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EXPERT REVIEWS

TLR9 and endogenous adjuvants of the whole blood-stage malaria vaccine

Expert Rev. Vaccines 9(7), 775–784 (2010)

Cevayir Coban[†1], Toshihiro Horii[2], Shizuo Akira[1] and Ken J Ishii[1,3]

[1]*Immunology Frontier Research Center, World Premier Institute for Immunology, Osaka University, Suita, Osaka 565-0871, Japan*
[2]*Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan*
[3]*Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation, Ibaraki City, Osaka 567-0085, Japan*
[†]*Author for correspondence: Tel.: +81 668 798 301 ccoban@biken.osaka-u.ac.jp*

Vaccination has been a successful tool in the protection against many infectious diseases, and recent advances in biotechnology have created new techniques and strategies to produce safe and efficacious vaccines for human use. However, developing a protective vaccine against malaria has been a challenge. In this article, we focus on an old approach with some new modifications, the so-called whole-parasite vaccination strategy against blood-stage *Plasmodium falciparum*, the deadliest human malarial agent. In addition, we discuss recent developments in our understanding of how the endogenous adjuvant activity in the parasites, which functions via Toll-like receptor 9, acts as a double-edged sword between protective vaccination and pathological responses against malaria infection.

KEYWORDS: adaptive immunity • adjuvant • hemozoin • innate immunity • malaria • TLR9

Malaria is an ancient disease caused by Apicomplexan *Plasmodium* parasites. According to the 2009 report from the WHO, half of the world's population is at risk of malaria. In 2008, 243 million cases were encountered with approximately 863,000 deaths [101]. Malaria control currently relies on the use of anti-parasite drugs and vector control, and while very effective if used properly, the parasite's ability to develop resistance to these drugs, combined with the development of insecticide resistance in mosquitoes, makes the development of an effective vaccine of paramount importance. However, it seems that our understanding of the *Plasmodium* parasites and their interaction with the host innate immune system is very limited and that we are still a long way from creating a successful vaccine against malaria [1]. Accumulating data on the biology of *Plasmodium* parasites indicate that several points need to be considered for vaccine design. First, *Plasmodium* parasites have a complex lifecycle both in vertebrates and invertebrates (mosquito), and mosquitoes can transmit disease from an individual host to hundreds of individuals very rapidly. Second, such a complex lifecycle in the host requires the sequestration of different cell types (i.e., hepatocytes and erythrocytes) and effector mechanisms from the immune system. The organisms then enter these cells and proliferate. Third, *Plasmodium* parasites can persist for months or even years in infected individuals and a single exposure does not confer lifelong protection against new infections, such is the case for some viral infections. Fourth, *Plasmodium* parasites undergo rapid antigenic variation and polymorphism in the host, presumably to prevent immune recognition.

Vaccination has been proven to be a key procedure for the eradication and/or control of many infectious organisms. Initial vaccination techniques often include empirical approaches, such as killing the whole organism or attenuating live microorganisms. Indeed, smallpox, diphtheria, pertussis, tetanus, polio and measles vaccinations are a few such successful examples of these types of primary vaccines. Similarly, in the case of malaria, it was established many years ago that host protective immunity against malaria parasites could be achieved in animal models as well as in humans following whole-parasite vaccinations [2–4]. However, along with advances in molecular biology over the last 20 years, vaccine development against malaria has mainly focused on the investigation of potentially protective antigens and the development of subunit vaccines [5,6]. This is mainly because immunization by protective antigens produced using Good Manufacturing Practices (GMP) as recombinant proteins had been shown to protect against some infectious

 10.1586/ERV.10.60 ISSN 1476-0584

diseases (i.e., hepatitis B virus). However, it is now clear from several animal and human clinical studies that many subunit vaccines fail to confer sterile protection against malaria, although some *Plasmodium* antigens were shown to reduce disease severity in immunoepidemiological studies [4,7–12]. Owing to the failure of many clinical trials with recombinant vaccines, the whole-parasite vaccine strategy against malaria has been regaining attention. There are several reasons for this: recombinant proteins require powerful adjuvants for successful protection and very limited numbers of potent adjuvants are available for human use [13]; recombinant protein immunizations are mostly strain specific and are successful only against challenge with homologous parasite strains, whereas whole-parasite vaccines may protect against heterologous parasites [14]; whole-parasite vaccines may help to avoid problems arising from antigenic variation and polymorphism, which are commonly found in *Plasmodium* parasites; and targeting multiple antigens by using whole parasites may be more effective than a single antigen vaccine to reduce disease severity. Furthermore, recent developments in the field of immunology have changed our understanding of the innate immune response to microbial products [15] and it is important to take these advances into consideration, along with new techniques and innovative ideas, to develop novel vaccines against malaria.

In this article, we explore recent advances in our understanding of the innate immune system and its role during malaria vaccination. We focus primarily on whole-parasite immunizations with blood-stage parasites and the role of the MyD88/Toll-like receptor (TLR)9 signaling pathway during vaccination with *Plasmodium falciparum* parasites. We hope that the culmination of this knowledge will be another building block in the wall towards the development of an effective vaccine against malaria.

Adjuvants in malaria vaccines

Successful adjuvants help protective antigens to stimulate potent and long-lasting immune responses. Therefore, adjuvants have become an essential component of vaccine formulations. Given the technological advances in the production of pure and safe recombinant antigens over the last few decades, adjuvants are now introduced (exogenous adjuvants) into vaccine formulations to initiate and/or enhance their immunogenicity. So far, aluminum salts (Alum) are the most effective exogenous adjuvants among the few adjuvants licensed for human use [16]. However, owing to the weakness of Alum to stimulate T-cell and B-cell responses, as well as unwanted side effects such as granuloma formation at the injection site, there was an urgent need to develop other potent exogenous adjuvants [17]. MF59, monophosphoryl derivative of lipid A (MPL) combinations, saponins, CpG oligodeoxynucleotides, imidazoquinolines and montanides are examples of new exogenous adjuvants that have been under investigation in many clinical trials for recombinant malaria vaccines [13]. RTS,S (currently in Phase III clinical trials in Africa) is one of the successful pre-erythrocytic stage vaccine candidates against *P. falciparum*, which is formulated in adjuvant combinations (i.e., saponins) with MPL [18].

In addition to exogenous adjuvants, recent advances in innate immune system research have improved our understanding and identification of 'endogenous' adjuvants and how they might work in vaccine formulations [16,19]. It has long been observed that inactivated virus or bacterial vaccines often do not require the addition of external adjuvants. Recently, it was postulated that whole-microbe vaccines harbor not only protective antigens, but also 'built-in' endogenous adjuvant components capable of activating the innate immune system [15,20,21]. The built-in adjuvant components include ligands for innate immune system receptors, such as TLRs, NOD-like receptors (NLRs) and RIG-I-like receptors. Contrary to what was previously thought, the viral or bacterial nucleic acids packaged inside microbial cells were found not to be inert; rather, they are potent stimuli for the production of proinflammatory cytokines and type I interferons (IFNs) through receptor-mediated immune recognition and signaling pathways [19,22]. Furthermore, DNA vaccines that closely mimic live pathogen infections were found to contain built-in adjuvants that stimulate the innate immune system, recognized by as yet unidentified DNA sensors via an endoplasmic reticulum-anchored adaptor molecule called STING and a non-canonical IκB kinase called TRAF family member-associated-binding kinase 1 (TBK1) [23–25].

Based on the increasing body of evidence that whole-microbe vaccines contain endogenous built-in adjuvants that could be ligands for innate immune system receptors, here we pose the intriguing question of which, if any, endogenous adjuvants would be contained within *Plasmodium* whole-parasite vaccines and how would this endogenous built-in adjuvant operate during *Plasmodium* parasite infection? We will now discuss why whole-parasite vaccines against malaria would potentially be effective due to the potency of the built-in adjuvant(s).

Whole-parasite vaccine approach for malaria: clues from history

Plasmodium parasites have a complex lifecycle in vertebrate and invertebrate hosts (Figure 1). They mainly live in three different compartments of the vertebrate body: the skin, liver and blood. The pre-erythrocytic stage consists of skin and liver forms of the parasites, while the erythrocytic stage contains the blood-stage parasites. Whole-parasite vaccine approaches mainly target these two stages: the pre-erythrocytic and erythrocytic blood stages (Figure 1 shows the stages of the lifecycle of *Plasmodium* parasites and whole-parasite vaccine strategies). The erythrocytic stage is further divided into two different stages: the asexual and sexual stages. Female and male gametocytes, which are responsible for the transmission of parasites to the mosquito vector, are called the 'sexual erythrocytic stage'. Naturally acquired immune responses to malaria and the role of pre-erythrocytic and erythrocytic-stage whole-parasite vaccine strategies have recently been reviewed [2,3,26,27].

Whole sporozoite immunizations

The main aim of vaccines against the pre-erythrocytic stage of *Plasmodium* is to either block the invasion of sporozoites into hepatocytes or destroy the sporozoite-infected hepatocytes,

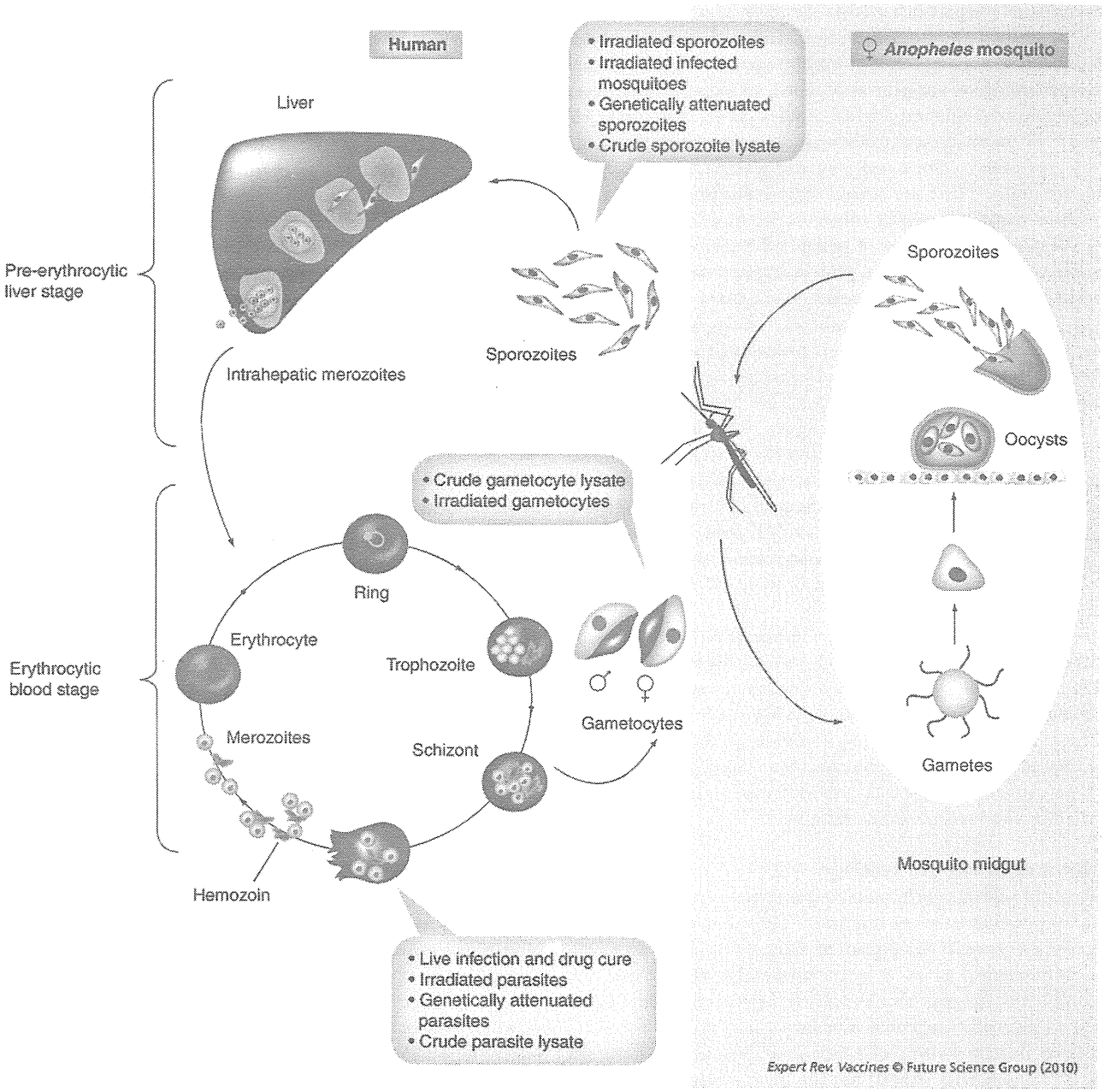

Figure 1. Lifecycle of *Plasmodium* parasites and whole-parasite vaccine approaches. Infected female *Anopheles* mosquitoes transmit *Plasmodium* parasites into a vertebrate host through their bites during blood meals. After a bite from an infected mosquito, sporozoites invade the liver hepatocytes within minutes, mature in the liver for approximately 1 week and transform into hepatocytic merozoites. Once the hepatic merozoites are released into the bloodstream, they begin to invade erythrocytes and initiate the erythrocytic blood-stage lifecycle of *Plasmodium* and the clinical symptoms of malaria such as fever attacks. During this stage, some of the merozoites develop into sexual stages, and form female and male gametocytes, which are central to malaria transmission. Finally, gametocytes taken up by the mosquito blood meal start to exflagellate and form gametes and oocysts. Within 10 days, sporozoites can be transferred to the salivary glands of mosquitoes, ready to be transmitted to humans by mosquito bites. Callout boxes show the stages that have been used as whole-parasite vaccines.

potentially leading to complete protection and prevention of transmission of *Plasmodium* parasites and malaria disease. Thus, the use of whole sporozoites for vaccination against malaria has been an attractive strategy and has been extensively investigated for over 50 years using animal models. Immunization with sporozoites inactivated by UV irradiation or killed by freeze-thawing, formalin treatment or drying has been shown to lead to reduced mortality rates [3,4]. Furthermore, immunization

with radiation-attenuated sporozoites via irradiated infected mosquitoes in humans induced sterile species-specific protective immunity against sporozoite challenge [27,28]. The underlying mechanism responsible for inducing protective immunity with irradiated sporozoites is not completely understood, although it was found that irradiated sporozoites can invade hepatocytes but do not reach full maturation and therefore do not progress into the blood stage [29]. Recent studies have highlighted that not only radiation-attenuated, but also genetically-attenuated sporozoites (lacking the genes essential for liver stage development) and chemically-attenuated sporozoites can stop liver stage development and provide sterile immunity and protection against malaria infection [26,30,31]. A more recent study showed that intact sporozoites could also induce protective immunity under drug treatment, without the need for multiple irradiated mosquito bites [32–35].

Whole blood-stage immunizations

The complexity of acquired immunity to malaria infection has been well recognized, especially in humans. Epidemiological studies have suggested that 'natural immunity' to malaria infection can only be acquired after several infections and can limit the severity of disease by lowering parasitemia rather than by clearing it. This evidence suggests the possibility of developing a blood-stage vaccine to reduce malaria disease severity, even though parasitemia might not be cleared completely [3]. In an early study, nearly 60 years ago, Freund *et al.* investigated the vaccination effect of whole formalin-killed erythrocytic stages formulated in complete Freund's adjuvant and found protection in ducks [36] and later in rhesus macaques [37]. These studies have recently been reviewed [38]. Their findings revealed that successful immunization and protection against homologous or heterologous parasite challenge required very high doses of infected erythrocytes or a potent adjuvant such as complete Freund's adjuvant. Later, Su *et al.* achieved successful vaccination using CpG DNA as a strong adjuvant against mouse malaria [39]. Very few studies have addressed the possibility of developing genetically-attenuated blood-stage parasites for vaccination. Recently, some studies have shown that genetically-attenuated blood-stage parasites were incapable of developing in the mosquito midgut, therefore preventing transmission of *Plasmodium* parasites from mosquitoes to mice. When mice were immunized with genetically-attenuated blood-stage parasites and challenged with a normally lethal parasite, complete sterile protection was achieved [40,41]. These studies highlighted the overall probability of developing a blood-stage malaria vaccine by using genetically-attenuated parasites.

Gametocytes, the sexual stage of malaria parasites, are part of the erythrocytic stage of *Plasmodium* infection. Evidence suggests that in endemic areas, natural immunity to gametocytes can rapidly develop with age, although adults can still continue to infect mosquitoes [42]. Initial immunizations with whole-killed, formalin-treated or irradiated gametocytes (mixed with other stages or purified) in several animal models, including chickens, mice and rhesus monkeys, have been shown to induce successful transmission-blocking immunity, which can effectively block transmission of malaria parasites into mosquitoes (reviewed in [3]).

A recent study introduced another whole-parasite vaccination strategy. It was reported that a subpatent infection with ultra-low doses of nonattenuated *Plasmodium*-infected erythrocytes terminated by drugs, could induce protective immunity by inducing mostly Th1 cellular responses in humans [43]. These vaccination strategies, which applied real infection-drug treatment regimens for both intact sporozoites and blood-stage parasites, indicated that protective immunity could be achieved in humans by intact whole parasites, at least against homologous strains [33,43], presumably via the recognition of a broad array of both pre-erythrocytic as well as erythrocytic-stage antigens by the host immune system. In addition, recent studies in mice have highlighted the possibility of cross-stage and/or species protection (reviewed in [14]).

The production of whole-parasite vaccines for humans has, inevitably, led to safety concerns. One of the main concerns raised is how to develop consistent GMP vaccine lot using human blood, and how to preserve such a vaccine prior to administration. In addition, it is also important to evaluate any possible drawbacks of using genetically-attenuated parasites for whole blood-stage vaccine.

What are the endogenous built-in adjuvants in the whole blood-stage malaria vaccine?

The challenge to make whole-parasite blood-stage vaccines against malaria, discussed earlier, has been a quiet success and the evidence now strongly suggests the possibility of built-in adjuvant component(s) in malaria parasites. There are several candidates in *Plasmodium* parasites that may act as endogenous adjuvant(s), such as *P. falciparum* glycosylphosphatidylinositols (GPIs; TLR2 ligand), heat-labile parasite fraction (TLR9 ligand), malarial hemozoin (TLR9 ligand) and DNA derived from *P. falciparum* (TLR9 ligand) [44–48]. The adjuvant components within blood-stage *P. falciparum* parasites have recently been explored by our group [49]. We reported that immunization of mice with large numbers of *P. falciparum* blood-stage parasites killed by freeze-thawing resulted in strong immunogenicity, owing to the enhanced built-in adjuvant activity of the parasites. A single immunization with large numbers of killed parasites (~10^8 infected erythrocytes) could induce high titers of parasite-specific antibodies (i.e., IgG, IgG_{2a}, IgG_{2b} and IgG_{2c}) in the serum and secretion of IFN-γ from spleens [49]. We also carefully analyzed whether innate immune system receptors (i.e., TLRs) mediate this adjuvant activity. *Plasmodium* GPIs were originally suggested to be a proinflammatory endotoxin of parasitic origin and synthetic GPIs were proposed as a potential anti-toxic vaccine against malaria [50]. However, we found no involvement of TLR2 [Coban C, Unpublished Data], which recognizes parasite-originated GPIs, in *P. falciparum* crude extract immunizations, even though crude extracts contain abundant GPIs of *P. falciparum* [51]. Furthermore, we found that, rather than TLR2, TLR9 controls the built-in adjuvant activity of the parasite. The main candidate ligand for TLR9 could be parasite-originated DNA, given that TLR9 is a canonical receptor for DNA. Furthermore, the recognition of malarial metabolites by TLR9 has been a matter of controversy [46–47]. However, DNA was not critical for the adjuvant effect of *P. falciparum*, because DNase-I

treatment did not alter the TLR9-dependent adjuvant effect of *P. falciparum* crude extract. We carefully performed *Plasmodium* species-typing PCR [52] to detect parasite DNA in the DNase-treated *P. falciparum* crude extract and could not amplify any parasite DNA, at least not any fragments over 100 bp in size. We confirmed, using *in vitro* binding studies, that DNAse-treated *P. falciparum* crude extract could still bind to recombinant TLR9. Overall, these experiments clarified that *Plasmodium* DNA is not required for the adjuvant effect of the *P. falciparum* crude extract and confirmed that the TLR9 ligand in the *P. falciparum* crude extract is not *P. falciparum* DNA. Instead, a non-DNA TLR9 ligand probably exists, such as hemozoin and/or some other heat labile components as previously reported [44,46].

To investigate whether the malarial heme metabolite hemozoin could possess TLR9-dependent adjuvant properties in *P. falciparum*, we prepared pure synthetic hemozoin from hemin chloride and examined its adjuvant effect on model antigens as well as on *P. falciparum* crude antigens. The pure synthetic hemozoin showed a strong adjuvant effect, however, its optimal response was quite variable and dependent on its size, method of synthesis and structural appearance [49]. Further analysis at the molecular and atomic levels revealed that TLR9 binds to hemozoin directly and specifically in a manner that depends on unique cysteine motifs (CysXXCys or Cys) and amino acid sequences, similar to its binding to CpG DNA, a well-known TLR9 ligand. However, in contrast to the TLR9-dependent adjuvant effect of natural hemozoin, the adjuvant effect of synthetic hemozoin was completely MyD88-dependent, but TLR9-independent. A possible explanation for this difference could be that although chemically synthetic and natural hemozoin are similar, cellular uptake and internalization (i.e., modification of the integrity of the phagosomal and/or endosomal membrane) may differ between the two types of hemozoin. Eventually, this may result in different receptor (and/or coreceptor) activations, such as via NLRP3, as recently suggested (reviewed in [53]). On the contrary, one may still consider the possibility that the *P. falciparum* crude extract contains a non-DNA, non-hemozoin TLR9 ligand as reported previously [44]. It should be noted that it is technically difficult to remove hemozoin from the *P. falciparum* crude extract without disturbing the integrity of other components, especially proteins. Thus, hemozoin under natural conditions might form a complex with DNA and parasite proteins to activate TLR9 [54]. Nevertheless, as we have shown that both natural and synthetic hemozoin specifically bind to TLR9 and change its conformation, this fulfills the definition of a TLR9 ligand [49].

TLR9 modulation during malaria infection *in vivo*

Toll-like receptor 9 has also been proposed to play important roles *in vivo* during malaria infection both in humans and in mice, although this has been the subject of some controversy (Table 1). The first *in vivo* study using a mouse model of malaria reported that the liver injury induced by *Plasmodium berghei* infection was mediated through MyD88, an adaptor molecule for TLR signaling (with the exception of TLR3), although precise identification of the individual TLRs involved was not reported (except that TLR4 and TLR6 were not involved) [55]. Subsequent studies in mice suggested an important role for TLR9 (as well as TLR2) in the pathogenesis of cerebral malaria, which might be mediated through the TLR9-dependent recruitment of immune cells to the brain [56,57], although it is controversial with other reports [58,59]. The different findings reported by independent research groups might be attributable to differences in the parasites used and/or housing of the mouse lines in different environments, as recently proposed [60]. However, evidence from other mouse malaria parasites suggested that although the adaptor molecule MyD88 has the most profound effect on the immunological responses to *Plasmodium* parasites, the immunomodulating role of TLR9 during mouse infections with *Plasmodium chabaudi* [48,61–63] and *Plasmodium yoelii* [64] via induction of, or synergy with, several cytokines (i.e., IFN-γ signaling) and interferons (i.e., IFN-α) has also inevitably been taken into consideration. Furthermore, TLR9 signaling may not solely play a role in the inflammatory response during malaria infections, but may also be essential for activation of regulatory T cells, which might help malaria parasites evade the immune response, leading to severe disease [65,66]. It might therefore be through TLR9-dependent dendritic cell (DC) activation [48,65] that TLR9 deficiency in mice leads to increased survival from malaria infection.

TLR9 modulation in malaria-endemic areas: implications for vaccine design

Acquired immunity to malaria parasites and their specific antigens occurs only after repeated exposures, but still might not be complete (i.e., continuous asymptomatic parasitemia). Therefore, it is important to understand the underlying mechanisms as to why people in endemic regions cannot develop sterile immunity. In particular, it is important to develop blood-stage malaria vaccines, because antibody responses elicited via immunizations probably in cooperation with monocytes block erythrocyte invasion by parasites [67]. Although it is under debate whether B-cell responses to malaria are persistent [68,69], the hypothesis that *P. falciparum* infection interferes with normal B-cell responses (activation, generation and maintenance of memory) [70] has certain implications. It was found in malaria-naive individuals by recombinant subunit *P. falciparum* immunizations formulated with CpG DNA adjuvant, a TLR9 agonist, that antigen-specific memory B cells and antibody levels correlated well [71]. However, a recent study from Mali, an endemic region, suggested that the same TLR9 agonist adjuvant together with the same recombinant subunit vaccine did not enhance memory B cells [72]. These data might be related to the findings of another study from Thailand, which found no significant difference in TLR9 expression in B cells between falciparum malaria patients and healthy controls [73]. These data may suggest the suppression of B-cell responses after chronic malaria infection. In addition, it is also possible that plasma cells may be more important and circulatory memory B cells may not be responsible for the maintenance of serum antibody titers as suggested by the recent study [69]; this requires further investigation.

An interesting phenomenon was observed with circulating plasmacytoid DCs (pDCs). In one report, *P. falciparum* malaria infected individuals had reduced numbers of circulating pDCs,

Table 1. *In vivo* modulation of Toll-like receptor 9 during malaria infection.

Outcome	Relevance to malaria infection	*Plasmodium* spp.	Ref.
Mouse/rodent malaria			
Cerebral malaria	Pathological	*Plasmodium berghei* ANKA	[56,57]
Lethal malaria	Pathological (induction of regulatory T cells)	*Plasmodium yoelii*	[65]
Hyper-responsiveness to malaria infection	Beneficial or pathological	*Plasmodium chabaudi AS*	[63]
Helps infected erythrocytes towards DC internalization	Helps antigen-specific antibody responses/ no effect on survival	*P. chabaudi AS* *P. berghei* ANKA	[48]
Effective vaccination against malaria	Required for effective vaccine against malaria	*P. yoelii*	[81]
Increase of pDC and IFN-α during infection	No effect on survival or parasitemia	*P. chabaudi*	[48]
Cerebral malaria	No effect	*P. berghei* ANKA	[58,59]
Parasitemia, splenic architecture	No effect	*P. chabaudi AS*	[62,82]
Lethal malaria	No effect	*P. yoelii*	[64]
Human malaria			
Increased risk for low-birth-weight in term infants in malaria-endemic regions	SNP/high-risk correlation	*Plasmodium falciparum*	[76]
Differential inflammatory responses to malaria infection	rSNP/modulatory effect	*P. falciparum*	[78]
Acute malaria infection	Decreased expression on pDC of patients	*P. falciparum*	[73]
Risk of severe malaria	SNP/no correlation	*P. falciparum*	[77]
Refractoriness to TLR9 agonists in vaccines against malaria in endemic region	Failure of usage of TLR9 agonists as vaccine adjuvant to recombinant antigens in endemic region	*P. falciparum*	[72]
Requirement for vaccine efficacy	Controls 'built-in' adjuvant properties in whole-parasite vaccinations	*P. falciparum*	[49]

DC: Dendritic cell; pDC: Plasmacytoid dendritic cell; rSNP: Rare single nucleotide polymorphism; SNP: Single nucleotide polymorphism; TLR: Toll-like receptor.

but higher secreted levels of IFN-α [44]. In *Plasmodium vivax* infection in humans it was reported that reduced pDC numbers could lead to poor antibody responses as well as activation of regulatory T cells [74]. Overall, these studies may suggest that continuous exposure and accumulation of hemozoin, and other related products which act through TLR9, may promote exhausted T- and B-cell responses to further TLR9 stimulation by an endogenous TLR9 ligand acting as an adjuvant, at least in certain populations. On the contrary, malarial infection may act in synergy with external stimuli to enhance innate immune responses [63,75]. Currently, it is not clear which hypothesis is relevant to the TLR9-dependent adjuvant properties of *Plasmodium* parasites.

The role of TLR9 during whole parasite vaccination studies under drug prophylaxis needs to be evaluated. As discussed earlier, sterile protection achieved by certain forms of blood-stage whole parasite vaccines, which require not only B cells but also CD4 and CD8 T cells specific to parasite antigen(s), may be attributed to its endogenous TLR9 agonistic adjuvant activity. It may be highly beneficial to use endogenous TLR9 adjuvants during immunizations as they may facilitate the establishment of complete sterile immunity to parasite challenges. However, the applicability of this approach to human vaccination in malaria-endemic areas warrants further investigations.

Recent human single nucleotide polymorphism studies from different malaria-endemic populations also warrant attention. Studies from Africa have suggested that a TLR9–1486T/C allele is associated with hemozoin-accumulated placental malaria and perhaps increases the risk of low birth weight [76], with some contradictory reports that might be due to rare cis-regulatory variants [77,78]. The same allele has been found to be associated with high parasitemia in the Amazonian region of Brazil [79]. In addition, some, but not all, African ethnic groups were found to be resistant to malaria infection due to a lower frequency of regulatory T cells and lower levels of TGF-β [80]. Further investigations via *TLR9* gene variant analysis are warranted in these populations. Overall, these polymorphism studies suggest that TLR9 modulates malaria pathogenesis and its genetics contribute to the clinical manifestations of malaria. Further studies are needed to understand how genetic polymorphism, including polymorphisms in TLR9, may affect the efficacy and safety of vaccinations in particular populations in different endemic regions.

Expert commentary

Problems with the recombinant technology in malaria vaccine trials in endemic regions have emerged, leading to an urgent need for the development of novel vaccine strategies against malaria based on an in-depth understanding of *Plasmodium* pathogenesis and host–pathogen interactions. Hence, whole-parasite vaccine approaches have been regaining attention for blood-stage malaria vaccines, along with potent and safe adjuvants. However, whole-microbe vaccines usually contain built-in adjuvants for potent activity. We have found that the whole blood-stage vaccine for malaria contains TLR9-dependent built-in adjuvant properties that can induce parasite-specific antibody responses. Careful analysis has suggested that hemozoin is responsible for this adjuvant effect. Accumulating evidence suggests that hemozoin may be used in future vaccine development against malaria and will help to develop new therapeutic targets for treating malaria infection.

Five-year view

The finding that the whole blood-stage vaccine for *P. falciparum* contains TLR9-dependent built-in adjuvant properties and that the component responsible is probably hemozoin, highlights several important questions to consider in future vaccine development against malaria. First, there is a great need to analyze whether high levels of TLR9-dependent parasite-specific antibodies induced by whole blood-stage vaccines confer long-term protective immunity to malaria. Furthermore, will these antibodies overcome the problems associated with vaccine design against an organism displaying rapid antigenic variation and polymorphism? Recently, the immunomodulatory role of TLR9 during malaria infection has been investigated in both human and mouse studies. However, the mechanisms by which TLR9 may contribute to the pathogenesis of *Plasmodium* species and the protection against malaria remain to be elucidated *in vivo*, especially in humans. Recent vaccination studies in humans from malaria-free and endemic regions [71,72] urge us to look more deeply at the roles of endogenous TLR9 adjuvants during continuous malaria exposure. This is because if exogenous TLR9 stimuli are less effective in exposed individuals than in malaria-naive humans at stimulating T and B cells, it may pose a problem for vaccination and necessitate the use of adjuvants. Thus, the findings of such studies will be crucial to the future design of successful malaria vaccines.

Acknowledgements

The authors thank members of the Akira Laboratory and the Horii Laboratory for discussions and valuable comments and R Culleton for critical reading of the manuscript.

Financial & competing interests disclosures

Cevayir Coban, Toshihiro Horii, Shizuo Akira and Ken J Ishii were supported by Japan Science and Technology as well as by grants from the Ministry of Education, Culture, Sports, Science and Technology in Japan, the RT Fund for Technology Development and Core Research for Evolutional Science and Technology (CREST). Cevayir Coban is also supported by the Bill and Melinda Gates Foundation (round 1 recipient of the Grand Challenges and Explorations grant) and the Osaka University Career Design Laboratory for Women. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Key issues

- In endemic regions, acquired immunity to *Plasmodium falciparum* parasites and their specific antigens requires time and repeated exposure to the organism as antibody responses develop slowly.
- The feasibility of whole-parasite vaccines against malaria was confirmed many years ago, however, subunit vaccine technology has now been incorporated.
- Single immunization of mice with high numbers of killed blood-stage *P. falciparum* parasites can induce high levels of parasite-specific antibodies via a Toll-like receptor (TLR)9-dependent mechanism, suggesting the existence of 'built-in' adjuvant properties.
- Hemozoin in *P. falciparum* crude extracts is the candidate molecule responsible for the TLR9-dependent adjuvant effect.
- Evidence suggests that TLR9 plays an immunomodulatory role during malaria infection, however, the mechanism(s) remain to be elucidated.
- TLR9- and hemozoin-based vaccine design may contribute to the development of successful vaccines against malaria in the future.

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INFECTIOUS DISEASE

Plasmacytoid Dendritic Cells Delineate Immunogenicity of Influenza Vaccine Subtypes

Shohei Koyama,[1,2] Taiki Aoshi,[3] Takeshi Tanimoto,[4] Yutaro Kumagai,[1] Kouji Kobiyama,[3] Takahiro Tougan,[3] Kazuo Sakurai,[5] Cevayir Coban,[1] Toshihiro Horii,[3] Shizuo Akira,[1]* Ken J. Ishii[1,3]*

(Published 31 March 2010; Volume 2 Issue 25 25ra24)

A variety of different vaccine types are available for H1N1 influenza A virus infections; however, their immunological mechanisms of action remain unclear. Here, we show that plasmacytoid dendritic cells (pDCs) and type I interferon (IFN)–mediated signaling delineate the immunogenicity of live attenuated virus, inactivated whole-virus (WV), and split virus vaccines. Although Toll-like receptor 7 acted as the adjuvant receptor for the immunogenicity of both live virus and WV vaccines, the requirement for type I IFN production by pDCs for the immunogenicity of the vaccines was restricted to WV. A split vaccine commonly used in humans failed to immunize naïve mice, but a pDC-activating adjuvant could restore immunogenicity. In blood from human adults, however, split vaccine alone could recall memory T cell responses, underscoring the importance of this adjuvant pathway for primary, but not secondary, vaccination.

INTRODUCTION

Vaccination is considered to be the best prophylaxis for influenza virus infection (*1*). There are three different types of influenza virus vaccines: live attenuated influenza virus (LAIV), formalin-inactivated whole-virus (WV) vaccine, and ether-treated hemagglutinin (HA) antigen–enriched virion-free "split" virus (SV) or "subunit" virus (SU) vaccine (*2*). Among them, SV and SU are the most commonly used in clinics because there are fewer reactogenicities, although LAIV and WV have been shown to have superior immunogenicity, especially in children (*3–5*). The immunogenicity and efficacy of these vaccines can be affected by host factors, such as age and immunological status [such as immunodeficiency (*6–8*)], and viral factors, including the antigenic mismatch between the vaccines and the circulating virus strains (*9*). However, the exact mechanisms used by the three types of vaccine compositions to achieve immunogenicity and how these mechanisms differ are not fully understood.

It is known that most, if not all, successful vaccines that induce strong protection against pathogens contain adjuvant components that activate the innate immune system via specific receptors, including Toll-like receptors (TLRs), retinoic acid–inducible gene-I (RIG-I)–like receptors (RLRs), and Nod-like receptors (NLRs) (*10–12*). These receptors are expressed by antigen-presenting cells, such as dendritic cells (DCs), which mediate the subsequent adaptive immune response by releasing specific cytokines, such as interferons (IFNs) and interleukins (ILs), and activating antigen-specific T and B cells. The nature of the adjuvant and the specific receptors that are activated on DCs can determine the type of immune response that is generated (*10–12*). Recent studies suggest that TLR7 and a certain NLR that activates the inflammasome—a collection of components that spur the innate immune system—are involved in controlling adaptive immune responses to influenza A virus infection (*13, 14*). Our previous work has characterized a key role for TLR signaling in the immune response to inactivated WV vaccination (*13*); however, no comprehensive study directly compares the role of TLRs, NLRs, and RLRs in either live virus or inactivated WV vaccinations (*15*). Here, we decipher the mechanism(s) by which the various virus preparations drive development of immunogenicity.

[1]Laboratory of Host Defense, WPI Immunology Frontier Research Center, Osaka University, Suita, Osaka 565–0871, Japan. [2]Department of Respiratory Medicine, Tohoku University Graduate School of Medicine, Sendai, Miyagi 980–8575, Japan. [3]Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565–0871, Japan. [4]The Research Foundation for Microbial Diseases of Osaka University, Kan-on-ji, Kagawa 768–0061, Japan. [5]Department of Chemical Processes and Environments, University of Kitakyushu, Kitakyushu, Fukuoka 808–0135, Japan.
*To whom correspondence should be addressed. E-mail: kenishii@biken.osaka-u.ac.jp (K.J.I.); sakira@biken.osaka-u.ac.jp (S.A.)

RESULTS

Inactivated WV requires TLR7-mediated, but not RLR- or NLR-mediated, immune activation for its immunogenicity

We first examined whether an inactivated WV preparation of the A/New Caledonia/20/1999 (NC) (H1N1) strain can immunize mice lacking TLR7, IPS-1 (IFN-β promoter stimulator–1; an adaptor protein for RIG-I–mediated type I IFN production), and ASC (apoptotic speck protein; containing a caspase recruitment domain, an adaptor molecule required for NLRP3 inflammasome activation). All three of these genes have been shown to be involved in innate immune recognition of live influenza virus infection (*14, 16–19*).

We immunized TLR7-deficient or IPS-1–deficient mice intranasally (i.n.) with WV of NC H1N1 twice at a 2-week interval. One week after the second injection, the vaccination-induced, virus-specific B cell and $CD4^+$ T cell adaptive immune responses were analyzed. TLR7-deficient mice showed virtually no virus-specific immunoglobulin A (IgA) in bronchoalveolar lavage fluid (BALF), no virus-specific IgG in serum (Fig. 1A), and no IFN-γ production from $CD4^+$ T cells specific to a viral nucleoprotein (NP) antigen epitope ($NP_{260–283}$ specific to $I\text{-}A^b$) (Fig. 1B), whereas IPS-1–deficient mice mounted comparable levels of these responses to wild-type mice (Fig. 1, A and B). Consistently, the immunized TLR7-deficient mice were not protected against a lethal challenge with the A/Puerto Rico/8/34 (PR) (H1N1) strain, whereas the immunized IPS-1–deficient mice were protected to a level comparable to vaccinated wild-type mice (Fig. 1C). We

obtained similar results with intramuscular (i.m.) immunization of these mice, with respect to total IgG titer and IgG2a titer (fig. S1). In contrast to i.n. immunization, i.m. immunization failed to elicit BALF IgA in either wild-type or TLR7-deficient mice. There was an enhanced serum IgG1 response in TLR7-deficient mice relative to wild-type mice, but this response failed to protect the TLR7-deficient mice from lethal challenge with PR H1N1 (fig. S1, A and C). Thus, the inactivated WV vaccine requires TLR7-mediated, but not RIG-I–mediated, IPS-1 for its immunogenicity, which is consistent with findings for the live virus vaccination (*13*).

We also immunized ASC-deficient mice with WV (NC H1N1) because a role for the ASC inflammasome in the adaptive immune response to influenza virus infection is controversial (*14*, *19*). ASC-deficient mice did not show any defects in their adaptive immune responses to WV immunization relative to wild-type mice (Fig. 1, D and E). Notably, ASC was not involved in the immunogenicity of live virus immunization, except for serum IgG1 production, whereas $CD8^+$ T cell responses were not observed with WV immunization (fig. S2). Immunized wild-type and ASC-deficient mice were similarly protected against lethal PR H1N1 virus challenge relative to naïve wild-type mice (Fig. 1F). Therefore, ASC inflammasome activation was not essential for the induction of virus-specific B and CD4 T cell responses to WV.

The type I IFN receptor is important for the immunogenic response to inactivated WV vaccine but not to the live virus

Because both the inactivated WV and the live virus require TLR7 for their immunogenicity, we sought to identify the downstream effector molecule(s) involved. Type I IFNs, such as IFN-α and IFN-β, are known to have potent adjuvant activity (*20*, *21*), and a recent study showed that WV immunization substantially up-regulated the expression of IFN-inducible genes, such as *Cxcl10* (*22*). Therefore, as a systemic indicator of type I IFN responses, we examined the amounts of the cytokine CXCL10 in the sera of mice 24 hours after vaccination. The induction of CXCL10 was significantly reduced in TLR7-deficient mice relative to wild-type, IPS-1–deficient, and ASC-deficient mice (Fig. 2A). Similar results were obtained for messenger RNA (mRNA) analyses of IFN-β and CXCL10 in the lung (fig. S3A), suggesting that type I IFNs might be dominant effector molecules in this TLR7-dependent system. To test this hypothesis directly, we i.n. immunized mice deficient in the IFN-α and IFN-β receptor 2 (IFNAR2) with WV as in Fig. 1. IFNAR2-deficient mice failed to induce virus-specific antibodies (including BALF IgA and serum IgG) and $CD4^+$ T cell responses (Fig. 2B and fig. S3, B and C) relative to the heterozygous IFNAR2 knockout mice. As a result, mortality was increased and a significantly larger body weight loss was observed after lethal PR H1N1 challenge in the IFNAR2-deficient mice (Fig. 2C). Naïve wild-type and IFNAR2-deficient mice showed similar susceptibilities to PR H1N1 challenge, consistent with a previous study (*23*). Similar results were also observed after i.m. immunization (fig. S3, D and E). When naïve IFNAR2-deficient mice were immunized with live virus, there was no alteration of the virus-specific serum IgG and IFN-γ secretion by virus NP antigen-specific $CD4^+$ T cells ($NP_{260–283}$ specific to $I\text{-}A^b$) and $CD8^+$ T cells ($NP_{366–374}$ specific to $H\text{-}2D^b$) (fig. S3, F to I), consistent with a previous study (*23*). Together, these results suggest that TLR7, but not RLRs or NLRs, is required for immunogenicity of inactivated as well as live influenza virus vaccination. In addition, the type I IFN receptor–mediated signaling pathway was critical for the immunogenic response to WV but not to the live virus.

Live virus and inactivated WV vaccines induce type I IFNs through distinct DC types

DCs are a critical component of the innate immune system that recognize vaccines and mediate the adaptive immune response. There are different subtypes of DCs that can be divided loosely into conventional myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), which express different receptors and secrete different cytokines on activation. To characterize in more detail the immune response triggered by the WV vaccine and live virus, we stimulated two types of bone marrow–derived DCs—Flt3

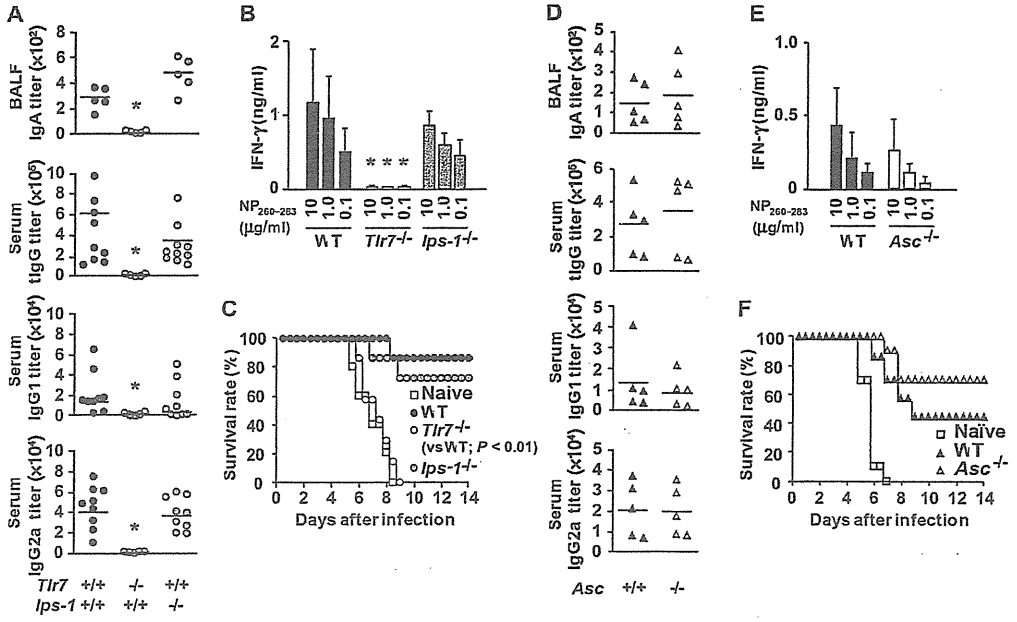

Fig. 1. TLR7-dependent, but not IPS-1–dependent or ASC-dependent, signaling is required for the induction of protective immune responses by inactivated WV. (**A** to **C**) Wild-type (WT), *Tlr7*-deficient, and *Ips-1*–deficient mice ($n = 9$ per group) were i.n. vaccinated with WV of NC (1.5 μg per mouse) twice, with a 2-week interval. One week after the second vaccination, we measured titers of antigen-specific mucosal (BALF) IgA and serum total IgG (tIgG), IgG1, and IgG2a (A) and IFN-γ production by $CD4^+$ T cells (B) by ELISA as described in Materials and Methods. $^*P < 0.05$ versus WT mice. (C) Vaccinated mice ($n = 9$ per group) were challenged with $10 \times LD_{50}$ (median lethal dose) (2×10^4 PFU per mouse) of lethal influenza virus PR, and their survival was monitored. $^*P < 0.05$ versus vaccinated WT mice. (**D** to **F**) WT and *Asc*-deficient mice ($n = 5$, each group) were similarly vaccinated, and their antigen-specific antibody responses (D), IFN-γ production by $CD4^+$ T cells (E), and survival (WT, $n = 7$; *Asc*-deficient, $n = 10$; naïve, $n = 10$) (F) were determined by ELISA as described in Materials and Methods. Each bar represents the mean (A and D) or mean ± SD (B and E). These results are representative of two independent experiments.

ligand–generated DCs (FL-DCs; which contain pDCs) and granulocyte-macrophage colony-stimulating factor (GM-CSF)–generated DCs (GM-DCs; which contain conventional DCs but no pDCs)—with the live virus or WV and then measured their IFN-β production by enzyme-linked immunosorbent assay (ELISA). The live virus strongly stimulated FL-DCs to produce IFN-β in a TLR7-dependent manner, whereas production by GM-DCs was in a TLR7-independent manner (Fig. 2D), consistent with our previous study (*13*). In contrast, WV-activated FL-DCs, but not GM-DCs, produced IFN-β, a process entirely dependent on TLR7 signaling (Fig. 2D), indicating that there is a clear distinction between the live virus and WV in terms of their abilities to activate DCs to secrete type I IFNs.

To confirm these in vitro observations in vivo, we next depleted pDCs in vivo to examine the role of these cells in the induction of adaptive immune responses to live virus and WV, as pDCs are known to play a key role in bridging the innate and adaptive immune responses (*24*). Wild-type mice were treated intravenously with an antibody to mPDCA-1 (*25*) and then immunized 24 hours later with the live virus or WV. Depletion of pDCs was confirmed in the spleen (fig. S4). After live virus vaccination, the concentrations of mRNAs derived from genes involved in induction of the adaptive immune response, specifically *Ifnb*, *Cxcl10*, *Il6*, and *Ccl2*, were clearly elevated in the lung irrespective of treatment with antibody to mPDCA-1 (Fig. 2E). In contrast, these transcriptional responses were severely impaired after WV vaccination in pDC-depleted mice relative to isotype control antibody–treated mice (Fig. 2E). Serum CXCL10 was also reduced in pDC-depleted mice treated with WV but not the live virus (Fig. 2F). Thus, both live virus and WV induced type I IFNs predominantly through pDCs in vivo; however, WV was dominantly recognized by pDCs, whereas live virus could also stimulate other cell types to activate innate immune responses.

Although it was previously reported that pDC activation is not essential for the induction of adaptive immune responses in live influenza virus infection (*26, 27*), pDC depletion specifically rendered the inactivated WV nonimmunogenic, as measured by virus-specific IgG concentrations in serum (Fig. 3A). In these experiments, mice were treated with an antibody to mPDCA-1 twice at both the primary and the secondary vaccinations. To further examine the more detailed role of pDCs in the primary and/or secondary vaccinations, we treated mice with an antibody to mPDCA-1 at either the primary or the secondary (boost) vaccination. Virus-specific mucosal IgA, serum IgG (Fig. 3B), and $CD4^+$ T cell IFN-γ (Fig. 3C) were significantly impaired when pDCs were depleted in the primary, but not in the secondary, vaccination with the inactivated WV. Thus, pDC activation is essential for inducing B cell and $CD4^+$ T cell responses to the inactivated WV during primary, but not secondary, vaccination. By sharp contrast, pDC activation at priming was not required for inducing B cell and $CD4^+$ T cell responses with the live virus.

WV-loaded pDCs were sufficient to transfer immunogenicity to naïve mice, which requires intrinsic as well as extrinsic type I IFN signaling

To further examine the role of pDCs, we performed cell transfer experiments. FL-DCs from wild-type mice were separated into two populations, namely, a B220 (CD45R)–enriched population containing pDCs and a B220-depleted population containing virtually no pDCs (as indicated in Fig. 3D). The cell populations were pulsed with WV and injected intravenously into wild-type mice. The virus-specific IgG concentrations elicited by the B220-enriched FL-DCs were significantly higher than those elicited by the B220-depleted population (Fig. 3D). In addition, when we transferred B220-enriched FL-DCs derived from the IFNAR2-deficient mice (lacking type I IFN) into wild-type mice or vice versa, virus-specific IgG induction was significantly impaired in both cases (Fig. 3E). We also confirmed that IFNAR2-deficient FL-DCs secreted significantly less type I IFN relative to that of FL-DCs from the heterozygous IFNAR2 knockout mice (fig. S5A). When we tested TLR7 deficiency with

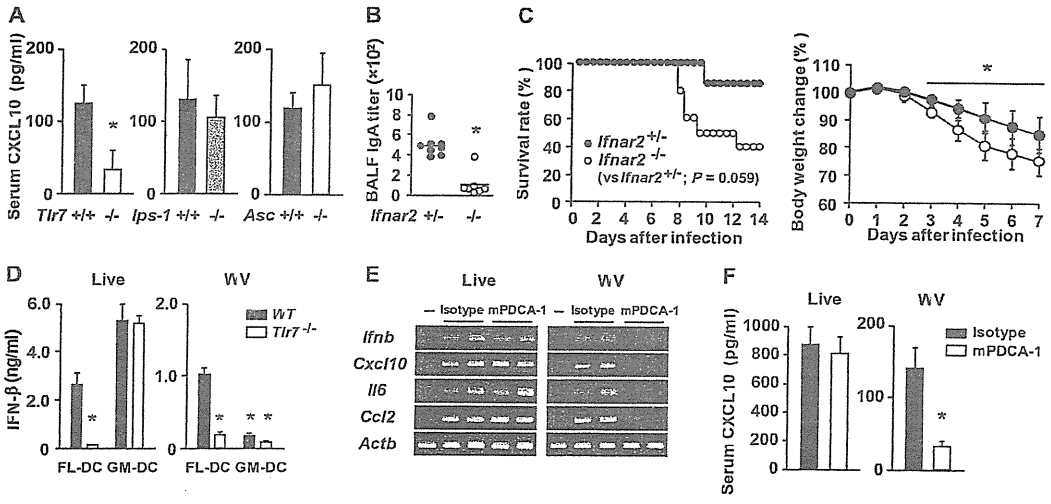

Fig. 2. Critical roles of type I IFN receptor–mediated signaling and pDC activation in inducing adaptive immune responses to the inactivated WV vaccine but not to the live virus. (**A**) *Tlr7*-deficient, *Ips-1*–deficient, *Asc*-deficient, and control mice ($n = 3$ per group) were i.n. vaccinated with WV (3.0 μg per mouse), and CXCL10 production in sera was measured by ELISA after 24 hours. *$P < 0.05$ versus control mice. (**B** and **C**) Control ($Ifnar2^{+/-}$) and type I IFN receptor–deficient ($Ifnar2^{-/-}$) mice ($n = 8$, each group) were i.n. vaccinated with WV (1.5 μg per mouse) as in Fig. 1, and BALF IgA (B) was measured by ELISA. *$P < 0.05$ versus control mice. The mice were then infected with lethal influenza PR at $10 \times LD_{50}$, and their survival and body weight (C) were monitored. *$P < 0.05$ versus control mice. (**D**) Type I IFN production by FL-DCs and GM-DCs from WT and *Tlr7*-deficient mice in response to the live virus [multiplicity of infection (MOI) = 10] and WV (5 μg/ml). IFN-β production was measured by ELISA 24 hours after the stimulation. *$P < 0.05$ versus WT FL-DC. (**E** and **F**) Innate immune responses to WV in pDC-depleted mice. Mice were treated with an antibody to mPDCA-1 24 hours before inoculation and then i.n. challenged with the live virus (1×10^5 PFU per mouse) or WV vaccine (5 μg per mouse). The expression of IFN-β, CXCL10, IL-6, and CCL2 mRNA in the lungs (E) and CXCL10 in sera (F) 24 hours after vaccination was measured by RT-PCR and ELISA. *$P < 0.05$ versus control mice. These results are representative of at least two independent experiments.

the same approach, we observed a substantial TLR7 signaling dependency in these FL-DC transfer experiments (fig. S5B). These results suggest that type I IFN–mediated signaling in pDCs, as well as in the recipient as yet unidentified cell type(s), is indispensable for eliciting the adaptive immune response to WV.

Split vaccine does not protect naïve mice, but immunogenicity can be improved with a pDC-activating adjuvant while it recalls memory T cells in human adult blood

Currently, the most widely used influenza vaccines in many countries comprise SV or SU, which mainly consist of purified protein antigens such as HA and neuraminidase. As mentioned earlier, vaccination of mice with SV led to significantly lower production of type I IFNs and related chemokines, such as CXCL10, at both the mRNA and the protein levels in the lung and serum, respectively (Fig. 4, A and B). SV also failed to activate DCs to produce type I IFNs in vitro (fig. S6A). These data suggest that the intrinsic TLR7 ligand (that is, viral genomic RNA) was lost during the SV production process. In support of this idea, removal of the RNA content from WV by ribonuclease treatment significantly decreased the TLR7-mediated type I IFN production by pDCs (fig. S6B). The reduced immunostimulatory activity of SV was associated with its diminished immunogenicity. When naïve mice were immunized with SV at the same dose, adjusted to the HA content (fig. S6C), as WV, the HA-specific IgG and CD4 T cell responses were significantly lower than those elicited by WV (Fig. 4, C and D). Together, these data strongly suggest that SV loses its built-in TLR7 adjuvant (viral genome RNA) during purification of WV, consistent with a recent study for the H5N1 virus (*28*).

Our results thus far raise the possibility of improving SV immunogenicity by adding a pDC-activating TLR ligand. Because pDCs express both TLR7 and TLR9, we examined whether addition of a TLR9 ligand to the "adjuvant-lost" split vaccine would replace the natural TLR7-mediated pDC activation. We used a second-generation TLR9 ligand of CpG DNA complexed with β-(1→3)-D-glucan, namely, schizophyllan (SPG) (*29*). This new TLR9 ligand is more potent and durable than naked CpG DNA, and it still retains the TLR9 ligand activity. Mice were i.n. immunized with WV, SV, or SV plus the SPG-CpG DNA conjugate (SV+SPG-CpG) and then evaluated for their adaptive immune responses. The SV+SPG-CpG induced robust type I IFN responses independently of TLR7 (Fig. 4, A and B). Correspondingly, the SV+SPG-CpG successfully enhanced HA-specific B cell and $CD4^+$ T cell responses to levels comparable to those from WV immunization of wild-type mice (Fig. 4, C and D). Immunization of TLR9-deficient mice provided further evidence that the responses induced by the SV+SPG-CpG were dependent on TLR9 but not on TLR7 (fig. S7A).

The protective efficacies of these three types of vaccines were also examined in mice. WV conferred protection against lethal PR H1N1 virus challenge in a TLR7-dependent and TLR9-independent manner, whereas the SV+SPG-CpG provided protection in a TLR9-dependent and TLR7-independent manner (Fig. 4E and fig. S7B). Notably, the original SV failed to provide protection against lethal PR H1N1 virus challenge in any of the groups of mice examined (Fig. 4E and fig. S7B). We also confirmed that the restored protective effect of the SPG-CpG adjuvant was mediated by type I IFN responses because IFNAR2-deficient mice failed to mount virus-specific B and T cell responses (fig. S7, C and D) and demonstrated no improved protection against infection (fig. S7E).

Although the usefulness of SV vaccination in the healthy adult human population has been recognized in many studies, our results are somewhat contradictory. Therefore, we tested the relevance of these observations in a human system. Human peripheral blood mononuclear cells (PBMCs) from healthy volunteers were stimulated with H1N1 live virus, inactivated H1N1 WV, and H1N1 SV for 24 hours, and then IFN-α and IFN-γ secretion was measured by ELISA. Consistent with the mice data, IFN-α was secreted with live virus and WV but not with SV stimulation (Fig. 4F). Depletion of pDCs with BDCA4 microbeads revealed that this IFN-α secretion was totally dependent on pDC in WV and partially in live virus stimulation (Fig. 4F). On the

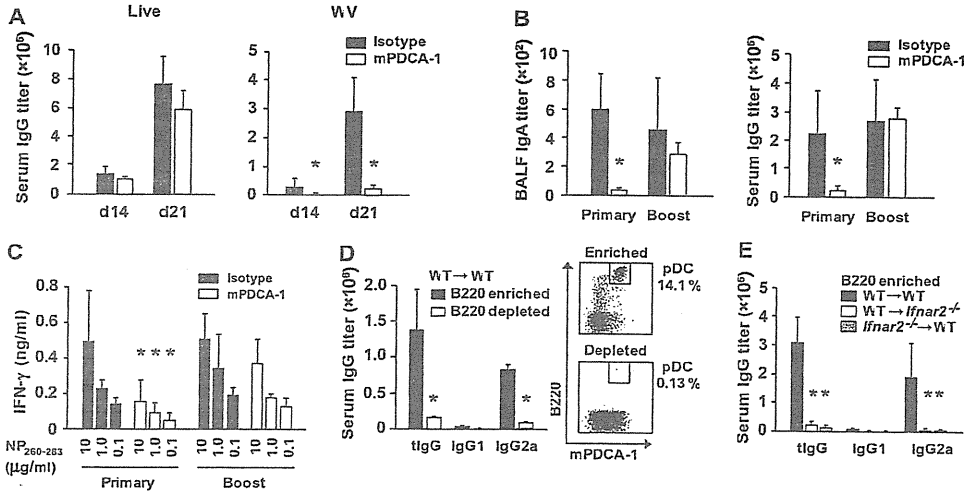

Fig. 3. Immunogenicity of the inactivated WV, but not of the live virus, depends on pDC activation at the primary vaccination. (**A**) The adaptive immune responses to WV (i.n.) were monitored in pDC-depleted mice ($n = 3$ per group). Mice were injected with an antibody to mPDCA-1 or isotype control antibody 24 hours before each vaccination, and serum IgG concentrations were measured by ELISA at the indicated times. *$P < 0.05$ versus control mice. (**B** and **C**) To determine whether pDCs are more important for priming or boosting after vaccination with inactivated WV, we treated one group of mice ($n = 3$) only with an antibody to mPDCA-1 before the primary vaccination (priming) and another group ($n = 3$) was treated only before the secondary vaccination (boosting). BALF IgA and serum IgG 1 week after the boost (B) and IFN-γ production from influenza-specific $CD4^+$ T cells (C) were measured by ELISA. *$P < 0.05$ versus control mice. (**D** and **E**) FL-DCs were separated into $B220^+$ and $B220^-$ DCs by MACS. Each DC was pulsed with WV in vitro, and $B220^+$ or $B220^-$ cells (5×10^5) were intravenously injected ($n = 3$ per group). Serum IgG was measured 2 weeks after the injection in WT mice. *$P < 0.05$ versus B220-enriched cells. (D) $B220^+$ cells (1×10^5) from WT and *Ifnar2*-deficient mice were intravenously transferred into untreated WT and *Ifnar2*-deficient mice ($n = 3$ per group), and serum IgG was measured by ELISA. *$P < 0.05$ versus WT to WT. (E) These results are representative of at least two independent experiments.

other hand, both WV and SV induced IFN-γ secretion comparably in the PBMC preparations even when the SV made from the swine-origin H1N1 A/California/04/2009 strain was used. Results obtained after $CD4^+$ and $CD8^+$ T cell depletion of the PBMC preparations revealed that virus-specific IFN-γ secretion was produced mainly by $CD4^+$ T cells in live virus and WV stimulation (Fig. 4G). These results suggest that SV could efficiently stimulate memory T cell responses without type I IFNs in a naturally (or seasonally) influenza virus–exposed human population.

DISCUSSION

Although TLR7 and certain NLRs have been shown previously to be involved in the induction of adaptive immune responses to influenza A virus infection (*13*, *14*), the current work represents a comprehensive study that directly compares the functions of TLRs, NLRs, and RLRs in the immunogenicity and efficacy of influenza inactivated WV vaccinations (Fig. 1 and fig. S1). We identified pDCs as an innate immune cell and type I IFNs as humoral factors that are essential for the immunogenicity of the inactivated WV vaccine (Figs. 2 and 3). Although our results demonstrate an essential role for pDCs in inactivated WV vaccination, other studies have identified a redundant role for pDCs in antiviral responses to live virus vaccination such as influenza virus (*26*, *27*). In addition, although TLR7 is expressed in a variety of cell types, including B cells and macrophages, our results strongly suggest an essential role for pDCs in mediating TLR7-induced innate and adaptive immune responses to inactivated influenza WV vaccination but not to live virus vaccination.

The critical role of pDCs in vaccine priming, but not in boosting, is apparent from results of the pDC depletion study, in which pDCs were removed from mice before vaccination with inactivated WV (Fig. 3, A to C). These findings parallel our in vitro data, in which the pDC-containing FL-DC preparation, but not the mDC-dominant GM-DC preparation, responded to inactivated WV to produce type I IFNs (Fig. 2D); however, these data do not necessarily exclude the involvement of other antigen-presenting cells for either priming or boosting. For example, pDCs pulsed with inactivated WV in vitro can prime naïve mice (Fig. 3D), but the type I IFNs produced by these pDCs were required not only for stimulating the pDCs themselves but also for priming as yet unidentified cell types of the recipient mouse (Fig. 3E). This suggests that cross talk exists between pDCs and the other as yet unidentified cells via type I IFN–mediated signaling. In addition, it will be of interest to examine whether other DCs, such as mDCs, are involved in this intercellular cross talk in such a way that pDCs can transfer the flu antigens to mDCs or that pDCs can present antigens directly to T cells and/or B cells. We note the distinct regulation of type I IFN induction by inactivated WV vaccination, which differed from IFN induction by live virus vaccination. Indeed, although type I IFNs were induced by both inactivated and live virus vaccinations, inactivated WV vaccines activated only TLR7 on pDCs,

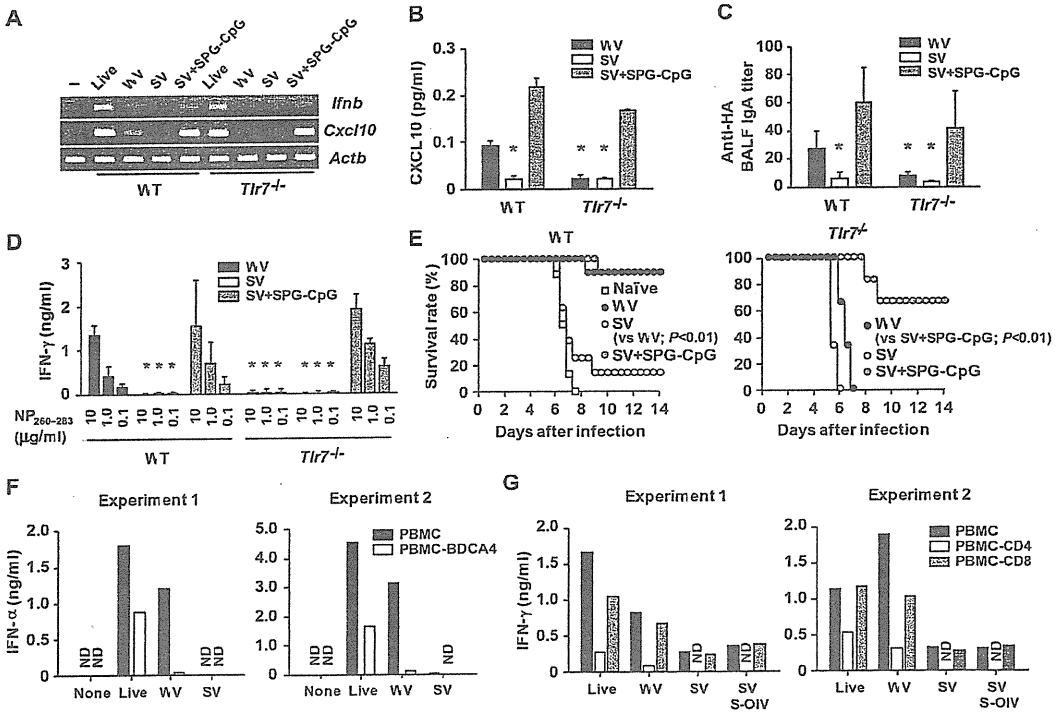

Fig. 4. Immunogenicity differences between WV and SV depend on type I IFN production via pDC activation in naïve hosts but not in primed hosts. (**A** and **B**) WT and *Tlr7*-deficient mice were i.n. vaccinated with three different vaccines as described in Materials and Methods. After 24 hours, IFN-β and CXCL10 expression in the lungs (A) and CXCL10 production in the sera (B) was measured by RT-PCR and ELISA. $^*P < 0.05$ versus WT mice immunized with WV. (**C** and **D**) To compare the immunogenicities of the three vaccines, we i.n. vaccinated WT ($n = 9$) and *Tlr7*-deficient ($n = 6$) mice as described in Fig. 1 and determined antigen (HA)–specific BALF IgA, serum total IgG (C), and IFN-γ production by $CD4^+$ T cells (D) by ELISA. $^*P < 0.05$ versus WT mice immunized with WV. (**E**) Vaccinated mice were challenged with the PR strain at $10 \times LD_{50}$, and their survival rates were determined as described in Materials and Methods. $^*P < 0.05$ versus WT mice immunized with WV or control mice. These results are representative of at least two independent experiments. (**F** and **G**) IFN-α and IFN-γ production in response to inactivated influenza vaccines in human PBMCs. PBMCs from healthy volunteers were stimulated with live NC virus, WV, and SV. Total PBMCs and pDC-depleted PBMCs (PBMC-BDCA4) were stimulated with live NC virus (0.01 MOI), WV (1.0 μg/ml), and SV (0.5 μg/ml), and IFN-α production was measured by ELISA (F). Total PBMCs and CD4- or CD8-depleted PBMCs were stimulated with WV and SV of NC (10 and 5 μg/ml, respectively) and SV of swine-origin influenza virus (SV S-OIV) (5 μg/ml). IFN-γ production was measured by ELISA (G). These results are from 2 representatives of 10 volunteers.

whereas live virus activated both TLR7 on pDCs and other TLR7-independent pathways in the other cells, possibly mDCs (Fig. 2, D and E). Therefore, although TLR7, pDCs, and type I IFNs all were essential for inactivated WV vaccination, pDCs and type I IFNs were not essential for live virus vaccination.

The adaptor ASC is a critical component of NLRP3 inflammasome (*30*). In contrast to type I IFN responses, ASC-dependent inflammasome activation has been shown to play a critical role in the survival of the mice challenged with live influenza virus (*14*, *18*, *19*). However, the requirement for inflammasome activation to induce influenza-specific adaptive immune responses has been controversial (*14*, *19*). Our data indicate that ASC-dependent inflammasome activation is dispensable for inducing adaptive immune responses to WV and live virus, except for IgG1 production in live virus vaccination (Fig. 1 and fig. S2). Concurrent analysis comparing three innate immune signaling pathways, TLR, NLR, and RLR, enabled us to elucidate that the TLR-dependent pathway dominantly controlled the T helper 1–type protective immunity elicited by WV and live virus vaccination.

Although SV, which is now used as the first choice for influenza vaccination in many countries, was not protective in naïve mice, its decreased immunogenicity was fully restored by adding a new TLR9 ligand that stimulates pDCs to secrete type I IFNs (Fig. 4, A to E, and fig. S7). These data above further support the notion that pDC activation and their type I IFN production play a critical role in the induction of inactivated influenza vaccine immunogenicity in naïve hosts. These results might explain in part the well-known fact that the efficacy of adjuvant-less SV is lower in young children than in adults (*7*), in which SV is simply boosting the memory T and/or B cell responses. This is further supported by our results obtained using human PBMCs (Fig. 4, F and G), which suggest that most human adults have virus-specific $CD4^+$ T cells that produce IFN-γ in response not only to seasonal flu viruses but also to the novel swine H1N1 virus. Our results also indicate that memory T cells react to both internal proteins, such as those in SV, and a wide spectrum of influenza virus surface antigens, such as those on swine-origin H1N1 (*31*, *32*) and H5N1 (*33*). The age distribution of the affected population in swine-origin H1N1 and H5N1 infections, which was limited to the young, might reflect the importance of memory T cells established by recurrent exposure to seasonal influenza live viruses and vaccines (*34*, *35*)

LAIVs activate both influenza-specific IgA-secreting B cells and cytotoxic $CD8^+$ T cells (*36*), which provides certain advantages over inactivated vaccines including WV and SV. Although WV is now unavailable for seasonal influenza, it is cost-effective and can induce heterosubtypic protection not only against a challenge by H1N1 (Fig. 1C and fig. S1C) but also against H5N1 (*37*, *38*), as with LAIV (*39*). In addition, recent progress in manufacturing techniques could reduce the adverse event rate in i.m. WV immunization (*37*, *38*) to yield results that are quite different from those of past clinical trials (*3*, *40*). An i.n. WV immunization may produce a sufficient combination of efficacy, safety, and utility for both seasonal and pre-pandemic vaccines (*41*–*45*).

Together, analysis of the molecular and cellular mechanisms of different influenza vaccines provides useful information for improving vaccine immunogenicity and efficacy, as well as for choosing an appropriate form of influenza vaccine with a rational safety approach.

MATERIALS AND METHODS

Animals, cells, viruses, and reagents

The generation of *Tlr7*-, *Ips-1*–, *Ifnar2*-, and *Tlr9*-deficient mice, either on a 129/Ola × C57/BL6 or on a C57/BL6 background, has been described previously (*13*, *46*). ASC-deficient mice were a gift from V. M. Dixit (*47*).

All animal experiments were performed in accordance with the institutional guidelines for the Osaka University animal facility.

Purified influenza viruses, H1N1 (PR and NC), a recombinant HA protein of PR, and both inactivated WV and split vaccines of NC were prepared as previously described (*48*). Both types of vaccines were derived from the NC strain. Briefly, the viruses were purified from allantoic fluid by filtration (0.45 μm) followed by sedimentation through a linear sucrose gradient. For formalin-inactivated WV vaccines, purified viruses were treated with 0.1 to 0.2% formalin at 4°C for a week. For the ether-split vaccines (SV), the viruses were mixed with an equal volume of ether and then incubated for 30 min at room temperature with stirring. The mixture was centrifuged (3000 rpm, 15 min), and the aqueous phase was collected and evaporated. CpG DNA forming a triple helix with SPG, a natural polysaccharide composed of β-(1→3)-D-glucan, was used as the second-generation TLR9 ligand as previously described (*29*, *49*, *50*). DCs were prepared as described previously. Briefly, bone marrow cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 mM 2-mercaptoethanol, and human Flt3 ligand (100 ng/ml) (PeproTech) or murine GM-CSF (10 ng/ml) (PeproTech) for 7 to 9 days to use as FL-DCs and GM-DCs.

Influenza virus infection and vaccination

For influenza virus infection or vaccination, mice were anesthetized and administered i.n. with 30 μl of phosphate-buffered saline (PBS) (15 μl for each nares) containing serial amount of influenza NC viruses and vaccines. Mice were infected with 1×10^5 to 2×10^5 plaque-forming units (PFU) of virus per mouse or vaccinated with WV (1.5 to 3.0 μg per mouse) or SV (0.75 μg per mouse) with or without SPG-CpG (30 μg per mouse) twice at a 2-week interval. For the analysis of protection, mice were infected with the indicated doses of lethal PR strain.

Measurement of innate immune responses

Reverse transcription polymerase chain reaction (RT-PCR) was performed to measure mRNA expression levels of type I IFNs, cytokines, and chemokines using the RNA of the stimulated cells as previously described (*13*). Protein concentrations of IFN-α, IFN-β, and CXCL10 in the culture supernatants of the stimulated cells were measured using ELISA kits (IFN-α and IFN-β, PBL Biomedical Laboratories; CXCL10, R&D Systems).

Plasmacytoid DC depletion and cell transfer

Plasmacytoid DCs were depleted by intravenous injection of antibody to mPDCA-1 (500 μg) (Miltenyi Biotec) 24 hours before live virus infection or inactivated WV vaccination.

FL-DCs were separated into two populations, B220-enriched and B220-depleted population, by B220 antibody MACS microbeads (Miltenyi Biotec) according to the manufacturer's protocol to obtain B220-enriched FL-DC. Each cell population was incubated with WV (5 to 10 μg/ml) for 3 hours, and 1×10^5 to 5×10^5 cells per mouse

were injected intravenously into each type of mice. Immunological assays were performed 2 weeks after injection.

Confirmation of pDC depletion in spleen by flow cytometric analysis

After Fc blocking with an antibody to CD16/32, isolated spleen cells were stained with fluorescein isothiocyanate (FITC)–conjugated antibody to CD11c, phycoerythrin (PE)–conjugated antibody to CD45R/B220, and allophycocyanin-conjugated antibody to mPDCA-1 (Miltenyi Biotec) for 30 min at room temperature and washed with PBS containing 1% bovine serum albumin. Just before fluorescence-activated cell sorting (FACS) analysis using FACSCalibur and CellQuest software (BD Biosciences), 7-aminoactinomycin D (BD Biosciences) was added.

Measurement of antigen-specific T and B cell responses

After two i.n. vaccinations, B cell–mediated humoral responses were measured as immunoglobulin production by ELISA using goat antibody to mouse total IgG, IgG1, IgG2a, and IgA conjugated to horseradish peroxidase (Southern Biotech) as previously described (*1*). T cell–mediated cellular responses were monitored by measuring $NP_{260-283}$/I-A^b–specific or $NP_{366-374}$/H-$2D^b$–specific IFN-γ secretion of splenocytes and the frequency and cytotoxicity of H-$2D^b$–specific CD8 T cells as described previously (*13*).

Preparation of human PBMCs for cytokine analysis

PBMCs were obtained from 10 healthy adult volunteers (30 to 50 years old, 6 males and 4 females). All of the experiments using human PBMCs were approved by the Institutional Review Board of the Research Institute for Microbial Diseases, Osaka University. Cells were purified from heparinized blood by density centrifugation using Ficoll-Paque Plus (Amersham). Human pDCs, CD4, or CD8 T cells were depleted with BDCA4 and CD4 or CD8 antibody MACS microbeads (Miltenyi Biotec), respectively, according to the manufacturer's protocol. Plasmacytoid DC depletion was confirmed by FACS analysis staining with FITC-conjugated antibody to BDCA2 and PE-conjugated antibody to CD123 (Miltenyi Biotec). PBMCs or pDC-depleted PBMCs (1×10^6 to 2×10^6 cells) were stimulated with each influenza vaccine at the indicated concentration. Twenty-four hours later, IFN-α and IFN-γ (R&D Systems) were measured in supernatants by ELISA according to their manufacturers' protocol.

Statistical analysis

Statistical significance ($P < 0.05$) between groups was determined using the Student's *t* test. A survival curve was generated using Kaplan-Meier methodology, and the susceptibility of mice after infection was compared using the log-rank test.

SUPPLEMENTARY MATERIAL

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Materials and Methods
Fig. S1. TLR7-dependent, but not IPS-1–dependent, signaling is required for the induction of protective immune responses with inactivated WV vaccine by i.m. immunization.
Fig. S2. ASC-dependent inflammasome activation was dispensable for adaptive immune response to influenza virus infection, except for systemic IgG1 production.
Fig. S3. Type I IFN receptor–mediated signaling was indispensable for adaptive immune response to WV but not to the live virus.
Fig. S4. Plasmacytoid DC depletion by mPDCA-1 antibody was confirmed in spleen.
Fig. S5. Type I IFN interaction between pDCs and other immune cells was required for WV vaccine immunogenicity.
Fig. S6. Different manner of type I IFN response to WV vaccine and split vaccine is dependent on the presence of the viral genome RNA.
Fig. S7. Indispensable role of type I IFN–mediated signaling in vaccination with split vaccine plus SPG-CpG.
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51. **Acknowledgments:** We thank T. Nukiwa, S. Uematsu, F. Takeshita, H. Kumar, T. Kawai, Y. Kawaoka, and T. Nakaya for providing critical materials and helpful suggestions. **Funding:** Japan Society for the Promotion of Science Research Fellowships for Young Scientists (S.K.); Knowledge Cluster Initiative (K.J.I. and T.H.); Grant-in-Aid for Scientific Research (KAKENHI) (K.J.I. and C.C.); Target protein project (K.J.I. and S.A.); Ministry of Education, Culture, Sports, Science and Technology; and Core Research for Evolutional Science and Technology, Japan Science and Technology Agency (K.S. and K.J.I.). **Competing interests:** Some of the authors have filed a patent application: Immunostimulatory DNA complex with β-1,3-D-glucan. Inventors: K.S., S. Shinkai, N. Shimada, K.J.I., and S.A. Applicant ERATO, SORST/JST JP2006-282148. The authors declare no other competing interests.

Submitted 22 December 2009
Accepted 11 March 2010
Published 31 March 2010
10.1126/scitranslmed.3000759

Citation: S. Koyama, T. Aoshi, T. Tanimoto, Y. Kumagai, K. Kobiyama, T. Tougan, K. Sakurai, C. Coban, T. Horii, S. Akira, K. J. Ishii, Plasmacytoid dendritic cells delineate immunogenicity of influenza vaccine subtypes. *Sci. Transl. Med.* **2**, 25ra24 (2010).

JOURNAL OF INTERFERON & CYTOKINE RESEARCH
Volume 31, Number 11, 2011

DOI: 10.1089/jir.2010.0132

Interferon-Inducible Antiviral Protein MxA Enhances Cell Death Triggered by Endoplasmic Reticulum Stress

Akiko Numajiri Haruki,* Tadasuke Naito,* Tomomi Nishie, Shoko Saito, and Kyosuke Nagata

Human myxovirus resistance gene A (MxA) is a type I interferon-inducible protein and exhibits the antiviral activity against a variety of RNA viruses, including influenza virus. Previously, we reported that MxA accelerates cell death of influenza virus-infected cells through caspase-dependent and -independent mechanisms. Similar to other viruses, influenza virus infection induces endoplasmic reticulum (ER) stress, which is one of cell death inducers. Here, we have demonstrated that MxA enhances ER stress signaling in cells infected with influenza virus. ER stress-induced events, such as expression of *BiP* mRNA and processing of *XBP1* mRNA, were upregulated in cells expressing MxA by treatment with an ER stress inducer, tunicamycin (TM), as well as influenza virus infection. TM-induced cell death was also accelerated by MxA. Furthermore, we showed that MxA interacts with BiP and overexpression of BiP reduces MxA-promoted ER stress signaling. Because cell death in virus-infected cells is one of ultimate anti-virus mechanisms, we propose that MxA-enhanced ER stress signaling is a part of the antiviral activity of MxA by accelerating cell death.

Introduction

THE INTERFERON (IFN) SYSTEM plays a central role in host defense against virus infection. IFN-inducible proteins exhibit antiviral roles through translation inhibition, viral RNA metabolism, and so on (García-Sastre and Biron 2006; Sadler and Williams 2008). Furthermore, it was shown that cell death of virus-infected cells, which is one of the ultimate host defense systems, was promoted by some of IFN-inducible proteins, including human myxovirus resistance gene A (MxA) protein (MxA) (Castelli and others 1997; Gil and others 2002; Numajiri and others 2006). MxA is one of the major IFN-inducible proteins and plays a distinct role in the IFN type I-mediated response in cells infected with a variety of viruses such as orthomyxovirus, paramyxovirus, rhabdovirus, togavirus, bunyavirus, coxsackie virus, and hepatitis B virus (Haller and others 2007; Sadler and Williams 2008). A variety of antiviral activities are associated with MxA, although the exact action mechanism is unclear. The anti-viral activity appears to vary depending on the nature of the infecting viruses (Haller and others 2007; Sadler and Williams 2008). MxA may also promote cell death infected with influenza virus (Numajiri and others 2006).

Virus infection causes destruction of infected cells, and induces apoptosis. Most cells undergo cell death while also producing pro-inflammatory cytokines to spread the alarm to their neighboring cells. In turn, viruses employ strategies to regulate the mitochondrial checkpoint for apoptosis, in particular by altering the balance of pro-apoptotic and pro-survival protein levels, by either producing pro-survival inhibitors to lead to cellular death or expressing proteins to maintain cellular survival such as viral Bcl-2 homologs (Galluzzi and others 2008; Postigo and Ferrer 2009).

Several viral proteins have been shown to induce apoptosis through direct effect on mitochondrial compartment or cellular factors. The influenza virus PB1-F2 protein enhances cell death by interaction with ANT3 and VDAC1 proteins at inner and outer mitochondrial membranes, respectively (Zamarin and others 2005). The transforming growth factor-β (TGF-β) activity increases in cells infected with influenza virus. Viral neuraminidase (NA) activates TGF-β, a known inducer of apoptosis, by elimination of sialic acid residues attached to carbohydrates on the latent TGF-β binding protein, which is associated with pro-TGF-β (Schultz-Cherry and Hinshaw 1996; Morris and others 1999). This allows the subsequent removal of the carbohydrate, a pre-determinant for the proteolytic cleavage of pro-TGF-β and release of the active molecule. Viral NS1 and M1 proteins are implicated in modulating apoptotic responses in infected cells. NS1 appears to downregulate apoptosis, although it can induce apoptosis when expressed from a plasmid in absence of virus replication (Morris and others 2002). M1 binds directly with and may inhibit caspase-8 (Zhirnov and others 1999; Timofeeva and others 2001). However, M1 can induce apoptosis in cells expressing this protein from a plasmid. In addition, efficient viral

Department of Infection Biology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan.
*These two authors contributed to this work equally.