

サイトカイン濃度の解析などとならび、本研究のマイクロ RNA チップ解析が分担研究として含まれる。

昨年度の成果としては、15 症例 30 検体におけるマイクロ RNA チップ解析の粗解析の結果、接種によって変動する miRNA、発熱の有無に相関する miRNA、抗体価上昇の高低に相関する miRNA などを認めていた。これらの機能については未解明であるが、発熱や有効性を接種前に予測できるバイオマーカーとしての可能性が示唆された。

そこで本年度は、これらの miRNA について定量的 PCR 解析を行って、本研究手法を検証すること、および新たな解析対象に 15 症例 30 検体を選出して同様のマイクロ RNA チップ解析を行い、昨年度認められた miRNA のふるまいを検証することを目指した。

## B.研究方法

### 1. 定量的 PCR

昨年度の解析で得られた RNA をもとに、

- A) 接種によって変動する miRNA 2 種
  - B) 発熱と相関する miRNA 1 種
  - C) 抗体価上昇と相関する miRNA 2 種
  - D) 年齢と相関する miRNA 1 種
  - E) 内因性コントロールとして miRNA 2 種
- の計 8 種の miRNA について既存のプライマーを利用して定量的 PCR を行った。

内因性コントロールには、過去の文献で報告のあったものおよび昨年度のアレイ解析において平均して高い発現が認められたものを選択した。

### 2. 2011 年度追加チップ解析(症例選択)

昨年度と同様に、上記治験の被験者のうち①臨床試験参加同意が得られた者または血清検体の利用について倫理審査委員会等の承認および組織の代表者などの許可が得られた者、かつ②血清検体が十分量保存されている者を対象とした。

そのうち、発熱に関連すると思われる臨床情報(年齢・接種ワクチン・過去のインフルエンザ罹患歴・過去のインフルエンザワクチン接種歴)をできる限り統一して交絡因子を排除し、発熱グレード(治験実施計画書で定義される、「37.5℃以上をグレード 1、38℃以上をグレード 2、39℃以上 1 日以内をグレード 3、39℃以上 2 日以上をグレード 4」)の代表的な症例を選択した。

RNA 抽出及び miRNA 測定に必要な 500 μL の血清を、2012 年 1 月 28 日に業務委託先である東レ株式会社に発送した。解析は昨年度と同様に、血清からの RNA 抽出およびチップ解析を東レ株式会社が行い、各 miRNA の発現は global normalization として表現された。Global normalization を各臨床データと照合して研究分担者が解析を行った。

## C.結果

### 1. 定量的 PCR

A~E の定量的 PCR では、アレイ解析で認められたサンプル間の傾向は、E を除き再現されなかった。A~D についてはアレイ解析において発現量(global normalization)が平均 5 ないし 20 と低い発現量であったために、アレイ解析と PCR との結果が整合しなかったと推定される。発熱および抗体価上昇によって A~D に群分けして行った群間比較で認められた興味深いふるまいは、特に global normalization の低い miRNA について、再現性に問題があると考えられた。

### 2. チップ解析

そこで、昨年度及び今年度実施したアレイ解析においては、群分けを行わず被験者個人の臨床情報と相関する miRNA の選出を行った。選出にあたってはアレイ解析における発現量が 50 前後以上であるものを対象とし、被験者で認められた発熱グレード、治験終了時の HI 抗体価、年齢などの臨床情報と miRNA の発現量との相関を検討したところ、ワクチン接種後血清においては興味深い相関を示す miRNA は認められなかったが、接種前血清中には、1 回目接種発熱グレードと有意な相関を示す miRNA を 2 種認めた。

## D.E.結論及び考察

今回検討症例数を 15 例から 30 例に拡大して実施した網羅的アレイ解析では、発熱と弱い相関を示す miRNA の検証を、予備的ではあるが行うことができた。これらはワクチン関連発熱の予測因子として有用である可能性がある。

今後、他の本治験被験者や他の臨床治験被験者サンプルなどでこの検証を行っていく予定である。

## F.文献

なし

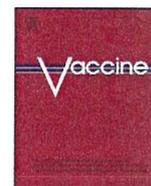
**G.研究発表**

なし

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

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### 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 WIV, plain H5 WIV, and H5 split vaccine. Alum adjuvant did not induce cytokine production, but H5 split induced IFN- $\gamma$  and TNF- $\alpha$ . H5 WIV induced IL-6, IL-17, TNF- $\alpha$ , MCP-1, IFN- $\gamma$ , and IFN- $\alpha$ . An extremely low level of IL-1 $\beta$  was produced in response to H5 WIV, and alum-adjuvanted H5 WIV enhanced IL-1 $\beta$  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.

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### 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

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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  $\geq$  1:40, and 53% seropositivity of NT  $\geq$  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  $\geq$  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, Uppsala, Sweden). They were adjusted to  $1 \times 10^6$  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- $\alpha$  was measured using an EIA kit (Verikine™ Human IFN-Alpha Serum Sample ELISA kit, pbl interferon, USA) and IL-1 $\beta$  and IL-6 were also measured using Quantikine Human IL-1 $\beta$  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 the 15 µg group. No serious systemic adverse reaction was observed: febrile reaction  $\geq$  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  $\geq$  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  $\geq$  38.0 °C was demonstrated in recipients aged less than 6 years and the incidence of febrile reaction ( $\geq$  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+	$\geq$ 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%)
$\geq$ 13 years	53	10 (19%)	3 (6%)
Total	374	203 (54%)	147 (39%)

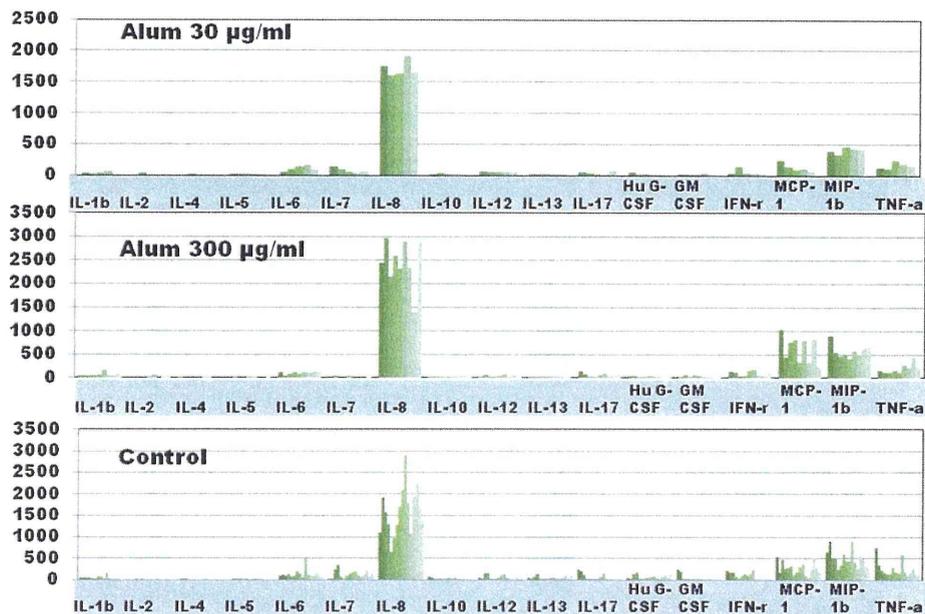


Fig. 1. Cytokine profile in PBMC cultures stimulated with aluminum solution. PBMC were stimulated with 0.1 ml of Alum adjuvants of 300 µg/ml (similar concentration as alum-adjuvanted H5 vaccine) and 30 µg/ml (1:10 dilution).

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 aluminum 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.

## 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

Table 2  
Relationship between acute febrile reactions and antibody response.

	N	Mean ± SD <sup>d</sup>	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

<sup>a</sup> Mean NT titers were significantly different between subjects with febrile reactions after immunization and those without febrile reactions ( $p < 0.01$ ).

<sup>b</sup> Significant difference was noted between NT titers in subjects with high body temperature  $\geq 37.5$ –38.0 C and in those with 38.0–39.0 C ( $p < 0.05$ ).

<sup>c</sup> Significant difference was noted between NT titers in subjects  $\geq 37.5$ –38.0 C and in those with  $\geq 39$  C ( $p < 0.01$ ).

<sup>d</sup> Mean titer of NT antibody expressed as  $10 \times 2^n$ .

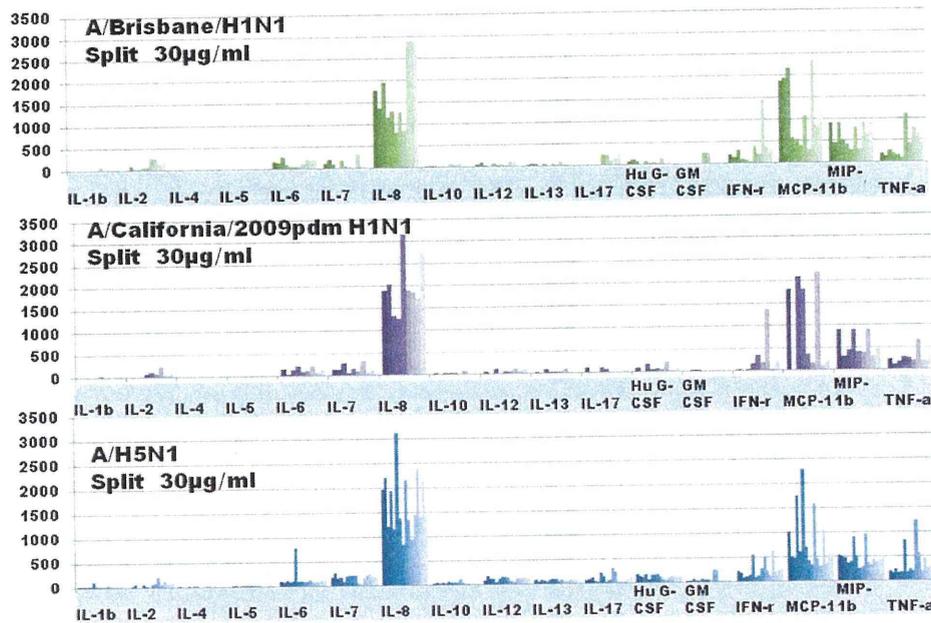


Fig. 2. Cytokine profile of PBMC cultures stimulated with split influenza vaccines. Split vaccine materials were used: H5N1 pandemic NIBRG-14 strain, originated from H5/A/Vietnam/1194/2004, A/H1N1/Brisbane/2007, and 2009 pandemic A/California/07/2009. Each antigen was prepared at the concentration of 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 ( $\geq 38^\circ\text{C}$ ) 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.

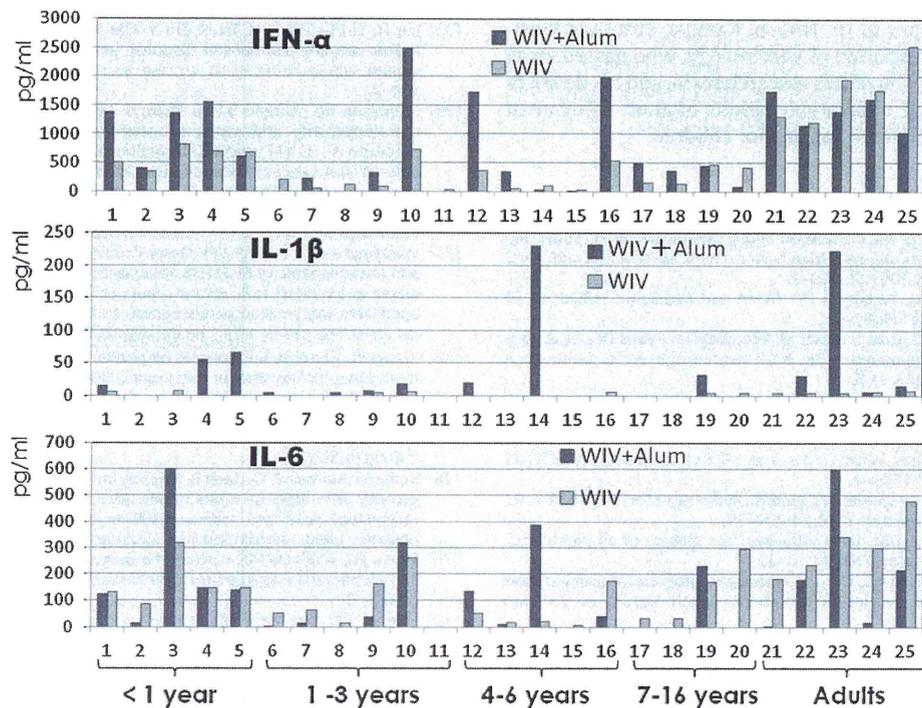
Many kinds of adjuvant have been developed, and they cause adverse reactions at the inoculation site or systemic reactions. Alum-based adjuvant was first approved for human use and continues to be widely used in many vaccines as an immuno-potentiator [29–31]. Two potential mechanisms are basically considered: (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-γ	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- $\alpha$ , IL-1 $\beta$ , and IL-6 production. IFN- $\alpha$ , IL-1 $\beta$ , 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 H5WIV, 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-1 $\beta$ , 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 E<sub>2</sub> (PGE<sub>2</sub>) and IL-1 $\beta$ . PGE<sub>2</sub> production was independent of NLRP3, ASC, and the caspase-1 inflammasome complex, and PGE<sub>2</sub> expression depended on cyclooxygenase (COX) and PGE synthase, regulated by spleen tyrosin kinase (Syk) and p38 MAP kinase in macrophages. PGE<sub>2</sub> was found to suppress Th1 responses with a reduced production of IL-2 and IFN- $\gamma$ , but facilitated the differentiation of Th1 cells in the presence of IL-12 and, thus, cytokine species and their balance regulated PGE<sub>2</sub> function on antibody production [18,42,43]. WIV and alum-adjuvanted WIV induced the production of the endogenous cytokines IL-1 $\beta$ , IFN- $\alpha$ , IL-6, and TNF- $\alpha$ , and they induced PGE<sub>2</sub> 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- $\alpha$  [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- $\alpha$ , IL-1 $\beta$ , 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.

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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.

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## Innate and adaptive immune responses to viral infection and vaccination

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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

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### 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<sup>o</sup>,9,10]; although it is still not clear whether all innate responses contribute equally to the induction of adaptive responses [8<sup>o</sup>,11<sup>o</sup>,12<sup>o</sup>]. 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.

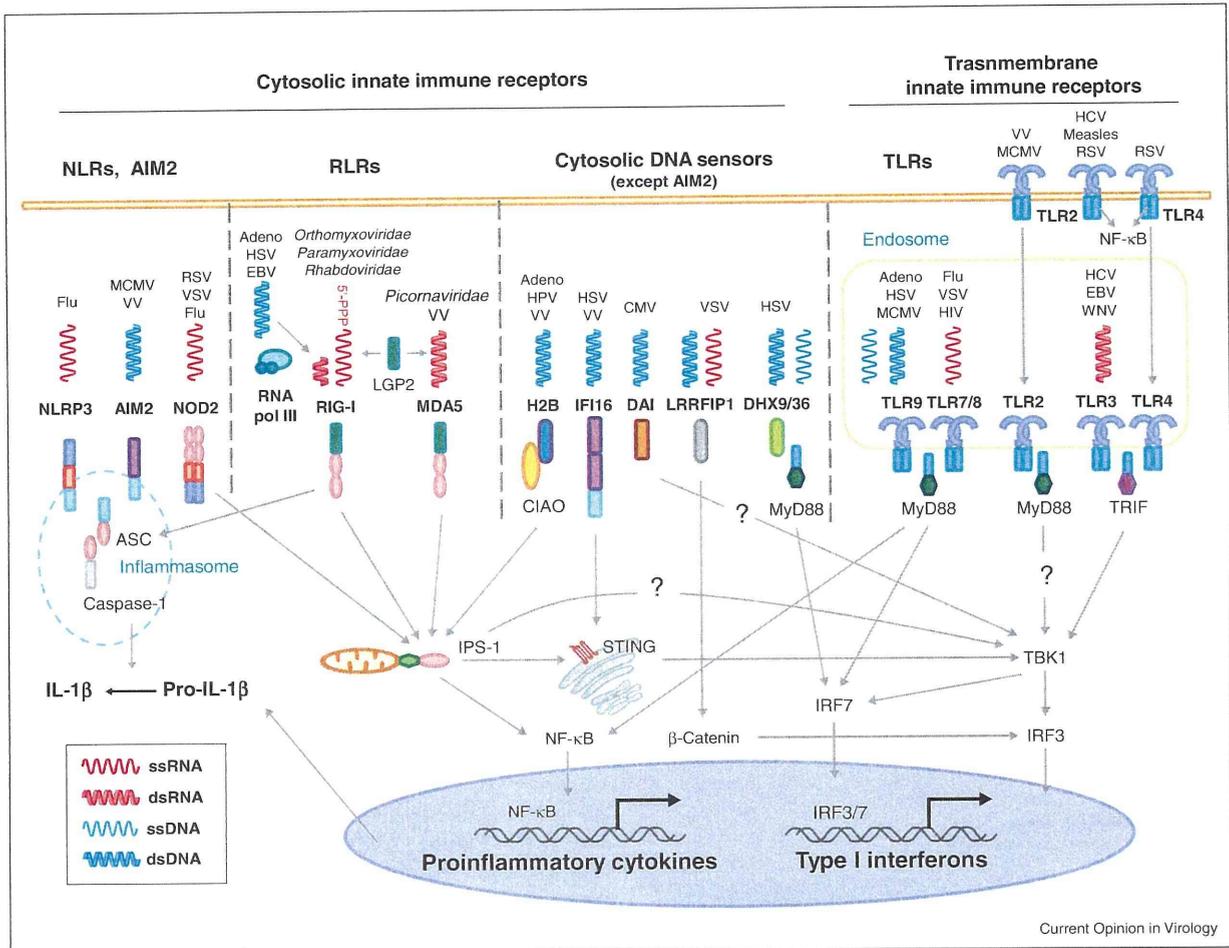
### The innate immune system may distinguish between the presence of a virus and viral infection

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- $\kappa$ B)-dependent cytokine responses, interferon regulatory factor (IRF)-dependent IFN- $\alpha/\beta$  responses, and inflammasome/caspase-1-dependent IL-1 $\beta$  responses. IFN- $\alpha/\beta$  are the major cytokines that limit viral replication, while other cytokines, including IL-6, TNF- $\alpha$  and IL-1 $\beta$ , recruit immune cells to the site of infection and elicit inflammation. NF- $\kappa$ B-dependent and IRF-dependent cytokines are transcriptionally regulated, whereas inflammasome-dependent IL-1 $\beta$  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-1 $\beta$ . 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- $\kappa$ B translocation. RIG-I can activate the inflammasome by interacting with the CARD domains of RIG-I and ASC, and produce IL-1 $\beta$ . **Cytosolic DNA sensor pathways:** extra-chromosomal histone H2B binds DNA virus-like HPV through its  $\alpha$ -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  $\beta$ -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- $\kappa$ B 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- $\kappa$ B and IRF7 activation via MyD88. TLR9 detects CpG-ODNs and DNAs derived from DNA viruses, leading to NF- $\kappa$ B 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- $\beta$ , which was not observed upon stimulation with Pam3SK4 (a well-known bacterial TLR2 agonist) [17<sup>\*\*</sup>]. Interestingly, this TLR2-mediated IFN- $\beta$  production was restricted in Ly6C(hi) inflammatory monocytes, and was dependent on TLR2 recruitment from the cell surface to the endosome [17<sup>\*\*</sup>]. 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- $\beta$  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- $\alpha$  production. Importantly, expression of these endosomal TLRs is restricted to certain types of dendritic cells (DCs). TLR3 is preferentially expressed by CD8 $\alpha$ (+)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- $\kappa$ B 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-1 $\beta$  production during VSV (ssRNA virus) infection [24<sup>\*\*</sup>].

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- $\kappa$ B and IRF. Although Nod2 was initially characterized as a cytosolic sensor for the bacterial cell wall component, muramyl dipeptide, which induces NF- $\kappa$ B activation, a recent report suggests that Nod2 also functions as a virus sensor [26<sup>\*\*</sup>] 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- $\beta$  responses [26<sup>\*\*</sup>]. Activation of NLRs, such as NLRP1, NLRP3, and NLRC4, leads to inflammatory formation, which results in caspase-1-mediated IL-1 $\beta$  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<sup>\*</sup>,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- $\beta$  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- $\beta$  induction *in vitro* [38]; however, gene knockout mice do not show the same phenotype, suggesting the presence of redundant DNA sensor mechanisms [39]. Lrrfp1 recognizes cytosolic dsDNA (and dsRNA), subsequently interacting with  $\beta$ -catenin and enhancing IRF3-mediated IFN- $\beta$  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- $\alpha$  [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- $\beta$  expression. However, this H2B-mediated dsDNA-dependent IFN- $\beta$  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-1 $\beta$  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
Lopez <i>et al.</i> [50]	A/PR8	IFN-I, TNF $\alpha$ /IL-6 CD4(IFN $\gamma$ ) $\rightarrow$ , CD4(IL-4)T, CD8 $\rightarrow$ , Ab $\rightarrow$	Not examined	Inflammasome (IL-1/IL-18) Not examined
Heer <i>et al.</i> [51]	A/PR8	CD4 $\rightarrow$ , CD8 $\rightarrow$ , Ab(IgG2a) $\downarrow$	Not examined	Not examined
Koyama <i>et al.</i> [52]	A/PR8, A/NC	CD4(IFN $\gamma$ ) $\downarrow$ , CD8 $\rightarrow$ , Ab(IgG2a) $\downarrow$	CD4(IFN $\gamma$ ) $\rightarrow$ , CD8 $\rightarrow$ , Ab $\rightarrow$	Not examined
Seo <i>et al.</i> [53]	A/PR8	CD4(Th1) $\downarrow$ , CD4(Th2) $\uparrow$ , CD8 $\rightarrow$ , Ab $\rightarrow$	CD4(Th1) $\rightarrow$ , CD8 $\rightarrow$ , Ab $\rightarrow$	Not examined
Ichinohe <i>et al.</i> [58]	A/PR8	Not examined	Not examined	CD4(IFN $\gamma$ ) $\downarrow$ CD8 $\downarrow$ , Ab(IgG, IgA) $\downarrow$
Allen <i>et al.</i> [59]	A/PR8	Not examined	Not examined	Intact adaptive responses (CD8 $\rightarrow$ , Ab $\rightarrow$ )
Thomas <i>et al.</i> [60]	A/PR8	Not examined	Not examined	CD8 $\rightarrow$ , Ab $\rightarrow$
Koyama <i>et al.</i> [48*]	A/NC	Not examined	Not examined	CD4(IFN $\gamma$ ) $\rightarrow$ , CD8 $\rightarrow$ , Ab(IgG1) $\downarrow$
	Inactivated WV(A/NC)	CD4 $\rightarrow$ , Ab $\downarrow$	CD4 $\rightarrow$ , Ab $\rightarrow$	CD4 $\rightarrow$ , Ab $\rightarrow$
Geeraedts <i>et al.</i> [54*]	Inactivated WV(H5N1)	CD4 $\downarrow$ , Ab $\downarrow$	Not examined	Not examined

groups. Ichinohe *et al.* demonstrated that NLRP3-independent, but ASC-dependent, inflammasome responses were important for both CD4<sup>+</sup> and CD8<sup>+</sup> responses, as well as IgA and IgG responses [58] (it is noteworthy that Pocock *et al.* also reported NLRP3-independent, but RIG-I and ASC-dependent, inflammasome activation by VSV [24<sup>••</sup>]). 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<sup>\*</sup>]. At present, the reason for these contradictory results is not clear [11<sup>\*</sup>,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- $\alpha/\beta$  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<sup>\*</sup>,54<sup>\*</sup>].

### 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 CD8<sup>+</sup> 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.

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## Novel Strategies to Improve DNA Vaccine Immunogenicity

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**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].

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

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[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 plasmacytoid 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 IFN $\gamma$  and cytotoxic T lymphocyte (CTL) responses) as their wild-type counterparts [32-33], although others suggest that TLR9 signaling is critical for inducing CD8<sup>+</sup> 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 over-expression strategy using adapter molecules such as the Myeloid Differentiation Primary Response Gene (MyD88) or Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing interferon- $\beta$  (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- $\kappa$ B and IRF3 to stimulate type I IFN production after intracellular dsDNA stimulation [42]. Upon dsDNA stimulation, STING relocates 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 I $\kappa$ B 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 im-

mune 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-1 $\beta$  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-1 $\beta$  release, which could be responsible for alum's adjuvanticity, independent of ASC or Caspase-1 (<sup>1</sup>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

<sup>1</sup> 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.