not to induce Th1 cellular immunity.

Aluminum salts are generally thought to catch antigens and keep them at the local injection site for periods of days to weeks, such that the antigen is slowly presented and processed by the immune system. This "depot effect" was shown historically in diphtheria toxin experiments, in which immunity was impaired when the injection site was removed, while animals with transplantation of the injection site showed transferred immunity in parallel [2]. In addition, inflammation and cell damage caused by aluminum salts were recently shown to be a critical step in their Th2-biased adjuvanticity.

MF59 is still known to be effective when it is administered in advance of a vaccine antigen. However, when MF59 is administered

at 24 h after an antigen, it is not sufficiently immunogenic. These observations show MF59 does not act via a "depot effect", but instead is supposed to condition the immune system to respond effectively. At 2 days after injection, MF59 is found in lymph node mature macrophages and the gene profile of the "adjuvant core response genes" found in microarray analyses of the injected muscle of mice suggests that the mechanism of action of MF59 involves strong recruitment of antigen-presenting cells to the injection site as early as 12 h after injection [36].

A recent comparison study between aluminum salts and MF59 in mice [37] has suggested that the degree of cell recruitment may represent the current description of adjuvanticity. Specifically, in the first 24 h, MF59 recruited significantly more neutrophils, monocytes, eosinophils, macrophages, and dendritic cells than aluminum salts.

MF59 is composed of 0.5% Tween-80 as a water-soluble surfactant, 0.5% Span85 as an oil-soluble surfactant, 4.3% squalene oil, and water. It is an oil-in-water preparation and its emulsion droplet size is approximately 130 nm. Experience with nanoparticle adjuvants suggests that the particle size may be a key factor for adjuvanticity, since microspheres with diameters of <10 nm activate antigen-presenting cells, while those with diameters of 30–100 nm act via a "depot effect". A study comparing the sizes of silica particles showed that 30-nm-diameter particles induced the most inflammation and toxicity compared with 70-nm- or 300-nm-diameter particles [38]. If this situation is universal, the cell recruitment by MF59 may not depend on its size, but on its components. A recent study [39] compared several kinds of oil for particle size, emulsion stability, and adjuvanticity in a malaria vaccine candidate and an influenza vaccine, and found that the physical/chemical characters were similar among squalene, sesame oil, grape seed oil, and soybean oil, and that squalene oil showed the highest adjuvanticity in both vaccines.

5. Concluding remarks

Adjuvanticity of MF59 and AS03 has been shown in various studies, but their mechanisms of action still remain unclear. Regardless of how MF59 and AS03 act as vaccine adjuvants, there appears to be more to do to achieve social agreement on the importance of influenza vaccines. Vaccines that are "safer and more immunogenic" and "for the high-risk population" are the goals for vaccine development.

Conflicts of interest

None declared.

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Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination

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Abstract | The demand is currently high for new vaccination strategies, particularly to help combat problematic intracellular pathogens, such as HIV and malarial parasites. In the past decade, the identification of host receptors that recognize pathogen-derived nucleic acids has revealed an essential role for nucleic acid sensing in the triggering of immunity to intracellular pathogens. This Review first addresses our current understanding of the nucleic acid-sensing immune machinery. We then explain how the study of nucleic acid-sensing mechanisms not only has revealed their central role in driving the responses mediated by many current vaccines, but is also revealing how they could be harnessed for the design of new vaccines.

Adjuvants
Substances that facilitate, enhance and/or modulate the host immune response to an antigen.

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Along with improved sanitary conditions and antibiotics, vaccines undoubtedly are one of the greatest successes of medicine against infectious diseases. However, most current vaccines were developed rather empirically, with limited knowledge of their immunological mechanisms of action[1,2]. These empirical approaches are proving rather impractical for the development of vaccines against many emerging diseases and current pandemics, such as AIDS and malaria. Consequently, there currently is a strong impetus towards improving our understanding of the mechanisms of action of existing vaccines. Indeed, this may hold the key to the rational design of better vaccination strategies. The demand is also high for the development of innovative, rationally designed vaccine adjuvants. Although the efficiency of vaccines is currently mainly evaluated from their induction of neutralizing antibodies[3], T helper 1 (T_H1) and $CD8^+$ T cell responses are increasingly considered as essential (or desirable) components of vaccine-elicited protection against intracellular pathogens[2]. Therefore, investigators are looking for adjuvants that can also induce sustainable cellular responses.

With research intensifying in the field of vaccine immunology, a common theme has emerged as to the mechanisms underlying all efficient vaccines. This premise is that the triggering of innate immune mechanisms is the initial event that crucially determines the outcome of the adaptive immune response[1,2]. Vaccines are thought to use mainly two types of immune triggers. First, they may contain pathogen-associated molecular patterns (PAMPs) derived from the target pathogen (BOX 1). Second, vaccine components (such as certain adjuvants) may induce the release of endogenous damage-associated molecular patterns (DAMPs), although this mechanism is less well studied. PAMPs and DAMPs can stimulate the innate immune system by activating conserved receptors that are often referred to as pattern-recognition receptors (PRRs). PRR-derived signals are integrated directly or indirectly at the level of antigen-presenting cells (APCs) and in this way crucially condition the adaptive immune responses to the vaccine[4] (FIG. 1).

Microbial nucleic acids are an important class of PAMPs, especially in the recognition of pathogens such as viruses that otherwise present few conserved molecular patterns. Microbial nucleic acids are discriminated from self nucleic acids based on different parameters, such as their sequence, structure, molecular modifications and localization[5–7]. On the other hand, mislocalized self nucleic acids — such as extranuclear DNA or extracellular RNA — can be recognized as DAMPs, probably because they are reliable indicators of cellular damage[6,8].

Recent research is giving centre stage to the immune sensing of nucleic acids as PAMPs and DAMPs in current vaccination strategies and supports the idea that nucleic acid sensors may be harnessed in the design of new vaccines. In this Review, we first provide an overview of the current understanding of the nucleic acid-sensing machinery. We next focus on

Box 1 | **PAMPs, DAMPs and PRRs as initial triggers of immunity**

More than two decades ago, Charles Janeway Jr anticipated that the induction of adaptive immune responses against pathogens requires not only antigen recognition by the adaptive immune system, but also the sensing of 'stranger' signals associated with the pathogen. He termed these signals pathogen-associated molecular patterns (PAMPs), and proposed that they are detected by germline-encoded receptors of the innate immune system, which were in turn named pattern-recognition receptors (PRRs)[123]. PAMPs were predicted to be conserved molecular structures present in pathogens but absent from host cells. Several types of PAMP were subsequently identified, all of which broadly fall into two categories: molecular structures associated with microbial envelopes (such as bacterial lipopolysaccharide, flagellin and lipoproteins); and microbial nucleic acids[6]. An alternative theory was later proposed by Polly Matzinger, suggesting that the triggering of adaptive immunity essentially depends on the sensing of endogenous 'danger' signals that indicate damage to host cells and tissues[124]. These signals were collectively termed damage-associated molecular patterns (DAMPs). In theory, any host molecule that becomes exposed or is altered following damage so that it becomes recognizable by receptors of the innate immune system is potentially a DAMP. Identified DAMPs include cleaved matrix proteins (such as low-molecular-weight hyaluronan), liberated intracellular proteins (such as heat-shock proteins, histones and high-mobility group box proteins) and extracellular host nucleic acids[8]. Although some DAMPs bind to non-PRR receptors, most DAMPs were proposed to activate PRRs[8]. In the context of infection and vaccination, parts of the 'stranger' and 'danger' models are probably complementary, in that PRR-mediated detection of both PAMPs and DAMPs might cooperate or synergize to activate innate and adaptive immune responses.

recent attempts at deconstructing the role of nucleic acid-sensing PRRs in current vaccines — including live attenuated vaccines, aluminium salt-adjuvanted vaccines and DNA vaccines — and on the valuable insights this is starting to offer into their mechanisms of action. We finally illustrate how recent research is harnessing nucleic acid-sensing PRRs in the rational design of new vaccine adjuvants.

Nucleic acid-sensing PRRs: a growing family

With new components being regularly identified, the study of nucleic acid-sensing PRRs and their downstream effectors is revealing a rather complex molecular machinery (FIG. 2). In this section, we provide a snapshot of the known and emerging nucleic acid-sensing PRRs, their ligands and their associated downstream signalling pathways. Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) have been the subject of excellent recent reviews[7,9–11] and will be addressed only briefly.

Nucleic acid-sensing TLRs. Out of the ten human TLRs and their twelve well-characterized mouse counterparts, four TLRs (TLR3, TLR7, TLR8 and TLR9) are nucleic acid sensors that recognize diverse pathogen-derived nucleic acids and synthetic ligands[10] (TABLE 1). Expression of the different TLRs is cell type-specific, resulting in a partition of PAMP recognition among different APCs[12,13]. TLR3 is expressed by conventional dendritic cells (cDCs) and macrophages, but not by plasmacytoid dendritic cells (pDCs). In humans, TLR7 and TLR9 expression is mostly restricted to pDCs and B cells, whereas the expression pattern of TLR8 is much broader and includes monocytes, macrophages and cDCs, but not pDCs.

TLR3, TLR7, TLR8 and TLR9 are intracellular TLRs and react to pathogen-derived nucleic acids that are taken up by endocytosis or derived from autophagy and transferred to the endolysosomal compartment[9]. This compartmentalization of nucleic acid-sensing TLRs seems to be essential to avoid cross-reactivity with host nucleic acids[7,9].

With the exception of TLR3, all nucleic acid-sensing TLRs depend on the adaptor protein myeloid differentiation primary-response protein 88 (MYD88) for signalling. MYD88-dependent TLR signalling results in the activation of the transcription factors activator protein 1 (AP1), nuclear factor κB (NF-κB), interferon-regulatory factor 1 (IRF1) and IRF5. This leads to the subsequent expression of pro-inflammatory cytokines that are essential for the recruitment and activation of immune cells[14]. TLR3 signalling uniquely depends on TIR-domain-containing adaptor protein inducing IFNβ (TRIF) and leads to the activation of AP1 and NF-κB, with the subsequent expression of pro-inflammatory cytokines. Through the activation of TANK-binding kinase 1 (TBK1) and IκB kinase-ε (IKKε), TRIF-dependent signalling also activates the transcription factor IRF3, which induces the expression of type I interferons (IFNs), which are essential in inducing antiviral responses (BOX 2). Of note, pDCs have an additional and unique wiring of MYD88 signalling, which, following TLR7 and TLR9 activation, leads to the IRF7-dependent expression of large quantities of type I IFNs[9].

RLRs and related helicases. RLRs — namely, retinoic acid-inducible gene I (RIG-I; also known as DDX58), melanoma differentiation-associated protein 5 (MDA5; also known as IFIH1) and laboratory of genetics and physiology 2 (LGP2; also known as DHX58) — are members of the DExD/H-box helicase superfamily that act as cytosolic RNA sensors[7,11]. RLRs are expressed broadly by immune and non-immune cells *in vivo*.

The prototypical natural ligand of RIG-I is short RNA with blunt-ended base pairing and an uncapped 5′ triphosphate end, although RIG-I has been shown to bind to various double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) ligands[7,11,15]. RIG-I may also be indirectly activated by cytosolic viral and bacterial double-stranded DNA (dsDNA), as pathogen AT-rich dsDNA can be transcribed by RNA polymerase III to generate dsRNA with 5′ triphosphate ends[16,17]. MDA5 generally responds to long dsRNA molecules[18]. Furthermore, RIG-I and MDA5 may be activated by self RNAs that are cleaved by RNase L[19]. The function of LGP2 has been little studied so far, but recent studies in LGP2-deficient mice indicate that it may positively participate in RIG-I- and MDA5-dependent antiviral responses[20,21].

As reviewed recently, MDA5 and RIG-I are important inducers of innate immunity to viruses[11]. In addition, RIG-I and MDA5 have been implicated in the sensing of bacteria[17,22,23], suggesting that RLR function extends beyond the roles of these receptors in antiviral immunity.

Conventional dendritic cells (cDCs). Phagocytes that are resident in lymphoid and non-lymphoid tissues and are specialized in the presentation of antigens to T cells.

Plasmacytoid dendritic cells (pDCs). A DC subtype specialized in producing large amounts of type I interferons in response to nucleic acids from pathogens.

RNase L
A ribonuclease that is induced in response to type I interferons and degrades all the RNA within the cell.

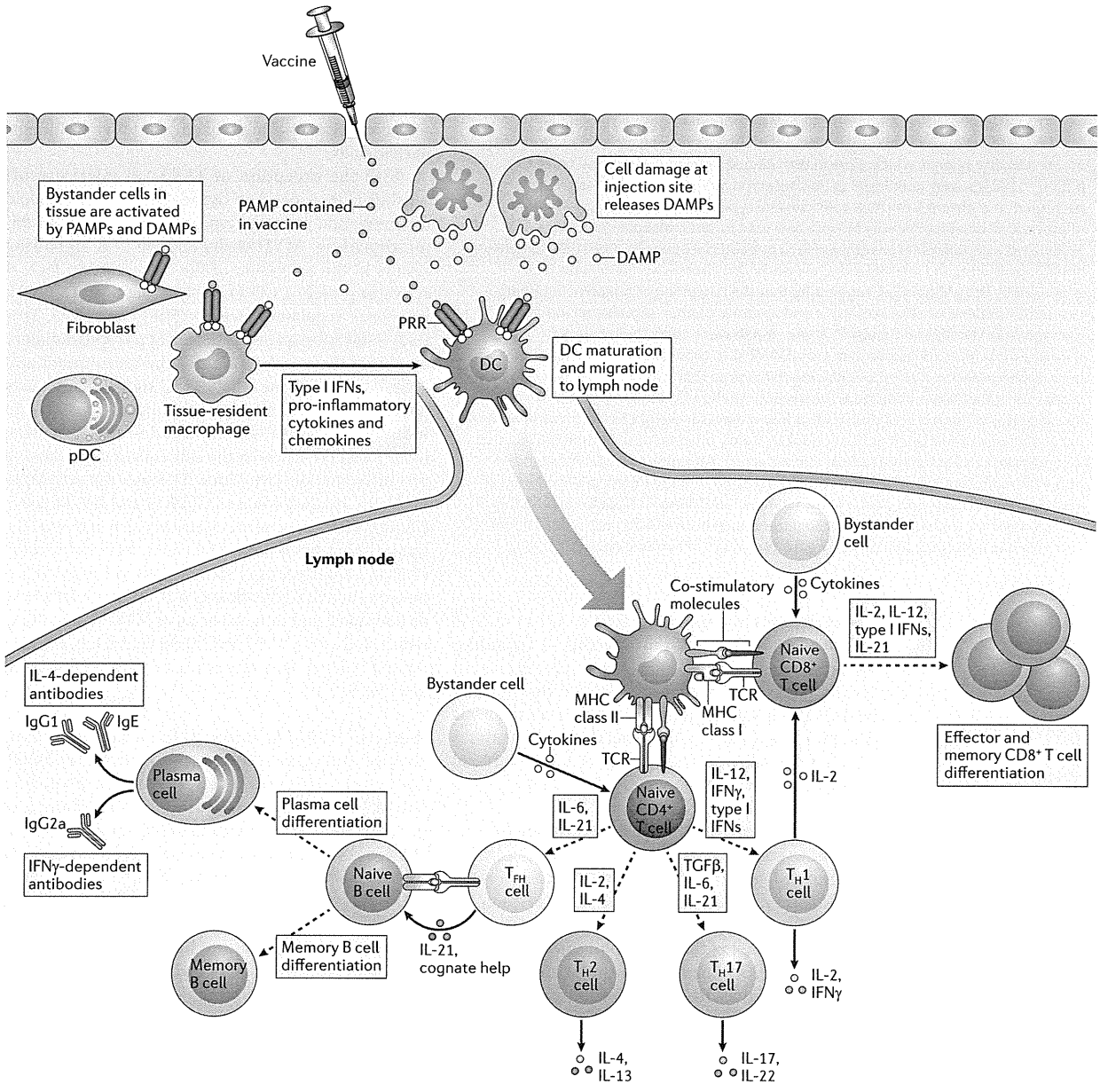

Figure 1 | **Induction of adaptive immune responses to vaccines through PRR-mediated dendritic cell activation.** Vaccines may contain pathogen-associated molecular patterns (PAMPs) or may induce the local release of damage-associated molecular patterns (DAMPs). These PAMPs and DAMPs are detected directly by pattern-recognition receptors (PRRs) expressed by dendritic cells (DCs), leading to DC activation, maturation and migration to the lymph nodes. Alternatively, PRR-mediated recognition of PAMPs and DAMPs by bystander cells may induce the release of tissue-derived factors, such as cytokines, that may cooperate in the activation and orientation of the DC response. In the lymph nodes, the activated DCs may present antigens to T cells, provide them with co-stimulatory signals and stimulate their differentiation by providing a favourable cytokine milieu. Some cytokines — such as interleukin-4 (IL-4) and type I interferons (IFNs) — may be provided by bystander cells. Depending on the cytokine milieu, $CD4^+$ T cells may differentiate into various T helper (T_H) cell subtypes. T_H cells may also acquire a T follicular helper (T_{FH}) cell phenotype and help in the activation of cognate B cells, thereby promoting the entry of these B cells into the plasma cell pathway or the germinal centre pathway. In addition, the cytokine expression profile of T_{FH} cells can dictate B cell isotype switching. Depending on the balance between activating cytokines (and most often with the help of T_H1 cell-derived IL-2), activated $CD8^+$ T cells differentiate into effector and memory $CD8^+$ T cells. pDC, plasmacytoid dendritic cell; TCR, T cell receptor; TGFβ, transforming growth factor-β.

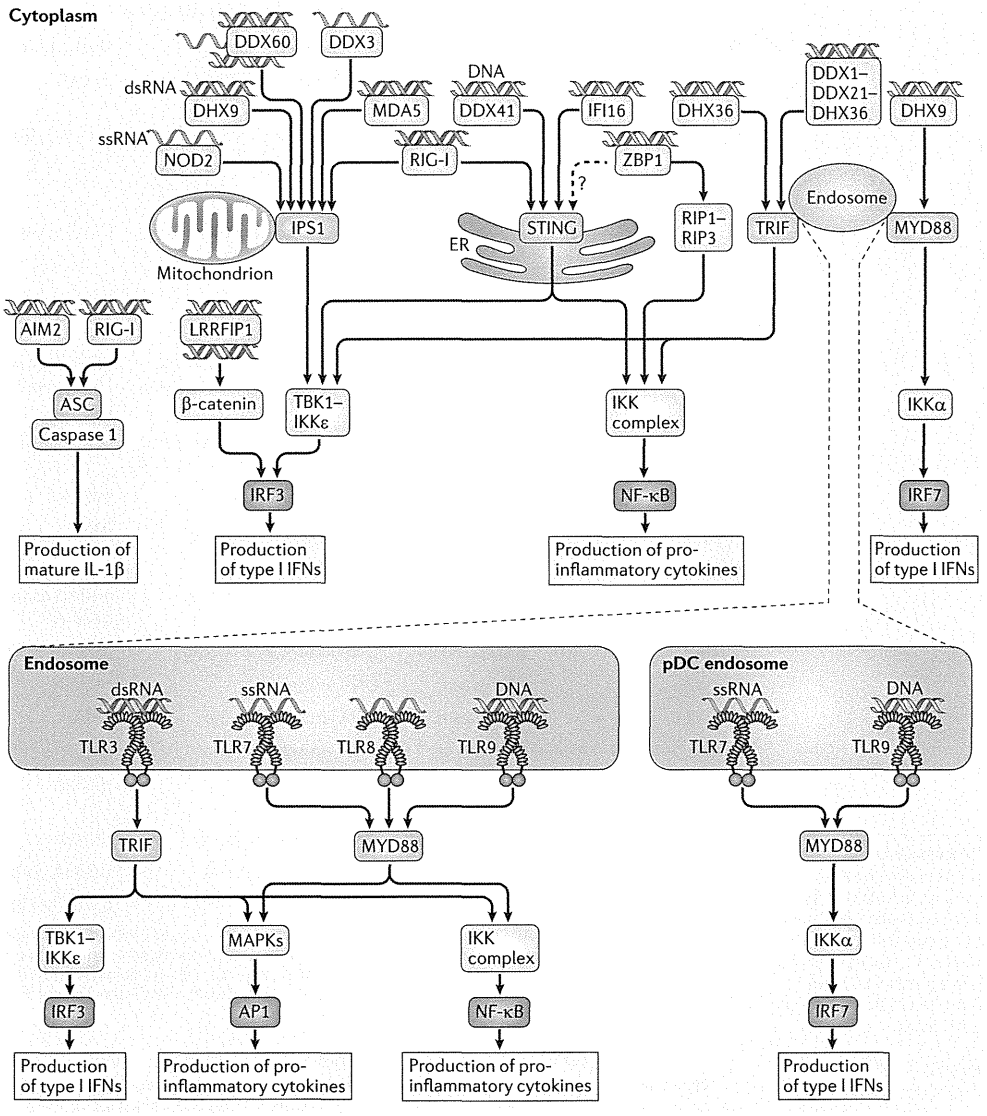

Figure 2 | **Overview of the nucleic acid-sensing machinery.** Endosomal Toll-like receptor 7 (TLR7), TLR8 and TLR9 initiate downstream signalling through the adaptor protein myeloid differentiation primary-response protein 88 (MYD88) in the cytosol. This leads to the activation of mitogen-activated protein kinases (MAPKs) and the IκB kinase (IKK) complex and subsequent activation of the transcription factors activator protein 1 (AP1) and nuclear factor-κB (NF-κB), promoting the expression of pro-inflammatory cytokines. In plasmacytoid dendritic cells (pDCs), the activation of TLR7 and TLR9 also leads to the expression of high levels of type I interferons (IFNs) by promoting the activation of interferon-regulatory factor 7 (IRF7) via IKKα. Endosomal TLR3 signals through TIR-domain-containing adaptor protein inducing IFNβ (TRIF), which in addition to activating NF-κB and AP1 may activate IRF3 through TANK-binding kinase 1 (TBK1) and IKKε, leading to the expression of type I IFNs. Various cytosolic receptors — including nucleotide-binding oligomerization domain protein 2 (NOD2), the RIG-I-like receptors (RLRs) retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), and some other DExD/H-box helicases — may induce the expression of pro-inflammatory cytokines and type I IFNs through the *IFNB*-promoter stimulator 1 (IPS1)-mediated activation of TBK1 and IKKε or through the activation of the IKK complex. The proposed cytosolic DNA receptors DDX41, IFNγ-inducible protein 16 (IFI16) and possibly Z-DNA-binding protein 1 (ZBP1) interact with stimulator of IFN genes (STING) to activate TBK1, IKKε and the IKK complex. ZBP1 was also shown to directly interact with receptor-interacting protein 1 (RIP1) and RIP3 to induce NF-κB activation. The helicases DDX1, DDX21 and DHX36 have been proposed to form a TRIF-interacting complex, and LRRFIP1 (leucine-rich repeat flightless-interacting protein 1) was suggested to potentiate IRF3 transcriptional activity through β-catenin. In pDCs, DHX36 and DHX9 activate TRIF-dependent and MYD88-dependent signalling, respectively. Finally, RIG-I and absent in melanoma 2 (AIM2) may induce inflammasome formation and caspase 1 activation through the adaptor protein ASC, leading to the release of mature interleukin-1β (IL-1β). dsRNA, double-stranded RNA; ER, endoplasmic reticulum; ssRNA, single-stranded RNA.

Table 1 | **Nucleic acid-sensing PRRs: localization, sensed pathogens and agonists**

PRR	Localization	Sensed pathogens	Natural agonists	Synthetic agonists
TLR3	Endolysosomal compartment	dsRNA viruses, ssRNA viruses, dsDNA viruses	dsRNA	Polyl:C, polyU
TLR7	Endolysosomal compartment	ssRNA viruses, bacteria, fungi, protozoan parasites	GU-rich ssRNA	Imidazoquinolines (R848, imiquimod, 3M001), guanosine analogues
TLR8	Endolysosomal compartment	ssRNA viruses, bacteria, fungi, protozoan parasites	GU-rich ssRNA	Imidazoquinolines (R848, 3M002), guanosine analogues
TLR9	Endolysosomal compartment	dsDNA viruses, bacteria, protozoan parasites	DNA	CpG ODNs
RIG-I	Cytoplasm	ssRNA viruses, DNA viruses, *Flaviviridae*, reovirus, bacteria	Short RNA with 5′ppp and/or base pairing	Short polyI:C
MDA5	Cytoplasm	*Picornaviridae*, vaccinia virus, *Flaviviridae*, reovirus, bacteria	Long dsRNA	Polyl:C
NOD2	Cytoplasm	RNA viruses	ssRNA	–
DDX3	Cytoplasm	RNA viruses	RNA	–
DDX1–DDX21–DHX36	Cytoplasm	RNA viruses	dsRNA	Polyl:C
DDX60	Cytoplasm	RNA viruses, DNA viruses	ssRNA, dsRNA, dsDNA	–
DHX9	Cytoplasm	DNA viruses, RNA viruses	dsDNA, dsRNA	CpG-B ODNs
DHX36	Cytoplasm	DNA viruses	dsDNA	CpG-A ODNs
DDX41	Cytoplasm	DNA viruses, bacteria	DNA	–
AIM2	Cytoplasm	DNA viruses, bacteria	DNA	–
IFI16	Cytoplasm and nucleus	DNA viruses	dsDNA	–
ZBP1	Cytoplasm	DNA viruses, bacteria	dsDNA	–
LRRFIP1	Cytoplasm	DNA viruses, bacteria	dsDNA, dsRNA	–
STING	Cytoplasm	Bacteria	Cyclic di-GMP	–

5′ppp, 5′ triphosphate end; AIM2, absent in melanoma 2; dsRNA, double-stranded RNA; IFI16, IFNγ-inducible protein 16; LRRFIP1, leucine-rich repeat flightless-interacting protein 1; MDA5, melanoma differentiation-associated protein 5; NOD2, nucleotide-binding oligomerization domain protein 2; ODN, oligodeoxynucleotide; polyI:C, polyinosinic–polycytidylic acid; PRR, pattern-recognition receptor; RIG-I, retinoic acid-inducible gene I; ssRNA, single-stranded RNA; STING, stimulator of IFN genes; TLR, Toll-like receptor; ZBP1, Z-DNA-binding protein 1.

Inflammasome
A multiprotein signalling complex, the activation and assembly of which leads to the recruitment and activation of caspase 1, resulting in the cleavage of pro-IL-1β and pro-IL-18 into their biologically active forms.

PolyI:C
(Polyinosinic–polycytidylic acid). A substance that is used as a mimic of viral double-stranded RNA.

CpG-B and CpG-A oligodeoxynucleotides
Synthetic oligodeoxynucleotides that contain immunostimulatory unmethylated dinucleotide CpG motifs. CpG-A oligodeoxynucleotides are based on a mixed phosphodiester–phosphorothioate backbone, contain a single CpG motif within a palindromic sequence and have a 3′ polyG tail, whereas CpG-B oligodeoxynucleotides are based on a phosphorothioate backbone and contain multiple CpG motifs.

RLR signalling depends on the adaptor *IFNB*-promoter stimulator 1 (IPS1; also known as MAVS, CARDIF and VISA). Interactions between RLRs and IPS1 lead to the activation of the transcription factors IRF1, IRF3, IRF7 and NF-κB, resulting in the expression of type I IFNs and pro-inflammatory cytokines[7,11]. In addition, RIG-I may interact with the adaptor protein ASC, resulting in inflammasome-dependent caspase 1 activation and the subsequent production of active interleukin-1β (IL-1β)[24]. RIG-I, but not MDA5, was also shown to interact with stimulator of IFN genes (STING; also known as MITA, MPYS and ERIS), which is an adaptor protein that is encoded by *Tmem173* and is predominantly found in the endoplasmic reticulum[25,26]. This interaction potentiates RIG-I signalling through TBK1 following RNA virus infection via as-yet-unclear mechanisms that potentially involve IPS1.

In addition to RLRs, several other members of the DExD/H-box helicase superfamily have recently been proposed to participate in sensing pathogen-derived nucleic acids. One report suggested that DDX3 might directly bind to viral RNA and associate with RIG-I, MDA5 and IPS1 (REF. 27). In a different study, DDX1, DDX21 and DHX36 were proposed to form a polyI:C-binding complex that interacts with TRIF in a mouse cDC cell line[28]. Silencing of DDX1, DDX21 or DHX36 expression reduced the production of type I IFNs by cells stimulated with long or short forms of polyI:C as well as during infection with RNA viruses. Another study suggested that DDX60 binds to viral ssRNA, dsRNA and dsDNA and associates with RIG-I, MDA5 and LGP2 (REF. 29). Silencing of DDX60 expression led to reduced type I IFN secretion following infection with RNA and DNA viruses, presumably owing to reduced RLR signalling and IRF3 activation.

A role has also been proposed for DHX9 and DHX36 as cytoplasmic sensors of CpG-B and CpG-A oligodeoxynucleotides, respectively, in a human pDC cell line[30]. Moreover, silencing of DHX9 or DHX36 expression in pDCs infected with a DNA virus led

Box 2 | **Type I interferons in adaptive immunity**

Type I interferons (IFNs) are a family of cytokines that comprises 12 IFNα subtypes, IFNβ1, IFNε, IFNκ and IFNω and has essential roles in the immune responses against viruses and other intracellular pathogens[125]. Type I IFNs are mostly known for their capacity to generate an innate antiviral state by inducing the expression of IFN-stimulated genes[126]. In addition to this essential function, type I IFNs may also profoundly affect adaptive immune responses, most often by contributing to the induction of T helper 1 (T_H1)-type responses[125]. Indeed, type I IFNs may directly favour the differentiation and modulate the effector function of T_H1 cells. Furthermore, type I IFNs promote the cross-presentation of antigens to $CD8^+$ T cells by conventional dendritic cells and may directly stimulate the proliferation of $CD8^+$ T cells. Finally, they have been shown to stimulate antibody production and isotype switching in B cells.

to decreased expression of tumour necrosis factor (TNF) and IFNβ1, respectively. It has been suggested that DHX9 and DHX36 may bind directly to MYD88. In keeping with this, silencing of DHX9 expression reduces the nuclear translocation of NF-κB in response to CpG-B-mediated stimulation, whereas silencing of DHX36 expression reduces the nuclear localization of IRF7 following CpG-A-mediated stimulation. Together, these observations suggest that DHX9 and DHX36 might trigger distinct MYD88-dependent signalling pathways in pDCs. Intriguingly, DHX9 and DHX36 do not appear to intervene in the response of cDCs to dsDNA[31], and this might point towards a pDC-specific role of these proteins. By contrast, DHX9 has been proposed to sense dsRNA in cDCs[32].

Finally, DDX41 was shown to bind dsDNA and to directly interact with STING and TBK1, but not IPS1 (REF. 31). Indeed, silencing of DDX41 expression led to a marked inhibition of type I IFN production by DCs following transfection with DNA or during infection with DNA viruses or *Listeria monocytogenes*.

NLRs and ALRs. NOD-like receptors (NLRs) are a family of cytosolic proteins with diverse functions in the immune system[33]. Despite their denomination, most NLRs actually seem to act as adaptor molecules rather than as receptors *per se*, and only some NLRs have been shown to directly bind PAMPs or DAMPs so far. Nevertheless, a recent report suggests that nucleotide-binding oligomerization domain protein 2 (NOD2) — which is already known as a receptor for the bacterial envelope component muramyl dipeptide — could also be implicated in the production of type I IFNs in response to viral infection through the sensing of ssRNA[34]. The proposed pathway involves signalling via IPS1 and subsequent activation of IRF3. NLRP3 (NOD-, LRR- and pyrin domain-containing 3), which is another NLR, is indirectly activated by viral and synthetic ssRNA and dsRNA, resulting in ASC-dependent inflammasome formation and the secretion of biologically active IL-1β[35,36]. Very recently, NLRP3 was also shown to directly sense oxidized mitochondrial DNA that is released into the cytosol during macrophage apoptosis, leading to inflammasome-dependent IL-1β production[37].

AIM2-like receptors (ALRs) are a newly proposed group of nucleic acid-sensing PRRs that comprises two members of the pyrin and HIN domain-containing protein family (PYHIN family): absent in melanoma 2 (AIM2) and IFNγ-inducible protein 16 (IFI16)[38]. AIM2 has been shown to detect cytoplasmic dsDNA and to induce the ASC-dependent formation of inflammasomes, resulting in the activation of caspase 1 and the production of biologically active IL-1β[39–42]. IFI16 was recently identified as a cytoplasmic protein able to bind to an IFNβ1-inducing fragment of the vaccinia virus dsDNA genome in human monocytes[43]. Gene-silencing experiments indicate that IFI16 promotes type I IFN production in response to transfected DNA and DNA virus infection. IFI16 signalling to induce type I IFNs involves STING, TBK1 and IRF3. IFI16 was also proposed to mediate the recognition of viral infection in the nucleus, resulting in the activation of inflammasomes[44]. Whether direct sensing of the viral nucleic acids is involved in this particular situation currently remains unknown.

Other nucleic acid-sensing PRRs. ZBP1 (Z-DNA-binding protein 1; also known as DAI and DLM1) is a type I IFN-inducible DNA-binding protein of poorly understood function. Silencing of ZBP1 expression *in vitro* decreases type I IFN production in response to transfected DNA or infection with a dsDNA virus[45]. ZBP1 may associate with TBK1 and IRF3 (REF. 45), and it has also been implicated in the activation of NF-κB through receptor-interacting protein 1 (RIP1) and RIP3 (REF. 46). However, ZBP1-deficient mice still respond to DNA vaccination and DNA virus infection in a similar manner to their wild-type counterparts[47]. This apparent discrepancy between *in vitro* and *in vivo* data has been attributed to a possible redundancy of DNA-sensing receptors and to cell type-specific effects. The contribution of ZBP1 to DNA sensing *in vivo* thus remains to be established.

LRRFIP1 (leucine-rich repeat flightless-interacting protein 1) is a leucine-rich motif-containing protein that was identified in a gene-silencing screen in macrophages as a cytosolic receptor involved in the production of type I IFNs in response to transfected DNA or bacterial infection[48]. LRRFIP1 is thought to be able to directly bind dsDNA and dsRNA, and to potentiate IRF3 transcriptional activity at the *IFNB1* promoter through β-catenin-dependent signalling.

STING is mostly known as an important adaptor protein downstream of many TBK1-activating PRRs. However, STING was also recently shown to directly bind to the bacterial nucleic acid signalling molecules cyclic di-GMP and cyclic di-AMP[49]. This finding indicates that STING could also be considered as a nucleic acid-sensing PRR.

Deconstructing current vaccines

As is apparent from their respective downstream effectors, nucleic acid-sensing PRRs can activate the key pathways of the innate immune system and, as such, may potentiate antigen-specific adaptive immune responses. Recent studies are starting to highlight the role of nucleic acids as 'built-in' adjuvants in important

classes of vaccines, such as live attenuated vaccines and DNA vaccines. Emerging evidence also supports the concept that nucleic acids and their metabolites are important endogenous mediators of the adjuvant effects of aluminium salt-based adjuvants (commonly referred to as alum), an important class of vaccine adjuvants. This knowledge could provide useful hints for the design and optimization of future vaccines.

Deconstructing live vaccines. Some live attenuated vaccines are among the most efficient vaccines ever developed. Although live attenuated vaccines cannot be generated against all types of pathogen, deconstructing the responses they induce may offer valuable clues for the design of new vaccines that mimic their mechanisms of action. Few studies have addressed this so far, but the data are starting to point towards a central role of nucleic acid-sensing PRRs in the response to live attenuated vaccines.

The yellow fever vaccine YF-17D is one of the most efficient antiviral vaccines ever developed, and it is able to induce protective immunity that lasts for decades. Evidence in mice indicates that YF-17D activates DCs through the concomitant stimulation of several TLRs (namely, TLR2, TLR7, TLR8 and TLR9), which results in the induction of $CD8^+$ T cell responses and a mixed T_H1- and T_H2-type immune response[50]. Although TLR2 signalling, which depends on MYD88, appears to downregulate the T_H1 and $CD8^+$ T cell responses elicited by the vaccine, MYD88-dependent signalling is required for these responses. Without ruling out a potential contribution of IL-1 and related cytokines or other MYD88-dependent PRRs, these results suggest an important role for nucleic acid-sensing TLRs in the induction of adaptive T_H1-type responses to YF-17D. In support of this assumption, DCs from mice deficient for either TLR7 or TLR9 secrete less IL-12 than wild-type DCs following infection with YF-17D[50]. In vaccinated humans, gene expression profiling indicates that YF-17D activates a prominent type I IFN response (which is probably controlled by IRF7) at the time the primary adaptive immune response is established[51,52]. Furthermore, YF-17D upregulates the expression of TLR7 (REF. 51) and activates RIG-I and MDA5 (REF. 52), although the contribution of these receptors to adaptive immune responses in this context is currently unknown. Finally, a recent study in humans indicates that YF-17D induces innate immune gene expression profiles that functionally overlap with those elicited by an experimental adjuvant that is based on a modified polyI:C agonist of TLR3 and MDA5 (REF. 53).

Vaccinia virus is the attenuated virus that formed the basis of the vaccine that allowed the eradication of smallpox. It is now used as a vector in other vaccines. Vaccinia virus may activate several APC-expressed PRRs, including RIG-I, MDA5, TLR2, TLR6, TLR9 and NLRP3- and AIM2-dependent inflammasomes[41,54,55]. Studies in knock-out mice have revealed that the activation of innate immune responses and the induction of $CD8^+$ T cell population expansion and memory formation in response to vaccinia virus crucially depend on TLR2 (REF. 56), but also require type I IFN production[56,57]. Moreover, a recent report suggests that, in mice, type I IFN production following vaccinia virus infection may result from TLR8-dependent activation of pDCs, possibly through the recognition of AT-rich DNA[58]. Whether this mechanism also occurs in humans, whose pDCs do not express TLR8, is not yet certain. In addition, cDCs may produce type I IFNs following vaccinia virus infection in a TLR-independent manner, probably through RLR-dependent signalling[55,56].

In the case of influenza A virus, a variety of vaccine compositions have been developed, including live attenuated, killed whole-virion and subunit vaccines. The influenza virus ssRNA genome has been shown to activate pDCs through TLR7 (REFS 59,60) and cDCs and stromal cells through RIG-I-dependent sensing[61,62]. Influenza virus RNA also indirectly triggers inflammasome activation[35,36,63]. Subunit vaccines, which are devoid of viral RNA, were shown to be ineffective at immunizing naive mice owing to their inability to stimulate pDCs, although they could still boost memory T cell responses[64]. This evidence underscores the importance of viral nucleic acid sensing in influenza vaccination. By contrast, live attenuated and killed vaccines induce robust primary adaptive immune responses through TLR7, a process that requires the production of type I IFNs by pDCs in the case of killed vaccines[64,65].

Very few studies so far have investigated the role of nucleic acid-sensing PRRs in live attenuated bacterial vaccines. The immunogenicity of such vaccines — which include the *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) vaccine — is usually attributed to the innate recognition of bacterial cell wall components, mostly by TLR2 and TLR4. However, live bacteria may also activate APCs through nucleic acid-sensing PRRs[17,22,66,67]. Recent research indicates that nucleic acid sensing could actually be key to the success of live bacterial vaccines.

One possible explanation for the higher efficiency of live attenuated bacterial vaccines over killed vaccines could be that the immune system is able somehow to sense general bacterial viability. This possibility has recently received support from an elegant study that compared the innate and adaptive immune responses induced by live and killed non-replicating non-virulent bacteria[68]. Live bacteria, but not killed bacteria, were shown to induce pronounced expression of type I IFNs and the release of mature IL-1β from infected macrophages and DCs. The augmented response to live bacteria was shown to depend on the sensing of bacterial mRNA, which is lost following the killing of the bacteria and was therefore termed a viability-associated PAMP ('vita-PAMP'). The cytosolic PRR responsible for vita-PAMP sensing in this context has not been identified, but the induction of type I IFNs by IRF3 and the generation of IL-1β by the NLRP3 inflammasome were impaired in TRIF-deficient cells. The recognition of this vita-PAMP was proposed to depend on the absence of 3′-polyadenylation in bacterial mRNA. Consistent with the idea that vita-PAMP sensing may boost adaptive immune responses, killed bacteria mixed with bacterial mRNA were shown to induce humoral responses similar to those induced by live bacteria in mice.

Molecular mechanisms of DNA vaccination. DNA vaccines are one example of vector-based vaccines that are currently in development[69]. What is considered a major advantage of DNA vaccines is their ability to induce the local expression of target antigens and to subsequently elicit T_H1 and $CD8^+$ T cell responses along with T_H1-biased antibody production. DNA vaccines are currently used in veterinary medicine, and attempts in humans indicate a good tolerability and safety profile[69,70]. However, DNA vaccines tend to display low immunogenicity in humans and this has hindered their development, although different approaches have been proposed to address this issue. The reasons for this lower responsiveness of humans compared with other mammals are currently unclear. Possible explanations could involve lower expression levels of certain components of the DNA-sensing machinery, differing expression patterns of nucleic acid-sensing PRRs or issues related to DNA delivery and processing in different cell types[69,70]. It is likely that a more accurate characterization of the cellular and molecular mechanisms involved in nucleic acid sensing during DNA vaccination would help us to understand these issues and improve the design of such vaccines.

The plasmids used in DNA vaccination may contain CpG motifs, which would provide a built-in adjuvant because these PAMPs activate TLR9. However, TLR9 deficiency does not appear to affect the cellular or humoral immune responses to repeated DNA vaccination in mice[47,71,72], although TLR9 could participate in $CD8^+$ T cell induction following the initial immunization[73]. Instead, T_H1 and $CD8^+$ T cell responses, as well as antibody production, in response to DNA vaccination in mice have been shown to crucially depend on the induction of type I IFNs through the STING–TBK1 axis[47,74]. Although the PRR implicated in DNA detection in this context remains to be identified, this suggests that cytoplasmic receptors for DNA have a more prominent role than intracellular TLRs in mediating the effect of DNA vaccines. Given that STING engagement may also lead to NF-κB activation[74], it could be worthwhile investigating the potential contribution of this pathway in DNA vaccination.

DNA vaccine administration may lead to the direct transfection of APCs or to the transfection of other tissue-resident cells, such as muscle cells. In the latter case, antigens may be indirectly acquired by DCs for presentation[69]. Bone marrow transfer experiments in mice support the idea that antibody responses to DNA vaccination require TBK1 activation in haematopoietic cells (presumably DCs)[47]. By contrast, TBK1 activity in non-haematopoietic cells (presumably stromal cells) is essential for $CD8^+$ T cell activation. Finally, the activation of antigen-specific $CD4^+$ T cells requires TBK1 activity in both the haematopoietic and non-haematopoietic compartments. Altogether, direct presentation, cross-presentation and bystander cytokine production are all likely to be essential for the adaptive immune response to DNA vaccines (FIG. 3).

Cross-presentation
A process by which certain antigen-presenting cells may take up and process extracellular antigens and present them on MHC class I molecules to $CD8^+$ T cells.

Nucleic tricks of an old adjuvant. Alum is the oldest but most widely used of the few vaccine adjuvants that are licensed for human use[1,75]. Alum mostly potentiates IgG1 and IgE production through the promotion of T_H2 cell responses, although the induction of $CD8^+$ T cells by alum has also been reported[76]. For decades, little attention has been given to the immunological mechanisms that drive the adjuvant activity of alum[77]. Renewed interest was sparked by the discovery that alum activates the NLRP3 inflammasome[78,79]. However, studies on the contribution of NLRP3 to the effects of alum on adaptive immune responses have generated conflicting results[76,80], suggesting that the NLRP3 inflammasome is not, in general, essential for the adjuvant activity of alum and that additional mechanisms are involved.

Dead lysed cells have been repeatedly observed at sites of alum injection[81,82], implying that alum may induce the release of DAMPs. Research in mouse models recently reported a role for two DAMPs, which were both connected to nucleic acid biology, in the adjuvant activity of alum[83–85]. Uric acid is the end product of the degradation of purines, and may be rapidly released by injured cells following DNA and RNA degradation. Alum induces the accumulation of uric acid at sites of injection, and reducing uric acid levels *in vivo* through treatment with uricase was shown to inhibit T cell responses and the production of IgG1 and IgE[83,84]. Uric acid has not been shown to form crystals (its usual form for recognition as a DAMP[8]) at sites of alum injection, and the signalling pathways activated in this context remain to be identified. Alum also induces the rapid release of host cell DNA at sites of injection[82,85], and the elimination of extracellular DNA using DNase I treatment decreases alum-induced T cell responses and the production of IgG1 and IgE[85]. Although the PRRs (or PRR) triggered by host DNA in alum immunization were not identified, IRF3 was shown to control the IgE response. However, any contribution of TLRs, RLRs or inflammasomes to this response was ruled out.

Harnessing nucleic acid sensors

With the increased recognition of the impact of nucleic acid-sensing PRRs on APC function, research is well underway to directly harness these PRRs using novel adjuvants. Several candidates, mostly TLR agonists so far, are now in the preclinical or early clinical stages of development[75].

TLR3 and RLR agonists. The activation of TLR3 in cDCs induces the production of IL-12, type I IFNs and pro-inflammatory cytokines by these cells and upregulates their expression of MHC class II and co-stimulatory molecules, as well as their cross-presentation activity[86–89]. Of note, cDCs with strong cross-presentation activity — such as $CD8\alpha^+$ and $CD103^+$ cDCs in mice and $DNGR1^+CD114^+BDCA3^+$ cDCs in humans — express the highest levels of TLR3 (REFS 88–90).

In preclinical models, co-administration of TLR3 agonists with soluble or DC-targeted antigens was shown to induce durable T_H1 cell[91–93] and $CD8^+$ T cell[89] responses, as well as augmented antibody responses[93–95], which could confer protection against subsequent intracellular pathogen infection[89,95].

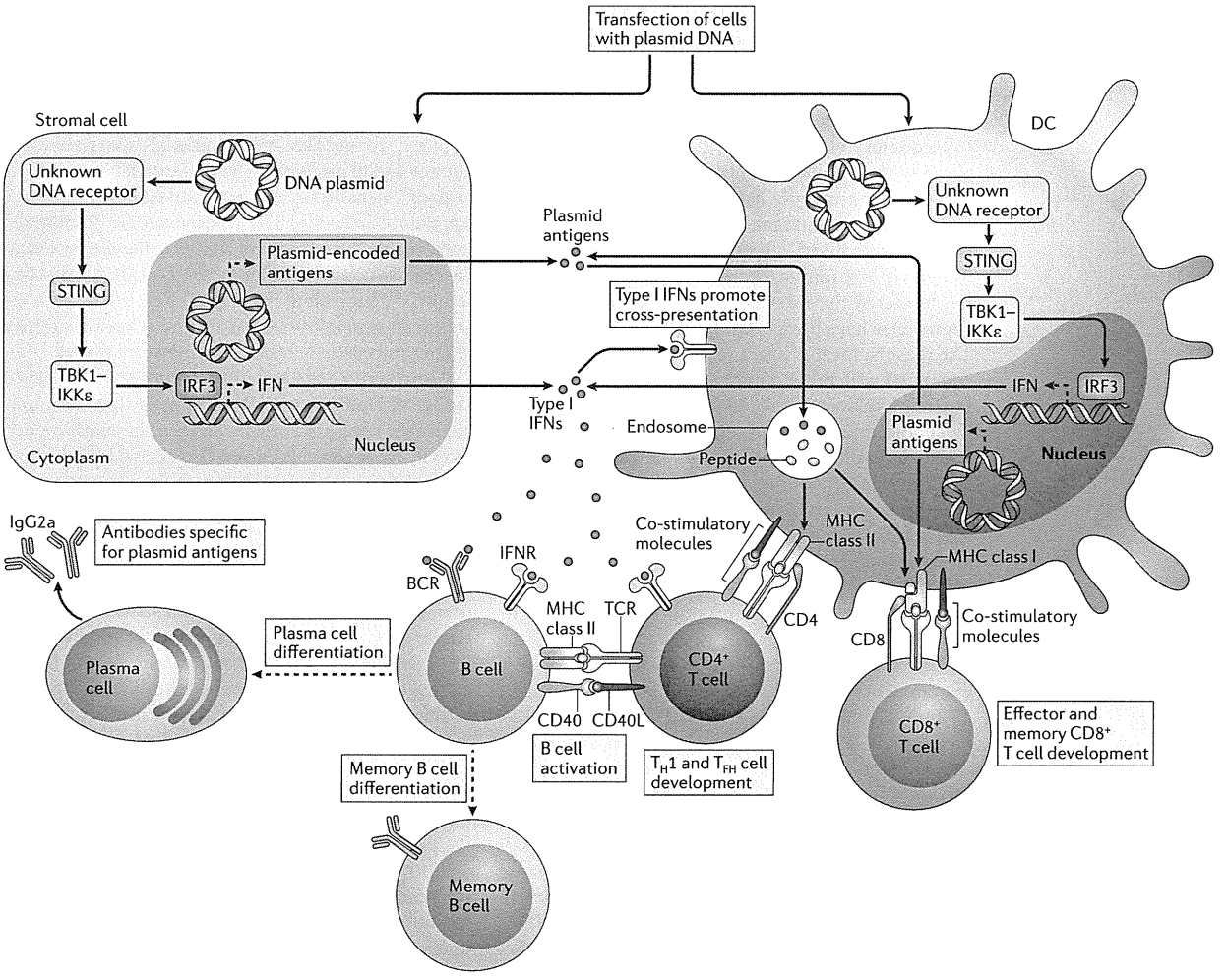

Figure 3 | **Mechanisms of DNA vaccination.** The plasmid DNA used in DNA vaccination may directly transfect stromal cells (such as muscle cells) or dendritic cells (DCs). In these cells, a cytosolic DNA receptor that has not yet been identified induces the activation of TANK-binding kinase 1 (TBK1) and IκB kinase-ε (IKKε) through stimulator of IFN genes (STING), leading to the activation of interferon-regulatory factor 3 (IRF3) and resulting in the production of type I interferons (IFNs). The antigens encoded by the transfected plasmid DNA can also be expressed in stromal cells and DCs. In DCs, these antigens may be directly processed and presented on MHC class I molecules to naive $CD8^+$ T cells. Alternatively, antigens may be indirectly acquired by DCs from stromal cells and then cross-presented to $CD8^+$ T cells or presented to naive $CD4^+$ T cells on MHC class II molecules. Type I IFN expression by stromal cells and DCs seems to be important for promoting the cross-presentation activity of DCs, as well as for the differentiation of T helper 1 (T_H1) cells and the promotion of T_H1-type isotype switching in B cells. BCR, B cell receptor; CD40L, CD40 ligand; TCR, T cell receptor; T_{FH}, T follicular helper.

Most TLR3 agonists, such as polyI:C, also activate MDA5 in DCs and stromal cells. Both TLR3 and MDA5 were proposed to participate in the induction of type I IFN production[92,94,96], which is essential for the development of polyI:C-induced T_H1 and $CD8^+$ T cell responses[92,96]. MDA5-dependent production of type I IFNs by stromal cells seems to be especially important for the generation of memory $CD8^+$ T cells in such models[96]. PolyI:C-induced activation of MDA5, but not TLR3, was also shown to be essential for the production of antibodies specific for a co-administered antigen in alum[94].

Even though the aforementioned immunization studies were performed in mice and nonhuman primates, data are emerging as to the potential adjuvant effects of ligands for TLR3 and MDA5 in humans. As mentioned above, a pilot systems biology study in human subjects compared the innate immune response induced by the YF-17D vaccine to that of an RNase-resistant analogue of polyI:C (polyI:C stabilized with poly-L-lysine and carboxymethylcellulose (polyICLC))[53]. The gene expression profile of blood cells from polyICLC-treated subjects showed the induction of a type I IFN response as well as signatures associated with

NF-κB signalling, inflammasomes and DC activation. However, the response was faster than that observed with YF-17D. TLR3 and MDA5 agonists are thus emerging as promising adjuvants in the development of vaccines that promote a T_H1-type response against viruses and other intracellular pathogens.

TLR7 and TLR8 agonists. A preferred option to target TLR7 and TLR8 are the small synthetic compounds imidazoquinolines. Given that the expression patterns of TLR7 and TLR8 differ between mice and humans, caution should be exerted when extrapolating results obtained with TLR7 and TLR8 agonists from mice to humans.

In human pDCs, which express TLR7, the activation of this receptor leads to the expression of type I IFNs, IL-12 and pro-inflammatory cytokines, as well as to the upregulation of co-stimulatory molecules[86,97]. Human cDCs express TLR8, and agonists of this TLR induce the expression of IL-12 and pro-inflammatory cytokines and the upregulation of co-stimulatory molecules[90,98].

In mice, the administration of an antigen together with a TLR7 or TLR8 agonist promotes T_H1 and $CD8^+$ T cell responses[99–101] and antibody production[99]. Data from mice and nonhuman primates indicate that conjugation of the TLR7 or TLR8 agonist with the antigen and protein aggregation may result in a more efficient induction of T_H1 and $CD8^+$ T cell responses[102,103]. In mice immunized subcutaneously with an antigen–TLR7/8 agonist conjugate, the improvement in these responses has been attributed to more efficient antigen uptake by multiple DC subsets[103]. TLR7-dependent production of type I IFNs has been implicated in this increased antigen uptake, as well as in the promotion of DC migration to the lymph nodes. Together with IL-12, type I IFNs appear to be required for optimal T_H1 and $CD8^+$ T cell responses following the administration of TLR7 and TLR8 agonists[101,103]. Thus, TLR7 and TLR8 agonists are emerging as promising candidate adjuvants for promoting T_H1-type immune responses, although the development of improved formulation and delivery strategies is likely to be key for their efficiency in humans.

TLR9 agonists. TLR9 agonists (mostly different types of CpG oligodeoxynucleotides) are the most studied and probably the most advanced nucleic acid-sensing PRR agonists in development as potential immune response-biasing vaccine adjuvants[75,104]. Again, it should be kept in mind when interpreting rodent studies that TLR9 expression is restricted in humans, being highest in pDCs and B cells, whereas mice have a broader expression pattern[105].

In human pDCs, stimulation of TLR9 leads to strong expression of type I IFNs, IL-12 and pro-inflammatory cytokines, as well as to the upregulation of co-stimulatory molecules[86]. In B cells, TLR9 activation leads to the expression of pro-inflammatory cytokines and, in conjunction with CD40 engagement, synergistically promotes the production of antibodies and IL-12, which allows B cells to promote the differentiation of T_H1 cells[106]. Concomitant stimulation of TLR9 in pDCs may further promote B cell antibody production and memory B cell differentiation in the absence of T cell help through type I IFN production[107]. In addition, TLR9 triggering synergizes with B cell receptor activation in the induction of antigen-specific B cell responses and promotes T_H1-biased isotype switching[108]. In mice, TLR9 agonists very potently induce T_H1 and $CD8^+$ T cell responses as well as T_H1-type B cell responses[104].

TLR9 agonists have entered clinical trials as adjuvants in hepatitis B, influenza and anthrax vaccines and have been shown to boost and accelerate protective antibody responses[75,104].

STING agonists. The discovery that STING may directly respond to cyclic di-GMP supports the idea that it could be targeted directly by novel adjuvant molecules. So far, this potential can only be inferred from data on cyclic di-GMP, which has immunostimulatory and adjuvant activities that are being increasingly documented[109]. For instance, treatment with cyclic di-GMP may upregulate the expression of MHC class II molecules, co-stimulatory molecules, pro-inflammatory cytokines and type I IFNs by human and mouse cDCs[110,111]. Furthermore, cyclic di-GMP has adjuvant effects on adaptive responses to soluble antigens in mice[110,111]. It remains to be determined whether the adjuvant activity of cyclic di-GMP *in vivo* is entirely due to STING activation or also a result of other activities of this molecule. Either way, it is likely that STING has an important role, given that mice with an inactivating point mutation in the gene encoding STING display impaired type I IFN responses to cyclic di-GMP[112].

Combined adjuvants. In line with the observation that efficient live attenuated vaccines target multiple PRRs[50,55], combining multiple PRR agonists appears to be a promising rationale for the design of effective new adjuvants. This approach is already being applied, for instance in the clinically approved adjuvant AS04 (a combination of alum and a TLR4 ligand). Similar strategies aim to couple the potential of nucleic acid-sensing PRRs with that of other PRRs. To date, most studies have combined TLR ligands.

MYD88-dependent and TRIF-dependent TLR ligands synergistically activate cDCs. Thus, a combination of these ligands strongly increases the secretion of IL-12, type I IFNs and pro-inflammatory cytokines by cDCs, resulting in efficient activation of T_H1 cells and $CD8^+$ T cells[113,114]. A recent *in vivo* study in mice using such a combined adjuvant strategy indicated that combining aggregated TLR2–TLR6, TLR3 and TLR9 ligands could boost not only the number of antigen-specific $CD8^+$ T cells, but also their avidity and functionality, providing a qualitative advantage over combinations of two agonists[115]. This difference has been linked to activation of the expression of IL-15 and IL-15 receptor subunit-α (IL-15Rα) by cDCs in a type I IFN-dependent manner[115]. In another study, a TLR4 agonist and a TLR7 agonist, which were combined in nanoparticles, were shown to have synergistic effects in increasing the levels of neutralizing antibodies and promoting the generation of memory B cells and long-lived plasma cells[116]. These effects were dependent on TLR triggering in

both DCs and B cells, and also on T cell help. Experimental immunizations using this combined adjuvant were shown to protect mice from lethal influenza virus infection and to boost neutralizing antibody responses in nonhuman primates[116]. Again, such studies highlight the benefit of optimizing formulation and delivery strategies in vaccines containing this type of adjuvant.

Conclusions and perspectives

Nucleic acid-sensing PRRs are taking centre stage in the induction of adaptive immune responses to many existing vaccines. Preclinical and clinical evidence indicates that the triggering of these receptors by selective agonists may suffice in mediating efficient immunization against co-administered antigens. Even though considerable progress has been made in the past decade since the discovery of the first nucleic acid-sensing PRR, much remains to be elucidated concerning the role of these receptors in adaptive immunity in general and in vaccination in particular.

A robust and comprehensive characterization of the nucleic acid-sensing machinery is likely to be key not only to a more complete understanding of antimicrobial immunity, but also for elucidating the mechanisms of action of many current vaccines. For instance, the monopoly of TLR9 on DNA sensing has recently been challenged by the discovery of cytosolic DNA-sensing mechanisms. However, the PRRs that mediate the response to nucleic acids in several important vaccination strategies — including DNA vaccination and alum-adjuvanted immunization — remain to be identified. A few novel DNA- and RNA-sensing PRRs have been proposed using *in vitro* approaches, and we expect that mice (conditionally) deficient for individual nucleic acid sensors should soon help to establish the respective contributions of these PRRs to antimicrobial immunity and vaccination. Moreover, a more advanced characterization of the expression patterns of these receptors and of their ligand-binding specificities could provide new molecular targets for experimental adjuvants or help to optimize delivery strategies. Notably, this could help us to understand the origin of human hyporesponsiveness to DNA vaccines, which deserves more scrutiny.

Another potentially important question is the extent to which host nucleic acids contribute to vaccination, in line with recent data suggesting a role for host DNA and uric acid in mediating the adjuvant effects of alum. In the context of alum-adjuvanted immunization, these two DAMPs induce T_H2-type responses independently of type I IFNs[83–85]. This is in contrast to most nucleic acid PAMPs, which induce T_H1-type responses that most often require type I IFN signalling. As it increasingly appears that PRR engagement may result in the active release of host nucleic acids[117], we propose that it may be worthwhile studying the potential adjuvant or immunomodulatory effects of host nucleic acids and their metabolites in vaccination. This investigation would probably benefit from the identification of the receptors for uric acid and host DNA that are involved in alum-adjuvanted immunization.

Finally, achieving a more precise understanding of the APCs and the PRRs that are targeted by nucleic acids in different vaccination strategies is likely to be of utmost importance. Indeed, APCs, especially cDCs, are highly heterogeneous, and multiple distinct subsets are present at the various sites potentially used for vaccination and in the lymphoid organs that drain such sites[118]. The improving characterization of the functional specialization and plasticity of each DC subset provides opportunities for tailoring vaccines to preferentially target specific DC subsets[119]. Notably in this regard, the expression patterns of intracellular TLRs indicate a distinct distribution among DC subsets that correlates with the functional specialization of each subset[13,88–90]. It is likely that further characterization of the contribution of pDCs to nucleic acid sensing will be of particular importance. Being 'professional' type I IFN producers, pDCs may at least be important bystander contributors to the triggering of T_H1-type immune responses by nucleic acid sensing in vaccination[65,120]. Furthermore, recent data suggest that pDCs could directly participate in the activation of $CD8^+$ T cells *in vivo*[121], although this notion remains controversial[122]. Determining the main PRRs through which pDCs react to nucleic acids in different settings could also provide valuable information. Although most research to date has focused on TLRs, there is evidence, for instance, that pDCs may respond to immunostimulatory dsDNA via STING[74]. Emerging mouse models that allow for the deletion of specific DC subsets or of genes encoding nucleic acid-sensing PRRs within these subsets are likely to help in deconstructing the relative contributions of pDCs and other DC subsets in the immune responses to different vaccines. This knowledge could be key to refining the formulation and delivery strategies for new vaccine adjuvants tailored to elicit specific types of adaptive immune response.

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Competing interests statement
The authors declare no competing financial interests.

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Synthetic Lipophilic Antioxidant BO-653 Suppresses HCV Replication

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The influence of the intracellular redox state on the hepatitis C virus (HCV) life cycle is poorly understood. This study demonstrated the anti-HCV activity of 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran (BO-653), a synthetic lipophilic antioxidant, and examined whether BO-653's antioxidant activity is integral to its anti-HCV activity. The anti-HCV activity of BO-653 was investigated in HuH-7 cells bearing an HCV subgenomic replicon (FLR3-1 cells) and in HuH-7 cells infected persistently with HCV (RMT-tri cells). BO-653 inhibition of HCV replication was also compared with that of several hydrophilic and lipophilic antioxidants. BO-653 suppressed HCV replication in FLR3-1 and RMT-tri cells in a concentration-dependent manner. The lipophilic antioxidants had stronger anti-HCV activities than the hydrophilic antioxidants, and BO-653 displayed the strongest anti-HCV activity of all the antioxidants examined. Therefore, the anti-HCV activity of BO-653 was examined in chimeric mice harboring human hepatocytes infected with HCV. The combination treatment of BO-653 and polyethylene glycol-conjugated interferon-α (PEG-IFN) decreased serum HCV RNA titer more than that seen with PEG-IFN alone. These findings suggest that both the lipophilic property and the antioxidant activity of BO-653 play an important role in the inhibition of HCV replication. ***J. Med. Virol. 85:241–249, 2013.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: BO-653; antioxidant activity; chemical structure; HCV replication; chimeric mice

INTRODUCTION

Hepatitis C virus (HCV) causes persistent infection, leading to chronic liver diseases including chronic hepatitis, cirrhosis, and hepatocellular carcinoma. In 2009, the number of patients with HCV infection worldwide was estimated to be 130–170 million [Lavanchy, 2009]. Recent years have seen the development of several promising treatments for patients infected with HCV. The addition of a protease inhibitor (boceprevir or telaprevir) to polyethylene glycol-conjugated interferon-α (PEG-IFN) and ribavirin improved dramatically the sustained virological response rates in treatment-naive patients with genotype 1 infections. However, the sustained virological response rate of triple therapy with a telaprevir-based regimen in null responders treated with PEG-IFN/ribavirin is only 30% [Fontaine and Pol, 2011; Kumada et al., 2012]. There is concern that high-risk groups such as patients with the *IL28B* minor allele (re8099917 SNP; GT/GG), the elderly, or those with fibrosis will be resistant to the triple therapy [Suppiah et al., 2009; Tanaka et al., 2009]. Therefore, new therapeutic strategies are required to treat HCV infection.

Chronic HCV infection is closely associated with oxidative stress. Oxidative stress reflects an imbalance between the production of reactive oxygen species (ROS) and the activity of intracellular antioxidant systems. The cumulative evidence from experimental

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and clinical studies demonstrates that HCV infection causes excessive ROS production and decreased activity of antioxidant enzymes [Kato et al., 2001; Levent et al., 2006]. In addition, previous studies showed that aggravation of oxidative stress in hepatocytes infected with HCV is correlated with the iron overload, while phlebotomy improves oxidative stress markers and liver pathology [Seronello et al., 2007]. Therefore, oxidative stress is a deleterious factor involved in the development of various hepatic diseases ranging from chronic hepatitis to hepatocellular carcinoma. In contrast, the influence of the intracellular redox state on HCV replication is controversial. Exogenous addition of either hydrogen peroxide or unsaturated fatty acid has been shown to induce oxidative stress and inhibit HCV replication in cell culture models [Choi et al., 2004; Huang et al., 2007]. Yano et al. [2007] reported previously that any of several nutrients (including vitamin E, a hydrophobic antioxidant) enhance HCV RNA replication. In contrast, overproduction of the antioxidant enzyme heme oxygenase-1 decreases HCV RNA replication in both full-length and subgenomic replicons [Zhu et al., 2008]. Despite these in vitro results, there have been no reports on the effect of antioxidant or pro-oxidant reagents on the life cycle of HCV in any animal models, such as chimeric mice harboring human hepatocytes infected with HCV.

BO-653 (2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran), a lipophilic (hydrophobic) antioxidant, was previously a clinical candidate for potential treatment of atherosclerosis and the prevention of post-angioplasty restenosis [Cynshi et al., 1998; Meng, 2003]. This compound is an effective inhibitor of lipid peroxidation and inhibits potently oxidation of lipids such as low-density lipoprotein [Noguchi et al., 1997; Tamura et al., 2003]. The present study examined the anti-HCV activity of BO-653 both in vitro and in vivo, and sought to clarify whether the antioxidant activity of the molecule was integral to the observed anti-HCV activity.

MATERIALS AND METHODS

Chemicals

BO-653 (molecular weight [MW], 388.6) was a gift of the Chugai Pharmaceutical company (Tokyo, Japan). Probucol [4,4′-(isopropylidenedithio)bis(2,6-di-*tert*-butylphenol)] was purchased from Wako Pure Chemical Industries (Osaka, Japan). *N*-acetyl cysteine and ascorbic acid (vitamin C) were obtained from Sigma–Aldrich (St. Louis, MO). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and D-α-tocopherol (vitamin E) were obtained from Calbiochem (San Diego, CA) and MP Biomedical LLC (Solon, OH), respectively.

Viruses and Cells

Patients provided written informed consent prior to blood sample collection.

HuH-7 cells harboring a HCV subgenomic replicon (FLR3-1 cells; genotype 1b, Con-1 strain) were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium GlutaMAX-I (DMEM-GlutaMax I; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) and 0.5 mg/ml G418 [Inoue et al., 2007].

HuH-7 cells infected persistently with HCV (RMT-tri cells; genotype 1a) were generated in the laboratory as described below and were maintained in DMEM containing 10% FCS, nonessential amino acids, 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], and 0.4% glucose. Complementary DNA (cDNA) of the full-genome HCV (nucleotides 1–9,598; GenBank accession number AB520610) was prepared from the serum of a patient with acute hepatitis infected with HCV genotype 1a [Inoue et al., 2007]. The resulting serum harbored HCV at a titer of $10^{8.6}$ copies/ml, as detected by a quantitative real-time polymerase chain reaction (qRT-PCR) as described previously [Takeuchi et al., 1999]. The sequence of the final cDNA construct was determined from a consensus of more than 10 clones, and was subcloned under the control of a T7 promoter (pHCV-RMT). The construct of HCV subgenomic replicon was generated from pHCV-RMT. HCV subgenomic RNA was transcribed using T7 RNA polymerase and the MEGAscript in vitro transcription kit (Ambion, Austin, TX) according to the manufacturer's instructions. The resulting synthetic RNA of the HCV subgenomic replicon was transfected into HuH-7 cells by electroporation. Following transfection, the HCV subgenomic replicon-bearing HuH-7 cells was established. Total RNA was extracted by the acid guanidinium–phenol–chloroform method from a sample of the HCV subgenomic replicon-bearing HuH-7 cells and reverse transcribed. Sequence of the resulting cDNA has three nonsynonymous substitutions compared to that of the original HCV subgenomic replicon. Next, three nonsynonymous substitutions were inserted into the original full-length HCV sequence to enhance the replication rate in HuH-7 cells. Full-length RNA was also transcribed as described above. The resulting synthetic RNA of full-length HCV was transfected into HuH-7 cells by electroporation. Following transfection, the HCV RNA level in the transfected cells was measured on a weekly basis, revealing persistent infection at a level of $10^{6.5}$–$10^{6.8}$ copies/μg total cellular RNA over the course of 50 days. Finally, the transfected cell line was designated as RMT-tri.

Analysis of Anti-HCV Effect of BO-653 in FLR3-1 Cells

The anti-HCV activity of BO-653 in FLR3-1 cells was measured by inhibiting luciferase activity [Inoue et al., 2007]. In brief, FLR3-1 cells were seeded at 4×10^3 cells/well in 96-well white plates. After 24 hr, the culture medium was replaced with fresh medium containing various concentrations of BO-653

(12–1,000 μM). The culture medium containing 1% MeOH was used as the negative control. After 72 hr incubation, the luciferase activity of the cells was measured using the Bright-Glo luciferase assay (Promega, Madison, WI) according to the manufacturer's instructions.

Analysis of Anti-HCV Effect of BO-653 in RMT-Tri Cells

RMT-tri cells were seeded at 2.5×10^4 cells/well in 24-well plates. After 24 hr, the culture medium was replaced with fresh medium containing various concentrations of BO-653 (12–1,000 μM). The culture medium containing 1% MeOH was used as the negative control. After 72 hr incubation, the cell monolayer was harvested by adding 400 μl of 5 M guanidine-isocyanate solution containing 5.6 μl of 2-mercaptoethanol. The total RNA was extracted as above; HCV RNA was quantified by qRT-PCR.

Comparison of Anti-HCV Activity of Lipophilic and Hydrophilic Antioxidants

The anti-HCV activity of various antioxidants, including hydrophilic and lipophilic compounds, was compared in FLR3-1 cells. BO-653, α-tocopherol, and probucol were used as lipophilic antioxidants; *N*-acetyl cysteine, ascorbic acid, and trolox were used as hydrophilic antioxidants. The anti-HCV activities of these compounds were determined by luciferase assays as described above.

Cytotoxicity Testing

Simultaneously with the luciferase assays, the cell viability was measured by using a WST-8 cell counting kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Western Blot Analysis

FLR3-1 cells were treated with BO-653 as described above. After 96 hr, the cells were lysed with lysis buffer (protease inhibitor cocktail [Complete, Roche Diagnostics, IN] formulated according to the manufacturer's instructions in 10 mM Tris [pH 7.4], 150 mM NaCl, 1% sodium dodecyl sulfate (SDS), 0.5% Nonidet P-40). The cell lysates were resolved by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After incubation with a blocking buffer consisting of 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 [TBS-T], the membranes were incubated with rabbit polyclonal anti-NS3 antibody (R212 clone) and goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated IgG (GE Healthcare, Little Chalfont, Buckinghamshire, UK) as the primary and secondary antibodies, respectively. Labeling was visualized using the Immobilon Western system (Millipore, Billerica, MA). To provide a loading control, β-actin was detected using mouse anti-β-actin monoclonal antibody (Sigma-Aldrich) and sheep anti-mouse IgG HRP-conjugated IgG (GE Healthcare) as the primary and secondary antibodies, respectively.

Immunofluorescent Staining

FLR3-1 cells treated with 111 μM BO-653 for 96 hr were probed with the primary antibody (anti-NS3) after blocking with TNB blocking buffer (PerkinElmer, Waltham, MA). An anti-rabbit IgG Alexa-Fluor 488 conjugate (Invitrogen, Grand Island, NY) was then applied as the secondary antibody.

Measurement of Antioxidant Activity of BO-653, α-Tocopherol, and Probucol in Lipid Peroxidation

Oxidation of methyl linoleate (10 mM) was carried out at 37°C under air in acetonitrile solution by adding 0.2 mM AMVN (2,2′-azobis-2,4-dimethylvaleronitrile) as a radical initiator in the presence of various concentrations of BO-653, α-tocopherol, and probucol. These antioxidants were added at concentrations of 0.2–20 μM to the reaction mixture; after 60 min incubation at 37°C, the reactions were stopped by chilling on ice.

The levels of lipid peroxidation were determined by the ferrous oxidation-xylenol orange (FOX) method [Nourooz-Zadeh et al., 1994]. In brief, completed methyl linoleate oxidation reactions were diluted 10-fold with MeOH containing 4.4 mM 2,6-di-*tert*-butyl-4-methylphenol (BHT). Each diluted solution (1.8 ml) was mixed with 0.1 ml of 2 mM xylenol orange solution (in 250 mM H_2SO_4) and 0.1 ml of 5 mM ferrous chloride solution (in 250 mM H_2SO_4). The mixture was incubated at room temperature for 60 min, at which point the absorbance at 570 nm was measured using a UV/visible light spectrophotometer. Cumene hydroperoxide was used to generate a standard curve for lipid hydroperoxidation.

Pharmacokinetics of BO-653 in uPA/SCID Mice Harboring Human Hepatocytes

Chimeric uPA/SCID mice harboring human hepatocytes were purchased from PhoenixBio (Hiroshima, Japan). All animal experiments were approved by the Ethics Committee of Tokyo Metropolitan Institute of Medical Science and were performed in accordance with the guidelines of the Animal Experimental Committee of Tokyo Metropolitan Institute of Medical Science. Two chimeric mice were administered BO-653 (at 800 or 2,000 mg/kg in 3% gum arabic solution) by single oral gavage. At 24 hr after administration, blood was collected and the plasma concentration of BO-653 was measured by high-performance liquid chromatography (HPLC). Aliquots of plasma (100 μl) were mixed with 50 μl MeOH containing 10 mM ascorbic acid and 100 μl acetonitrile containing 30 μg/ml MeO-BO-653 as the internal control. The mixtures were centrifuged at 9,100*g* for 5 min, and the resulting supernatants of 30 μl each were separated using

an octadecyl column (Capcell Pak C18 UG120, 3 μm, 4.6 mm × 50 mm; Shiseido, Tokyo, Japan) at 30°C, a detection wavelength of 300 nm, and an eluent (acetonitrile) flow rate of 1.0 ml/min.

Treatment of HCV-Infected Chimeric Mice With BO-653 and/or PEG-IFN

Chimeric mice also were used as an in vivo model of persistent HCV infection, as described previously [Inoue et al., 2007]. uPA/SCID mice were engrafted with human hepatocytes; 6 weeks later, the chimeric mice were infected by intravenous (IV) injection with patient serum containing 10^6 copies of HCV genotype 1b (HCR6; GenBank accession no. AY045702). By 4 weeks after infection, the HCV RNA levels reached a plateau of 10^6–10^7 copies/ml of mouse serum. To determine anti-HCV activity of BO-653 in the early phase of the treatment, the chimeric mice (n = 2–5 per group) infected with HCV were given once-daily oral gavage with 2,000 mg/kg BO-653 in 3% gum arabic, and/or twice weekly subcutaneous injection with 30 μg/kg PEG-IFNα-2a (Chugai Pharmaceutical) as shown in Table I. Body weights were monitored daily, and blood for serum was collected prior to the start of treatment (Day-1) and once weekly thereafter (Days 8 and 14). Following the terminal bleed, animals were sacrificed and liver specimens were collected.

Quantitation of HCV RNA by qRT-PCR

After completion of the treatment, total RNA was purified from the serum and liver specimens by the acid guanidinium-phenol-chloroform method and qRT-PCR was used to quantify HCV RNA from the RNA samples corresponding to 1 μl serum and about 5 mm^3 of liver.

Quantitation of Serum Human Albumin

The human albumin concentration in the blood of chimeric mice was measured in 2-μl serum samples by using an Alb-II kit (Eiken Chemical, Tokyo, Japan) according to the manufacturer's instructions.

Statistical Analysis

Data are presented as mean ± standard deviations (SDs). Statistical analysis was performed by using either Student's *t*-test or ANOVA, followed by Tukey's test or Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Inhibitory Effect of BO-653 on HCV Replication In Vitro

The anti-HCV activity of BO-653 (Fig. 1A) was investigated in cells harboring HCV subgenomic replicons (FLR3-1 cells). BO-653 suppressed the replication of HCV subgenomic replicons in a concentration-dependent manner (Fig. 1B). The half-maximal inhibitory concentration (IC_{50}) of BO-653 in FLR3-1 cells was 36.0 μM. In contrast, no cytotoxicity was observed with up to 1,000 μM of BO-653 in FLR3-1 cells (Fig. 1B). Western blotting and immunofluorescent staining of FLR3-1 cells demonstrated that the level of HCV NS3 protein, but not that of β-actin, was reduced as the concentrations of BO-653 increased (Fig. 1C and D). A similar trend was seen in RMT-tri cells for the replication of full-genome HCV genotype 1a (Fig. 1E).

Comparison of Anti-HCV Activity of Lipophilic Antioxidants

The antioxidant activity of BO-653 has been compared previously with that of probucol and α-tocopherol [Cynshi et al., 1998]. Therefore, the anti-HCV activity of these three lipophilic antioxidants was compared in FLR3-1 cells. At a concentration >37 μM, BO-653 exhibited stronger inhibitory effects against HCV replication than did the two other compounds (Fig. 2A). In addition, the antioxidant activity of these compounds was determined by an in vitro lipid peroxidation system. BO-653 had the strongest antioxidant activity against lipid peroxidation in this in vitro assay (Fig. 2C).

Comparison of Anti-HCV Activity of Hydrophilic and Lipophilic Antioxidants

The anti-HCV activities of some representative antioxidants were investigated further (Fig. 3A). As noted above, lipophilic antioxidants exhibited anti-HCV activity in cell culture; however, hydrophilic antioxidants (*N*-acetyl cysteine, ascorbic acid, and trolox) did not inhibit the replication of HCV subgenomic replicons at comparable concentrations. None of the

TABLE I. Schedule of Blood Collection and Drug Administration for Chimeric Mice Infected With HCV

	Day															
	−1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Collection of blood	B									B						B
BO-653		BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	
PEG-IFN		I			I				I			I				
BO-653 + PEG-IFN		BO/I	BO	BO	BO/I	BO	BO	BO	BO/I	BO	BO	BO/I	BO	BO	BO	

B, sampling of blood; BO, orally administrated BO-653 (2,000 mg/kg); I, subcutaneous injection of PEG-IFN (30 μg/kg).