[34] Clifford HD, Yerkovich ST, Khoo SK, Zhang G, Upham J, Le Souëf PN, et al. Toll-like receptor 7 and 8 polymorphisms: associations with functional effects and cellular and antibody responses to measles virus and vaccine. Immunogenetics 2012;64:219–28.
[35] Caskey M, Lefebvre F, Filali-Mouhim A, Cameron MJ, Goulet JP, Haddad EK, et al. Synthetic double-stranded RNA induces innate immune responses similar to a live viral vaccine in humans. J Exp Med 2011;208:2357–66.
[36] Mosca F, Tritto E, Muzzi A, Monaci E, Bagnoli F, Iavarone C, et al. Molecular and cellular signatures of human vaccine adjuvants. Proc Natl Acad Sci U S A 2008;105:10501–6.
[37] Calabro S, Tortoli M, Baudner BC, Pacitto A, Cortese M, O'Hagan DT, et al. Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. Vaccine 2011;29:1812–23.
[38] Lu X, Tian Y, Zhao Q, Jin T, Xiao S, Fan X. Integrated metabonomics analysis of the size-response relationship of silica nanoparticles-induced toxicity in mice. Nanotechnology 2011;22:055101.
[39] Fox CB, Baldwin SL, Duthie MS, Reed SG, Vedvick TS. Immunomodulatory and physical effects of oil composition in vaccine adjuvant emulsions. Vaccine 2011;29:9563–72.

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

Christophe J. Desmet[1] *and Ken J. Ishii*[2,3]

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.

[1]Laboratory of Cellular and Molecular Immunology, GIGA-Research and Faculty of Veterinary Medicine, B34, University of Liege, 1 Avenue de l'Hopital, B-4000 Liège, Belgium.
[2]Laboratory of Vaccine Science, WPI Immunology Frontier Research Center (IFREC), Osaka University, 3-1 Yamadaoka, Suita, 565-0871 Osaka, Japan.
[3]Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation (NIBIO), 7-6-8 Asagi Saito Ibaraki-City Osaka, Japan.
e-mails: christophe.desmet@ulg.ac.be; kenishii@biken.osaka-u.ac.jp
doi:10.1038/nri3247
Published online 22 June 2012

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

© 2012 Macmillan Publishers Limited. All rights reserved

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.

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.

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.

© 2012 Macmillan Publishers Limited. All rights reserved

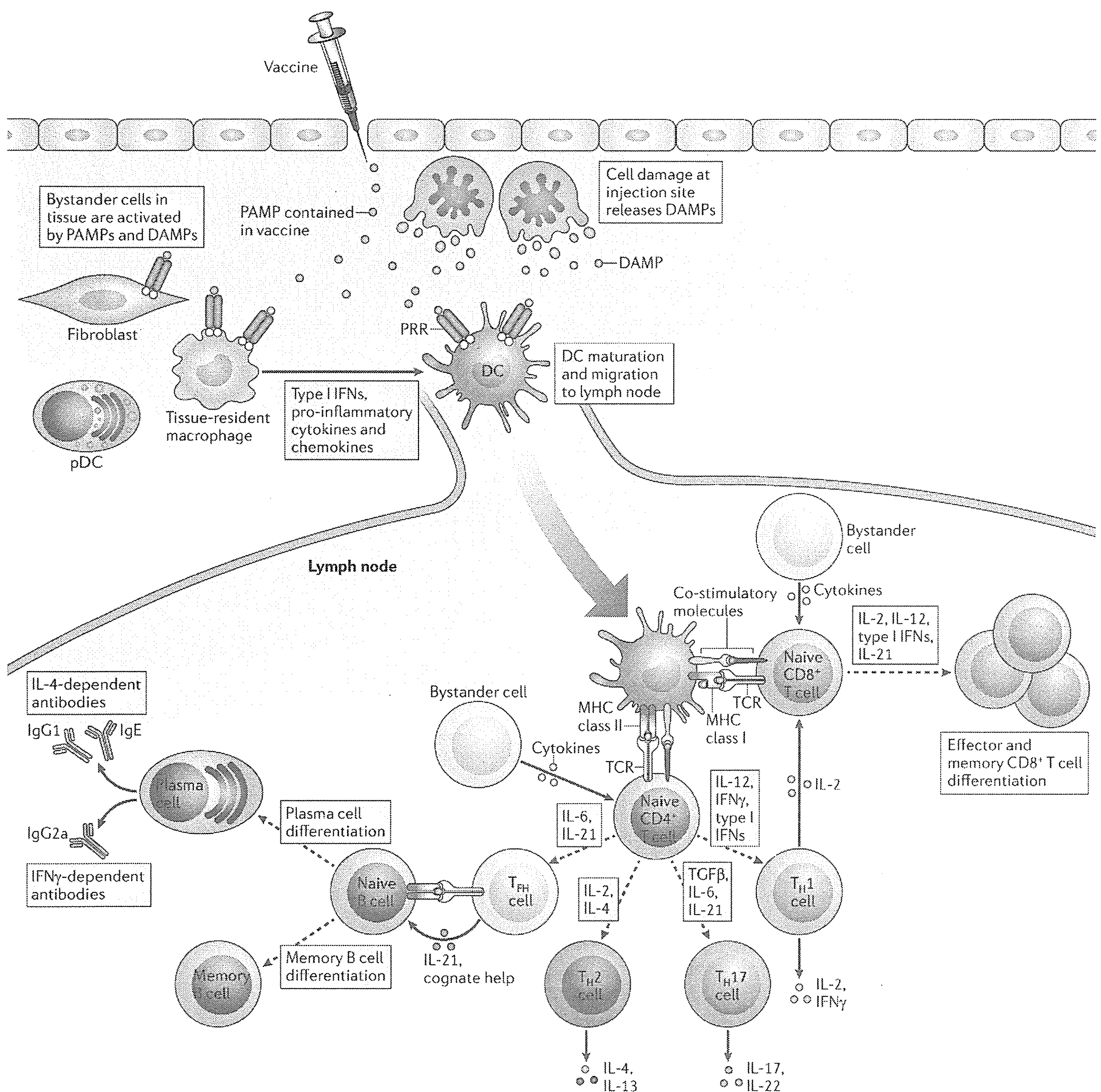

Figure 1 | **Induction of adaptive immune responses to vaccines through PRR-mediated dendritic cell activation.** Vaccines may contain pathogen-associated molecular patterns (PAMPs) or may induce the local release of damage-associated molecular patterns (DAMPs). These PAMPs and DAMPs are detected directly 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.

© 2012 Macmillan Publishers Limited. All rights reserved

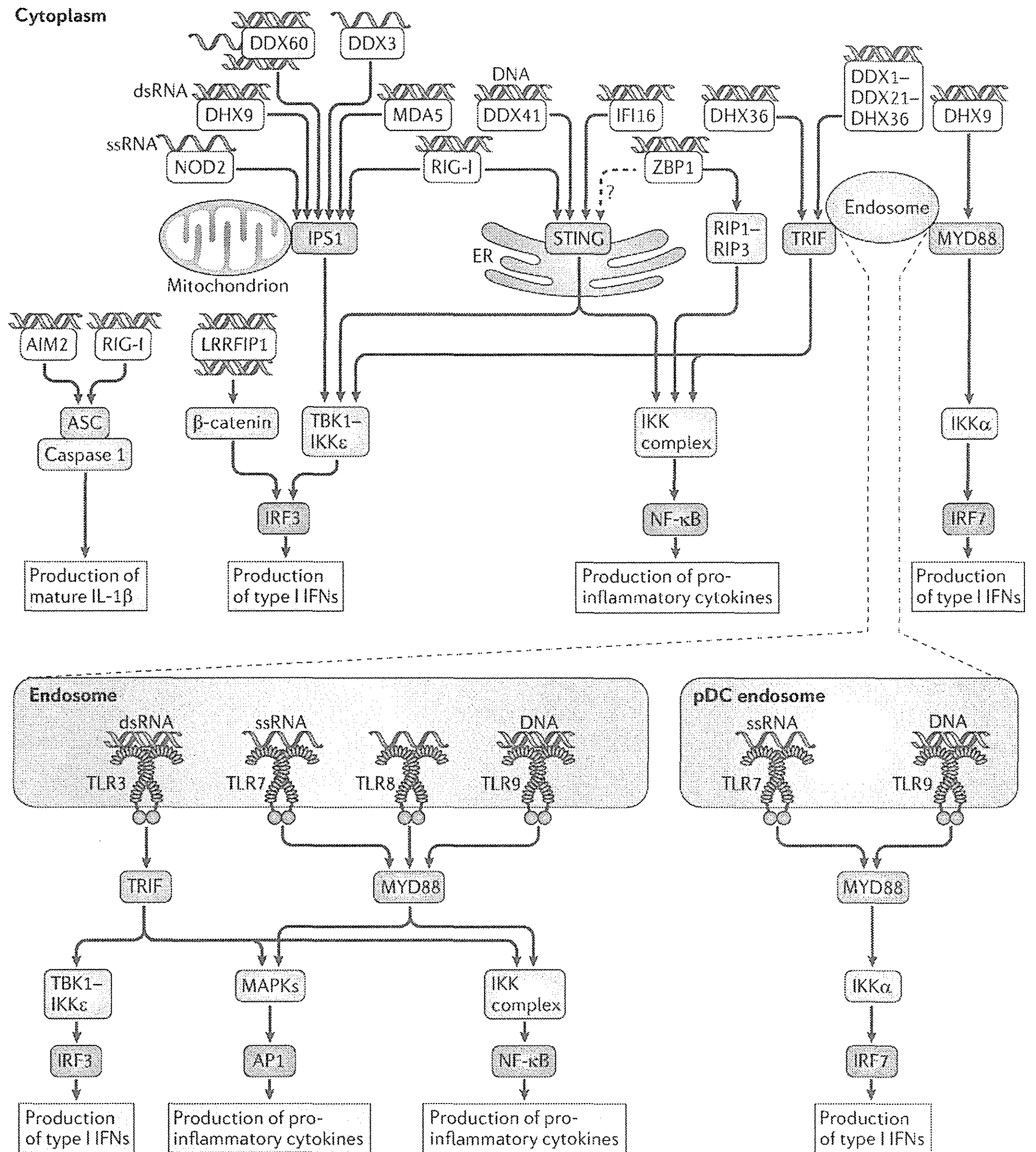

Figure 2 | **Overview of the nucleic acid-sensing machinery.** Endosomal Toll-like receptor 7 (TLR7), TLR8 and TLR9 initiate downstream signalling through the adaptor protein myeloid differentiation primary-response protein 88 (MYD88) in the cytosol. This leads to the activation of mitogen-activated 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.

© 2012 Macmillan Publishers Limited. All rights reserved

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	PolyI: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	PolyI:C
NOD2	Cytoplasm	RNA viruses	ssRNA	–
DDX3	Cytoplasm	RNA viruses	RNA	–
DDX1–DDX21–DHX36	Cytoplasm	RNA viruses	dsRNA	PolyI: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

© 2012 Macmillan Publishers Limited. All rights reserved

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

© 2012 Macmillan Publishers Limited. All rights reserved

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.

© 2012 Macmillan Publishers Limited. All rights reserved

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].

© 2012 Macmillan Publishers Limited. All rights reserved

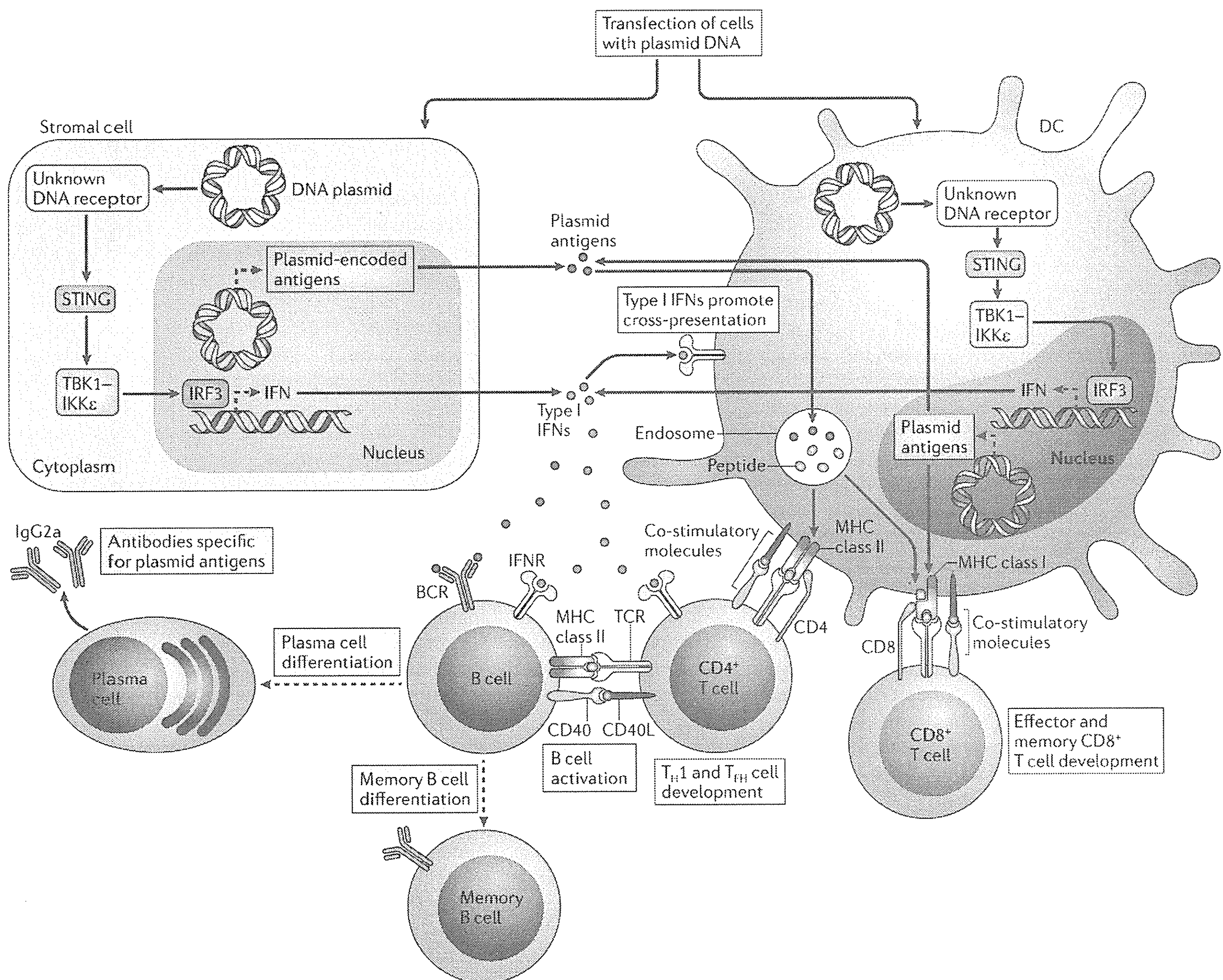

Figure 3 | **Mechanisms of DNA vaccination.** The plasmid DNA used in DNA vaccination may directly transfect stromal cells (such as muscle cells) or dendritic cells (DCs). In these cells, a cytosolic DNA receptor that has not yet been identified induces the activation of TANK-binding kinase 1 (TBK1) 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

© 2012 Macmillan Publishers Limited. All rights reserved

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

© 2012 Macmillan Publishers Limited. All rights reserved

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.

1. Coffman, R. L., Sher, A. & Seder, R. A. Vaccine adjuvants: putting innate immunity to work. *Immunity* **33**, 492–503 (2010).
2. Pulendran, B. & Ahmed, R. Immunological mechanisms of vaccination. *Nature Immunol.* **131**, 509–517 (2011).
3. Plotkin, S. A. Vaccines: correlates of vaccine-induced immunity. *Clin. Infect. Dis.* **47**, 401–409 (2008).
4. Iwasaki, A. & Medzhitov, R. Regulation of adaptive immunity by the innate immune system. *Science* **327**, 291–295 (2010).
5. Pichlmair, A. & Reis e Sousa, C. Innate recognition of viruses. *Immunity* **27**, 370–383 (2007).
6. Takeuchi, O. & Akira, S. Pattern recognition receptors and inflammation. *Cell* **140**, 805–820 (2010).
7. Barbalat, R., Ewald, S. E., Mouchess, M. L. & Barton, G. M. Nucleic acid recognition by the innate immune system. *Annu. Rev. Immunol.* **29**, 185–214 (2011).
8. Chen, G. Y. & Nunez, G. Sterile inflammation: sensing and reacting to damage. *Nature Rev. Immunol.* **10**, 826–837 (2010).
9. Blasius, A. L. & Beutler, B. Intracellular Toll-like receptors. *Immunity* **32**, 305–315 (2010).
10. Kawai, T. & Akira, S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* **34**, 637–650 (2011).
11. Loo, Y. M. & Gale, M. Jr. Immune signaling by RIG-I-like receptors. *Immunity* **34**, 680–692 (2011).
12. Kadowaki, N. *et al.* Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* **194**, 863–869 (2001).
13. Iwasaki, A. & Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nature Immunol.* **5**, 987–995 (2004).
14. Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunol.* **11**, 373–384 (2010).
15. Schlee, M. & Hartmann, G. The chase for the RIG-I ligand — recent advances. *Mol. Ther.* **18**, 1254–1262 (2010).
16. Ablasser, A. *et al.* RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. *Nature Immunol.* **10**, 1065–1072 (2009).

© 2012 Macmillan Publishers Limited. All rights reserved

17. Chiu, Y.-H., MacMillan, J. B. & Chen, Z. J. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. *Cell* **138**, 576–591 (2009).
18. Kato, H. *et al.* Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *J. Exp. Med.* **205**, 1601–1610 (2008).
19. Malathi, K., Dong, B., Gale, M. & Silverman, R. H. Small self-RNA generated by RNase L amplifies antiviral innate immunity. *Nature* **448**, 816–819 (2007).
20. Venkataraman, T. *et al.* Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses. *J. Immunol.* **178**, 6444–6455 (2007).
21. Satoh, T. *et al.* LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. *Proc. Natl Acad. Sci. USA* **107**, 1512–1517 (2010).
22. Monroe, K. M., McWhirter, S. M. & Vance, R. E. Identification of host cytosolic sensors and bacterial factors regulating the type I interferon response to *Legionella pneumophila*. *PLoS Pathog.* **5**, e1000665 (2009).
23. Li, X. D. *et al.* Mitochondrial antiviral signaling protein (MAVS) monitors commensal bacteria and induces an immune response that prevents experimental colitis. *Proc. Natl Acad. Sci. USA* **108**, 17390–17395 (2011).
24. Poeck, H. *et al.* Recognition of RNA virus by RIG-I results in activation of CARD9 and inflammasome signaling for interleukin 1β production. *Nature Immunol.* **11**, 63–69 (2010).
25. Ishikawa, H. & Barber, G. N. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* **455**, 674–678 (2008).
26. Zhong, B. *et al.* The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity* **29**, 538–550 (2008).
27. Oshiumi, H., Sakai, K., Matsumoto, M. & Seya, T. DEAD/H BOX 3 (DDX3) helicase binds the RIG-I adaptor IPS-1 to up-regulate IFN-β-inducing potential. *Eur. J. Immunol.* **40**, 940–948 (2010).
28. Zhang, Z. *et al.* DDX1, DDX21, and DHX36 helicases form a complex with the adaptor molecule TRIF to sense dsRNA in dendritic cells. *Immunity* **34**, 866–878 (2011).
29. Miyashita, M., Oshiumi, H., Matsumoto, M. & Seya, T. DDX60, a DEXD/H box helicase, is a novel antiviral factor promoting RIG-I-like receptor-mediated signaling. *Mol. Cell. Biol.* **31**, 3802–3819 (2011).
30. Kim, T. *et al.* Aspartate-glutamate-alanine-histidine box motif (DEAH)/RNA helicase A helicases sense microbial DNA in human plasmacytoid dendritic cells. *Proc. Natl Acad. Sci. USA* **107**, 15181–15186 (2010).
31. Zhang, Z. *et al.* The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. *Nature Immunol.* **12**, 959–965 (2011).
32. Zhang, Z., Yuan, B., Lu, N., Facchinetti, V. & Liu, Y. J. DHX9 pairs with IPS-1 to sense double-stranded RNA in myeloid dendritic cells. *J. Immunol.* **187**, 4501–4508 (2011).
33. Elinav, E., Strowig, T., Henao-Mejia, J. & Flavell, R. A. Regulation of the antimicrobial response by NLR proteins. *Immunity* **34**, 665–679 (2011).
34. Sabbah, A. *et al.* Activation of innate immune antiviral responses by Nod2. *Nature Immunol.* **10**, 1073–1080 (2009).
35. Kanneganti, T. D. *et al.* Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J. Biol. Chem.* **281**, 36560–36568 (2006).
36. Allen, I. C. *et al.* The NLRP3 inflammasome mediates *in vivo* innate immunity to influenza A virus through recognition of viral RNA. *Immunity* **30**, 556–565 (2009).
37. Shimada, K. *et al.* Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* **36**, 401–414 (2012).
38. Keating, S. E., Baran, M. & Bowie, A. G. Cytosolic DNA sensors regulating type I interferon induction. *Trends Immunol.* **32**, 574–581 (2011).
39. Burckstummer, T. *et al.* An orthogonal proteomic–genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. *Nature Immunol.* **10**, 266–272 (2009).
40. Fernandes-Alnemri, T., Yu, J.-W., Datta, P., Wu, J. & Alnemri, E. S. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature* **458**, 509–513 (2009).
41. Hornung, V. *et al.* AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* **458**, 514–518 (2009).
42. Roberts, T. L. *et al.* HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. *Science* **323**, 1057–1060 (2009).
43. Unterholzner, L. *et al.* IFI16 is an innate immune sensor for intracellular DNA. *Nature Immunol.* **11**, 997–1004 (2010).
44. Kerur, N. *et al.* IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi sarcoma-associated herpesvirus infection. *Cell Host Microbe* **9**, 363–375 (2011).
45. Takaoka, A. *et al.* DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* **448**, 501–505 (2007).
46. Kaiser, W. J., Upton, J. W. & Mocarski, E. S. Receptor-interacting protein homotypic interaction motif-dependent control of NF-κB activation via the DNA-dependent activator of IFN regulatory factors. *J. Immunol.* **181**, 6427–6434 (2008).
47. Ishii, K. J. *et al.* TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature* **451**, 725–729 (2008).
48. Yang, P. *et al.* The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type I interferon via a β-catenin-dependent pathway. *Nature Immunol.* **11**, 487–494 (2010).
49. Burdette, D. L. *et al.* STING is a direct innate immune sensor of cyclic di-GMP. *Nature* **478**, 515–518 (2011).
50. Querec, T. *et al.* Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *J. Exp. Med.* **203**, 413–424 (2006).
51. Gaucher, D. *et al.* Yellow fever vaccine induces integrated multilineage and polyfunctional immune responses. *J. Exp. Med.* **205**, 3119–3131 (2008).
52. Querec, T. D. *et al.* Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans. *Nature Immunol.* **10**, 116–125 (2009).
53. Caskey, M. *et al.* Synthetic double-stranded RNA induces innate immune responses similar to a live viral vaccine in humans. *J. Exp. Med.* **208**, 2357–2366 (2011).
References 51–53 illustrate how systems biology may help to deconstruct the mechanisms of action of current vaccines in humans.
54. Samuelsson, C. *et al.* Survival of lethal poxvirus infection in mice depends on TLR9, and therapeutic vaccination provides protection. *J. Clin. Invest.* **118**, 1776–1784 (2008).
55. Delaloye, J. *et al.* Innate immune sensing of modified vaccinia virus Ankara (MVA) is mediated by TLR2-TLR6, MDA-5 and the NALP3 inflammasome. *PLoS Pathog.* **5**, e1000480 (2009).
56. Zhu, J., Martinez, J., Huang, X. & Yang, Y. Innate immunity against vaccinia virus is mediated by TLR2 and requires TLR-independent production of IFN-β. *Blood* **109**, 619–625 (2007).
57. Quigley, M., Martinez, J., Huang, X. & Yang, Y. A critical role for direct TLR2–MyD88 signaling in CD8 T-cell clonal expansion and memory formation following vaccinia viral infection. *Blood* **113**, 2256–2264 (2009).
58. Martinez, J., Huang, X. & Yang, Y. Toll-like receptor 8-mediated activation of murine plasmacytoid dendritic cells by vaccinia viral DNA. *Proc. Natl Acad. Sci. USA* **107**, 6442–6447 (2010).
59. Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S. & Reis e Sousa, C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303**, 1529–1531 (2004).
60. Lund, J. M. *et al.* Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc. Natl Acad. Sci. USA* **101**, 5598–5603 (2004).
61. Yoneyama, M. *et al.* The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nature Immunol.* **5**, 730–737 (2004).
62. Kato, H. *et al.* Cell type-specific involvement of RIG-I in antiviral response. *Immunity* **23**, 19–28 (2005).
63. Thomas, P. G. *et al.* The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. *Immunity* **30**, 566–575 (2009).
64. Koyama, S. *et al.* Plasmacytoid dendritic cells delineate immunogenicity of influenza vaccine subtypes. *Sci. Transl. Med.* **2**, 25ra24 (2010).
65. Aoshi, T., Koyama, S., Kobiyama, K., Akira, S. & Ishii, K. J. Innate and adaptive immune responses to viral infection and vaccination. *Curr. Opin. Virol.* **1**, 226–232 (2011).
66. Mancuso, G. *et al.* Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. *Nature Immunol.* **10**, 587–594 (2009).
67. von Meyenn, F. *et al.* Toll-like receptor 9 contributes to recognition of *Mycobacterium bovis* Bacillus Calmette-Guerin by Flt3-ligand generated dendritic cells. *Immunobiology* **211**, 557–565 (2006).
68. Sander, L. E. *et al.* Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. *Nature* **474**, 385–389 (2011).
This study supports the idea that bacterial nucleic acids may be recognized as a signal of microbial viability and may contribute to an enhanced adaptive immune response against the pathogen.
69. Liu, M. A. Immunologic basis of vaccine vectors. *Immunity* **33**, 504–515 (2010).
70. Coban, C. *et al.* Novel strategies to improve DNA vaccine immunogenicity. *Curr. Gene Ther.* **11**, 479–484 (2011).
71. Spies, B. *et al.* Vaccination with plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. *J. Immunol.* **171**, 5908–5912 (2003).
72. Babiuk, S. *et al.* TLR9$^{-/-}$ and TLR9$^{+/+}$ mice display similar immune responses to a DNA vaccine. *Immunology* **113**, 114–120 (2004).
73. Rottembourg, D. *et al.* Essential role for TLR9 in prime but not prime–boost plasmid DNA vaccination to activate dendritic cells and protect from lethal viral infection. *J. Immunol.* **184**, 7100–7107 (2010).
74. Ishikawa, H., Ma, Z. & Barber, G. N. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* **461**, 788–792 (2009).
This study identifies STING as an essential adapter protein in the signalling pathways of TBK1-activating cytosolic DNA sensors.
75. Mbow, M. L., De Gregorio, E., Valiante, N. M. & Rappuoli, R. New adjuvants for human vaccines. *Curr. Opin. Immunol.* **22**, 411–416 (2010).
76. McKee, A. S. *et al.* Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. *J. Immunol.* **183**, 4403–4414 (2009).
77. Marrack, P., McKee, A. S. & Munks, M. W. Towards an understanding of the adjuvant action of aluminium. *Nature Rev. Immunol.* **9**, 287–293 (2009).
78. Hornung, V. *et al.* Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nature Immunol.* **9**, 847–856 (2008).
79. Eisenbarth, S. C., Colegio, O. R., O'Connor, W., Sutterwala, F. S. & Flavell, R. A. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* **453**, 1122–1126 (2008).
80. Spreafico, R., Ricciardi-Castagnoli, P. & Mortellaro, A. The controversial relationship between NLRP3, alum, danger signals and the next-generation adjuvants. *Eur. J. Immunol.* **40**, 638–642 (2010).
81. Goto, N. *et al.* Local tissue irritating effects and adjuvant activities of calcium phosphate and aluminium hydroxide with different physical properties. *Vaccine* **15**, 1364–1371 (1997).
82. Munks, M. W. *et al.* Aluminum adjuvants elicit fibrin-dependent extracellular traps *in vivo*. *Blood* **116**, 5191–5199 (2010).
83. Kool, M. *et al.* Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J. Exp. Med.* **205**, 869–882 (2008).
84. Kool, M. *et al.* An unexpected role for uric acid as an inducer of T helper 2 cell immunity to inhaled antigens and inflammatory mediator of allergic asthma. *Immunity* **34**, 527–540 (2011).
85. Marichal, T. *et al.* DNA released from dying host cells mediates aluminum adjuvant activity. *Nature Med.* **17**, 996–1002 (2011).
86. Lore, K. *et al.* Toll-like receptor ligands modulate dendritic cells to augment cytomegalovirus- and HIV-1-specific T cell responses. *J. Immunol.* **171**, 4320–4328 (2003).
87. Schulz, O. *et al.* Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* **433**, 887–892 (2005).
88. Jongbloed, S. L. *et al.* Human CD141^{+} (BDCA-3)$^{+}$ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J. Exp. Med.* **207**, 1247–1260 (2010).

© 2012 Macmillan Publishers Limited. All rights reserved

89. Jelinek, I. *et al.* TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection. *J. Immunol.* **186**, 2422–2429 (2011).
90. Poulin, L. F. *et al.* Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8α+ dendritic cells. *J. Exp. Med.* **207**, 1261–1271 (2010).
91. Trumpfheller, C. *et al.* The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. *Proc. Natl Acad. Sci. USA* **105**, 2574–2579 (2008).
92. Longhi, M. P. *et al.* Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. *J. Exp. Med.* **206**, 1589–1602 (2009).
93. Stahl-Hennig, C. *et al.* Synthetic double-stranded RNAs are adjuvants for the induction of T helper 1 and humoral immune responses to human papillomavirus in rhesus macaques. *PLoS Pathog.* **5**, e1000373 (2009).
94. Kumar, H., Koyama, S., Ishii, K. J., Kawai, T. & Akira, S. Cutting edge: cooperation of IPS-1- and TRIF-dependent pathways in poly IC-enhanced antibody production and cytotoxic T cell responses. *J. Immunol.* **180**, 683–687 (2008).
95. Tewari, K. *et al.* Poly(I:C) is an effective adjuvant for antibody and multi-functional CD4+ T cell responses to *Plasmodium falciparum* circumsporozoite protein (CSP) and αDEC-CSP in non human primates. *Vaccine* **28**, 7256–7266 (2010).
96. Wang, Y., Cella, M., Gilfillan, S. & Colonna, M. Cutting edge: polyinosinic:polycytidylic acid boosts the generation of memory CD8 T cells through melanoma differentiation-associated protein 5 expressed in stromal cells. *J. Immunol.* **184**, 2751–2755 (2010).
97. Russo, C. *et al.* Small molecule Toll-like receptor 7 agonists localize to the MHC class II loading compartment of human plasmacytoid dendritic cells. *Blood* **117**, 5683–5691 (2011).
98. Levy, O., Suter, E. E., Miller, R. L. & Wessels, M. R. Unique efficacy of Toll-like receptor 8 agonists in activating human neonatal antigen-presenting cells. *Blood* **108**, 1284–1290 (2006).
99. Hamm, S. *et al.* Immunostimulatory RNA is a potent inducer of antigen-specific cytotoxic and humoral immune response *in vivo*. *Int. Immunol.* **19**, 297–304 (2007).
100. Zhang, W. W. & Matlashewski, G. Immunization with a Toll-like receptor 7 and/or 8 agonist vaccine adjuvant increases protective immunity against *Leishmania major* in BALB/c mice. *Infect. Immun.* **76**, 3777–3783 (2008).
101. Rajagopal, D. *et al.* Plasmacytoid dendritic cell-derived type I interferon is crucial for the adjuvant activity of Toll-like receptor 7 agonists. *Blood* **115**, 1949–1957 (2010).
102. Wille-Reece, U. *et al.* HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8+ T cell responses in nonhuman primates. *Proc. Natl Acad. Sci. USA* **102**, 15190–15194 (2005).
103. Kastenmuller, K. *et al.* Protective T cell immunity in mice following protein-TLR7/8 agonist-conjugate immunization requires aggregation, type I IFN, and multiple DC subsets. *J. Clin. Invest.* **121**, 1782–1796 (2011).
References 102 and 103 show how optimizing the formulation of agonists for nucleic acid sensors may affect quantitative and qualitative aspects of the response to subunit vaccines adjuvanted with such molecules.
104. Huang, X. & Yang, Y. Targeting the TLR9–MyD88 pathway in the regulation of adaptive immune responses. *Expert Opin. Ther. Targets* **14**, 787–796 (2010).
105. Campbell, J. D. *et al.* CpG-containing immunostimulatory DNA sequences elicit TNF-α-dependent toxicity in rodents but not in humans. *J. Clin. Invest.* **119**, 2564–2576 (2009).
106. Wagner, M. *et al.* IL-12p70-dependent Th1 induction by human B cells requires combined activation with CD40 ligand and CpG DNA. *J. Immunol.* **172**, 954–963 (2004).
107. Poeck, H. *et al.* Plasmacytoid dendritic cells, antigen, and CpG-C license human B cells for plasma cell differentiation and immunoglobulin production in the absence of T-cell help. *Blood* **103**, 3058–3064 (2004).
108. Krieg, A. M. *et al.* CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549 (1995).
109. Chen, W., Kuolee, R. & Yan, H. The potential of 3',5'-cyclic diguanylic acid (c-di-GMP) as an effective vaccine adjuvant. *Vaccine* **28**, 3080–3085 (2010).
110. Karaolis, D. K. *et al.* Bacterial c-di-GMP is an immunostimulatory molecule. *J. Immunol.* **178**, 2171–2181 (2007).
111. McWhirter, S. M. *et al.* A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP. *J. Exp. Med.* **206**, 1899–1911 (2009).
112. Sauer, J. D. *et al.* The *N*-ethyl-*N*-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the *in vivo* interferon response to *Listeria monocytogenes* and cyclic dinucleotides. *Infect. Immun.* **79**, 688–694 (2011).
113. Trinchieri, G. & Sher, A. Cooperation of Toll-like receptor signals in innate immune defence. *Nature Rev. Immunol.* **7**, 179–190 (2007).
114. Zhu, Q. *et al.* Toll-like receptor ligands synergize through distinct dendritic cell pathways to induce T cell responses: implications for vaccines. *Proc. Natl Acad. Sci. USA* **105**, 16260–16265 (2008).
115. Zhu, Q. *et al.* Using 3 TLR ligands as a combination adjuvant induces qualitative changes in T cell responses needed for antiviral protection in mice. *J. Clin. Invest.* **120**, 607–616 (2010).
116. Kasturi, S. P. *et al.* Programming the magnitude and persistence of antibody responses with innate immunity. *Nature* **470**, 543–547 (2011).
References 115 and 116 illustrate how combining nucleic acid sensor agonists and optimizing their delivery strategies could allow fine-tuning of the responses to subunit vaccines.
117. Remijsen, Q. *et al.* Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. *Cell Death Differ.* **18**, 581–588 (2011).
118. Hashimoto, D., Miller, J. & Merad, M. Dendritic cell and macrophage heterogeneity *in vivo*. *Immunity* **35**, 323–335 (2011).
119. Palucka, K., Banchereau, J. & Mellman, I. Designing vaccines based on biology of human dendritic cell subsets. *Immunity* **33**, 464–478 (2010).
120. Gilliet, M., Cao, W. & Liu, Y.-J. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nature Rev. Immunol.* **8**, 594–606 (2008).
121. Takagi, H. *et al.* Plasmacytoid dendritic cells are crucial for the initiation of inflammation and T cell immunity *in vivo*. *Immunity* **35**, 958–971 (2011).
This study suggests a direct role of pDCs in the priming of CD8+ T cell responses *in vivo*.
122. Reizis, B., Colonna, M., Trinchieri, G., Barrat, F. & Gilliet, M. Plasmacytoid dendritic cells: one-trick ponies or workhorses of the immune system? *Nature Rev. Immunol.* **11**, 558–565 (2011).
123. Janeway, C. A. Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* **54** (Pt 1), 1–13 (1989).
124. Matzinger, P. Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* **12**, 991–1045 (1994).
125. González-Navajas, J. M., Lee, J., David, M. & Raz, E. Immunomodulatory functions of type I interferons. *Nature Rev. Immunol.* **12**, 125–135 (2012).
126. Sadler, A. J. & Williams, B. R. Interferon-inducible antiviral effectors. *Nature Rev. Immunol.* **8**, 559–568 (2008).

Acknowledgements
The authors thank F. Bureau, C. Coban and T. Marichal for critical reading of the manuscript. C.J.D. is supported by the Fonds National de la Recherche Scientifique (FRS-FNRS, Belgium; Fonds pour la Recherche Scientifique Médicale grant). K.J.I. is supported by a Health and Labour Sciences Research Grant of the Japanese Ministry of Health, Labour and Welfare and by the Core Research Evolutionary Science and Technology (CREST) programme at the Japan Science and Technology Agency.

Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
Christophe J. Desmet's homepage:
http://www.giga.ulg.ac.be/pcm
Ken J. Ishii's homepage: http://www.ifrec.osaka-u.ac.jp/en/laboratory/vaccinescience/index.php
ALL LINKS ARE ACTIVE IN THE ONLINE PDF

© 2012 Macmillan Publishers Limited. All rights reserved

REVIEW

Oncolmmunology 1:6, 917-923; September 2012; © 2012 Landes Bioscience

TLR3/TICAM-1 signaling in tumor cell RIP3-dependent necroptosis

Tsukasa Seya,* Hiroaki Shime, Hiromi Takaki, Masahiro Azuma, Hiroyuki Oshiumi and Misako Matsumoto

Department of Microbiology and Immunology; Hokkaido University Graduate School of Medicine; Sapporo, Japan

Keywords: interferon-inducing pathway, necroptosis, RIP signaling, TLR3, TICAM-1, TLR3, TRIF

Abbreviations: CTL, cytotoxic T lymphocyte; DAI, DNA-dependent activator of IFN-regulatory factors; DAMP, damage-associated molecular pattern; HMGB1, high-mobility group box 1; HSP, heat shock protein; mDC, myeloid dendritic cell; NK, natural killer; NLR, NOD-like receptor; PAMP, pathogen- associated molecular pattern; PRR, pattern-recognition receptor; RIP, receptor-interacting protein kinase; TICAM-1, Toll-IL-1-homology domain-containing adaptor molecule 1; TLR, Toll-like receptor; TNFα, tumor necrosis factor α; TNFR1, TNFα receptor 1

The engagement of Toll-like receptor 3 (TLR3) leads to the oligomerization of the adaptor TICAM-1 (TRIF), which can induces either of three acute cellular responses, namely, cell survival coupled to Type I interferon production, or cell death, via apoptosis or necrosis. The specific response elicited by TLR3 determines the fate of affected cells, although the switching mechanism between the two cell death pathways in TLR3-stimulated cells remains molecularly unknown. Tumor necrosis factor α (TNFα)-mediated cell death can proceed via apoptosis or via a non-apoptotic pathway, termed necroptosis or programmed necrosis, which have been described in detail. Interestingly, death domain-containing kinases called receptor-interacting protein kinases (RIPs) are involved in the signaling pathways leading to these two cell death pathways. Formation of the RIP1/RIP3 complex (called necrosome) in the absence of caspase 8 activity is crucial for the induction of necroptosis in response to TNFα signaling. On the other hand, RIP1 is known to interact with the C-terminal domain of TICAM-1 and to modulate TLR3 signaling. In macrophages and perhaps tumor cell lines, RIP1/RIP3-mediated necroptotic cell death can ensue the administration of the TLR agonist polyI:C. If this involved the TLR3/TICAM-1 pathway, the innate sensing of viral dsRNA would be linked to cytopathic effects and to persistent inflammation, in turn favoring the release of damage-associated molecular patterns (DAMPs) in the microenvironment. Here, we review accumulating evidence pointing to the involvement of the TLR3/TICAM-1 axis in tumor cell necroptosis and the subsequent release of DAMPs.

Introduction

Cell death is an important process for both development and homeostasis in multicellular organisms. The mode of cell death is closely associated with other biological responses occurring within the host, including inflammation. Cell death has been categorized as apoptotic or necrotic and, until recently, apoptosis had been considered as a synonym of programmed cell death.[1] Caspases are a family of cysteine proteases that mediate apoptotic cell death in response to ligands of death receptors, including tumor necrosis factor α (TNFα), FAS ligand (FASL) and TRAIL, as well as to intracellular damage, upon the induction of pro-apoptotic BH3-only members of the Bcl-2 family. However, it is now clear that apoptosis is not the only cellular mechanism that mediates programmed cell death. Necrotic cell death, which has traditionally been viewed as a form of passive cell death, may also be regulated, and in this case has been called necroptosis or programmed necrosis.[2] Necroptosis may be induced by TNFα receptor 1 (TNFR1) agonists, but also by innate pattern-recognition receptors (PRRs) such as Toll-like receptor (TLR) 3 and TLR4.[1,4] These two TLRs can recruit the adaptor TICAM-1 (also known as TRIF), leading to Type I interferon (IFN) signaling.[3] In line with this notion, the TLR3 ligand polyI:C (a synthetic double-stranded RNA, dsRNA) can activate either apoptosis or necrosis, depending on the cell lines tested. Cell death induced by the TLR3-TICAM-1 axis may therefore be executed through two distinct subroutines.[5] The mechanisms that dictate the cellular decision to undergo apoptosis or necroptosis in response to TLR3 signaling, as well as the mechanisms that mediate the execution of necroptosis, are the subject of intense investigation.

Toll-like receptors and other PRRs harbor the ability to specifically recognize microbal molecules, known as pathogen-associated molecular patterns (PAMPs).[6] PAMPs trigger the maturation of myeloid dendritic cells (mDCs) through the activation of TLR and/or other pathways, eventually eliciting cellular immunity.[7] In mDCs, nucleic acid-recognizing TLRs (i.e., TLR3, TLR7, TLR8 and TLR9) reside in endosomes and sense their ligands only when they are internalized.[8] The uptake of DNA or RNA of microbial origin therefore allows cross-presentation to T cells and the exposure of natural killer (NK) cell-activating ligands. Besides this extrinsic maturation route, it is known that the formation of autophagosomes may deliver cytoplasmic nucleic acids of viral origin to the endosome via autophagy.[9] In either route, TLR signaling links immunological events to pathological cell death.

Recently accumulated evidence suggests that TLRs serve as receptors not only for foreign PAMPs but also for cellular

*Correspondence to: Tsukasa Seya; Email: seya-tu@pop.med.hokudai.ac.jp
Submitted: 05/28/12; Accepted: 06/22/12
http://dx.doi.org/10.4161/onci.21244

Table 1. Host response to nucleic acids and other DAMPs

PAMP/DAMP	Receptors
Microbial nucleic acids(PAMP)	
cytosolic long dsRNA	MDA5
cytosolic 5'-PPP-RNA	RIG-I
endosomal >140 bp dsRNA	TLR3
nonmethylated CpG DNA	TLR9
cytosolic dsDNA	DNA sensors*
Self molecular patterns(DAMP)	
HMGB1	RAGE, TLR2/4
Uric acid	CD14, TLR2/4
HSPs	CD14, TLR2/4,**
S100 proteins	RAGE
Self nucleic acids (DAMP)	
Self DNA	DNA sensors*
Self mRNA	TLR3

*See **Table 2**; ** D40, CD91, Scavenger receptors etc.

Table 2. RNA·DNA recognition molecules in vertebrates

Receptors	Adaptors	Ligands	Induction of Type I IFN
		TLR family	
TLR3	TICAM-1	dsRNA, stem RNA	+
TLR7/8	MyD88	ssRNA	+
TLR22	TICAM-1	dsRNA	+
PKR	?	dsRNA	-
		RLR family	
RIG-I	MAVS	5'-PPP RNA, dsRNA	+
MDA5	MAVS	dsRNA (long)	+
		NLR family	
NALP3	ASC	dsRNA	+
NOD2	MAVS	ssRNA	+
		DDX family	
DDX1	TICAM-1	dsRNA	+
DDX21	TICAM-1	dsRNA	+
DHX36	TICAM-1	dsRNA	+
		DNA sensors	
TLR9	MyD88	CpG DNA	+
DAI	TBK1	dsDNA	+
Pol3/RIG-I	MAVS	dsDNA	+
IFI16	TBK1	dsDNA	+
DDX41	STING	dsDNA	+
DHX9	MyD88	dsDNA	+
DDX36	MyD88	dsDNA	+
ZAPS	?	dsDNA	+

constituents that are liberated from damaged or necrotic cells.[10] Thus, innate pattern-recognition is not only a mechanism for discriminating pathogens from the host, but also a means for inspecting cellular homeostasis. Molecules that, upon release from damaged/necrotic cells, activate the immune system are called damage-associated molecular patterns (DAMPs).[11] The most popular TLR adaptor MYD88 is known to contain death domains, and some reports have suggested that TLR signaling may be involved in cell death secondary to PAMP/DAMP-stimulation. Necroptotic or damaged cells may thus represent a result of TLR death signaling, and generate a functional complex consisting of sources of DAMPs as well as of the phagocytic response.[11,12]

DAMPs refer to intracellular molecules that acquire inflammation-inducing capacities when released from cells. DAMPs do not belong to the cytokine family but rather resemble PAMP in their functional properties, in particular with regard to mDC and macrophages. The functions of DAMPs may be associated with responses including regeneration and tumorigenesis. During the past 5 years, necroptotic cell death has been closely connected with innate immune responses involving pattern-sensing.[12,13] DAMPs include a large number of cytosolic or nuclear molecules (**Table 1**), as well as, surprisingly, self nucleic acids.[14] This implies that, like viral DNA and RNA, autologous nucleic acids can evoke inflammation. Here, we discuss the importance of the immune modulation induced by nucleic acids and necroptotic host cells.

Necroptosis: Programmed Necrosis Induced by TNFα

TNFα has been reported to induce two different types of cell death, apoptosis and necrosis, in a cell type-specific manner.[15,16] Through TNFR1, TNFα is implicated in NFκB activation and contributes to cell growth in many cancer cell lines. In parallel TNFα-induced hemorrhagic necrosis has been observed in several cancer cell lines, but the molecular mechanisms underlying these differential responses to TNFα remain poorly understood. Recently, several reports have suggested that the formation of a supracomplex containing the receptor-interacting protein kinase 1 (RIP1) and its homolog RIP3 (which has been named "necrosome") is responsible for the switch from apoptosis to necroptosis.[17,18] RIP1 and RIP3 can assemble only in the absence of functional caspase-8, indicating that this enzyme acts as a key protease for blocking the formation of the necrosome.[5,19] Many viral factors, as well as the genomic instability that frequently characterizes tumor cells, can compromise caspase-8 function, thereby facilitating the induction of necroptosis. Hence, TNFα can promote cell death by signaling through its receptors, including TNRF1 and downstream via RIP1/RIP3, although the output of TNFα signaling is ultimately determined by cell type.

Virus-Mediated Necroptosis

It is notable that a necrotic phenotype has been observed in polyI:C-stimulated bone marrow-derived murine macrophages and other cell lines.[13] TICAM-1 and RIP3 are involved in this process, suggesting the implication of the necrosome pathway in dsRNA-mediated cell death.[12,13] It has been reported that viral

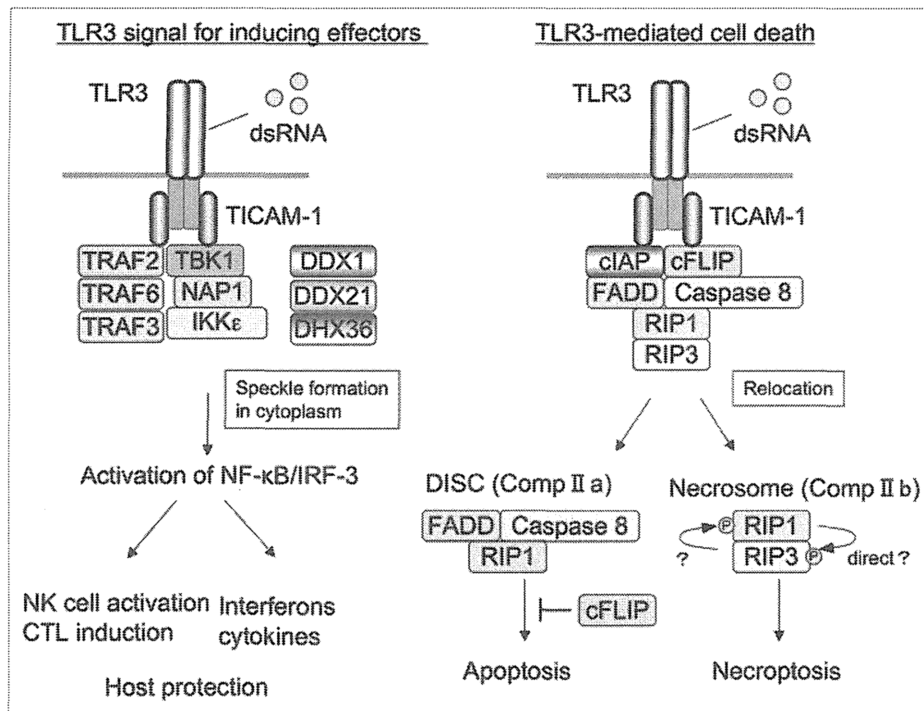

Figure 1. TLR3 signals inducing cell death or effector functions in myeloid cells. Cell survival (left panel) and cell death (right panel) signals are schematically depicted. TICAM-1 assembles in a supramolecular complex around oligomerized Toll-like receptor 3 (TLR3) in the endosome. The complex (named Speckle) then dissociates from TLR3, tranlocating to the cytoplasm. IRF-3 and NFκB are activated by Speckle, leading to their nuclear translocation and induction of Type I interferon (IFN) and inflammatory cytokines, respectively. In dendritic cells (DCs), natural killer (NK) cell-activating ligands and factors for cross-presentation are induced downstream of IRF-3/7 (left panel). In contrast, cell death signaling culminates in apoptosis and/or necrosis depending on downstream signal transducers (right panel). TLR3-dependent apoptosis has been reported in several cancer cell lines,[7] while TLR3-dependent necroptosis has been observed in mouse bone marrow-derived macrophages.[13] These events rely on RIP1/RIP3 activation, similar to those elicited upon ligation of the tumor necrosis factor α receptor 1 (TNFR1). Whether or not the translocation of the TICAM-1 complex is required for the cell death signaling, as well as the mechanisms determining either cytokine secretion or cell death, remain unknown.

dsRNA frequently induces apoptosis in infected cells, a process that in general is known as cytopathic effect.[20] TICAM-1 and RIPs, mainly RIP1, may also be involved in virus-derived necrotic cell death.[5,13] This is relatively rare compared with apoptosis since it occurs only when the viral genome encodes caspase-8 inhibitors.[19] Furthermore, this process requires viral dsRNA to be delivered from the cytosol to the endosomes (where TLR3 is situated) of infected cells. This may happen if the dsRNA is engulfed by autophagosomes, which ensure its transfer to endosomes. The possible involvement of another PRR that sense viral RNA, RIG-I/MDA5, in cell death as induced by viral infection cannot be always ruled out. TNFα can be produced downstream of the TLR3- and RIG-I-mediated RNA-sensing pathways and may induce necrotic cell death. Many RNA viruses trigger cell death,[20] but the factors determining the induction of necroptosis in virus-infected cells remain to be clarified.

DNA viruses can induce necroptosis via another mechanism, which involves the DNA-dependent activator of IFN-regulatory factors (DAI, also known as DLM-1/ZBP1).[21] DAI is a DNA sensor[22] and directly activates RIP3 in the absence of Type I IFN induction.[21] This said, the sensing of DNA in the cytoplasm of virus-infected cells is complex, and it may be that DAI is not the only molecule linked to such a necroptotic response. It is unknown whether RIP3-mediated necroptosis can be induced even if caspase-8 is blocked upon the recognition of viral DNA by DAI or via other mechanisms.[20] In fact, this type of virus-derived necrosis has been reported with DNA viruses that encode caspase inhibitors including vaccinia virus (VV), which encodes B13R/Spi2, poxvirus, encoding CrmA, the Kaposi sarcoma-associated herpesvirus (KSHV), encoding K13 and the molluscum contagiosum virus (MCV), which encodes MC159.[20,23] Generally speaking, the mode of cell death secondary to virus infection differ as a function of viral species. The physiological role of TLR3- and DAI-mediated necroptosis should therefore be analyzed in a virus-specific fashion.

Necroptosis in Inflammation

Apoptosis plays a major role in physiological contexts, while necrosis is very common under pathological conditions.[1] Necroptosis differs from accidental necrosis in its programmed nature, and differs from apoptosis in that necroptosis often stimulates inflammation. When virus-infected cells undergo apoptosis, they are removed by phagocytosis. Viral genomes, be they either

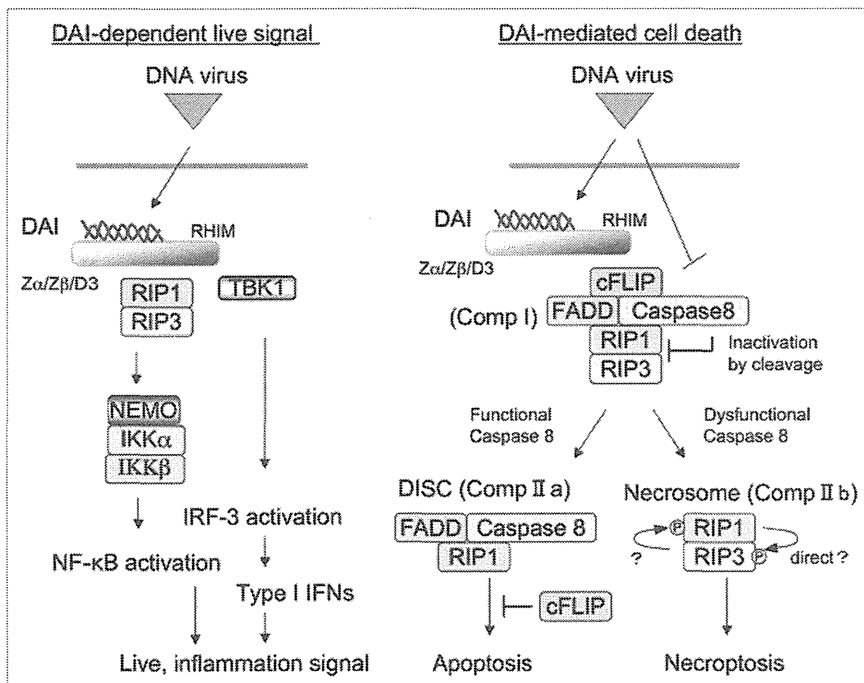

Figure 2. Necroptosis induced by the DAI pathway. Cell survival (left panel) and cell death (right panel) signals transmitted by the DNA-dependent activator of IFN-regulatory factors (DAI) are schematically depicted. Pro-survival signaling involves the activation of IRF-3 and NFκB to support antiviral responses (left panel). Type I IFNs and inflammatory cytokines are the main effectors induced by IRF-3/NFκB activation. In contrast, DAI activates RIP3 to induce necroptosis during viral infection, provided that caspases are inhibited. When viruses express caspase inhibitors, the RIP1/RIP3 necrosome plays a dominant role in the activation of cell death via necroptosis (right panel). If caspase-8 is active, RIP3 should get inactivated and apoptosis should be the dominant phenotype, though this scheme has not yet been experimentally confirmed. The mechanisms determining the choice between these two signaling pathways are unknown.

DNA- or RNA-based, are degraded in infected cells, thus being able neither to stimulate phagocytes including macrophages and DCs, nor to favor the liberation of DAMPs. In contrast, non-apoptotic cell death is accompanied by the release of DAMPs and viral products, resulting in the activation of macrophages,[13] as it occurs during chronic infection, in which viruses produce caspase inhibitors or render infected cells resistant to apoptosis.[24] A typical model of necroptosis evokes two effectors, namely, viral nucleic acids and DAMPs, to modulate immune and bystander cells of the host. In the context of necroptosis, these effectors allow for the amplification of inflammatory responses by myeloid phagocytes (mDCs and macrophages). These cells accumulate in inflammation as induced by persistent viral infection, and mediate the secondary release of cytokines and other biologically active molecules. In addition, viral factors can result in incipient inflammation, as observed in chronic infections by the hepatitis B or C virus.[24] This, in conjunction with viral nucleic acids and DAMPs, may modify the features of the infectious milieu. Further studies are needed to clarify the importance of viral nucleic acids and DAMPs in the context of virus-dependent chronic inflammation, as it may facilitate tumor progression.

Necroptosis and Oncogenesis

Accumulating evidence indicates that pro-inflammatory signals, including those following the activation of NFκB, are crucial for oncogenesis. Moreover, DAMPs have been associated with tumorigenesis as well as with antitumor immune responses.[25,26] Tumor progression is not always accompanied by viral infections, and it remains unclear whether DAMPs released from non-infected tumor cells are sufficient to support tumor growth. It has been reported that self mRNA acts as a TLR3 ligand[14] and that self DNA can stimulate host cell sensors.[22,27] Due to the uncomplete identification and functional characterization of DNA sensors and their signaling pathways, however, it is unknown whether host nucleic acids are potent inducers of inflammation as compared with viral RNA or unmethylated CpG DNA of bacterial origin. Moreover, the role of RNA sensors in the tumor microenvironment has not yet been clarified (**Table 2**).

DAMPs have recently been characterized at the molecular level[11] and representative DAMPs (**Table 1**) include HMGB1,[28] uric acid crystal,[10] S100 proteins,[29] naked actin[30,31] and heat-shock proteins (HSPs).[32] The functional features of DAMPs and the mechanisms whereby they provoke inflammation have been delineated,[11,28,29] and these studies have introduced the concept of "inflammasome" in the field of innate immunity.[33] Caspase-1 is activated upon the administration of NOD-like receptor (NLR) ligands, which include some DAMPs as well as inorganic PAMPs. Active caspase-1, together with the upregulation of the immature variants of IL-1 family proteins that ensues TLR stimulation, accelerates the robust release of IL-1β, IL-18 and IL-33.[34] There are many kinds of NLRs as well as TLRs, and the common pathways (including those centered around the adaptor ASC) can be activated by a variety of cytoplasmic DAMPs and PAMPs.[33,34] The cytoplasmic immature forms of the abovementioned cytokines are activated by limited caspase-1-mediated proteolysis, and then are secreted into the extracellular microenvironment.[34] Hence, IL-1 family proteins require two DAMPs/PAMP signals for their upregulation and activation.[35] Of note, the tumorigenic properties of asbestos and silica are in part attributable to the activation of the inflammasome, leading to the secreton of IL-1 family proteins. However, not all DAMPs operate as inflammasome activators, even in the broad sense of this term.

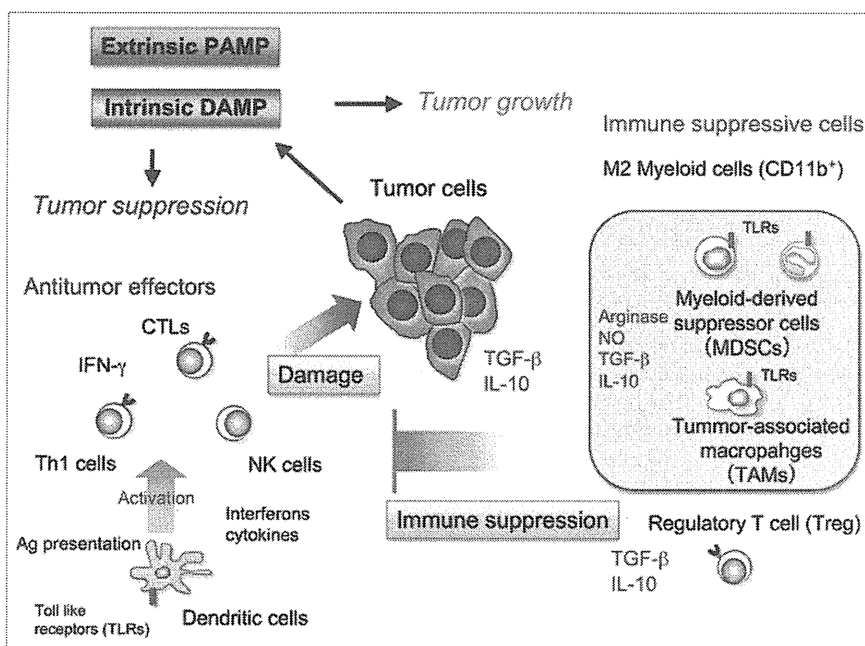

Figure 3. Inflammation provides the microenvironment for infection-related cancer. Immune cells infiltrating the tumor mass may modulate the local microenvironment upon the recognition of pathogen- or damage-associated molecular patterns (PAMP/DAMPs). Cancer cells undergoing necrosis liberate DAMPs and debris containing nucleic acids, which recruit immune cells stimulating an inflammatory response. In some cases, tumors benefit from the inflammatory response, while in other cases they regress following inflammation. The mechanisms determining this switch remain to be clarified.

Immune Response Elicited by the Phagocytosis of Dead Cells

Phagocytosis of dead cells involves not only cell clearance but also the initiation of an immune response. Dead cell antigens are rapidly presented on MHC Class II molecules after internalization by DCs, driving the recruitment and activation of various CD4⁺ T cell subsets, including Th1, Th2, Th17 and regulatory T cells (Tregs) (**Fig. 1**). In the presence of a second co-stimulatory signal provided by TLRs, working as an adjuvant, DCs cross-present antigens on MHC Class I molecules to induce the proliferation of CD8⁺ cytotoxic T lymphocytes (CTLs).[36] The presentation of exogenous antigens by DCs is therefore dependent on the presence of PAMPs/DAMPs.[36] Accordingly, necrotic debris appears to result in CTL cross-priming more efficiently than apoptotic bodies. Cross-presentation is enhanced by molecules such as Type I IFN and CD40, and by immune cells including CD4⁺ T, NK and NKT cells. Hence, the use of adjuvants to affect many cell types of the immune system other than antigen-presenting cells, and a precise evaluation of the total cross-priming activity appear to be indispensable for the development of efficient adjuvant therapies.

The TLR3/TICAM-1 axis is best known as an inducer of cross-presentation in vivo.[37] The cross-presentation activity of the TLR3 ligands polyI:C and viral dsRNA was first described by Schulz et al. in 2005.[38] While the potency of polyI:C as an adjuvant has been reported by Steinman and colleagues,[37,39] the precise identity of the DAMPs participating in cross-presentation and possessing latent cross-priming (CTL-inducing) capacities has not yet been determined.

It is known that phagocytosis induces functional changes in mDCs and macrophages (**Fig. 2**): phagocytes are skewed toward a regulatory phenotype accompanied by the production of IL-10 and TGFβ during the phagocytosis of apoptotic cell debris, even in the presence of PAMP.[40,41] This suggests that material that cannot be taken up exerts different effects on mDCs than internalizable material during their phagocytic interactions. Phagocytes undergo cytoskeletal rearrangement when they take up cell debris, involving cell adhesion molecules that accelerate the interaction between the phagocyte membrane and cell debris. The opsonization of dead cells further enhances phagocytosis as well as the induction of an immune outcome.[42] Complement-mediated opsonization of dead cells strongly alters the functional properties of mDCs and macrophages.[43] Yet, it has been impossible to discriminate apoptotic and necroptotic cells based on this.[44] Thus, the mechanisms whereby necroptotic cells initiate an immune response upon phagocytosis by mDCs and macrophages, compared with apoptotic cells, remain largely uncharacterized. Elucidating the role of necroptotic cells and DAMPs as adjuvants for NK-cell activation and antigen presentation is highly relevant for antitumor therapy. Since the phagocytosis of dead cells by mDCs usually leads to the generation of tolerogenic mDCs, additional adjuvants appear to be required for mDCs to present tumor antigens in an immunogenic fashion, leading to the induction of an effective immune response against cancer.

Termination of Inflammation

Inflammation often drives tissue repair and regeneration, and the microenvironment formed during inflammation serves a basis for assembling cells that initiate tissue development and reorganization (**Fig. 3**). The pro-inflammatory microenvironment facilitates cell growth as well as genome instability, thus being prone to the accumulation of cells with multiple mutations. Furthermore, incipient inflammation compromises the immune system so that the abnormal proliferation of transformed cells is tolerated. Thus, malignant cells build up a tissue that involves tumor-associated macrophages serving a scaffold for invasion and metastasis.[45] In this context, a region harboring DAMP-mediated persistent inflammation provides a perfect nest for tumor progression (**Fig. 3**). Therapeutics for suppressing inflammation, such as aspirin, may constitute an immune therapy irrespective of the presence of infection.[46] We surmise that two types of inflammation exist, namely tumor-supporting and tumor-suppressing, implying that inflammation is a complex phenomenon consisting of multiple distinct aspects. We have shown that some adjuvants can induce tumor-suppressing inflammation, thereby limiting tumor proliferation by DAMPs.[47] The adjuvant-induced switch of cell death/inflammation signals to an antitumor outcome is an intriguing approach for cancer therapy, particularly in view of the fact that the mechanisms of adjuvant signaling are being increasingly characterized at the molecular level.[48,49] The clarification of the role of adjuvant signaling in compromising tumor progression will lead to the discovery of non-toxic synthetic tumor-regressing molecules with potential as novel anticancer therapeutics.[50]

Acknowledgements

We thank Drs H.H. Aly, R. Takemura, A. Maruyama, Sayuri Yamazaki and J. Kasamatsu in our laboratory for their fruitful discussions. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture (Specified Project for Advanced Research, MEXT) and the Ministry of Health, Labor and Welfare of Japan and by the Takeda and the Waxmann Foundations. Financial supports by a MEXT Grant-in-Project "the Carcinogenic Spiral," "the National Cancer Center Research and Development Fund (23-A-44)" and the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) are gratefully acknowledged.

References

1. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, et al.; Nomenclature Committee on Cell Death 2009. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 2009; 16:3-11; PMID:18846107; http://dx.doi.org/10.1038/cdd.2008.150.
2. Tait SW, Green DR. Caspase-independent cell death: leaving the set without the final cut. Oncogene 2008; 27:6452-61; PMID:18955972; http://dx.doi.org/10.1038/onc.2008.311.
3. Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, Seya T. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. J Biol Chem 2003; 278:49751-62; PMID:14519765; http://dx.doi.org/10.1074/jbc.M305820200.
4. Ermolaeva MA, Michallet MC, Papadopoulou N, Utermöhlen O, Kranidioti K, Kollias G, et al. Function of TRADD in tumor necrosis factor receptor 1 signaling and in TRIF-dependent inflammatory responses. Nat Immunol 2008; 9:1037-46; PMID:18641654; http://dx.doi.org/10.1038/ni.1638.
5. Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M, et al. cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol Cell 2011; 43:449-63; PMID:21737330; http://dx.doi.org/10.1016/j.molcel.2011.06.011.
6. Medzhitov R, Janeway CA Jr. Innate immunity: the virtues of a nonclonal system of recognition. Cell 1997; 91:295-8; PMID:9363937; http://dx.doi.org/10.1016/S0092-8674(00)80412-2.
7. Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. Science 2010; 327:291-5; PMID:20075244; http://dx.doi.org/10.1126/science.1183021.
8. Kawai T, Akira S. Toll-like receptor and RIG-I-like receptor signaling. Ann N Y Acad Sci 2008; 1143:1-20; PMID:19076341; http://dx.doi.org/10.1196/annals.1443.020.
9. Morris S, Swanson MS, Lieberman A, Reed M, Yue Z, Lindell DM, et al. Autophagy-mediated dendritic cell activation is essential for innate cytokine production and APC function with respiratory syncytial virus responses. J Immunol 2011; 187:3953-61; PMID:21911604; http://dx.doi.org/10.4049/jimmunol.1100524.
10. Chen CJ, Kono H, Golenbock D, Reed G, Akira S, Rock KL. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. Nat Med 2007; 13:851-6; PMID:17572686; http://dx.doi.org/10.1038/nm1603.
11. Kono H, Rock KL. How dying cells alert the immune system to danger. Nat Rev Immunol 2008; 8:279-89; PMID:18340345; http://dx.doi.org/10.1038/nri2215.
12. Cavassani KA, Ishii M, Wen H, Schaller MA, Lincoln PM, Lukacs NW, et al. TLR3 is an endogenous sensor of tissue necrosis during acute inflammatory events. J Exp Med 2008; 205:2609-21; PMID:18838547; http://dx.doi.org/10.1084/jem.20081370.
13. He S, Liang Y, Shao F, Wang X. Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. Proc Natl Acad Sci U S A 2011; 108:20054-9; PMID:22123964; http://dx.doi.org/10.1073/pnas.1116302108.
14. Karikó K, Ni H, Capodici J, Lamphier M, Weissman D. mRNA is an endogenous ligand for Toll-like receptor 3. J Biol Chem 2004; 279:12542-50; PMID:14729660; http://dx.doi.org/10.1074/jbc.M310175200.
15. Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, et al. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 2009; 325:332-6; PMID:19498109; http://dx.doi.org/10.1126/science.1172308.
16. Vandenabeele P, Declercq W, Van Herreweghe F, Vanden Berghe T. The role of the kinases RIP1 and RIP3 in TNF-induced necrosis. Sci Signal 2010; 3:re4; PMID:20354226; http://dx.doi.org/10.1126/scisignal.3115re4.
17. He S, Wang L, Miao L, Wang T, Du F, Zhao L, et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 2009; 137:1100-11; PMID:19524512; http://dx.doi.org/10.1016/j.cell.2009.05.021.
18. Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 2009; 137:1112-23; PMID:19524513; http://dx.doi.org/10.1016/j.cell.2009.05.037.
19. Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, et al. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 2011; 471:368-72; PMID:21368762; http://dx.doi.org/10.1038/nature09857.
20. Galluzzi L, Brenner C, Morselli E, Touat Z, Kroemer G. Viral control of mitochondrial apoptosis. PLoS Pathog 2008; 4:e1000018; PMID:18516228; http://dx.doi.org/10.1371/journal.ppat.1000018.
21. Upton JW, Kaiser WJ, Mocarski ES. DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. Cell Host Microbe 2012; 11:290-7; PMID:22423968; http://dx.doi.org/10.1016/j.chom.2012.01.016.
22. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, Ban T, et al. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature 2007; 448:501-5; PMID:17618271; http://dx.doi.org/10.1038/nature06013.
23. Shisler JL, Moss B. Immunology 102 at poxvirus U: avoiding apoptosis. Semin Immunol 2001; 13:67-72; PMID:11289801; http://dx.doi.org/10.1006/smim.2000.0297.
24. Saeed M, Shiina M, Date T, Akazawa D, Watanabe N, Murayama A, et al. In vivo adaptation of hepatitis C virus in chimpanzees for efficient virus production and evasion of apoptosis. Hepatology 2011; 54:425-33; PMID:21538444; http://dx.doi.org/10.1002/hep.24399.
25. Salaun B, Romero P, Lebecque S. Toll-like receptors' two-edged sword: when immunity meets apoptosis. Eur J Immunol 2007; 37:3311-8; PMID:18034428; http://dx.doi.org/10.1002/eji.200737744.
26. Piccinini AM, Midwood KS. DAMPening inflammation by modulating TLR signalling. Mediators Inflamm. 2010; pii: 672395.