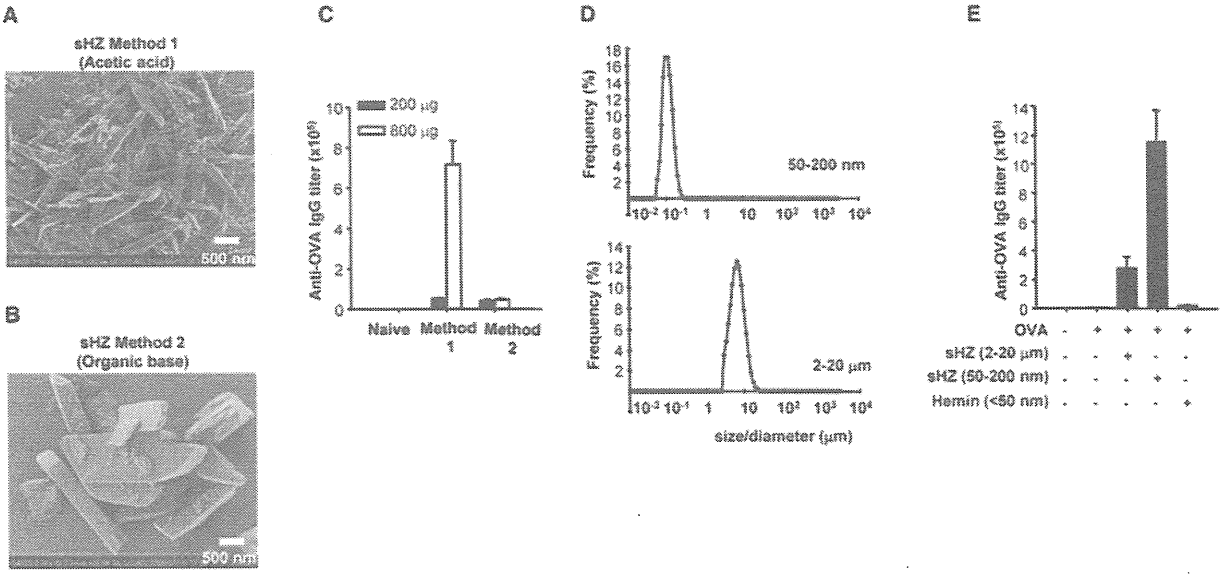

Figure 5. Adjuvanticity of Synthetic Hemozoin Crystals Depends on Their Size, with Smaller Crystals Showing a Better Adjuvant Effect
(A and B) FESEM images of sHZ synthesized by two different methods as described in Experimental Procedures. Scale bars, 500 nm.
(C) Serum OVA-specific IgG responses of C57B/6 mice s.c. immunized with OVA with or without sHZ purified by method 1 or method 2. See also Figure S4A–S4C.
(D) sHZ crystals produced by method 1 were size-differentiated using a laser-scattering particle size distribution analyzer.
(E) Mice were immunized to analyze the adjuvant effects of different sizes of sHZ (50–200 nm and 2–20 μm) and hemin (<50 nm). See also Figure S7. Results are representative of at least two independent experiments (mean ± SD; n = 3–5 mice per group).

Hemozoin Acts as a Potent Adjuvant, and Its Adjuvanticity Depends on Its Size

Is pure synthetic HZ a good adjuvant in vivo? Given the fact that various hemin chloride sources and methods can be used to synthesize sHZ, we first employed several production protocols to investigate the adjuvant properties of sHZ (Egan, 2008) using ultrapure hemin chloride. We found that two commonly used methods for synthesizing sHZ from hemin chloride produced crystals with a distinct appearance that differed in their adjuvant effects. With one method, HZ polymerized in the presence of acetic acid (method 1) and produced crystals that ranged in diameter from 50 nm to 1 μm, while the other method, involving an organic base (method 2), produced crystals that ranged in size from 1 to 5 μm as visualized by field emission scanning electron microscopy (FESEM) (Figures 5A and 5B). When mice were immunized with the model protein antigens ovalbumin (OVA) (or in some cases human serum albumin [HSA]) in the presence or absence of sHZ produced by method 1 or 2, the OVA-specific total IgG responses were significantly higher with sHZ produced by method 1 than with sHZ produced by method 2 (Figure 5C). Interestingly, when we separated the HZ produced by method 1 into two size distributions using a laser-scattering particle size distribution analyzer (Figure 5D), larger sHZ particles (up to 20 μm in size) were counted. It should be noted that sHZ crystals tend to make aggregates that the larger HZ particles might be attributed to the aggregation. Nevertheless, sHZ with sizes between 50 and 200 nm had optimal adjuvant effects compared with larger sHZ molecules (2–20 μm) and the smaller hemin monomer (<50 nm) (Figure 5E). We also examined different administration routes to investigate the adjuvant effects of sHZ such as subcutaneous (s.c.) and intranasal (i.n.) routes, and found that antigen-specific antibody responses were induced in a dose-dependent manner (Figures S4A and S4B and Figure 6A). There were no detectable OVA-specific IFNγ or interleukin (IL)-17 production by spleen cells, but substantial amounts of OVA-specific IL-13 and IL-5 were detected (Figure S4C). Analysis of the IgG isotypes elevated by sHZ coadministration showed that mainly IgG1 isotypes were elevated, followed by IgG2b and IgG2c, in mice (Figure 6B); this response was very potent when compared with those elicited by the adjuvants alum or CpG DNA (Figure 6C). On the other hand, sHZ administration elevated mainly IgG1 while CpG DNA elevated mainly IgG2a (Figure S4D) sHZ is indeed a potent adjuvant for protein vac optimal sizes of 20–200 nm, which coincide with t other particle adjuvants taken up by antigen-presenting cells via receptor-mediated endocytosis (McGee et al., 1997; Xiang et al., 2006).

Synthetic Hemozoin's Adjuvant Effect Is Mediated by MyD88, but Not TLR9 or the Inflammasome/IL-1β

We further investigated how the HZ adjuvant effect was regulated. To examine whether the potent adjuvant effect of sHZ was mediated by TLR9 and through MyD88, mice lacking TLR9 and MyD88 were immunized with OVA plus sHZ. MyD88-deficient mice failed to elicit a serum anti-OVA antibody titer (Figure 6D). By contrast, however, TLR9-deficient mice showed comparable anti-OVA titers to those in wild-type mice, suggesting that there are distinct mechanisms for the adjuvant effects of sHZ other than TLR9, any of which seem to culminate in

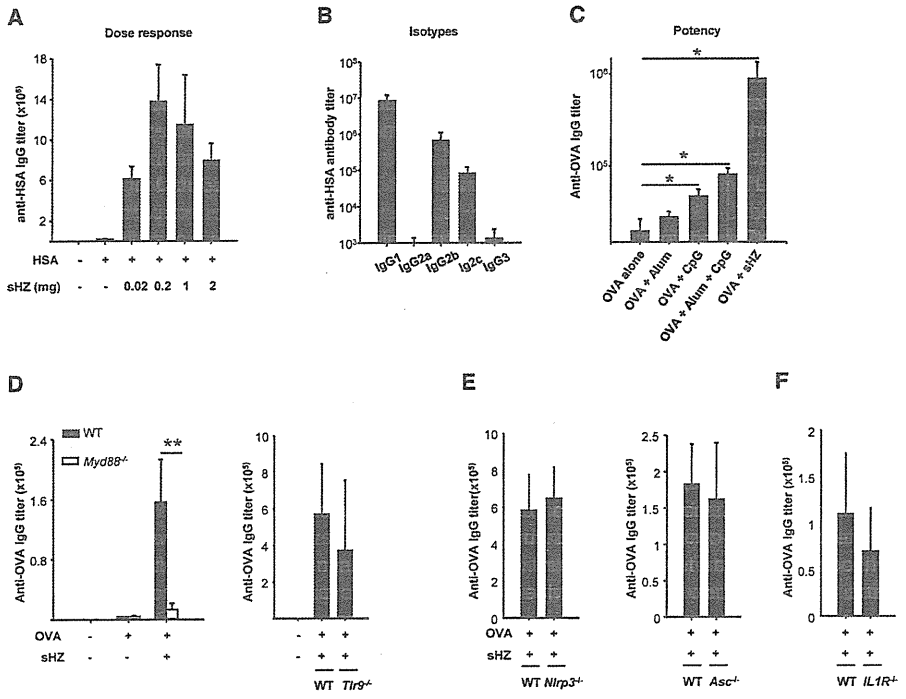

Figure 6. Synthetic Hemozoin's Potent Adjuvant Effect Is Mediated by MyD88 but Independently of TLR9 and the Inflammasome

(A and B) Mice were immunized with HSA and various concentrations of sHZ. Serum HSA-specific IgG (A) and IgG isotypes (B) were measured by ELISA (mean ± SD).

(C) Comparison of the adjuvant effects of alum, CpG DNA, and sHZ. Balb/c mice were immunized s.c. with OVA and alum (200 μg), CpG DNA (50 μg), and sHZ (800 μg), and serum OVA-specific IgG responses were determined after boost immunizations by ELISA (mean ± standard error; n = 3). *$p < 0.05$ compared with OVA-alone group. See also Figure S4D.

(D–F) Serum OVA-specific IgG responses of *Myd88*$^{-/-}$ and *Tlr9*$^{-/-}$ mice (D), *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice (E), and *IL1R*$^{-/-}$ mice (F) after boost immunizations were determined by ELISA (**$p < 0.01$; mean ± SD). Wild-type (n = 5–6); *Myd88*$^{-/-}$ (n = 6); *TLR9*$^{-/-}$ (n = 6); *Nlrp3*$^{-/-}$ (n = 3); *Asc*$^{-/-}$ (n = 6) and *IL1R*$^{-/-}$ (n = 5). See also Figures S5A–S5C and Figure S6. Experiments are representative of at least two independent experiments.

MyD88-dependent signals. We confirmed these findings by obtaining similar results with HSA (Figures S5A and S5B).

Particles like MSU crystals have recently been shown to be recognized by NOD-like receptors (NLRP3 in the case of MSU) and form large cytosolic complexes, collectively called the inflammasome. We were interested in clarifying whether the adjuvanticity of sHZ, whose appearance resembles MSU crystals (Figure 5A and Figure S2D), could involve the inflammasome. In the same set of OVA or HSA immunization protocols, mice deficient in ASC or NLRP3 elicited normal antibody responses (Figure 6E and Figure S5B) as well as IL1R-deficient mice (Figure 6F and Figure S5C). These results suggest that inflammasome/ IL-1β pathway have a minimal role in the adjuvant effect of sHZ.

To examine whether sHZ-induced inflammation occurs in vivo, we measured neutrophil recruitment. When sHZ was injected intraperitoneally, significant numbers of neutrophils were recruited into the peritoneum within 24 hr (Figure S6A), and the neutrophil recruitment occurred normally in mice lacking either ASC or TLR9 (Figure S6B). However, mice lacking MyD88 failed to exhibit neutrophil recruitment in response to sHZ injection (Figure S6A).

Synthetic Hemozoin Is a Potent Adjuvant for Malarial Antigens as Well as for an Allergen

It has been previously shown that subclinical infection of healthy human volunteers with red cells infected with *Pf* or immunization of mice with a small number of dead parasites in the presence of CpG DNA or alum can induce cellular immunity and protection (Pombo et al., 2002; Good, 2009). To examine whether sHZ would have a potent adjuvant effect on immunization with *Pf* crude extract from a few parasites, we immunized mice s.c. with 10 μg total *Pf* crude extract together with sHZ, boosted twice, and measured anti-*Pf* crude extract specific antibody responses. sHZ significantly improved IgG levels (mainly IgG2c and IgG1) against *Pf* crude extract (Figure 7A and data not shown).

To examine whether sHZ could be used in animal species other than mice, we immunized Beagle dogs with house dust mite allergen (Derf2) together with alum and sHZ, and boosted that elevated IgG2 type (but not IgG1) antibodies, which may resemble Th1-like immune responses in dogs (Figure 7B and 7C) (Hou et al., 2006). Accordingly, Derf2-specific IgE responses after Ag challenges were significantly reduced in sHZ coadministered dogs (Figure 7C). It is of note, however, that antibody

responses elicited in mice were dominant with IgG1 isotype, which was different from those in dogs (Figure S5D). These data further suggest that sHZ may serve as a potent Th1-like adjuvant, at least in the canine model, while it acts like a Th2-dominant adjuvant in a murine model.

DISCUSSION

Malaria is still one of the leading pathogens, affecting 40% of the world's population and killing over one million people each year, but a successful vaccine against it not yet available. Owing to the failure of many clinical trials for recombinant vaccines, and recent progress in our understanding of innate immune adjuvant effect of microbial products (Kaisho and Akira, 2002), the whole-parasite vaccine strategy has been regaining attention (Good, 2009; Doolan et al., 2009). Successful vaccination with erythrocytic parasites was achieved using powerful adjuvants, such as CpG DNA (Su et al., 2003). We further demonstrated that an increased dose of a crude extract of *Pf* parasites without adjuvant successfully displayed strong immunogenicity, owing to the enhanced "built-in adjuvant" activity of the parasite. Surprisingly, this adjuvant effect was heavily dependent on TLR9. Because this has been a matter of controversy, the TLR9 ligand in malaria parasite was carefully analyzed again, and we found that DNA is not required for this TLR9-mediated adjuvant effect of the *Pf* crude extract (Figure 2). It is thus reasonable to interpret that the built-in adjuvant in *Pf* crude extract is a non-DNA TLR9 ligand, such as HZ and/or some other heat-labile components as previously reported (Coban et al., 2005; Pichyangkul et al., 2004).

HZ is a heme crystalline dimer generated by parasite digestion of hemoglobin as a byproduct of the heme detoxification system in malaria infection (Arese and Schwarzer, 1997; Hanscheid et al., 2007). It has been proposed to play important roles in pathophysiology during malaria infection, because it activates macrophages and dendritic cells to produce both proinflammatory and anti-inflammatory cytokines and chemokines (Engwerda and Good, 2005; Keller et al., 2006; Hanscheid et al., 2007; Coban et al., 2007a). Although immune recognition of malarial HZ by TLR9 has been demonstrated in vitro and in vivo (Coban et al., 2005), another study suggested that HZ itself was immunologically inert, and that TLR9-dependent immune activation is instead caused by HZ-conjugated malarial DNA (Parroche et al., 2007).

Parroche et al. suggested that DNase-I treatment of *Pf* crude extract did not remove *Plasmodium* DNA from *Pf*HZ shown by *Pf*-specific PCR (Parroche et al., 2007). When we performed PCR by using the same *Pf*-specific primers, 205 bp PCR product of *Pf* DNA was completely lost after DNase-I treatment (Figure S2B). We further confirmed the absence of *Pf* DNA after treatment of *Pf* crude extract by PCR using the other primers (101 bp product) (data not shown). In vivo, this DNase-free *Pf* crude extract displayed potent adjuvant activity via TLR9 (Figure 2A), strongly suggesting that the built-in adjuvant of *Pf* crude extract is not DNA.

What in *Pf* crude extract would be a TLR9 ligand rather than *Pf* DNA? We went on to reconfirm our previous finding that HZ is a TLR9 ligand by examining the definition of a ligand for a receptor, in which a ligand is to directly bind to a receptor in a specific manner. TLR9 binds directly both HZ and hemin, and change its conformation following binding to HZ (Figure 3). Moreover, competition for TLR9 binding occurs among *Pf* crude extract, CpG DNA, and sHZ, further supporting the possibility that HZ fulfills the definition of TLR9 ligand specificity. We also identified the binding motifs in the extracellular domain of TLR9, where cysteine residues play a crucial role in controlling the binding to ligands including *Pf* crude extract, heme, and CpG DNA (Figure 4). Taken together, these findings suggest that the adjuvant component of erythrocytic stage *Pf* parasites contains a TLR9 ligand, most likely HZ, but not DNA.

Involvement of the inflammasome pathways in inflammation during malaria infection, as has been implicated by a recent report (Orengo et al., 2008), was not apparent in the built-in adjuvant effect of whole-parasite vaccination with erythrocytic stage *Pf* crude extract (Figure 1). Consistently, the effect of synthetic HZ was also independent of the NLRP3-ASC inflammasome, as well as IL-1 receptor. It is of note that, while natural HZ in the *Pf* crude extract acts mostly as a TLR9 ligand, sHZ has an adjuvant effect independent of TLR9, but still utilizes the MyD88-dependent pathway. One possible explanation for this difference is that the uptake or ability to break the integrity of phagosomal and/or endosomal membrane of natural and synthetic HZ might differ given that the natural HZ is often covered by *Pf*-derived lipid, protein, or nucleic acids, compared with sHZ as a naked sharp crystal. Another explanation is that, although natural HZ purified from *Pf* cultures is known to be identical to sHZ (Pagola et al., 2000), several purification protocols give different crystal sizes ranging from 50 nm to 20 μm, which displayed quite different adjuvant activities (Figure 5). The studies are under investigation to examine whether different sizes of sHZ make distinct delivery and interaction with the innate immune cells and their immune receptors.

In addition, recent reports have suggested that immune recognition of and inflammatory response by sHZ is mediated by NLRP3 (Dostert et al., 2009; Griffith et al., 2009; Jaramillo et al., 2009). Although all of these three reports showed that IL-1β production by macrophages as well as neutrophil recruitment in response to sHZ was reduced in mice lacking NLRP3, precise mechanism of NLRP3-inflammasome activation are yet unclear. Griffith et al. suggested that uric acid is induced by sHZ and that the uric acid is the NLRP3 ligand (Griffith et al., 2009), but Dostert et al. suggested that uric acid is not involved in NLRP3 activation by sHZ (Dostert et al., 2009). Moreover, Jaramillo et al. showed elegantly that sHZ activates Lyn/Syk-mediated intracellular signaling pathway at the upstream of NLRP3 or ASC, indicating the existence of other HZ receptor(s) such as Dectin1, TREM family members, Siglec, or DAP12 (Jaramillo et al., 2009). Although the involvement of NLRP3 in sHZ-induced IL-1β or the following inflammatory response may play an important role in pathogenesis of malaria infections, we clearly demonstrate that NLRP3 as well as ASC, IL-1 receptor is not involved in sHZ-adjuvant activity (Figure 6), suggesting that there may be additional receptor(s) as well as pathway(s) for HZ-induced innate and adaptive immune activations.

Finally we found that sHZ acts as a potent adjuvant only when its synthesis method and size were optimized (Figure 5). This adjuvant effect of HZ for protein vaccines was observed with different model antigens, such as OVA and HSA, and via different

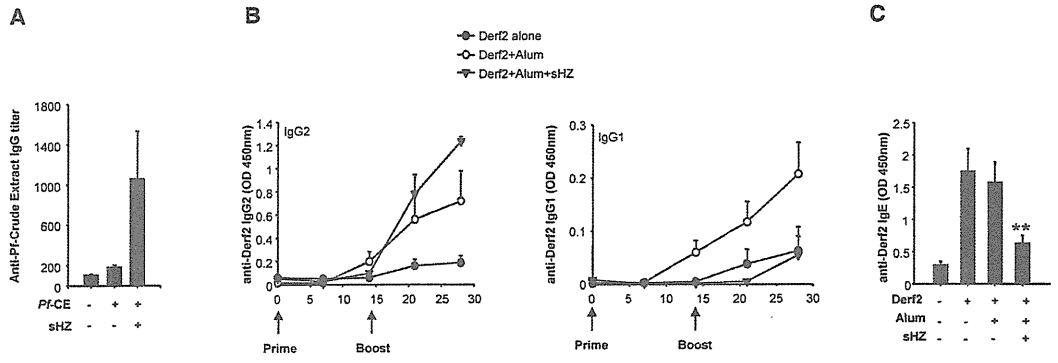

Figure 7. Synthetic Hemozoin Could Be Used as Adjuvant for Whole-Blood Malaria Antigens and Against Canine Allergy
(A) WT mice were immunized s.c. with 10 μg *Pf* crude extract and sHZ and were boosted twice. Serum *Pf* crude extract-specific IgG responses were analyzed by ELISA (mean ± SD; n = 4 per group).
(B and C) ELISA serum anti-Derf2-specific IgG2, IgG1 (B), and IgE (C) responses of Beagle dogs immunized with Derf2 antigen formulated with or without alum and alum + sHZ. See also Figure S5D. Experiments were repeated twice using four animals per group (**$p < 0.01$).

immunization routes, such as s.c. and i.n. immunization. It is of note that unlike CpG DNA, which has species-specific activity, HZ displayed its adjuvant effect in several animal models for vaccines, including murine, canine and non-human primate models (Figures 5–7, data not shown). In mice, a predominantly IgG1-dominant antibody adjuvant effect observed after HZ-adjuvanted immunization and T cell specific immune responses were mainly IL-13 and IL-5, but not IFNγ or IL-17, suggesting that HZ elicits Th2-type immune responses in mice (Figures 5, 6, and S4). However, by contrast, dog immunization with HZ elicited IgG2-dominant antibody responses, which is considered a Th1-type immune response (Reed and Scott, 1993). sHZ administration in the dog-allergy model was nonetheless very potent to reduce allergen-specific IgE responses (Figure 7), suggesting successful usage of sHZ as an adjuvant in dogs against allergy. Similarly, our data suggested that sHZ could be introduced externally as an adjuvant to improve immunogenicity of whole-malaria vaccines (Figure 7A). Our preliminary data also suggest that, in non-human primates, sHZ could elevate malarial antigen-specific B and T cell responses, and could be used as a vaccine adjuvant for whole-blood stage vaccines (K.J.I., unpublished data). Overall, our results clearly show that HZ possesses inflammatory and adjuvant activities through differential regulation by TLR9 pathways, thereby potentiating its ability to act as a therapeutic target for treating malaria infection, and its usefulness in vaccine adjuvant development.

EXPERIMENTAL PROCEDURES

Reagents

Hemin chloride was from Fluka (HPLC purified, >98% pure), whereas OVA and HSA (low endotoxin) were from Sigma. A synthetic CpG DNA (D35) was from Gene Design Incorporation. DNase-I was from Invitrogen. Zymosan was from Invivogen. MSU crystals were prepared as described elsewhere (Martinon et al., 2006).

Mice and Immunizations

Mice deficient for *MyD88*, *TLR9*, *Asc* or *Nlrp3*, and *Asc* on a C57BL/6 background were generated and used for experiments as described previously (Coban et al., 2005; Franchi and Nunez, 2008). All animal experiments were performed according to the institutional guidelines approved by the Research Institute for Microbial Diseases and Immunology Frontier Research Center of Osaka University and the University of Michigan.

Wild-type and *Tlr9*$^{-/-}$ mice were immunized intraperitoneally with the *Pf* crude extract (100 μl, approximately ~1 × 10^8 parasites). Alternatively, mice were immunized with a DNase-I-treated *Pf* crude extract. At 3 weeks after immunization, antibodies against *Pf* crude extract antigens in the sera of mice were detected by ELISA.

Various immunization schedules were conducted using either OVA or HSA as antigens. For s.c. immunizations, mice were injected on day 0 with 50 μg OVA (or 10 μg HSA) in a total of 200 μl sHZ solution at various concentrations and boosted on day 10 with 25 μg OVA (or 10 μg HSA) in the same sHZ solution. Blood samples were collected from the mice on day 17 for analysis of OVA- or HSA-specific antibody responses. For i.n. immunizations, mice were anesthetized and administered i.n. with 5 μg OVA with or without 80 μg sHZ (15 μl/nose), twice, at 2-week intervals. The mice were sacrificed 10 days after the boost, and serum, bronchoalveolar lavage fluid, and nasal fluid secretion samples were collected for antibody measurements.

Dogs and Allergy Model

Five-month-old Beagle dogs from the breeding colony at Zenoaq Animal Experiment Center (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) were used. All immunization and sensitization protocols were performed in accordance with the institutional guidelines of and approved by Zenoaq Animal Center Laboratories Committee. Dogs were immunized s.c. on day 0 with 100 μg Derf2 protein produced in silkworms by a recombinant Baculovirus system, formulated in 500 μg aluminum hydroxide in a 200 μg sHZ solution, and boosted on day 14 with the same dose. Four weeks after the boost immunization, dogs were challenged with Derf2 s.c. four times biweekly. Blood samples were collected on days 7, 14, 21, and 28 for analysis of Derf2-specific IgG1 and IgG2 antibody responses, and on day 90 for the measurement of antigen-specific IgE responses.

Preparation of *Pf* Crude Extract, Natural HZ, Synthetic HZ, and *Pf* DNA

Mycoplasma-free *Pf* parasites (3D7) were maintained in culture medium as described previously (Aoki et al., 2002). After synchronization, mature and HZ-rich mostly trophozoite and schizont stage parasites (~4%–5%) were purified by 63% Percoll density centrifugation, washed and resuspended in incomplete medium, freeze-thawed three or four times, and stored at −80°C until use. Each milliliter of HZ-rich *Pf* crude extract preparation contained approximately 1 × 10^9 infected erythrocytes. As controls, uninfected human erythrocytes were treated similarly. Pure natural HZ was extensively purified from *Pf*-infected erythrocytes as described previously (Coban et al., 2005).

sHZ was purified from hemin chloride using two alternative methods (Egan, 2008). Method 1 (acid-catalyzed method) is known to produce smaller and homogenous crystals and was performed as previously described (Coban et al., 2005; Jaramillo et al., 2005). Briefly, 45 mg hemin chloride was dissolved in 4.5 ml 1 N NaOH and neutralized with 1 N HCl. Then, acetic acid was added until the pH reached 4.8, at a constant temperature of 60°C with magnetic stirring. The mixture was allowed to precipitate at room temperature overnight. The precipitate was subjected to washes with 2% SDS-buffered with 0.1 M sodium bicarbonate (pH 9.1) and subsequent extensive washes with 2% SDS, and then six to eight washes with distilled water. Method 2 (anhydrous method in methanol) is known to produce much bigger crystals and was performed as previously described (Bohle et al., 2002). Briefly, hemin chloride (52.2 mg) was dissolved completely in 2 ml 2,6-lutidine L, diluted with 10 ml dimethyl sulfoxide:methanol (1:1 mixture), tightly wrapped with parafilm and left at room temperature for 2 weeks in the dark, before being centrifuged and washed with 10 ml 0.1 M $NaHCO_3$ for 3 hr. The final product was washed three times with water and methanol, and dried. A stock solution in distilled water was prepared for use in in vivo studies and stored at 4°C. The HZ concentration was calculated as either mM or mg/ml after weighing the dried amount of sHZ. Experiments were mostly performed with sHZ prepared by method 1, except for the results shown in Figure 5B. All solutions were prepared and resuspended in endotoxin-free water (double-processed tissue culture water, Sigma-W3500) and stored in the dark at 4°C. There was no detectable level of endotoxin by LAL assay in any HZ preparation. None of the sHZ purified had any other contaminants, such as hemin, as determined by TLC (Figure S7A) or protein/DNA determined by SDS-PAGE at pH 11 and agarose gel, respectively (data not shown). FT-IR and powder X-ray diffraction pattern of the sHZ confirmed that the sample showed the same unit cell parameters as reported previously (Pagola et al., 2000) (Figures S7B and S7C). *Pf* genomic DNA was isolated as reported previously (Kongkasuriyachai et al., 2004).

Recombinant TLR Proteins and Peptides

Recombinant fusion proteins consisting of the extracellular domains of the human TLRs TLR9 (amino acids 1–818) and TLR2 (amino acids 1–588) fused to mouse IgG2b-Fc were constructed by amplifying the corresponding extracellular domain and ligating the fragment in frame into a pCIneo vector containing the coding sequence for mouse IgG2b-Fc. Fusion proteins were stably expressed in 293-F cells and purified from cell lysates by protein G affinity chromatography. Peptides were derived from unique regions of human *Tlr9* ectodomain sequence and synthesized by Operon Technologies.

Intraperitoneal Neutrophil Recruitment

sHZ (1000 μg) was resuspended in 200 μl PBS and intraperitoneally administered to various mice. At 16–18 hr after injection, the peritoneum was washed with a 30 ml cold PBS containing EDTA (3 mM) and heparin (10 U/ml) and the cells were counted. Neutrophils were identified using PE-conjugated anti-Gr-1 and APC-conjugated anti-CD11b antibodies (Becton Dickinson) in the presence of an anti-CD16 antibody. The stained cells were washed and analyzed using a FACSCalibur system.

Antibody and Cytokine ELISA

The plates were coated with OVA (1 μg/ml) or HSA (10 μg/ml), Derf2 (1 μg/ml) or *Pf* SE36 antigen (3 μg/ml) and analyzed by ELISA using a previously described procedure (Coban et al., 2005; Okech et al., 2006).

Spleens were extracted 3 weeks after the prime immunization and then stimulated with *Pf* crude extract. Seventy-two hours later, the cell culture supernatants were collected and analyzed for IFNγ, IL-13, IL-5, and IL-17 by ELISA (DuoSet ELISA Kit, R&D Systems).

Binding Assay and ELISA

Zymosan (5 μg/ml), CpG DNA (D35; 10 μg/ml), *Pf* crude extract (10 μg/ml), and *Pf* DNA (2 μg/ml) were coated on 96-well plates in PBS overnight at 4°C and then blocked with PBS containing 1% bovine serum albumin. The binding of TLR9 or TLR2 fusion proteins was detected with HRP-labeled mouse anti-IgG (Southern Biotech.). For competition ELISAs, rTLR9 protein was incubated with sHZ, MSU or CpG DNA for 1 hr and then incubated on *Pf* crude extract-coated plates for an additional 1 hr.

CD Spectroscopy

CD spectra were recorded with a J-820 spectropolarimeter (Jasco) using a 1 mm cell at 20°C. Each sample containing 1 μM TLR9 protein with or without a ligand (1 or 10 μM CpG DNA; 10 or 100 μM sHZ, or 10 or 100 μM MSU), was prepared in 20 mM sodium acetate buffer containing 150 mM NaCl at pH 5.5. Twenty spectra were acquired for each sample with a 1 nm bandwidth, a scanning speed of 50 nm per min, and a response time of 2 s, and then averaged. The resultant spectra were calculated after the spectra of rTLR9 were subtracted.

NMR Spectroscopy

^{1}H-1D NMR measurements were carried out using an AV400M NMR machine (Bruker BioSpin, Rheinstetten, Germany) with a 5 mm Shigemi tube (Shigemi, Tokyo, Japan) at 25°C. Each sample was prepared at a peptide concentration of 250 μM in 20 mM sodium phosphate buffer (pH 7.0) containing 10% (v/v) D_2O for the signal lock. For the measurements with hemin, various concentrations of hemin were prepared and the pH was set to 7.0 to prevent precipitation of hemin. For the measurements with CpG DNA (D35), because the signals from CpG DNA were larger than those from the peptides, the spectrum of D35 was subtracted from the spectra of the peptide-CpG DNA mixture to more easily judge the difference. All NMR data were processed and analyzed using NMRPipe (Delaglio et al., 1995) and Igor Pro (WaveMetrics, Lake Oswego, OR).

FESEM and Particle Size Analysis

Slides were coated with different dilutions of sHZ and images were captured using ultra-high-resolution FESEM (S-4800; Hitachi). The particle size and distribution in the sHZ solution were analyzed using a laser-scattering particle size distribution analyzer (LA-950; Horiba).

Statistical Analysis

Statistical differences between groups were analyzed using the Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at doi:10.1016/j.chom.2009.12.003.

ACKNOWLEDGMENTS

The authors sincerely thank Drs. N. Palacpac, T. Kanneganti, R. Jerala, M. Treeby, H. Kumar, and Y. Kumagai as well as the other members of Akira Lab and Horii Lab for their comments and for providing reagents, and Drs. S. Takahashi, T. Inoue, T. Inui, Y. Hara, Y. Fujita, K. Murase, M. Nakamura, and S. Itagaki for technical support. We thank Drs. T Tsuboi and K. Tanabe for their kind support and for providing critical reagents, such as *Pf*MSP1. This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology in Japan; from the RT Fund for Technology Development and CREST, JST, Japan; and from the National Institutes of Health. T.R. was supported by a fellowship from the Swiss National Science Foundation. C.C, T.T, K.J.I., and S.A. filed a patent application related to the method and usage of hemozoin as an adjuvant. Y.I., K.O., and T.T. are employees for Nihon ZENOAQ, which develops GMP lot of hemozoin, and were funded by JST.

Received: July 29, 2009
Revised: October 20, 2009
Accepted: December 7, 2009
Published: January 20, 2010

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Review

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EXPERT REVIEWS

Innate immune control of nucleic acid-based vaccine immunogenicity

Expert Rev. Vaccines 8(8), 1099–1107 (2009)

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Optimal vaccine efficacy requires not only a protective antigen, but also a strong immune activator as an adjuvant. Most viral vaccines, such as influenza vaccines and nonviral genetic vaccines (e.g., DNA vaccines), contain nucleic acids, which appear to act as essential 'built-in' adjuvants. Specific receptors, including Toll-like receptors, retinoic acid-inducible protein-I-like receptors, and nucleotide-binding oligomerization domain-like receptors can detect specific nucleic acid patterns, depending on the immunized tissue, cell type and intracellular localization. The resulting immune activation is uniquely regulated by intra- and intercellular signaling pathways, which are indispensable for the ensuing vaccine immunogenicity, such as antigen-specific T- and B-cell responses. Thus, elucidation and manipulation of immune signaling and interactions by nucleic acid adjuvants are essential for maximizing the immunogenicity and safety of viral and DNA vaccines.

KEYWORDS: innate immunity • nucleic acid • vaccine adjuvant

An adjuvant is a compound that can promote and modulate vaccine immunogenicity. The word adjuvant is derived from the Latin word '*adjuvare*,' meaning 'to help or facilitate.' Current vaccine formulations, such as subunit vaccines and split vaccines, are used in very pure forms, mainly consisting of target antigens. This is done to reduce the incidence of reactions, such as fever, swelling and pain, which are some of the safety concerns regarding live-attenuated or whole-killed vaccines. However, such subunit or split vaccines often suffer from lower immunogenicity compared with whole-microbe vaccines. Therefore, adjuvants are necessary, and have been used to improve the immunogenicity of these 'clean' vaccines. Among other advantages, an adjuvant can reduce required dosages of the target antigens and modulate antigen-specific immune responses in a qualitative manner, such as Th1 and Th2 responses [1,2].

It has long been believed that adjuvants improve immunogenicity by retaining immunized antigens through covalent and/or noncovalent conjugation, to prevent their immediate dissemination and degradation in what is called the 'depot effect'. However, adjuvants are known to increase inflammatory cell infiltration at the injection site. It has only recently been shown that vaccine immunogenicity depends on adjuvant components within the vaccine formulations, controlling the magnitude and specificity of the innate immune activations following vaccination [3–5].

Most, if not all, adjuvants use immune receptors expressed on antigen-presenting cells (APCs), including dendritic cells (DCs) and macrophages [6,7]. We now know that some adjuvants are nucleic acids that had been thought for a long time to be inert in the immune system; accumulating evidence suggests that RNA and DNA packed in both viral and nonviral genetic vaccines are in fact 'built-in' adjuvants. Synthetic RNA (e.g., polyinosinic:polycytidylic acid [poly I:C]) and DNA (e.g., CpG oligodeoxynucleotide [ODN]) are the best examples of adjuvants that induce the production of proinflammatory cytokines and type I interferons (IFNs) through immune recognition and signaling (FIGURE 1). In this review, we discuss how the innate immune response to nucleic acid adjuvants controls the immunogenicity of viral and DNA vaccines, and what strategies can be provided to improve their efficacy and safety.

Innate immune recognition of nucleic acids

Nucleic acids are essential components of all living organisms. During viral or other nucleic acid-based vaccinations, nucleic acids are introduced

 10.1586/ERV.09.57 ISSN 1476-0584

to, and recognized by, the host's immune receptors. Immune receptors that recognize nucleic acids include Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs).

Nucleic acid receptors in endosomes

After vaccination with viral vectors, or nonviral vectors containing nucleic acids, both DNA and RNA are mainly taken up into the endosomes of APCs, including macrophages, DCs and B cells, where they are seen as foreign nucleic acids by endosomal immune

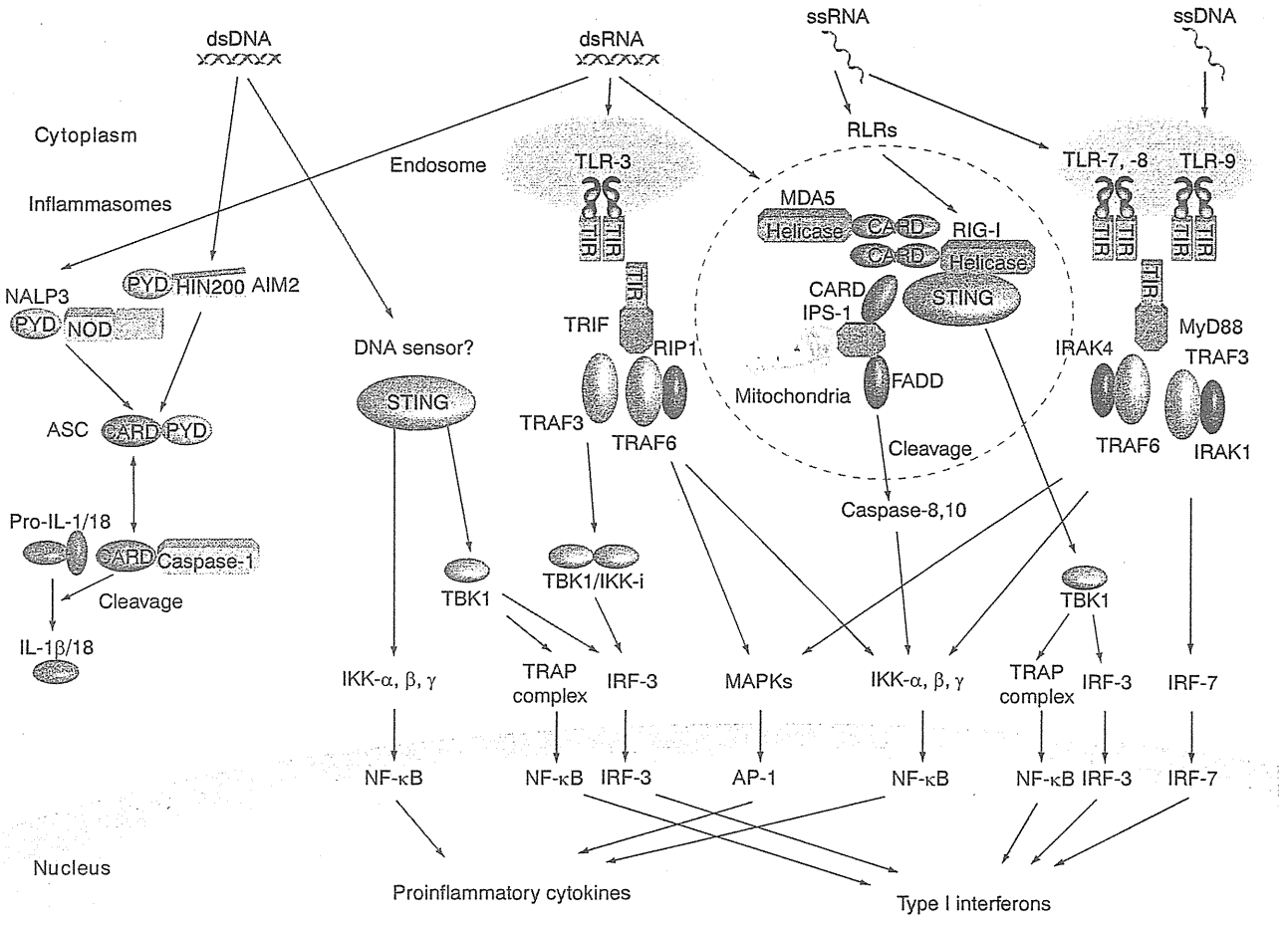

Figure 1. Immune receptors for nucleic acid recognition. TLR-3, -7, -8 and -9 are located in endosomes. TLR-3 signals through TRIF (also known as TICAM-1). TRIF associates with TRAF-3, TRAF-6 and RIP1. TRAF3 activates TBK1 and IKK-i, while TRAF6 and RIP1 activate MAPKs and NF-κB. TLR-7, -8 and -9 signal through an adaptor, MyD88. MyD88 forms a complex with IRAK-4, IRAK-1, TRAF3, TRAF6, and activates IRF-3, IRF-7, NF-κB and MAPKs. RIG-I, MDA5, NALP3 and AIM2 are located in the cytoplasm. Both RIG-I and MDA5 associate with the adapter protein IPS-1, and with STING. STING induces IRF-3 phosphorylation via TBK1, and NF-κB activation via the TRAP complex. IPS-1 also interacts with FADD and leads to activation of NF-κB through cleavage of caspase-8 and -10. NALP3 and AIM2 associate with ASC. These receptors form inflammasomes with ligands and ASC to activate caspase-1 and produce IL-1β and IL-18. Besides the inflammasome-dependent signaling pathway, STING- and TBK1-dependent pathways also exist in dsDNA sensing, although their sensors are still unknown.
AIM: Absent in melanoma; ASC: Apoptosis-associated speck-like protein; CARD: Caspase recruitment domain; FADD: Fas-associated death domain-containing protein; IKK: Inducible IκB kinase; IRAK: IL-1R-associated kinase; IRF: Interferon regulatory factor; MAPK: Mitogen-activated protein kinase; MDA5: Melanoma differentiation-associated gene-5; MyD88: Myeloid differentiation primary response gene 88; NOD: Nucleotide-binding oligomerization domain; PYD: Pyrin domain; RIG: Retinoic acid-inducible gene; RIP: Receptor interacting protein; RLR: Retinoic acid-inducible gene I-like receptor; STING: Stimulator of interferon genes; TANK: TRAF family member-associated NF-κB activator; TBK1: TANK-binding kinase 1; TLR: Toll-like receptor; TRAF: Tumor necrosis factor receptor-associated factor; TRAP: Translocon-associated protein; TRIF: Toll/IL-1R domain-containing adaptor-inducing IFN-β.

receptors. Four kinds of TLRs – TLR-3, -7, -8 and -9 – are known as endosomal nucleic acid receptors (Figure 1). Their distributions within the host tissues and cells are quite varied, enabling them to create an array of immune responses. Among human DCs, the expression of TLR-7 and -9 is mostly restricted to plasmacytoid DCs, which specialize in the production of type I IFNs, which are potent antiviral cytokines. TLR-8 and -3 are expressed on various types of cells, including conventional DCs and macrophages, producing mainly proinflammatory cytokines. TLR-3 and -7 can be upregulated in other cell types in response to type I IFNs.

TLR-3 is a receptor for dsRNA, a common viral signature generated during viral infection (including vaccination) [8] and for siRNAs, which are involved in angiogenesis suppression [9]. TLR-7 and -8 recognize ssRNA derived from either the microbe or host, depending on sequence preferences [10]. TLR-9 has been shown to recognize specific sequence motifs – so-called CpG motifs – that have been postulated as typical pathogen-associated molecular patterns [11]; however, a recent report suggests that this may be true only when DNA is modified with a phosphorothioate linkage to be resistant to nucleases. The authors suggest instead that TLR-9 recognizes the sugar backbone of ssDNAs with natural phosphodiester linkages, independent of specific sequence and base modifications, while its recognition of ssDNAs with phosphorothionate backbones requires unmethylated CpG motifs [12]. Moreover, TLR-9 activation by synthetic ODNs was shown to depend on cell types and aggregated forms of ODNs, rather than their sequences [13].

Thus, TLR-3, -7, -8 and -9 are expressed in the endosomes of various immune cells that sense unique signatures within RNA and DNA (e.g., sequences, modifications and/or structures), triggering robust but specific immune activation through association with specific adapter proteins and downstream signaling pathways (Figure 1) [7,14].

Cytoplasmic nucleic acid receptors

In the case of viral infection or administration of synthetic nucleic acids by liposome-mediated transfection or electroporation, foreign nucleic acids can enter the cytoplasm of host cells, where they are subject to cytoplasmic immune receptors.

Cytoplasmic immune receptors for RNA include RIG-I, melanoma differentiation-associated gene-5 (MDA5) and laboratory of genetics and physiology-2, all of which are now categorized as RLRs. RIG-I and MDA5 are expressed in both immune cells and nonimmune cells (such as fibroblasts), and are strongly upregulated by type I IFNs. RIG-I recognizes both 5′-triphosphorylated ssRNAs [15] and short (~1 kb) dsRNAs [16]. By contrast, MDA5 preferentially recognizes longer (>2 kb) dsRNAs, including synthetic dsRNA polyI:C [16].

The molecular basis of these distinct ligand specificities for RIG-I and MDA5 is still unclear. However, both RIG-I and MDA5 are associated with the adapter protein IFN-β promoter stimulator-1 (IPS-1; also known as MAVS, VISA or CARDIF) [17–20] and stimulator of IFN genes (STING; also known as MITA or ERIS) [21,22,23]. IPS-1 localizes on the outer membrane of the mitochondria and activates TANK-binding kinase 1 (TBK1) and inducible IκB kinase (IKK-i) via TNF receptor-associated factor 3; it also activates NF-κB through Fas-associated death domain-containing protein and initiates the cleavage of caspase-8 and -10 (Figure 1) [7,14]. STING induces IFN regulatory factor-3 phosphorylation via TBK1 and IKK-i, and NF-κB activation via the translocon-associated protein complex.

Another cytoplasmic immune receptor for RNA is the NLR. It comprises three motifs: a C-terminal leucine-rich repeat, a central nucleotide-binding domain and a N-terminal signaling domain. The latter contains a caspase recruitment domain (CARD), pyrin domain or baculovirus inhibitor of apoptosis repeats [24]. The majority of NLRs use a CARD-containing apoptosis-associated speck-like protein (ASC) in their signaling pathways [25] as an adapter to activate caspase-1. Activation of caspase-1 and subsequent processing of pro-IL-1β is mediated through the formation of a complex with an NLR (such as NALP3), ASC and procaspase-1, collectively called an inflammasome (Figure 1) [26]. NALP3/cryopyrin was recently shown to sense RNA [27,28]. NALP3/cryopyrin is essential for caspase-1 activation and for IL-1β and IL-18 production in response to bacterial RNA, and to the imidazoquinoline compounds R837 and R848. For certain NALP3 ligands, a brief pulse with ATP was required to enhance caspase-1 activation and IL-1β maturation by inducing a potassium efflux through a selective channel. The secretion of TNF-α and IL-6, as well as activation of NF-κB and mitogen-activated protein kinases, were unaffected by cryopyrin deficiency [27]. Synthetic dsRNA, polyI:C, and viral infections with Sendai and influenza viruses also trigger the secretion of IL-1β and IL-18 cryopyrin – dependently in macrophages but normally in TLR-3 or TLR-7-deficient macrophages [28].

Cytoplasmic DNA recognition is quite distinct from cytoplasmic RNA recognition. As initially studied by Isaacs *et al.* [29] and re-discovered by Suzuki *et al.* [30], DNA has been shown to be immunomodulatory, especially in its double-stranded form. Ishii *et al.* refined their findings that transfection of natural DNA, or of synthetic polynucleotides that form double-stranded structures, stimulates cells to produce type I IFN and induces autonomous cell protection from viral replication, independently of TLR-9 [31]. Unlike the CpG motifs needed for TLR-9 activation, methylation of such dsDNA had no effect on activity. Rather, poly(dA-dT) • poly(dT-dA) induced higher levels of IFN-I compared with poly(dG-dC) • poly(dC-dG), suggesting that the right-handed helical structure of B-form DNA (B-DNA) is essential for cellular activation of IFN-I production; this is mediated through TLR-independent, TBK1-dependent means [31]. Thus, TLR-independent, TBK1-dependent cytoplasmic DNA recognition plays an important role in immune responses during viral and bacterial infections [31,32], and in controlling the ensuing adaptive immune responses [33].

Moreover, TLR-independent, TBK1-independent, NLR-mediated, ASC-dependent signaling appears to be involved in DNA sensing [34]. NALP3/cryopyrin can detect an adenovirus – a nonenveloped DNA virus – but not liposome-mediated transfections of viral, bacterial or human DNAs [34]. Therefore, NLRs may also be expected to be involved in DNA sensing. Recent

Table 1. Nucleic acid adjuvants target innate immune activation.

Innate immune receptors–adaptors	Ligands/adjuvants	Adjuvant combinations	Ref
TLR-3–TRIF	dsRNA/synthetic dsRNA (poly I:C, poly I:C[12]U)	Emulsions, liposomes	[40,62,71–74]
TLR-7/8–MyD88	ssRNA/imidazoquinolines (imiquimod, resiquimod)	Emulsions, liposomes	[75–78]
TLR-9–MyD88	ssDNA/synthetic unmethylated CpG oligonucleotides	Emulsions, liposomes	[48,77,79,80]
RIG-I–IPS-1	ssRNA and short dsRNA /unknown		[81]
MDA5–IPS-1	Long dsRNA/synthetic dsRNA (poly I:C)		[40]
NALP3–ASC	Adenoviral DNA/unknown		[34]
AIM2–ASC	Synthetic DNA/unknown		[35–38]
Unknown receptor – STING	Synthetic dsDNA (poly dA:dT)/DNA vaccine (plasmid dsDNA)		[31,33]

AIM2: Absent in melanoma 2; IPS-1: IFN-β promoter stimulator-1; MDA5: Melanoma differentiation-associated gene-5; MyD88: Myeloid differentiation primary response gene 88; Poly I:C: Polyinosinic:polycytidylic acid; PYD: Pyrin domain; RIG:Retinoic acid-inducible gene; STING: Stimulator of interferon genes; TLR: Toll-like receptor; TRIF: Toll/IL-1R domain-containing adapter inducing IFN-β.

studies have reported a new molecule, absent-in-melanoma 2, which forms a complex with ASC and procaspase-1; it can directly interact with dsB-DNA in the cytoplasm and induce IL-1β secretion (Figure 1) [35–38].

Adjuvant effects of nucleic acids mediated by immune receptors

As mentioned previously, immunostimulatory activity of nucleic acids through specific receptors and signaling pathways has been demonstrated to be a potent adjuvant. Some mechanisms of efficacy in nucleic acid adjuvants have been clarified recently (Table 1). Three kinds of nucleic acids adjuvants – polyI:C (dsRNA), influenza virus genome (ssRNA) and DNA vaccine (dsDNA) – are discussed here.

Recent evidence suggests that DCs and macrophages are activated by dsRNAs such as polyI:C through TLR-3 and MDA5, and their specific adaptors TRIF and IPS-1, thereby resulting in the production of proinflammatory cytokines, particularly IL-12 and type I IFN, respectively [39,40]. These pathways contribute to the adjuvant effects of polyI:C [40]. In this study, the polyI:C-enhanced, antigen-specific antibody (Ab) response was severely decreased in IPS-1-deficient mice but not in TRIF-deficient mice; no antibody production was elicited in TRIF– IPS-1-doubly-deficient mice [40]. In addition, antigen-specific $CD8^+$ T-cell expansion was reduced in both IPS-1 and TRIF mice and completely abolished in the IPS-1–TRIF-doubly deficient mice. Therefore, the adjuvant effect of poly I:C appeared to be coordinately regulated by the TLR-3–TRIF and MDA5–IPS-1 signaling pathways [40].

Influenza virus genome ssRNA can stimulate TLR-7 and RIG-I. In influenza virus infection, the contribution of the TLR-7-dependent pathway to adaptive immunity is the subject of some debate [41,42]. However, a recent study has shown that the immune response to influenza A virus is cooperatively regulated by the TLR-7/myeloid differentiation primary response gene 88 (MyD88) and RIG-I/IPS-1 pathways *in vivo*. Their concurrency was apparent as mice lacking both pathways – but not mice lacking only one of these pathways – failed to induce antiviral responses via type I IFNs, resulting in increased viral load in the lung. The adaptive immune response, including influenza-specific Th1-type antibody response and $CD4^+$ T-cell activation, was strictly regulated by TLR-7/MyD88, but not RIG-I/IPS-1 signaling; the induction of influenza-specific $CD8^+$ T cells was unimpaired by the absence of either MyD88 or IPS-1 [43]. Some recent reports suggest that NLR-mediated recognition of influenza viruses is also involved in antiviral responses, as ASC/caspase-1-dependent inflammasomes and secreted IL-1β appeared to be involved in adaptive immune responses to influenza virus infection, especially $CD8^+$ T-cell activation. However, it is as yet unclear how TLRs, RLRs and NLRs are differentially regulated for influenza virus recognition [44–46].

In vaccinations with inactivated whole influenza virus, the immune response has been shown to be dominantly controlled by the TLR-7/MyD88-dependent signaling pathway [43]. Moreover, a recent report demonstrated that whole-virus vaccines induce a much better adaptive immune response than split vaccines. Split vaccine is commonly used as a seasonal influenza vaccine, and mostly consists of the surface protective antigens hemagglutinin and neuraminidase to reduce reactogenicity. By contrast, the whole-virus vaccine contains viral genome ssRNA in addition to the several viral proteins lost during the purification process of split vaccine, which might function as built-in adjuvants [47]. These results indicate that influenza genome ssRNA could play an important role as an adjuvant in influenza vaccination, and that its adjuvant efficacy is dominantly controlled by the TLR-7/MyD88 signaling pathway.

DNA vaccines are DNA plasmids encoding the target antigen genes that become expressed in the administered host cells, resulting in both humoral and cellular antigen-specific immune responses. Unmethylated CpG motifs within plasmid DNA had been thought to be built-in adjuvants for DNA vaccine immunogenicity [48,49]. Recent evidence, however, has changed earlier opinions that TLR-9 was essential for the adjuvant effect of the plasmid DNA backbone towards a new one that plasmid dsDNA activates the immune system by a distinct recognition mechanism in the cytoplasm involving type I IFN production using TBK1 [33,50], but

not TLR-9/MyD88 or DLM-1/ZBP1 (later renamed DAI) [33,51]. Using *in vivo* DNA vaccination by transfection methods such as electroporation, IFNαR2 and TBK1 mice could not induce optimal DNA vaccine immunogenicity, including antigen-specific T- and B-cell activation. By contrast, MyD88–TRIF-doubly deficient mice and DAI mice could induce normal responses to DNA vaccine, as well as wild-type mice. These results indicated that DNA vaccine immunogenicity is dependent on TBK1 and secreted type I IFNs [33]. Moreover, bone marrow-transfer experiments demonstrated that in DNA vaccination, TBK1-dependent signaling in hematopoietic cells was essential for B-cell and $CD4^+$ T-cell activation, whereas nonhematopoietic cells require it for $CD8^+$ T-cell activation [33].

Are nucleic acid adjuvants enough?

As described previously, several kinds of synthetic nucleic acids possess potent adjuvant effects through immune mechanisms of action. Are we using them efficiently and safely enough? Here we discuss potential ways to improve their potency and safety in terms of the immunogenicity of vaccines, whether nucleic acids are used as added adjuvant or as a built-in adjuvant.

Vaccine delivery systems

It is very important to manipulate drug delivery systems by vaccine formations such as particle size and charge for improvement of vaccine efficacy. The ideal goal is to ensure delivery of antigens and adjuvants of interest to the key immune cells, such as DCs, in order to increase the vaccines' specific effect with their adjuvants, while decreasing dosages and unnecessary side effects. With the development of biotechnology, nano-sized vaccine delivery systems, including virus-like particles, immunostimulating complexes, inert nanobeads, and so on, are becoming useful in vaccine delivery [52]. Virus-like particles are self-assembling particles consisting of several viral proteins manufactured by recombinant technology. These assembling proteins form subviral particles ranging in size from approximately 20–100 nm and can mimic viral infections [53]. Immunostimulating complexes appear as hollow particles of approximately 40 nm in diameter, and initially have negative charges owing to their components, which include Quil-A saponin, phospholipids and cholesterol. Therefore, positively charged antigens can be electrically and structurally embedded into such particles [54]. Nanobeads, which are 40–50 nm in diameter and positively charged, have been shown to be more efficiently taken up by DCs. Moreover, 40–100-nm-sized beads have been found to be more efficiently taken up by $DEC205^+$ DCs, whereas larger 1-μm beads are preferentially taken up by $F4/80^+$ macrophages in mice [55]. Cationized gelatin nanobeads containing CpG ODN strongly induced type I IFN responses in human plasmacytoid DCs [56]. Thus, nano-sized vaccine delivery systems are available not only as mimics of viruses or inducers of the depot effect, but also as uptake modulators for DCs and macrophages according to particle size or charge.

However, for several vaccine-targeted diseases, little is known about which cells are essential for vaccine immunogenicity and adjuvant effect, and where interactions between such key cells and antigens occur in each tissue and organ. The more such immunological mechanisms are elucidated, the more effectively drug delivery systems can be utilized.

Adjuvant combinations

It is known that some combinations of immune receptor ligands can stimulate immune signaling synergistically and/or competitively with each other [57]. Synergy was observed in combinations between TRIF-dependent and TRIF-independent APCs, as with MyD88-dependent and MyD88-independent TLRs, such as TLR-3 and -9 [58,59]. These combinations increase the induction of the Th1 cell-linked cytokines, IL-12 and IL-23, especially in DCs [59]. On the other hand, combining agonists that share a single pathway appears to induce tolerance [60]. Synergy can occur between TLRs but also between TLRs and NLRs, such as NOD2–TLR-9 [61]; others are currently unknown. In the case of nucleic acids, combination with TLR agonists that do not share the same signaling pathway or noninnate immune receptor agonists such as mineral salts, emulsions and liposomes can induce synergic effects (Table 1). For example, combining the TLR-3/MDA5 ligand poly I:C with the TLR-9 ligand CpG induces synergistic activation of antiviral innate immune responses [62] and antibody. Similarly, CD4 T-cell response to the influenza vaccine was enhanced by immunizations with MF59, or in those with alum and CpG compared with CpG alone [63]. While the synergy between these different adjuvants promotes vaccine immunogenicity, they can also amplify vaccine reactivity. In a clinical trial of AS04 (monophosphoryl lipid A [MPLA] and alum) and AS02 (MPLA and QS21 formulated with liposomes), both local and general adverse events were more severe, and detected more frequently in combination than in CpG alone (although AS04 and AS02 are not nucleic acid adjuvants). While no serious adverse events were observed even in these combination adjuvants [64], vaccines are mostly administered to healthy populations. Therefore, it is necessary to weigh the risk–benefit ratio in selecting an appropriate adjuvant combination.

Routes of administration

Administration route is one of the keys to vaccine efficacy and safety. Although intramuscular and subcutaneous administrations are the most common routes of vaccination, there is an increasing call to reduce the use of needles and their related pain, as well as other adverse events. Moreover, as most infectious diseases occur at the mucosal surfaces of airways and gastrointestinal tracts (where mucosal IgA is known to play a major role in immune protection), mucosal vaccines may be considered safer, more efficient and more cost effective against many infectious diseases, such as influenza viruses and pneumococcal infections. Among mucosal adjuvants, CpG-ODNs and MPLA may be useful against ADP-ribosylating enterotoxin (such as cholera toxin) and heat-labile enterotoxins. However, both efficacy and safety are not well-defined, except for MPLA in humans [65]. A recent study indicated that several synthetic TLR ligands could work as intranasal vaccine adjuvants in mice simply by mixing with antigens [66]. It means that every innate immune receptor ligand could be a mucosal adjuvant; their

modification might be dispensable in mucosal administration. Although intranasal administration is a very common form of mucosal administration [67], sublingual, intravaginal and intrarectal vaccines are also indicated to be efficient in protection against influenza virus infection [68], sexually transmitted diseases [69] and cancer [70] in animal models. However, their efficacy and safety remain to be evaluated for human use.

Conclusion

Recent research on innate immunity has shed light on the mechanisms of some empirically used adjuvants. Advances in molecular biology and biotechnology have produced various immunostimulants and formulations for vaccine adjuvants, including some that incorporate nucleic acids. Researchers should characterize the action of these drugs, select the proper routes and combine them rationally to increase or modulate vaccine immunogenicity. Several possibilities now exist for clinical development, although more research is needed to design the safest and most efficient vaccines and adjuvants for each targeted disease.

Expert commentary & five-year view

Recent advances in molecular biology and biotechnology have enabled the production of various immunostimulants and formulations for vaccine adjuvants suitable for vaccine delivery. However, in spite of this progress, most vaccine immunogenicity mechanisms are still not fully understood. Future development in this area is required for the development of new, safe and efficacious vaccines – especially the identification of the types of immune cells active in vaccine uptake and antigen presentation, and the location and other details of their interactions with B and T cells.

Acknowledgements

The authors thank all members of the Department of Host Defense and Molecular Protozoology at the Research Institute for Microbial Diseases, Osaka University (Japan).

Financial & competing interests disclosure

This work was supported by JSPS Research Fellowships for Young Scientists (Shohei Koyama), the Knowledge Cluster Initiative (Toshihiro Horii and Ken J Ishii), KAKENHI (Shohei Koyama, Toshihiro Horii, Ken J Ishii, Cevayir Coban and Shizuo Akira), the Target Protein Project (Ken J Ishii and Shizuo Akira), the Ministry of Education, Culture, Sports, Science and Technology, CREST, and JST (Ken J Ishii). 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

- Optimal vaccine efficacy not only needs a protective antigen, but also an innate immune receptor ligand as an adjuvant.
- Some viral and nonviral genetic vaccines contain nucleic acids as 'built-in' adjuvants and those nucleic acids play a key role in vaccine immunogenicity.
- Recent progress in innate immunity has shown that Toll-like receptors, retinoic acid-inducible gene I-like receptors, and nucleotide-binding oligomerization domain-like receptors are essential for the recognition and adjuvant activity of nucleic acids.
- Besides nucleic acids, many kinds of innate immune activators are available for vaccine adjuvants.
- Optimal use of nucleic acids as adjuvants requires a combination of approaches, including drug-delivery systems, routes of administration, adjuvant combinations and appreciation of species-specific differences.
- A more detailed understanding of vaccine immunogenicity mechanisms is indispensable for evidence-based vaccine development.

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Predicting the Antigenic Structure of the Pandemic (H1N1) 2009 Influenza Virus Hemagglutinin

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Abstract

The pandemic influenza virus (2009 H1N1) was recently introduced into the human population. The hemagglutinin (HA) gene of 2009 H1N1 is derived from "classical swine H1N1" virus, which likely shares a common ancestor with the human H1N1 virus that caused the pandemic in 1918, whose descendant viruses are still circulating in the human population with highly altered antigenicity of HA. However, information on the structural basis to compare the HA antigenicity among 2009 H1N1, the 1918 pandemic, and seasonal human H1N1 viruses has been lacking. By homology modeling of the HA structure, here we show that HAs of 2009 H1N1 and the 1918 pandemic virus share a significant number of amino acid residues in known antigenic sites, suggesting the existence of common epitopes for neutralizing antibodies cross-reactive to both HAs. It was noted that the early human H1N1 viruses isolated in the 1930s–1940s still harbored some of the original epitopes that are also found in 2009 H1N1. Interestingly, while 2009 H1N1 HA lacks the multiple *N*-glycosylations that have been found to be associated with an antigenic change of the human H1N1 virus during the early epidemic of this virus, 2009 H1N1 HA still retains unique three-codon motifs, some of which became *N*-glycosylation sites via a single nucleotide mutation in the human H1N1 virus. We thus hypothesize that the 2009 H1N1 HA antigenic sites involving the conserved amino acids will soon be targeted by antibody-mediated selection pressure in humans. Indeed, amino acid substitutions predicted here are occurring in the recent 2009 H1N1 variants. The present study suggests that antibodies elicited by natural infection with the 1918 pandemic or its early descendant viruses play a role in specific immunity against 2009 H1N1, and provides an insight into future likely antigenic changes in the evolutionary process of 2009 H1N1 in the human population.

Citation: Igarashi M, Ito K, Yoshida R, Tomabechi D, Kida H, et al. (2010) Predicting the Antigenic Structure of the Pandemic (H1N1) 2009 Influenza Virus Hemagglutinin. PLoS ONE 5(1): e8553. doi:10.1371/journal.pone.0008553

Editor: Robert Belshaw, University of Oxford, United Kingdom

Received August 25, 2009; **Accepted** December 4, 2009; **Published** January 1, 2010

Funding: This work was supported by Grants-in-Aid for Scientific Research (B) (19300041) from the Japan Society for the Promotion of Science (JSPS), and for Young Scientists (B) (21780272) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (http://www.mext.go.jp/english/index.htm), and in part, by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases (05021011) from MEXT, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

In April 2009, pandemic (H1N1) 2009 influenza virus (2009 H1N1) was first found in patients with febrile respiratory illness in the United States and Mexico, and has spread rapidly across the world by human-to-human transmission. On the 11th of June 2009, the World Health Organization declared a global pandemic of 2009 H1N1 infection. H1N1 influenza virus caused a pandemic in 1918 (1918 H1N1) [1], and its descendant virus with highly altered antigenicity of the viral surface protein, hemagglutinin (HA) has been causing "seasonal flu" in humans.

The 2009 H1N1 resulted from genetic reassortment between the recently circulating swine H1 viruses in North America and the avian-like swine viruses in Europe [2]. Phylogenetic analysis showed that the HA gene of 2009 H1N1 was derived from the so-called "classical swine H1N1" virus, which likely shares a common ancestor with the recent human H1N1 virus [2]. Accordingly, it has been reported that the early strains of the classical swine H1N1 virus, which was first identified in North America in 1930, were antigenically similar to the prototype strain of 1918 H1N1, A/South Carolina/1/1918 (SC1918), detected from a few victims of the pandemic in 1918 [3,4]. Since antigenic changes occur more slowly in swine than in the human population [5], HA of the classical swine H1N1 virus was antigenically highly conserved until the late 1990s [4,6], raising the possibility that the recently emerged 2009 H1N1 may still retain an antigenic structure similar to that of SC1918 and the early isolates of its descendants.

In this study, we generated three-dimensional (3D) structures of the HA molecules of 1918 H1N1, its descendent, recent seasonal H1N1 viruses, and 2009 H1N1, and compared their antigenic structures to look for evidence for the existence of shared epitopes for neutralizing antibodies. Since the 2009 H1N1 HA antigenic sites will be targeted by antibody-mediated selection pressure in humans in the near future, we further discuss possible directions of antigenic changes in the evolutionary process of this pandemic virus.

Results and Discussion

It is known that the H1 HA molecules have four distinct antigenic sites: Sa, Sb, Ca, and Cb [7,8,9,10] (Figure 1). As a result, these sites consist of the most variable amino acids in the HA molecule of the seasonal human H1N1 viruses that have been subjected to antibody-mediated immune pressure since its

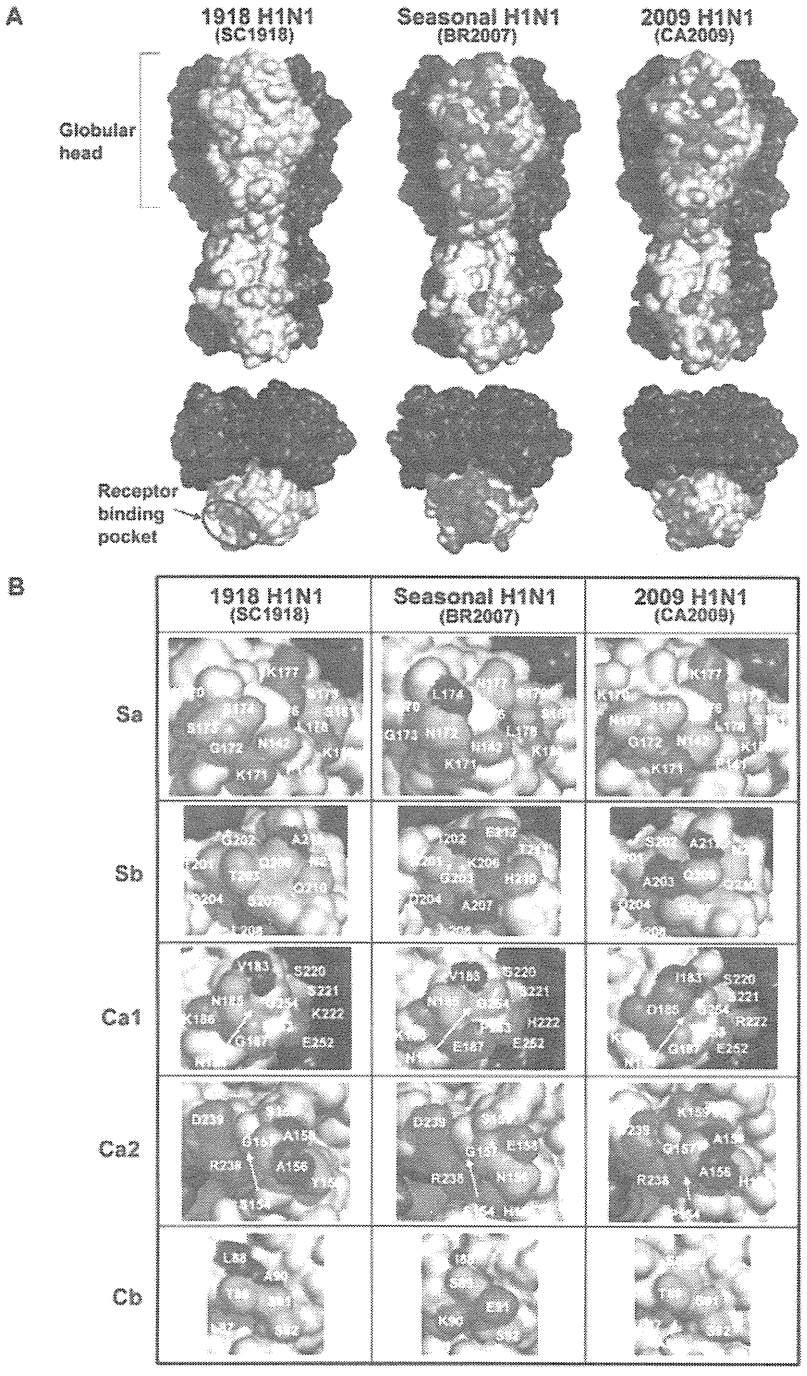

Figure 1. Comparison of the structures of antigenic sites on the HA molecules among 1918 H1N1 (SC1918), recent seasonal H1N1 (BR2007), and 2009 H1N1 (CA2009). Three-dimensional models of the H1 HA molecules of SC1918, BR2007, and CA2009 were constructed based on the HA crystal structures of A/South Carolina/1/18, A/Puerto Rico/8/34, and A/swine/Iowa/30, respectively (PDB codes: 1RUZ, 1RU7, and 1RUY, respectively). Models with solvent-accessible surface representation were generated by a molecular modeling method as described in the Methods section. Molecular surface of the HA trimers viewed on its side (upper) and top (lower) are shown (A). One monomer (center) is colored gray and the others are colored dark gray. The antigenic sites, Sa (light pink), Sb (light blue), Ca (pale green), and Cb (light orange) are indicated on the model of SC1918 HA. The spatial locations of amino acid residues that are distinct from those of SC1918 HA are shown in red on the models of BR2007 and CA2009 HAs. Each amino acid residue is mapped on the close-up views of each antigenic site of SC1918, BR2007, and CA2009 HAs (B). The Ca site is divided into subregions, Ca1 and Ca2. Amino acids are colored by the default ClustalX color scheme [29]: Trp, Leu, Val, Ile, Met, Phe, and Ala (blue); Lys and Arg (red); Thr, Ser, Asn, and Gln (green); Cys (pink); Asp and Glu (magenta); Gly (orange); His and Tyr (cyan); Pro (yellow).
doi:10.1371/journal.pone.0008553.g001

Table 1. Amino acid similarity in the HA antigenic sites among recent seasonal H1N1 (BR2007), 2009 H1N1 (CA2009), and 1918 H1N1 (SC1918).

Antigenic sites	No. amino acids involved	No. of amino acids identical to SC1918	
		BR2007	CA2009
Sa	13	8	12
Sb	12	4	10
Ca	19	13	13
Cb	6	2	5

doi:10.1371/journal.pone.0008553.t001

emergence in 1918 [3]. To investigate the structures of these antigenic sites of 2009 H1N1, 3D structures of the HA molecules of SC1918, the recent seasonal human H1N1 virus A/Brisbane/59/2007 (BR2007), and 2009 H1N1 A/California/04/2009 (CA2009) [2] were constructed by a homology modeling approach, and compared by mapping all the amino acid residues that were distinct from those of SC1918 HA (Figure 1 and Table S1). We found that most of these antigenic sites of BR2007 HA predominantly contained altered amino acid residues if compared with SC1918. By contrast, amino acid residues at these positions were relatively conserved in CA2009 HA. Notably, the Sa and Sb sites that contain many amino acids involved in neutralizing epitopes near the receptor binding pockets [8,10] remain almost intact in CA2009 HA (Table 1), suggesting that antibodies raised by natural infection with SC1918 or its antigenically related descendant viruses play a role in specific immunity against CA2009.

We then constructed 3D structures of the representative strains of seasonal H1 viruses that had been isolated since 1934, and tracked the amino acid substitutions on their HA molecules (Figure 2 and Figure S1). We confirmed that amino acid substitutions associated with the antigenic changes gradually accumulated on the globular head region of HA and were distributed over four distinct antigenic sites. However, it was noted that the early isolates represented by the A/Puerto Rico/8/1934 and A/Bellamy/1942 strains, but not the strains isolated after the

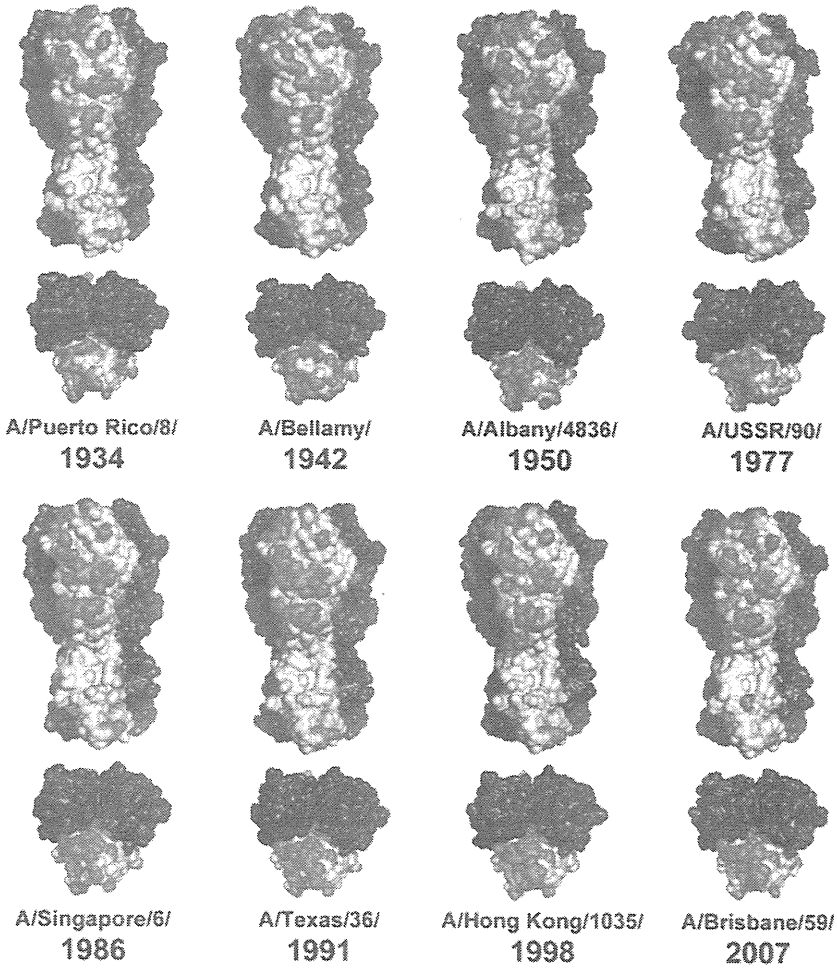

Figure 2. Amino acid substitutions associated with antigenic changes of seasonal human H1N1 virus HAs. All models were generated and shown by a molecular modeling method as described in the Methods section and the legend of Figure 1.
doi:10.1371/journal.pone.0008553.g002

1950s, still harbored unchanged amino acids forming potential neutralizing epitopes in the Sa and Sb sites (Figure 2). It seems likely that most of the amino acids on these antigenic sites were eventually substituted in the late 1940s (Figure S1).

It is well-documented that antigenic changes of HA occasionally result in the acquisition of carbohydrate side chains on the HA molecule [8,11]. Since the carbohydrate side chains in the vicinity of antigenic sites mask the neutralizing epitopes on the HA surface, amino acid substitutions associated with acquisition of carbohydrate chains are believed to efficiently generate antigenic variants. Accordingly, recent seasonal H1N1 viruses have acquired 4–5 *N*-glycosylation sites (Asn-Xaa-Ser/Thr, where Xaa is any amino acid except Pro) in the globular head region of HA [12,13], whereas SC1918 HA had only one site, at Asn 104 (Figure 3).

Interestingly, CA2009 also has a single potential *N*-glycosylation site at the same position in the globular head region of HA (Figure 3), despite the fact that the classical swine H1N1 virus emerged in the early 1900s and was circulating in the pig population until recently. This prompted us to estimate the potential of 2009 H1N1 to acquire additional *N*-glycosylation sites on its HA, which may be related to its future evolutionary process in the human population. We previously defined a three-codon motif that becomes an *N*-glycosylation site with a single-nucleotide mutation as "*Cand1*", and suggested that the presence of the *Cand1* sites in the HA sequence is one of the key factors for human influenza A viruses to rapidly acquire *N*-glycosylation sites during the early epidmic in the human population [13]. We compared the number of the *Cand1* sites in the HA globular head region between SC1918 and CA2009 (Figure 3 and Table S1). We found that CA2009 HA possessed three *Cand1* sites on the antigenic sites Sa and Ca, all of which were also present at the same position in SC1918 HA (positions of the first Asn residue, 177, 179, and 184). Of these, the *Cand1* sites with positions at 177 and 179 had actually become potential *N*-glycosylation sites in human H1N1 viruses, although these two sites did not exist concurrently [12]. It is noted that these two *Cand1* sites are still present on the surface of CA2009 HA, suggesting the likelihood of additional *N*-glycosylation at these sites during future antigenic changes of 2009 H1N1 HA.

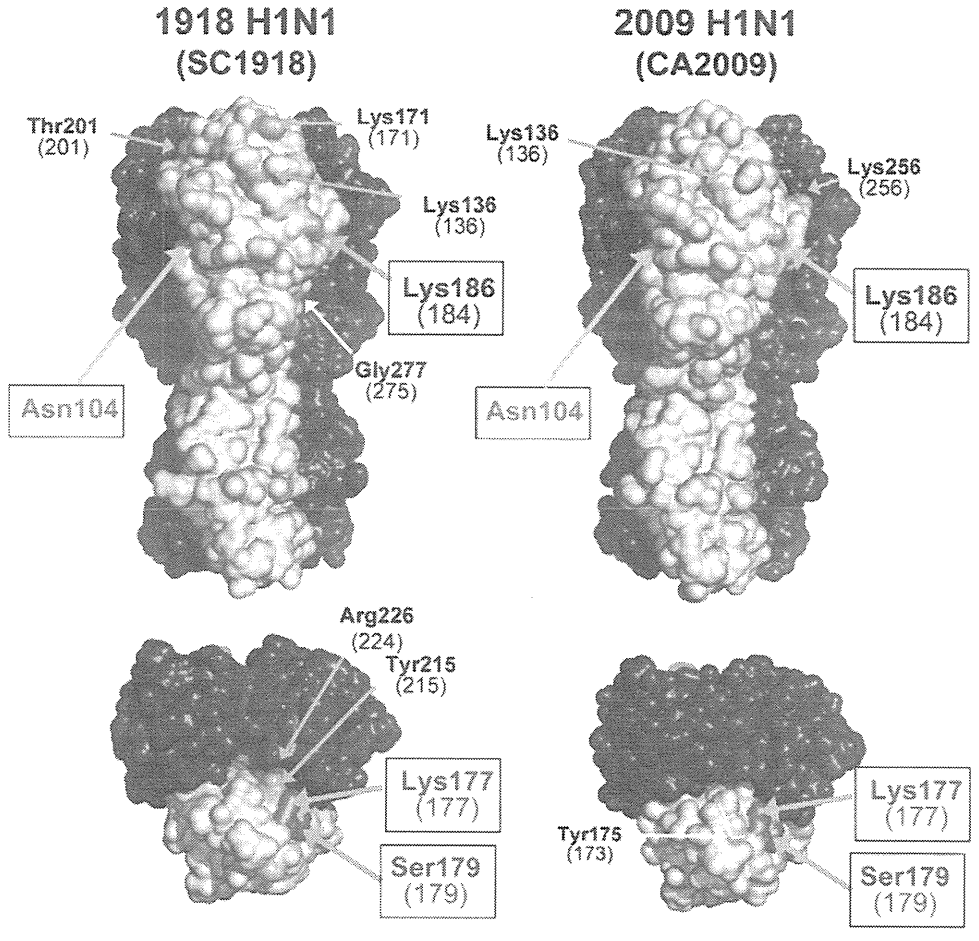

Figure 3. Comparison of the *N*-glycosylation potential of HA between SC1918 and CA2009. Residues shown in green represent Asn at the actually existing *N*-glycosylation sites. Residues shown in orange or blue represent the amino acids in *Cand1* sites that require a nucleotide substitution to produce *N*-glycosylation sites. Residues shown in blue represent the amino acids that were actually substituted, resulting in the acquisition of *N*-glycosylation sites during the antigenic evolution of human H1N1 viruses. Numbers in parentheses show the positions of Asn residues that may be linked to carbohydrate chains, if respective *Cand1* sites mutate to have *N*-glycosylation sites. All models were generated as described in the Methods section and the legend of Figure 1.
doi:10.1371/journal.pone.0008553.g003