In vivo CTL assay. *In vivo* CTL assay was carried out as described previously by Suvas et al. [17]. Briefly, spleen cells from naive HHD mice were equally split into two populations. One population was pulsed with 10 mM of peptide M1 58-66 and labeled with a high concentration (2.5 μM) of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR). The other popula-

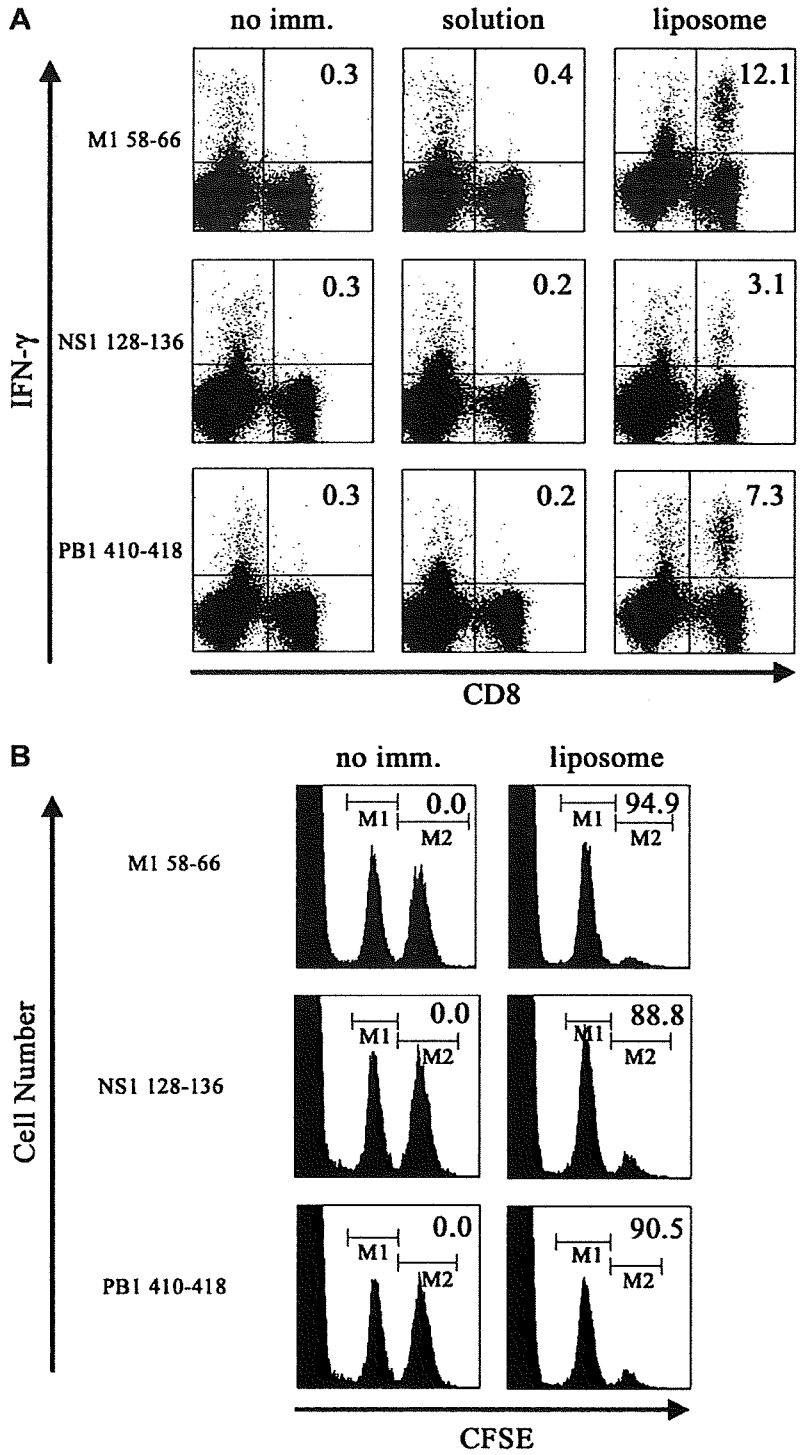

Fig. 1. Induction of antigen-specific CD8⁺ T-cell and CTL responses. Mice received immunization with the liposome-coupled peptides M1 58-66, NS1 128-136, and PB1 410-418 (liposome) or liquefied peptides in the same amounts as liposome conjugates (solution) in the presence of CpG. Control mice received no immunization (no imm.). One week after the immunization, ICS (A) and *in vivo* CTL assay (B) were performed as described in Materials and methods. (A), ICS of antigen-specific CD8⁺ T-cells among spleen cells of A2Tg mice. Cells were stained for their surface expression of CD8 (*x*-axis) and their intracellular expression of IFN-γ (*y*-axis). The numbers shown indicate the percentage of CD8⁺ cells that were positive for intracellular IFN-γ. (B), induction of CTLs by liposome-coupled peptides. The numbers shown indicate the percentage of total cells killed. The data shown are representative of three independent experiments.

tion was unpulsed and labeled with a lower concentration (0.25 μM) of CFSE. An equal number (1×10^7) of cells from each population was mixed together and adoptively transferred intravenously (i.v.) into mice that had been immunized once with a liposomal peptide two weeks earlier. Twelve hours later, spleen cells were prepared and analyzed by flow cytometry. To calculate specific lysis, the following formula was used: % specific lysis = (1 − {(number of $CFSE^{low}$ cells in normal mice)/(number of $CFSE^{high}$ cells in normal mice)}/{(number of $CFSE^{low}$ cells in immunized mice)/(number of $CFSE^{high}$ cells in immunized mice)}) × 100.

Viral challenge experiment. Mice were anesthetized by an intraperitoneal injection of ketamine (175 mg/g weight) (Sigma-Aldrich) and xylazine (3.5 mg/g weight) (Bayer Holding Ltd., Tokyo, Japan), and were challenged intranasally (i.n.) with either $5 \times LD_{50}$ (1×10^4 $TCID_{50}$) of H1N1 (A/PR/8/34) virus or 1×10^4 $TCID_{50}$ of H3N2 (A/Aichi/2/68) virus resuspended in 40 ml of PBS per animal. The mice were weighed daily and monitored for mortality for two weeks. For virus titration, mice were sacrificed on day 2, 4, or 6 after the virus challenge, and the virus titers in their lungs were determined by calculating $TCID_{50}$ using MDCK cells as described [15]. In brief, the lungs were homogenized in 1 ml of PBS and the homogenate was clarified by centrifugation at 2000 rpm for 10 min. The lung homogenates were then serially 10-fold diluted in 96-well U-bottomed plates, 5 wells per dilution, starting from 10^{-1} to 10^{-7} in DMEM with 5% FCS. MDCK cells in D-5 were added to all wells (2.5×10^4 cells/well) and incubated at 35 °C in 5% CO_2. One day later, the culture medium in each well was replaced by DMEM containing 2 mg/ml acetylated trypsin (Sigma-Aldrich), and the plates were incubated in a CO_2 incubator at 35 °C for 4 more days. After the addition of 50 ml of 0.5% chicken red blood cell suspension in PBS to each well, the agglutination pattern for each sample was observed and virus titers were determined by calculating $TCID_{50}$ [15]. Three to six mice were used in each experimental group. The limit of detection in this assay was 10^3 $TCID_{50}$/mouse.

Statistical analyses. Statistical analyses were performed using Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

Results

Induction of antigen-specific CD8⁺ T-cells and CTLs by liposome-coupled CTL epitopes derived from internal proteins of influenza viruses

HLA-A*0201 (A2)-binding epitopes were predicted among the amino-acid sequences of six coding regions—M1, NP, PA, PB1, PB2, and NS—in the H1N1 influenza virus strain A/PR/8/34 (PR8) using programs available on the Internet. The predicted epitopes were then synthesized and chemically coupled to liposomes to evaluate their abilities to induce antigen-specific $CD8^+$ T-cells and CTLs by means of ICS and an *in vivo* cytotoxicity assay, respectively. Immunization of the liposome-coupled, HLA-A2-restricted epitope peptides, M1 58-66, NS1 128-136, and PB1 410-418 (Table 1), induced significant levels of antigen-specific $CD8^+$ T-cells in HLA-A2-transgenic (A2Tg) mice, as evaluated by ICS (Fig. 1A). Moreover, as shown in Fig. 1B, all the peptide-liposome conjugates in Fig. 1A induced significant *in vivo* CTL responses, indicating that all the predicted CTL epitopes were loaded onto MHC class I and were recognized by CTLs. On the other hand, solutions of the predicted peptides did not induce antigen-specific $CD8^+$ T-cells in A2Tg mice immunized even in the presence of CpG (Fig. 1A). As shown in Table 1, all three peptides were preserved well not only in H1N1 (A/PR/8/34) and H3N2 (A/Aichi/2/68) seasonal influenza viruses but also in the pandemic H1N1 2009 virus (A/New York/4290/2009) and in a highly pathogenic avian influenza virus, H5N1 (A/Hong Kong/483/97), except that NS1 128-136 partially changed in H3N2 (A/Aichi/2/68) and H1N1 (A/New York/4290/2009). Among the CTL epitopes determined in the present study, peptide M1 58-66 was already reported by Gotch et al. in 1987 [14].

Virus challenge experiment

Among the above-described HLA-A2-restricted CTL epitopes, virus challenge experiments were performed using peptide M1 58-66. A2Tg mice were immunized subcutaneously with liposome-coupled, HLA-A2-restricted peptide M1 58-66. One week after the immunization, they were infected intranasally with the influenza virus H1N1 (A/PR/8/34) or H3N2 (A/Aichi/2/68) strain. As shown in Fig. 2, viral growth in the lung was suppressed significantly in the immunized mice 2–6 days after the infection with either H1N1 (A) or H3N2 (B) viruses. Thus, immunization with liposome-coupled peptides successfully induced protection against influenza viruses regardless of the influenza virus subtypes in A2Tg mice.

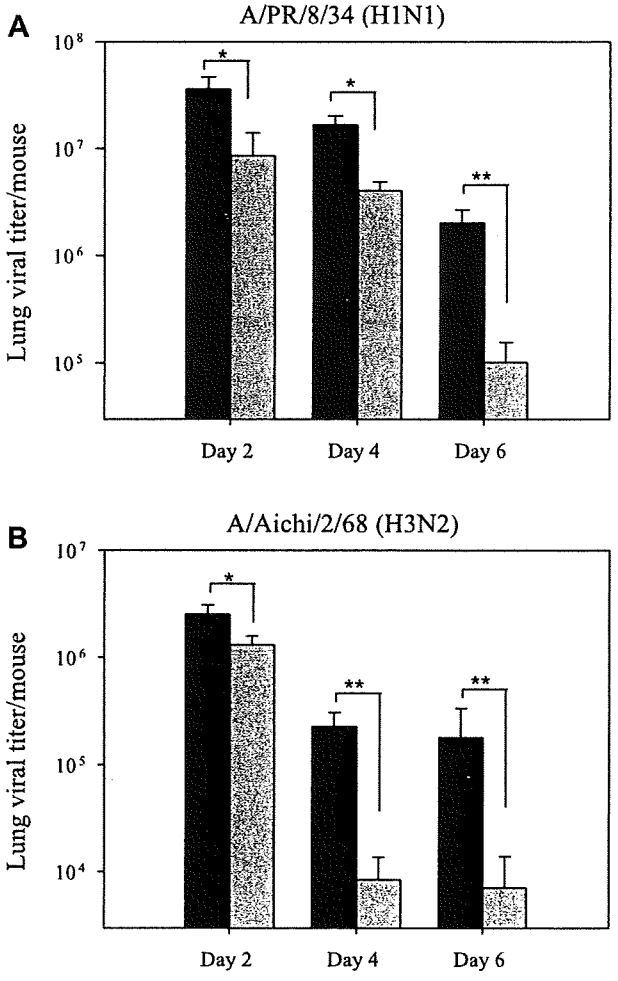

Fig. 2. Viral titers in lungs of mice following H1N1 or H3N2 virus challenge. Mice received immunization with either liposome-coupled peptide M1 58-66 liposome conjugates in the presence of CpG (gray bars) or none (black bars). One week later, mice were challenged i.n. with either the H1N1 (A) or the H3N2 virus (B). On day 2, 4, or 6 post-infection, viral titers in the lungs were determined by calculating $TCID_{50}$ using MDCK cells as described in Materials and methods. Data represent mean and SE of 5 mice per group. $^*p < 0.05$. $^{**}p < 0.01$.

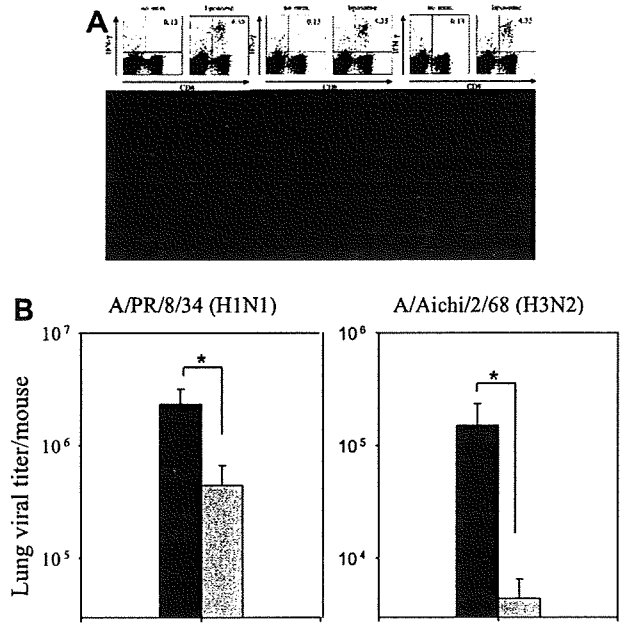

Fig. 3. Induction of long-lasting immunity by peptide-liposome conjugates. Mice were immunized with either liposome-coupled peptide M1 58-66 in the presence of CpG or none. 90 days after the immunization, ICS (A) and viral challenge experiments (B) were performed. (A), ICS of $CD8^+$ T-cells specific for peptide M1 58-66 among spleen cells of A2Tg mice immunized with liposome-coupled peptide (liposome) or none (no imm.). (B), viral replication in the lungs of mice infected with influenza viruses in mice immunized with liposome-coupled peptide (gray bars) or none (black bars). Data represent the mean and SE of 5 mice per group. $^*p < 0.05$.

Induction of memory $CD8^+$ T-cells

After confirming the induction of protective ability by liposomal peptide in the effector phase, we investigated whether or not this immunization induced memory $CD8^+$ T-cells. As shown in Fig. 3, $CD8^+$ T-cells specific to peptides M1 58-66 were detected significantly in immunized mice at 90 days after the immunization (Fig. 3A). In addition, viral growth in the lung was suppressed significantly after nasal challenge with either the H1N1 or H3N2 influenza virus (Fig. 3B). Thus, it was demonstrated that the immunization readily induced memory $CD8^+$ T-cells.

Induction of long-lasting protection against lethal doses of influenza viruses

We next investigated whether or not the long-lasting immunity demonstrated above would help protect mice against infection with lethal doses of influenza viruses. Six months after the immunization with liposome-coupled M1 58-66 peptides, mice were challenged with a lethal dose of influenza virus H1N1 PR8 strain. As shown in Fig. 4, although the immunized mice lost body weight up to 8 days after infection, the rate of loss was significantly lower than that in a non-immunized control group at 4–7 days, and the immunized mice recovered body weight thereafter (Fig. 4A). Finally, 5 out of 6 immunized mice stayed alive while all of the non-immunized control mice died within 7 days after infection with a significant loss of body weight and morbidity (Fig. 4B).

Discussion

The present study demonstrated that liposome-coupled CTL epitope peptides derived from internal antigens of influenza virus

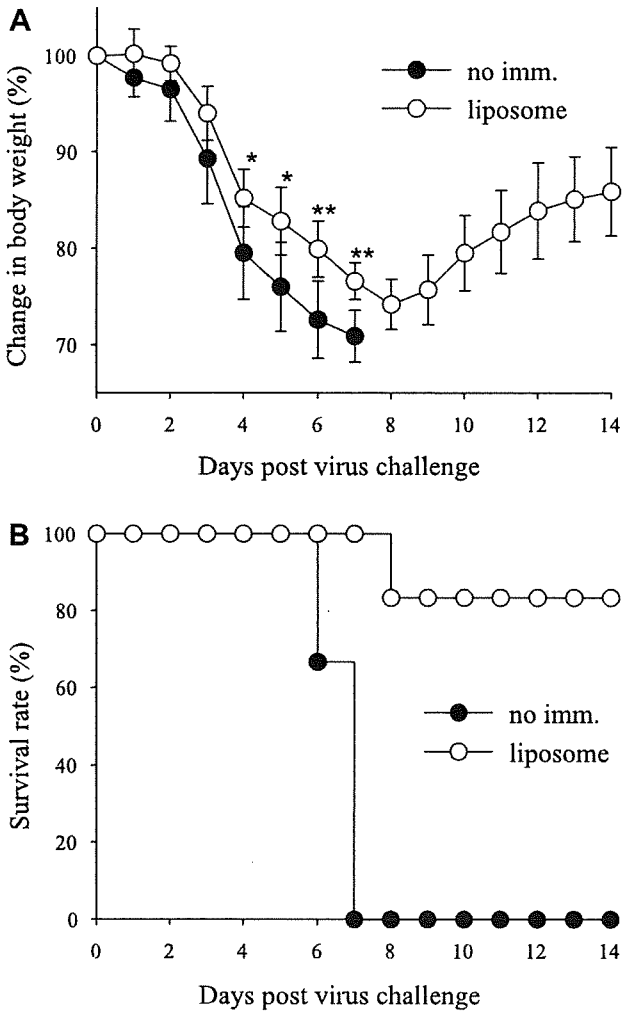

Fig. 4. Maintenance of protective ability against a lethal dose of H1N1 virus in mice immunized with liposome-coupled peptide. Mice were immunized with either liposome-coupled peptide M1 58-66 in the presence of CpG (open symbols) or none (solid symbols). Six months after the immunization, mice were challenged i.n. with $5 \times LD_{50}$ of H1N1 (A/PR/8/34) virus. The mice were weighed and monitored daily for mortality up to 14 days post-challenge. (A), changes in body weight of mice calculated as a percentage of the mean weight per group as compared with starting body weight. Data represent mean body weight and SE of 6 mice per group. $^*p < 0.05$ and $^{**}p < 0.01$ as compared with non-immunized control mice. (B), survival percentage. The mice were euthanized after they had lost 30% of their initial body weight.

protected against infection with heterosubtypic influenza viruses. Since the early finding that influenza A virus-specific CTLs are broadly cross-reactive for cells of the same MHC class I type infected with serologically distinct H1N1 and H3N2 viruses [4], numerous investigators have reported on immunodominant and cross-reactive CTL epitopes derived from conserved internal antigens, such as NP [18,19], M1 [14,19,20], and NS1 [5] of the influenza viruses. It was hoped that CTL epitopes could be used to develop a broadly protective influenza vaccine [21]. To date, candidate $CD8^+$ T-cell vaccines have been reported to induce protection even partially [22], suggesting that $CD8^+$ T-cells certainly help protect against influenza. Therefore, the $CD8^+$ T-cell vaccine strategy against influenza was expected to support antibody-focused vaccine strategies by "reducing" [21] or "dampening" [5] the impact of the next pandemic.

The liposome-coupled CTL epitope peptides efficiently induced antigen-specific $CD8^+$ T-cells and CTLs (Fig. 1), and suppressed viral

replication in the lungs of mice infected with either H1N1 or H3N2 influenza viruses (Fig. 2). In addition, since a single immunization just one week before infection successfully reduced viral replication in the lung, this vaccination protocol would be expected to counter the rapid spread of an influenza pandemic. The efficacy of the liposome-coupled peptides in inducing CTL-based protective immunity was likely due to the characteristics of liposome-coupled antigens, which are very readily recognized by APCs [23] and which effectively induce cross-presentation via MHC class I in the APCs [10], in addition to their safety, in that they are least likely to induce allergic responses [24]. Moreover, the liposome-coupled CTL epitope peptides were capable of inducing long-lived $CD8^+$ memory T-cells without including $CD4^+$ T-cell epitope in the composition of the vaccine (Fig. 3). In fact, mice immunized with liposome-coupled M1 58-66 peptides remained protected for at least 6 months after immunization (Fig. 4).

The CTL epitopes employed in the present study are contained not only in the seasonal influenza viruses but also in the currently emerging S-OIV and in the extremely virulent avian H5N1 influenza viruses (Table 1), suggesting that this liposomal peptide vaccine might be effective for protection against infection with both seasonal and pandemic influenza viruses. In addition, the liposome employed in the present study was originally developed as an antigenic carrier that effectively induces humoral immunity (i.e., antibody production) against liposome-coupled antigens [25]. Therefore, the antigen-liposome conjugates are capable of inducing both humoral and cellular immunity against influenza viruses, by combined coupling of antibody and CTL epitopes to the surfaces of liposomes.

Conclusions

The results obtained in the present study demonstrated that liposome-coupled CTL epitope peptides derived from highly conserved internal antigens of influenza viruses might be applicable to the development of a broadly protective influenza vaccine that could confer protective immunity against both seasonal and pandemic influenza.

Acknowledgments

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References

[1] CDC, Morbidity and Mortality Weekly Report, Serum cross-reactive antibody response to a novel influenza A (H1N1) virus after vaccination with seasonal influenza vaccine, MMWR 58 (2009) 521–524.
[2] D. Gatherer, The 2009 H1N1 influenza outbreak in its historical context, J. Clin. Virol. 45 (2009) 174–178.
[3] B. Olsen, V.J. Munster, A. Wallensten, et al., Global patterns of influenza A virus in wild birds, Science 312 (2006) 384–388.
[4] R.B. Effors, P.C. Doherty, W. Gerhard, et al., Generation of both cross-reactive and virus-specific T-cell populations after immunization with serologically distinct influenza A viruses, J. Exp. Med. 145 (1977) 557–568.
[5] J.H.C.M. Kreijtz, G. Mutsert, C.A. Baalen, et al., Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus, J. Virol. 82 (2008) 5161–5166.
[6] L.Y. Lee, D.L.A. Ha, C. Simmons, et al., Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals, J. Clin. Invest. 118 (2008) 3478–3490.
[7] W.R. Heath, F.R. Carbone, Cross-presentation, dendritic cells, tolerance and immunity, Annu. Rev. Immunol. 19 (2001) 47–64.
[8] A.T. Glenny, C.G. Pope, Immunology notes. XXIII. The antigenic value of toxoid precipitated by potassium alum, J. Pathol. Bacteriol. 29 (1926) 31–40.
[9] H. Aggrebeck, J. Wantzin, I. Heon, Booster vaccination against diphtheria and tetanus in man: comparison of three different vaccine formations-III, Vaccine 14 (1996) 1265–1272.
[10] M. Taneichi, H. Ishida, K. Kajino, et al., Antigen chemically coupled to the surface of liposomes are cross-presented to $CD8^+$ T cells and induce potent antitumor immunity, J. Immunol. 177 (2006) 2324–2330.
[11] T. Uchida, M. Taneichi, Clinical application of surface-linked liposomal antigens, Mini Rev. Med. Chem. 8 (2008) 184–192.
[12] T. Nagata, T. Toyota, H. Ishigaki, et al., Peptides coupled to the surface of a kind of liposome protect infection of influenza viruses, Vaccine 25 (2007) 4914–4921.
[13] S. Pascolo, N. Bervas, J.M. Ure, et al., HLA-A2.1-restricted education and cytolytic activity of $CD8^+$ T lymphocytes from β2 microglobulin (β2m) HLA-A2.1 monochain transgenic $H\text{-}2D^b$ β2m double knockout mice, J. Exp. Med. 185 (1997) 2043–2051.
[14] F. Gotch, J. Rothbard, K. Howland, et al., Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2, Nature 326 (1987) 881–882.
[15] R. Cottey, C.A. Rowe, B.S. Bender, Influenza virus, in: Current Protocols in Immunology, John Wiley & Sons Inc., 2001, pp. 19.11.1–19.11.32.
[16] M. Matsui, O. Moriya, M.L. Belladonna, Adjuvant activities of novel cytokines, interleukine (IL)-23 and IL-27 for induction of hepatitis C virus-specific cytotoxic T lymphocytes in HLA-A*0201 transgenic mice, J. Virol. 78 (2004) 9093–9104.
[17] S. Suvas, U. Kumaraguru, C.D. Pack, et al., $CD4^+CD25^+$ T Cells regulate virus-specific primary and memory $CD8^+$ T cell responses, J. Exp. Med. 198 (2003) 889–901.
[18] S.L. Epstein, W. Kong, J.A. Miplon, et al., Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein, Vaccine 23 (2005) 5404–5410.
[19] A.R.M. Townsend, J. Rothbard, F.M. Gotch, et al., The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides, Cell 44 (1986) 959–968.
[20] E.G.M. Berkhoff, D. Wit, M.M. Geelhoed-Mieras, et al., Functional constrains of influenza A virus epitopes limit escape from cytotoxic T lymphocytes, J. Virol. 79 (2005) 11239–11246.
[21] P.C. Doherty, A. Kelso, Toward a broadly protective influenza vaccine, J. Clin. Invest. 118 (2008) 3273–3275.
[22] P.G. Thomas, R. Keating, D.J. Hulse-Post, et al., Cell-mediated protection in influenza infection, Emerg. Infect. Dis. 12 (2006) 48–54.
[23] Y. Tanaka, M. Kasai, M. Taneichi, et al., Liposomes with differential lipid components exert differential adjuvanticity in antigen-liposome conjugates via differential recognition by macrophages, Bioconjug. Chem. 15 (2004) 35–40.
[24] M. Taneichi, S. Naito, H. Kato, et al., T cell-independent regulation of IgE antibody production induced by surface-linked liposomal antigen, J. Immunol. 169 (2002) 4246–4252.
[25] Y. Nakano, M. Mori, S. Nishinohara, et al., Surface-linked Liposomal antigen induces IgE-selective unresponsiveness regardless of the lipid components of liposomes, Bioconjug. Chem. 12 (2001) 391–395.

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

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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

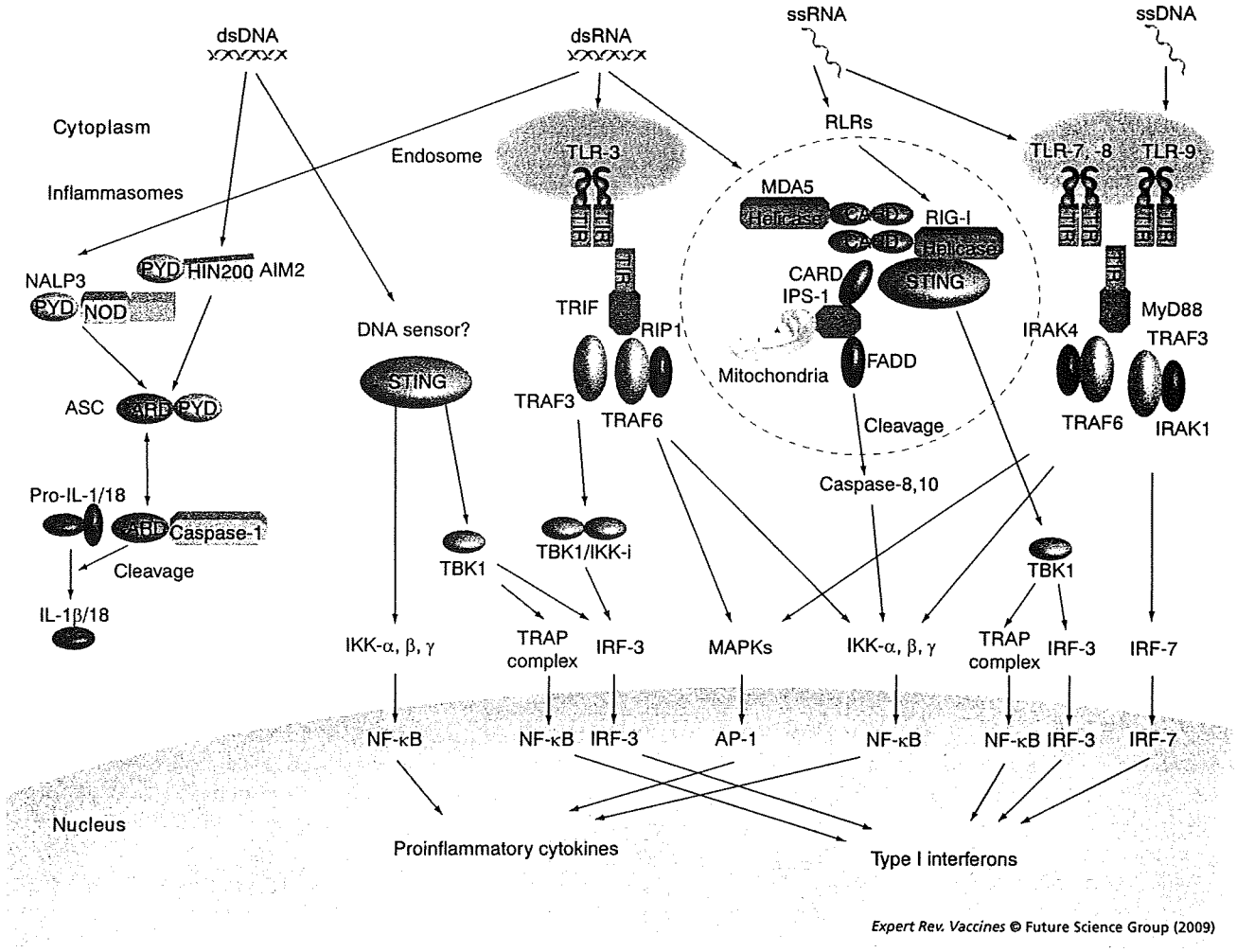

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Figure 1. Immune receptors for nucleic acid recognition. TLR-3, -7, -8 and -9 are located in endosomes. TLR-3 signals through TRIF (also known as TICAM-1). TRIF associates with TRAF-3, TRAF-6 and RIP1. TRAF3 activates TBK1 and IKK-i, while TRAF6 and 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 [6-], 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.

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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.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1 Hunter RL. Overview of vaccine adjuvants: present and future. *Vaccine* 20, S7–S12 (2002).

2 Rappuoli R. Bridging the knowledge gaps in vaccine design. *Nat. Biotechnol.* 25, 1361–1366 (2007).

3 Pulendran B, Ahmed R. Translating innate immunity into immunological memory: implications for vaccine development. *Cell* 124, 849–863 (2006).

•• **Discusses the importance of innate immunity manipulation for vaccine immunogenicity.**

4 Pashine A, Valiante NM, Ulmer JB. Targeting the innate immune response with improved vaccine adjuvants. *Nat. Med.* 11, S63–S68 (2005).

5 Steinman RM, Hemmi H. Dendritic cells: translating innate to adaptive immunity. *Curr. Top. Microbiol. Immunol.* 311, 17–58 (2006).

6 Ishii KJ, Akira S. Toll or Toll-free adjuvant path toward the optimal vaccine development. *J. Clin. Immunol.* 27, 363–371 (2007).

7 Ishii KJ, Koyama S, Nakagawa A *et al.* Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host Microbe* 3, 352–363 (2008).

8 Alexopoulou L, Holt AC, Medzhitov R *et al.* Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature* 413, 732–738 (2001).

9 Kleinman ME, Yamada K, Takeda A *et al.* Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. *Nature* 452, 591–597 (2008).

10 Diebold SS. Recognition of viral single-stranded RNA by Toll-like receptors. *Adv. Drug Deliv. Rev.* 60, 813–823 (2008).

11 Hemmi H, Takeuchi O, Kawai T *et al.* A Toll-like receptor recognizes bacterial DNA. *Nature* 408, 740–745 (2000).

12 Haas T, Metzger J, Schmitz F *et al.* The DNA sugar backbone 2′-deoxyribose determines Toll-like receptor 9 activation. *Immunity* 28, 315–323 (2008).

13 Hou B, Reizis B, Defranco AL. Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms. *Immunity* 29, 272–282 (2008).

14 Koyama S, Ishii KJ, Coban C *et al.* Innate immune response to viral infection. *Cytokine* 43, 336–341 (2008).

15 Pichlmair A, Schulz O, Tan CP *et al.* RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science* 314, 997–1001 (2006).

16 Kato H, Takeuchi O, Mikamo-Satoh E *et al.* Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *J. Exp. Med.* 205, 1601–1610 (2008).

17 Kawai T, Takahashi K, Sato S *et al.* IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat. Immunol.* 6, 981–988 (2005).

18 Seth RB, Sun L, Ea CK *et al.* Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-κB and IRF 3. *Cell* 122, 669–682 (2005).

19 Meylan E, Curran J, Hofmann K *et al.* Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437, 1167–1172 (2005).

20 Xu LG, Wang YY, Han KJ *et al.* VISA is an adapter protein required for virus-triggered IFN-β signaling. *Mol. Cell* 19, 727–740 (2005).

21 Ishikawa H, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signaling. *Nature* 455, 674–678 (2008).

22 Zhong B, Yang Y, Li S *et al.* The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity* 4, 538–550 (2008).

23 Sun W, Li Y, Chen L *et al.* ERIS, an endoplasmic reticulum IFN stimulator, activates innate immune signaling through dimerization. *Proc. Natl Acad. Sci. USA* 106(21), 8653–8658 (2009).

24 Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. *Nature* 442, 39–44 (2006).

25 Masumoto J, Zhou W, Chen FF *et al.* ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells. *J. Biol. Chem.* 274, 33835–33838 (1999).

26 Franchi L, Eigenbrod T, Muñoz-Planillo R *et al.* The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat. Immunol.* 10, 241–247 (2009).

27 Kanneganti TD, Ozoren N, Body-Malapel M *et al.* Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* 440, 233–236 (2006).

• **This report, together with [26], demonstrates that NALP3-dependent inflammasome activation is involved in microbial RNA recognition.**

28 Kanneganti TD, Body-Malapel M, Amer A *et al.* Critical role for cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J. Biol. Chem.* 281, 36560–36568 (2006).

29 Rotem Z, Cox RA, Isaacs A. Inhibition of virus multiplication by foreign nucleic acid. *Nature* 197, 564–546 (1963).

30 Suzuki K, Mori A, Ishii KJ *et al.* Activation of target-tissue immune-recognition molecules by double-stranded polynucleotides. *Proc. Natl Acad. Sci. USA* 96(5), 2285–2290 (1999).

31 Ishii KJ, Coban C, Kato H *et al.* A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nat. Immunol.* 7, 40–48 (2006).

32 Stetson DB, Medzhitov R. Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity* 24, 93–103 (2006).

33 Ishii KJ, Kawagoe T, Koyama S *et al.* TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature* 451, 725–729 (2008).

•• **Demonstrates that DNA vaccine immunogenicity is dominantly regulated by the TANK-binding kinase 1-dependent pathway, but not the Toll-like receptor-9-dependent pathway.**

34 Muruve DA, Petrilli V, Zaiss AK *et al.* The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. *Nature* 452, 103–107 (2008).

35 Roberts TL, Idris A, Dunn JA *et al.* HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. *Science* 323(5917), 1057–1060 (2009).

• **This report, together with [34,36] shows a novel molecule that is involved in DNA sensing and induction of NALP3-independent inflammasome activation.**

36 Hornung V, Ablasser A, Charrel-Dennis M *et al.* AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* 458(7237), 514–518 (2009).

37 Fernandes-Alnemri T, Yu JW, Datta P *et al.* AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature* 458(7237), 509–513 (2009).

38 Bürckstümmer T, Baumann C, Blüml S *et al.* An orthogonal proteomic–genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. *Nat. Immunol.* 3, 266–272 (2009).

39 Kato H, Takeuchi O, Sato S *et al.* Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441, 101–105 (2006).

40 Kumar H, Koyama S, Ishii KJ *et al.* Cooperation of IPS-1- and TRIF-dependent pathways in poly IC-enhanced antibody production and cytotoxic T-cell responses. *J. Immunol.* 180, 683–687 (2008).

41 Lopez CB, Moltedo B, Alexopoulou L *et al.* TLR-independent induction of dendritic cell maturation and adaptive immunity by negative-strand RNA viruses. *J. Immunol.* 173, 6882–6889 (2004).

42 Heer AK, Shamshiev A, Donda A *et al.* TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses. *J. Immunol.* 178, 2182–2191 (2007).

43 Koyama S, Ishii KJ, Kumar H *et al.* Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. *J. Immunol.* 179, 4711–4720 (2007).

44 Ichinohe T, Lee HK, Ogura Y *et al.* Inflammasome recognition of influenza virus is essential for adaptive immune responses. *J. Exp. Med.* 206, 79–87 (2009).

45 Thomas PG, Dash P, Aldridge JR Jr *et al.* The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. *Immunity* 4, 566–575 (2009).

46 Allen IC, Scull MA, Moore CB *et al.* The NLRP3 inflammasome mediates *in vivo* innate immunity to influenza a virus through recognition of viral RNA. *Immunity* 4, 556–565 (2009).

47 Geeraedts F, Goutagny N, Hornung V *et al.* Superior immunogenicity of inactivated whole virus H5N1 influenza vaccine is primarily controlled by Toll-like receptor signalling. *PLoS. Pathog.* 4(8), e1000138 (2008).

48 Krieg AM. Therapeutic potential of Toll-like receptor 9 activation. *Nat. Rev. Drug Discov.* 5, 471–484 (2006).

49 Gurunathan S, Klinman DM, Seder RA. DNA vaccines: immunology, application, and optimization. *Annu. Rev. Immunol.* 18, 927–974 (2000).

50 Coban C, Koyama S, Takeshita F *et al.* Molecular and cellular mechanisms of DNA vaccines. *Hum. Vaccin.* 4(6), 453–456 (2008).

51 Spies B, Hochrein H, Vabulas M *et al.* Vaccination with plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. *J. Immunol.* 171, 5908–5912 (2003).

52 Peek LJ, Middaugh CR, Berkland C. Nanotechnology in vaccine delivery. *Adv. Drug Deliv. Rev.* 60(8), 915–928 (2008).

53 Scheerlinck JP, Greenwood DL. Virus-sized vaccine delivery systems. *Drug Discov. Today* 19/20, 882–887 (2008).

54 Sanders MT, Brown LE, Deliyannis G *et al.* ISCOM-based vaccines: the second decade. *Immunol. Cell Biol.* 2, 119–128 (2005).

55 Fifis T, Gamvrellis A, Crimeen-Irwin B *et al.* Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. *J. Immunol.* 173, 3148–3154 (2004).

56 Zwiorek K, Bourquin C, Battiany J *et al.* Delivery by cationic gelatin nanoparticles strongly increases the immunostimulatory effects of CpG oligonucleotides. *Pharm. Res.* 3, 551–562 (2008).

57 Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. *Nat. Rev. Immunol.* 7, 179–190 (2007).

58 Roelofs MF, Joosten LA, Abdollahi-Roodsaz S *et al.* The expression of Toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of Toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. *Arthritis Rheum.* 52, 2313–2322 (2005).

59 Napolitani G, Rinaldi A, Bertoni F *et al.* Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat. Immunol.* 6, 769–776 (2005).

• **This report, together with [56,58] shows that innate immune receptor ligands could be combined to induce either synergy or tolerance.**

60 Bagchi A, Herrup EA, Warren HS *et al.* MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. *J. Immunol.* 178, 1164–1171 (2007).

61 van Heel DA, Ghosh S, Hunt KA *et al.* Synergy between TLR9 and NOD2 innate immune responses is lost in genetic Crohn's disease. *Gut* 54, 1553–1557 (2005).

62 He H, Genovese KJ, Nisbet DJ *et al.* Synergy of CpG oligodeoxynucleotide and double-stranded RNA (poly I:C) on nitric oxide induction in chicken peripheral blood monocytes. *Mol. Immunol.* 12, 3234–3242 (2007).

63 Wack A, Baudner BC, Hilbert AK *et al.* Combination adjuvants for the induction of potent, long-lasting antibody and T-cell responses to influenza vaccine in mice. *Vaccine* 4, 552–561 (2008).

64 Pichichero ME. Improving vaccine delivery using novel adjuvant systems. *Hum. Vaccine* 4, 262–270 (2008).

65 Casella CR, Mitchell TC. Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. *Cell Mol. Life Sci.* 20, 3231–3240 (2008).

66 Oma K, Jizi Z, Hirokazu E *et al.* Intranasal immunization with a mixture of PspA and a Toll-like receptor agonist induces specific antibodies and enhances bacterial clearance in the airways of mice. *Vaccine* (2009) (In Press).

67 Oliveira ML, Arêas AP, Ho PL. Intranasal vaccines for protection against respiratory and systemic bacterial infections. *Expert Rev. Vaccines* 6(3), 419–429 (2007).

68 Song JH, Nguyen HH, Cuburu N *et al.* Sublingual vaccination with influenza virus protects mice against lethal viral infection. *Proc. Natl Acad. Sci. USA* 105(5), 1644–1649 (2008).

69 Genescà M, Skinner PJ, Bost KM *et al.* Protective attenuated lentivirus immunization induces SIV-specific T cells in the genital tract of rhesus monkeys. *Mucosal Immunol.* 3, 219–228 (2008).

70 Kim-Schulze S, Kim HS, Wainstein A *et al.* Intrarectal vaccination with recombinant vaccinia virus expressing carcinoembryonic antigen induces mucosal and systemic immunity and prevents progression of colorectal cancer. *J. Immunol.* 11, 8112–8119 (2008).

71 Adams M, Navabi H, Jasani B *et al.* Dendritic cell (DC) based therapy for cervical cancer: use of DC pulsed with tumour lysate and matured with a novel synthetic clinically non-toxic double stranded RNA analogue poly [I]:poly [C(12)U] (Ampligen R). *Vaccine* 21, 787–790 (2003).

72 Ichinohe T, Tamura S, Kawaguchi A *et al.* Cross-protection against H5N1 influenza virus infection is afforded by intranasal inoculation with seasonal trivalent inactivated influenza vaccine. *J. Infect. Dis.* 196, 1313–1320 (2007).

73 Jin B, Wang RY, Qiu Q *et al.* Induction of potent cellular immune response in mice by hepatitis C virus NS3 protein with double-stranded RNA. *Immunology* 122, 15–27 (2007).

74 Fujimura T, Nakagawa S, Ohtani T *et al.* Inhibitory effect of the polyinosinic-polycytidylic acid/cationic liposome on the progression of murine B16F10 melanoma. *Eur. J. Immunol.* 36, 3371–3380 (2006).

75 Adams S, O'Neill DW, Nonaka D *et al.* Immunization of malignant melanoma patients with full-length NY-ESO-1 protein using TLR7 agonist imiquimod as vaccine adjuvant. *J. Immunol.* 181, 776–784 (2008).

76 Wu JJ, Huang DB, Tyring SK. Resiquimod: a new immune response modifier with potential as a vaccine adjuvant for Th1 immune responses. *Antiviral Res.* 64, 79–83 (2004).

77 Wille-Reece U, Flynn BJ, Loré K *et al.* Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime–boost immunization in nonhuman primates. *J. Exp. Med.* 203, 1249–1258 (2006).

78 Johnston D, Zaidi B, Bystryn JC. TLR7 imidazoquinoline ligand 3M-019 is a potent adjuvant for pure protein prototype vaccines. *Cancer Immunol. Immunother.* 56, 1133–1141 (2007).

79 Vajdy M, Selby M, Medina-Selby A *et al.* Hepatitis C virus polyprotein vaccine formulations capable of inducing broad antibody and cellular immune responses. *J. Gen. Virol.* 87, 2253–2262 (2006).

80 Zaks K, Jordan M, Guth A *et al.* Efficient immunization and crosspriming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes. *J. Immunol.* 176, 7335–7345 (2006).

81 Hornung V, Ellegast J, Kim S *et al.* 5′-triphosphate RNA is the ligand for RIG-I. *Science* 314, 994–997 (2006).

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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.

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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

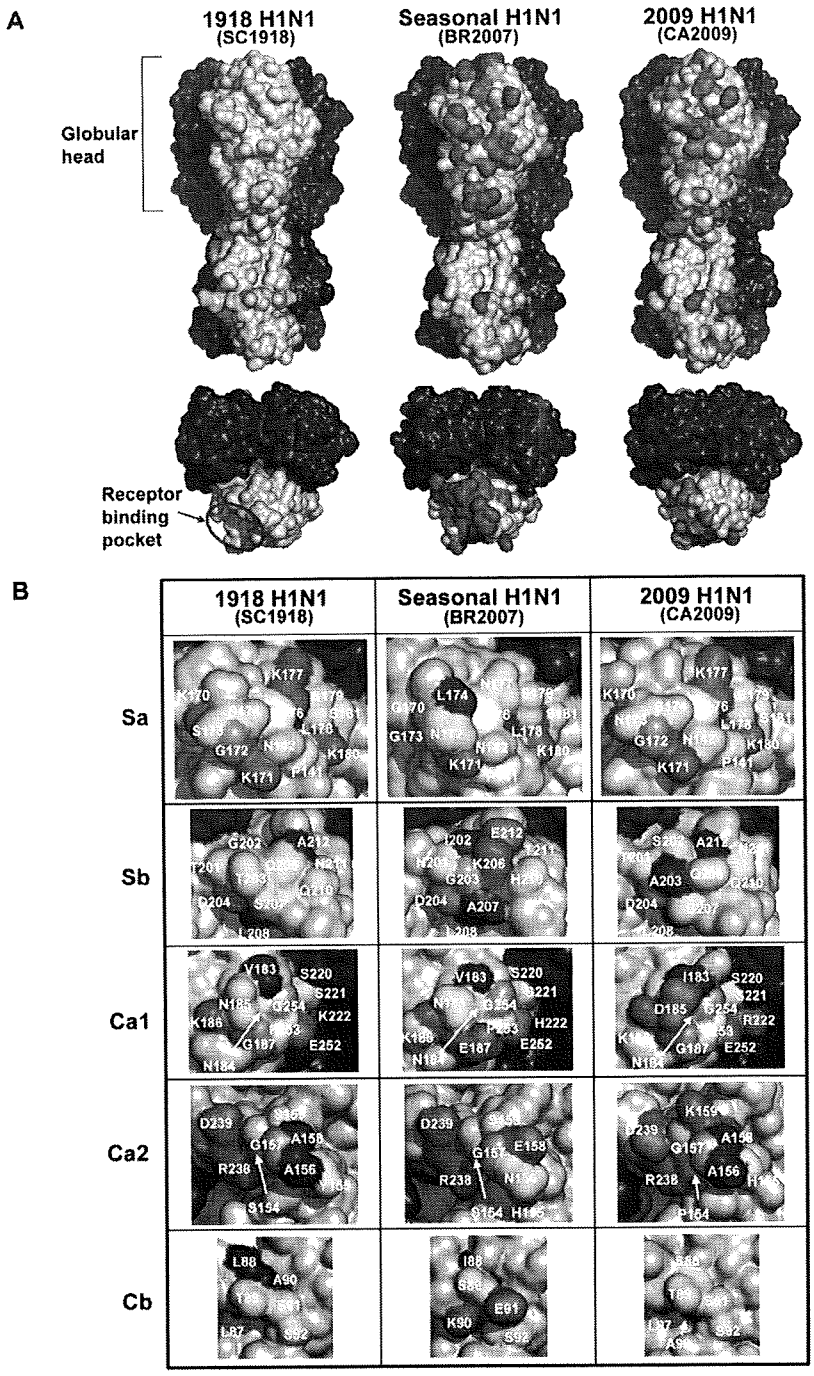

Figure 1. Comparison of the structures of antigenic sites on the HA molecules among 1918 H1N1 (SC1918), recent seasonal H1N1 (BR2007), and 2009 H1N1 (CA2009). Three-dimensional models of the H1 HA molecules of SC1918, BR2007, and CA2009 were constructed based on the HA crystal structures of A/South Carolina/1/18, 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).
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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

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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

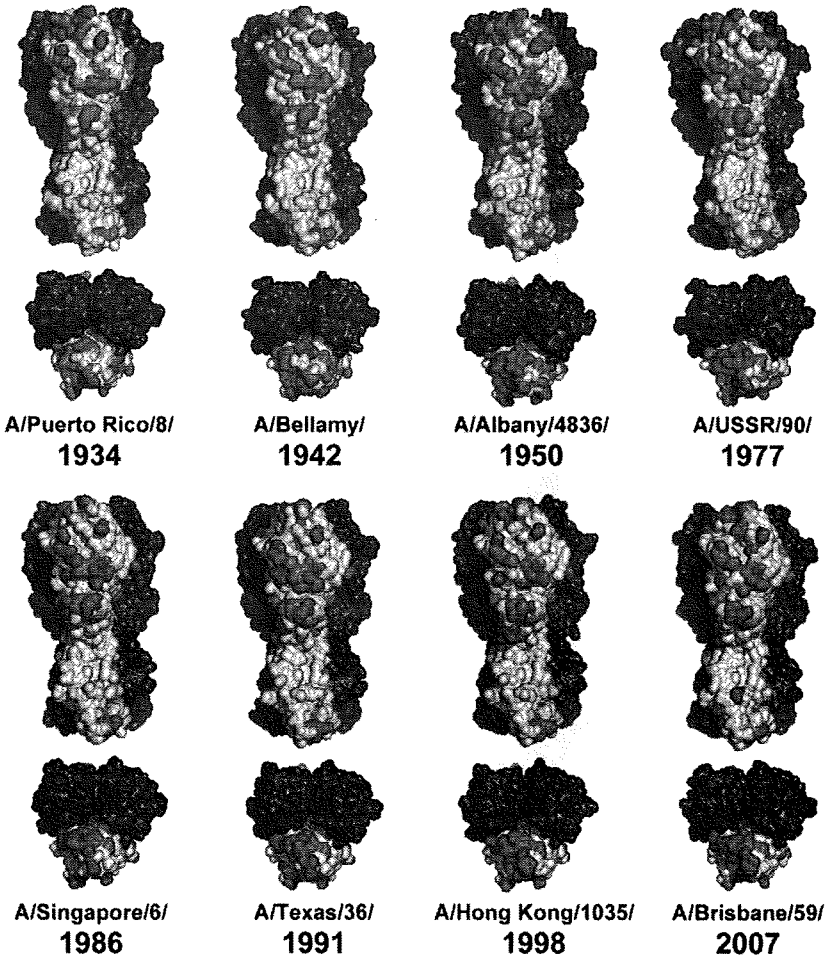

Figure 2. Amino acid substitutions associated with antigenic changes of seasonal human H1N1 virus HAs. All models were generated and shown by a molecular modeling method as described in the Methods section and the legend of Figure 1.
doi:10.1371/journal.pone.0008553.g002

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.

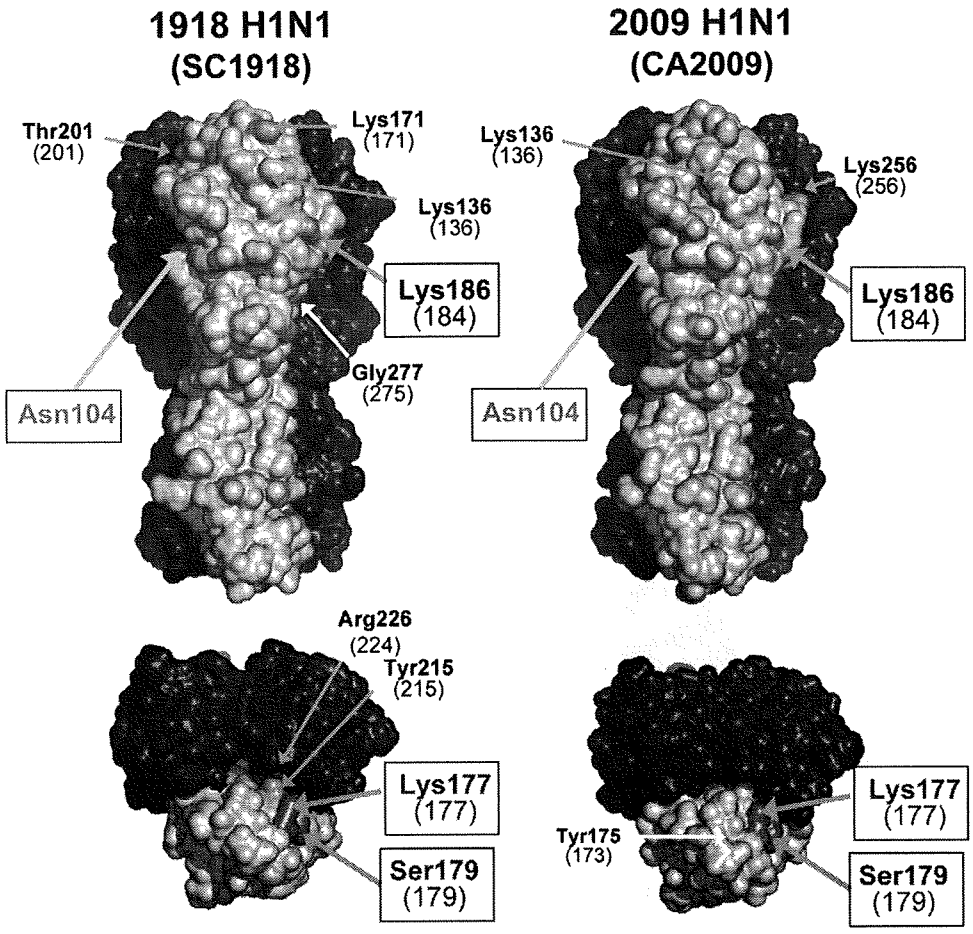

Figure 3. Comparison of the *N*-glycosylation potential of HA between SC1918 and CA2009. Residues shown in green represent Asn at the actually existing *N*-glycosylation sites. Residues shown in orange or blue represent the amino acids in *Cand1* sites that require a nucleotide substitution to produce *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

In this paper, we employed 3D structures constructed by a homology modeling method to map amino acid residues on the antigenic sites of HA. When compared to the presentation of simple primary sequences, the 3D presentation has following advantages: (a) There are several amino acid residues that are buried beneath the surface of the HA molecule, even if they are included in the antigenic sites described by the primary amino acid sequences. Since such amino acid residues do not directly contribute to the interaction with antibodies, the surface structures of antigenic sites that are accessible for antibodies can be compared more precisely in the presentation by 3D models than by the primary amino acid sequence. (b) An epitope likely consists of multiple amino acid residues belonging to different antigenic regions presented by the primary amino acid sequence. Such conformational epitopes can be illustrated only by the 3D presentation. (c) One of the purposes of this study is to provide a structural basis to confirm antigenic similarity between the 1918 H1N1 and the pandemic 2009 H1N1 viruses. For this purpose, we employed a homology modeling method rather than simply mapping on the existing crystal structure (e.g. 1918 H1N1 HA), since this method is generally used to generate a 3D structure of a protein molecule if there is no available crystal structure of the target protein [14]. Thus, we believe that this method produces more likely HA structure models of the viruses whose HA crystal structure are not available (e.g. CA2009). In fact, our homology modeling approach suggests that several amino acid residues were occasionally buried beneath or exposed to the surface of HA molecule, depending on the substitutions found in the viruses examined (Figure 1B and Figure S1). The homology modeling approach might enable us to analyze such dynamics of antigenic changes at molecular levels.

Our analysis indicated that 2009 H1N1 had undergone less significant antigenic changes of HA in the pig population than human H1N1 virus since their emergence in the early 1900s. The Centers for Disease Control and Prevention reported that vaccination with recent (2005–2009) human H1N1 viruses was unlikely to provide protection against 2009 H1N1 [15]; however, cross-reactive antibodies were detected in 33% of people aged 60 and over. Another report showed that appreciable neutralizing antibodies against CA2009 were present in the sera collected from individuals born before 1918 [16]. Our 3D models provide a protein-structural basis supporting these observations, and further suggest that infection with the 1918 H1N1 or early human H1N1 viruses (viruses present before the 1940s), but not with antigenically divergent human H1N1 viruses circulating after the 1950s, elicited cross-neutralizing antibodies to 2009 H1N1.

This virus will soon be subjected to complex immunological selection pressure by the antibody response that will be induced in the human population by vaccination and/or natural infection with homologous viruses, and pre-existing immunity cross-reactive to the early descendants of 1918 H1N1. In the present study, we showed that the antigenic structure of 2009 H1N1 HA might still be similar, at least in part, to that of the 1918 H1N1 HA. We speculate that the 2009 H1N1 HA antigenic sites involving the conserved amino acids will soon be targeted by neutralizing antibodies in humans. Thus, it is of interest to monitor whether these antigenic sites of 2009 H1N1 will undergo similar patterns of amino acid substitutions to those seen in seasonal H1N1 viruses during its epidemic period (Figure 4). Interestingly, we found that some of the recent variants of the 2009 H1N1 virus (as of November 3, 2009) have indeed undergone substitutions identical to those predicted in Figure 4. Although the present study still needs to be supported by experimental data, our approach may provide new perspectives on collective immunity against 2009 H1N1 and an insight into future antigenic changes of this new human pandemic influenza virus.

Methods

Sequence Data of HA Genes

Nucleotide sequences for HA genes of SC1918 (AF117241), BR2007 (CY030230), CA2009 (FJ966082), A/Puerto Rico/8/1934/Mount Sinai (AF389118), A/Bellamy/1942 (CY009276), A/Albany/4836/1950 (CY021701), A/USSR/90/1977 (DQ508897), A/Singapore/6/1986 (CY020477), A/Texas/36/1991 (AY289927), and A/Hong Kong/1035/1998 (AF386777) [2,3,17,18,19,20] were obtained from Influenza Virus Resource at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html).

Molecular Modeling

MODELLER 9v6 [21] was used for homology modeling of HA structures. After one hundred models of the HA trimer were generated, the model was chosen by a combination of the MODELLER objective function value and the discrete optimized protein energy (DOPE) statistical potential score [22]. After addition of hydrogen atoms, the model was refined by energy minimization (EM) with the minimization protocols in the Discovery Studio 2.1 software package (Accelrys, San Diego, CA) using a CHARMm force field. Steepest descent followed by conjugate gradient minimizations was carried out until the root mean square (rms) gradient was less than or equal to 0.01 kcal/mol/Å. The generalized Born implicit solvent model [23,24] was used to model the effects of solvation. The HA model was finally evaluated by using PROCHECK [25], WHATCHECK [26], and VERIFY-3D [27]. All figures are shown as a solvent-accessible surface representation prepared by PyMOL (DeLano Scientific LLC) [28]. All HA structures constructed by a homology modeling method are available in Supplementary Files S1, S2, S3, S4, S5, S6, S7, and S8.

Sequence Data Analyses for *N*-Glycosylation Sites

Custom-made programs were developed with the Ruby language and used for investigating the numbers of potential *N*-glycosylation sites and candidate codons (*Cand1*) in HA sequences. The programs are available upon request.

Supporting Information

Table S1

Found at: doi:10.1371/journal.pone.0008553.s001 (0.04 MB PDF)

Figure S1 Amino acid substitutions of seasonal human H1N1 virus HAs shown in close-up views of each antigenic site. The strains used in this analysis are corresponding to those shown in Figure 2. Amino acids are colored according to the scheme in the legend of Figure 1B.

Found at: doi:10.1371/journal.pone.0008553.s002 (1.02 MB PDF)

File S1 PDB file of the homology model of H1 HA (A/California/04/2009) after energy minimizations.

Found at: doi:10.1371/journal.pone.0008553.s003 (0.20 MB ZIP)

File S2 PDB file of the homology model of H1 HA (A/Bellamy/1942) after energy minimizations.

Found at: doi:10.1371/journal.pone.0008553.s004 (0.20 MB ZIP)

File S3 PDB file of the homology model of H1 HA (A/Albany/4836/1950) after energy minimizations.

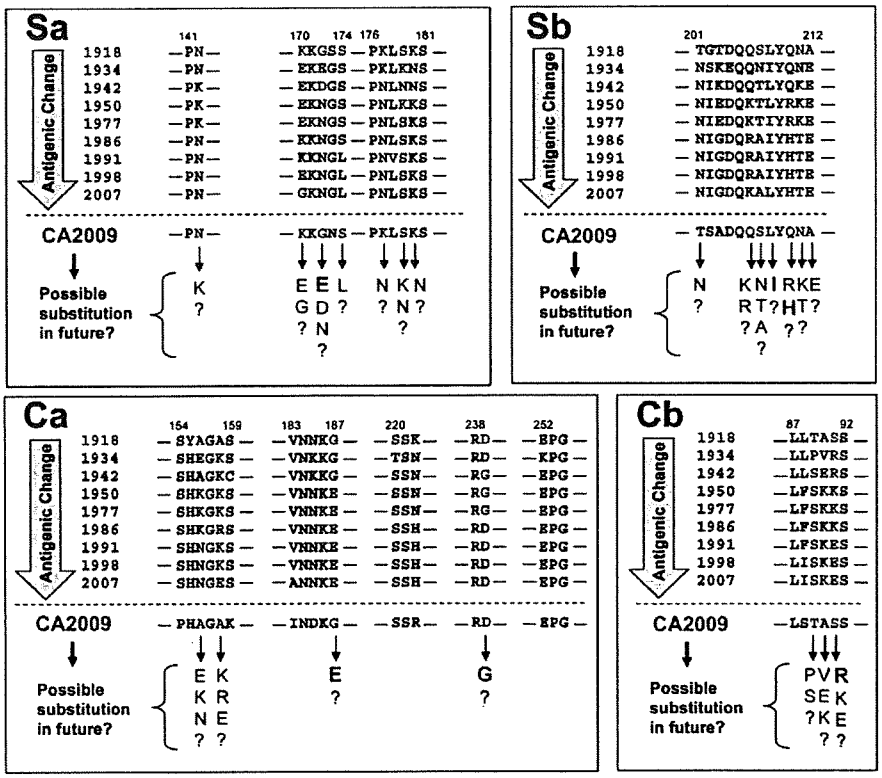

Figure 4. Prediction of the future amino acid substitutions on the antigenic sites of 2009 H1N1 HA. Amino acid sequences of HA antigenic sites of human H1N1 viruses are shown. Sequence data are corresponding to those of virus strains shown in Figures 1 and 2. Amino acid residues shared between 1918 H1N1 (SC1918) and 2009 H1N1 (CA2009) are shown in red, and those that have been substituted since 1934 are shown in blue. Amino acid residues indicated by arrows represent the predicted substitutions which might be associated with antigenic changes of 2009 H1N1 in the near future. The amino acid substitutions which have already been found in the recent variants of the 2009 H1N1 virus (as of November 3, 2009) are shown in bold pink letters.
doi:10.1371/journal.pone.0008553.g004

Found at: doi:10.1371/journal.pone.0008553.s005 (0.20 MB ZIP)

File S4 PDB file of the homology model of H1 HA (A/USSR/90/1977) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s006 (0.20 MB ZIP)

File S5 PDB file of the homology model of H1 HA (A/Singapore/6/1986) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s007 (0.20 MB ZIP)

File S6 PDB file of the homology model of H1 HA (A/Texas/36/1991) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s008 (0.20 MB ZIP)

File S7 PDB file of the homology model of H1 HA (A/Hong Kong/1035/1998) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s009 (0.20 MB ZIP)

File S8 PDB file of the homology model of H1 HA (A/Brisbane/59/2007) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s010 (0.20 MB ZIP)

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Author Contributions

Conceived and designed the experiments: MI KI HK AT. Analyzed the data: MI RY DT. Wrote the paper: MI AT.

References

1. Reid AH, Taubenberger JK (2003) The origin of the 1918 pandemic influenza virus: a continuing enigma. J Gen Virol 84: 2285–2292.
2. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, et al. (2009) Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325: 197–201.
3. Reid AH, Fanning TG, Hultin JV, Taubenberger JK (1999) Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. Proc Natl Acad Sci U S A 96: 1651–1656.
4. Vincent AL, Lager KM, Ma W, Lekcharoensuk P, Gramer MR, et al. (2006) Evaluation of hemagglutinin subtype 1 swine influenza viruses from the United States. Vet Microbiol 118: 212–222.
5. Sugita S, Yoshioka Y, Itamura S, Kanegae Y, Oguchi K, et al. (1991) Molecular evolution of hemagglutinin genes of H1N1 swine and human influenza A viruses. J Mol Evol 32: 16–23.
6. Sheerar MG, Easterday BC, Hinshaw VS (1989) Antigenic conservation of H1N1 swine influenza viruses. J Gen Virol 70(Pt 12): 3297–3303.
7. Luoh SM, McGregor MW, Hinshaw VS (1992) Hemagglutinin mutations related to antigenic variation in H1 swine influenza viruses. J Virol 66: 1066–1073.
8. Caton AJ, Brownlee GG, Yewdell JW, Gerhard W (1982) The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 31: 417–427.

9. Gerhard W, Yewdell J, Frankel ME, Webster R (1981) Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. Nature 290: 713–717.
10. Brownlee GG, Fodor E (2001) The predicted antigenicity of the haemagglutinin of the 1918 Spanish influenza pandemic suggests an avian origin. Philos Trans R Soc Lond B Biol Sci 356: 1871–1876.
11. Gallagher P, Henneberry J, Wilson I, Sambrook J, Gething MJ (1988) Addition of carbohydrate side chains at novel sites on influenza virus hemagglutinin can modulate the folding, transport, and activity of the molecule. J Cell Biol 107: 2059–2073.
12. Igarashi M, Ito K, Takada A Prediction of N-glycosylation potential of influenza virus hemagglutinin by a bioinformatic approach. Glycomicrobiology http://www.glycoforum.gr.jp/science/glycomicrobiology/GM04/GM04E.html.
13. Igarashi M, Ito K, Kida H, Takada A (2008) Genetically destined potentials for N-linked glycosylation of influenza virus hemagglutinin. Virology 376: 323–329.
14. Maurer-Stroh S, Ma J, Lee RT, Sirota FL, Eisenhaber F (2009) Mapping the sequence mutations of the 2009 H1N1 influenza A virus neuraminidase relative to drug and antibody binding sites. Biol Direct 4: 18; discussion 18.
15. Prevention CfDCa (2009) Serum cross-reactive antibody response to a novel influenza A (H1N1) virus after vaccination with seasonal influenza vaccine. MMWR Morb Mortal Wkly Rep 58: 521–524.
16. Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, et al. (2009) In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature: in press.
17. Taubenberger JK, Reid AH, Krafft AE, Bijwaard KE, Fanning TG (1997) Initial genetic characterization of the 1918 "Spanish" influenza virus. Science 275: 1793–1796.
18. Schickli JH, Flandorfer A, Nakaya T, Martinez-Sobrido L, Garcia-Sastre A, et al. (2001) Plasmid-only rescue of influenza A virus vaccine candidates. Philos Trans R Soc Lond B Biol Sci 356: 1965–1973.
19. Lee MS, Yang CF (2003) Cross-reactive H1N1 antibody responses to a live attenuated influenza vaccine in children: implication for selection of vaccine strains. J Infect Dis 188: 1362–1366.
20. Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, et al. (2009) Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 360: 2605–2615.
21. Eswar N, John B, Mirkovic N, Fiser A, Ilyin VA, et al. (2003) Tools for comparative protein structure modeling and analysis. Nucleic Acids Res 31: 3375–3380.
22. Shen MY, Sali A (2006) Statistical potential for assessment and prediction of protein structures. Protein Sci 15: 2507–2524.
23. Still WC, Tempczyk A, Hawley RC, Hendrickson T (1990) Semianalytical Treatment of Solvation for Molecular Mechanics and Dynamics. Journal of the American Chemical Society 112: 6127–6129.
24. Tsui V, Case DA (2000) Theory and applications of the generalized Born solvation model in macromolecular Simulations. Biopolymers 56: 275–291.
25. Laskowski RA, Macarthur MW, Moss DS, Thornton JM (1993) Procheck - a Program to Check the Stereochemical Quality of Protein Structures. Journal of Applied Crystallography 26: 283–291.
26. Hooft RW, Sander C, Scharf M, Vriend G (1996) The PDBFINDER database: a summary of PDB, DSSP and HSSP information with added value. Comput Appl Biosci 12: 525–529.
27. Eisenberg D, Luthy R, Bowie JU (1997) VERIFY3D: assessment of protein models with three-dimensional profiles. Methods Enzymol 277: 396–404.
28. DeLano WL (2002): The PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA).
29. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876–4882.