an exposed virulence factor found in virtually all pneumococcal strains [12,13]. Anti-PspA antibodies overcome the anticomplement effect of PspA, allowing for increased complement activation and C3 deposition on PspA-bearing bacteria [14,15]. Serum from humans immunized with PspA can passively protect mice against challenge with various pneumococcal strains [16]. Importantly, a recent study confirmed that the rabbit antibodies to PspA could mediate killing in the modified opsonophagocytosis killing assay [17].

PspA is composed of five domains: (i) a signal peptide, (ii) an α-helical highly charged (N-terminal) domain, (iii) a proline-rich region domain, (iv) a choline-binding domain, and (v) a short hydrophobic tail [18,19]. The α-helical domain of PspA has an antiparallel coiled-coil motif and is considered to be the most exposed part of the molecule [20]. The α-helical domain binds to protective monoclonal antibodies and inhibits killing of pneumococci by at least two host cationic peptides [21,22]. The proline-rich domain is composed of many repetitive sequences shared by other proline-rich domains making its inclusion important for achieving broad protection [23].

PspA proteins have been grouped into three families encompassing six different clades based on the C-terminal 100 amino acids of the α-helical region [24]. Family 1 comprises clades 1 and 2; family 2 comprises clades 3, 4 and 5; and family 3 comprises clade 6 [22,24]. Pneumococcal strains expressing family 1 or 2 PspA proteins constitute >96% of clinical isolates from patients with IPD [6,13,25]. Although different PspA proteins induce antibodies with different degrees of cross-reactivity in vitro and cross-protection of mice [26,27], our previous studies demonstrated that no single PspA construct can elicit complete protection against challenge by strains with all PspA clades and families [28]. To accommodate this variability, it was proposed that a combination of two PspA antigens, one from PspA family 1 and one from PspA family 2, should elicit protection against the vast majority of pneumococcal strains [29–31]. Thus, it is important to determine which PspA fragments show the broadest cross-reactivity. In this study, we prepared fusion proteins of three pairs of PspA molecules, and determined which provided the broadest cross-reaction with clinical isolates of *S. pneumoniae*.

2. Materials and methods

2.1. Pneumococcal strains

Six laboratory strains (all originally from patients), including BG9739 (serotype 4, PspA clade 1), D39 (serotype 2, PspA clade 2), WU2 (serotype 3, PspA clade 2), TIGR4 (serotype 3, PspA clade 3), EF5668 (serotype 4, PspA clade 4), and ATCC 6303 (serotype 3, PspA clade 5) were used to construct the fusion PspA proteins. These laboratory strains and a recent clinical isolate, KK1162 (serotype 3, PspA clade 4), were used for bacterial challenge. Sixty-eight clinical isolates, including KK1162 strain, from Japanese adult patients with IPD were also used [32]. These isolates were serotyped using agglutination assay, and their PspA clades were determined using a method published previously [32,33].

2.2. Construction of fusion PspA fragments

Our previous study demonstrated a significant protection against sepsis caused by WU2 strain (PspA clade 1) by immunization with full-length BG9739 derived PspA (clade 1) but only a weak protection against homologous challenge with BG9739 [28]. Therefore, we excluded PspA clade 1 derived from BG9739 strain from the fusion PspA proteins. In this study, we prepared the fusion proteins from three pairs of PspA clade 2 from family 1 and PspA clades 3, 4 and 5 from family 2. All cloning procedures were performed with *Escherichia coli* DH5α grown in Luria–Bertani medium (Sigma-Aldrich, St. Louis, MO) supplemented with kanamycin (30 μg/ml). DNA fragments encoding portions of the N-terminal regions (containing the α-helix domain and proline-rich region) of PspA clades 2 and 3 were amplified by PCR using strains D39 and TIGR4. The primers used in this procedure are available in Appendix 1. The resulting PCR products were digested with *Nde*I and *Eco*RI, and were ligated to the pET28a (+) vector (Novagen, Madison, WI), and the sequences were confirmed by DNA sequencing. The pET28a–PspA constructs digested with *Eco*RI and *Xho*I, and the resulting fragments, which encoded portions of the N-terminal regions of PspA clades 4, 5, or 2 were amplified by PCR using strains EF5668 (Accession no. U89711), ATCC6303 (Accession no. AF071820), or WU2 (Accession no. AF071814), respectively, and were ligated to the linearized vector. The fusion PspA proteins were obtained with primers that allowed the removal of the signal sequence. The fusion PspA2+4 was constructed by fusing the 3′ terminus of PspA clade 2 of D39 strain (Accession no. AF071814) with the 5′ terminus of PspA clade 4 of EF5668 strain, through the *Eco*RI ligated to pET28a–6 × His. The fusion PspA2+5 was constructed by fusing the 3′ terminus of PspA clade 2 of D39 strain with the 5′ terminus of PspA clade 5 of ATCC6303 strain, through the *Eco*RI ligated to pET28a–6 × His. The fusion PspA3+2 was constructed by fusing the 3′ terminus of PspA clade 3 of TIGR4 strain (Accession no. AE005672.3) with the 5′ terminus of PspA clade 2 of WU2 strain, through the *Eco*RI ligated to pET28a–6 × His.

2.3. PspA expression and purification

Competent *E. coli* BL21 (DE3) cells were transformed with pET28a (+) vectors containing the fusion PspA or the single PspA constructs. The recombinant proteins were purified and stored as described elsewhere [34].

2.4. Immunization of mice

Female C57/BL6j mice (6–8 weeks old) were purchased from CLA Japan. Mice were immunized subcutaneously three times at 7-days intervals with 0.1 μg of recombinant fusion PspA derivatives in lipopolysaccharide-free phosphate-buffered saline (PBS) (Sigma) in combination with 2.5 μg of TLR9 ligand adjuvants K3 CpG oligonucleotides (CpG ODNs) and 5 μg of aluminum hydroxide gel (AHG) (A gift from The Research Foundation for Microbial Diseases of Osaka University) or CpG ODNs alone (final volume of 200 μl per mouse). A subcutaneous route of immunization was chosen because our preliminary study demonstrated the levels of PspA-specific IgG in mice subcutaneously immunized with 0.1 μg of PspA plus 2.5 μg of CpG ODNs were significantly higher than those in mice nasally immunized with 0.1 μg of PspA plus 2.5 μg of CpG ODNs (data not shown). CpG ODNs were prepared as described previously [35]. Because the PspA clade-specific IgG levels tended to be higher in mice immunized with each PspA fusion protein with CpG ODNs plus AHG than in those immunized with PspA fusion protein with CpG ODNs alone (see Appendix 2), we used the CpG ODNs plus AHG (define as the double adjuvants), for the immunization of mice with PspA fusion proteins in this study. These double adjuvants were safe in nonhuman primate models, and were applicable to humans [36]. Serum was collected from mice by retro-orbital bleeding 1 week after the third immunization. All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University, Japan (Permit Number: Biken-AP-H23-05-0).

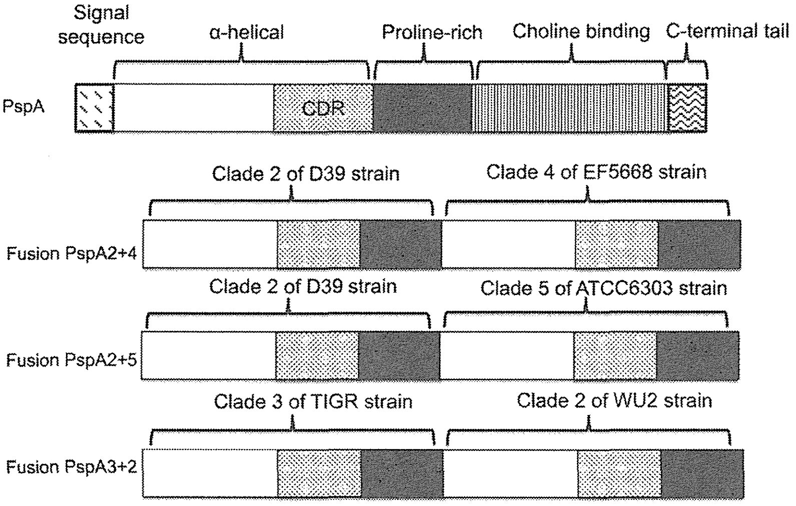

Fig. 1. Schematic diagram of PspA and three fusion PspA proteins. The entire PspA molecule containing the N-terminal α-helical domain, which contains the clade-defining region (CDR), the proline-rich region, the choline-binding domain, and the C-terminal tail (upper column). Each recombinant fusion protein is shown with its different composition (three lower rows).

2.5. Binding of PspA-specific IgG to pneumococcal strains by flow cytometry

Five pneumococcal strains for bacterial challenge and 68 clinical isolates were grown in blood agar plates overnight and then subcultured again on blood agar plates for 4–5 h. The bacteria were collected in PBS, harvested by centrifugation, and washed once with PBS. Ninety microliters of the bacterial suspension at a concentration of 1×10^8 colony-forming units (cfu)/ml in PBS was incubated with 10 μl of mouse antisera for 30 min at 37 °C. After incubation, the suspension was washed once with PBS, resuspended in 100 μl of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:100), and incubated for 30 min on ice. After the incubation, the bacterial suspension was washed twice with PBS and suspended in 500 μl of 1% formaldehyde. The samples were kept on ice in the dark until analyzed by flow cytometry using a BD FACSCalibur™ with CellQuest software (BD Sciences, San Jose, CA), and the percentage of fluorescent bacteria (>1 fluorescence intensity unit) in each group was determined. Sera from mice immunized with double adjuvants only were used as the negative controls.

2.6. Protection against pneumococcal challenge

The mice immunized with the PspA fusion protein plus double adjuvants were challenged intranasally with 2×10^7 cfu of strain BG9739 (clade 1), 2×10^7 cfu of strain WU2 (clade 2), 5×10^6 cfu of strain TIGR4, 2×10^7 cfu of strain KK1162 (clade 4), or 5×10^5 cfu of strain ATCC6303 (clade 5). Bacterial challenges were performed 2 weeks after the final immunization. Mortality was monitored for 2 weeks following pneumococcal challenge. The mice immunized with double adjuvants alone were used as a control.

2.7. Statistical analysis

Analysis of variance followed by an unpaired Mann–Whitney *U* test was used to evaluate differences in antibody titer. The percent binding by immune sera to each pneumococcal strain was compared by paired *t*-test. Survival rates were analyzed by the Kaplan–Meier log-rank test. All analyses were performed using GraphPad Prism Software (GraphPad software, La Jolla, CA). *p* values <0.05 were considered significant.

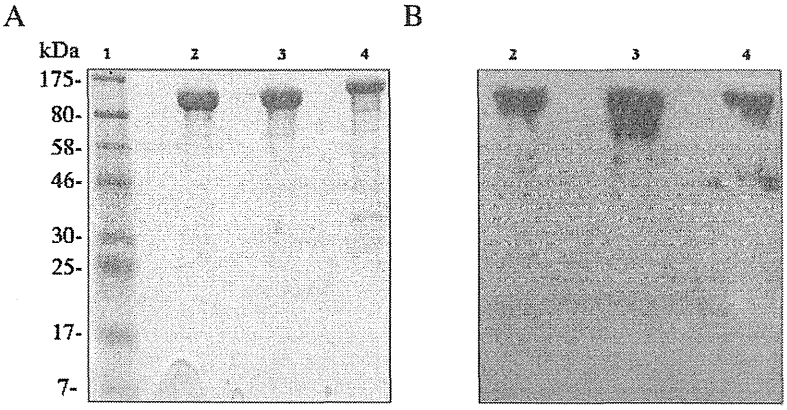

Fig. 2. Characterization of three purified fusion PspA proteins by SDS–PAGE (A) and Western blot analysis (B). The proteins were subjected to SDS–PAGE and detected by direct staining with Coomassie brilliant blue. Lane 1, standard molecular weight markers; lane 2, PspA2+4; lane 3, PspA2+5; 4, lane PspA3+2. The values on the left are molecular sizes in kilodaltons. Mouse antiserum against PspA recombinant protein (clade 2) was used for Western blot analysis. Lane 2, PspA2+4; lane 3, PspA2+5; lane 4, PspA3+2.

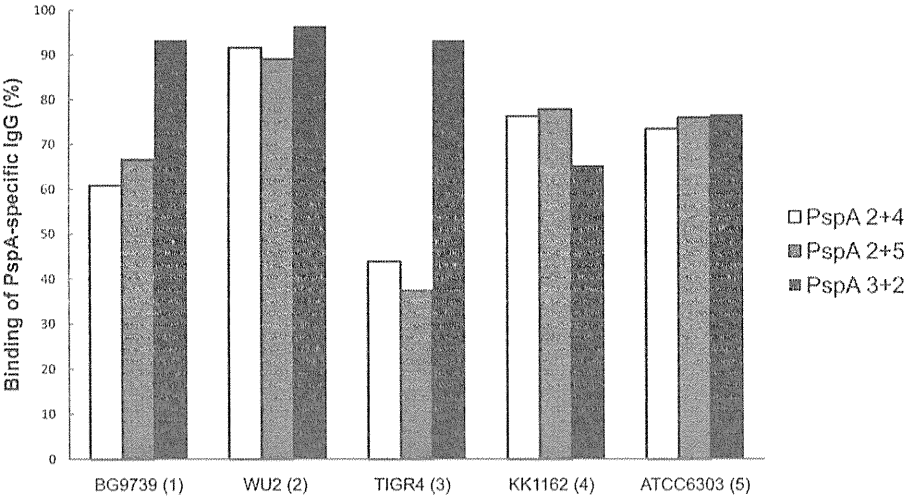

Fig. 3. The binding of PspA-specific IgG by antisera with PspA fusion proteins to the challenge strains with different clades. The mean percentages of fluorescent bacteria positive for IgG binding by antisera from mice immunized with PspA2+4, PspA2+5, or PspA3+2 in combination CpG ODNs plus AHG (double adjuvants) are shown for five pneumococcal strains with PspA clades 1–5 used in the challenge experiments. The numbers in parentheses represents the PspA clade.

3. Results

A schematic diagram of PspA and the three PspA fusion proteins constructed from PspA families 1 and 2 are shown in Fig. 1. The purified recombinant fusion proteins were electrophoresed on sodium dodecyl sulfate–polyacrylamide (SDS–PAGE) gels and evaluated by Coomassie blue staining (Fig. 2A) and by Western blotting using mouse anti-PspA/Rx1 sera (PspA/Rx1 and PspA/D39 are identical clade 2 PspA molecules) (Fig. 2B).

PspA-specific IgG binding >60% was found in antiserum raised by PspA2+4 or PspA2+5 plus double adjuvants for the challenge strains expressing PspA clades 1, 2, 4, and 5, but not for the strain expressing clade 3 (Fig. 3). By contrast, PspA-specific IgG binding > 60% was found for the challenge strains expressing all five PspA clades in antiserum raised by PspA3+2 plus double adjuvants.

For the challenge with the bacterial strain BG9739 with PspA clade 1, the survival rate was greater in mice immunized with PspA3+2 plus double adjuvants ($p < 0.01$) compared with mice

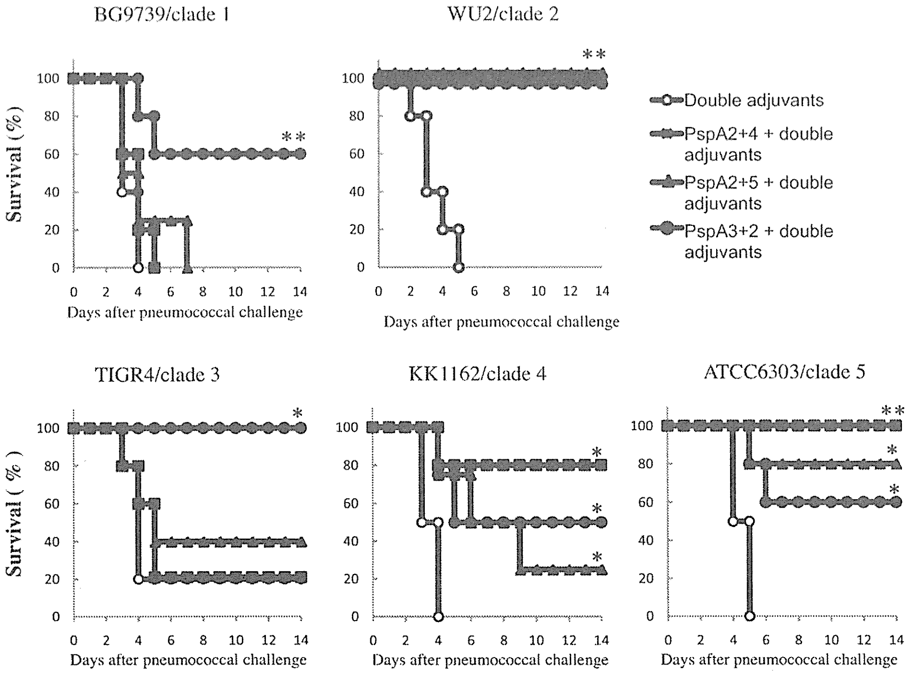

Fig. 4. Protective effects of immunization with fusion PspA proteins against pneumococcal challenge in mice. Mice were immunized subcutaneously with PspA2+4 (closed squares), PspA2+5 (closed triangles), or PspA3+2 (closed circles) in combination with CpG ODNs plus AHG (double adjuvants) or double adjuvants alone (open circles) three times at 1-week intervals. Two weeks after the last immunization, the immunized mice were challenged intranasally with pneumococcal strains with PspA clades 1–5. Mortality was monitored for 2 weeks. Eight to 10 mice per group were examined in each challenge experiment using pneumococcal strain with five different clades. $^{*}p < 0.05$ (vs double adjuvants alone), $^{**}p < 0.01$ (vs double adjuvants alone).

Table 1
Serotypes and PspA clades of 68 isolates from adults with invasive pneumococcal disease.

Serotype	No. strain	No. strain				
		Family 1		Family 2		
		Clade 1	Clade 2	Clade 3	Clade 4	Clade 5
1	1	1				
3	10	9		1		
4	4			4		
6A	2		1			1
6B	10	7		3		
6C	1		1			
7F	2			2		
9V	1			1		
10A	3	3				
11A	2				2	
12F	1			1		
14	5	5				
15A	1				1	
15B	1			1		
16	1			1		
18B	1	1				
18C	1	1				
19A	3			3		
19F	3	1		2		
20	1	1				
22F	3	3				
23A	1				1	
23F	5					5
33	1	1				
34	1	1				
35	2				2	
38	1		1			
Total	68 (100%)	34 (50%)	3 (4%)	19 (28%)	6 (9%)	6 (9%)

immunized with double adjuvants alone (Fig. 4). By contrast, the survival rate did not differ between mice immunized with PspA2+4 or PspA2+5 plus double adjuvants compared with mice immunized with double adjuvants alone. For the bacterial challenge with the WU2 strain with PspA clade 2, the survival rate was significantly higher in mice immunized with PspA2+4, PspA2+5, or PspA3+2 plus double adjuvants ($p < 0.01$) compared with mice immunized with double adjuvants alone. For the bacterial challenge with the TIGR4 strain with PspA clade 3, the survival rate was significantly higher in mice immunized with PspA3+2 plus double adjuvants ($p < 0.05$) compared with mice immunized with double adjuvants alone. The survival rate did not differ between mice immunized with PspA2+4 or PspA2+5 plus double adjuvants compared with mice immunized with double adjuvants alone. In the case of challenge with clade 4 and 5 strains, all three PspA fusion vaccines showed significant protection compared with mice immunized with double adjuvants alone ($p < 0.01$ or $p < 0.05$). These data indicate that immunization with the PspA3+2 vaccine conferred significant protection of mice against pneumococcal challenge by all of the strains expressing PspA clades 1–5. The other two PspA fusion proteins failed to elicit protection against two of the challenge strains (PspA clades 1 and 3).

The distribution of serotypes and PspA clades of 68 clinical isolates from adult patients with IPD are shown in Table 1. The major serotypes were serotype 3 (15%) and 6B (15%), followed by serotypes 14 (7%) and 23F (7%). The major PspA clades were clade 1 (50%) and clade 3 (28%), followed by clade 4 (9%), clade 5 (9%), and clade 2 (4%). All the clinical isolates belonged to PspA clades 1–5, which is in agreement with previous studies [6,13,25].

The binding of PspA-specific IgG in antiserum raised by PspA2+4, PspA2+5, or PspA3+2 plus double adjuvants was examined for the 68 clinical isolates (Fig. 5). The binding of PspA-specific IgG for clade 3 strains ($n = 19$) in antiserum raised by PspA3+2 was significantly higher than in that raised by PspA2+4 or PspA2+5 ($p < 0.05$). By contrast, the binding of PspA-specific IgG for clade 5 strains ($n = 6$) in antiserum raised by PspA3+2 was significantly lower than that by PspA2+4 ($p < 0.05$) or PspA2+5 ($p < 0.05$). No significant difference was found in the binding of PspA-specific IgG for 34 clade 1 strains, three PspA clade 2 strains, or six PspA clade 4 strains between the three types of antiserum raised by PspA2+4, PspA2+5, or PspA3+2.

4. Discussion

In this study, we have demonstrated >60% binding of PspA-specific IgG in the antiserum raised in mice by PspA2+4 or PspA2+5 to four challenge strains expressing clades 1, 2, 4, and 5, but low binding of PspA-specific IgG to the strain expressing clade 3 (Fig. 3). By contrast, >60% binding of PspA-specific IgG in antiserum raised in mice by PspA3+2 was found to all five challenge strains expressing PspA clades 1–5. Immunization with PspA3+2 provided significant protection against pneumococcal challenge by these five strains expressing clades 1–5, but PspA2+4 or PspA2+5 protected mice against only three of the strains expressing clades 2, 4 and 5 in this study (Fig. 4). Therefore, it may be speculated that the binding of PspA-specific IgG closely correlates with the protective effects of PspA fusion protein against pneumococcal challenge in mice. These findings are supported by a recent report on the ability of opsonophagocytic killing and protection of mice against pneumococcal infection by human antiserum to PspA [17]. Only one exception for this speculation is that no protection was found against pneumococcal challenge by the clade 1 strain BG9739 (serotype 4) in mice immunized with PspA2+4 or PspA2+5 plus double adjuvants despite of >60% binding of PspA-specific IgG in antiserum raised by PspA2+4 or PspA2+5 for this clade 1 strain. One possible reason for the inefficient immunization with PspA2+4 or PspA2+5 in mice infected with BG9739 strain may be the presence of serotype 4 capsular polysaccharide. Our previous study demonstrated that the difficulty in protecting against serotype 4 strains was eliminated when mice were immunized with a homologous PspA of the same PspA family [37]. However, only weak protection against infection with strain BG9739 was observed by immunization of mice with the homologous PspA clade 1 [28]. Therefore, it remains uncertain whether immunization with PspA2+4 or PspA2+5 plus double adjuvants did not protect against pneumococcal challenge by the clade 1 strain BG9739 in mice.

No differences were found in the binding of PspA-specific IgG to the clinical isolates belonging to the major clade 1 ($n = 34$) and the two minor clades 2 ($n = 3$) and 4 ($n = 6$) between the types of antiserum raised by the three PspA fusion proteins. For the clinical isolates belonging to the second major clade 3 ($n = 19$), antiserum raised by PspA3+2 demonstrated the greatest binding between the three types of antiserum raised by the PspA fusion proteins (Fig. 5). These findings are in agreement with those showing the binding of PspA-specific IgG to the TIGR4 strain expressing clade 3 for the three types of antiserum raised by each PspA fusion protein (Fig. 3). However, antiserum raised by PspA3+2 demonstrated the lowest binding to six clinical isolates belonging to the minor clade 5 between three types of antiserum raised by each PspA fusion protein. Collectively, PspA3+2 appears to be advantageous in terms of its cross-reactivity with clinical isolates and cross-protection against pneumococcal challenge in mice compared with the other two PspA fusion proteins.

Darrieux et al. reported that immunization with fusion proteins containing fragments of PspA from families 1 and 2 provided cross-protection against pneumococcal strains from families 1 and 2 in mice [30]. The fusion proteins containing PspA clade 1 and PspA clade 3 or 4 fragments provided significant protection against the A66.1 strain (PspA clades 1, and 2), but the protection against strains from clades 3 and 4 was of borderline significance. In another

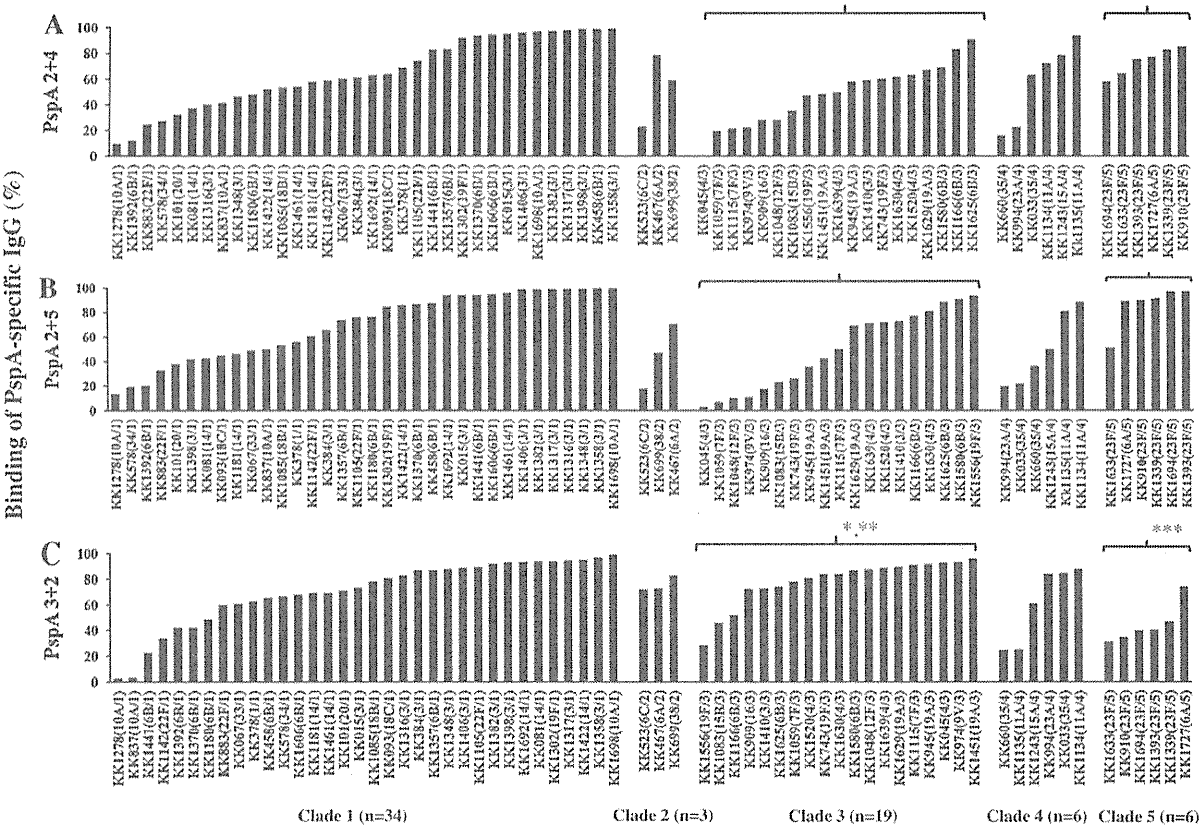

Fig. 5. Comparison of PspA-specific IgG binding by antisera with PspA2+4 (A), PspA2+5 (B), or PspA3+2 (C) in combination with CpG ODNs plus AHG (double adjuvants) to 68 pneumococcal isolates (34 for clade1, three for clade 2, 19 for clade 3, six for clade 4, and six for clade 5). The serotypes and PspA clades are shown in parentheses after the strain names. $^*p < 0.01$ (vs PspA2+4), $^{**}p < 0.05$ (vs PspA2+5), $^{***}p < 0.05$ (vs PspA2+4 or PspA2+5).

study, these authors reported that antiserum against fusion protein PspA1+4 demonstrated strong cross-reactivity with PspA clades 1 and 5 but low cross-reactivity with PspA clade 2 or 3 [29]. Consequently, Darrieux et al. failed to demonstrate significant protection against pneumococcal challenge by strains with PspA clades 1–5, although they demonstrated limited cross-protection by immunization with the fusion proteins containing fragments of PspA from families 1 and 2.

A limitation of our study is that we generated and examined only three PspA fusion proteins, which contained one clade each from PspA families 1 and 2. Another limitation is that the binding of PspA-specific IgG was assessed in a small number of clinical isolates from adult patients with IPD.

The antiserum raised by PspA3+2 demonstrated relatively weak binding capacity to the clinical isolates expressing PspA clade 5 in this study. Further studies are required to generate the other types of PspA fusion proteins that can induce PspA-specific IgG with a high affinity to strains expressing PspA clades 5, as well as to strains expressing PspA clade 1–4. In addition, immunization with PspA2+4 or PspA2+5 provided better protection than PspA3+2 against bacterial challenge of clade 4 or clade 5 strain in this study. Therefore, the combined immunization with PspA3+2 with PspA2+4 or PspA2+5 simultaneously or sequentially may have the potential to improve the breadth of immunity against pneumococcal isolates.

In conclusion, immunization of mice with PspA3+2 induced antiserum exhibiting a high binding capacity to the clinical isolates expressing PspA clades 1–4, but not clade 5. Among the three PspA fusion proteins examined in this study, PspA3+2 was found to be advantageous over the other two PspA fusion proteins because PspA3+2 induced a broad range of cross-reactivity with clinical isolates and afforded a cross-protection against pneumococcal challenge in mice.

Author contributions

K.O., Y.A., K.J.I., K.U. and K.T. conceived and designed the experiments. Z.P. and Y.A. performed the experiments. Z.P. and D.T. analyzed the data. K.O., Z.P., Y.A., and D.E.B. wrote the paper.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

We thank Michiyo Hayakawa for technical assistance. This work was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation and by the Biomedical Cluster Kansai project, which is promoted by the Regional Innovation Cluster Program and is subsidized by the Japanese Government; and by research grants from the Ministry of Health, Labor and Welfare of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.07.108.

References

[1] O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet 2009;374:893–902.

[2] Pilishvili T, Lexau C, Farley MM, Hadler J, Harrison LH, Bennett NM, et al. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. J Infect Dis 2010;201:32–41.

[3] Singleton RJ, Hennessy TW, Bulkow LR, Hammitt LL, Zulz T, Hurlburt DA, et al. Invasive pneumococcal disease caused by nonvaccine serotypes among Alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. JAMA 2007;297:1784–92.

[4] Ardanuy C, Tubau F, Pallares R, Calatayud L, Domínguez MA, Rolo D, et al. Epidemiology of invasive pneumococcal disease among adult patients in Barcelona before and after pediatric 7-valent pneumococcal conjugate vaccine introduction, 1997–2007. Clin Infect Dis 2009;48:57–64.

[5] Miller E, Andrews NJ, Waight PA, Slack MPE, Gorge RC. Herd immunity and serotype replacement 4 years after seven-valent pneumococcal conjugate vaccination in England and Wales: an observation cohort study. Lancet Infect Dis 2011;11:760–8.

[6] Croney CM, Coats MT, Nahm MH, Briles DE, Crain MJ. PspA family distribution, unlike capsular serotype, remains unaltered following introduction of the heptavalent pneumococcal conjugate vaccine. Clin Vaccine Immunol 2012;19:891–6.

[7] Rev-Yochay G, Rahav G, Riesenberg K, Wiener-Well Y, Strahilevitz J, Stein M, et al. Initial effects of the national PCV7 childhood immunization program on adult invasive pneumococcal disease in Israel. PLOS ONE 2014;9:e88406.

[8] Briles DE, Tart RC, Swiatlo E, Dillard JP, Smith P, Benton KA, et al. Pneumococcal diversity: considerations for new vaccine strategies with emphasis on pneumococcal surface protein A (PspA). Clin Microbiol Rev 1998;11:645–57.

[9] Briles DE, Ades E, Paton JC, Sampson JS, Carlone GM, Huebner RC, et al. Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. Infect Immun 2000;68:796–800.

[10] Ogunniyi AD, Folland RL, Briles DE, Hollingshead SK, Paton JC. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. Infect Immun 2000;68:3028–33.

[11] Tai SS. *Streptococcus pneumoniae* protein vaccine candidates: properties, activities and animal studies. Crit Rev Microbiol 2006;32:139–53.

[12] Daniels CC, Briles TC, Mirza S, Håkansson AP, Briles DE. Capsule does not block antibody binding PspA, a surface virulence protein of *Streptococcus pneumoniae*. Microb Pathog 2006;40:228–33.

[13] Hollingshead SK, Baril L, Ferro S, King J, Coan P, Briles DE, Pneumococcal Proteins Epi Study Group. Pneumococcal surface protein A (PspA) family distribution among clinical isolates from adults over 50 years of age collected in seven countries. J Med Microbiol 2006;55:215–21.

[14] Tu A-H, Fulgham RL, McCrory MA, Briles DE, Szalai J. Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. Infect Immun 1999;67:4720–4.

[15] Ren B, Szalai AJ, Hollingshead SK, Briles DE. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. Infect Immun 2004;72:114–22.

[16] Briles DE, Hollingshead SK, King J, Swift A, Braun PA, Park MK, et al. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. J Infect Dis 2000;182:1694–701.

[17] Daniels CC, Kim K-H, Burton RL, Mirza S, Walker M, King J, et al. Modified opsonization, phagocytosis, and killing assays to measure potentially protective antibodies against pneumococcal surface protein A. Clin Vaccine Immunol 2013;20:1549–58.

[18] Yother J, Briles DE. Structural properties and evolutionary relationships of PspA, a surface protein of *Streptococcus pneumoniae*, as revealed by sequence analysis. J Bacteriol 1992;174:601–9.

[19] Yother J, White JM. Novel surface attachment mechanism of the *Streptococcus pneumoniae* protein PspA. J Bacteriol 1994;176:2976–85.

[20] Senkovich O, Cook WJ, Mirza S, Hollingshead SK, Protasevich II, Briles DE, et al. Structure of a complex of human lactoferrin N-lobe with pneumococcal surface protein a provides insight into microbial defense mechanism. J Mol Biol 2007;370:701–13.

[21] McDaniel LS, Ralph BA, McDaniel DO, Briles DE. Localization of protection-eliciting epitopes on PspA of *Streptococcus pneumoniae* between amino acid residues 192 and 260. Microb Pathog 1994;17:323–37.

[22] Shaper M, Hollingshead SK, Benjamin Jr WH, Briles DE. PspA protects *Streptococcus pneumoniae* from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin. Infect Immun 2004;72:5031–40.

[23] Daniels CC, Coan P, King J, Hale J, Benton KA, Briles DE, et al. The proline-rich region of pneumococcal surface proteins A and C contains surface-accessible epitopes common to all pneumococci and elicits antibody-mediated protection against sepsis. Infect Immun 2010;78:2163–72.

[24] Hollingshead SK, Becker R, Briles DE. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. Infect Immun 2000;68:5889–900.

[25] Qian J, Yao K, Xue L, Xie G, Zheng Y, Wang C, et al. Diversity of pneumococcal surface protein A (PspA) and relation to sequence typing in *Streptococcus pneumonia* causing invasive diseases in Chinese children. Eur J Clin Microbiol Infect Dis 2012;31:217–23.

[26] Oma K, Zhao J, Ezoe H, Akeda Y, Koyama S, Ishi KJ, 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;27:3181–8.

[27] Moreno AT, Oliveira ML, Ferreira DM, Ho PL, Darrieux M, Leite LC, et al. Immunization of mice with single PspA fragments induces antibodies capable of mediating complement deposition on different pneumococcal strains and cross-protection. Clin Vaccine Immunol 2010;17:439–46.

[28] Briles DE, Hollingshead SK, Nabors GS, Paton JC, Brooks-Water A. The potential for using protein vaccines to protect against otitis media caused by *Streptococcus pneumoniae*. Vaccine 2000;19(Suppl. 1):S87–95.

[29] Darrieux M, Moreno AT, Ferreira DM, Pimenta FC, de Andrade AL. Recognition of pneumococcal isolates by antisera raised against PspA fragments from different clades. J Med Microbiol 2008;57:273–8.

[30] Darrieux M, Miyaji EN, Ferreira DM, Lopes LM, Lopes A, Ren B, et al. Fusion proteins containing family 1 and family 2 PspA fragments elicit protection against *Streptococcus pneumoniae* that correlates with antibody-mediated enhancement of complement deposition. Infect Immun 2007;75:5930–8.

[31] Xin W, Li Y, Mo H, Roland KL, Curtiss III R. PspA family fusion proteins delivered by attenuated *Salmonella enterica* serovar Typhmilium extend and enhance protection against *Streptococcus pneumoniae*. Infect Immun 2009;77:4518–28.

[32] Chiba N, Morozumi M, Shouji M, Wajima T, Iwata S, Sunakawa K, et al. Rapid decrease of 7-valent conjugate vaccine coverage for invasive pneumococcal diseases in pediatric patients in Japan. Microb Drug Resist 2013;19:308–15.

[33] Pimenta FC, Ribeiro-Dias F, Brandileone MC, Miyaji EN, Leite LC, Sgambatti de Andrade AL. Genetic diversity of PspA types among nasopharyngeal isolates collected during an ongoing surveillance study of children in Brazil. J Clin Microbiol 2006;44:279–84.

[34] Ezoe H, Akeda Y, Piao Z, Aoshi T, Koyama S, Tanimoto T, et al. Intranasal vaccination with pneumococcal surface protein A plus poly(I:C) protects against secondary pneumococcal pneumonia in mice. Vaccine 2011;29:1754–61.

[35] Takeshita F, Leifer CA, Gursel I, Ishii KJ, Takeshita S, Gursel M, et al. Cutting edge: role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. J Immunol 2001;167:3555–8.

[36] Tougan T, Aoshi T, Coban C, Katakai Y, Kai C, Yasumoti Y, et al. TLR9 adjuvants enhance immunogenicity and protective efficacy of the SE36/AHG malaria vaccine in nonhuman primate models. Hum Vaccine Immunother 2013;9:283–90.

[37] Roche H, Ren B, McDaniel LS, Håkansson A, Briles DE. Relative roles of genetic background and variation in PspA in the ability of antibodies to PspA to protect against capsular type 3 and 4 strains of *Streptococcus pneumoniae*. Infect Immun 2003;71:4498–505.

Vaccine 32 (2014) 5295–5300

Contents lists available at ScienceDirect

Vaccine

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

Hemozoin as a novel adjuvant for inactivated whole virion influenza vaccine

Ryuta Uraki[a], Subash C. Das[b], Masato Hatta[b], Maki Kiso[a], Kiyoko Iwatsuki-Horimoto[a], Makoto Ozawa[c,d], Cevayir Coban[e], Ken J. Ishii[f,g], Yoshihiro Kawaoka[a,b,h,i,*]

[a] *Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan*
[b] *Influenza Research Institute, Department of Pathobiological Sciences, University of Wisconsin-Madison, Madison, WI 53711, USA*
[c] *Laboratory of Animal Hygiene, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima 890-0065, Japan*
[d] *Transboundary Animal Diseases Center, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima 890-0065, Japan*
[e] *Laboratory of Malaria Immunology, Immunology Frontier Research Center (IFReC), Osaka University, Osaka, Japan*
[f] *Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation, Osaka, Japan*
[g] *Laboratory of Vaccine Science, IFReC, Osaka University, Osaka, Japan*
[h] *ERATO Infection-Induced Host Responses Project (JST), Saitama 332-0012, Japan*
[i] *Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan*

ARTICLE INFO

Article history:
Received 31 January 2014
Received in revised form 27 May 2014
Accepted 22 July 2014
Available online 6 August 2014

Keywords:
Influenza virus
Vaccine
Hemozoin
Adjuvant
Antibody

ABSTRACT

Because vaccination is an effective means to protect humans from influenza viruses, extensive efforts have been made to develop not only new vaccines, but also for new adjuvants to enhance the efficacy of existing inactivated vaccines. Here, we examined the adjuvanticity of synthetic hemozoin, a synthetic version of the malarial by-product hemozoin, on the vaccine efficacy of inactivated whole influenza viruses in a mouse model. We found that mice immunized twice with hemozoin-adjuvanted inactivated A/California/04/2009 (H1N1pdm09) or A/Vietnam/1203/2004 (H5N1) virus elicited higher virus-specific antibody responses than did mice immunized with non-adjuvanted counterparts. Furthermore, mice immunized with hemozoin-adjuvanted inactivated viruses were better protected from lethal challenge with influenza viruses than were mice immunized with non-adjuvanted inactivated vaccines. Our results show that hemozoin improves the immunogenicity of inactivated influenza viruses, and is thus a promising adjuvant for inactivated whole virion influenza vaccines.

1. Introduction

Despite the worldwide surveillance network of influenza viruses, the incidence and prevalence of influenza are hard to predict, as exemplified by the influenza (H1N1) 2009 pandemic [1,2]. Vaccination stands on the frontlines of influenza infection control: both live attenuated and inactivated influenza vaccines are currently available [3,4]. The live attenuated vaccines are more efficient than inactivated vaccines at inducing the mucosal immune responses that play an important role in combating influenza virus infection [5,6]. However, because of the safety concerns such as the emergence of revertant and/or reassortant viruses, these live vaccines are licensed in a limited number of countries. By contrast, inactivated vaccines have few safety concerns and are globally available. While they efficiently induce humoral immune responses, a high dose (usually 15 μg) of the inactivated vaccine is required to provide adequate immunity [7,8]. Therefore, there is room for improvement in the current influenza vaccines.

Vaccine is generally assessed on the basis of immunogenicity, safety, and costs [9]. To enhance the immunogenicity of the inactivated vaccines, adjuvants, such as aluminum compounds and salts, have been considered [10]. Adjuvants are defined as immune modulators that are added to inactivated vaccines to boost the immune responses, enable the use of lower amounts of antigens, and thus expand the vaccine supply [10,11]. Although most of the inactivated influenza vaccines currently used are injected via the intramuscular or subcutaneous routes, previous studies have shown that intranasal vaccinations induce antibodies more effectively than do intramuscular or subcutaneous vaccinations [12–14]. However, the

---

* Corresponding author at: Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo, Japan. Tel.: +81 3 5449 5504; fax: +81 3 5449 5408.
E-mail addresses: kawaoka@ims.u-tokyo.ac.jp, kawaokay@svm.vetmed.wisc.edu (Y. Kawaoka).

http://dx.doi.org/10.1016/j.vaccine.2014.07.079
0264-410X/

alum compounds that are generally used as adjuvants for intramuscular administration do not enhance the efficacy of intranasal vaccines; therefore, to improve the efficacy of intranasal vaccines, novel intranasal adjuvants are required [15].

Malaria parasites digest hemoglobin in red blood cells, resulting in the production of potentially toxic heme metabolites [16]. To protect themselves from oxidative damage, the parasites polymerize toxic heme enzymatically into a safer insoluble substance, hemozoin [17]. Recently, hemozoin and a chemically identical synthetic version of hemozoin (called β-hematin) have been investigated for their potency as novel adjuvants, and the molecular pathway underlying their immunological function has also been studied. Such studies have demonstrated that purified hemozoin is a non-DNA ligand for Toll-like receptor 9 (TLR9) that may activate innate immune cells via TLR9 [18–20]. This latter point has been a subject of debate, however, because the adjuvant effect of synthetic hemozoin is dependent on MyD88 and not TLR9 [21]. Recently, we reported that hemozoin enhances the protective efficacy of a subcutaneously administered influenza HA split vaccine in a ferret model [22].

We speculated that synthetic hemozoin (hereafter referred to only as hemozoin) could serve as a novel intranasal adjuvant for the inactivated influenza vaccine. Accordingly, here we evaluated the adjuvanticity of hemozoin on the vaccine efficacy of intranasally administered inactivated whole virion influenza vaccines in a murine lethal infection model. The results indicate that hemozoin is a promising adjuvant for inactivated whole virion influenza vaccines.

2. Materials and methods

2.1. Cells and viruses

Human embryonic kidney HEK293T cells were maintained in Dulbecco's modified Eagle medium (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA). Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) (Invitrogen) supplemented with 5% newborn calf serum (NCS) (Sigma, St. Louis, MO). All cells were maintained in a humidified incubator at 37 °C in 5% CO_2.

A/California/04/2009 (H1N1; Ca04), which is an early isolate of influenza (H1N1) 2009 pandemic viruses, and mouse-adapted Ca04 (MACa04) [23] viruses were propagated in MDCK cells as previously described [24]. A/Vietnam/1203/2004 (H5N1; VN1203) virus, a representative strain of highly pathogenic avian influenza viruses, was grown in MDCK cells and in 10-day-old embryonated chicken eggs to use as challenge viruses and as vaccine and ELISA antigens, respectively. All work involving live VN1203 virus was carried out at the ABSL-3 laboratory of the Influenza Research Institute, UW-Madison, following the protocol designed by Institutional Animal Care and Use Committee (IACUC).

2.2. Inactivated influenza virus and adjuvant

To inactivate MDCK cell-propagated Ca04 virus and egg-propagated VN1203 virus, formalin (final concentration, 0.1%) was added to the viruses, which were then incubated at 4 °C for 1 week. The inactivated viruses were purified through a 10–50% sucrose density gradient and resuspended in phosphate-buffered saline (PBS) as described previously [25]. Inactivation of Ca04 viruses was confirmed by passaging them twice in MDCK cells and examining their cytopathic effect; inactivation of VN1203 viruses was confirmed by passaging them twice in embryonated chicken eggs followed by hemagglutination assays.

Synthetic hemozoin, was purified from hemin chloride (>98% pure, Fluka) by using the acid-catalyzed method described previously [21] and was re-suspended in endotoxin-free water with no detectable levels of endotoxin. The synthetic hemozoin concentration was calculated in mM (1 mg of hemozoin in 1 ml of water was equal to 1 mM).

2.3. Immunization and protection studies

For the immunization and protection studies with Ca04 virus, six-week-old female BALB/c mice ($n = 13$ per group) were anesthetized with isoflurane and intranasally administered with 50 μl of PBS, 9 mM hemozoin only, inactivated Ca04 only [5×10^6 plaque-forming unit (PFU), which corresponds to 0.1 μg when the total amount of viral protein was measured by using a BCA protein assay (Thermo Scientific)], or inactivated Ca04 adjuvanted with 9 mM hemozoin, twice with a 2-week interval between the immunizations. Three weeks after the final administration, three mice from each group were euthanized for collection of bronchoalveolar lavage fluid (BALF) and nasal washes. The remaining mice ($n = 10$ per group) were intranasally challenged with 10-fold 50% mouse lethal doses (MLD_{50}) of MACa04 virus. On days 3 and 6 post-challenge, three mice each were euthanized and their lungs were collected, homogenized with MEM containing 0.3% BSA, and examined for virus titers by using plaque assays in MDCK cells. The body weight and survival of the remaining challenged mice ($n = 4$ per group) were monitored daily for 14 days.

For VN1203 virus, four-week-old female BALB/c mice ($n = 16$ per group) were immunized as described above. Two weeks after the last immunization, five mice from each group were euthanized for collection of BALF and nasal washes. The remaining mice ($n = 11$ per group) were challenged with 100 MLD_{50} of VN1203 virus. On days 3 and 6 post-challenge, three mice each were euthanized and their lungs were collected, homogenized with MEM containing 0.3% BSA, and examined for virus titers by using plaque assays in MDCK cells. The body weight and survival of the remaining challenged mice ($n = 5$ per group) were monitored daily for 14 days.

2.4. Detection of virus-specific antibodies

Virus-specific antibodies in nasal washes, BALF, and serum were detected by using an ELISA as previously described [25–27]. Briefly, 96-well ELISA plate wells were coated with approximately 0.3 μg (in 50 μl) of purified Ca04 or VN1203 virus treated with disruption buffer (0.5 M Tris-HCl [pH 8.0], 0.6 M KCl, and 0.5% Triton X-100) or sarkosyl, respectively. After incubation of the virus-coated plates with the test samples, virus-specific IgA and IgG antibodies in the samples were detected by using anti-mouse IgA and IgG goat antibodies conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratory Inc., Gaithersburg, MD, Rockland), respectively.

2.5. Hemagglutination inhibition assay (HI assay)

To detect HI antibodies against Ca04 and VN1203, an HI assay was performed as described previously [28,29]. Briefly, serum samples were treated with receptor-destroying enzyme (RDE; Denka Seiken Co., Ltd.) by incubating at 37 °C for 16–18 h followed by inactivation at 56 °C for 30 min. One volume of turkey or horse red blood cells (RBCs) was then added to 20 volumes of serum and the sera were incubated for 1 h on ice with intermittent mixing. The samples were then centrifuged at 900 × *g* for 5 min, and the supernatants were transferred to new tubes for use in the HI assay. Serially diluted sera (2-fold dilutions) were mixed with 4 HA units of virus antigen and incubated with 0.5% turkey RBCs or 1% horse RBCs to determine the extent of hemagglutination inhibition.

2.6. Statistical analysis

Statistically significant differences in the virus-specific titers ($P < 0.05$ and $P < 0.01$) and the survival rates of the challenged mice ($P < 0.05$) were assessed by use of a one-way ANOVA followed by a Dunnett's test and Log-rank statistical analysis, respectively.

3. Results

3.1. Hemozoin enhances influenza virus-specific antibody responses in mice

To examine the effect of hemozoin on antibody responses elicited by immunization with inactivated influenza viruses, we intranasally administered BALB/c mice with hemozoin-adjuvanted inactivated virus (Ca04 or VN1203 virus, 5×10^6 plaque-forming units (PFU), the total amount of viral protein was 0.1 μg) twice with a 2-week interval between the immunizations. At three or two weeks after the final administration, we examined the antibody responses to the administered Ca04 or VN1203 virus by using an ELISA to measure the amount of IgG in the serum and IgA in the BALF and nasal washes (Fig. 1). Neither IgG nor IgA against Ca04 or VN1203 virus was appreciably detected in any samples from the PBS- or hemozoin-administered mice. Under these conditions, although one mouse immunized with non-adjuvanted inactivated Ca04 (Fig. 1A upper panel) and one mouse immunized with non-adjuvanted inactivated VN1203 virus (Fig. 1B upper panel) produced virus-specific IgG in the serum at a detectable level, all of the mice immunized with hemozoin-adjuvanted inactivated Ca04 ($n = 3$) or VN1203 ($n = 5$) virus elicited significantly higher levels of virus-specific IgG in the serum. We also examined the functional properties of the elicited antibodies by using hemagglutination inhibition (HI) assays. For both the Ca04 and VN1203 viruses, greater HI titers were obtained after vaccination with hemozoin-adjuvanted inactivated viruses than with non-adjuvanted inactivated viruses (Fig. 1A and B upper, right panel), although the titer difference for Ca04 virus between the hemozoin group and the control groups was not statistically significant (Fig. 1A upper, right panel). Of note, although the addition of hemozoin did not enhance IgA production in the nasal washes or BALF of the inactivated Ca04 virus-immunized mice, some of the mice immunized with the hemozoin-adjuvanted inactivated VN1203 virus did produce high levels of virus-specific IgA in their nasal washes and BALF (Fig. 1B lower panels). Taken together, these results indicate that hemozoin enhanced the immunogenicity of inactivated influenza viruses, resulting in more efficient production of virus-specific antibodies.

3.2. Hemozoin enhances the efficacy of inactivated influenza vaccine against lethal challenge in mice

To further assess the adjuvanticity of hemozoin, mice immunized twice with hemozoin-adjuvanted inactivated Ca04 or VN1203 virus were challenged with a lethal dose of MACa04 (10 MLD_{50}) [23] or VN1203 (100 MLD_{50}) virus (Fig. 2). In the MACa04 challenge group, although all of the PBS-administered mice and 75% of the hemozoin-administered or inactivated Ca04 virus-immunized mice died, all of the mice immunized with hemozoin-adjuvanted inactivated Ca04 virus survived (Fig. 2A). Intriguingly, no significant difference was found in Ca04 virus titers in the lungs among the mouse groups tested (Table 1). These results suggest that the adjuvanticity of hemozoin was sufficient to protect mice from lethal challenge with MACa04 virus.

For VN1203 virus, all PBS- and hemozoin-administered and inactivated VN1203 virus-immunized mice died following the lethal challenge (Fig. 2B). By contrast, 60% of the mice immunized with hemozoin-adjuvanted inactivated VN1203 virus survived although mice of all groups experienced body weight loss (Fig. 2B). In accordance with the results of the MACa04 virus challenge, the addition of hemozoin to inactivated VN1203 virus immunization did not affect the virus titers in the lungs of VN1203 virus-challenged mice (Table 2). These results suggest that hemozoin enhanced the vaccine efficacy of the inactivated influenza viruses by modulating host responses, but not by directly inhibiting virus replication. Overall, these results suggest that hemozoin is a promising adjuvant for inactivated influenza vaccines.

Table 1
Virus titers in the lungs of immunized mice challenged with mouse-adapted Ca04 virus.[a]

Immunization	Day after challenge	Virus titer (mean $\log_{10}$ PFU ± SD/g) in: lungs
PBS	3	8.1 ± 0.03
	6	6.5 ± 0.3
Hemozoin	3	8.2 ± 0.03
	6	6.6 ± 0.06
Inactivated Ca04 virus	3	8.1 ± 0.2
	6	5.7 ± 1.0
Hemozoin-adjuvanted inactivated Ca04 virus	3	8.0 ± 0.2
	6	6.2 ± 0.4

[a] Mice were intranasally immunized twice with the indicated agents (50 μl per mouse) and challenged with 10 MLD_{50} of MACa04 virus (50 μl per mouse) 3 weeks after the final immunization. Lungs were collected from mice ($n = 3$) on days 3 and 6 after challenge and examined for virus titers by use of plaque assays in MDCK cells.

Table 2
Virus titers in the lungs of immunized mice challenged with VN1203 virus.[a]

Immunization	Day after challenge	Virus titer (mean $\log_{10}$ PFU ± SD/g) in: lungs
PBS	3	6.3 ± 0.2
	6	6.3 ± 0.2
Hemozoin	3	6.6 ± 0.2
	6	6.3 ± 0.2
Inactivated VN1203 virus	3	6.7 ± 0.3
	6	5.6 ± 0.4
Hemozoin-adjuvanted inactivated VN1203 virus	3	6.4 ± 0.3
	6	6.0 ± 0.4

[a] Mice were intranasally immunized twice with the indicated agents (50 μl per mouse) and challenged with 100 MLD_{50} of VN1203 virus (50 μl per mouse) 4 weeks after the final immunization. Lungs were collected from mice ($n = 3$) on days 3 and 6 after challenge and examined for virus titers by use of plaque assays in MDCK cells.

4. Discussion

Here, we examined the effect of an adjuvant candidate, hemozoin, on the vaccine efficacy of inactivated whole virion influenza vaccines against lethal challenge in a mouse model. Significantly better virus-specific antibody responses were induced by hemozoin-adjuvanted inactivated virus than by inactivated viruses (Fig. 1). We further demonstrated that the hemozoin-adjuvanted inactivated viruses protected mice from lethal challenges more efficiently than did their non-adjuvanted counterparts with no effect of virus titers in the lungs (Fig. 2, Tables 1 and 2). These results indicate that hemozoin is a promising candidate as an effective adjuvant for inactivated whole virion influenza vaccines.

We observed significantly higher levels of IgA specific for VN1203 virus in the BALF and nasal washes, and of serum IgG, in mice immunized with hemozoin-adjuvanted inactivated VN1203 virus than in mice immunized with non-adjuvanted inactivated VN1203 virus-immunized mice (Fig. 1B). These results suggest that hemozoin enhanced the mucosal immune responses and

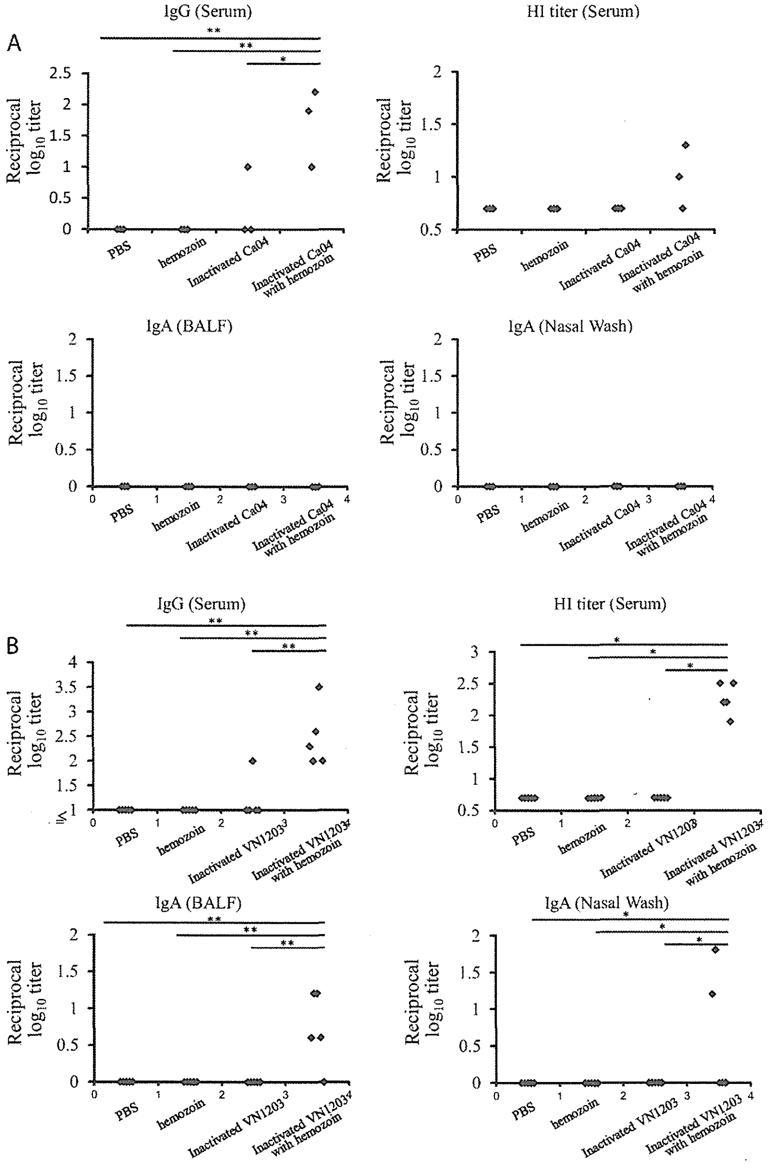

Fig. 1. Virus-specific antibody responses in immunized mice. Virus-specific antibodies were detected by means of ELISA and HI assays with purified Ca04 (A) or VN1203 (B) virus as a viral antigen. IgG antibody titers (upper, left panels) and HI titers (upper, right panels) in serum and IgA antibody titers in the BALF (lower, left panels), and nasal washes (lower, right panels) from mice intranasally mock-immunized with PBS or hemozoin or immunized with non-adjuvanted or hemozoin-adjuvanted inactivated virus were measured. Values represent antibody titers in individual mice (A: $n = 3$, B: $n = 5$). Statistically significant differences (*: $P < 0.05$, **: $P < 0.01$) are indicated.

may potentially compensate for the well-recognized weakness of inactivated vaccines [30,13,31]. By contrast, enhanced IgA production by the hemozoin addition was not observed with the Ca04 virus counterparts (Fig. 1A). This contradiction may reflect a difference in immunogenicity between the Ca04 and VN1203 viruses. Further study is required to clarify the mechanisms by which hemozoin promotes IgA responses after immunization with inactivated vaccines. In addition, hemozoin-adjuvanted inactivated virus protected mice better than non-adjuvanted inactivated viruses although virus titers in lungs were similar between animals immunized with and without the adjuvant (Fig. 2, Tables 1 and 2). This finding suggests that hemozoin enhanced the vaccine efficacy of the inactivated influenza viruses by modulating host responses. In the current study, we measured viral loads only in respiratory organs, which are the primary sites of influenza virus replication even for strains that cause systemic infection (e.g., VN1203 virus). A further study to examine the inhibitory effect of hemozoin on systemic spread of influenza viruses may explain the better protection afforded by hemozoin-adjuvanted vaccine.

Although hemozoin is a ligand for TLR9 [18–20], studies using TLR9- or MyD88-deficient mice suggest that the potent adjuvant effect of synthetic hemozoin is mediated not via TLR9, but through MyD88 [21]. In addition, previous studies have demonstrated that hemozoin stimulates innate inflammatory responses, inducing neutrophil recruitment via MyD88 [21,32]. Thus, one of the possible mechanisms underlying the hemozoin-mediated enhanced efficacy of inactivated influenza vaccine may be that hemozoin induces the balanced Th1/Th2 responses in a MyD88-dependent manner, leading to the improved immunogenicity of the inactivated influenza viruses and to the better protection against lethal challenge with influenza viruses. Of note, one of four mice administered with only hemozoin survived after the lethal challenge with

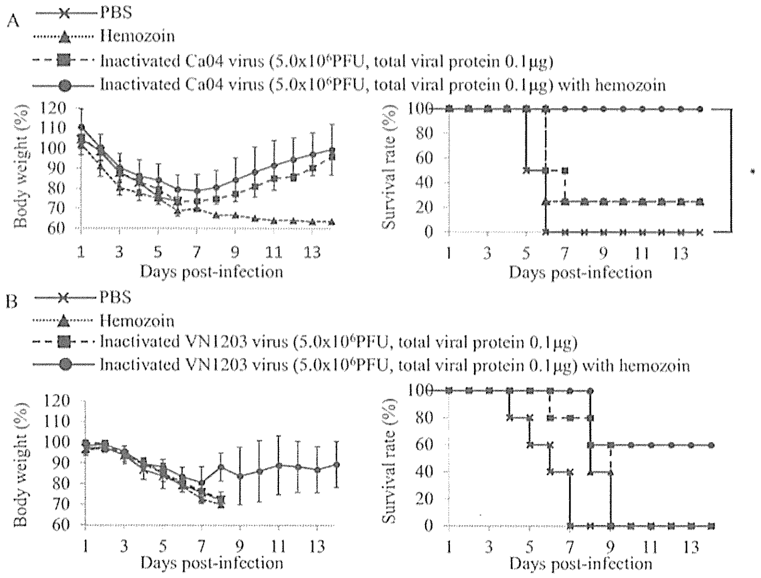

Fig. 2. Body weight changes and survival of mice challenged with lethal doses of viruses. Mice were mock-immunized with PBS or hemozoin, or immunized with non-adjuvanted or hemozoin-adjuvanted inactivated virus twice with a 2-week interval in between the immunizations. Three or four weeks after the final immunization, mice were intranasally challenged with 10 MLD_{50} of MACa04 virus (A: $n = 4$) or 100 MLD_{50} of VN1203 virus (B: $n = 5$), respectively. Body weight (left panels) and survival (right panels) were monitored for 14 days after challenge. Values are expressed as mean changes in body weight ± SD (left panels). Statistically significant differences in the survival rate of immunized mice (*: $P < 0.05$) are indicated (A: right panel).

MACa04 virus (Fig. 2A), suggesting that hemozoin itself might have protective effects against influenza virus infection. Additional study is required to clarify the inhibitory effect of hemozoin on influenza virus infection.

In conclusion, here, we demonstrated the potential of hemozoin as a novel whole virion influenza vaccine adjuvant. Because the mechanism by which hemozoin enhances immunogenicity remains unclear, we should continue to evaluate the adjuvanticity of hemozoin in the context of influenza vaccination. In addition, to establish the efficacy of hemozoin as an adjuvant, further studies are needed including studies in an additional animal model such as ferrets.

Acknowledgements

We thank Dr. Susan Watson for editing the manuscript and Y. Igari and T. Tsukui from Nihon Zenoaq, Co., Ltd. for providing synthetic hemozoin. This work was supported, by a Grant-in-Aid for Specially Promoted Research, by the Japan Initiative for the Global Research Network on Infectious Diseases from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, by grants-in-aid from the Ministry of Health, Labour, and Welfare, Japan, by ERATO (Japan Science and Technology Agency), by Strategic Basic Research Programs of Japan Science and Technology Agency, by National Institute of Allergy and Infectious Diseases Public Health Service research grants, and by an NIAID-funded Center for Research on Influenza Pathogenesis (CRIP, HHSN266200700010C). R.U. is supported by JSPS Research Fellowships for young scientists.

References

[1] Glezen WP. Cold-adapted, live attenuated influenza vaccine. Expert Rev Vaccines 2004;3(April (2)):131–9.
[2] Neumann G, Kawaoka Y. The first influenza pandemic of the new millennium. Influenza Other Respir Viruses 2011;5(May (3)):157–66.
[3] Nabel GJ, Fauci AS. Induction of unnatural immunity: prospects for a broadly protective universal influenza vaccine. Nat Med 2010;16(December (12)):1389–91.
[4] Lambert LC, Fauci AS. Influenza vaccines for the future. N Engl J Med 2010;363(November (21)):2036–44.
[5] Rimmelzwaan GF, Fouchier RA, Osterhaus AD. Influenza virus-specific cytotoxic T lymphocytes: a correlate of protection and a basis for vaccine development. Curr Opin Biotechnol 2007;18(December (6)):529–36.
[6] Cox RJ, Brokstad KA, Ogra P. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scand J Immunol 2004;59(January (1)):1–15.
[7] Brokstad KA, Cox RJ, Olofsson J, Jonsson R, Haaheim LR. Parenteral influenza vaccination induces a rapid systemic and local immune response. J Infect Dis 1995;171(January (1)):198–203.
[8] Cox RJ, Brokstad KA, Zuckerman MA, Wood JM, Haaheim LR, Oxford JS. An early humoral immune response in peripheral blood following parenteral inactivated influenza vaccination. Vaccine 1994;12(August (11)):993–9.
[9] Tetsutani K, Ishii KJ. Adjuvants in influenza vaccines. Vaccine 2012;30(December (52)):7658–61.
[10] Reed SG, Orr MT, Fox CB. Key roles of adjuvants in modern vaccines. Nat Med 2013;19(December (12)):1597–608.
[11] Dey AK, Srivastava IK. Novel adjuvants and delivery systems for enhancing immune responses induced by immunogens. Expert Rev Vaccines 2011;10(February (2)):227–51.
[12] van Riet E, Ainai A, Suzuki T, Hasegawa H. Mucosal IgA responses in influenza virus infections; thoughts for vaccine design. Vaccine 2012;30(August (40)):5893–900.
[13] Tumpey TM, Renshaw M, Clements JD, Katz JM. Mucosal delivery of inactivated influenza vaccine induces B-cell-dependent heterosubtypic cross-protection against lethal influenza A H5N1 virus infection. J Virol 2001;75(June (11)):5141–50.
[14] Ainai A, Tamura S, Suzuki T, Ito R, Asanuma H, Tanimoto T, et al. Characterization of neutralizing antibodies in adults after intranasal vaccination with an inactivated influenza vaccine. J Med Virol 2012;84(February (2)):336–44.
[15] Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. Immunol Cell Biol 2004;82(October (5)):488–96.
[16] Francis SE, Sullivan Jr DJ, Goldberg DE. Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. Annu Rev Microbiol 1997;51:97–123.
[17] Arese P, Schwarzer E. Malarial pigment (haemozoin): a very active 'inert' substance. Ann Trop Med Parasitol 1997;91(July (5)):501–16.
[18] Coban C, Ishii KJ, Kawai T, Hemmi H, Sato S, Uematsu S, et al. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. J Exp Med 2005;201(January (1)):19–25.
[19] Parroche P, Lauw FN, Goutagny N, Latz E, Monks BG, Visintin A, et al. Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9. Proc Natl Acad Sci U S A 2007;104(February (6)):1919–24.
[20] Wu X, Gowda NM, Kumar S, Gowda DC. Protein-DNA complex is the exclusive malaria parasite component that activates dendritic cells and triggers innate immune responses. J Immunol 2010;184(April (8)):4338–48.

[21] Coban C, Igari Y, Yagi M, Reimer T, Koyama S, Aoshi T, et al. Immunogenicity of whole-parasite vaccines against *Plasmodium falciparum* involves malarial hemozoin and host TLR9. Cell Host Microbe 2010;7(January (1)): 50–61.

[22] Onishi M, Kitano M, Taniguchi K, Homma T, Kobayashi M, Sato A, et al. Hemozoin is a potent adjuvant for hemagglutinin split vaccine without pyrogenicity in ferrets. Vaccine 2014;32(May (25)):3004–9.

[23] Sakabe S, Ozawa M, Takano R, Iwastuki-Horimoto K, Kawaoka Y. Mutations in PA, NP, and HA of a pandemic (H1N1) 2009 influenza virus contribute to its adaptation to mice. Virus Res 2011;158(June (1–2)):124–9.

[24] Yamada S, Hatta M, Staker BL, Watanabe S, Imai M, Shinya K, et al. Biological and structural characterization of a host-adapting amino acid in influenza virus. PLoS Pathog 2010;6(8):e1001034.

[25] Uraki R, Kiso M, Iwatsuki-Horimoto K, Fukuyama S, Takashita E, Ozawa M, et al. A novel bivalent vaccine based on a PB2-knockout influenza virus protects mice from pandemic H1N1 and highly pathogenic H5N1 virus challenges. J Virol 2013;87(July (14)):7874–81.

[26] Kida H, Brown LE, Webster RG. Biological activity of monoclonal antibodies to operationally defined antigenic regions on the hemagglutinin molecule of A/Seal/Massachusetts/1/80 (H7N7) influenza virus. Virology 1982;122(October (1)):38–47.

[27] Das SC, Hatta M, Wilker PR, Myc A, Hamouda T, Neumann G, et al. Nanoemulsion W805EC improves immune responses upon intranasal delivery of an inactivated pandemic H1N1 influenza vaccine. Vaccine 2012;30(November (48)):6871–7.

[28] Jia N, Wang SX, Liu YX, Zhang PH, Zuo SQ, Lin Z, et al. Increased sensitivity for detecting avian influenza-specific antibodies by a modified hemagglutination inhibition assay using horse erythrocytes. J Virol Methods 2008;153(October (1)):43–8.

[29] Kayali G, Setterquist SF, Capuano AW, Myers KP, Gill JS, Gray GC. Testing human sera for antibodies against avian influenza viruses: horse RBC hemagglutination inhibition vs. microneutralization assays. J Clin Virol 2008;43(September (1)):73–8.

[30] Clements ML, Betts RF, Tierney EL, Murphy BR. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. J Clin Microbiol 1986;24(July (1)):157–60.

[31] Benton KA, Misplon JA, Lo CY, Brutkiewicz RR, Prasad SA, Epstein SL. Heterosubtypic immunity to influenza A virus in mice lacking IgA, all Ig, NKT cells, or gamma delta T cells. J Immunol 2001;166(June (12)):7437–45.

[32] Shio MT, Kassa FA, Bellemare MJ, Olivier M. Innate inflammatory response to the malarial pigment hemozoin. Microbes Infect 2010;12(November (12–13)): 889–99.

System Vaccinology for the Evaluation of Influenza Vaccine Safety by Multiplex Gene Detection of Novel Biomarkers in a Preclinical Study and Batch Release Test

Takuo Mizukami[1], Haruka Momose[1], Madoka Kuramitsu[1], Kazuya Takizawa[1], Kumiko Araki[1], Keiko Furuhata[1], Ken J. Ishii[2,3], Isao Hamaguchi[1]*, Kazunari Yamaguchi[1]

1 Laboratory of Blood and Vaccine safety, Department of Safety Research on Blood and Biologicals, National Institute of Infectious Diseases, Tokyo, Japan, **2** Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation (NIBIO), Osaka, Japan, **3** Laboratory of Vaccine Science, WPI Immunology Frontier Research Center (WPI-IFREC), Osaka University, Osaka, Japan

Abstract

Vaccines are beneficial and universal tools to prevent infectious disease. Thus, safety of vaccines is strictly evaluated in the preclinical phase of trials and every vaccine batch must be tested by the National Control Laboratories according to the guidelines published by each country. Despite many vaccine production platforms and methods, animal testing for safety evaluation is unchanged thus far. We recently developed a systems biological approach to vaccine safety evaluation where identification of specific biomarkers in a rat pre-clinical study evaluated the safety of vaccines for pandemic H5N1 influenza including *Irf7, Lgals9, Lgalsbp3, Cxcl11, Timp1, Tap2, Psmb9, Psme1, Tapbp, C2, Csf1, Mx2, Zbp1, Ifrd1, Trafd1, Cxcl9, β2m, Npc1, Ngfr* and *Ifi47*. The current study evaluated whether these 20 biomarkers could evaluate the safety, batch-to-batch and manufacturer-to-manufacturer consistency of seasonal trivalent influenza vaccine using a multiplex gene detection system. When we evaluated the influenza HA vaccine (HAv) from four different manufactures, the biomarker analysis correlated to findings from conventional animal use tests, such as abnormal toxicity test. In addition, sensitivity of toxicity detection and differences in HAvs were higher and more accurate than with conventional methods. Despite a slight decrease in body weight caused by HAv from manufacturer B that was not statistically significant, our results suggest that HAv from manufacturer B is significantly different than the other HAvs tested with regard to *Lgals3bp, Tapbp, Lgals9, Irf7* and *C2* gene expression in rat lungs. Using the biomarkers confirmed in this study, we predicted batch-to-batch consistency and safety of influenza vaccines within 2 days compared with the conventional safety test, which takes longer. These biomarkers will facilitate the future development of new influenza vaccines and provide an opportunity to develop *in vitro* methods of evaluating batch-to-batch consistency and vaccine safety as an alternative to animal testing.

Citation: Mizukami T, Momose H, Kuramitsu M, Takizawa K, Araki K, et al. (2014) System Vaccinology for the Evaluation of Influenza Vaccine Safety by Multiplex Gene Detection of Novel Biomarkers in a Preclinical Study and Batch Release Test. PLoS ONE 9(7): e101835. doi:10.1371/journal.pone.0101835

Editor: Sang-Moo Kang, Georgia State University, United States of America

Received March 10, 2014; **Accepted** June 11, 2014; **Published** July 10, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data are included within the paper.

Funding: This work was supported by Grants-in-Aid from Ministry of Health, Labor, and Welfare of Japan (201132001 and 201208036, respectively). 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.

* Email: 130hama@nih.go.jp

Introduction

Vaccination is a beneficial and universal tool to prevent infectious disease [1]. Because most vaccines are derived from inactivated virus, bacteria or toxoids, contamination by incomplete inactivation can cause serious adverse events. Thus, historically, the safety of vaccines is strictly regulated by law and each batch of vaccine must be tested by the National Control Laboratories according to the guidelines published in each country, *e.g.* the European Pharmacopeia, United States Pharmacopeia and World Health Organization guidelines [2]. After the diphtheria toxoid (DT) immunization incident in Japan in 1950 that caused the death of 68 children and illness in over 600 infants owing to contamination by incomplete inactivation of DT [3], the abnormal toxicity test (ATT) (also known as general safety test) was introduced to the Japanese guidelines. This stated that the minimum requirement of biological products (MRBP) and all inactivated vaccines and toxoids was mandatory safety evaluation by ATT and other specific toxicity tests.

Influenza vaccine is one of the most widely used commercially available vaccines worldwide for preventing seasonal influenza and its complications. Influenza virus vaccine is mainly produced using embryonated fertilized chicken eggs and inactivated with formaldehyde. Whole particle influenza virus vaccine [WPv] was first licensed as an influenza vaccine in the US in 1945 [4] and is still used in some countries. Although WPv contains all the components of the influenza virus and induces strong immunity in the vaccinated individual, a high incidence of adverse events, including local reactions at the site of injection and febrile illness, particularly among children have been reported [5,6]. Thus, most recent vaccines manufactured since the 1970s have been subvirion vaccines. The subvirion influenza HA vaccine [HAv] showed a marked reduction of pyrogenicity compared with WPv [7]. The trivalent influenza vaccine [TIV] is a recently developed subvirion influenza vaccine with components selected and updated each year to protect against one of the three main groups of circulating influenza virus strains in humans. TIV may be administered every year. Vaccine adjuvant, e.g. alum, MF59 and AS03, was also used to enhance immunity in preparation for the H5N1 pandemic [8]. To improve immunogenicity and reduce toxicity in addition to batch-to-batch quality assurance of influenza vaccine, seed lot systems, recombinant DNA technology, as well as animal and insect cell culture inactivated vaccine production systems were introduced. Despite the increase in many vaccine production platforms, adjuvants, additives and vaccine types, safety evaluation tests in the preclinical phase and batch release have been unchanged in most countries, including in Japan.

We previously reported that improved ATT could evaluate and assure the batch-to-batch consistency of vaccines more strictly compared with conventional methods [9]. In addition, we recently introduced a system biological approach to vaccine safety evaluation and demonstrated that specific biomarkers could be used to evaluate batch-to-batch consistency and safety of vaccines to diphtheria-pertussis-tetanus (DPT) [10,11] and Japanese encephalitis virus (JEV) [12]. Most recently, we showed that a system biological approach could evaluate the safety of pandemic H5N1 influenza vaccine [13]. We found 20 biomarkers for the evaluation of batch-to-batch consistency and the safety of H5N1 vaccine compared with HAv.

In this study, we tested whether these biomarkers could evaluate batch-to-batch consistency and the safety of seasonal HAv, as well as adjuvanted whole virion-derived influenza vaccine, using a multiplex gene detection system. This method might facilitate the evaluation of batch-to-batch consistency of HAv and reduce the time required for batch release compared with conventional ATT. These biomarkers will help the future development of new *in vitro* methods to evaluate vaccine safety as an alternative to animal testing.

Materials and Methods

1. Animals and Ethics statement

Eight-week-old male Fischer (F334/N) rats weighing 160–200 g were obtained from SLC (Tokyo, Japan). All animals were housed in rooms maintained at 23±1°C, with 50±10% relative humidity, and 12-h light/dark cycles for at least 1 week prior to the test use. All animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases (NIID), Tokyo, Japan. The study was approved by the Institutional Animal Care and Use Committee of NIID.

2. Vaccines

The following vaccines were used in this study: (1) PDv: inactivated monovalent A/H5N1 whole-virion influenza vaccine (derived from NIBRG-14: A/Vietnam/1194/2004) adjuvanted with aluminum hydroxide, containing 30 μg HA/ml; (2) WPv: inactivated whole trivalent influenza vaccine (A/Newcaledonia/20/99 (H1N1), A/Hiroshima/52/2005 (H3N2), and B/Malaysia/2506/2004); HAV: trivalent HA influenza vaccine (A/Solomon Island/3/2006 (H1N1), A/Hiroshima/52/2005 (H3N2), and B/Malaysia/2506/2004), containing 30 μg HA/ml each strain. For evaluation of commercially distributed HAV in Japan, we used trivalent HA influenza vaccine (A/Solomon Island/3/2006 (H1N1), A/Hiroshima/52/2005 (H3N2) and B/Malaysia/2506/2004), containing 30 μg HA/ml per strain. PDv and WPv were produced, and manufactured by the Chemo-Sero-Therapeutic Research Institute, Kaketsuken (Kumamoto, Japan). Licensed and authorized HAvs were purchased from four different manufactur-

Table 1. Biomarkers to evaluate influenza vaccine safety.

Official Symbol	Official Full Name	Gene ID
Irf7	Interferon regulatory factor 7	293624
Lgals9	Lectin, galactoside-binding, soluble, 9	25476
Lgalsbp3	Lectin, galactoside-binding, soluble, 3 binding protein	245955
Cxcl11	Chemokine (C-X-C motif) ligand 11	305236
Timp1	TIMP metallopeptidase inhibitor 1	116510
Tap2	Transporter 2, ATP-binding cassette, sub-family B	24812
Psmb9	Proteasome (prosome, macropain) subunit, beta type, 9	24967
Psme1	Proteasome (prosome, macropain) activator subunit 1	29630
Tapbp	TAP binding protein (tapasin)	25217
C2	Complement component 2	24231
Csf1	Colony stimulating factor 1 (macrophage)	78965
Mx2	Myxovirus (influenza virus) resistance 2	286918
Zbp1	Z-DNA binding protein 1	171091
Ifrd1	Interferon-related developmental regulator 1	29596
Trafd1	TRAF type zinc finger domain containing 1	114635
Cxcl9	Chemokine (C-X-C motif) ligand 9	246759
β2m	Beta-2 microglobulin	24223
Npc1	Niemann-Pick disease, type C1	266732
Ngfr	Nerve growth factor receptor	24596
Ifi47	Interferon gamma inducible protein 47	246208

doi:10.1371/journal.pone.0101835.t001

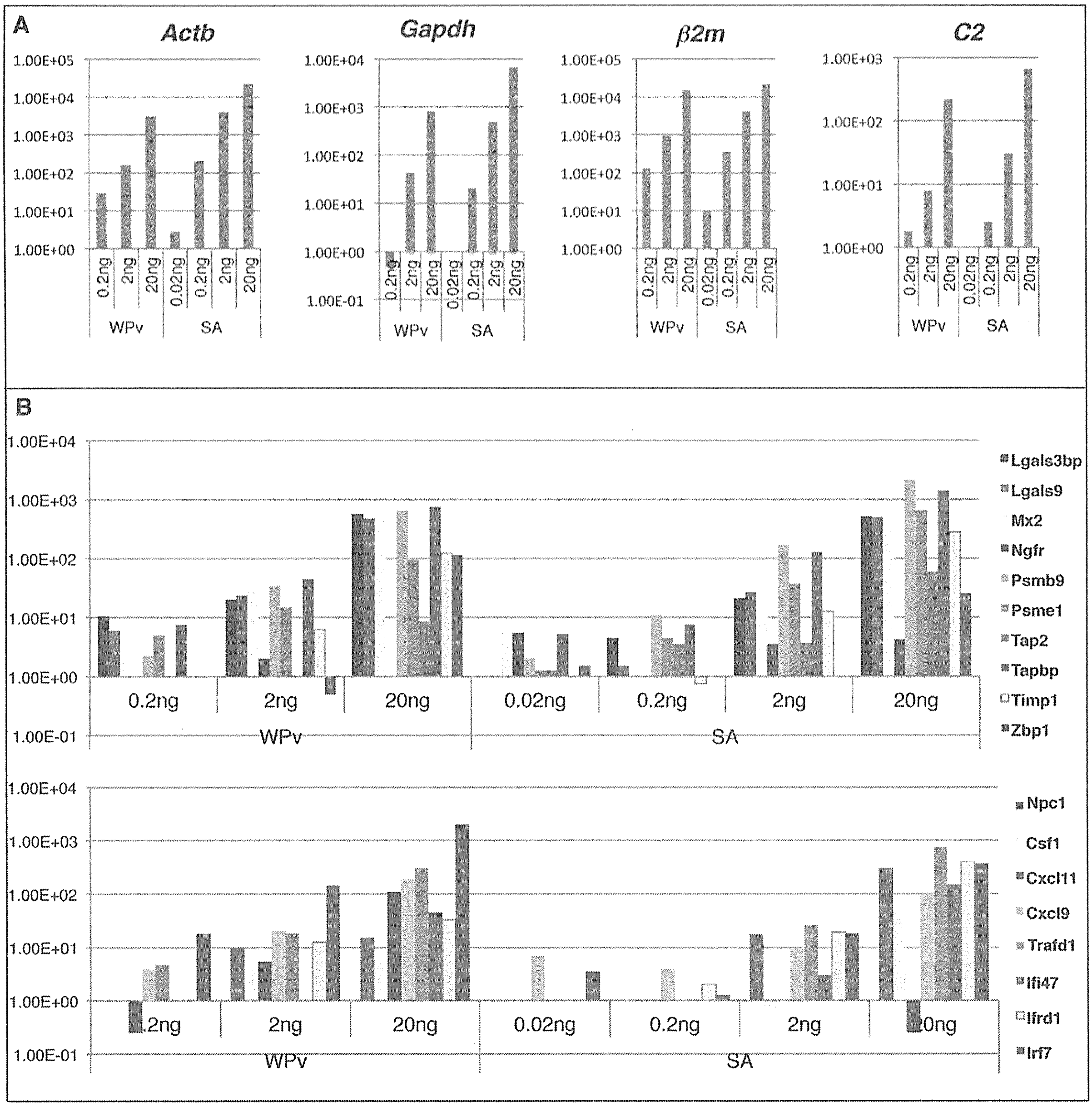

Figure 1. Optimization of QGP in influenza vaccine safety evaluation. A) Gene expression of *Actb*, *Gapdh*, *B2m* and *C2* and B) biomarkers, in 0.2, 2 and 20 ng RNA-containing samples from SA- and WPv-treated rat lungs. Relative expression levels of the *Gapdh* gene are indicated. SA: saline, WPv: Whole particle virion influenza vaccine.
doi:10.1371/journal.pone.0101835.g001

ers [HAv (Lot L03A) from Kaketsuken (Kumamoto), HAv (Lot 309) from Kitasato Institute (Saitama), HAv (Lot 343-A) from Denka Seiken Co., Ltd. (Tokyo), HAv (Lot HA082D) from Biken (Kagawa)] in Japan. All vaccines complied with the MRBP in Japan. HAv used in this study was tested and authorized by NCL (National Control Laboratory) for distribution in Japan.

3. Abnormal toxicity test

ATT was performed according to the MRBP [http://www.nih.go.jp/niid/en/mrbp-e.html] using rats with a slight modification. Each 5 ml of vaccine was intra-peritoneally (*i.p.*) injected into rats. Five milliliters of saline (SA) (Otsuka normal saline; Otsuka Pharmaceutical Factory Inc., Naruto, Tokushima, Japan) was *i.p.* injected as a control. One day after the injection, rat body weight was measured and peripheral blood was collected. The number of white blood cells was counted with a hemocytometer (Nihon Kohden, Japan).

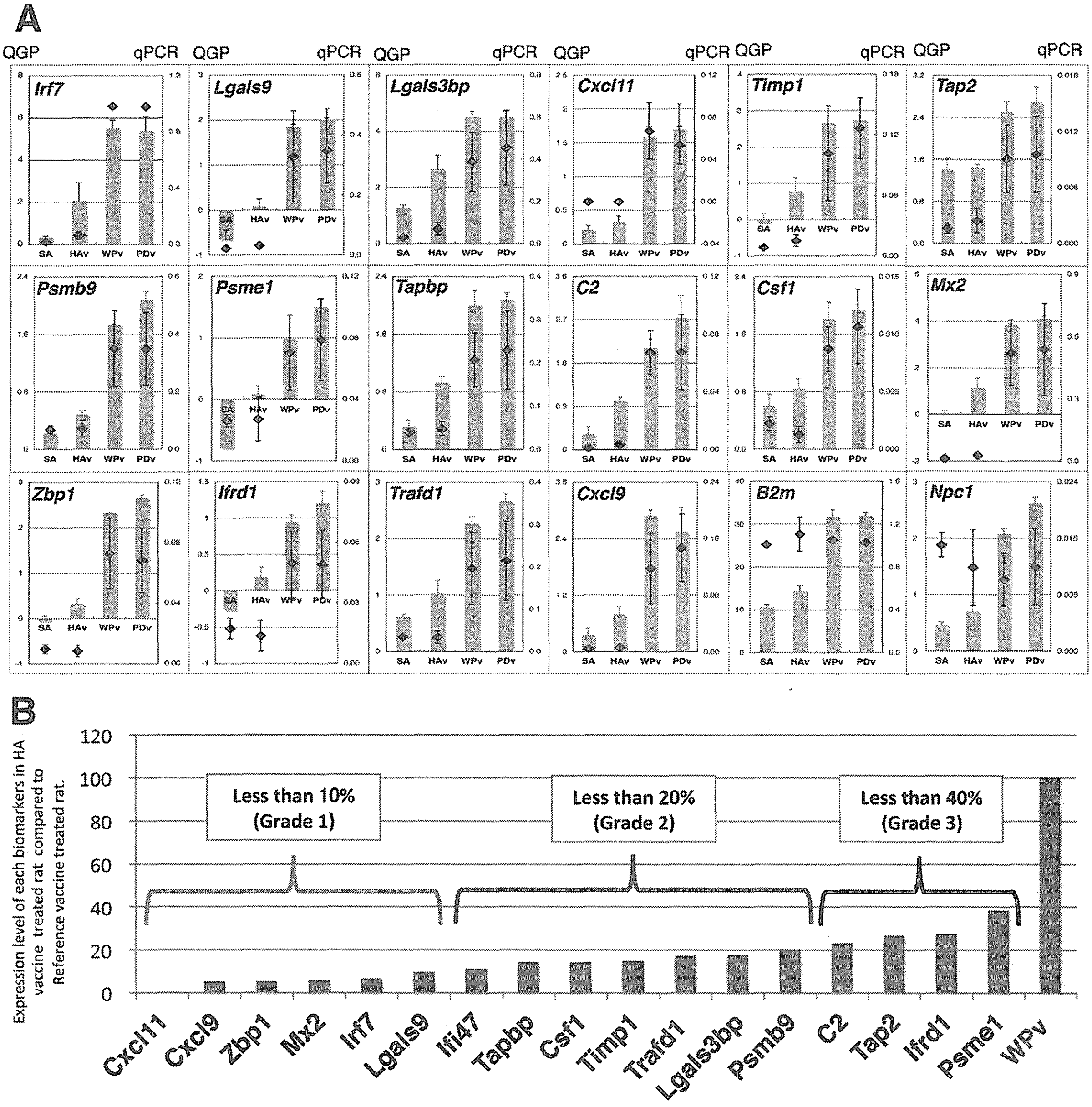

Figure 2. Validation of QGP with real-time PCR methods. A) GQP result was validated with real-time PCR methods. Bar graph indicates the real-time PCR results and dot blot indicates QGP results. B) Biomarkers were classified into three grades according to the relative expression level compared with WPv-treated rats.
doi:10.1371/journal.pone.0101835.g002

4. RNA preparation

One day after injection, rats were sacrificed to obtain whole lung tissues. Organs were immediately frozen in liquid nitrogen for storage. Thawed tissue was homogenized and mixed with an Isogen reagent (Nippon Gene, Tokyo, Japan). Total RNA was prepared from the lysate in accordance with the manufacturer's instructions. Poly (A)+ RNA was prepared from total RNA with a Poly (A) Purist Kit (Ambion, Austin, TX), according to the manufacturer's instructions.

5. Quantitative RT-PCR analysis

Poly (A)+ RNA was used to synthesize first-strand cDNA using a First-strand cDNA Synthesis Kit (Life Science Inc., St. Petersburg, FL), according to the manufacturer's instructions. Expression levels of biomarkers (**Table 1**) were analyzed by real-time polymerase chain reaction (PCR) using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with 7500 Fast System SDS Software Version 1.3. cDNA was amplified for real-time PCR using SYBR Green I (Molecular Probes Inc.) to detect the PCR products. One microliter of 6-fold diluted cDNA was

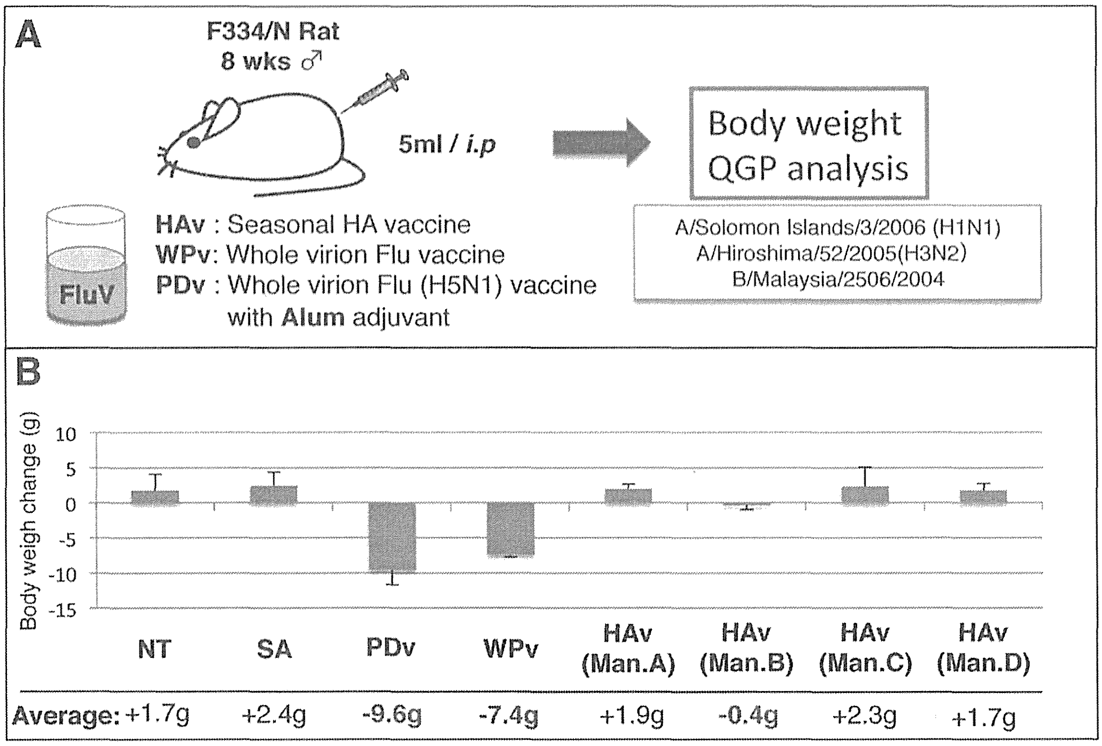

Figure 3. Evaluation of seasonal influenza vaccine with conventional animal safety test. A) The abnormal toxicity test was performed according to the Minimum Requirements of Biological Products. Each 5 ml vaccine was *i.p.* injected into rats, the body weight measured and lung tissues collected at day 1 after injection. B) Body weight change at day 1 after injection. NT: nontreated rat, SA: saline, PDv: pandemic H5N1 whole virion-derived vaccine with alum adjuvant, WPv: whole particle virion influenza vaccine, HAv: influenza HA vaccine, Man: manufacturer.
doi:10.1371/journal.pone.0101835.g003

used in a 20-μl final volume reaction containing 10 μl SYBR Green PCR Master Mix (Applied Biosystems), and forward and reverse primers were as described previously [13]. The 7500 Fast System was programmed to run an initial polymerase activation step at 95°C for 10 min followed by 40 cycles of denaturation (95°C for 15 s) and extension (60°C for 1 min). Product synthesis was monitored at the end of the extension step of each cycle. Gene expression values were normalized against rat GAPDH.

6. QuantiGene Plex assays

QuantiGene Plex (QGP) assays were performed according to the QuantiGene Plex Reagent System instructions (Panomics Inc., Fremont, CA), as described previously [11]. Briefly, 10 μl of starting poly (A)+RNA (50 ng) was incubated for 10 min at 65°C, then mixed with 33.3 μl of lysis mixture, 40 μl of capture buffer, 2 μl of capture beads, and 2 μl of the target gene-specific probe set. Probe sets were heated for 5 min prior to use. Each sample mixture was then dispensed into an individual well of a capture plate, sealed with foil tape and incubated at 54°C for 16–20 h. The hybridization mixture was transferred to a filter plate, and the wells were washed three times with 200 μl of wash buffer. Signals for the bound target mRNA were developed by sequential hybridization with branched DNA (bDNA) amplifier, and biotin-conjugated label probe, at 48°C for 1 h each. Two washes with wash buffer were used to remove unbound material after each hybridization step. Streptavidin-conjugated phycoerythrin was added to the wells and incubated at room temperature for 30 min. The luminescence of each well was measured using a Luminex 100 microtiter plate luminometer (Luminex). Two replicate assays measuring RNA directly (independent sampling n = 6 for mRNA, n = 3–5 for lysate) were performed for all described experiments. The 20 target genes and GAPDH mRNA were quantified, and the ratio of the target genes to GAPDH mRNA was calculated.

7. Statistical analysis

Multiple comparisons were performed for SA, PDv, WPv and HA. To determine differences between manufacturers, multiple comparisons were performed for SA and HA from manufacturers A, B, C and D. Statistical analysis was performed in GraphPad Prism 6 (GraphPad Software, La Jolla, CA) using an ordinary one-way analysis of variance test followed by a Tukey multiple comparison test.

Results

Optimization of multiple gene detection system, QuantiGene Plex, for safety evaluation of the influenza vaccine

We previously reported that 20 selected genes (**Table 1**), from 76 differentially expressed genes in adsorbed PDv-treated rats, could be used as biomarkers to evaluate H5N1 influenza vaccine safety compared with other types of influenza vaccine using conventional real-time PCR [13]. To establish faster and more convenient methods to detect these biomarkers in one-step as a new vaccine safety test, we used QuantiGene Plex (QGP)

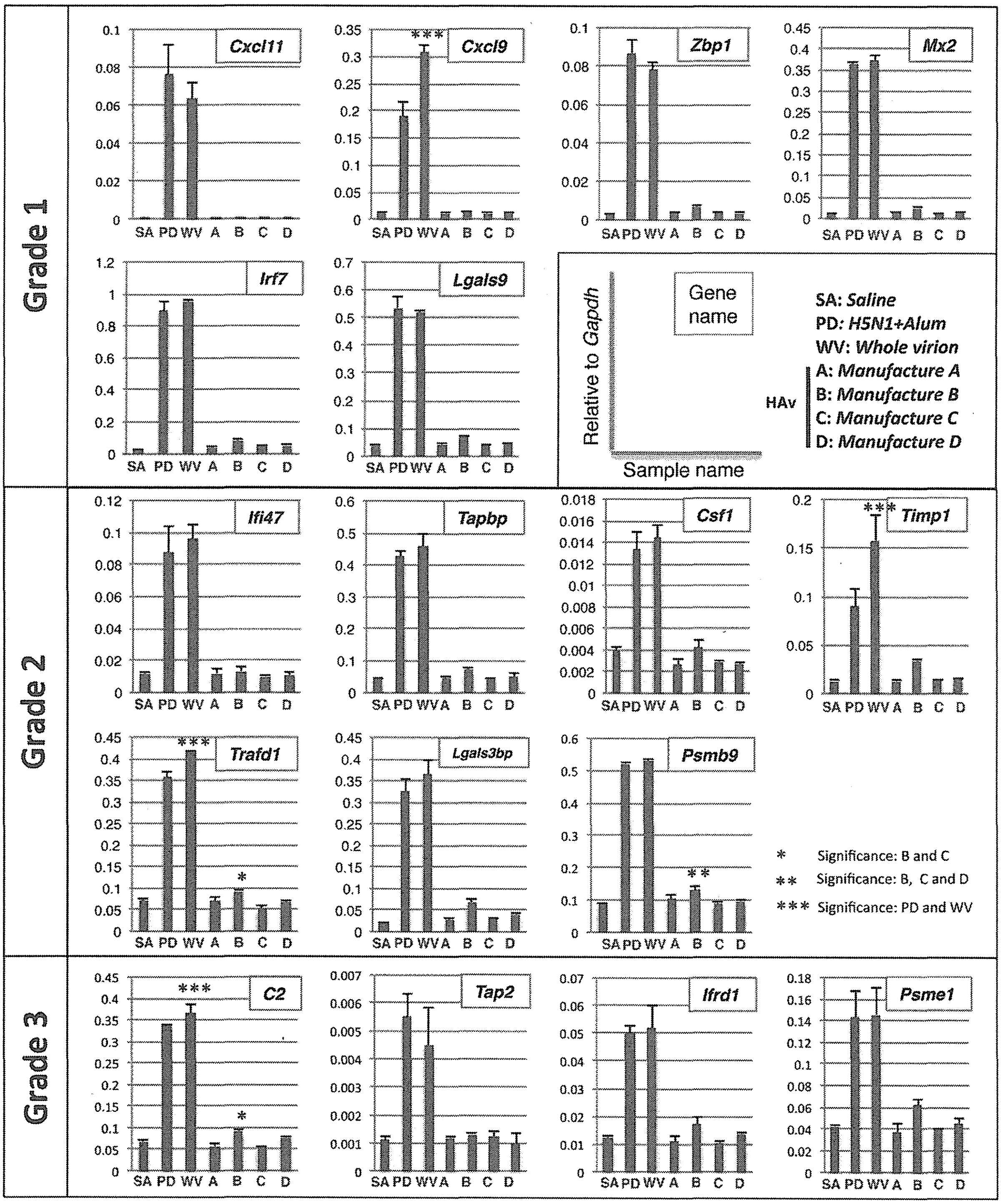

Figure 4. Evaluation of seasonal influenza vaccine with QGP. The relative gene expression levels of the *Gapdh* gene are indicated in each column (grades 1, 2 and 3, respectively). *Significant difference between B and C. **Significant difference between B, C and D, ***Significant difference between PD and WPv.
doi:10.1371/journal.pone.0101835.g004

technology (Panomics Inc., Fremont, CA). We designed a custom QGP 2.0 assay to enable the measurement of expression levels of identified biomarkers. The Panomics QGP 2.0 assays provided quantitative measurements of 3 to 80 target RNAs per well by

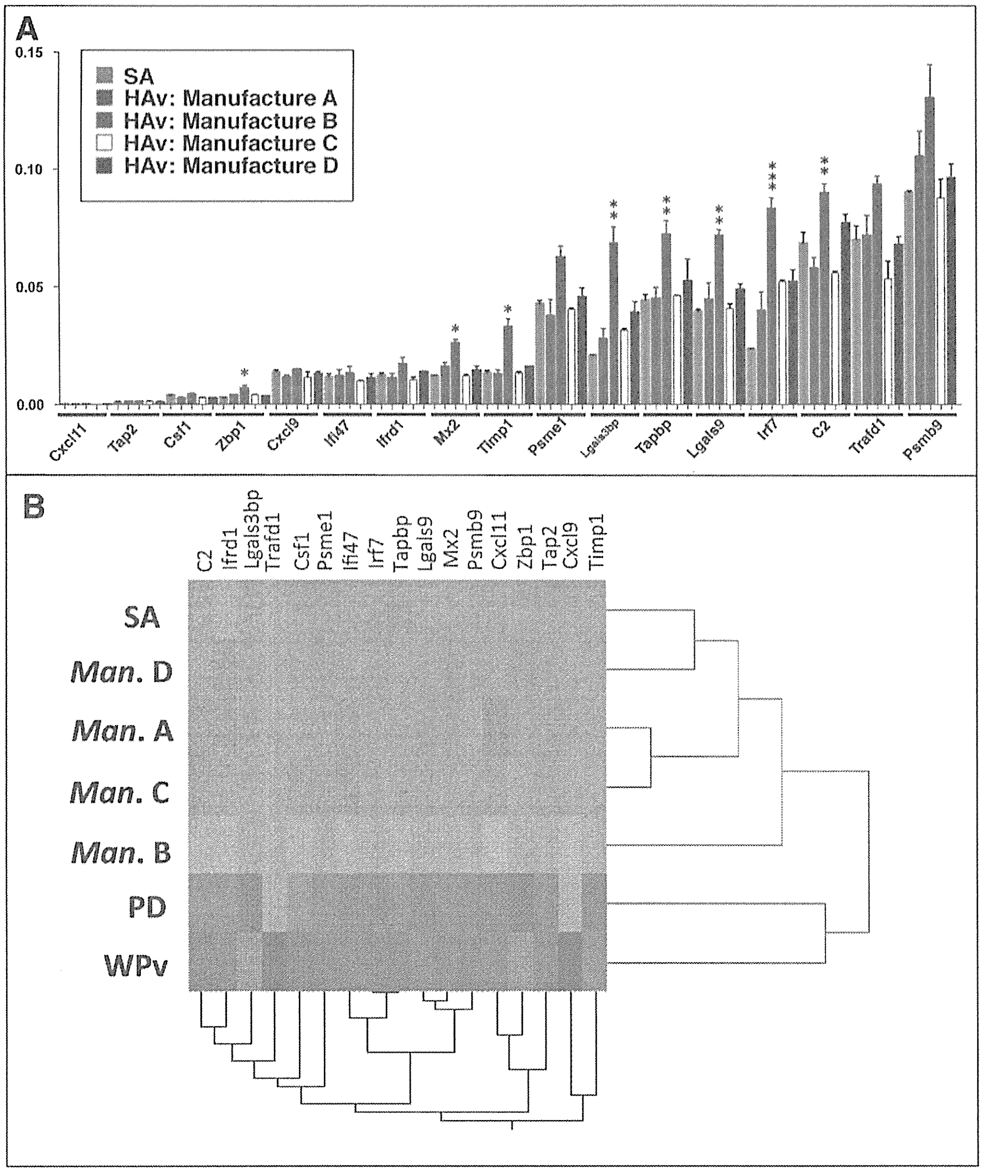

Figure 5. Evaluation of seasonal influenza vaccine with QGP and cluster analysis. A) Relative gene expression in HAv-treated rat lungs to *Gapdh* is indicated in the bar graph. B) Hierarchical clustering analysis with biomarkers could predict differences in HAv manufacturers as B is located in a separate cluster from other HAvs.
doi:10.1371/journal.pone.0101835.g005

using bDNA technology in conjunction with multi-analyte magnetic beads to provide the detection and quantitation of multiple mRNA targets simultaneously. bDNA technology is a hybridization-based methodology that uses labeled DNA probes to amplify the signal rather than the target mRNA. Here, we produced probes for 20 genes and two control genes (*Actb* and *Gapdh*) for the one-step detection and quantification of these biomarkers. To check the sensitivity of probes and dynamic range of our biomarkers, we prepared 0.02, 0.2, 2 and 20 ng total RNA samples from WPv and SA-treated rat lungs and performed QGP analysis. Two control genes and two biomarkers (*β2m* and *C2*) reacted in a dose-dependent manner (**Figure 1A**). We re-evaluated all probes with the same sample. Each biomarker reacted in a dose-dependent manner (**Figure 1B**) except *Ngfr* and *Npc1*. Therefore, 20 ng of RNA sample was used for multiplex gene detection. All biomarkers except *β2m* reacted in a dose-dependent manner. *β2m* was saturated when using 20 ng RNA sample; thus *β2m* could not be used for QGP analysis.

Validation of QGP with real-time PCR

To validate QGP, we performed real-time PCR analysis using the same samples. As a result, most biomarker gene expression data from the QGP correlated with the real-time PCR result except for *β2m*, *Npc1* (Figure 2) and *Ngfr* (data not shown). Finally,

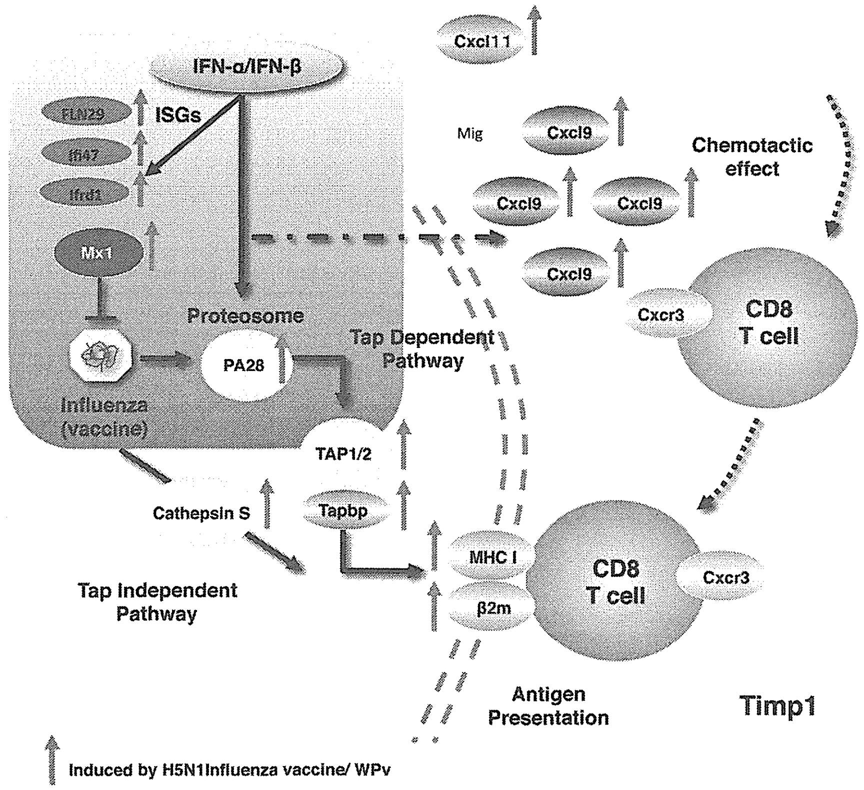

Figure 6. Summary of biomarker studies. Biomarkers used in this study were strongly correlated with immune responses after influenza infection.
doi:10.1371/journal.pone.0101835.g006

17 genes were selected as the multiplex detection biomarker set. We next determined the relative biomarker expression levels in HAv-treated rat lungs compared with WPv used as a reference toxicity vaccine in the leukopenic toxicity test (LTT) in Japan. We classified *Cxcl11*, *Cxcl9*, *Zbp1*, *Mx2*, *Irf7* and *Lgals9* as a "Grade 1" gene set where relative expression levels in HAv compared with WPv were less than 10%. Likewise, we classified *Ifi47*, *Tapbp*, *Csf1*, *Timp1*, *Trafd1*, *Lgals3bp* and *Psmb9* as a "Grade 2" gene set where relative expression levels were less than 20% and *C2*, *Tap2*, *Ifrd1* and *Psme1* as a "Grade 3" gene set where relative expression levels were less than 40% in HAv compared with WPv. In Japan, it is acceptable for leukopenic toxicity levels of HAv to be not more than 20% of WPv by LTT. We applied LTT criteria for selecting and subdividing these biomarkers into three grades with expression levels below 20% of WPv and others.

Evaluation of HAv safety in Japan using ATT and QGP

To evaluate the toxicity of seasonal HAv using biomarkers, we purchased market authorized seasonal influenza vaccines distributed in Japan from four different manufacturers (Kaketsuken, Denka Seiken, Kitasato, and Biken). Although the vaccines have been evaluated and passed ATT by the NCL according to the Japanese guidelines for MRBP, the reactogenicity of the vaccine to animals (rats, mice and guinea pigs) was varied. To evaluate these differences, we performed ATT and checked the body weight changes of rats after *i.p.* injection of each HAv (Figure 3A). Although treatment with PDv or WPv (toxic reference whole virion-derived vaccines) significantly decreased the body weight of rats, HAvs from three different manufacturers had no effect on body weight. HAv from manufacture B reduced the body weight of rats at day 1 (Figure 3B). However, there was no significant difference in rat body weight change for the other HAvs; thus HAv from manufacturer B might be slightly different, when comparing the mean body weight at day 1. In addition, there was no significant difference in leukocyte numbers following administration of HAv from the four manufacturers (data not shown). To evaluate the differences of each HAv, we next performed multiplex biomarker detection by QGP. No biomarkers were significantly up-regulated in HAv-treated rats compared with controls (Figure 4) except for *Psmb9*. Furthermore, *Psmb9* expression was significantly up-regulated following administration of HAv from manufacturer B compared with the control SA-treated and HAvs from the other manufacturers. The expression levels of *C2* and *Trafd1* were also significantly up-regulated in the HAv from manufacturer B compared with the HAv from manufacturer C.

Biomarkers to evaluate safety of adjuvanted influenza vaccine

Both PDv and WPv contain the whole virion influenza vaccine and alum adjuvant is only added to PDv to enhance its immunogenicity. There was no difference in body weight change between WPv- and PDv-treated rats (Figure 3B). However, among the 17 biomarkers, the expression level of three genes, *Cxcl9*, *Timp1* and *Trafd1* in PDv-treated rats were significantly decreased compared with WPv-treated rats (Figure 4). Thus, these biomarkers could potentially evaluate the aluminum adjuvant effect.