

effector T cells, DC clustering was followed by T cell accumulation. Therefore, we propose that these perivascular dDC clusters may provide antigen-presentation sites for efficient activation of effector T cells. This is suggested by the observations that CHS responses and intracutaneous T cell activation were attenuated substantially in the absence of these clusters, in conditions of macrophage depletion or inhibition of integrin function, IL-1R signaling<sup>22,23</sup> or CXCR2 signaling<sup>24</sup>.

In contrast to antigen presentation in the skin, antigen presentation in other peripheral barrier tissues is relatively well understood. In submucosal areas, specific sentinel lymphoid structures (mucosa-associated lymphoid tissue (MALT)) serve as peripheral antigen-presentation sites<sup>25</sup>, and lymphoid follicles are present in non-inflammatory bronchi (bronchus-associated lymphoid tissue (BALT)). These structures serve as antigen-presentation sites in non-lymphoid peripheral organs. By analogy, the concept of skin-associated lymphoid tissue (SALT) was proposed in the early 1980s, on the basis of findings that cells in the skin are able to capture, process and present antigens<sup>26,27</sup>. However, the role of cellular skin components as antigen-presentation sites has remained uncertain. Here we have identified an inducible structure formed by dermal macrophages, dDCs and effector T cells, which seemed to accumulate sequentially. Because formation of this structure was essential for efficient activation of effector T cells, these inducible leukocyte clusters may function as SALTs. Unlike leukocyte clusters in MALT, these leukocyte clusters were not found in the steady state but were induced during the development of an adaptive immune response. Therefore, these clusters might be better called 'inducible SALTs', similar to inducible BALTs in the lung<sup>28</sup>. In contrast to the cells present in inducible BALT, we did not identify naive T cells or B cells in SALT (data not shown), which suggested that the leukocyte clusters in the skin may be specialized for the activation of effector T cells but not for the activation of naive T cells. Our findings suggest that approaches for the selective inhibition of this structure may have novel therapeutic benefit in inflammatory disorders of the skin.

## METHODS

Methods and any associated references are available in the online version of the paper.

**Accession codes.** GEO: microarray data, GSE53680.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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## AUTHOR CONTRIBUTIONS

Y.N., G.E. and K.K. designed this study and wrote the manuscript; Y.N., G.E., S. Nakamizo, S.O., S.H., N.K., A.O., A.K., T. Honda and S. Nakajima performed the experiments and analyzed data; S.T. and Y.S. did experiments related to microarray analysis; K.J.I., H.T., H.Y., Y.I., M.K. and L.g.N. developed experimental reagents and gene-targeted mice; J.F. and E.G.-Y. did experiments related to immunohistochemistry of human samples; T.O., T. Hashimoto, Y.M. and K.K. directed the project and edited the manuscript; and all authors reviewed and discussed the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## ONLINE METHODS

**Mice.** 8- to 12-week-old female C57BL/6 mice were used in this study. C57BL/6N mice were from SLC. Langerin-eGFP-DTR mice<sup>29</sup>, CD11c-DTR mice<sup>30</sup>, CD11c-YFP mice (that express CD11c tagged with YFP)<sup>31</sup>, LysM-DTR mice<sup>32</sup>, RAG-2-deficient mice<sup>33</sup>, Mas-TRECK mice<sup>13,14</sup>, Bas-TRECK mice<sup>13,14</sup>, ALY/Nscjcl-aly/aly mice<sup>12</sup>, IL-1 $\alpha$ / $\beta$ -deficient mice<sup>34</sup>, IL-1R1-deficient mice<sup>35</sup>, NLRP3-deficient mice<sup>36</sup> and caspase-1/11-deficient mice<sup>37</sup> have been described. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

**Human subjects.** Biopsy samples of human skin were obtained from a nickel-reactive patch after 48 h after placement of nickel patch tests in patients with previously proven allergic contact dermatitis. A biopsy of petrolatum-occluded skin was also obtained as a control. Informed consent was obtained under protocols approved by the Institutional Review Board at the Icahn School of Medicine at Mount Sinai School Medical Center, and the Rockefeller University in New York.

**Induction of CHS responses.** Mice were sensitized on shaved abdominal skin with 25  $\mu$ l 0.5% (wt/vol) DNFB (1-fluoro-2,4-dinitrofluorobenzene; Nacalai Tesque) dissolved in acetone and olive oil (at a ratio of 4:1). Five days later, the ears were challenged with 20  $\mu$ l 0.3% DNFB. For adoptive transfer, T cells were magnetically sorted, with an autoMACS (Miltenyi Biotec), from the draining LNs of sensitized mice and then were transferred intravenously ( $1 \times 10^7$  cells) into naive mice.

**Depletion of cutaneous DC subsets, macrophages and neutrophils.** For depletion of all cutaneous DC subsets (including LCs), 6-week-old Langerin-DTR mice were irradiated (two doses of 550 rads given 3 h apart) and were given transfer of  $1 \times 10^7$  BM cells from CD11c-DTR mice. Eight weeks later, 2  $\mu$ g DT (Sigma-Aldrich) was injected intraperitoneally. For selective depletion of LCs, irradiated Langerin-DTR mice were given transfer of BM cells from C57BL/6 mice, and 1  $\mu$ g DT was injected. For selective depletion of dDCs, irradiated C57BL/6 mice were given transfer of BM cells from CD11c-DTR mice, and 2  $\mu$ g DT was injected. For depletion of macrophages, irradiated C57BL/6 mice were given transfer of BM cells from LysM-DTR mice and 800 ng DT was injected. For depletion of neutrophils, anti-Ly6G (1A8; BioXCell) was administered to mice intravenously at a dose of 0.5 mg per mouse 24 h before experiments.

**Time-lapse imaging of cutaneous DCs, macrophages and T cells.** Cutaneous DCs were observed in CD11c-YFP mice. For labeling of cutaneous macrophages *in vivo*, 5 mg TRITC-dextran (Sigma-Aldrich) was injected intravenously and mice were allowed to 'rest' for 24 h. At that time, cutaneous macrophages became fluorescent because they had incorporated extravasated dextran. For labeling of skin-infiltrating T cells, T cells from DNFB-sensitized mice were labeled with CellTracker Orange (CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl) amino)tetramethylrhodamine); Invitrogen) and were adoptively transferred into recipient mice. Keratinocytes and sebaceous glands were visualized by subcutaneous injection of isolectin B4 (Invitrogen) and BODIPY (Molecular Probes), respectively. Mice were positioned on a heating plate on the stage of a two-photon IX-81 microscope (Olympus) and their ear lobes were fixed beneath a cover slip with a single drop of immersion oil. Stacks of ten images, spaced 3  $\mu$ m apart, were acquired at intervals of 1–7 min for up to 24 h. For calculation of T cell and DC velocities, movies were processed and analyzed with Imaris 7.2.1 software (Bitplane).

**Histology and immunohistochemistry.** For histological examination, tissues were fixed with 10% formalin in phosphate-buffered saline, then were embedded in paraffin. Sections with a thickness of 5  $\mu$ m were prepared and then were stained with hematoxylin and eosin. For whole-mount staining, the ears were split into dorsal and ventral halves and were incubated for 30 min at 37 °C with 0.5 M ammonium thiocyanate. Then the dermal sheets were separated and fixed in acetone for 10 min at –20 °C. After treatment with Image-iT FX Signal Enhancer (Invitrogen), the sheets were incubated with antibody to mouse MHC class II (M5/114.15.2; eBioscience) followed by incubation

with antibody to rat IgG conjugated to Alexa Fluor 488 (A-11006; Invitrogen) or Alexa Fluor 594 (A-11007; Invitrogen). The slides were mounted with a ProLong Antifade kit with the DNA-binding dye DAPI (4',6-diamidino-2-phenylindole; Molecular Probes) and were observed with a fluorescent microscope (BZ-900; KEYENCE). The number and size of DC clusters were evaluated in ten fields of 1 mm<sup>2</sup> per ear and were assigned scores according to the criteria in **Supplementary Figure 5a**.

**Cell isolation and flow cytometry.** For the isolation of skin lymphocytes, the split ears were incubated for 1 h at 37 °C in digestion buffer (RPMI medium supplemented with 2% FCS, 0.33 mg/ml of Liberase TL (Roche) and 0.05% DNase I (Sigma-Aldrich)). After that incubation, the tissues were disrupted by passage through a 70- $\mu$ m cell strainer and stained with the appropriate antibodies (identified below). For analysis of intracellular cytokine production, cell suspensions were obtained in the presence of 10  $\mu$ g/ml of brefeldin A (Sigma-Aldrich) and were fixed with Cytofix Buffer and permeabilized with Perm/Wash Buffer according to the manufacturer's protocol (BD Biosciences). Cells were stained with the following: antibody to mouse CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-CD11c (N418), anti-B220 (RA3-6B2), antibody to MHC class II (M5/114.15.2), anti-F4/80 (BM8), anti-IFN- $\gamma$  (XMG1.2), anti-Gr1 (RB6-8c5) and 7-amino-actinomycin D (all from eBioscience); anti-mouse CD45 (30-F11) and anti-TCR- $\beta$  (H57-597; both from BioLegend); and anti-CD16-CD32 (2.4G2; BD Biosciences). Flow cytometry was done with an LSR Fortessa (BD Biosciences) and data were analyzed with FlowJo software (TreeStarA).

**Chemotaxis assays.** Chemotaxis was assessed as described with some modifications<sup>38</sup>. The dermis of the ear skin was minced and then was digested for 30 min at 37 °C with 2 mg/ml collagenase type II (Worthington Biochemical) containing 1 mg/ml hyaluronidase (Sigma-Aldrich) and 100  $\mu$ g/ml DNase I (Sigma-Aldrich). DDCs and macrophages were isolated with an autoMACS. Alternatively, BM-derived DCs and macrophages were prepared.  $1 \times 10^6$  DCs were added to a Transwell insert with a pore size of 5  $\mu$ m (Corning), and  $5 \times 10^5$  macrophages were added to the lower wells, and the cells were incubated for 12 h at 37 °C. A known number of fluorescent reference beads (FlowCount Fluospheres; Beckman Coulter) were added to each sample to allow accurate quantification of cells that had migrated to the lower wells by flow cytometry.

**Cell proliferation assay.** Mice were sensitized with 25  $\mu$ l 0.5% DNFB or 7% trinitrochlorobenzene (Chemical Industry). Five days later, T cells were magnetically separated from the draining LNs of each group of mice and were labeled with CellTrace Violet according to the manufacturer's protocol (Invitrogen).  $1 \times 10^6$  T cells were adoptively transferred into naive mice, and the ears of the recipient mice were challenged with 20  $\mu$ l of 0.5% DNFB. 24 h later, ears were collected and analyzed by flow cytometry.

**In vitro differentiation of DCs and M1 and M2 macrophages from BM cells.** BM cells from the tibiae and fibulae were plated at a density of  $5 \times 10^6$  cells per 10-cm dish on day 0. For DC differentiation, cells were cultured at 37 °C in 5% CO<sub>2</sub> in cRPMI medium (RPMI medium supplemented with 1% L-glutamine, 1% HEPES, 0.1% 2-mercaptoethanol and 10% FBS) containing 10 ng/ml granulocyte-macrophage colony-stimulating factor (Peprotech). For macrophage differentiation, BM cells were cultured in cRPMI medium containing 10 ng/ml macrophage colony-stimulating factor (Peprotech). The medium was replaced on days 3 and 6 and cells were harvested on day 9. For the induction of M1 macrophages or M2 macrophages, cells were stimulated for 48 h with IFN- $\gamma$  (10 ng/ml; R&D Systems) or with IL-4 (20 ng/ml; R&D Systems), respectively.

**In vitro IL-1 $\alpha$ -stimulation assay of dermal macrophages.** Dermal macrophages were separated from mice deficient in IL-1 $\alpha$  and IL-1 $\beta$ <sup>34</sup> to avoid preactivation during cell preparations. Split ears were treated for 30 min at 37 °C with 0.25% trypsin and EDTA for removal of the epidermis, then were minced and then incubated with collagenase as described above. CD11b<sup>+</sup> cells were separated by magnetic-activated cell sorting, and  $2 \times 10^5$  cells per well in 96-well plates were incubated for 24 h with or without 10 ng/ml IL-1 $\alpha$  (R&D Systems).

**Blocking assay.** For the LFA-1-blocking assay, mice were given intravenous injection of 100  $\mu$ g KBA (neutralizing antibody to LFA-1; a gift from H. Yagita) 12–14 h after challenge with 20  $\mu$ l 0.5% DNFB. For blockade of IL-1R, mice were given subcutaneous injection of 10  $\mu$ g recombinant mouse IL-1ra (PROSPEC) 5 h before challenge. For blockade of CXCR2, mice were given intraperitoneal treatment with 50  $\mu$ g CXCR2 inhibitor<sup>17</sup> (SB265610; Tocris Bioscience) 6 h before and at the time of painting of the skin with hapten.

**Quantitative PCR analysis.** Total RNA was isolated with an RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was synthesized with a PrimeScript RT reagent kit and random hexamers according to the manufacturer's protocol (TaKaRa). A LightCycler 480 and LightCycler SYBR Green I Master mix were used according to the manufacturer's protocol (Roche) for quantitative PCR (primer sequences, **Supplementary Table 2**). The expression of each gene was normalized to that of the control gene *Gapdh*.

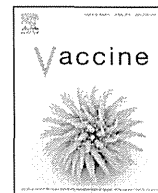
**Microarray analysis.** Total RNA was isolated with an RNeasy Mini Kit according to the manufacturer's protocol (Qiagen). An amplified sense-strand DNA product was synthesized with the Ambion WT Expression Kit (Life Technologies), was fragmented and labeled by the WT Terminal Labeling and Controls Kit (Affymetrix) and was hybridized to a Mouse Gene 1.0 ST Array (Affymetrix). We used the robust multiarray average algorithm for log transformation ( $\log_2$ ) and normalization of the GeneChip data.

**General experimental design and statistical analysis.** For animal experiments, a sample size of three to five mice per group was used on the basis of past experience in generating statistical significance. Mice were randomly assigned to study groups and no specific randomization or blinding protocol was used. Sample or mouse identity was not masked for any of these studies.

Prism software (GraphPad) was used for statistical analyses. Normal distribution was assumed a priori for all samples. Unless indicated otherwise, an unpaired parametric *t*-test was used for comparison of data sets. In cases in which the data-point distribution was not Gaussian, a nonparametric *t*-test was also applied. *P* values of less than 0.05 were considered significant.

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# Protective properties of a fusion pneumococcal surface protein A (PspA) vaccine against pneumococcal challenge by five different PspA clades in mice

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## ABSTRACT

An increase in the appearance of nonvaccine serotypes in both children and adults with invasive pneumococcal disease (IPD) after introduction of pneumococcal conjugate vaccine represents a limitation of this vaccine. In this study, we generated three recombinant pneumococcal surface protein A (PspA) proteins comprising PspA families 1 and 2, and we examined the reactivity of antisera raised in mice immunized with a PspA fusion protein in combination with CpG oligonucleotides plus aluminum hydroxide gel. The protective effects of immunization with PspA fusion proteins against pneumococcal challenge by strains with five different PspA clades were also examined in mice. Flow cytometry demonstrated that PspA3+2-induced antiserum showed the greatest binding of PspA-specific IgG to all five challenge strains with different clades. PspA2+4- or PspA2+5-induced antiserum showed the lowest binding of PspA-specific IgG to clade 3. Immunization with PspA3+2 afforded significant protection against pneumococcal challenge by five strains with different clades in mice, but immunization with PspA2+4 or PspA2+5 failed to protect mice from pneumococcal challenge by strains with clades 1 and 3. The binding of PspA-specific IgG in antisera raised by three PspA fusion proteins was examined in 68 clinical isolates from adult patients with IPD. Immunization of mice with PspA3+2-induced antiserum with a high binding capacity for clinical isolates expressing clades 1–4, but not clade 5. Our results suggest that the PspA3+2 vaccine has an advantage over the PspA2+4 or PspA2+5 vaccine in terms of a broad range of cross-reactivity with clinical isolates and cross-protection against pneumococcal challenge in mice.

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## 1. Introduction

*Streptococcus pneumoniae* is a major cause of morbidity and mortality caused by pneumonia, bacteremia, and meningitis worldwide [1]. After introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) in children, significant declines in the incidence of invasive pneumococcal disease (IPD) caused by vaccine serotypes were reported in children and adults [2,3]. However, an increase

in the incidence of IPD caused by non-PCV7 serotypes has been also observed in children and adults [3–5]. In addition, after introduction of a 13-valent pneumococcal conjugate vaccine (PCV13) in children, serotypes not included in PCV13 have been isolated with increasing frequency in pediatric and adult patients with IPD [6,7]. Because there are >90 different pneumococcal capsular serotypes, continuous supplementation of pneumococcal conjugate vaccines with new serotypes for serotype replacement may not be a practical strategy.

Previous studies have demonstrated that several pneumococcal proteins are potential vaccine candidates [8–11]. One candidate protein antigen is pneumococcal surface protein A (PspA), which is

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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  $\alpha$ -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  $\alpha$ -helical domain of PspA has an antiparallel coiled-coil motif and is considered to be the most exposed part of the molecule [20]. The  $\alpha$ -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  $\alpha$ -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

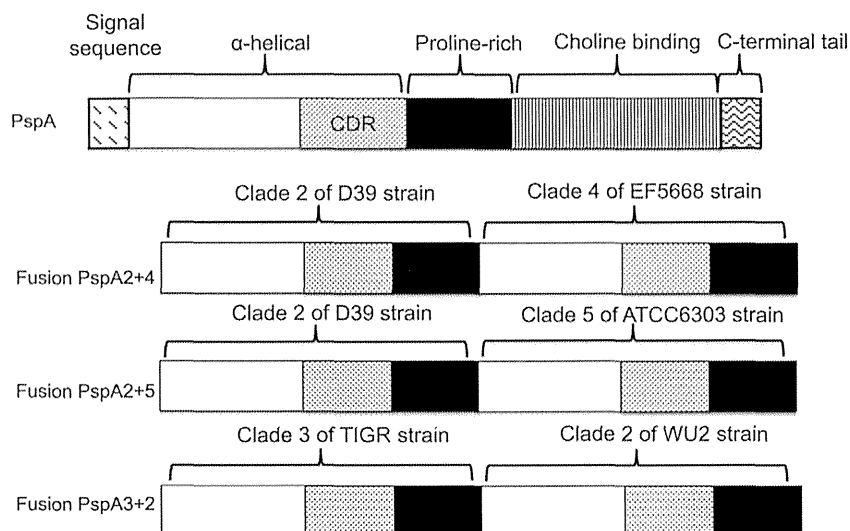
3, 4 and 5 from family 2. All cloning procedures were performed with *Escherichia coli* DH5 $\alpha$  grown in Luria–Bertani medium (Sigma-Aldrich, St. Louis, MO) supplemented with kanamycin (30  $\mu$ g/ml). DNA fragments encoding portions of the N-terminal regions (containing the  $\alpha$ -helix domain 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  $\times$  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  $\times$  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  $\times$  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  $\mu$ g of recombinant fusion PspA derivatives in lipopolysaccharide-free phosphate-buffered saline (PBS) (Sigma) in combination with 2.5  $\mu$ g of TLR9 ligand adjuvants K3 CpG oligonucleotides (CpG ODNs) and 5  $\mu$ g 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  $\mu$ l 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  $\mu$ g of PspA plus 2.5  $\mu$ g of CpG ODNs were significantly higher than those in mice nasally immunized with 0.1  $\mu$ g of PspA plus 2.5  $\mu$ g 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  $\alpha$ -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

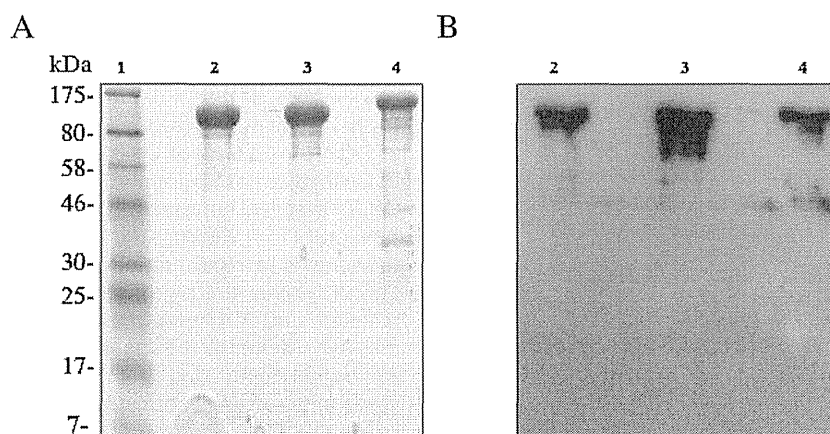
Five pneumococcal strains for bacterial challenge and 68 clinical isolates were grown in blood agar plates overnight and then subcultured again on blood agar plates for 4–5 h. The bacteria were collected in PBS, harvested by centrifugation, and washed once with PBS. Ninety microliters of the bacterial suspension at a concentration of  $1 \times 10^8$  colony-forming units (cfu)/ml in PBS was incubated with  $10 \mu\text{l}$  of mouse antisera for 30 min at  $37^\circ\text{C}$ . After incubation, the suspension was washed once with PBS, resuspended in  $100 \mu\text{l}$  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  $500 \mu\text{l}$  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

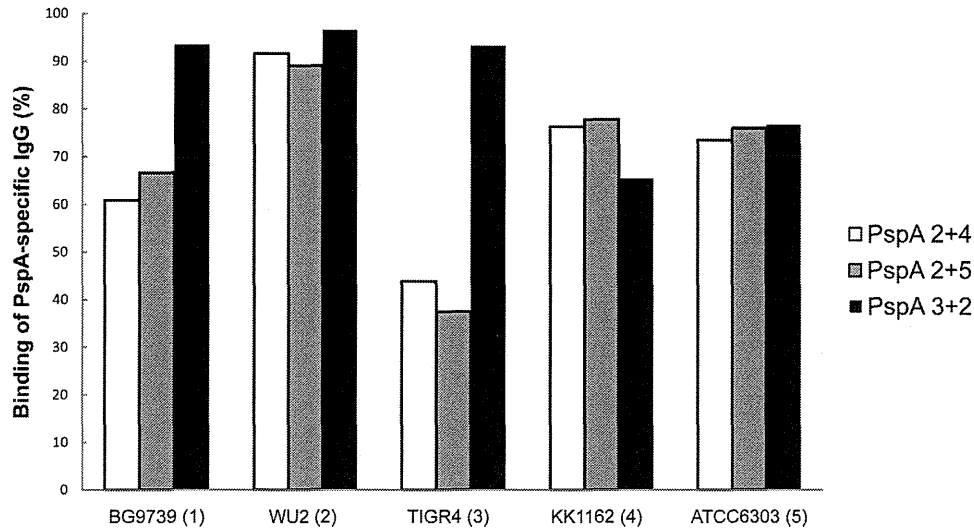
The mice immunized with the PspA fusion protein plus double adjuvants were challenged intranasally with  $2 \times 10^7$  cfu of strain BG9739 (clade 1),  $2 \times 10^7$  cfu of strain WU2 (clade 2),  $5 \times 10^6$  cfu of strain TIGR4,  $2 \times 10^7$  cfu of strain KK1162 (clade 4), or  $5 \times 10^5$  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; lane 4, 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.



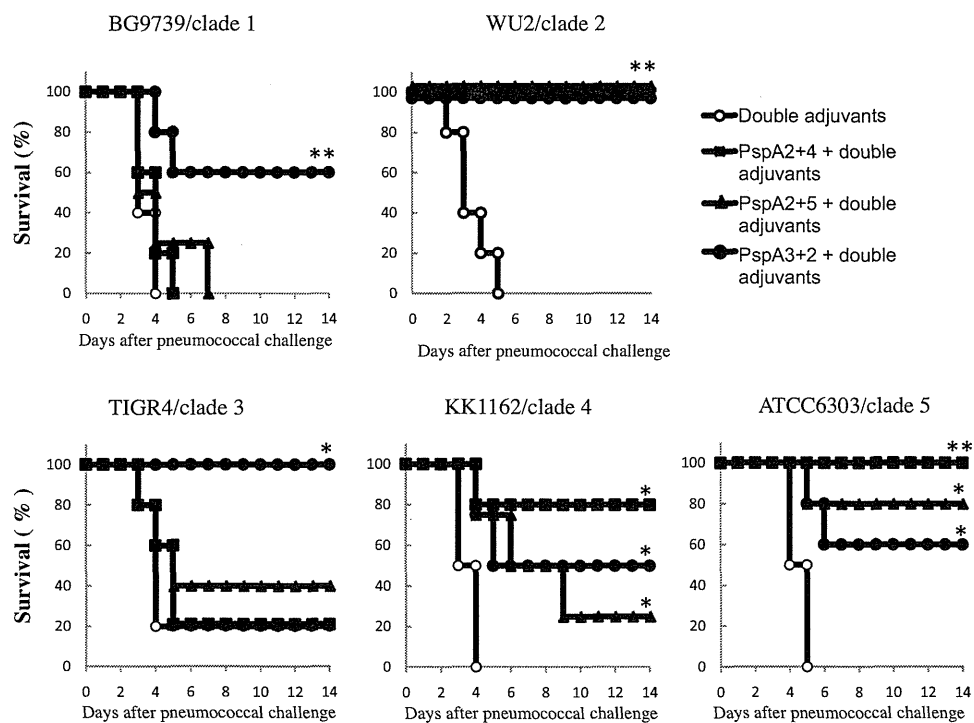
**Fig. 3.** The binding of PspA-specific IgG by antisera with PspA fusion proteins to the challenge strains with different clades. The mean percentages of fluorescent bacteria positive for IgG binding by antisera from mice immunized with PspA2+4, PspA2+5, or PspA3+2 in combination CpG ODNs plus AHG (double 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.

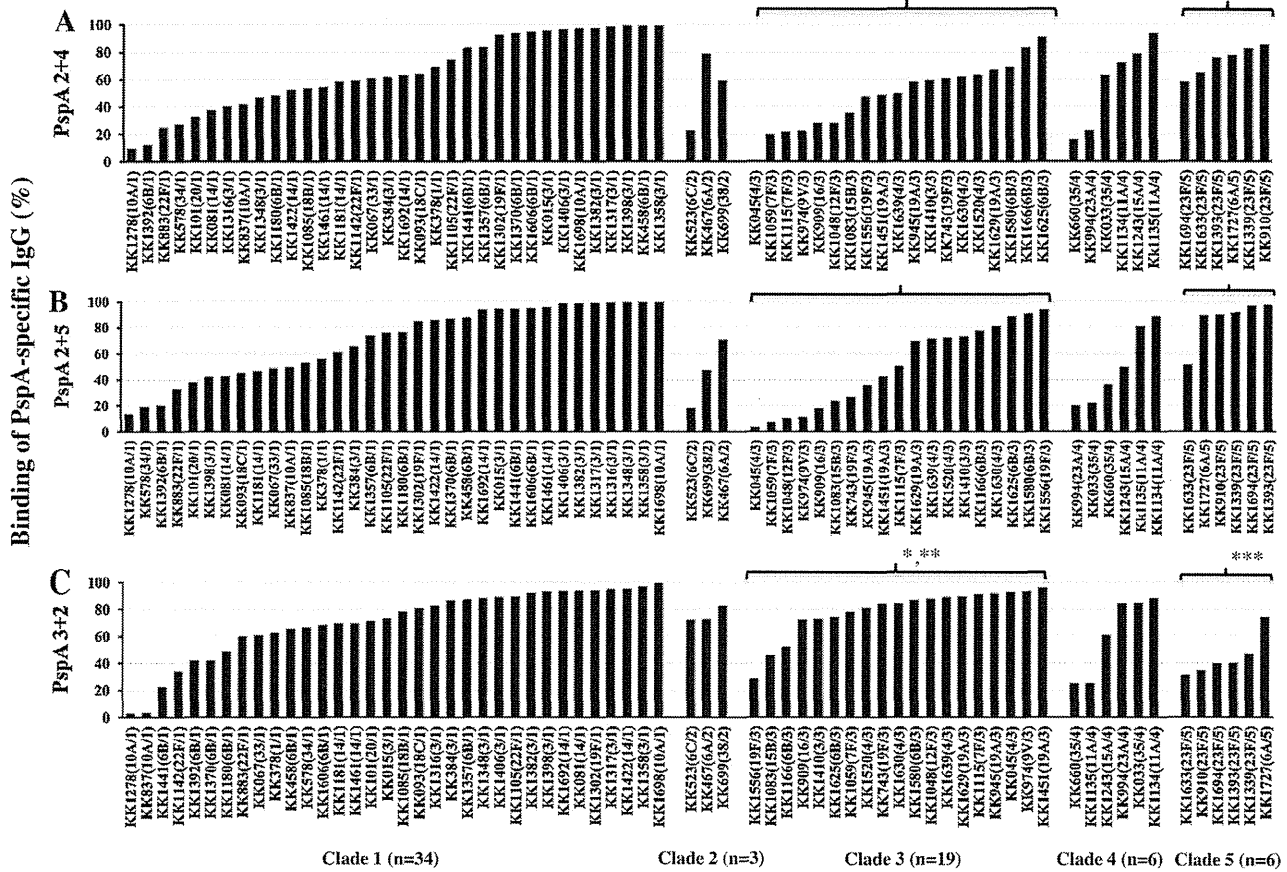
#### 4. Discussion

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.L., 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

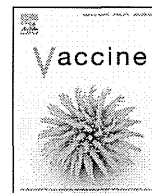
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#### Appendix A. Supplementary data

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>.

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## Hemozoin as a novel adjuvant for inactivated whole virion influenza vaccine



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### 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.

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### 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

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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  $\beta$ -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<sub>2</sub>.

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 passing them twice in MDCK cells and examining their cytopathic effect; inactivation of VN1203 viruses was confirmed by passing 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  $\mu$ l of PBS, 9 mM hemozoin only, inactivated Ca04 only [ $5 \times 10^6$  plaque-forming unit (PFU), which corresponds to 0.1  $\mu$ g 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<sub>50</sub>) 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<sub>50</sub> 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  $\mu$ g (in 50  $\mu$ l) 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  $\times$  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,  $5 \times 10^6$  plaque-forming units (PFU), the total amount of viral protein was  $0.1 \mu\text{g}$ ) 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<sub>50</sub>) [23] or VN1203 (100 MLD<sub>50</sub>) 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

**Table 1**

Virus titers in the lungs of immunized mice challenged with mouse-adapted Ca04 virus.<sup>a</sup>

Immunization	Day after challenge	Virus titer (mean log <sub>10</sub> 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

<sup>a</sup> Mice were intranasally immunized twice with the indicated agents (50 μl per mouse) and challenged with 10 MLD<sub>50</sub> 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.<sup>a</sup>

Immunization	Day after challenge	Virus titer (mean log <sub>10</sub> 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

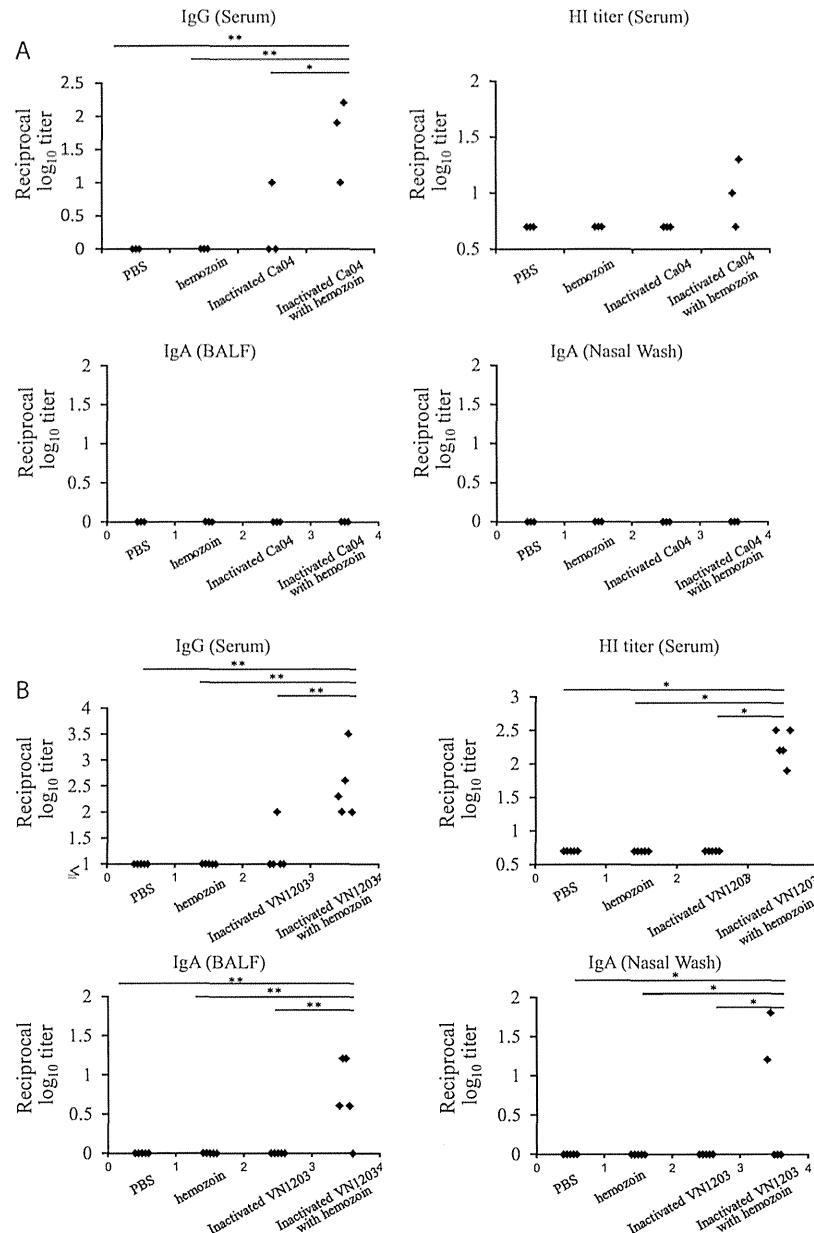
<sup>a</sup> Mice were intranasally immunized twice with the indicated agents (50 μl per mouse) and challenged with 100 MLD<sub>50</sub> of VN1203 virus (50 μl per mouse) 4 weeks after the final immunization. Lungs were collected from mice ( $n=3$ ) on days 3 and 6 after challenge and examined for virus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

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.

## 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

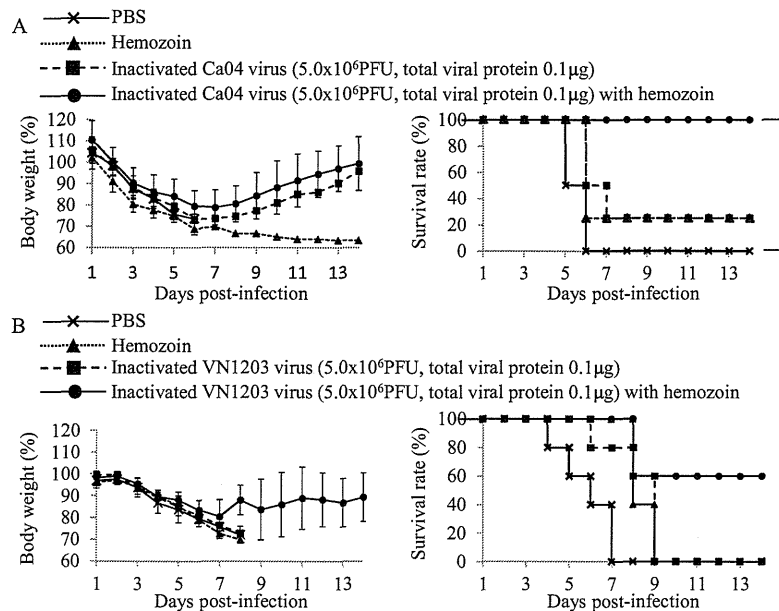


**Fig. 1.** Virus-specific antibody responses in immunized mice. Virus-specific antibodies were detected by means of ELISA and HI assays with purified Ca04 (A) or VN1203 (B) virus as a viral antigen. IgG antibody titers (upper, left panels) and HI titers (upper, right panels) in serum and IgA antibody 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<sub>50</sub> of MACa04 virus (A:  $n=4$ ) or 100 MLD<sub>50</sub> 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  $\pm$  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.

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

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## 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*, *Lgals3bp*, *Cxcl11*, *Timp1*, *Tap2*, *Psmb9*, *Psme1*, *Tapbp*, *C2*, *Csf1*, *Mx2*, *Zbp1*, *Ifrd1*, *Trafd1*, *Cxcl9*,  $\beta$ 2m, *Npc1*, *Ngfr* and *Irf47*. 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.

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## 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 Pharmacopoeia, United States Pharmacopoeia 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 multiplexed 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  $23 \pm 1^\circ\text{C}$ , with  $50 \pm 10\%$  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.

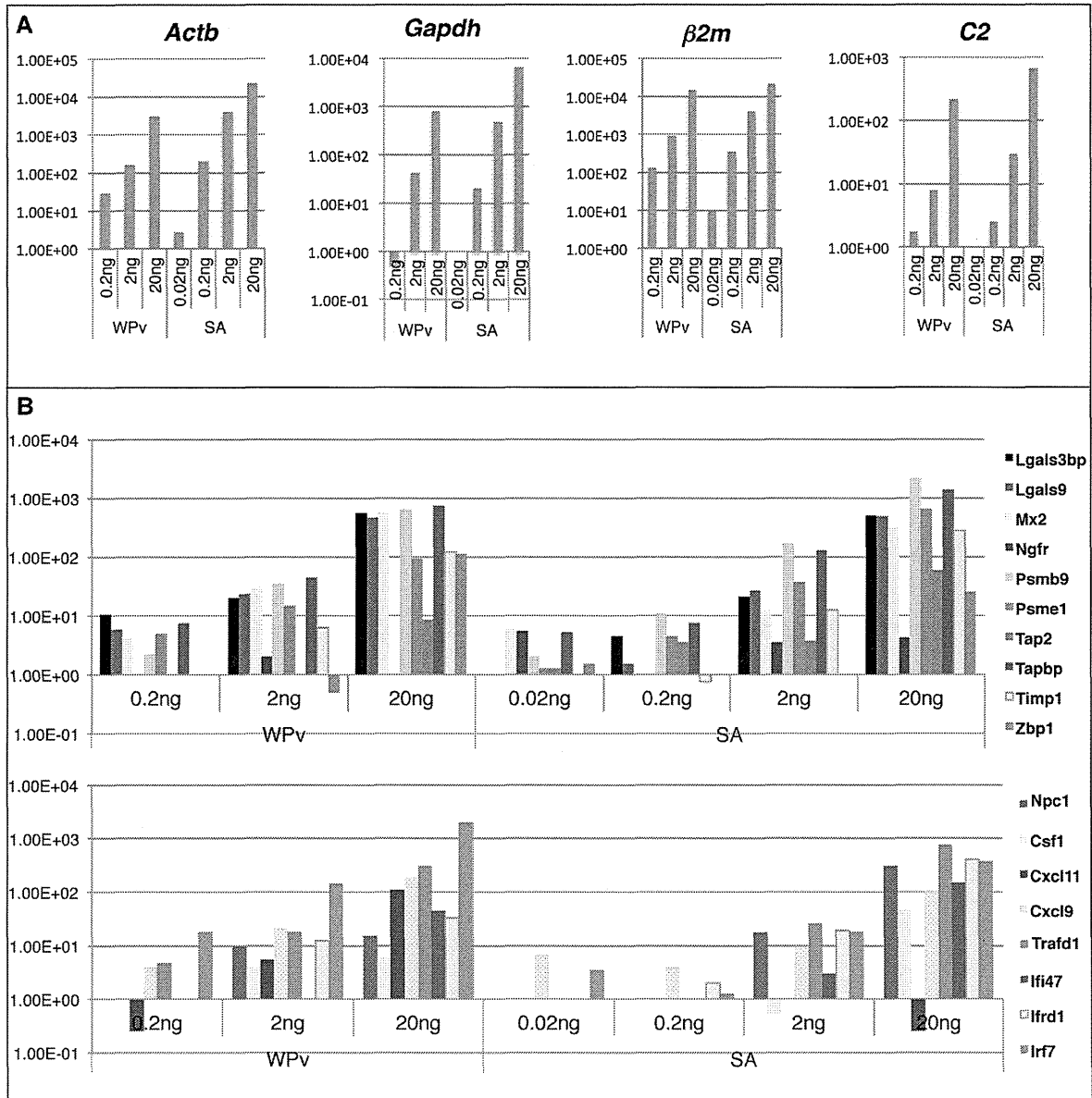
### 2. Vaccines

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  $\mu\text{g}$  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  $\mu\text{g}$  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  $\mu\text{g}$  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
<i>Irf7</i>	Interferon regulatory factor 7	293624
<i>Lgals9</i>	Lectin, galactoside-binding, soluble, 9	25476
<i>Lgalsbp3</i>	Lectin, galactoside-binding, soluble, 3 binding protein	245955
<i>Cxcl11</i>	Chemokine (C-X-C motif) ligand 11	305236
<i>Timp1</i>	TIMP metalloproteinase inhibitor 1	116510
<i>Tap2</i>	Transporter 2, ATP-binding cassette, sub-family B	24812
<i>Psmb9</i>	Proteasome (prosome, macropain) subunit, beta type, 9	24967
<i>Psmc1</i>	Proteasome (prosome, macropain) activator subunit 1	29630
<i>Tapbp</i>	TAP binding protein (tapasin)	25217
<i>C2</i>	Complement component 2	24231
<i>Csf1</i>	Colony stimulating factor 1 (macrophage)	78965
<i>Mx2</i>	Myxovirus (influenza virus) resistance 2	286918
<i>Zbp1</i>	Z-DNA binding protein 1	171091
<i>Irfd1</i>	Interferon-related developmental regulator 1	29596
<i>Trafd1</i>	TRAF type zinc finger domain containing 1	114635
<i>Cxcl9</i>	Chemokine (C-X-C motif) ligand 9	246759
<i><math>\beta</math>2m</i>	Beta-2 microglobulin	24223
<i>Npc1</i>	Niemann-Pick disease, type C1	266732
<i>Ngfr</i>	Nerve growth factor receptor	24596
<i>Iff47</i>	Interferon gamma inducible protein 47	246208

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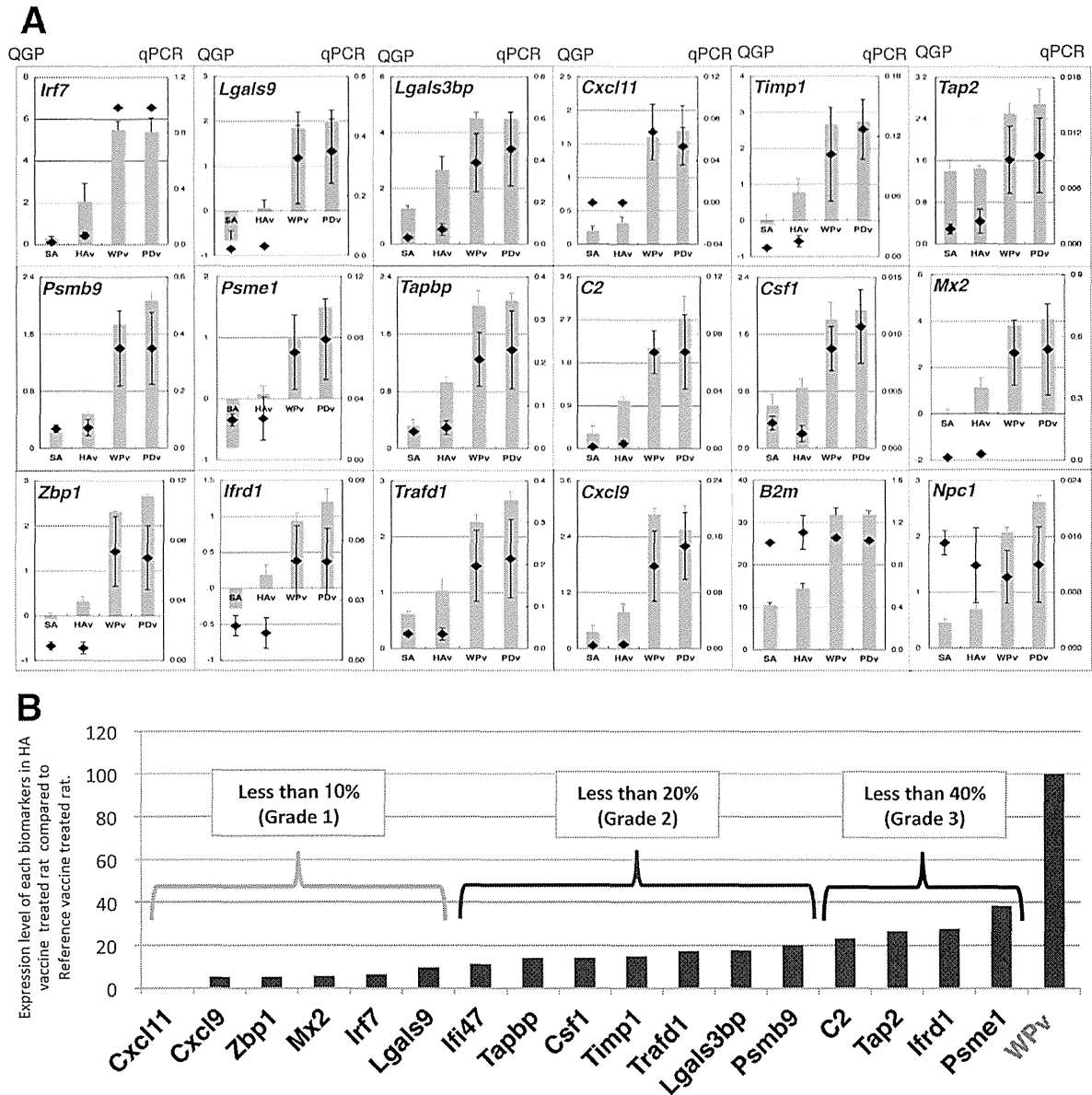
**Figure 1. Optimization of QGP in influenza vaccine safety evaluation.** A) Gene expression of *Actb*, *Gapdh*, *B2m* and *C2* and B) biomarkers, in 0.2, 2 and 20 ng RNA-containing samples from SA- and WPv-treated rat lungs. Relative expression levels of the *Gapdh* gene are indicated. SA: saline, WPv: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