Vaccine 30 (2012) 3885–3890

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Alum-adjuvanted H5N1 whole virion inactivated vaccine (WIV) enhanced inflammatory cytokine productions

Tetsuo Nakayama[a,*], Yasuyo Kashiwagi[b], Hisashi Kawashima[b], Takuji Kumagai[c], Ken J. Ishii[d,e], Toshiaki Ihara[f]

[a] Kitasato Institute for Life Sciences, Laboratory of Viral Infection, Tokyo, Japan
[b] Tokyo Medical University, Department of Pediatrics, Tokyo, Japan
[c] Kumagai Pediatric Clinic, Sapporo, Japan
[d] National Institute of Biomedical Innovation, Laboratory of Adjuvant Innovation, Osaka, Japan
[e] Osaka University, Immunology Frontier Research Center, Laboratory of Vaccine Science, Osaka, Japan
[f] National Mie Hospital, Department of Pediatrics, Tsu, Mie Prefecture, Japan

ARTICLE INFO

Article history:
Received 11 October 2011
Received in revised form 29 March 2012
Accepted 1 April 2012
Available online 13 April 2012

Keywords:
H5N1
Whole virion inactivated influenza vaccine
Aluminum adjuvant
IFN-α
IL-1β
IL-6
Inflammasome

ABSTRACT

Alum-adjuvanted H5 whole virion inactivated vaccine (WIV) was licensed for adults in Japan but induced marked febrile reactions with significantly stronger antibody responses in children. In this study, the mechanisms behind the different responses were investigated. Lymphocytes were obtained from 25 healthy subjects who were not immunized with H5 vaccine, to examine the innate immune impact of the various vaccine formulations, analyzing the cytokine production profile stimulated with alum adjuvant alone, alum-adjuvanted H5 WIIV, plain H5 WIV, and H5 split vaccine. Alum adjuvant did not induce cytokine production, but H5 split induced IFN-γ and TNF-α. H5 WIV induced IL-6, IL-17, TNF-α, MCP-1, IFN-γ, and IFN-α. An extremely low level of IL-1β was produced in response to H5 WIV, and alum-adjuvanted H5 WIV enhanced IL-1β production, with similar levels of other cytokines stimulated with H5 WIV. Enhanced production of cytokines induced by alum-adjuvanted H5 WIV may be related to the higher incidence of febrile reactions with stronger immune responses in children but it should be further investigated why efficient immune responses with febrile illness were observed only in young children.

1. Introduction

In 2009, swine H1N1 influenza virus caused rapid global human-to-human transmission and was initially suspected as a new pandemic strain [1]. However, it actually emerged from swine influenza virus, which was first isolated in North America, genetically combined with human, swine, and avian genome compartments [2,3]. In this sense, pandemic A/H1N1 2009 was not a new pandemic strain [4,5]. Pre-existing antibody levels were reportedly low in young generations and most patients were young adults and children, not elderly [6]. A 2009 pandemic H1N1 vaccine seed was obtained after adaptation to egg, but the virus yield was poor in comparison with seasonal seeds. In Japan, egg-derived pandemic split vaccine was produced and introduced just after the peak of the outbreak. This pandemic raised several pressing issues: vaccine development, prompt supply and distribution, antigen saving, and vaccine efficacy to prepare for the unknown forthcoming pandemic.

In the 20th century, three pandemics of influenza occurred. The most devastating pandemic dated back to 1918, known as Spanish flu, caused by a highly pathogenic H1N1 influenza virus transmitted through some animals from avian pathogenic virus, estimated to have killed 40–50 million people [7]. In 1957, Asian influenza A/H2N2 caused the second pandemic, and Hong Kong influenza A/H3N2 appeared as the third pandemic in 1968. Seasonal influenza outbreaks or epidemics are caused by an antigenic drift of A/H1N1 or A/H3N2, whereas the pandemics appeared as antigenic shift, leading to new strains which are thought to be recombination with non-preexisting features of hemagglutinin (HA) and neuraminidase (NA) in human influenza viruses. After the 1968 pandemic of A/H3N2, several cases and small local outbreaks were reported, caused by new strains, H5, H7, or H9, and they were considered to be from poultry, and H5 is very close to human as a target for vaccine development [8–13]. There was a regional outbreak of H5 in Hong Kong in 1997, and six of 18 patients died, causing an H5 pandemic threat [9]. Sporadic H5 transmission on

* Corresponding author at: Kitasato Institute for Life Sciences, Laboratory of Viral Infection, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan.
Tel.: +81 3 5791 6269; fax: +81 3 5791 6130.
E-mail address: tetsuo-n@lisci.kitasato-u.ac.jp (T. Nakayama).

0264-410X/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2012.04.004

poultry farms and in migratory birds has spread across Asia to the EU and Africa, and approximately 550 cases of human H5 infection have been reported since 2004, showing a high mortality rate of approximately 60%. Most cases have involved close and direct contact with poultry, with no definite case of human-to-human transmission [14]. There are several barriers to human-to-human transmission: receptor usage of HA protein, cleavage efficiency by cellular protease, and host factors. Now, H5 is very close to the human, and the primary strategy to prevent and control influenza pandemics is the development of an effective and safe vaccine to mitigate the uneasiness, uncertainty, and pandemic threat.

Split vaccine has been used for more than 40 years and H5 is known to be poorly immunogenic. A two-dose schedule of 90 μg split vaccine of H5/Vietnam/1203/2004 induced 57% seropositivity of HI ≥1:40, and 53% seropositivity of NT ≥1:40 without adjuvant [15]. The addition of alum adjuvant improved the immunogenicity and could reduce the antigen usage to 30 μg with a similar immunogenicity to plain split, 90 μg [16].

In Japan, alum-adjuvanted H5N1 whole inactivated virion (WIV) (alum concentration: 300 μg/ml) was developed using a genetically engineered reassortant, the NIBRG-14 strain, originated from H5/A/Vietnam/1194/2004. In a clinical phase II trial in healthy adults, alum-adjuvanted 15 μg HA protein of WIV led to favorable immunogenicity (>70% sero-conversion rate in NT test) without demonstrating any serious systemic illnesses [17]. Whereas, when it was administered to young infants and children with a reduction in antigen doses, 7.5 or 3 μg, a high fever ≥37.5 °C was observed in over 60% of the recipients at less than six years of age, but, unexpectedly, NT antibody titers were higher than those observed in a clinical trial in adults. Recent detailed insights into the mechanisms of adjuvant effect on innate immunity and inflammasome have led to the better understanding of immunogenicity and immunotoxicity [18–20]. In this study, cytokine and chemokine responses were investigated to analyze the reason why a high incidence of febrile reactions was observed after the administration of alum-adjuvanted whole inactivated H5 vaccine to children.

2. Materials and methods

2.1. Study design and subjects

Twenty-five healthy subjects were enrolled in this study, aged 3 months to 59 years, who were not immunized with H5 vaccine. Among them, 20 subjects were under 20 years of age. The study design and protocol were discussed and approved by the ethical committee of Tokyo Medical University. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation through Ficoll-Paque™ Plus (GE Healthcare Bio-science, Uppssala, Sweden). They were adjusted to 1×10^6 cells in a 24-well plate in 1 ml of RPMI 1640 medium supplemented with 4% FBS and adequate antibiotics. They were stimulated with 100 μl of vaccine preparations or alum adjuvant alone.

2.2. Vaccine antigens

The NIBRG-14 strain, a genetically reassortant vaccine seed strain, originated from H5/A/Vietnam/1194/2004 and PR-8, was grown in MDCK and purified through zonal ultracentrifugation. Purified virus particles were inactivated by formalin treatment and used as whole inactivated vaccine (WIV). Alum-adjuvanted WIV was produced by adding alum adjuvant (1:1 mixture of Al phosphate and hydroxide) at a final alum concentration of 300 μg/ml. Purified virus particles were split by treatment with ether and Tween 80 and inactivated with formalin, and used as split vaccine material. Other strains were employed to compare the immunological responses: seasonal A/Brisbane/H1N1 and 2009 pandemic A/California/07/2009, produced by Kitasato Institute for Biologicals, Saitama. All vaccine materials were adjusted to 30 μg/ml HA protein concentration.

H5 WIV pandemic vaccine for clinical trial was produced from egg-derived WIV materials by Kitasato Institute for Biologicals, Saitama and Biken Intitutes, Kannonji.

2.3. Cytokine assay

Culture supernatants were harvested at 24 hr after stimulation with influenza vaccine materials and subjected to Bio-Plex Pro™ Human Cytokine Assay 17-plex, using Bio-Plex 200 (Bio-Rad, USA). The concentration of IFN-α was measured using an EIA kit (Verikine™ Human IFN-Alpha Serum Sample ELISA kit, pbl interferon, USA) and IL-1β and IL-6 were also measured using Quantikine Human IL-1β and Quantikine IL-6, respectively (R&D Systems, USA), following the instruction manual.

3. Results

3.1. Summary of alum-adjuvanted vaccine trial in children

An alum-adjuvanted H5N1 WIV clinical study was conducting involving 337 subjects aged 20–59 years. Two doses were given at 21–28 day intervals, and HI and NT antibodies were examined before immunization, just before the second dose, and one month after the second dose. NT antibodies became sero-converted in 260/337 (77%) in the15 μg group. No serious systemic adverse reaction was observed: febrile reaction ≥37.5 °C was reported in 3%. Alum-adjuvanted H5N1 WIV was licensed for stockpiling to prepare for a pandemic.

Using the same vaccine, a clinical trial was performed involving 374 subjects aged 6 months to 19 years. 0.1 ml was given to those less than one year, 0.25 ml for those 1–6 years, and 0.5 ml for those over six years of age. Febrile illness ≥37.5 °C was observed in 203/374 (54%) after the first dose, but decreased to 33/367 (9.0%) after the second dose. Unexpectedly, a high incidence of febrile reaction ≥38.0 °C was demonstrated in recipients aged less than 6 years and the incidence of febrile reaction (≥38 °C) after vaccination reduced by age: 5/5 (100%) in those less than one year, 52/92 (57%) in those 1–3 years, 48/90 (53%) in those 4–6 years, 39/134 (29%) in those 7–12 years, and 3/53 (6%) in those 13–19 years (Table 1).

NT titers after two-dose vaccination were compared in subjects who had a febrile reaction and those without febrile illness. The mean NT titer was $10 \times 2^{3.56 \pm 1.30}$ in those with febrile illness, being significantly higher than those without febrile illness, $10 \times 2^{2.76 \pm 1.26}$ ($p < 0.01$). Higher NT antibody titers seemed to be induced in those with a higher body temperature after vaccination (Table 2).

3.2. Cytokine induction by alum adjuvant

Alum adjuvant was prepared at the same concentration of 300 μg/ml. PBMCs were stimulated with 3 μg or 30 μg of

Table 1
Incidence of febrile reactions in different age groups.

	n	Fever+	≥38.0 °C
<1 year	5	5 (100%)	5 (100%)
1–3 years	92	68 (74%)	52 (57%)
4–6 years	90	57 (63%)	48 (53%)
7–12 years	134	63 (47%)	39 (29%)
≥13 years	53	10 (19%)	3 (6%)
Total	374	203 (54%)	147 (39%)

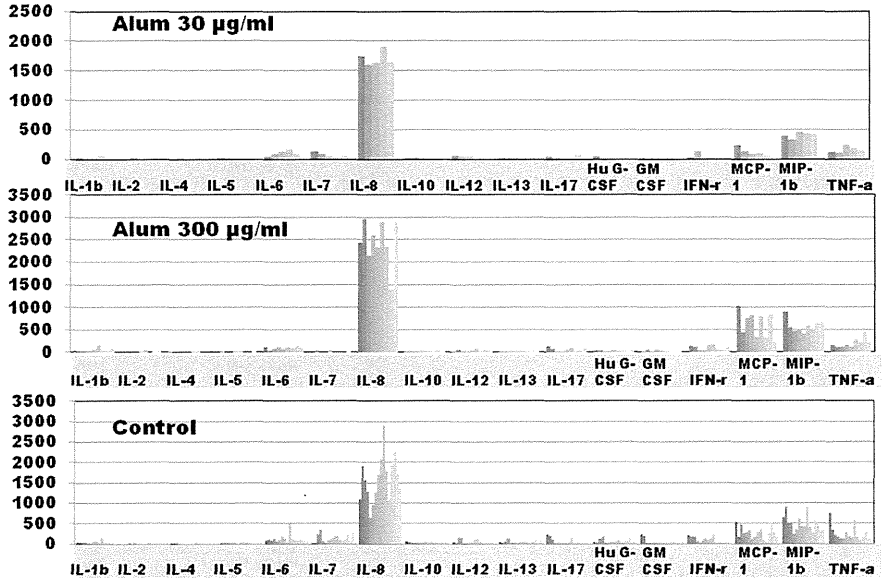


Fig. 1. Cytokine profile in PBMC cultures stimulated with aluminum solution. PBMC were stimulated with 0.1 ml of Alum adjuvants of 300 μg/ml (similar concentration as alum-adjuvanted H5 vaccine) and 30 μg/ml (1:10 dilution).

aluminum, and the results of cytokine profiles are shown in Fig. 1. Culture fluids were assayed using human 17plex. In control cultures of 25 subjects, IL-6, IL-7, IL-8, IFN-γ, MCP-1, MIP-1β, and TNF-α were produced at the baseline without any stimuli, and no additionally enhanced cytokine production was noted when stimulated with 30 μg alum adjuvant.

3.3. Cytokine production in response to different formulations of H5 influenza vaccines

H5 split materials were prepared and cytokine production profile was compared to those in response to the seasonal A/H1N1/Brisbane and A/H1N1/California/04/2009. IFN-γ was produced when stimulated with each split antigen, showing different levels of IFN-γ (Fig. 2). There was no significant difference in the other cytokine profiles among three split materials.

Alum-adjuvanted WIV, plain WIV, and the split formulation of the H5 vaccine antigen were adjusted to 30 μg/ml HA protein concentration. PBMC were stimulated with 3 μg of HA antigen. Through the analysis of 17 cytokines and chemokines, the productions of IL-1β, IL-6, IL-17, IFN-γ, TNF-α, and MCP-1 showed different profiles from control culture or when stimulated with aluminum alone. Results of cytokine profiles are shown in Table 3. IFN-γ and TNF-α were produced when stimulated with H5 split material. H5 WIV induced the higher production of IL-6, IL-17, TNF-α, and MCP-1 than control culture or those stimulated with Alum or H5 split materials. There was no increase in IL-1β production when stimulated with aluminium alone and H5 split antigen, but slightly higher levels of IL-1β production were observed in response to plain WIV. When stimulated with alum-adjuvanted WIV, the enhanced production of IL-1β was demonstrated and the other cytokines were produced similar to the stimulation with H5 WIV.

The 17-plex human cytokine assay demonstrates the cytokine profile and does not reflect the actual concentrations of the cytokines. As shown in Table 3, enhanced production of IL-1β was noted but IFN-α is not assayed in 17-plex kits. IL-1β, IL-6, and IFN-α were evaluated using EIA, and the results are shown in Fig. 3. IFN-α was produced when stimulated with WIV, and higher levels of IFN-α were demonstrated in subject numbers 21–25. In younger subjects less than one year of age (subject numbers 1–5), the enhanced production of IFN-α was shown in response to alum-adjuvanted WIV. A very low level of IL-1β was produced in response to WIV, and IL-1β production was enhanced when stimulated with alum-adjuvanted WIV. IL-6 was also produced in response to both WIV and alum-adjuvanted WIV, and alum-adjuvanted WIV enhanced the production of IFN-α, IL-1β, and IL-6. The production pattern of IFN-α in different age groups was similar to that of IL-6. IL-1β production profile was different from the others. Production of these cytokines seemed to be prominent in young infants at less than one year of age (subject Numbers 1–5) and adults (subject Numbers 21–25). Cytokine productions seemed to be different in each individual.

Table 2
Relationship between acute febrile reactions and antibody response.

	N	Mean ± SD[d]	95% C.I.	
Fever−	170	2.76 ± 1.26	2.58–2.95	
Fever+	200	3.56 ± 1.30	3.38–3.74	[a]
37.5–<38.0 °C	56	3.11 ± 1.27	2.77–3.45	
38.0–<39.0 °C	79	3.53 ± 1.32	3.24–3.82	[b]
≥39.0 °C	65	3.98 ± 1.17	3.70–4.27	[c]

[a] Mean NT titers were significantly different between subjects with febrile reactions after immunization and those without febrile reactions ($p < 0.01$).
[b] Significant difference was noted between NT titers in subjects with high body temperature ≥37.5–38.0 °C and in those with 38.0–39.0 °C ($p < 0.05$).
[c] Significant difference was noted between NT titers in subjects ≥37.5–38.0 °C and in those with ≥39 °C ($p < 0.01$).
[d] Mean titer of NT antibody expressed as 10×2^n.

4. Discussion

High-level immunogenicity is primarily required for a highly pathogenic pandemic, such as H5N1. Current split H5 was poor immunogenic and the WIV vaccine formulation has been reconsidered to have renewed merits concerning immunogenicity and cross-reaction [21–25]. Besides alum adjuvant, squalene oil emulsion adjuvants (MF59 and AS03) were used in H5 pandemic investigational split vaccines and induced

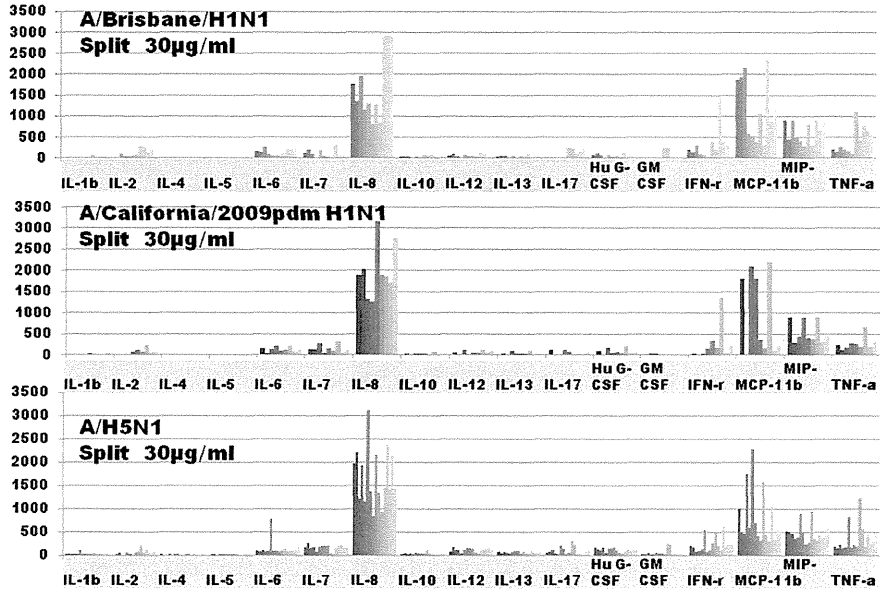


Fig. 2. Cytokine profile of PBMC cultures stimulated with split influenza vaccines. Split vaccine materials were used: H5N1 pandemic NIBRG-14 strain, originated from H5/A/Vietnam/1194/2004, A/H1N1/Brisbane/2007, and 2009 pandemic A/California/07/2009. Each antigen was prepared at the concentration of 30 μg/ml of HA antigen, and PBMC were stimulated with 0.1 ml (3 μg/test).

high-level immunogenicity with allowing for antigen saving, along with cross protective broad antibody responses [26,27]. This type of adjuvant was also applied for the 2009 pandemic vaccines, and resulted in efficient immunogenicity [23,24,28].

WIV was originally considered to induce high-level reactogenicity, and it was replaced by a split formulation in the 1960s [29–31]. H5 split vaccine was poorly immunogenic, and most European companies used oil emulsion adjuvants such as MF59 or AS03. Waddington et al. [25] reported the immunogenicity and reactogenicity of H1N1 pandemic vaccine comprising different formulations of AS03 oil-in-water emulsion adjuvanted and WIV in children at 6 months to 12 years of age. Seroconversion rates were nearly 98–99% in the AS03-adjuvanted vaccine group, but 80.6% at <5 years, and 95.9% at 5–12 years after immunization with WIV. An important finding was that WIV showed a strong age-dependent response in terms of immunogenicity, probably influenced by a past history of influenza infection. As for systemic adverse illness, febrile reaction was observed in approximately 10% of recipients aged <5 years, and in 3% of those aged 5–12 years after the administration of WIV. Wu et al. [21] reported that 5–15 μg of alum-adjuvanted H5 split vaccines were tolerated by children aged 3–11 years and 5–30 μg alum-adjuvanted split and 5 μg WIV vaccines were also tolerated by those aged 12–17 years. 10–15 μg of alum-adjuvanted split vaccine induced a 55% seroconversion and seroprotection rate in those aged 3–11 years, and 5 μg of alum-adjuvanted WIV induced a higher immunogenicity than 10 μg of adjuvanted split vaccine. When alum-adjuvanted WIV was used in young infants, a high incidence of febrile reactions (50–60%) was reported in a study in China although the number of recipients was very small [21].

In Japan, alum-adjuvanted WIV was licensed for adults but not for children. In a clinical trial of alum-adjuvanted WIV in a pediatric group, the incidence of febrile reactions (≥38 °C) after vaccination reduced by age: 100% in those less than one year, 50–60% in those 1–6 years, 29% in those 7–12 years, and 6% in those 13–19 years. The cytokine response was investigated in lymphocyte cultures stimulated with different H5 vaccine formulations to identify the reason for the immunogenicity and immunotoxicity of alum-adjuvanted H5 WIV. Cytokine production by PBMC was higher in young infants, but some teenagers and adults demonstrated a high-level cytokine response.

Many kinds of adjuvant have been developed, and they cause adverse reactions at the inoculation site or systemic reactions. Alum-based adjuvant was first approved for human use and continues to be widely used in many vaccines as an immuno-potentiator [29–31]. Two potential mechanisms are basically considered: (a) the formation of a depot from which the antigen is gradually released; (b) soluble antigen is converted to a particle form easily phagocytosed by antigen presenting cells (APC) such as dendritic cells or macrophages [31].

Recently, the stimulation on the innate immunity has been found to modulate the development of an acquired immune response through the production of cytokines [19,20]. The innate immune system consists of Toll-like receptors (TLRs), retinoic

Table 3
Production of IL-1β, IL-6, IL-17, IFN-γ, TNF-α, and MCP-1 when stimulated with Alum, H5 split, H5WIV and Alum adjuvanted H5 WIV.

	IL-1β	IL-6	IL-17	IFN-Y	TNF-α	MCP-1
Control	26.8 (13.3–40.3)	86.9 (46.4–127.3)	26.4 (13.3–39.5)	73.5 (45.7–101.3)	224.1 (148.4–299.9)	194.1 (120.8–267.4)
Alum	36.3 (21.6–51.0)	71.8 (50.7–92.9)	40.3 (26.1–54.5)	75.1 (56.6–93.7)	151.4 (114.4–188.4)	294.8 (154.5–435.0)
H5 split	21.6 (12.3–30.8)	145.4 (88.3–202.5)	69.3 (38.0–100.6)	182.3 (118.8–245.7)	328.5 (226.9–430.2)	544.3 (299.9–788.6)
H5WIV	50.1 (38.1–62.2)	503.6 (370.8–636.3)	180.0 (154.8–215.3)	354.4 (226.2–482.5)	843.4 (681.4–1005.4)	1452.5 (927.2–1977.8)
H5WIV + Alum	142.7 (63.0–22.4)	467.6 (306.3–628.8)	159.2 (133.5–185.0)	274.8 (169.0–380.5)	624.0 (424.3–823.7)	1023.2 (576.5–1469.9)

Lymphocytes were obtained from 25 healthy individuals who were not immunized with H5 vaccine. Mean values (pg/ml) are shown and ranges of 95% CI are in the parenthesis.

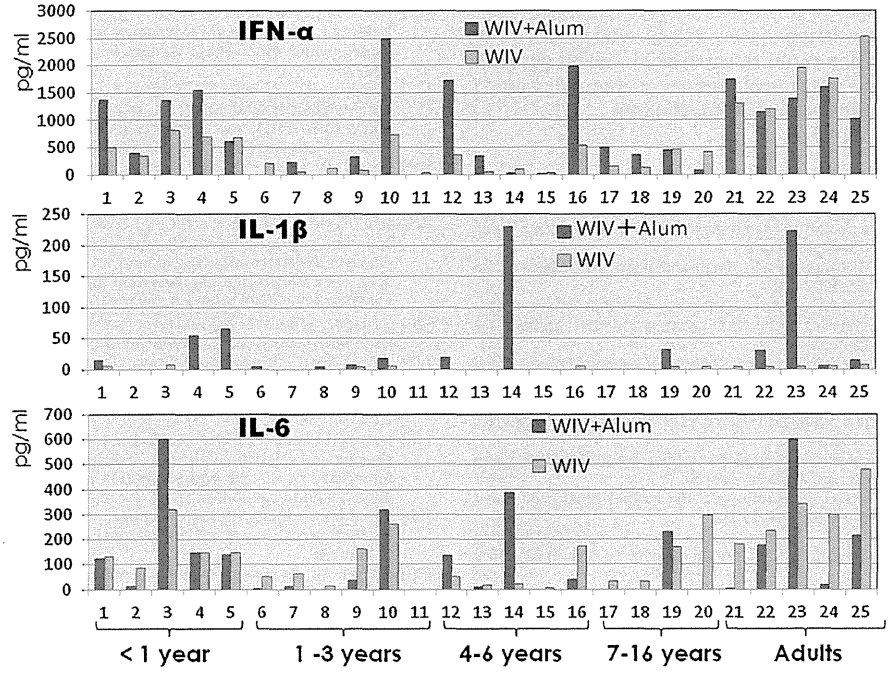


Fig. 3. IFN-α, IL-1β, and IL-6 production. IFN-α, IL-1β, and IL-6 were measured by EIA in PBMC cultures. PBMC were stimulated with H5 WIV and alum-adjuvanted WIV vaccine materials. Samples 1–5 were obtained from healthy individuals less than one year, those 6–11 from 1 to 3 years of age, those 12–16 from 4 to 6 years, those 17–20 from 7 to 16 years, and those 21–25 from adults. Black columns are cytokine productions stimulated with adjuvanted H5WI, and grey columns show those stimulated with H5 WIV.

acid inducible gene-based (RIG)-like receptors, and nucleotide oligomerization domain (NOD)-like receptors (NLRs), known as inflammasome [20,32–34]. Inflammasome consists of NLRP3, apoptosis-associated speck-like protein (ASC), which is thought to be an adaptor molecule of NLRP-3, resulting in the recruitment of caspase. It stimulates the production of inflammatory cytokines, IL-1β, IL-6, and IL-18 from proinflammatory molecules through the enzymatic activity of caspase [34]. Alum adjuvant induced cellular lysosomal damage or tissue damage and stimulated NLRP3 inflammasome through increased levels of uric acid caused by tissue damage [35,36]. The mechanisms of immunogenicity induced by Alum adjuvant have remained poorly understood regarding whether the stimulation of NLRP3 inflammasome is dispensable or not [37–39].

The activation of innate immunity increased antigen-specific adaptive immunity through TLRs induced by influenza vaccine without influencing NLRP3 inflammasome [40]. WIV influenza virus induced antigen-specific antibodies through the production of type I IFN involving the activation of TLR7 in mice [32,41]. Kuroda et al. [42] reported that alum induced LPS-primed macrophages to produce prostaglandin E2 (PGE2) and IL-1β. PGE_2 production was independent of NLRP3, ASC, and the caspase-1 inflammasome complex, and PGE_2 expression depended on cyclooxygenese (COX) and PGE synthase, regulated by spleen tryrosin kinase (Syk) and p38 MAP kinase in macrophages. PGE_2 was found to suppress Th1 responses with a reduced production of IL-2 and IFN-γ, but facilitated the differentiation of Th1 cells in the presence of IL-12 and, thus, cytokine species and their balance regulated PGE2 function on antibody production [18,42,43]. WIV and alum-adjuvanted WIV induced the production of the endogenous cytokines IL-1β, IFN-α, IL-6, and TNF-α, and they induced PGE2 in circumventricular organs through capillary fenestration, which is a well-known pyrogen [20,44].

WIV has genomic RNA that is recognized by TLR-7, inducing IFN-α [40]. In the clinical trial of alum-adjuvanted WIV, the incidence of febrile reactions (>38 °C) after vaccination reduced by age: 100% at less than one year, 50–60% at 1–6 years, 29% at 7–12 years, and 6% at 13–19 years. However, there was no comparative control group who received non-adjuvanted H5 plain WIV to discuss the incidence of febrile reactions. Cytokine production by PBMC was higher in young infants, some teenagers and adults in response to WIV. Enhanced productions of IFN-α, IL-1β, and IL-6 were demonstrated in very young subjects, and were suggested to be associated with a higher incidence of febrile reactions (immunotoxicity) and high immunogenicity (adjuvantogenicity). Cytokine profiles should be checked in serum from those who had high fever after immunization with alum-adjuvanted H5 WIV to observe the direct relationship between the enhanced cytokine level and febrile illness. Lymphocytes from adults also produced high levels of cytokines in response to alum-adjuvanted H5 WIV. Even though, sufficient immune responses were not observed in adults with lower incidence of febrile illness. It should be further investigated to clarify the different responsiveness to cytokines by aging.

Acknowledgements

This study was supported by Research on Regulatory Science of Pharmaceuticals and Medical Devices Grants, and funding for Research on the Accumulation of Evidence for Effective Vaccine Use and Vaccine Policy, from the Ministry of Health, Labour, and Welfare.

The clinical trial of H5N1 pandemic influenza vaccine for children was performed under the chief investigator, Dr. Hitoshi Kamiya, and was supported by a clinical trial promotion project of Japan Medical Association, which was funded by a Health Labour Sciences Research Grant from the Ministry of Health, Labour, and Welfare.

We dedicate this paper to Dr. Hitoshi Kamiya, Emeritus President of the Japanese Association of Vaccinology, who passed away in 2011, for his organization of this research team, and his desire to know the reasons for the unfavorable results of alum-adjuvanted H5N1 whole virion inactivated vaccine for children.

References

[1] Centers for Disease Control and Prevention. Swine influenza A (H1N1) infection in two children-Southern California, March–April 2009. MMWR Morbidity and Mortality Weekly Report 2009;58:400–2.
[2] Poland GA, Jacobson RM, Targonski PV. Avian and pandemic influenza: an overview. Vaccine 2007;25:3057–61.
[3] Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 2009;459:1122–5.
[4] Novel swine-origin influenza A (H1N1) virus investigation team. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. New England Journal of Medicine 2009;360:2605–15.
[5] Cohen J. Swine flu pandemic, What's old is new: 1918 virus matches 2009 H1N1 strain. Science 2010;327:1563–4.
[6] WHO. Seroepidemiological studies of pandemic influenza A (H1N1) 2009 virus. Weekly Epidemiological Record 2010;24:229–36.
[7] Taubenberger JK, Morens DM. 1918 influenza: the mother of all pandemics. Emerging Infectious Diseases 2006;12:15–22.
[8] Leroux-Roels I, Leroux-Roels G. Current status and progress of prepandemic and pandemic influenza vaccine development. Expert Review of Vaccines 2009;8:401–23.
[9] Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, et al. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 1998;351:472–7.
[10] Tran TH, Nguyen TL, Nguyen TD, Luong TS, Pham PM, Nguyen VC, et al. Avian influenza A (H5N1) in 10 patients in Vietnam. New England Journal of Medicine 2004;350:1179–88.
[11] Tiensin T, Chaitaweesub P, Songserm T, Chaisingh A, Hoonsuwan W, Buranathai C, et al. Highly pathogenic avian influenza H5N1, Thailand, 2004. Emerging Infectious Diseases 2005;11:1664–72.
[12] Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, et al. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proceedings of the National Academy of Sciences of the United States of America 2004;101:1356–61.
[13] Moon HJ, Song MS, Cruz DJ, Park KJ, Pascua PN, Lee JH, et al. Active reassortment of H9 influenza viruses between wild birds and live-poultry markets in Korea. Archives of Virology 2010;155:229–41.
[14] Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, et al. Probable person-to-person transmission of avian influenza A (H5N1). New England Journal of Medicine 2005;352:333–40.
[15] Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine. New England Journal of Medicine 2006;354:1343–51.
[16] Bresson JL, Perronne C, Launay O, Gerdil C, Saville M, Wood J, et al. Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomized trial. Lancet 2006;367:1657–64.
[17] Conference report: report of the 6th meeting on the evaluation of pandemic influenza vaccines in clinical trials. Vaccine 2010;28:6811–20.
[18] Philbin VJ, Levy O. Developmental biology of the innate immune response: implications for neonatal and infant vaccine development. Pediatric Research 2009;65:98–105.
[19] Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. Immunity 2010;33:492–503.
[20] Batista-Duharte A, Lindblad EB, Oviedo-Orta E. Progress in understanding adjuvant immunotoxicity mechanisms. Toxicology Letters 2010.
[21] Wu J, Fang HH, Chen JT, Zhou JC, Feng ZJ, Li CG, et al. Immunogenicity, safety, and cross-reactivity of an inactivated, adjuvanted, prototype pandemic influenza (H5N1) vaccine: a phase II, double-blind, randomized trial. Clinical Infectious Diseases 2009;48:1087–95.
[22] Lin JT, Li CG, Wang X, Su N, Liu Y, Qiu YZ, et al. Antibody persistence after 2-dose priming and booster response to a third dose of an inactivated, adjuvanted, whole-virion H5N1 vaccine. Journal of Infectious Diseases 2009;199:184–7.
[23] Nicholson KG, Abrams KR, Batham S, Clark TW, Hoschler K, Lim WS, et al. Immunogenicity and safety of whole-virion and $AS03_A$-adjuvanted 2009 influenza A (H1N1) vaccines: a randomized, multicentre, age-stratified, head to head trial. Lancet Infectious Diseases 2011;11:91–101.
[24] Wu J, Li W, Wang HQ, Chen JT, Lv M, Zhou JC, et al. A rapid immune response to 2009 influenza A(H1N1) vaccines in adults: a randomized, double-blind, controlled trial. Journal of Infectious Diseases 2010;202:675–80.
[25] Waddington CS, Walker WT, Oeser C, Reiner A, John T, Wilkins S, et al. Safety and immunogenicity of AS03B adjuvanted split virion versus non-adjuvanted whole virion H1N1 influenza vaccine in UK children aged 6 months–12 years: open label, randomised, parallel group, multicentre study. British Medical Journal 2010;340:c2649, http://dx.doi.org/10.1136/bmj.c2649.
[26] Atmar RL, Keitel WA. Adjuvants for pandemic influenza vaccines. Current Topics in Microbiology and Immunology 2009;333:323–44.
[27] Leroux-Roels I, Borkowski A, Vanwolleghem T, Drame M, Clement F, Hons E, et al. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. Lancet 2007;370:580–9.
[28] Roman F, Vaman T, Gerlach B, Markendorf A, Gillard P, Devaster JM. Immunogenicity and safety in adults of one dose of influenza A H1N1v 2009 vaccine formulated with and without AS03A-adjuvant: preliminary report of an observer-blind, randomised trial. Vaccine 2010;28:1740–5.
[29] Wood JM, Williams MS. History of inactivated influenza vaccine. In: Nicholson KG, Webster RG, Hay AJ, editors. Textbook of Influenza. Blackwell Science; 1998. p. 317–23.
[30] Hunter RL. Overview of vaccine adjuvants: present and future. Vaccine 2002;20:S7–12.
[31] HogenEsch H. Mechanism of stimulation of the immune response by aluminum adjuvants. Vaccine 2002;20:S34–9.
[32] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783–801.
[33] Ichinohe T. Perspective roles of TLR, RIG-1 and NLRP3 in influenza virus infection and immunity: impact on vaccine design. Expert Review of Vaccines 2010;9:1315–24.
[34] Schroder K, Tschopp J. The inflammasomes. Cell 2010;140:821–32.
[35] Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature 2008;453:1122–6.
[36] Kool M, Soullié T, Nimwegen MV, Willart MAM, Muskens F, Jung S, et al. Alum ajuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. Journal of Experimental Medicine 2008;205:869–82.
[37] Kool M, Pétrilli V, De Smedt T, Rolaz A, Hammad H, van Nimwegen M, et al. Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. Journal of Immunology 2008;181:3755–9.
[38] Franchi L, Núñez G. The NLRP3 inflammasome is critical for alum-mediated IL-1β secretion but dispensable for adjuvant activity. European Journal of Immunology 2008;38:2085–9.
[39] Spreafico R, Ricciardi-Castagnoli P, Mortellaro A. The controversial relationship between NLRP3, alum, danger signals and the next-generation adjuvants. European Journal of Immunology 2010;40:595–653.
[40] Ishii KJ, Akira S. Toll or toll-free adjuvant path toward the optimal vaccine development. Journal of Clinical Immunology 2007;27:363–71.
[41] Uematsu S, Akira S. Toll-like receptors and type I interferons. Journal of Biological Chemistry 2007;282:15319–23.
[42] Kuroda E, Ishii KJ, Uematsu S, Ohata K, Coban C, Akira S, et al. Silica crystals and aluminium salts regulate the production of prostagrandin in macrophages via NALP3 inflammasome-independent mechanisms. Immunity 2011;34:514–26.
[43] Yao C, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K, et al. Prostagradin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. Nature Medicine 2009;15:636–40.
[44] Conti B, Tabarean L, Andrei C, Bartfai T. Cytokines and fever. Frontiers in Bioscience 2004;9:1433–49.

Available online at www.sciencedirect.com
ScienceDirect
Current Opinion in Virology

Innate and adaptive immune responses to viral infection and vaccination

Taiki Aoshi[1,2], Shohei Koyama[3], Kouji Kobiyama[1,2], Shizuo Akira[4] and Ken J Ishii[1,2]

Recent accumulating evidence suggests that the human immune system possesses a variety of innate receptors that recognize, distinguish, and respond to viral infections and to vaccination. These include Toll-like receptors, C-type lectin receptors, RIG-I-like receptors, Nod-like receptors and possibly AIM2-like receptors. However, the precise mechanisms by which these receptors exert their critical roles in the induction of virus-specific adaptive immune responses have not been fully elucidated. In this review, we discuss recent advances in our understanding of the innate immune recognition of viruses and the differential connection to the adaptive immune responses induced by infection or vaccination, with a particular focus on the influenza virus.

Addresses
[1] Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation, Osaka, Japan
[2] Laboratory of Vaccine Science, WPI Immunology Frontier Research Center, Osaka University, Osaka, Japan
[3] Department of Respiratory Medicine, Tohoku University Graduate School of Medicine, Miyagi, Japan
[4] Laboratory of Host Defense, WPI Immunology Frontier Research Center, Osaka University, Osaka, Japan

Corresponding author: Ishii, Ken J (kenishii@biken.osaka-u.ac.jp)

Current Opinion in Virology 2011, 1:226–232

This review comes from a themed issue on Vaccines
Edited by Hildegund Ertl and Bali Pulendran

Available online 30th July 2011

1879-6257/$ – see front matter

DOI 10.1016/j.coviro.2011.07.002

Introduction

Several families of innate immune receptors, including Toll-like receptors (TLRs) [1], C-type lectin receptors [2], RIG-I-like receptors (RLRs) [3], Nod-like receptors (NLRs) [4], and AIM2-like receptors (ALRs) [5] have been identified over the last decade. Generally, these germ-line-encoded receptors recognize 'non-self' molecules derived from a variety of microbes. Some of these receptors also recognize danger signals sent out by damaged cells/tissues [6]. These innate immune receptors are critical for the initiation and regulation of host immune responses against infection and autoimmunity [7]. Furthermore, it is evident that innate immune responses are extremely important for establishing effective adaptive immune responses to infection and vaccination [8•,9,10]; although it is still not clear whether all innate responses contribute equally to the induction of adaptive responses [8•,11•,12•]. In the following sections, we briefly review the current knowledge about virus recognition by innate immune receptors, and discuss the connections between the innate and adaptive immune responses, using influenza virus as an example.

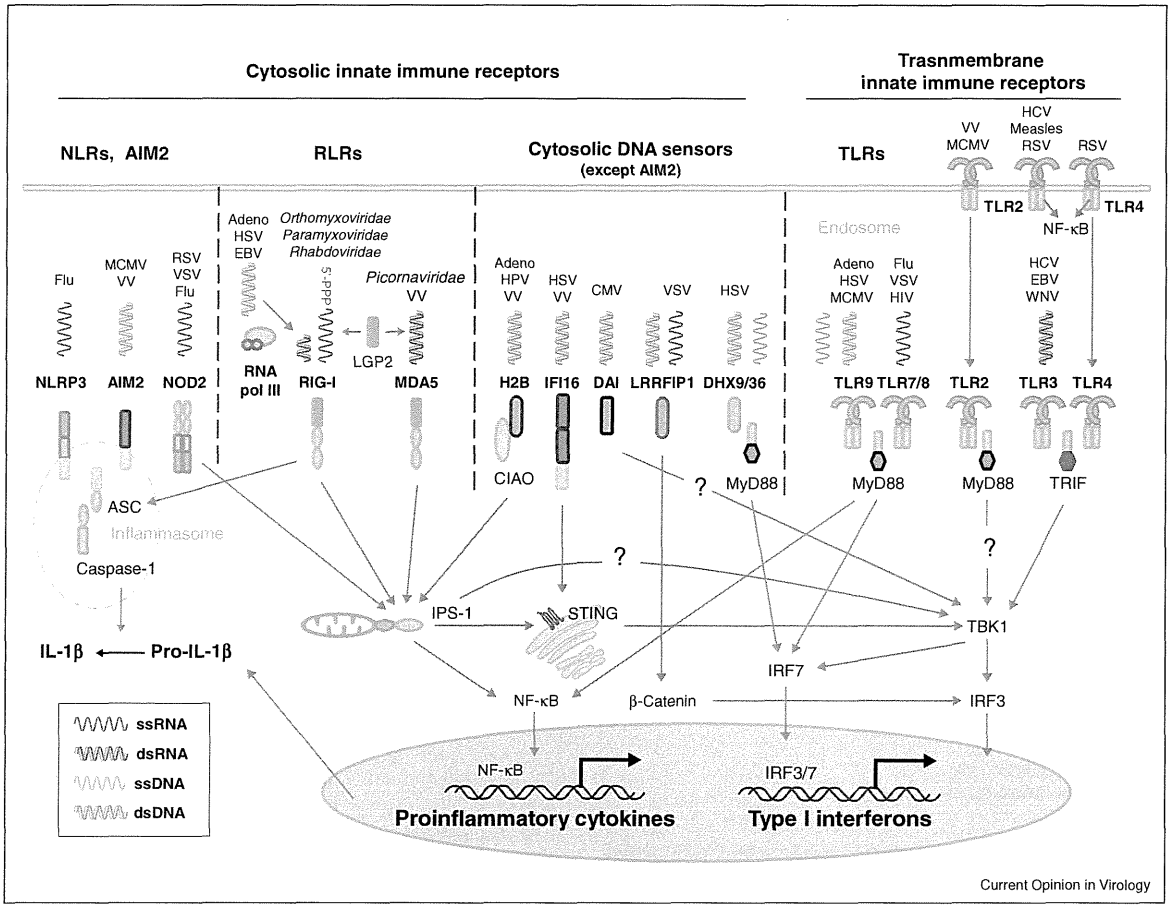
The innate immune system may distinguish between the presence of a virus and viral infection

In addition to bacteria and parasites, viruses are a major cause of infectious diseases. Because of their diverse organ/tissue tropisms, genomic structure (positive or negative stranded, single or double stranded, RNA or DNA) and pathogenic lifecycles, host cells can recognize viruses through a variety of innate immune receptors. Extracellular viruses are detected by transmembrane receptors such as TLRs, and cytosolic viral infections are detected by cytosolic receptors such as RLRs, NLRs, and ALRs (Figure 1). This diverse set of innate receptors may also allow the host immune system to determine viral status — live or dead, replicating or not replicating, pathogenic or non-pathogenic — in a manner similar to that recently proposed for bacterial infection [13]. These innate immune receptors trigger signaling cascades that are generally integrated with innate responses, such as nuclear factor kappa B (NF-κB)-dependent cytokine responses, interferon regulatory factor (IRF)-dependent IFN-α/β responses, and inflammasome/caspase-1-dependent IL-1β responses. IFN-α/β are the major cytokines that limit viral replication, while other cytokines, including IL-6, TNF-α and IL-1β, recruit immune cells to the site of infection and elicit inflammation. NF-κB-dependent and IRF-dependent cytokines are transcriptionally regulated, whereas inflammasome-dependent IL-1β secretion is regulated both transcriptionally and post-transcriptionally (Figure 1). Importantly, many viruses can suppress these innate responses at the 'sensing' and/or transcriptional level upon replication within infected cells [14].

Immune recognition of viruses by transmembrane innate receptors

Transmembrane innate receptors, such as TLRs, recognize extracellular viruses, and their activation does not

Figure 1



Innate immune receptors involved in virus recognition. **NLR and AIM2 pathways**: NLRP3 is activated by a wide variety of stimuli, including RNA viruses. Foreign cytoplasmic dsDNA is also detected by AIM2 via the HIN200 domain. Their activation induces the recruitment of the adaptor protein, ASC, via the pyrin domain. Procaspase-1 is also recruited to ASC via the CARD domain (inflammasome formation). This interaction leads to the auto-cleavage of caspase-1 and results in the activation of caspase-1, which cleaves pro-IL-1β. NOD2 is involved in the recognition of ssRNA viruses. NOD2 activates IPS-1, a mitochondrial membrane-anchored protein, through the NBD and LRR domains, which leads to IRF3 activation. **RLR pathway**: RIG-I is essential for IFN responses to several ssRNA viruses such as *Orthomyxoviridae* and *Paramyxoviridae*. However, MDA5 is necessary for responses to a different set of viruses, such as *Picornaviridae*. LGP2 can act as a positive regulator, making viral RNP complexes more accessible to RIG-I and MDA5. Some viral DNAs are transcribed into 5′ tri-phosphate RNA (the RIG-I ligand) by cytosolic RNA polymerase III (pol III). RIG-I and MDA5 signal via the adaptor protein, IPS-1, which leads to type I IFN production through the TBK1-IRF3-dependent pathway, and proinflammatory cytokine production through NF-κB translocation. RIG-I can activate the inflammasome by interacting with the CARD domains of RIG-I and ASC, and produce IL-1β. **Cytosolic DNA sensor pathways**: extra-chromosomal histone H2B binds DNA virus-like HPV through its α-helical region and interacts with IPS-1 via association with the adaptor protein CIAO. IFI16 binds DNA viruses via the HIN200 domains. They then activate the STING-TBK1-IRF3-dependent signaling pathway, resulting in the production of type I IFN. DAI detects DNA viruses and induces TBK1-IRF3-dependent type I IFN production. LRRFIP1 detects both bacterial DNA and viral RNA from VSV and induces type I IFN production via the β-catenin-IRF3 transactivator pathway. The DExD/H box helicase, DHX9/36, detects CpG-ODNs and DNA viruses such as HSV, leading to MyD88-IRF7-dependent type I IFN production. **TLR pathway**: some RNA viruses are detected by cell surface TLR2 and TLR4, which induce MyD88-dependent NF-κB activation. TLR4 is also recruited to the endosome, leading to TRIF-dependent type I IFN production. TLR3 and TLR7/8 recognize dsRNA and ssRNA, respectively, from RNA viruses. TLR3 induces TRIF-TBK1-dependent type I IFN production, whereas TLR7/8 induces NF-κB and IRF7 activation via MyD88. TLR9 detects CpG-ODNs and DNAs derived from DNA viruses, leading to NF-κB and IRF7 activation via MyD88. Some DNA viruses are also recognized by TLR2 in the endosome, which then induces IRF3/7-dependent type I IFN production.

necessarily require infection of the receptor-expressing cells. Based on cellular localization, TLRs can be grouped in two types: cell surface TLRs (TLR1,2,4,5,6) and endosomal TLRs (TLR3,7,8,9) [1]. Cell surface TLRs recognize bacterial/fungal cell wall components. However, many reports show that some viral proteins are also recognized by cell surface TLR2 and TLR4 [15,16]. A recent report by Barbalat *et al.* identified another interesting example of viral recognition by cell surface TLRs. Mouse cytomegalovirus and vaccinia virus (both dsDNA viruses) were recognized via TLR2. This led to the production of IFN-β, which was not observed upon stimulation with Pam3SK4 (a well-known bacterial TLR2 agonist) [17••]. Interestingly, this TLR2-mediated IFN-β production was restricted in Ly6C(hi) inflammatory monocytes, and was dependent on TLR2 recruitment from the cell surface to the endosome [17••]. However, the exact molecular mechanism(s) underlying virus recognition by cell surface TLRs is the subject of future research. The endosomal TLRs, TLR3, TLR7/8, and TLR9 recognize virus-derived dsRNA, ssRNA, and DNA, respectively [18]. Many viruses are recognized by these endosomal TLRs (Figure 1). TLR3 signaling is mediated by the adaptor molecule TRIF, which induces IRF3 phosphorylation leading to IFN-β production. TLR7/8/9 signaling is mediated by another adaptor molecule, MyD88 (an adaptor commonly used by other TLRs, except TLR3) leading to IRF7-mediated IFN-α production. Importantly, expression of these endosomal TLRs is restricted to certain types of dendritic cells (DCs). TLR3 is preferentially expressed by CD8α(+)DCs, and TLR7/9 is preferentially expressed by plasmacytoid DCs (pDCs). Overall, the recognition of the presence of viruses seems to be mediated by limited types of host cells that express these transmembrane innate immune receptors.

Immune recognition of viruses by cytosolic innate receptors

In contrast to transmembrane receptors, cytosolic innate receptors are expressed by all host cells. RLRs and NLRs mainly recognize viral RNAs, and the recently identified ALRs (and other cytosolic DNA sensors) detect viral DNA in the cytosol of infected cells. This cytosolic receptor-mediated virus recognition is critically important for the host innate immune responses to contain viral replication within the infected cells before the adaptive immune responses are fully developed. In contrast, the contribution of this form of cytosolic virus recognition to adaptive responses is varied and more controversial, as discussed later in this review.

RLRs comprise retinoic acid inducible gene-I (RIG-I), melanoma differentiation associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). Both RIG-I and MDA5 recognize viral RNAs within the cytoplasm of infected cells. However, the exact molecular signatures of the RIG-I and MDA5 ligands are still not fully understood [19–22]. Owing to the lack of a caspase recruitment domain (CARD), which is important for interactions with IPS-1, LGP2 was assumed to function as a negative regulator of RIG-I and MDA5. However, a recent study suggests that LGP2 positively regulates RIG-I and MDA5 signaling, possibly by modifying the viral RNA structure before detection by these two receptors [23]. Virus recognition by RIG-I and MDA5 is mediated by a single adaptor molecule, IPS-1 (or MAVS), and leads to NF-κB and IRF3/IRF7 activation. Interestingly, a recent report demonstrated that RIG-I can directly activate ASC in an NLRP3-independent manner, leading to caspase-1-dependent IL-1β production during VSV (ssRNA virus) infection [24••].

NLRs comprise a large number of family member proteins that contain a conserved NOD motif [25], and can be classified into two groups. Activation of Nod1 and Nod2 leads to the activation of NF-κB and IRF. Although Nod2 was initially characterized as a cytosolic sensor for the bacterial cell wall component, muramyl dipeptide, which induces NF-κB activation, a recent report suggests that Nod2 also functions as a virus sensor [26••] and activates a non-classical NLR signaling pathway [27]. Sabbah *et al.* showed that Nod2 can directly sense cytosolic ssRNA from RSV and influenza virus, leading to MAVS(IPS-1)-IRF3-mediated IFN-β responses [26••]. Activation of NLRs, such as NLRP1, NLRP3, and NLRC4, leads to inflammasome formation, which results in caspase-1-mediated IL-1β and IL-18 secretion (Figure 1). The NLRP3 inflammasome is one of the best characterized inflammasomes, and is activated by bacterial toxins, LPS, and viral RNAs, as well as uric acid and alum [11•,28]. Interestingly, it appears that many RLRs and NLRs sense virus infections by detecting viral genomic, or replication-intermediate, RNA. This might indicate that the presence of viral nucleic acids provides the stronger proof of active viral infection, rather than general danger signals.

Cytosolic DNA sensors

AIM2 and IFI16 are both recently identified cytosolic DNA sensors and are involved in DNA-dependent inflammasome activation and IFN-β production, respectively [5,29–34]. Because both proteins contain a PYHIN domain [35–37], it has been proposed that they be referred to as ALRs [5]. However, several other molecules are also known to be involved in DNA sensing within the cytosol. DAI (ZBP-1) is the first reported DNA sensor molecule that triggers TBK1-IRF3-dependent IFN-β induction *in vitro* [38]; however, gene knockout mice do not show the same phenotype, suggesting the presence of redundant DNA sensor mechanisms [39]. Lrrfip1 recognizes cytosolic dsDNA (and dsRNA), subsequently interacting with β-catenin and enhancing IRF3-mediated IFN-β expression [40]. DHX36 and DHX9, present in human pDC, are cytosolic CpG-A and CpG-B binding

proteins, respectively. These proteins mediate the MyD88/IRF7-dependent production of IFN-α [41]. AT-rich DNA is also recognized indirectly by RNA polymerase III. AT-rich DNA is transcribed into 5-triphosphate dsRNA, which is then recognized by the RIG-I pathway [42,43]. The cytosolic histone, H2B, is also involved in DNA sensing. The dsDNA/H2B complex activates IPS-1 via CIAO (an adaptor molecule that links histone H2B and IPS-1) in human cell lines leading to IFN-β expression. However, this H2B-mediated dsDNA-dependent IFN-β production is not observed in mice, most likely because of the lack of the interaction between mouse CIAO and mouse IPS-1 [44].

Signaling via which innate immune receptors leads to adaptive immune responses: TLRs, RLRs, NLRs, or others?

Activation of the innate immune system is critical for establishing adaptive immune responses. This is simply demonstrated by the fact that immunization with a highly purified recombinant protein is usually unsuccessful owing to the lack of innate responses [45]. On the other hand, viral infections are usually sensed by multiple innate receptors. The live attenuated yellow fever vaccine 17D has been shown to activate multiple TLRs, resulted in CD8T and a mixed Th1/Th2 immune responses [46,47]. In the case of influenza virus, infection can be detected by three different receptors: TLR7, RIG-I, and NLRP3 [48•,49]. However, conflicting results have been reported, particularly in terms of the adaptive immune responses examined in these receptors/adaptors deficient mice (Table 1).

The involvement of TLR7/MyD88 has been examined by four independent studies [50–53]. It is very difficult to generalize the results, which range from almost nothing to identifying a prominent phenotype, except that all of the studies consistently agreed that CD8T responses were not affected by the absence of the TLR7/MyD88 pathway. However, two independent studies consistently demonstrated that, in contrast to live virus, the immunogenicity of a chemically killed (inactivated) whole virus was completely dependent on TLR7/MyD88 signaling [48•,54•].

The RIG-I/IPS-1 pathway was also examined in two independent studies [52,53]. They concluded that although RIG-I/IPS-1 signaling induces almost overlapping cytokine responses to those induced by TLR7/MyD88 (Figure 1), IPS-1-deficiency had no substantial effect upon adaptive responses to influenza virus infection [52,53]. This may reflect differential cellular expression of these receptors. RIG-I is ubiquitously expressed by most cells, whereas TLR7 is preferentially expressed by pDCs. It may also reflect the fact that RIG-I sensing requires viral replication within the cell, whereas TLR7 recognizes viruses in the endosome, which is not dependent upon virus infection (Figure 1). Differential regulation of adaptive immune responses by TLRs and RLRs has also been reported in another virus infection system. Jung *et al.* demonstrated that during LCMV infection, CD8T responses in MyD88-deficient mice were significantly reduced, whereas IPS-1-deficient mice showed comparable CD8T responses to those of wild-type mice [55].

NLRP3 can be triggered by viral RNA [56] and/or ionic perturbation caused by the influenza M2 protein [57]. NLRP3 triggers ASC-mediated NLRP3 inflammasome formation, leading to caspase-1-dependent IL-1β and IL-18 secretion. Inflammasome involvement in influenza virus infection has been studied by four independent

Table 1

Adaptive immune responses in mice deficient in innate immune receptors/adaptors against influenza virus infection and vaccination.

	Virus	TLRs(Myd88) deficiency	RLRs(IPS-1) deficiency	NLRs(NLRP3, ASC, caspase-1) deficiency
		IFN-I, TNFα/IL-6		Inflammasome (IL-1/IL-18)
Lopez *et al.* [50]	A/PR8	CD4(IFNγ)→, CD4(IL-4)T, CD8→, Ab→	Not examined	Not examined
Heer *et al.* [51]	A/PR8	CD4→, CD8→, Ab(IgG2a)↓	Not examined	Not examined
Koyama *et al.* [52]	A/PR8, A/NC	CD4(IFNγ)↓, CD8→, Ab(IgG2a)↓	CD4(IFNγ)→, CD8→, Ab→	Not examined
Seo *et al.* [53]	A/PR8	CD4(Th1)↓, CD4(Th2)↑, CD8→, Ab→	CD4(Th1)→, CD8→, Ab→	Not examined
Ichinohe *et al.* [58]	A/PR8	Not examined	Not examined	CD4(IFNγ)↓ CD8↓, Ab(IgG, IgA)↓
Allen *et al.* [59]	A/PR8	Not examined	Not examined	Intact adaptive responses (CD8→, Ab→)
Thomas *et al.* [60]	A/PR8	Not examined	Not examined	CD8→, Ab→
Koyama *et al.* [48•]	A/NC	Not examined	Not examined	CD4(IFNγ)→, CD8→, Ab(IgG1)↓
	Inactivated WV(A/NC)	CD4→, Ab↓	CD4→, Ab→	CD4→, Ab→
Geeraedts *et al.* [54•]	Inactivated WV(H5N1)	CD4↓, Ab↓	Not examined	Not examined

groups. Ichinohe *et al.* demonstrated that NLRP3-independent, but ASC-dependent, inflammasome responses were important for both CD4T and CD8T responses, as well as IgA and IgG responses [58] (it is noteworthy that Poeck *et al.* also reported NLRP3-independent, but RIG-I and ASC-dependent, inflammasome activation by VSV [24••]). In contrast, Allen *et al.* and Thomas *et al.* showed that NLRP3 inflammasome responses were not involved in adaptive responses, but play a more important role in the innate phase of host defense and in tissue healing [59,60]. We also examined ASC-deficient mice and found that inflammasome activation had almost no impact on the adaptive response to live influenza virus infection [48•]. At present, the reason for these contradictory results is not clear [11•,61].

Viral subversion of innate immune responses may affect adaptive immune responses

These controversies may be explained by differences in the types of virus used; especially the different subversion mechanisms used by the viruses. Influenza virus (and other viruses) possesses an immune evasion protein that modulates the innate immune signaling cascades of the host [14]. Even though most studies used a mouse-adapted PR8 virus, Heynisch *et al.* reported that two variants of A/PuertoRico/8/34 show very different activation patterns for cellular signaling molecules in MDCK cells [62]. This most likely reflects the fact that these variant viruses modulate cytosolic signaling systems in different ways. Influenza NS-1 is the most well-characterized of the proteins that subvert RIG-I mediated IFN-α/β responses at multiple steps [63]. A recent report suggests that the inflammasome is also an evasion target of a herpes virus [64]. Intriguingly, no direct viral mechanism that antagonizes TLR signaling has been described for influenza A virus [63]. Taken together, these data suggest that the same PR8 virus may induce very different host immune responses. Furthermore, they may also suggest that subverting the infection-dependent cytosolic innate system may be easier than subverting the infection-independent TLR system. In line with this hypothesis, once the virus is fixed with formalin (and killed), the host immune response is consistently TLR7/MyD88-dependent [48•,54•].

Conclusions

The existence of diverse innate immune receptors may reflect a redundancy that ensures sensitive detection of viruses in a variety of tissue and cell types, and the subsequent induction of host defense mechanisms. TLRs can detect extracellular viruses (either live or dead), and do not require viral infection of receptor-expressing cells. By contrast, detection by cytosolic receptors requires viral infection and replication, which can be easier evasion targets for many viruses. The innate immune response plays two roles in host defense: (1) it limits (or at least controls) viral replication during initial infection; and (2) it induces adaptive immune responses responsible for viral clearance and maintenance (memory). However, it is still not clear to what extent each innate immune receptor contributes to the adaptive immune responses. Owing to sophisticated immune evasion mechanisms, infection by live viruses may not provide a clear answer. However, immunization with an inactivated whole virion influenza vaccine clearly demonstrates that TLR-mediated innate signaling alone is sufficient to induce adaptive immune responses. Currently, it is difficult to examine the individual contribution of each RLR and NLR to the adaptive immune response because of the lack of selective activators. Recently, Kasturi *et al.* demonstrated that synthetic nanoparticle based vaccines composed of multiple TLR ligands induced persistent antibody and CD8T responses than single TLR activating vaccine [65]. It suggests that activations of multiple innate immune receptors may be required for long lasting memory responses but not necessarily required for mounting temporal effector responses. Further studies will clarify the more detailed coordination between innate and adaptive immune responses, and provide a more rational way of vaccine design.

Conflicts of interest statement

The authors have no conflicts of interest to declare.

Acknowledgements

The authors thank Drs. Cevayir Coban and Fumihiko Takeshita for helpful discussion, and all members of Ishii's and Coban's laboratory. This work was supported by Ministry of Health, Labour and Welfare (MHLW) (KJI), the Knowledge Cluster Initiative (KJI), a Grant-in-Aid for Scientific Research (KAKENHI) (TA, KK and KJI) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and by CREST, JST (KJI).

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Kawai T, Akira S: **The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.** *Nat Immunol* 2010, **11**:373-384.

2. Geijtenbeek TBH, Gringhuis SI: **Signalling through C-type lectin receptors: shaping immune responses.** *Nat Rev Immunol* 2009, **9**:465-479.

3. Yoneyama M, Fujita T: **RNA recognition and signal transduction by RIG-I-like receptors.** *Immunol Rev* 2009, **227**:54-65.

4. Franchi L, Wamer N, Viani K, Nunez G: **Function of Nod-like receptors in microbial recognition and host defense.** *Immunol Rev* 2009, **227**:106-128.

5. Unterholzner L, Keating SE, Baran M, Horan KA, Jensen SB, Sharma S, Sirois CM, Jin TC, Latz E, Xiao TS *et al.*: **IFI16 is an innate immune sensor for intracellular DNA.** *Nat Immunol* 2010, **11**:997-1004.

6. Chen GY, Nunez G: **Sterile inflammation: sensing and reacting to damage.** *Nat Rev Immunol* 2010, **10**:826-837.

7. Takeuchi O, Akira S: **Pattern recognition receptors and inflammation.** *Cell* 2010, **140**:805-820.

8. • Palm NW, Medzhitov R: **Pattern recognition receptors and control of adaptive immunity**. *Immunol Rev* 2009, **227**:221-233.
This (also Ref [11•,12•]) is an insightful review that discusses the connection between innate and adaptive immune responses.

9. Manicassamy S, Pulendran B: **Modulation of adaptive immunity with Toll-like receptors**. *Semin Immunol* 2009, **21**:185-193.

10. Koyama S, Coban C, Aoshi T, Horii T, Akira S, Ishii KJ: **Innate immune control of nucleic acid-based vaccine immunogenicity**. *Expert Rev Vaccines* 2009, **8**:1099-1107.

11. • Williams A, Flavell RA, Eisenbarth SC: **The role of NOD-like receptors in shaping adaptive immunity**. *Curr Opin Immunol* 2010, **22**:34-40.
See annotation to Ref [8•].

12. • Joffre O, Nolte MA, Sporri R, Sousa CRE: **Inflammatory signals in dendritic cell activation and the induction of adaptive immunity**. *Immunol Rev* 2009, **227**:234-247.
See annotation to Ref [8•].

13. Vance RE, Isberg RR, Portnoy DA: **Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system**. *Cell Host Microbe* 2009, **6**:10-21.

14. Versteeg GA, Garcia-Sastre A: **Viral tricks to grid-lock the type I interferon system**. *Curr Opin Microbiol* 2010, **13**:508-516.

15. Finberg RW, Wang JP, Kurt-Jones EA: **Toll like receptors and viruses**. *Rev Med Virol* 2007, **17**:35-43.

16. Carty M, Bowie AG: **Recent insights into the role of Toll-like receptors in viral infection**. *Clin Exp Immunol* 2010, **161**:397-406.

17. •• Barbalat R, Lau L, Locksley RM, Barton GM: **Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands**. *Nat Immunol* 2009, **10**:1200-1207.
This paper showed that mouse cytomegalovirus and vaccinia virus infection can induce MyD88-mediated IFN responses in Ly6C(hi)CD11b(+)CD11c(−)B220(−) inflammatory monocytes.

18. Blasius AL, Beutler B: **Intracellular Toll-like receptors**. *Immunity* 2010, **32**:305-315.

19. Schlee M, Roth A, Hornung V, Hagmann CA, Wimmenauer V, Barchet W, Coch C, Janke M, Mihailovic A, Wardle G *et al.*: **Recognition of 5′ triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus**. *Immunity* 2009, **31**:25-34.

20. Rehwinkel J, Tan CP, Goubau D, Schulz O, Pichlmair A, Bier K, Robb N, Vreede F, Barclay W, Fodor E *et al.*: **RIG-I detects viral genomic RNA during negative-strand RNA virus infection**. *Cell* 2010, **140**:397-408.

21. Pichlmair A, Schulz O, Tan CP, Rehwinkel J, Kato H, Takeuchi O, Akira S, Way M, Schiavo G, Sousa CRE: **Activation of MDA5 requires higher-order RNA structures generated during virus infection**. *J Virol* 2009, **83**:10761-10769.

22. Schmidt A, Endres S, Rothenfusser S: **Pattern recognition of viral nucleic acids by RIG-I-like helicases**. *J Mol Med* 2011, **89**:5-12.

23. Satoh T, Kato H, Kumagai Y, Yoneyama M, Sato S, Matsushita K, Tsujimura T, Fujita T, Akira S, Takeuchi O: **LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses**. *Proc Natl Acad Sci U S A* 2010, **107**:1512-1517.

24. •• Poeck H, Bscheider M, Gross O, Finger K, Roth S, Rebsamen M, Hannesschlager N, Schlee M, Rothenfusser S, Barchet W *et al.*: **Recognition of RNA virus by RIG-I results in activation of CARD9 and inflammasome signaling for interleukin 1 beta production**. *Nat Immunol* 2010, **11**:63-1824.
This paper showed that cytosolic RNA from VSV sensed by RIG-I can induce ASC/inflammasome activation in an NLRP3-independent manner.

25. Kanneganti TD: **Central roles of NLRs and inflammasomes in viral infection**. *Nat Rev Immunol* 2010, **10**:688-698.

26. •• Sabbah A, Chang TH, Harnack R, Frohlich V, Tominaga K, Dube PH, Xiang Y, Bose S: **Activation of innate immune antiviral responses by Nod2**. *Nat Immunol* 2009, **10**:1073-1080.
This paper showed that Nod2 directly recognizes ssRNA and activates the MAVS(IPS-1)/IRF3/IFN-β pathway before RIG-I mediated sensing.

27. Shaw MH, Kamada N, Warner N, Kim YG, Nunez G: **The ever-expanding function of NOD2: autophagy, viral recognition, and T cell activation**. *Trends Immunol* 2011, **32**:73-79.

28. Martinon F, Mayor A, Tschopp J: **The inflammasomes: guardians of the body**. *Ann Rev Immunol* 2009, **27**:229-265.

29. Burckstummer T, Baumann C, Bluml S, Dixit E, Durnberger G, Jahn H, Planyavsky M, Bilban M, Colinge J, Bennett KL *et al.*: **An orthogonal proteomic–genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome**. *Nat Immunol* 2009, **10**:266-272.

30. Fernandes-Alnemri T, Yu JW, Juliana C, Solorzano L, Kang S, Wu JH, Datta P, McCormick M, Huang L, McDermott E *et al.*: **The AIM2 inflammasome is critical for innate immunity to *Francisella tularensis***. *Nat Immunol* 2010, **11**:385-394.

31. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, Latz E, Fitzgerald KA: **AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC**. *Nature* 2009, **458**:514-518.

32. Roberts TL, Idris A, Dunn JA, Kelly GM, Burnton CM, Hodgson S, Hardy LL, Garceau V, Sweet MJ, Ross IL *et al.*: **HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA**. *Science* 2009, **323**:1057-1060.

33. Jones JW, Kayagaki N, Broz P, Henry T, Newton K, O'Rourke K, Chan S, Dong J, Qu Y, Roose-Girma M *et al.*: **Absent in melanoma 2 is required for innate immune recognition of *Francisella tularensis***. *Proc Natl Acad Sci U S A* 2010, **107**:9771-9776.

34. Rathinam VAK, Jiang ZZ, Waggoner SN, Sharma S, Cole LE, Waggoner L, Vanaja SK, Monks BG, Ganesan S, Latz E *et al.*: **The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses**. *Nat Immunol* 2010, **11**:395-403.

35. Goubau D, Rehwinkel J, Sousa CRE: **PYHIN proteins: center stage in DNA sensing**. *Nat Immunol* 2010, **11**:984-986.

36. Choubey D, Duan X, Dickerson E, Ponomareva L, Panchanathan R, Shen H, Srivastava R: **Interferon-inducible p200-family proteins as novel sensors of cytoplasmic DNA: role in inflammation and autoimmunity**. *J Interferon Cytokine Res* 2010, **30**:371-380.

37. Gariglio M, Mondini M, De Andrea M, Landolfo S: **The multifaceted interferon-inducible p200 family proteins: from cell biology to human pathology**. *J Interferon Cytokine Res* 2011, **31**:159-172.

38. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, Ban T, Lu Y, Miyagishi M, Kodama T, Honda K *et al.*: **DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response**. *Nature* 2007, **448**:501-505.

39. Ishii KJ, Kawagoe T, Koyama S, Matsui K, Kumar H, Kawai T, Uematsu S, Takeuchi O, Takeshita F, Coban C *et al.*: **TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines**. *Nature* 2008, **451**:725-729.

40. Yang PY, An HZ, Liu XG, Wen MY, Zheng YY, Rui YC, Cao XT: **The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type I interferon via a beta-catenin-dependent pathway**. *Nat Immunol* 2010, **11**:487-494.

41. Kim T, Pazhoor S, Bao MS, Zhang ZQ, Hanabuchi S, Facchinetti V, Bover L, Plumas J, Chaperot L, Qin J *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 U S A* 2010, **107**:15181-15186.

42. Ablasser A, Bauernfeind F, Hartmann G, Latz E, Fitzgerald KA, Hornung V: **RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate**. *Nat Immunol* 2009, **10**:1065-1072.

43. Chiu YH, MacMillan JB, Chen ZJJ: **RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway**. *Cell* 2009, **138**:576-591.

44. Kobiyama K, Takeshita F, Jounai N, Sakaue-Sawano A, Miyawaki A, Ishii KJ, Kawai T, Sasaki S, Hirano H, Ishii N *et al.*:

Extrachromosomal histone H2B mediates innate antiviral immune responses induced by intracellular double-stranded DNA. *J Virol* 2010, **84**:822-832.

45. Ishii KJ, Akira S: **Toll or toll-free adjuvant path toward the optimal vaccine development**. *J Clin Immunol* 2007, **27**:363-371.

46. Querec T, Bennouna S, Alkan SK, Laouar Y, Gorden K, Flavell R, Akira S, Ahmed R, Pulendran B: **Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity**. *J Exp Med* 2006, **203**:413-424.

47. Querec TD, Akondy RS, Lee EK, Cao WP, Nakaya HI, Teuwen D, Pirani A, Gernert K, Deng JS, Marzolf B *et al.*: **Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans**. *Nat Immunol* 2009, **10**:116-125.

48. • Koyama S, Aoshi T, Tanimoto T, Kumagai Y, Kobiyama K, Tougan T, Sakurai K, Coban C, Horii T, Akira S *et al.*: **Plasmacytoid dendritic cells delineate immunogenicity of influenza vaccine subtypes**. *Sci Transl Med* 2010, **2**:24-25.

This paper (also Ref [54•]) showed that adaptive immune responses induced by an inactivated whole influenza virus vaccine were dependent on TLR7 signaling.

49. Ichinohe T: **Respective roles of TLR, RIG-I and NLRP3 in influenza virus infection and immunity: impact on vaccine design**. *Expert Rev Vaccines* 2010, **9**:1315-1324.

50. Lopez CB, Moltedo B, Alexopoulou L, Bonifaz L, Flavell RA, Moran TM: **TLR-independent induction of dendritic cell maturation and adaptive immunity by negative-strand RNA viruses**. *J Immunol* 2004, **173**:6882-6889.

51. Heer AK, Shamshiev A, Donda A, Uematsu S, Akira S, Kopf M, Marsland BJ: **TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses**. *J Immunol* 2007, **178**:2182-2191.

52. Koyama S, Ishii KJ, Kumar H, Tanimoto T, Coban C, Uematsu S, Kawai T, Akira S: **Differential role of TLR- and RLR-signaling in the immune responses to influenza a virus infection and vaccination**. *J Immunol* 2007, **179**:4711-4720.

53. Seo SU, Kwon HJ, Song JH, Byun YH, Seong BL, Kawai T, Akira S, Kweon MN: **MyD88 Signaling Is Indispensable for Primary Influenza A Virus Infection but Dispensable for Secondary Infection**. *Journal of Virology* 2010, **84**:12713-12722.

54. • Geeraedts F, Goutagny N, Hornung V, Severa M, de Haan A, Pool J, Wilschut J, Fitzgerald KA, Huckriede A: **Superior immunogenicity of inactivated whole virus H5N1 influenza vaccine is primarily controlled by Toll-like receptor signalling**. *PLoS Pathogens* 2008, **4**:e1000138.

See annotation to Ref [48•].

55. Jung A, Kato H, Kumagai Y, Kumar H, Kawai T, Takeuchi O, Akira S: **Lymphocytoid choriomeningitis virus activates plasmacytoid dendritic cells and induces a cytotoxic T-cell response via MyD88**. *J Virol* 2008, **82**:196-206.

56. Kanneganti TD, Body-Malapel M, Amer A, Park JH, Whitfield J, Franchi L, Taraporewala ZF, Miller D, Patton JT, Inohara N *et al.*: **Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA**. *J Biol Chem* 2006, **281**:36560-36568.

57. Ichinohe T, Pang IK, Iwasaki A: **Influenza virus activates inflammasomes via its intracellular M2 ion channel**. *Nat Immunol* 2010, **11**:404-410.

58. Ichinohe T, Lee HK, Ogura Y, Flavell R, Iwasaki A: **Inflammasome recognition of influenza virus is essential for adaptive immune responses**. *J Exp Med* 2009, **206**:79-87.

59. Allen IC, Scull MA, Moore CB, Holl EK, McElvania-TeKippe E, Taxman DJ, Guthrie EH, Pickles RJ, Ting JPY: **The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA**. *Immunity* 2009, **30**:556-565.

60. Thomas PG, Dash P, Aldridge JR, Ellebedy AH, Reynolds C, Funk AJ, Martin WJ, Lamkanfi M, Webby RJ, Boyd KL *et al.*: **The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1**. *Immunity* 2009, **30**:566-575.

61. Pang IK, Iwasaki A: **Inflammasomes as mediators of immunity against influenza virus**. *Trends Immunol* 2011, **32**:34-41.

62. Heynisch B, Frensing T, Heinze K, Seitz C, Genzel Y, Reichl U: **Differential activation of host cell signalling pathways through infection with two variants of influenza A/Puerto Rico/8/34 (H1N1) in MDCK cells**. *Vaccine* 2010, **28**:8210-8218.

63. Schmolke M, Garcia-Sastre A: **Evasion of innate and adaptive immune responses by influenza A virus**. *Cell Microbiol* 2010, **12**:873-880.

64. Gregory SM, Davis BK, West JA, Taxman DJ, Matsuzawa S, Reed JC, Ting JPY, Damania B: **Discovery of a viral NLR homolog that inhibits the inflammasome**. *Science* 2011, **331**:330-334.

65. Kasturi SP, Skountzou I, Albrecht RA, Koutsonanos D, Hua T, Nakaya HI, Ravindran R, Stewart S, Alam M, Kwissa M *et al.*: **Programming the magnitude and persistence of antibody responses with innate immunity**. *Nature* 2011, **470**:543-547.

Novel Strategies to Improve DNA Vaccine Immunogenicity

Cevayir Coban[1], Kouji Kobiyama[2,3], Taiki Aoshi[2,3], Fumihiko Takeshita[2,3], Toshihiro Horii[4], Shizuo Akira[5] and Ken J. Ishii[2,3,*]

[1]*Laboratory of Malaria Immunology, Immunology Frontier Research Center, World Premier Institute for Immunology, Osaka University;* [2]*Laboratory of Vaccine Science, Immunology Frontier Research Center, World Premier Institute for Immunology, Osaka University;* [3]*Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation, Osaka;* [4]*Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University;* [5]*Laboratory of Host Defense, Immunology Frontier Research Center, World Premier Institute for Immunology, Osaka University*

Abstract: DNA vaccines can induce both humoral and cellular immune responses in animals. Some DNA vaccines are already licensed for infectious diseases such as West Nile virus encephalitis in horses. When used in humans, however, DNA vaccines suffer from lower immunogenicity profiles. Although the reasons for this are poorly understood, various hypotheses have been proposed. This review aims to provide better understanding of the molecular and immunological mechanisms by which DNA vaccines work and how such knowledge can be used to bring about improvements in their efficacy. Recent studies have provided evidence that the 'adjuvant effect' of plasmid DNA is mediated by its double-stranded structure. This structure activates stimulator of interferon genes/TANK-binding kinase 1 (STING/TBK1)-dependent innate immune signaling pathways in the absence of Toll-like receptors. Indeed, type-I interferons (IFNs), induced *in vivo* via the STING/TBK1 pathway, were found to be crucial for both direct- and indirect-antigen presentation via distinct cell types (i.e. dendritic cells (DC) and muscle cells, respectively). Importantly, incorporation of TBK1 into a DNA vaccine was found to enhance the antigen-specific humoral immune responses targeting the *Plasmodium falciparum* serine repeat antigen (SERA), a candidate vaccine antigen expressed in the blood-stages of human malaria parasites. Thus, the results of these studies may offer new ways to develop DNA vaccines, as well as delivering novel vaccine adjuvants against infectious diseases.

Keywords: Adjuvant, CpG motifs, DNA vaccine, innate immunity, STING, TBK1, TLR9, type I interferon.

INTRODUCTION

DNA vaccines represent a major advance in the fight against infectious diseases. Their mechanism of action enables the antigen of interest to be delivered to the host immune system in a manner that is similar to natural exposure to the pathogen. DNA vaccines comprise plasmid DNA encoding the antigen of interest whose expression is controlled by a mammalian promoter; following administration of the vaccine, the host immune system is exposed to the expressed antigen. Such *in vivo* introduction of genetic material usually elicits strong humoral and cellular (Th1 type CD4+ T cells and CD8+ cytotoxic T cells) responses, making DNA vaccines distinct from conventional protein or peptide vaccines. Indeed, DNA vaccines have shown remarkable success in most animal studies and clinical trials in humans [1-2]. For example, DNA vaccines for horses, salmonid fish and dogs have been licensed since 2005 against West Nile virus, Infectious hematopoietic necrosis virus and melanoma, respectively [3-5]. DNA vaccine clinical trials against HIV and malaria, at least showed good safety and tolerability profile in humans [6-7].

*Address correspondence to this author at the Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation, 7-6-8 Asagi, Saito, Ibaraki City, Osaka 567-0085, Japan; Tel: +81-72-641-8043; Fax: +81-72-641-8079; E-mail: kenishii@biken.osaka-u.ac.jp

However, low immunogenicity has proved a significant obstacle to efficacy for DNA vaccines, especially in higher primates and humans. To date, various approaches have been taken to improve the immunogenicity of such vaccines. For example, modifying the microenvironment of the vaccination site by co-administration of various genes (i.e. genes encoding the co-stimulatory molecules, cytokines and chemokines [8-9], and/or genes that induce apoptosis [10-12]), proteins or other immunologically active molecules is not unusual. Some other approaches that have been tried to improve DNA vaccine immunogenicity include: 'Prime-Boost' immunization with DNA followed by a viral vector encoding the antigen (or the protein antigen) [13]; various immunization techniques and DNA delivery systems such as electroporation [14-16]; and microparticles and tattoo-immunization [17-18]. However, we are a long way from a complete understanding of how DNA vaccines work.

Recent studies are starting to shed light on this subject, however. Crucial to this is the recent discovery of many innate immune system receptors such as Toll-like receptors (TLRs), Nucleotide binding oligomerization domain (NOD)-like receptors, and Retinoic acid inducible gene (RIG)-like receptors, which have revealed that the main components of successful vaccines (adjuvants) are the ligands for many innate immune system receptors. Use of such molecules has great potential to improve vaccine design and development

1 -5 3 /11 $58.00+.00

[19-21]. Indeed, many of the innate immune system receptor ligands are protein, nucleic acid, lipid, and carbohydrate in nature (i.e. lipid-A and poly-IC and CpG oligodeoxynucleotides (CpG ODNs) and have been used as adjuvants in vaccine trials for many years. Similarly, recent intensive research efforts searching for a cytosolic DNA sensor has improved our understanding [22-23]. Hence, this review will discuss some of the recent developments in this field and consider some of the strategies available for improving the immunogenicity of DNA vaccines. Attention will be directed towards the use of malarial antigens as anti-malaria vaccines.

1.1. Strategies to Improve DNA Vaccine Immunogenicity; Manipulation of Intrinsic Adjuvant Properties

It was shown that bacterial DNA contains immunostimulatory CpG motifs, which comprise an unmethylated C followed by G and specific flanking sequences that are recognized by TLR9 [24-26]. These findings suggest that DNA vaccine immunogenicity might be attributed to CpG motifs present in the plasmid backbone, which could act as an intrinsic "built in adjuvant" for DNA vaccines [27-28]. Therefore, incorporation of TLR9 and related TLR pathways has been a promising approach that could improve DNA vaccine immunogenicity.

Indeed, addition of several CpG motifs into a plasmid backbone has been shown to improve the immunogenicity of DNA vaccines [29-31]. In one study, DNA plasmid backbones were modified by the addition of two distinct types of human-specific CpG motifs (D and K types ODNs), and the effects of such modifications on various types of human immune cells (and in mice) were investigated for use in humans against malaria [31]. It was found that the modified plasmid DNA could induce maturation of human monocytes into DCs via activation of plasmocytoid DC (pDC); both modified plasmids (containing as few as three to five human CpG motifs) resulted in differential DC maturation in comparison with an unmodified plasmid. In addition, although expression of the encoded antigen (*P. falciparum* surface protein 25 (Pfs25), a *P. falciparum* transmission-blocking vaccine candidate) was not affected by introduction of additional immunostimulatory CpG motifs into the plasmid backbone, at least one of the plasmids (D type ODN incorporated Pfs25) induced higher levels of Pfs25-specific IgG with subtle differences in antibody isotypes. However, later studies found that TLR9-deficient mice had comparable levels of antigen-specific IgG, IgG1 and IgG2a antibody responses (including IFNγ and cytotoxic T lymphocyte (CTL) responses) as their wild-type counterparts [32-33], although others suggest that TLR9 signaling is critical for inducing CD8+ T cell responses after DNA vaccination at least after priming [34-35].

Other approaches to improve DNA vaccine immunogenicity include targeting TLR pathway signaling molecules, which can mimic a microbial infection in the absence of any microbial components. One such study investigated an overexpression strategy using adapter molecules such as the Myeloid Differentiation Primary Response Gene (MyD88) or Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing interferon-β (TRIF) and found that greater enhancement of humoral responses was achieved when the DNA vaccine incorporated the MyD88 genetic adjuvant [36]. In contrast, incorporation of the TRIF genetic adjuvant greatly enhanced cellular immune responses resulting in superior protection against influenza virus or tumor progression. Similarly, incorporation of the interferon regulatory factor (IRF) 1, 3 and 7 into DNA vaccines improved both humoral and cellular immune responses against viral infection [37-38].

Overall, these studies suggest that although the immunogenicity of DNA vaccines can be enhanced by additional CpG motifs, or over expression of TLR-mediated signaling molecules in the plasmid backbone, the 'basal' adjuvant effects of DNA vaccines are independent of TLR-mediated recognition. The results of another study using MyD88/TRIF-double deficient mice are consistent with this [39].

1.2. Double-Stranded Plasmid DNA is Recognized Via the DNA Recognition Machinery, STING and TBK1

If plasmid DNA is not recognized by TLRs (TLR9) and its adapter molecules, what is it recognized by? Recent work has shown that bacterial or synthetic CpG-DNAs are not the only molecules that can activate the innate immune system. In fact, double-stranded (ds) DNA derived from host cells in the B form (a right-handed helical structure) can also do this [40]. When transfected into the cytosol, dsDNA activated fibroblasts, macrophages and dendritic cells to produce robust amounts of type-I interferons (IFNs) independent of TLRs; but in this case, signaling is mediated through TANK-binding kinase-1 (TBK1) [40]. TBK1 is crucial for type-I IFN induction via TLR-dependent and TLR-independent pathways [21,41]. However, within the last few years, several studies have identified various cytosolic DNA-recognition molecules (recently reviewed by [23]). Briefly, STING (stimulator of interferon genes, alternatively designated MITA, MPYS and ERIS), which is localized in the endoplasmic reticulum (ER), was found to activate NF-kB and IRF3 to stimulate type I IFN production after intracellular dsDNA stimulation [42]. Upon dsDNA stimulation, STING relocalizes with TBK1 from the endoplasmic reticulum to perinuclear vesicles containing the subunit of the exocyst complex 5 (Sec5) (also called EXOC2) [43]. Quite recently, a new molecule designated the interferon-inducible tripartite-motif (TRIM) 56 was identified as an interferon-inducible E3 ubiquitin ligase that modulates STING dimerization upstream of TBK1 to confer double-stranded DNA-mediated innate immune responses [44]. At the final stage, TBK1 comprises inducible IκB kinase (IKK-i) and these two kinases directly phosphorylate interferon regulatory factor 3 (IRF3) and IRF7 [45-46], resulting the production of type I interferons (IFNs).

These components of the DNA recognition machinery (STING and TBK1) have collectively indicated that a double-stranded structure could be essential for DNA vaccine-induced immunogenicity. Accordingly, use of TBK1-deficient mice revealed that DNA vaccine immunogenicity was completely dependent on TBK1 [39]. Moreover, such immunogenicity occurs through the activation of type-I IFN-mediated innate immunity resulting in the adjuvant effect for the encoded antigen [39,41] Fig. (**1**). Furthermore, type I IFNs were found to be essential for optimal DNA vaccine immunogenicity (antigen-specific T and B cell induction) by

Table 1. Summary of DNA Vaccine Studies Using Mice Deficient for Innate Immune System Molecules

Knockout Mice	DNA Vaccine-Induced Antigen Specific-			Reference
	Ab Responses	CD4+ T Cells	CD8+ T Cells	
TLR9	→	→	→*	[33, 35]
MyD88/TRIF	→	→	→	[39]
IPS	→	→	→	[39]
IFNAR	↓	↓	↓	[39]
STING	↓	↓	↓	[47]
TBK1	↓	↓	↓	[39]
IRF3	→	↓	↓	[48]

*Some groups suggest that TLR9 signaling is critical for inducing CD8+ T cell responses after priming [30].

using interferon (alpha and beta) receptor 2 (IFNAR2)-deficient mice, but not attributable to TLR signaling (i.e. because MyD88/TRIF-double deficient mice normally respond to DNA vaccines [39]). Bone-marrow transfer experiments revealed that TBK1-mediated signaling in dendritic cells mainly involved the induction of both antigen-specific B cells and CD4+ T cells. Moreover, DNA-transfected non-immune cells, such as muscle cells, were found to cross-prime CD8+ T cells, which following DNA vaccination promoted adaptive immune responses Fig. (**1**).

Similar studies were performed with STING-deficient mice [47], which revealed the importance of STING for plasmid DNA immunogenicity Fig. (**1**). One possible drawback of this pathway is that RNA could be generated during DNA vaccination, which might act as an adjuvant by activating TBK1-dependent signaling [41]. Because ss- and dsRNA is recognized via TLR3/7/8, RIG-I and Melanoma differentiation-associated gene 5 (MDA5) and utilizes the adapter molecules TRIF, MyD88 and interferon-beta promoter stimulator 1 (IPS-1), respectively [21], this possibility was excluded using TRIF-, MyD88- and IPS-1-deficient mice; the results showed that DNA vaccine-induced immune responses against all of the adaptors were comparable with their WT counterparts [41].

These DNA vaccination studies in mice collectively revealed that DNA vaccination induces both antigen-specific B cells as well as CD4+ and CD8+ T cell responses entirely dependent on TBK1. However, interestingly, IRF3-deficient mice elicited strong antigen-specific humoral responses after DNA vaccinations, while CD4 and CD8 T cell responses (including the production of Th1, Th2 and Th17 cytokines) were severely impaired [48], suggesting other down-stream molecules of TBK1 leading production of antibodies, requires further investigation (see Table **1** for a summary of DNA vaccine studies as of current day by using mice deficient for immune system receptors).

1.3. Does the Inflammasome Play a Role in DNA Vaccine Immunogenicity?

In addition to TLR-independent and STING/TBK1-dependent cytosolic DNA recognition, another innate immune signaling mechanism has been suggested for cytosolic DNA recognition. That is, cytosolic DNA mediates the Apoptotic speck protein containing a caspase recruitment domain (ASC)/caspase-1-mediated secretion of IL-1β by AIM2 (absent in melanoma 2), a human IFN-inducible gene (HIN)-200 family member [49-52]. However, it should be noted that the AIM2 inflammasome is essential for caspase-1 activation, but not for type I IFN production in response to cytosolic dsDNA Fig. (**1**).

Although several studies have shown that DNA vaccine immunogenicity could be improved by incorporation of IL-1 and caspases [12,53], we do not know whether the inflammasome pathways, AIM2 and ASC, are required for DNA vaccine immunogenicity. However, recent evidence suggests that the AIM2/ASC inflammasome presumably has little impact on the immunogenicity/adjuvanticity of a DNA vaccine. We have recently shown that alum adjuvant (the only adjuvant licensed for human use) induces cell death and release of DNA; this in turn induces IL-1β release, which could be responsible for alum's adjuvanticity, independent of ASC or Caspase-1 ([1]Marichal T *et al,* in press). This hypothesis could be tested using AIM2 and/or ASC-deficient mice for DNA vaccination studies.

1.4. Can we Improve DNA Vaccine Immunogenicity by Incorporating TBK1 as a Novel adjuvant?

Having shown that plasmid DNA-induced immunogenicity was due to its adjuvant properties (mediated via the TBK1 kinase), we evaluated whether such immunogenicity could be improved by incorporating overexpressed TBK1 as an adjuvant. First, we confirmed that TBK1 protein expression was successful using *in vitro* transient transfection (*T. Aoshi, unpublished observations*). However, after many attempts to improve immunogenicity (i.e. mixing the TBK1 encoding plasmid with the antigen encoding plasmid, or using a plasmid co-expressing model of antigen plus TBK1 in a single backbone) we finally concluded that co-expressing

[1] Marichal T, Ohata K, Bedoret D, Mesnil C, Sabatel C, Kobiyama K, Lekeux P, Coban C, Akira S, Ishii KJ, Bureau F, Desmet CJ. DNA released from dying host cells mediates aluminum adjuvant activity. Nat Med In press 2011.

TBK1 in the same backbone do not enhance immunogenicity for both humoral and cellular immunity (*T. Aoshi, unpublished observations*). It is possible that the immunization route used (intramuscular electroporation) may not have been ideal (i.e. electroporation itself may release DNA) for evaluating the effect of the TBK1 molecule. Therefore, we optimized the plasmid doses and immunization routes using plasmids encoding one of the leading vaccine candidate antigens from the blood stages of *P. falciparum*, the serine repeat antigen 36 (PfSERA36) [54]. Targeting *Plasmodium* blood-stage antigens might have several benefits for humans living in malaria endemic regions [55-56]. Younger children would primarily benefit from such vaccines because they are disproportionately affected by the severity of the disease, which can result in death.

We tested intramuscular (i.m.) injection route which is milder way of introducing DNA vaccine. Equal amounts of the plasmids (50 μg) were introduced intramuscularly (i.m.)

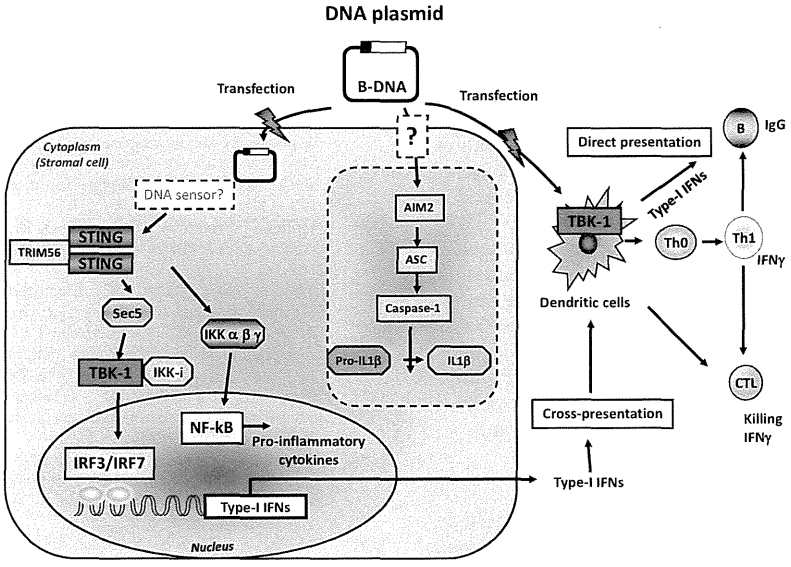


Fig. (1). Double-stranded plasmid DNA utilizes STING and TBK1 for production of type I IFN. The induction of both antigen-specific B cells and CD4+ T cells in hematopoietic cells (i.e. dendritic cells) and CD8+ T cells in non-hematopoietic cells (i.e. muscle cells) is mediated via type I IFNs controlled by STING/TBK1. Therefore, both direct priming and cross priming of the adaptive immunity occur after DNA vaccination. (Abbreviations: B-DNA; B form right-handed helical structure deoxyribonucleic acid, STING; stimulator of interferon genes, Sec5; subunit of the exocyst complex 5, TRIM56; interferon-inducible tripartite-motif 56, TBK1; TANK-binding kinase 1, IKK; IkB kinase, IKK-i; inducible IKK, IRF; interferon regulatory factor, Nf-κB; nuclear factor κB, AIM-2; absent in melanoma 2, ASC; Apoptotic speck protein containing a caspase recruitment domain).

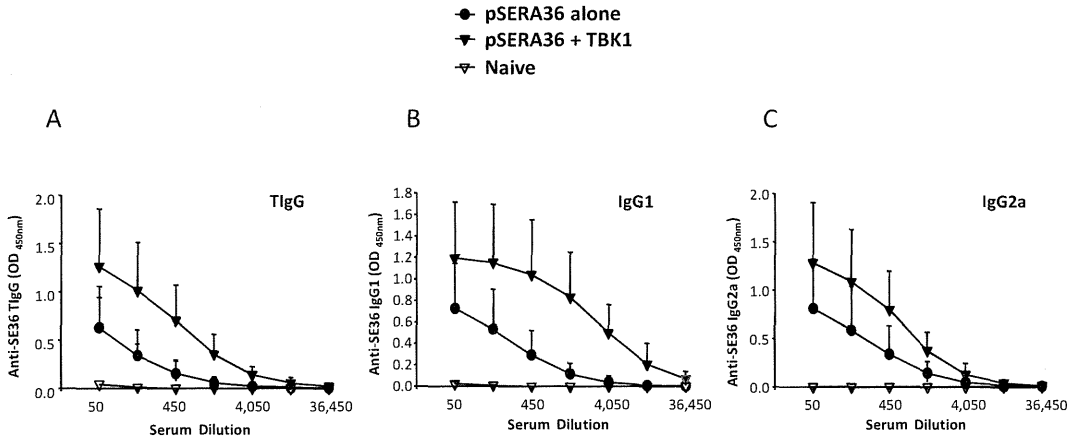


Fig. (2). Co-immunization with a TBK1-encoding plasmid improves DNA vaccine immunogenicity. Mice were immunized with 50 μg of each plasmid (encoding either the *P. falciparum* SERA36 or TBK1) via intramuscular (i.m.) immunization and boosted 4 weeks later with PfSERA36 antigen (1 μg) formulated in alum intradermally (i.d.). Total IgG (A), IgG1 (B), and IgG2a (C) responses for the *P. falciparum* SERA36 antigen were analyzed by ELISA 2 weeks after the booster immunizations.

into mice and boosted 4 weeks later with a PfSERA36 antigen intradermally (i.d.). The TBK1-encoding plasmids, while successfully improving antigen-specific antibody responses, both IgG1 and IgG2a isotypes, Fig. (**2 A-C**) in the plasmid cocktails of SERA36, failed to improve cellular immune responses (*K. Kobiyama, unpublished observations*). Our results suggested that simple injection of TBK1 expressing plasmid in DNA vaccine plasmid cocktail may improve, at least, anti-malarial humoral immunogenicity.

CONCLUSION

Recent attempts to identify the double-stranded DNA sensor have provided great insight into the molecular and cellular mechanisms contributing to DNA vaccine immunogenicity (Table **1**). Our current understanding highlights the importance of type-I IFN mediated innate immune activation via the STING/TBK1 intracellular detection machinery. Such activation confers the adjuvant effect to the encoded antigen. Evidence from our studies suggests that the TBK1-encoded DNA vaccine plasmids used for targeting intracellular signaling pathways might have enormous potential to modulate the innate immune system and increase the immunogenicity of DNA vaccines. A major goal now is to translate the findings of these studies into medical applications (i.e. develop a blood-stage malaria vaccine against *P. falciparum*). It is our hope that studies of this type may deliver new ways to develop safe and effective vaccine adjuvants.

ACKNOWLEDGEMENTS

These studies were supported by the Bill and Melinda Gates Foundation (to C.C. as a Round 1 recipient of Grand Challenges and Explorations grant). This work was supported by the Ministry of Health, Labour and Welfare (MHLW) (K.J.I.), the Knowledge Cluster Initiative (K.J.I.), a Grant-in-Aid for Scientific Research (KAKENHI) from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) (C.C., T.A., K.K. and K.J.I.), and by the Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST) (K.J.I.).

REFERENCES

[1] Liu MA. DNA vaccines: an historical perspective and view to the future. Immunol Rev 2011; 239: 62-84.

[2] Liu MA, Ulmer JB. Human clinical trials of plasmid DNA vaccines. Adv Genet 2005; 55: 25-40.

[3] Davis BS, Chang GJ, Cropp B, *et al.* West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses *in vitro* a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. J Virol 2001; 75: 4040-7.

[4] Corbeil S, Lapatra SE, Anderson ED, *et al.* Evaluation of the protective immunogenicity of the N, P, M, NV and G proteins of infectious hematopoietic necrosis virus in rainbow trout oncorhynchus mykiss using DNA vaccines. Dis Aquat Organ 1999; 39: 29-36.

[5] Bergman PJ, McKnight J, Novosad A, *et al.* Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. Clin Cancer Res 2003; 9: 1284-90.

[6] Kibuuka H, Kimutai R, Maboko L, *et al.* A phase 1/2 study of a multiclade HIV-1 DNA plasmid prime and recombinant adenovirus serotype 5 boost vaccine in HIV-Uninfected East Africans (RV 172). J Infect Dis 2010; 201: 600-7.

[7] Moorthy VS, Imoukhuede EB, Milligan P, *et al.* A randomised, double-blind, controlled vaccine efficacy trial of DNA/MVA ME-TRAP against malaria infection in Gambian adults. PLoS Med 2004; 1: e33.

[8] Ishii KJ, Weiss WR, Ichino M, Verthelyi D, Klinman DM. Activity and safety of DNA plasmids encoding IL-4 and IFN gamma. Gene Ther 1999; 6: 237-44.

[9] Applequist SE, Rollman E, Wareing MD, *et al.* Activation of innate immunity, inflammation, and potentiation of DNA vaccination through mammalian expression of the TLR5 agonist flagellin. J Immunol 2005; 175: 3882-91.

[10] Stevenson FK. DNA vaccines and adjuvants. Immunol Rev 2004; 199: 5-8.

[11] Sasaki S, Xin KQ, Okudela K, Okuda K, Ishii N. Immunomodulation by apoptosis-inducing caspases for an influenza DNA vaccine delivered by gene gun. Gene Ther 2002; 9: 828-31.

[12] Sasaki S, Amara RR, Oran AE, Smith JM, Robinson HL. Apoptosis-mediated enhancement of DNA-raised immune responses by mutant caspases. Nat Biotechnol 2001; 19: 543-7.

[13] Moore AC, Hill AV. Progress in DNA-based heterologous prime-boost immunization strategies for malaria. Immunol Rev 2004; 199: 126-43.

[14] Trollet C, Bloquel C, Scherman D, Bigey P. Electrotransfer into skeletal muscle for protein expression. Curr Gene Ther 2006; 6: 561-78.

[15] Sardesai NY, Weiner DB. Electroporation delivery of DNA vaccines: prospects for success. Curr Opin Immunol 2011.

[16] Broderick KE, Shen X, Soderholm J, *et al.* Prototype development and preclinical immunogenicity analysis of a novel minimally invasive electroporation device. Gene Ther 2011; 18: 258-65.

[17] Singh M, Chakrapani A, O'Hagan D. Nanoparticles and microparticles as vaccine-delivery systems. Expert Rev Vaccines 2007; 6: 797-808.

[18] Pokorna D, Rubio I, Muller M. DNA-vaccination via tattooing induces stronger humoral and cellular immune responses than intramuscular delivery supported by molecular adjuvants. Genet Vaccines Ther 2008; 6: 4.

[19] Kaisho T, Akira S. Toll-like receptors as adjuvant receptors. Biochim Biophys Acta 2002; 1589: 1-13.

[20] Ishii KJ, Akira S. Toll or toll-free adjuvant path toward the optimal vaccine development. J Clin Immunol 2007; 27: 363-71.

[21] Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S. Host innate immune receptors and beyond: making sense of microbial infections. Cell Host Microbe 2008; 3: 352-63.

[22] Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010; 11: 373-84.

[23] Barber GN. Innate immune DNA sensing pathways: STING, AIMII and the regulation of interferon production and inflammatory responses. Curr Opin Immunol 2011; 23: 10-20.

[24] Klinman DM. Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat Rev Immunol 2004; 4: 249-58.

[25] Krieg AM. Therapeutic potential of Toll-like receptor 9 activation. Nat Rev Drug Discov 2006; 5: 471-84.

[26] Hemmi H, Takeuchi O, Kawai T, *et al.* A Toll-like receptor recognizes bacterial DNA. Nature 2000; 408: 740-5.

[27] Sato Y, Roman M, Tighe H, *et al.* Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science 1996; 273: 352-4.

[28] Klinman DM, Yamshchikov G, Ishigatsubo Y. Contribution of CpG motifs to the immunogenicity of DNA vaccines. J Immunol 1997; 158: 3635-9.

[29] Kojima Y, Xin KQ, Ooki T, *et al.* Adjuvant effect of multi-CpG motifs on an HIV-1 DNA vaccine. Vaccine 2002; 20: 2857-65.

[30] Pontarollo RA, Babiuk LA, Hecker R, Van Drunen Littel-Van Den Hurk S. Augmentation of cellular immune responses to bovine herpesvirus-1 glycoprotein D by vaccination with CpG-enhanced plasmid vectors. J Gen Virol 2002; 83: 2973-81.

[31] Coban C, Ishii KJ, Gursel M, Klinman DM, Kumar N. Effect of plasmid backbone modification by different human CpG motifs on the immunogenicity of DNA vaccine vectors. J Leukoc Biol 2005; 78: 647-55.

[32] Spies B, Hochrein H, Vabulas M, *et al.* Vaccination with plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. J Immunol 2003; 171: 5908-12.

[33] Babiuk S, Mookherjee N, Pontarollo R, *et al.* TLR9-/- and TLR9+/+ mice display similar immune responses to a DNA vaccine. Immunology 2004; 113: 114-20.
[34] Pavlenko M, Leder C, Moreno S, Levitsky V, Pisa P. Priming of CD8+ T-cell responses after DNA immunization is impaired in TLR9- and MyD88-deficient mice. Vaccine 2007; 25: 6341-7.
[35] Rottembourg D, Filippi CM, Bresson 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 2010; 184: 7100-7.
[36] Takeshita F, Tanaka T, Matsuda T, *et al.* Toll-like receptor adaptor molecules enhance DNA-raised adaptive immune responses against influenza and tumors through activation of innate immunity. J Virol 2006; 80: 6218-24.
[37] Sasaki S, Amara RR, Yeow WS, Pitha PM, Robinson HL. Regulation of DNA-raised immune responses by cotransfected interferon regulatory factors. J Virol 2002; 76: 6652-9.
[38] Castaldello A, Sgarbanti M, Marsili G, *et al.* Interferon regulatory factor-1 acts as a powerful adjuvant in tat DNA based vaccination. J Cell Physiol 2010; 224: 702-9.
[39] Ishii KJ, Kawagoe T, Koyama S, *et al.* TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. Nature 2008; 451: 725-9.
[40] Ishii KJ, Coban C, Kato H, *et al.* A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. Nat Immunol 2006; 7: 40-8.
[41] Coban C, Koyama S, Takeshita F, Akira S, Ishii KJ. Molecular and cellular mechanisms of DNA vaccines. Hum Vaccin 2008; 4: 453-6.
[42] Ishikawa H, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature 2008; 455: 674-8.
[43] Chien Y, Kim S, Bumeister R, *et al.* RalB GTPase-mediated activation of the IkappaB family kinase TBK1 couples innate immune signaling to tumor cell survival. Cell 2006; 127: 157-70.
[44] Tsuchida T, Zou J, Saitoh T, *et al.* The ubiquitin ligase TRIM56 regulates innate immune responses to intracellular double-stranded DNA. Immunity 2010; 33: 765-76.
[45] Fitzgerald KA, McWhirter SM, Faia KL, *et al.* IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 2003; 4: 491-6.
[46] Sharma S, tenOever BR, Grandvaux N, *et al.* Triggering the interferon antiviral response through an IKK-related pathway. Science 2003; 300: 1148-51.
[47] Ishikawa H, Ma Z, Barber GN. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. Nature 2009; 461: 788-92.
[48] Shirota H, Petrenko L, Hattori T, Klinman DM. Contribution of IRF-3 mediated IFNbeta production to DNA vaccine dependent cellular immune responses. Vaccine 2009; 27: 2144-9.
[49] Roberts TL, Idris A, Dunn JA, *et al.* HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. Science 2009; 323: 1057-60.
[50] Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature 2009; 458: 509-13.
[51] Burckstummer T, Baumann C, Bluml S, *et al.* An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. Nat Immunol 2009; 10: 266-72.
[52] Hornung V, Ablasser A, Charrel-Dennis M, *et al.* AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature 2009; 458: 514-8.
[53] Okuda K, Kawamoto S, Fukushima J. Cytokine and costimulatory factor-encoding plasmids as adjuvants for DNA vaccination. Methods Mol Med 2000; 29: 197-204.
[54] Horii T, Shirai H, Jie L, *et al.* Evidences of protection against blood-stage infection of Plasmodium falciparum by the novel protein vaccine SE36. Parasitol Int 2010; 59: 380-6.
[55] Coban C, Igari Y, Yagi M, *et al.* Immunogenicity of whole-parasite vaccines against Plasmodium falciparum involves malarial hemozoin and host TLR9. Cell Host Microbe 2010; 7: 50-61.
[56] Coban C, Horii T, Akira S, Ishii KJ. TLR9 and endogenous adjuvants of the whole blood-stage malaria vaccine. Expert Rev Vaccines 2010; 9: 775-84.

Received: May 25, 2011 **Revised: July 22, 2011** **Accepted: July 26, 2011**

PMID: 22023477

nature medicine

DNA released from dying host cells mediates aluminum adjuvant activity

Thomas Marichal[1,2], Keiichi Ohata[3], Denis Bedoret[1,2], Claire Mesnil[1,2], Catherine Sabatel[1,2], Kouji Kobiyama[3,4], Pierre Lekeux[1,2], Cevayir Coban[3], Shizuo Akira[3], Ken J Ishii[3–5], Fabrice Bureau[1,2,5] & Christophe J Desmet[1,2,5]

Aluminum-based adjuvants (aluminum salts or alum) are widely used in human vaccination, although their mechanisms of action are poorly understood. Here we report that, in mice, alum causes cell death and the subsequent release of host cell DNA, which acts as a potent endogenous immunostimulatory signal mediating alum adjuvant activity. Furthermore, we propose that host DNA signaling differentially regulates IgE and IgG1 production after alum-adjuvanted immunization. We suggest that, on the one hand, host DNA induces primary B cell responses, including IgG1 production, through interferon response factor 3 (Irf3)-independent mechanisms. On the other hand, we suggest that host DNA also stimulates 'canonical' T helper type 2 (T_H2) responses, associated with IgE isotype switching and peripheral effector responses, through Irf3-dependent mechanisms. The finding that host DNA released from dying cells acts as a damage-associated molecular pattern that mediates alum adjuvant activity may increase our understanding of the mechanisms of action of current vaccines and help in the design of new adjuvants.

Since the first report of its adjuvant activity in 1926 (ref. 1), it has been widely believed that alum creates an immunologically inert 'depot' that prolongs antigen exposure or facilitates antigen uptake by antigen-presenting cells (APCs). However, in line with the decades-old observation that alum triggers interleukin (IL)-1β production[2], recent reports indicated that alum may activate caspase 1 (encoded by *Casp1*)[3] through inflammasomes containing the cytosolic receptor Nlrp3 (NLR family, pyrin domain containing 3)[4–8]. Whether the Nlrp3 inflammasome consistently contributes to the adjuvant effect of alum on humoral responses *in vivo*, however, remains unclear[5–10]. Nevertheless, these studies suggested that signaling through specific receptors of innate immunity, commonly referred to as pattern recognition receptors (PRRs), might have a role in the adjuvant activity of alum. Notably, it has also been proposed that alum induces the local accumulation of uric acid, which would act as an endogenous adjuvant molecule boosting T cell responses[11].

It has actually long been known that alum exerts some level of cytotoxicity[12]. Moreover, dying cells release numerous molecules that act as endogenous danger signals, or damage-associated molecular patterns (DAMPs), and which can alert the innate immune system through the activation of various PRR signaling pathways[13,14]. We thus hypothesized that DAMPs released at immunization sites and the subsequent activation of PRR signaling could contribute to the adjuvant effects of alum. Here we report that alum-induced cytotoxicity results in the release of host DNA, which acts as a DAMP mediating the adjuvant activity of alum.

RESULTS

Host DNA boosts adaptive responses after alum immunization

We observed that endotoxin-free ovalbumin (OVA) adsorbed on Imject Alum (alum), administered intramuscularly (i.m.) or intraperitoneally (i.p.), induced the local accumulation of extracellular double-stranded DNA (dsDNA) in mice (**Fig. 1a** and **Supplementary Fig. 1a**), part of which was entrapped in macroscopic alum depots (**Fig. 1b** and **Supplementary Fig. 1b**). This dsDNA release correlated with the death of local cells (**Fig. 1c** and **Supplementary Fig. 1c**). Both alum and aluminum hydroxide gel (Alhydrogel) induced similar DNA release and cell death (**Supplementary Fig. 1d,e**).

Because released host cell DNA may trigger immune responses[13,15–17], we hypothesized that extracellular host DNA could act as an endogenous adjuvant in alum vaccination. Remarkably, i.p. or i.m. injections of OVA mixed with purified mouse genomic DNA (OVA and DNA), in quantities similar to those released by alum, boosted OVA-specific IgM, IgG1 and IgE responses as efficiently as OVA and alum (**Fig. 2a–c** and **Supplementary Fig. 2a–c**). IgG2c production was not significantly induced in these conditions (data not shown). Thus, like alum, host DNA may act as an adjuvant that preferentially boosts T_H2-dependent humoral responses. Digestion of extracellular DNA *in vivo* by treatment with DNase I decreased antigen-specific humoral responses in OVA and alum–treated mice (**Fig. 2d–f**). Furthermore, the transfer of OVA, together with acellular peritoneal lavage fluid from mice immunized i.p. with OVA and alum, triggered OVA-specific IgG1 and IgE production in naive recipient mice, a response

[1]Laboratory of Cellular and Molecular Physiology, Groupe Interdisciplinaire de Génoprotéomique Appliquée, University of Liège, Liège, Belgium. [2]Laboratory of Biochemistry, Faculty of Veterinary Medicine, University of Liège, Liège, Belgium. [3]World Premier International Immunology Frontier Research Center, Osaka University, Osaka, Japan. [4]Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation, Osaka, Japan. [5]These authors contributed equally to this work. Correspondence should be addressed to C.J.D. (christophe.desmet@ulg.ac.be) or K.J.I. (kenishii@biken.osaka-u.ac.jp).

Received 11 February; accepted 19 May; published online 17 July 2011; doi:10.1038/nm.2403