

### Acknowledgments

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# Dectin-2-Dependent NKT Cell Activation and Serotype-Specific Antibody Production in Mice Immunized with Pneumococcal Polysaccharide Vaccine

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## Abstract

Although thymus-independent type 2 antigens generally do not undergo Ig class switching from IgM to IgG, pneumococcal polysaccharide vaccine (PPV) induces the production of serotype-specific IgG. How this happens remains unclear, however. In the present study, PPV immunization induced production of IgG as well as IgM specific for a serotype 3-pneumococcal polysaccharide in the sera of wild-type (WT) mice, but this phenomenon was significantly reduced in Dectin-2 knockout (KO) mice. Immunization with PPV caused IL-12p40 production in WT mice, but this response was significantly reduced in Dectin-2KO mice. Likewise, immunization with PPV activated natural killer T (NKT) cells in WT mice but not in Dectin-2KO mice. Furthermore, administration of  $\alpha$ -galactosylceramide, recombinant (r)IL-12 or rIFN- $\gamma$  improved the reduced IgG levels in Dectin-2KO mice, and treatment with neutralizing anti-IFN- $\gamma$  mAb resulted in the reduction of IgG synthesis in PPV-immunized WT mice. Transfer of spleen cells from PPV-immunized WT mice conferred protection against pneumococcal infection on recipient mice, whereas this effect was cancelled when the transferred spleen cells were harvested from PPV-immunized Dectin-2KO mice. These results suggest that the detection of PPV antigens via Dectin-2 triggers IL-12 production, which induces IFN- $\gamma$  synthesis by NKT cells and subsequently the production of serotype-specific IgG.

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## Introduction

*Streptococcus pneumoniae* is a leading causative bacterium of community-acquired pneumonia [1–3]. The risk of serious *S. pneumoniae* infection is increased in patients with a deficiency of IgG against pneumococcal capsular polysaccharides and phosphorylcholine, which facilitates opsonophagocytic killing by neutrophils [4,5]. Immunization with 23-valent pneumococcal polysaccharide vaccine (PPV) results in a protective IgG response in vaccinated individuals who have a high risk of pneumococcal infection [6–8]. Thus, IgG production against pneumococcal polysaccharides is a key factor in protecting hosts from pneumococcal infection. However, it remains to be clarified how PPV causes the production of IgG.

Recently, C-type lectin receptors (CLRs), pattern recognition receptors (PRRs) for pathogen-derived polysaccharides, have garnered much attention from many investigators with respect to their role in host defense against fungal infection [9]. DC-associated C-type lectin-2 (Dectin-2), one of the CLRs, possesses a

carbohydrate recognition domain (CRD) for the Ca<sup>++</sup>-dependent recognition of mannose [10,11]. The triggering of Dectin-2-mediated stimulation by fungal hyphae or specific Ab leads to the activation of NF- $\kappa$ B and the production of proinflammatory cytokines [12,13].

Capsular polysaccharide of *S. pneumoniae* is classified as a thymus-independent type 2 (TI-2) antigen, which does not require T cell help for the activation of B cells. TI-2 antigens generally fail to induce Ig class switching from IgM to IgG, affinity maturation and memory B cell response because of the lack of a cognate CD40/CD40L interaction between T and B cells [14,15]. One notable exception to this is the fact that serotype-specific IgG2 is produced in individuals who receive PPV immunization [16,17], although it remains to be clarified which cells are involved in this response.

In earlier studies by Snapper and co-workers, IFN- $\gamma$  was found to stimulate the secretion of IgG3, a mouse homologue of human IgG2, by TI-2 antigen-stimulated B cells [18]. Natural killer (NK) T cells, which express both  $\alpha\beta$  T cell antigen receptors and NK

cell markers, have been identified as a novel lymphocyte population that acts in the innate stages of immune responses [19]. These cells recognize glycolipid antigens, such as  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer), in the context of CD1d molecules on dendritic cells (DCs) [20], which leads to the rapid production of IFN- $\gamma$  and IL-4 [21,22]. Kobrynski and co-workers demonstrated that NKT cells play a critical role in the Ab production caused by pneumococcal polysaccharides [23]. Recently, we reported evidence suggesting that NKT cells may be involved in the production of serotype-specific IgG after PPV immunization in clinical settings [24].

Given this background, in the present study, we addressed the question of whether Dectin-2 and NKT cells contributed to the Ab production caused by PPV immunization. We found that Dectin-2 was absolutely required for the production of IL-12p40 by dendritic cells upon in-vitro stimulation with PPV, and that immunization with PPV caused IFN- $\gamma$  synthesis by NKT cells, which was possibly activated by the secretion of IL-12 from DCs leading to the production of serotype-specific Ab.

## Materials and Methods

### Mice

Dectin-2KO mice were generated by homologous recombination of the *Clectn* gene as described previously [13]. These mice were back-crossed for seven generations to C57BL/6J. Male or female mice at 6 to 12 weeks of age were used for the experiments. Wild-type (WT) littermate mice for Dectin-2KO mice were used as controls. The experiments were approved by the ethics committees of Tohoku University (Permit Number: 2011 idou-201). We took the utmost care to alleviate any pain and suffering on the part of the mice.

### Reagents and vaccination

Twenty-three-valent pneumococcal polysaccharide vaccine (PPV; Pneumovax<sup>®</sup>NP) was purchased from MSD K.K., Tokyo, Japan. The PPV contained 25  $\mu$ g each of 23 different types of pneumococcal polysaccharide antigen. WT or Dectin-2KO mice were vaccinated intraperitoneally with 20  $\mu$ L of PPV diluted in 200  $\mu$ L normal saline. Serum samples were collected at various time intervals post-vaccination. Alpha-GalCer was purchased from Funakoshi (Tokyo, Japan) and prepared according to the manufacturer's instructions. For in-vitro experiments, RPMI1640 medium and fetal calf serum (FCS) were obtained from Nippro (Osaka, Japan) and BioWest (Nuaille, France), respectively. Lipopolysaccharide (LPS) prepared from *Escherichia coli* O-111 (Sigma-Aldrich, St. Louis, MO, USA) and mannan from *Saccharomyces cerevisiae* (Sigma-Aldrich) were used as a control.

### Bacteria

A serotype-3 clinical strain of *S. pneumoniae*, designated as URF918, was established from a patient with pneumococcal pneumonia [25]. The bacteria were cultured in Todd-Hewitt broth (Difco, Detroit, MI, USA) at 37°C in a 5% CO<sub>2</sub> incubator, harvested at 6 h, at the mid-log phase of growth, and then washed twice in PBS. The inoculum was prepared at 3.3 $\times$ 10<sup>8</sup> colony forming units (CFU)/ml and then stored at -80°C until use.

### Preparation and culture of dendritic cells

Bone marrow-derived dendritic cells (BM-DCs) were prepared as previously described [26]. In brief, BM cells from WT mice and Dectin-2KO mice were cultured at 2 $\times$ 10<sup>5</sup>/ml in 10 ml RPMI1640 medium supplemented with 10% FCS, 100 U/ml penicillin G, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, and

50  $\mu$ M 2-mercaptoethanol (Sigma-Aldrich) containing 20 ng/ml murine granulocyte-macrophage colony-stimulating factor (GM-CSF; Wako Pure Chemical Industries, Ltd., Osaka, Japan). On day 8, non-adherent cells were collected and used as BM-DCs. BM-DCs were stimulated at 1 $\times$ 10<sup>5</sup>/ml for 24 h at 37°C in 5% CO<sub>2</sub> with various concentrations of PPV or other stimuli. Methyl- $\alpha$ -D-mannopyranoside (ManP) (Sigma-Aldrich) was used for a competitive inhibition assay against mannose binding to Dectin-2.

### Deletion of ConA-binding fraction of PPV

In order to delete the Concanavalin A (ConA)-binding fraction, PPV was incubated with ConA Sepharose4B (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for 15 min at room temperature. The ConA unbound fraction was used to stimulate BM-DCs.

### Measurement of serotype-specific Ab and cytokines

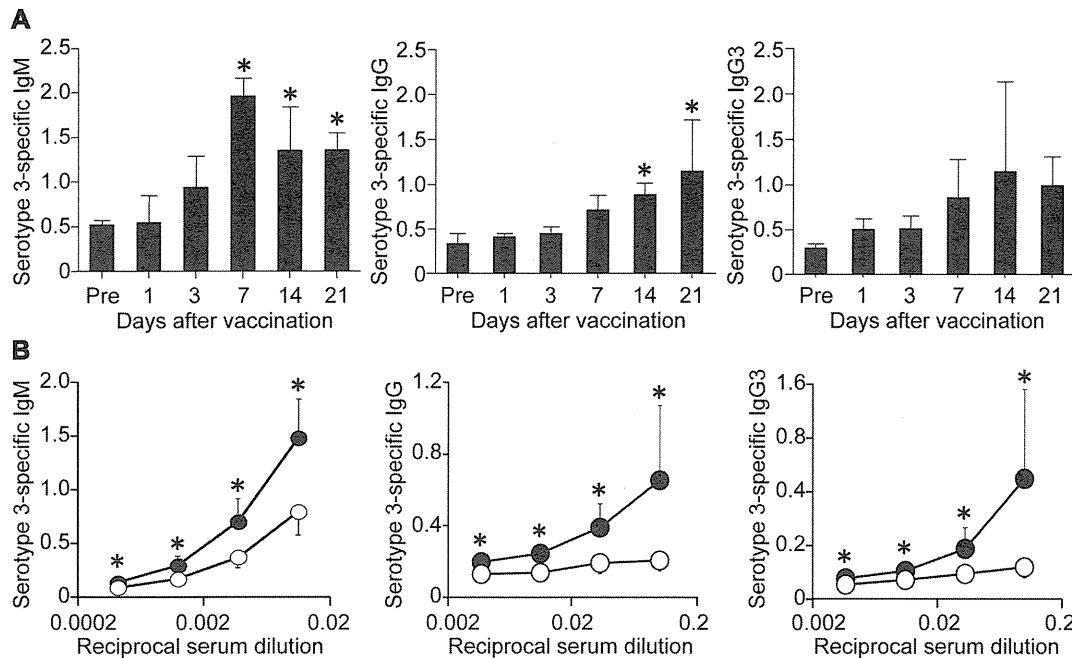
The quantities of serotype-specific Ab against pneumococcal polysaccharide type 3 (PPS3) in sera were measured by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Nunc A/S, Roskilde, Denmark) were coated with 3  $\mu$ g/ml of each polysaccharide [American Type Culture Collection (ATCC), Manassas, VA, USA] in PBS for 1 h at 37°C. Prior to testing, serum samples were diluted with an absorption buffer of 0.05% skim milk PBS to 1:10, and incubated at room temperature for 30 min to allow the adsorption of non-specific Ab to cell wall polysaccharide (CWP: Statens Serum Institute, Copenhagen, Denmark) and PPS22F (ATCC). HRP-conjugated goat anti-mouse IgM, IgG or IgG3 antibodies (Southern Biotechnology Associates, Birmingham, AL, USA) diluted with 1:4000 were used as detection Ab. The concentrations of IgM, IgG and IgG3 were determined based on the absorbance at 450 nm. The concentration of IL-12p40 and TNF- $\alpha$  in the culture supernatants was determined by ELISA using capture and biotinylated developing antibodies (BD Biosciences, Franklin Lakes, NJ, USA). The detection limit was 15 and 9.8 pg/ml, respectively. The concentration of IL-6 was assayed using an ELISA kit (BioLegend, San Diego, CA, USA) in which the detection limit was 7.8 pg/ml.

### Transfer of spleen cells and *S. pneumoniae* infection

Spleen cells were prepared from WT or Dectin-2KO mice 14 days after PPV immunization, and the obtained cells (2 $\times$ 10<sup>7</sup>/mouse) were transferred into WT mice. One day later, mice were inoculated with live *S. pneumoniae* (1.6 $\times$ 10<sup>6</sup> CFU/mouse) at 50  $\mu$ l per mouse by insertion of a 24G I.V catheter (TERUMO, Tokyo, Japan) into the trachea.

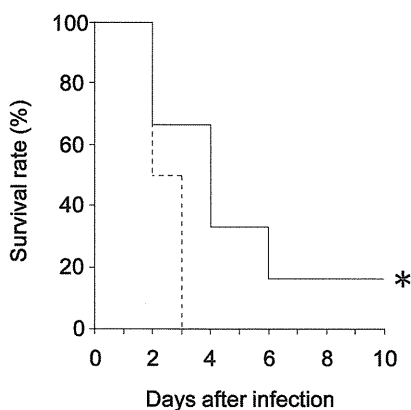
### Assay for CD69 expression and intracellular IFN- $\gamma$ production

For evaluation of CD69 expression on NK, NKT and T cells, spleen cells obtained from WT or Dectin-2KO mice on day 9 after vaccination were stained with FITC-conjugated anti-CD3 (Clone 145-2C11; BioLegend), PE-conjugated anti-NK1.1 (clone PK136; BioLegend) and APC-conjugated anti-CD69 mAb (clone H1.2F3; BioLegend). The isotype-matched control IgG for each Ab was used as a reference. For intracellular IFN- $\gamma$  staining, spleen cells were incubated at 4 $\times$ 10<sup>5</sup>/ml with 5 ng/ml of phorbol 12-myristate 13-acetate, 500 ng/ml of ionomycin and 2  $\mu$ M of monensin (Sigma-Aldrich) for 4 hours at 37°C before the cell surface was stained. Then, cells were incubated in the presence of cytofix/cytoperm (BD Biosciences), washed twice in BD perm/wash solution and stained with FITC-conjugated anti-IFN- $\gamma$  mAb (clone XMG1.2; BD Biosciences) or control rat IgG. The stained cells were analyzed using a FACS Canto II flow cytometer (BD



**Figure 1. Reduced production of PPS3-specific Ab in Dectin-2KO mice.** (A) WT mice received intraperitoneal injection of 20  $\mu$ L PPV diluted in 200  $\mu$ L normal saline. Sera were collected at indicated time points after PPV immunization, and concentrations of anti-PPS3 IgM, IgG and IgG3 were measured as OD450 values at  $\times 90$ ,  $\times 10$  and  $\times 10$  dilution, respectively. Data are shown as the mean  $\pm$  SD of four mice. \*,  $p < 0.05$ , compared with pre-vaccination level of each Ab. (B) Serum levels of anti-PPS3 IgM, IgG and IgG3 on day 14 after PPV immunization were compared between WT and Dectin-2KO mice. Data are shown as the mean  $\pm$  SD of six mice. Similar results were obtained in three experiments. \*,  $p < 0.05$ . Closed circles, WT mice; Open circles, Dectin-2KO mice. doi:10.1371/journal.pone.0078611.g001

Biosciences). Data were collected from 30,000 individual cells using parameters of forward scatter (FSC) and side scatter (SSC) to limit the lymphocyte population.



**Figure 2. Transfer of spleen cells from PPV-immunized Dectin-2KO mice fails to induce protection from pneumococcal infection.** Spleen cells obtained from WT or Dectin-2KO mice 14 days after PPV immunization were suspended in PBS. Recipient WT mice received intravenous injections of the spleen cells ( $2 \times 10^7$ /mouse) and, 24 h later, were infected intratracheally with *S. pneumoniae* ( $1.6 \times 10^6$ /mouse). The number of live mice was daily noted. Solid line, mice given spleen cells from PPV-immunized WT mice ( $n = 6$ ); dotted line, mice given spleen cells from PPV-immunized Dectin-2KO mice ( $n = 6$ ). Similar results were obtained in two experiments. \*,  $p < 0.05$ , compared with mice given spleen cells from PPV-immunized Dectin-2KO mice. doi:10.1371/journal.pone.0078611.g002

#### Administration of recombinant murine IFN- $\gamma$ and IL-12

Mice were injected intraperitoneally with recombinant (r)IFN- $\gamma$  (PeproTech, Inc., Rocky Hill, NJ, USA) at 20,000 IU/mouse or PBS once every four days, starting on day 7 post-vaccination. In the other experiment, rIL-12 (PeproTech) or PBS was given at 0.1  $\mu$ g/mouse/day via an intraperitoneal route for one week beginning on day 1 after PPV administration.

#### Activation of NKT cells

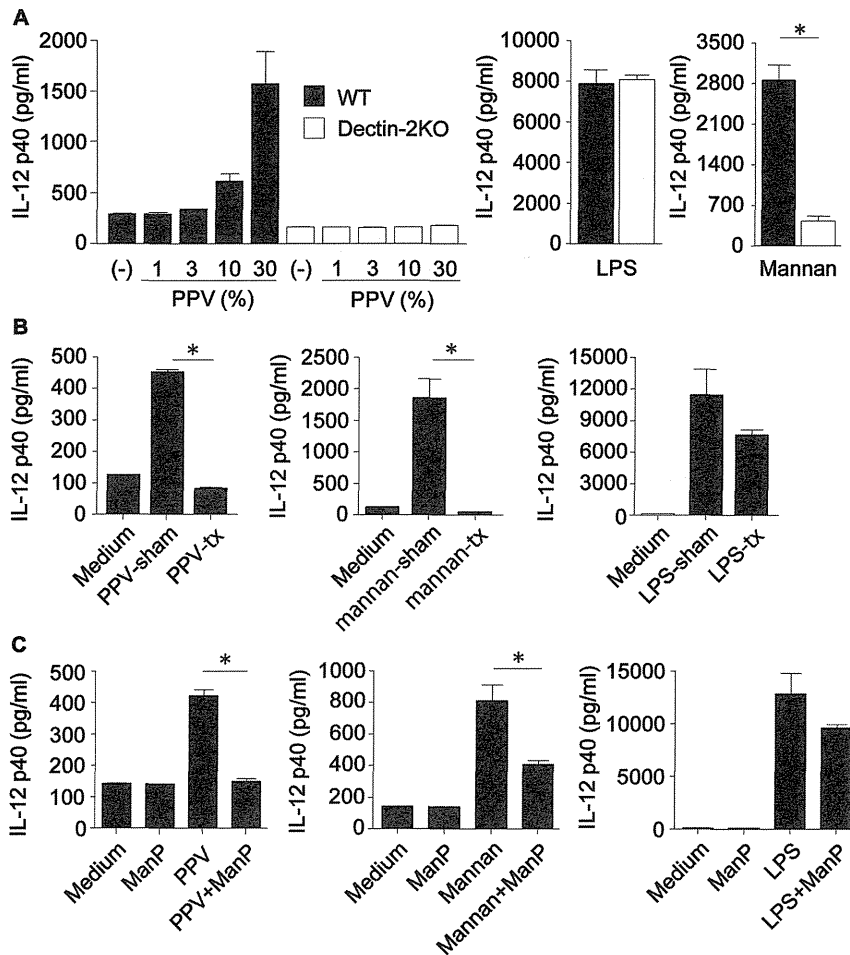
To activate NKT cells, WT or Dectin-2KO mice were injected intraperitoneally with  $\alpha$ -GalCer (1  $\mu$ g/mouse) or PBS containing 0.8% dimethyl sulfoxide (DMSO) on day 7 post-vaccination.

#### Neutralization of endogenous IFN- $\gamma$

Anti-IFN- $\gamma$  mAb was purified from culture supernatants of hybridoma (clone R4-6A2, ATCC) using a protein G column kit (Kirkegaard & Perry Lab.). To block endogenously synthesized IFN- $\gamma$ , mice were injected intraperitoneally with mAb against this cytokine at 200  $\mu$ g/mouse on days 6, 7 and 10 post-vaccination. Rat IgG (ICN Pharmaceuticals, Inc., Aurora, OH, USA) was used as a control.

#### Statistical analysis

Statistical analysis was conducted using JMP software (SAS Institute Inc., Cary, NC, USA) on a Windows computer. Differences between two groups were tested using two-tail analysis in the unpaired Student's t-test. Differences among three or more groups were tested using ANOVA with post-hoc analysis (Student-Newman-Keuls test). Survival data was analyzed using the Kaplan-Meier log rank test. A  $p$  value less than 0.05 was considered significant.



**Figure 3. Dectin-2 is essential for PPV-induced IL-12p40 production by BM-DCs.** (A) BM-DCs derived from WT or Dectin-2KO mice were cultured with indicated doses of PPV for 24 h. Concentration of IL-12p40 in the culture supernatants was measured. LPS and mannan were used at 1 μg/ml and 3 mg/ml, respectively, as controls. Data are shown as the mean±SD of triplicate cultures. Closed column, WT mice; Open column, Dectin-2KO mice. (B) BM-DCs derived from WT mice were stimulated with ConA-unbound fraction of PPV, mannan and LPS or sham-treated PPV, mannan and LPS for 24 h. Concentrations of IL-12p40 in the culture supernatants were measured. Data are shown as the mean±SD of triplicate cultures. PPV-sham, mannan-sham and LPS-sham: sham-treated PPV, mannan and LPS; PPV-tx, mannan-tx and LPS-tx, ConA-bound polysaccharide-deleted PPV, mannan and LPS. (C) BM-DCs derived from WT mice were stimulated with 30% PPV, 1 μg/ml LPS or 3 mg/ml mannan in the presence or absence of 50mM methyl- $\alpha$ -D-mannopyranoside (ManP) for 24 h. Similar results were obtained in three experiments. \*,  $p < 0.05$ . doi:10.1371/journal.pone.0078611.g003

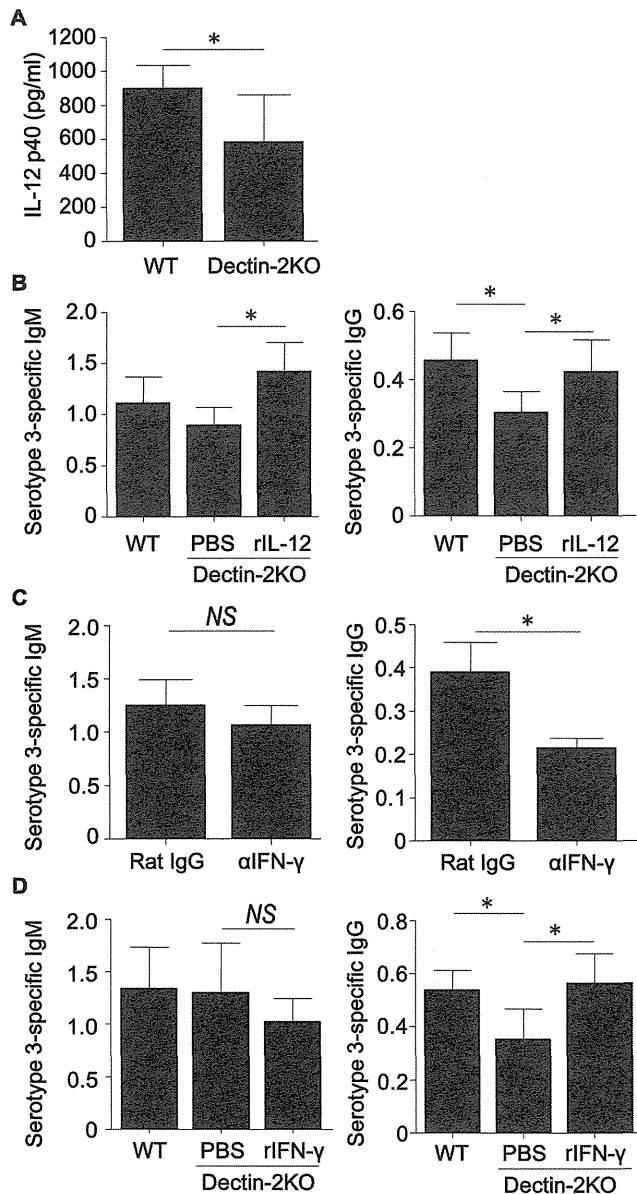
## Results

### Production of PPS3-specific Ab is reduced in Dectin-2KO mice

Initially, we measured the serum levels of anti-PPS3 IgM, IgG and IgG3 in WT mice at various time intervals after PPV immunization. As shown in Figure 1A, IgM began to increase on day 3, reached its peak level on day 7 and then decreased slightly on days 14 and 21 post-vaccination. Compared to the increase in IgM, that of IgG and IgG3 was delayed by one week. To assess the role of Dectin-2 in the Ab production caused by PPV immunization, WT and Dectin-2KO mice were injected with PPV, and the serum levels of anti-PPS3 IgM, IgG and IgG3 were measured on day 14. As shown in Figure 1B, IgM, IgG and IgG3 levels on day 14 were significantly reduced in Dectin-2KO mice compared to WT mice. Similarly, production of IgM against serotypes 6B, 14, 19F and 23F and of IgG against serotypes 6B and 19F was significantly lower in Dectin-2KO mice than in WT mice, although this difference was not observed in IgG production against serotypes 14 and 23F (see Figure S1).

### Dectin-2 is involved in the protective effect of PPV against pneumococcal infection

To elucidate whether Dectin-2 deficiency affected the host protection caused by PPV vaccination against pneumococcal infection, spleen cells were prepared from WT and Dectin-2KO mice on day 14 after vaccination and transferred into uninfected WT mice that were then infected with *S. pneumoniae* 24 h after the cell transfer (see Figure S2A). As shown in Figure 2, all of the mice that received the transfer from vaccinated Dectin-2KO mice were dead within three days post-infection; in contrast, among the mice that received the transfer from vaccinated WT mice, survival was prolonged and 17% remained alive throughout the observation period. In addition, body weight loss and hypothermia caused by pneumococcal infection were improved in the group that received spleen cells transferred from vaccinated WT mice compared to those that received spleen cells transferred from vaccinated Dectin-2KO mice (see Figure S2B).



**Figure 4. Involvement of IL-12 and IFN- $\gamma$  in the production of PPS3-specific Ab after PPV immunization.** (A) Sera were collected from WT or Dectin-2KO mice on day 9 after PPV immunization, and the concentrations of IL-12p40 were measured. Each column represents the mean  $\pm$  SD of five mice. (B) Mice received daily intraperitoneal injections of rIL-12 (0.1  $\mu$ g/mouse each time) or PBS for seven days beginning on day 1 after PPV immunization. (C) WT mice were given intraperitoneal injections of anti-IFN- $\gamma$  mAb or rat IgG (200  $\mu$ g/mouse each time) on days 6, 7 and 10. (D) PPV-immunized Dectin-2KO mice received daily intraperitoneal injections of rIFN- $\gamma$  (20,000 IU/mouse each time) or PBS for four days, starting on day 7 post-PPV immunization. On day 14, serum levels of IgM and IgG were measured as OD450 values at  $\times$ 90 and  $\times$ 30 dilution, respectively. Data are shown as the mean  $\pm$  SD of five to six mice. Similar results were obtained in two experiments. \*,  $p < 0.05$ ; NS, not significant.  
doi:10.1371/journal.pone.0078611.g004

#### Role of Dectin-2 in the activation of BM-DCs upon stimulation with PPV

To elucidate the role of Dectin-2 in the activation of immune cells by PPV, we compared the production of IL-12p40 due to stimulation with PPV in BM-DCs from WT and Dectin-2KO

mice. As shown in Figure 3A, IL-12p40 synthesis by WT BM-DCs was induced by PPV in a dose-dependent fashion, whereas such production was mostly abrogated in BM-DCs from Dectin-2KO mice. A similar pattern was observed upon stimulation with mannan, the detection of which is mediated by Dectin-2 [13]. By contrast, synthesis of this cytokine by BM-DCs was not affected when cells were stimulated with LPS, which is recognized by TLR4. Similarly, production of TNF- $\alpha$  and IL-6 by BM-DCs upon stimulation with PPV was also abrogated in Dectin-2KO mice (see Figure S3A and B).

ConA is widely used as an  $\alpha$ -mannose-binding lectin. To address the involvement of ConA-bound polysaccharides, we examined how depletion of the fraction bound to this lectin from PPV affected IL-12p40 synthesis by BM-DCs. As shown in Figure 3B, IL-12p40 synthesis was completely abolished by this depletion. ManP competes the binding of  $\alpha$ -mannose to ConA. In next experiments, we examined how addition of excessive amount of ManP affected the activation of BM-DCs caused by PPV. This treatment led to the complete diminution of IL-12p40 synthesis (Figure 3C). These results suggest that Dectin-2 may sense some sugar moieties bound to ConA, such as  $\alpha$ -mannose contained in PPV polysaccharides. In disagreement with this suggestion, however, we did not detect IL-12p40 synthesis by BM-DCs stimulated with ConA-bound fraction of PPV (data not shown).

#### Involvement of IL-12 in Ab production induced by PPV immunization

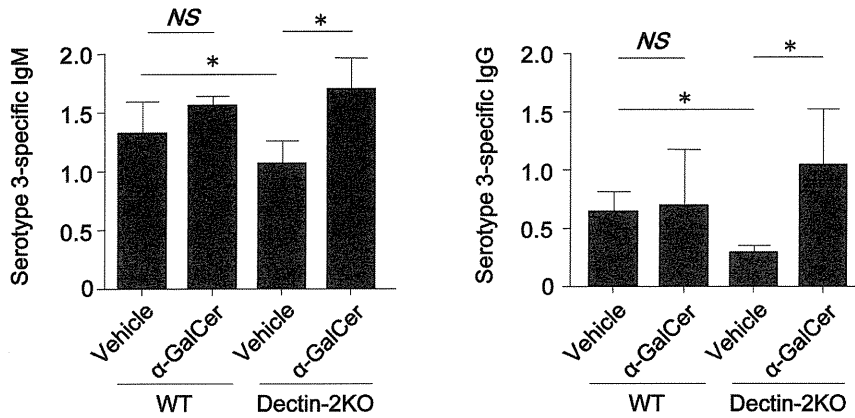
To address this possibility, we measured the serum concentration of IL-12p40 on day 9 after PPV immunization. As shown in Figure 4A, IL-12p40 levels were lower in Dectin-2KO mice than in WT mice. These results suggest that the impaired Ab production was due to attenuated IL-12 synthesis in Dectin-2KO mice; accordingly, we next asked whether the administration of rIL-12 altered the production of Ab in Dectin-2KO mice. As shown in Figure 4B, this treatment completely improved the reduced production of anti-PPS3 IgM and IgG in Dectin-2KO mice, which was consistent with this hypothesis.

#### Involvement of IFN- $\gamma$ in Ab production induced by PPV immunization

To elucidate the possible involvement of IFN- $\gamma$  in Ab production induced by PPV immunization and mediated by Dectin-2, we first examined the effect of treatment with neutralizing mAb against this cytokine on the production of anti-PPS3 Ab in WT mice. As shown in Figure 4C, this treatment significantly reduced the production of anti-PPS3 IgG, but not of IgM, on day 14 compared to treatment with control rat IgG. In addition, we sought to establish whether the administration of rIFN- $\gamma$  altered the production of Ab in Dectin-2KO mice. As shown in Figure 4D, this treatment completely improved the reduced production of anti-PPS3 IgG in Dectin-2KO mice, which was consistent with this hypothesis.

#### Involvement of NKT cells in Ab production induced by PPV immunization

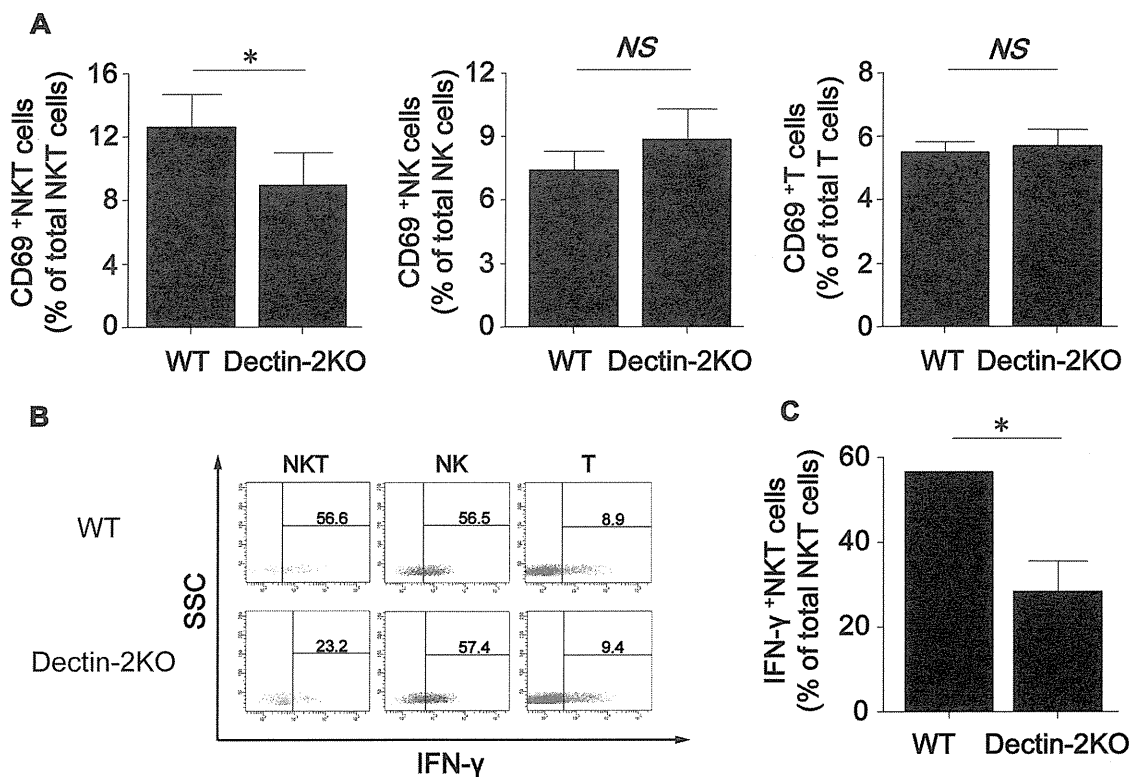
Finally, in order to elucidate the possible involvement of NKT cells in Ab production resulting from PPV immunization and mediated by Dectin-2, we initially asked if  $\alpha$ -GalCer treatment altered the production of anti-PPS3 Ab in Dectin-2KO mice. As shown in Figure 5, this treatment recovered the reduced production of IgM and IgG on day 14 post-vaccination in Dectin-2KO mice. Next, we compared the expression of CD69 in splenic NKT, NK and T cells obtained from WT and



**Figure 5. Effect of  $\alpha$ -GalCer treatment on the reduced production of PPS3-specific Ab in Dectin-2KO mice.** Sera were collected from WT or Dectin-2KO mice on day 14 after PPV immunization. These mice received an intraperitoneal injection of  $\alpha$ -GalCer (1  $\mu$ g/mouse) or vehicle on day 7 post-PPV immunization. Concentrations of PPS3-specific IgM and IgG in sera were measured as OD450 values at  $\times 90$  and  $\times 10$  dilution, respectively. Data are shown as the mean  $\pm$  SD of four mice. Similar results were obtained in two experiments. \*,  $p < 0.05$ . doi:10.1371/journal.pone.0078611.g005

Dectin-2KO mice on day 9. As shown in Figure 6A, the proportion of CD69<sup>+</sup> NKT cells was lower in Dectin-2KO mice than in WT mice, whereas this difference was not observed in NK and T cells. In addition, we examined the intracellular expression of IFN- $\gamma$  in splenic NKT, NK and T cells on the same day

(Figure 6B), and the proportion of IFN- $\gamma$ <sup>+</sup> NKT cells was reduced in Dectin-2KO mice compared to WT mice (Figure 6C). These results suggest that IFN- $\gamma$  secreted from NKT cells contributes to the Ab production induced by PPV immunization.



**Figure 6. NKT cell activation by PPV immunization.** Spleen cells were prepared from WT and Dectin-2KO mice on day 9 after PPV immunization. (A) The obtained cells were stained with FITC-conjugated anti-CD3, PE-conjugated anti-NK1.1, and APC-conjugated anti-CD69 mAbs. Expression of CD69 in NKT (CD3<sup>+</sup>NK1.1<sup>+</sup>), NK (CD3<sup>+</sup>NK1.1<sup>+</sup>) and T (CD3<sup>+</sup>NK1.1<sup>-</sup>) cells were analyzed using flow cytometer. Data are shown as the mean  $\pm$  SD of five mice. \*,  $p < 0.05$ ; NS, not significant compared with WT mice. (B) Intracellular expression of IFN- $\gamma$  in NKT, NK and T cells was analyzed using flow cytometer. Cut-off lines were determined on the basis of isotype-matched control IgG profile. Representative profile of the cytokine expression in each subset is shown. (C) Percent of IFN- $\gamma$ <sup>+</sup> population in NKT cells was analyzed in each group. Data are shown as the mean  $\pm$  SD. Similar results were obtained in three experiments. \*,  $p < 0.05$ ; NS, not significant. doi:10.1371/journal.pone.0078611.g006

## Discussion

In the present study, we measured the serotype 3-specific Ab and used this type of *S. pneumoniae* strain isolated from a patient with pneumonia. We have demonstrated, with this bacterial strain, that NKT cells play a critical role in the innate phase of host protection against pneumococcal infection [25,27]. Briles and co-workers demonstrated that the median time to death in mice which died of infection with the serotype 3 or 4 strain was significantly shorter than the other pneumococcal types [28]. Furthermore, a recent article reported that serotype 3 was highly correlated with case-fatality ratios in invasive pneumococcal infection [29]. Thus, serotype 3 pneumococcus is one of the clinically important bacteria in this infection [30]. In our further experiments, production of IgM against PPS6B, 14, 19F and 23F as well as PPS3 was significantly reduced in Dectin-2KO mice compared to WT mice, as was IgG production against PPS6B and 19F but not against PPS14 and 23F (see Figure S1), suggesting that not all polysaccharides in PPV may be detected solely through Dectin-2. In this respect, PPS3, 6B and 19F are reported to have relatively simple structures without side chains, in contrast to the more complicated structures of 14 and 23F [31]; the simpler structures might be related to the different effects of Dectin-2-deficiency on Ab responses. Furthermore, in an in-vitro binding assay, DC-SIGN interacts with *S. pneumoniae* serotypes 3 and 14, but not certain other serotypes including 19F [32]. SIGN-R1 plays an essential role in eliciting the host immune response and protecting against infection with serotypes 2 and 14 *S. pneumoniae* by mediating recognition and clearance of these bacteria [33,34], although it is not directly involved in PPS-specific production of IgM and IgG [35]. These findings suggest that the binding specificity of each PPS to Dectin-2 may not be uniform.

Earlier investigations reported an important role of IL-12 in the host defense against pneumococcal infection. Exogenous administration of IL-12 enhanced the innate immune response in lungs against *S. pneumoniae* infection by inducing IFN- $\gamma$  production [36] and increased the host protection against this infection [37,38]. Furthermore, in a study by Buchanan and co-workers, co-administration of IL-12 enhanced anti-PPS IgG production caused by PPV conjugated to diphtheria toxin CRM<sub>197</sub> [39]. We also previously showed that IL-12p40KO mice were highly susceptible to *S. pneumoniae* infection, which was due to the reduced production of IFN- $\gamma$  [40]. Thus, IL-12 has been identified as a key cytokine. We measured the serum level of IL-12p40 after PPV immunization because it was difficult to directly measure this cytokine at the local site where DCs were activated. This level was significantly reduced in Dectin-2KO mice compared with WT mice, although the reduction was less marked than we had expected based on the results of in-vitro experiments using BM-DCs, in which IL-12p40 production was completely abrogated in Dectin-2KO mice. In addition, replenishment of rIL-12 resulted in significant improvement of the reduced IgG production in Dectin-2KO mice. Although possible involvement of IL-23 that shares IL-12p40 [41] is not completely excluded, the current data suggest that IL-12 produced by DCs in a Dectin-2-dependent fashion may be involved in the Ab response caused by PPV immunization.

IL-12 promotes the production of IFN- $\gamma$  by various cells including NKT, NK and T cells [42,43]. Buchanan and co-workers demonstrated that IL-12 enhances the Ab response to thymus-independent polysaccharide antigens in the absence of T and NK cells [44]. In our clinical study, NKT cells were suggested to contribute to the production of serotype-specific IgG in humans after PPV immunization [24]. Furthermore, the role of NKT cells in supporting the proliferation and production of Ab by naïve and

memory B cells has been extensively investigated [45-47]. Kobrynski and co-workers demonstrated that CD1d-deficient mice are impaired in the production of IgG specific for pneumococcal capsular polysaccharides, but not for a protein antigen [23]. These earlier findings raise the possibility that the detection of PPV polysaccharides via Dectin-2 may lead to the activation of NKT cells, which contribute to the PPV-induced Ab response through the secretion of IFN- $\gamma$ . In keeping with this possibility, we observed activation of NKT cells, as evidenced by an increased expression of CD69 and the intracellular production of IFN- $\gamma$ , in the spleen after PPV immunization; their activation was reduced in the absence of Dectin-2. In further experiments, the replenishment of rIFN- $\gamma$  improved the reduced Ab production caused by PPV in Dectin-2KO mice and the administration of neutralizing anti-IFN- $\gamma$  mAb led to an attenuated Ab response in vaccinated WT mice. Interestingly, Snapper and co-workers previously demonstrated that IFN- $\gamma$  strongly induces the production of IgG3 by B cells upon stimulation with a thymus-independent type-2 antigen [48]. In addition, mice lacking IgG3 are susceptible to *S. pneumoniae* infection and not protected from this infection by immunization with PPS [49], suggesting that IFN- $\gamma$  may be involved in class switching of Ab to IgG under these conditions. In agreement with these earlier observations, production of PPS3-specific IgG3 was almost completely abolished in Dectin-2KO mice compared to WT mice. Considered together with the findings in previous investigations, the results of the present study indicate that IFN- $\gamma$  secreted from NKT cells as a downstream event of Dectin-2-mediated DC activation plays an important role in serotype-specific IgG production after PPV immunization.

To the best of our knowledge, these data are the first evidence showing that Dectin-2 is involved in IgM and IgG production against pneumococcal capsular polysaccharides after PPV immunization. It should be noted that Ab production against pneumococcal polysaccharides was accompanied by synthesis of IL-12p40 and activation of splenic NKT cells and their production of IFN- $\gamma$  in a Dectin-2-dependent fashion. These observations have important implications for understanding the precise mechanism of PPV's effects and for further improvement in the clinical effectiveness of this vaccine. Further investigations are necessary to establish in greater detail the mechanism of NKT cell activation during the Ab response after vaccination.

## Supporting Information

**Figure S1 Production of PPS6B, 14, 19F and 23F-specific Ab.** WT and Dectin-2KO mice received intraperitoneal injections of 20  $\mu$ L PPV diluted in 200  $\mu$ L normal saline. Sera were collected on day 14 after PPV immunization, and concentrations of anti-PPS6B, 14, 19F and 23F IgM and IgG were measured as OD450 values at  $\times 90$  and  $\times 30$  dilution, respectively. Data are shown as the mean  $\pm$  SD of six mice. Similar results were obtained in three experiments. \*,  $p < 0.05$ ; NS, not significant. (TIF)

**Figure S2 Effect of spleen cell transfer on body weight and temperature after pneumococcal infection.** (A) Schematic diagram of the spleen cell transfer experiment. Spleen cells were prepared from three WT or Dectin-2KO mice on day 14 after PPV immunization, and the obtained cells were transferred at  $2 \times 10^7$ /mouse to six WT mice each group. One day later, the recipient WT mice were infected intratracheally with *S. pneumoniae* ( $1.6 \times 10^6$  CFU/mouse). (B) The body weight and temperature of each mouse were measured daily. Body weight is expressed as a relative value to that before infection. Solid line,



mice given spleen cells from PPV-immunized WT mice; dotted line, mice given spleen cells from PPV-immunized Dectin-2KO mice. Similar results were obtained in two experiments. (TIF)

**Figure S3 Dectin-2 is essential for PPV-induced TNF- $\alpha$  and IL-6 production by BM-DCs.** BM-DCs derived from WT or Dectin-2KO mice were cultured with indicated doses of PPV for 24 h. Concentrations of TNF- $\alpha$  (A) and IL-6 (B) in the culture supernatants were measured. LPS and mannan were used at 1  $\mu$ g/ml and 3 mg/ml, respectively, as controls. Data are shown as the

mean $\pm$ SD of triplicate cultures. Similar results were obtained in three experiments. \*,  $p < 0.05$ . Closed column, WT mice; Open column, Dectin-2KO mice. (TIF)

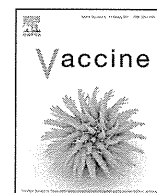
## Author Contributions

Conceived and designed the experiments: KK. Performed the experiments: TM YA MT NM. Analyzed the data: KK KI YK YM KO. Contributed reagents/materials/analysis tools: SS YI. Wrote the paper: KK.

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# Sustained functional serotype-specific antibody after primary and secondary vaccinations with a pneumococcal polysaccharide vaccine in elderly patients with chronic lung disease



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## ABSTRACT

An observational study was conducted to determine immunogenicity before and after primary and secondary vaccinations with 23-valent pneumococcal polysaccharide vaccine in a cohort of 40 elderly patients with chronic lung diseases. Safety of this vaccine was also compared between primary and secondary vaccination. We analyzed serotype-specific immunoglobulin G (IgG) and the opsonization index (OI) for serotypes 6B, 14, 19F, and 23F and compared adverse local and systemic reactions. The levels of serotype-specific IgG and the OIs significantly increased 1 month after primary and secondary vaccinations. Peak levels of IgG after secondary vaccination were 5–20% lower than those after primary vaccination, while serotype-specific OIs after secondary vaccination were comparable with those after primary vaccination. The levels of serotype-specific IgG required for 50% killing significantly decreased 1 month after vaccination. These values for serotypes 14, 19F, and 23F were slightly elevated immediately before secondary vaccination, but those for serotype 6B did not change. After secondary vaccination, these values declined slightly for serotypes 14, 19F, and 23F and remained low for serotype 6B. Although self-limited local and systemic reactions were more frequent after secondary vaccination compared with primary vaccination, no serious systemic reaction was found after either vaccination. Our data suggest a sustained functional serotype-specific IgG after primary and secondary vaccination and confirmed the safety of secondary vaccination among elderly individuals with chronic lung disease.

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## 1. Introduction

*Streptococcus pneumoniae* is a leading human pathogen that causes a variety of diseases, such as invasive pneumococcal disease (IPD) and non-bacteremic pneumonia, in children and adults. The rates of IPD are highest among children under 5 years of age and among adults who are older than 65 years of age [1–3]. Community-acquired pneumonia (CAP), which is most likely to be caused by *S. pneumoniae*, and the incidence of pneumococcal CAP is also high among the elderly [3–5].

The efficacy, immunogenicity, and safety of the 23-valent pneumococcal polysaccharide vaccine (PPV23; Pneumovax®, Merck Sharp & Dohme) has been extensively studied in adults [6,7]. Although *S. pneumoniae* is commonly responsible for 8–25% of

exacerbation in patients with chronic lung diseases such as chronic obstructive pulmonary disease, the immunogenicity studies of PPV23 in this population are scarce [8–12]. Consistent results from observational studies have demonstrated that PPV23 reduces the risk of IPD in immunocompetent older adults. Recent studies from Japan reported that PPV23 prevented pneumococcal pneumonia and reduced the death rate due to pneumococcal pneumonia among nursing home inhabitants in Japan and that PPV23 was effective for all-cause pneumonia for study subjects older than 75 years of age after routine immunization with the influenza vaccine [13,14]. As the percentage of the elderly population (aged 65 years and over) is 23.1% in Japan [15], the demand for receiving PPV23 revaccination 5 years or more after primary vaccination is increasing. The US Advisory Committee on Immunization Practices recommends a single revaccination for persons at increased risk, including adults ≥65-years old who had received their first vaccination ≥5 years previously and were less than 65 years old at the time of their first vaccination in 1997 [16]. Revaccination with

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PPV23 was contraindicated in Japan until October 2009 [17], but revaccination has been approved for adults who had received their first dose of PPV23 more than 5 years previously.

Jackson et al. reported that secondary vaccination with PPV23 induced local reactions more frequently than primary vaccination, but that these reactions were not serious and resolved within 3 days [18]. In addition, two recent studies from the United States demonstrated comparable functional antibody responses between primary and secondary vaccination in adults older than 65 years of age [19,20]. Hammitt et al. also demonstrated that repeat revaccination with PPV23, administered 6 years or more after the prior dose, was immunogenic and generally tolerated [21]. Furthermore, IgG concentrations were found to exceed vaccine-naïve levels for seven of eight serotypes tested 10 years after the first or second doses of PPV23 [22]. In this study, we examined the immunogenicity and safety of PPV23 in a cohort of patients with chronic lung disease (CLD) who were followed up through the time of primary to secondary vaccination at a single institution and report on the sustained and functional serotype-specific antibodies raised by primary and secondary vaccinations with PPV23.

## 2. Materials and methods

### 2.1. Study subjects

Between October 2001 and November 2002, 101 patients with CLD who were 65 years of age or older received primary vaccination with PPV23 at our outpatient clinic. Serum samples from these study subjects had been acquired before and 1 month after primary vaccination and had been preserved for antibody titer analyses [23]. Of 101 patients, 30 patients died and 31 patients were lost for follow-up at our outpatient clinic until September 2009. All patients provided written informed consent.

This study was reviewed and approved by the ethics committee of the National Hospital Organization, Tokyo National Hospital, and was conducted according to the principles expressed in the Declaration of Helsinki.

### 2.2. Samples

Blood samples were drawn from 40 study subjects before and 1 month after secondary vaccination with PPV23. Sera were separated by centrifugation and stored at  $-80^{\circ}\text{C}$  until used.

The levels of serotype-specific immunoglobulin G (IgG) and the opsonization index (OI) were measured in the serum samples obtained before and 1 month after primary vaccination and before and 1 month after secondary vaccination.

### 2.3. ELISA

Antipneumococcal IgG antibodies were measured with the World Health Organization (WHO)-approved ELISA methodology, using standard reference serum (89-SF or 007sp) and C-polysaccharide and 22F polysaccharide absorptions, as previously described [24,25]. The levels of serotype-specific IgG for four serotypes, 6B, 14, 19F, and 23F, were determined according to the WHO protocol (a detailed protocol is available at [www.vaccine.uab.edu/ELISAProtocol](http://www.vaccine.uab.edu/ELISAProtocol) [89SF]). These four serotypes are commonly found in adult patients with CAP in Japan [5].

### 2.4. Multiplexed opsonophagocytic killing assay

A multiplexed opsonophagocytic killing assay (MOPA) for the four serotypes, based on antibiotic-resistant target bacteria, was performed at the Research Institute for Microbial Diseases, Osaka

**Table 1**

Baseline characteristics of 40 patients with chronic pulmonary diseases.

| Characteristics                         | Values  |
|---|---------|
| Male sex: No. (%)                       | 18(45)  |
| Mean age: years of age (SD)             | 77(6.1) |
| 65–69 years of age: No. (%)             | 4(0.1)  |
| 70–79 years of age: No. (%)             | 22(55)  |
| $\geq 80$ years of age: No. (%)         | 14(35)  |
| Comorbid illness: No. (%)               |         |
| Sequela of pulmonary tuberculosis       | 13(33)  |
| Bronchiectasis                          | 7(18)   |
| Asthma                                  | 7(18)   |
| Nontuberculous mycobacterial infection  | 6(15)   |
| Aspergillosis                           | 3(8)    |
| Chronic obstructive pulmonary disease   | 3(8)    |
| Interstitial pneumonia                  | 1(3)    |
| Home oxygen therapy: No. (%)            | 15(38)  |
| Mean time to revaccination: months (SD) | 91(3.7) |

SD, standard deviation.

University, as previously described [26]. The quality control serum was prepared from the pooled sera of adults vaccinated with PPV23 and was used in each assay. The OI was defined as the serum dilution that killed 50% of bacteria, and the OI was determined using opsoTiter3 software according to the WHO protocol (a detailed protocol is available at [www.vaccine.uab.edu/UAB-MOPA](http://www.vaccine.uab.edu/UAB-MOPA)). Functional activity of serotype-specific IgG was expressed as the concentration of IgG required for 50% killing of the pneumococcal strain by dividing the IgG concentration of a test sample by the OI [27].

### 2.5. Adverse reactions

Subjects were provided a diary to record their body temperature and any local or systemic reactions that occurred from the day of secondary vaccination to day 14. They were instructed to assess the maximal diameter of any redness or swelling at the site of injection; this was expressed as mild for a maximum diameter of 1–5 cm, as moderate for a maximum diameter  $\geq 5$  cm, and as severe for a maximum diameter  $\geq 10$  cm. A systemic symptom was considered mild when the subjects felt a certain symptom but had no difficulty in daily life. A physical examination with an interview was conducted to record the condition of the study subject on the day of secondary vaccination and 14 days after. Data of adverse reactions after primary vaccination were used for comparison with those after secondary vaccination [23].

### 2.6. Statistical analysis

Average antibody concentrations and increases were expressed as geometric means. Differences in the geometric mean concentrations (GMCs) of serotype-specific IgG, the OIs, or the IgG required for 50% killing were assessed by the Wilcoxon matched-pairs signed-ranks test. The frequencies of adverse reactions were compared between primary and secondary vaccinations by the Student *t*-test. Differences with a *P* value  $< 0.05$  were considered to be statistically significant.

## 3. Results

The subject patient group comprised 18 males and 22 females, and all of them were Japanese (Table 1). Four subjects were in their 60s, 22 in their 70s, and 14 in their 80s; the mean age was 77 years. The mean interval between primary and secondary vaccinations was 7 years and 7 months. Their comorbid illnesses included sequelae of pulmonary tuberculosis (33%), bronchiectasis (18%), bronchial asthma (18%), nontuberculous mycobacterial infection

**Table 2**

Comparison of geometric mean concentrations (GMCs) and geometric increases (*n*-fold) in levels of serotype-specific IgG in sera and opsonization index (OI) of sera from 40 study subjects before and after primary and secondary vaccination.

| Serotype | Time point  | GMC of IgG (mg/ml) (95% CI)      | Geometric mean increase ( <i>n</i> -fold; post/pre) | GMT of OI (95% CI)           | Geometric mean increase ( <i>n</i> -fold; post/pre) |
|----------|-------------|----------------------------------|---|------------------------------|---|
| 6B       | Pre dose 1  | 1.29 (1.02–1.64)                 |   | 75 (44–129)                  |   |
|          | Post dose 1 | 2.99 (2.18–4.10) <sup>a</sup>    | 2.32  | 557 (327–949) <sup>a</sup>   | 7.43  |
|          | Pre dose 2  | 1.65 (1.20–2.28)                 |   | 267 (142–500)                |   |
|          | Post dose 2 | 2.73 (1.97–3.79) <sup>b</sup>    | 1.65  | 768 (489–1206) <sup>b</sup>  | 2.88  |
| 14       | Pre dose 1  | 3.11 (2.36–4.12)                 |   | 120 (67–216)                 |   |
|          | Post dose 1 | 8.75 (7.01–10.91) <sup>a</sup>   | 2.81  | 1028 (539–1958) <sup>a</sup> | 8.55  |
|          | Pre dose 2  | 4.31 (2.82–6.58) <sup>c</sup>    |   | 344 (182–648)                |   |
|          | Post dose 2 | 6.96 (4.70–10.32) <sup>b,e</sup> | 1.61  | 699 (384–1273) <sup>b</sup>  | 2.03  |
| 19F      | Pre dose 1  | 2.46 (2.05–2.94)                 |   | 34 (19–62)                   |   |
|          | Post dose 1 | 4.61 (3.81–5.59) <sup>a</sup>    | 1.87  | 538 (325–892) <sup>a</sup>   | 15.77   |
|          | Pre dose 2  | 2.81 (2.20–3.58) <sup>d</sup>    |   | 158 (91–275)                 |   |
|          | Post dose 2 | 4.38 (3.53–5.44) <sup>b</sup>    | 1.56  | 556 (349–887) <sup>b</sup>   | 3.51  |
| 23F      | Pre dose 1  | 1.31 (1.00–1.70)                 |   | 17 (10–27)                   |   |
|          | Post dose 1 | 3.10 (2.34–4.11) <sup>a</sup>    | 2.37  | 142 (74–271) <sup>a</sup>    | 8.57  |
|          | Pre dose 2  | 1.57 (1.12–2.20)                 |   | 47 (27–80)                   |   |
|          | Post dose 2 | 2.56 (1.75–3.75) <sup>b</sup>    | 1.63  | 179 (100–322) <sup>b</sup>   | 3.84  |

GMC, geometric mean concentration, GMT, geometric mean titer; CI, confidence interval; dose 1, primary vaccination; dose 2, secondary vaccination. Pre dose 1: before primary vaccination; post dose 1: after primary vaccination; pre dose 2: before secondary vaccination; post dose 2: after secondary vaccination. Sera were collected 1 month before and after primary and secondary vaccinations.

<sup>a</sup>  $P < 0.01$  (vs. pre dose 1).

<sup>b</sup>  $P < 0.01$  (vs. pre dose 2).

<sup>c</sup>  $P < 0.01$  (vs. pre dose 1).

<sup>d</sup>  $P < 0.05$  (vs. pre dose 1).

<sup>e</sup>  $P < 0.01$  (vs. post dose 1).

(15%), and others. Fifteen patients (38%) were on home oxygen therapy.

The GMCs of serotype-specific IgG for serotypes 14, 19F, 6B, or 23F of the study subjects were significantly elevated 1 month after primary vaccination ( $P < 0.01$ ) and remained slightly higher than that before primary vaccination, even at the time of prevaccination. The GMCs of serotype-specific IgG for serotypes 14 and 19F were significantly higher immediately before secondary vaccination compared with those before primary vaccination, whereas the GMCs of serotype-specific IgG for serotypes 6B and 23F before secondary vaccination did not significantly differ from those before primary vaccination. The GMCs of serotype-specific IgG for all serotypes significantly increased by secondary vaccination, but the GMCs of serotype-specific IgG for all serotypes 1 month after secondary vaccination were lower than those 1 month after primary vaccination. The proportions of the peak IgG levels 1 month after secondary vaccination compared with those 1 month after primary vaccination were 79.5–95.0% for all serotypes. The GMCs of serotype-specific OIs for all serotypes significantly increased 1 month after primary vaccination (Table 2). The GMTs of serotype-specific OIs 1 month after secondary vaccination were higher or comparable with those 1 month after primary vaccination for serotypes 6B, 19F, and 23F, but not for serotype 14. The GMTs of serotype-specific OIs for all serotypes decreased thereafter, but secondary vaccination significantly increased the GMTs of serotype-specific OIs at 1 month postvaccination. The GMCs of serotype-specific IgG required for 50% killing for all serotypes significantly decreased after primary vaccination ( $P < 0.01$ ; Fig. 1). The GMCs of serotype-specific IgG required for 50% killing was significantly elevated for serotype 19F but did not change for serotypes 6B, 14, and 23F during the interval between primary and secondary vaccinations. The GMCs of serotype-specific IgG required for 50% killing significantly decreased for serotypes 14, 19F, and 23F between before and after secondary vaccinations ( $P < 0.01$ ) but remained unchanged for serotype 6B.

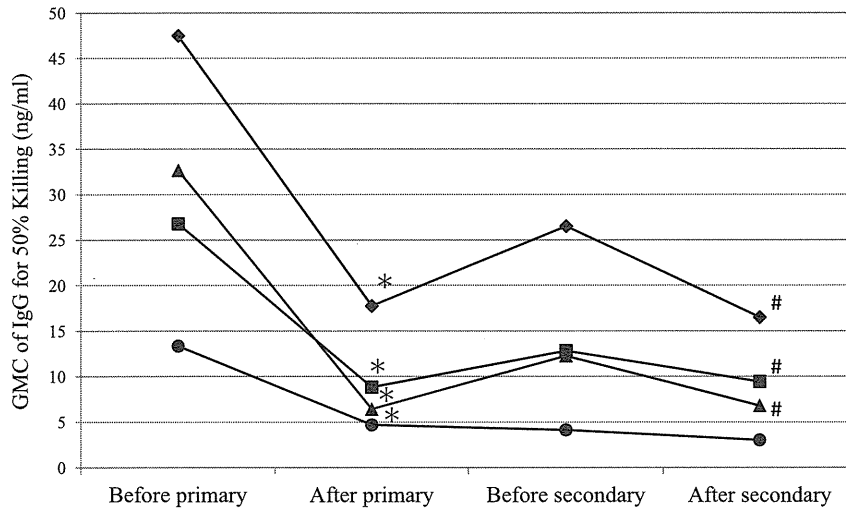
No serious adverse reaction, such as anaphylactic shock, was found after secondary vaccination. The frequencies of local

reactions, including local swelling, reddening, and pain, peaked 1 day after secondary vaccination and then gradually disappeared within 1 week (Table 3). Only one subject developed fever over 38 °C on the day of revaccination, while none of the other subjects developed fever. Several subjects developed systemic symptoms, including nausea, headache, muscle pain, joint pain, or malaise. These symptoms were found more frequently during the first 2 days after secondary vaccination and improved slowly thereafter. All adverse reactions disappeared within 2 weeks after secondary vaccination.

The frequency of local reactions, including pain and swelling or redness, was significantly higher in subjects after secondary vaccination than after primary vaccination (Fig. 2A). The frequency of systemic reactions, including headache, malaise, muscle pain, and joint pain, was also significantly higher in subjects after secondary vaccination than after primary vaccination (Fig. 2B).

#### 4. Discussion

In this study, we demonstrated immunogenicity during the period from before primary vaccination to 1 month after secondary vaccination in a cohort of elderly patients with CLD. Significant increases in the GMCs of serotype-specific IgG or serotype-specific OIs occurred after primary and secondary vaccinations. Peak levels of serotype-specific IgG after PPV23 secondary vaccination were only 5–20% lower than peak levels after primary vaccination, which is in agreement with previous studies [19,20,28]. This phenomenon of hyporesponsiveness induced by PPV23 was found to be associated with a depletion of the memory B-cell population [29]. In contrast, we found serotype-specific OIs after secondary vaccination to be comparable with those after primary vaccination. Therefore, hyporesponsiveness was not found in opsonic activity after secondary vaccination. The GMCs of serotype-specific IgG or the OIs after primary vaccination declined within several years, but these levels before secondary vaccination were still higher than those before primary vaccination. The values of OIs



**Fig. 1.** The GMCs of serotype-specific IgG required for 50% killing of bacteria for serotypes 6B (closed circle), 14 (closed square), 19F (closed triangle), and 23F (closed diamond) before and after primary and secondary vaccinations with 23-valent pneumococcal polysaccharide vaccine. The number of subjects examined for serotypes 6B, 14, 19F, and 23F were 30, 33, 23, and 21, respectively. \* $P < 0.05$  versus before primary vaccination; # $P > 0.05$  versus 1 month before secondary vaccination.

for serotypes 14 and 23F before and after primary and secondary vaccination were comparable to those reported in a previous paper [20].

A previous study reported that serotype-specific IgG 30 days after secondary vaccination was modestly lower than that after primary vaccination, but the difference was transient and was resolved within several years after secondary vaccination [19]. Levels of OI were similar after primary or secondary vaccination, although levels 30 days after secondary vaccination were insignificantly lower than those 30 days after primary vaccination [20]. A recent study also demonstrated that serotype-specific IgG levels for seven of eight serotypes tested during 10 years after secondary vaccination were higher than those after primary vaccination [22].

Although we determined the levels of serotype-specific IgG, but not IgM or IgA, the serum opsonic activity correlated best with levels of serotype-specific IgG in healthy adults after vaccination with PPV23 [30,31]. Significant decreases in IgG levels required for 50% killing for serotypes of 6B, 14, 19F, and 23F after primary vaccination indicated an improved efficiency of opsonic activity in sera. These values of IgG required for 50% killing slightly increased during the mean interval more than 7 years, except for serotype 6B. However, these values for all serotypes were still lower than those before primary vaccination, suggesting a sustained functional serotype-specific IgG after primary vaccination. The values of IgG

required for 50% killing in serotype 14, 19F, and 23F further declined after secondary vaccination. These findings suggest an improved efficiency of opsonic activity of serotype-specific IgG for these three serotypes after secondary vaccination. Our results may provide new insights for protective immunity more than five years after primary vaccination and after secondary vaccinations in the elderly population.

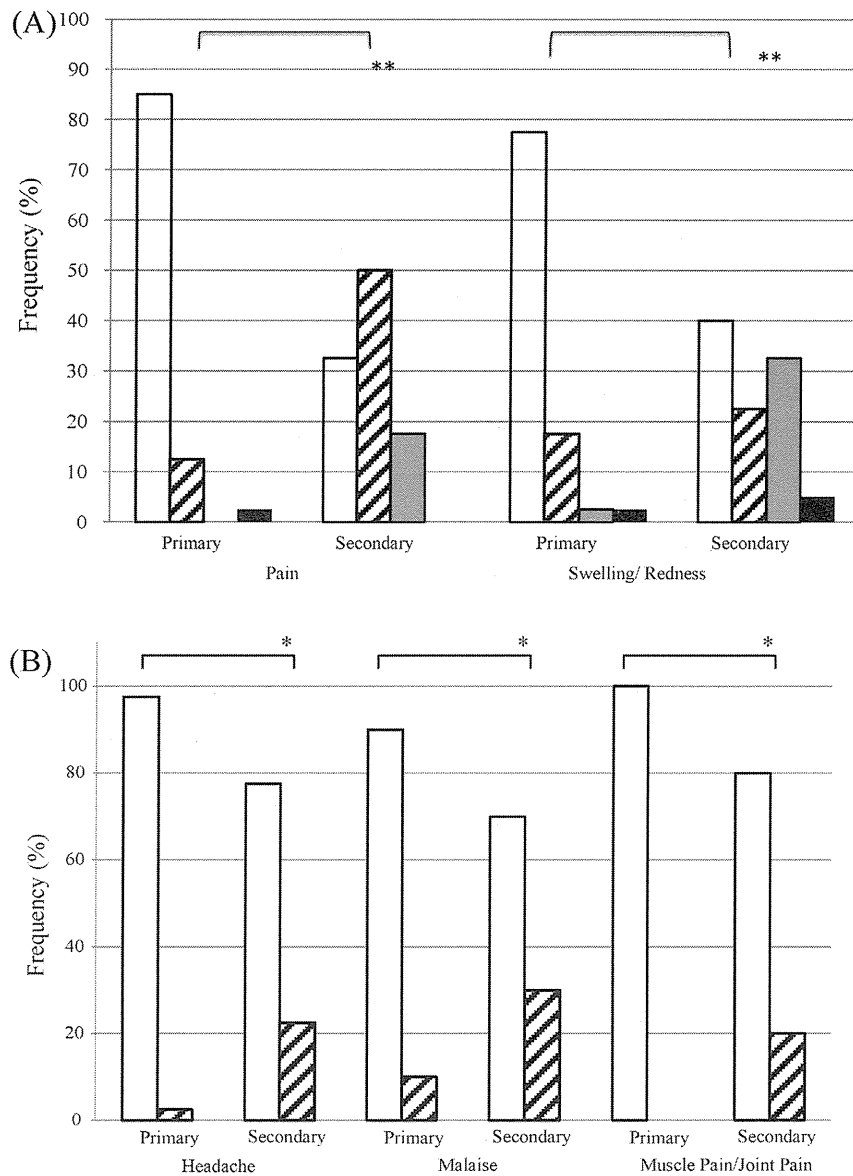
Among the previous studies of immunogenicity by PPV23 in patients with chronic lung diseases [8–12], we could directly compare the levels of serotype-specific IgG in the present study with those of our previous study in 13 hospitals, Japan [12], because the WHO approved ELISA [24,25] was used to determine the IgG levels for serotypes 6B, 14, 19F, and 23F in our previous study [12] and in the present study. The IgG levels in our previous study before primary vaccination with PPV23 were slightly higher for all serotypes than those after primary vaccination in the present study. Because of the higher prevaccination IgG level, the fold increases for all serotypes 1 month after primary vaccination in previous our study were lower than those in the present study.

It remains uncertain whether the immune response in adult patients with chronic lung diseases is inferior to that in healthy adults. We previously reported that 31% of 84 patients with chronic lung diseases were low responders to PPV23 [12]. Rubins et al. also reported that 20% of elderly patients with chronic illnesses

**Table 3**

Proportion of local and systemic adverse reactions after secondary vaccination among 40 subjects.

|          | Adverse reactions   | Day 0              | Day 1    | Day 2    | Day 3  | Day 4   | Day 5  | Day 6  |
|----------|---|--------------------|----------|----------|--------|---------|--------|--------|
|          |   | No. of subject (%) |          |          |        |         |        |        |
| Local    | Maximal diameter of redness or swelling at the injection site |                    |          |          |        |         |        |        |
|          | <5 cm   | 9(23)              | 11(8)    | 6(15)    | 6(15)  | 8(20)   | 5(13)  | 3(7.5) |
|          | ≥5 cm   | 7(17.5)            | 11(27.5) | 8(20)    | 6(15)  | 3(8)    | 1(3)   | 2(5)   |
|          | ≥10 cm  | 0(0)               | 1(2.5)   | 2(5)     | 0(0)   | 0(0)    | 0(0)   | 0(0)   |
|          | Mild soreness in arm  | 13(33)             | 23(58)   | 15(37.5) | 10(25) | 6(15)   | 1(2.5) | 2(5)   |
|          | Moderate soreness in arm                                      | 4(10)              | 4(10)    | 0(0)     | 0(0)   | 0(0)    | 0(0)   | 0(0)   |
|          | Severe soreness in arm  | 0(0)               | 0(0)     | 0(0)     | 0(0)   | 0(0)    | 0(0)   | 0(0)   |
| Systemic | Individual symptom  |                    |          |          |        |         |        |        |
|          | Nausea  | 1(3)               | 1(3)     | 1(3)     | 1(3)   | 1(3)    | 1(3)   | 3(8)   |
|          | Headache  | 3(8)               | 5(12.5)  | 1(2.5)   | 3(7.5) | 6(15)   | 4(10)  | 2(5)   |
|          | Myalgia   | 4(10)              | 5(12.5)  | 2(5)     | 1(2.5) | 1(2.5)  | 2(5)   | 3(8)   |
|          | Arthralgia  | 0(0)               | 0(0)     | 0(0)     | 2(5)   | 2(5)    | 1(3)   | 1(3)   |
|          | Fatigue   | 6(15)              | 9(23)    | 5(13)    | 7(18)  | 7(17.5) | 3(8)   | 4(10)  |
|          | Body temperature ≥38 °C                                       | 1(2.5)             | 0(0)     | 0(0)     | 0(0)   | 0(0)    | 0(0)   | 0(0)   |



**Fig. 2.** Comparison of adverse reactions between primary and secondary vaccinations among the study subjects. Local adverse reactions (A) and systemic symptoms (B) were compared between primary and secondary vaccinations. Data are shown as the frequencies of subjects who received primary and secondary vaccinations ( $n=40$ ). The grade of finding or symptom was expressed as none (open bar), mild (slashed bar), moderate (gray bar), or severe (closed bar).  $**P<0.01$ ,  $*P<0.05$  versus primary vaccination.

including chronic lung diseases were poor responders, while none of the healthy young adults were poor responder [32]. Therefore, the great heterogeneity of the study subjects as shown in Table 1 might influence on the results of immunogenicity in this study.

Although we found self-limited local and systemic reactions to be more frequent after secondary vaccination than after primary vaccination, no serious systemic reaction was found after secondary vaccination. These findings are in agreement with two previously published reports [18,22].

The limitations of this study include (1) a small number of study subjects for comparative investigation of safety and immunogenicity between primary and secondary vaccination; (2) only four serotypes examined for the serotype-specific IgG and OIs; and (3) the antibody levels were examined only 1 month after secondary vaccination. In addition, it should be noted that the increased levels of opsonic activity after primary or secondary vaccination found in this study are not directly associated with protection of patients with chronic lung disease from IPD or non-bacteremic

pneumococcal pneumonia, since the protective levels of OIs against such pneumococcal infections in adults remain unknown.

In conclusion, we demonstrated significant increases of GMCs of serotype-specific IgG and OIs after primary and secondary vaccinations with PPV23. The GMCs of serotype-specific IgG and OIs several years after primary vaccination were still higher than those before primary vaccination. Either primary or secondary vaccination conferred a long-term sustained and functional serotype-specific IgG. Our study also confirmed the safety of secondary vaccination with PPV23.

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## Original article

# Nationwide survey of rotavirus-associated encephalopathy and sudden unexpected death in Japan

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## Abstract

**Background:** Rotavirus can cause severe complications such as encephalopathy/encephalitis and sudden unexpected death. The incidence of rotavirus-associated encephalopathy/encephalitis or sudden unexpected death remains unknown. To clarify the clinical features of rotavirus-associated encephalitis/encephalopathy and sudden unexpected death, we conducted a nationwide survey in Japan. **Method:** A two-part questionnaire was designed to determine the number of the cases and the clinical features of severe cases of rotavirus infection, including encephalitis/encephalopathy and sudden unexpected death, between 2009 and 2011. **Result:** Of the 1365 questionnaires sent to hospitals, 963 (70.5%) were returned and eligible for analysis. We determined 58 cases of rotavirus-associated encephalitis/encephalopathy and 7 cases of sudden unexpected death. These patients were diagnosed with rotavirus infection by immunochromatography. Although 36/58 (62.1%) encephalitis/encephalopathy patients had no sequelae, 15/58 (25.9%) patients had neurological sequelae, and 7/58 (12.1%) patients had fatal outcomes. Pleocytosis was observed in 9/40 (22.5%) patients and cerebrospinal fluid protein levels were elevated in only 4/40 (10%) patients. Elevated lactate dehydrogenase (LDH) (>500 IU/L) or acidemia (pH < 7.15) were related to a poor prognosis. **Conclusion:** We estimate that annual cases of rotavirus-associated encephalitis/encephalopathy and sudden unexpected death were 44.0 and 4.9 cases in Japan, respectively. Elevated LDH (>500 IU/L) or acidemia (pH < 7.15) were related to a poor prognosis of the encephalitis/encephalopathy.

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**Keywords:** Rotavirus; Encephalopathy; Sudden unexpected death; Neurological sequelae

## 1. Introduction

Rotavirus is a common cause of gastroenteritis in infants and children. Severe dehydration caused by rotavirus-induced diarrhea and vomiting can be fatal in developing countries. Although gastroenteritis induced

by rotavirus infection causes a large economic burden in developed countries, it is generally a self-limiting benign infectious disease in these countries [1]. However, rotavirus can cause severe complications such as encephalopathy/encephalitis [2–4], myocarditis [5,6], and sudden unexpected death [7]. Initially, rotavirus replication was thought to be limited to the gastrointestinal tract in patients with gastroenteritis. Recent studies have shown that rotavirus infection causes antigenemia [8–10] and RNAemia [11,12], which suggests that viral spreading beyond the gastrointestinal tract contributes to sys-

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temic infection. Moreover, the detection of not only structural proteins but also nonstructural proteins of rotavirus in several tissues, including myocardium and brain, obtained from fatal case of rotavirus infection was indicative of viral replication in these tissues [13]. These data strongly suggest that rotavirus is associated with severe extra-intestinal complications.

The incidence of rotavirus-associated encephalopathy/encephalitis or sudden unexpected death remains unknown. One epidemiological analysis has demonstrated a fatality rate of 1 death per 1616 rotavirus-associated hospitalizations (0.06%) in the United States [14]. Similarly, Swedish surveillance demonstrated a fatality rate of 1 death per 984 children with rotavirus gastroenteritis (0.1%) [15]. A recent nationwide survey in Japan determined that rotavirus was the third most common pathogen associated with acute encephalopathy/encephalitis in Japanese children [16]. Unfortunately, detailed clinical features as well as the incidence of rotavirus encephalitis/encephalopathy and sudden unexpected death remain unclear. Herein, we performed a nationwide survey to clarify the incidence and clinical features of rotavirus-associated encephalitis/encephalopathy and sudden unexpected death in Japan.

## 2. Materials and methods

### 2.1. Study design for questionnaire

A two-part questionnaire was utilized to determine the number of the cases and the clinical features for severe cases of rotavirus infection, including encephalitis/encephalopathy or sudden unexpected death, seen over the two-year period between September 1st 2009 and August 31st 2011 that is prior to rotavirus vaccine introduction in Japan. The first part of the questionnaire included 5 questions. The first question asked whether the hospitals with pediatric wards had hospitalized cases of rotavirus encephalitis/encephalopathy or sudden unexpected death, because all patients with severe complications are expected to be admitted for intensive treatments. Rotavirus-associated encephalitis/encephalopathy was defined as rotavirus gastroenteritis patients with stupor and/or convulsions. In order to exclude the rotavirus patients with the severe form of febrile convulsions, patients with convulsions lacking stupor required the abnormal neuroimaging findings for definition of the rotavirus-associated encephalitis/encephalopathy. If an institute had a case of rotavirus-associated encephalitis, we included an additional 4 questions to determine: (1) the age of the patient, (2) the sex of the patient, (3) the presence or absence of virological examinations for the detection of rotavirus (immunochromatography), and (4) the prognosis of the patient. Meanwhile, the patients with cardiopulmonary arrest at the time of hospital admission without any specific abnormal find-

ings on the physical examinations and the past history to suggest underlying diseases were classified as sudden unexpected deaths. The criterion for entry into the study was confirmation of rotavirus infection using a rapid test for the detection of rotavirus antigen in a stool specimen.

The second part of the questionnaire was sent to the institutes that reported rotavirus-associated encephalitis/encephalopathy or sudden unexpected death cases. It focused on clinical features such as: (1) the results of hematological examinations including white blood cell count (WBC) (/ $\mu$ l), hemoglobin (Hb) (g/dl), platelet count (Plt) (/ $\mu$ l), aspartate aminotransferase (AST) (IU/L), alanine aminotransferase (ALT) (IU/L), lactic dehydrogenase (LDH) (IU/L), blood urea nitrogen (BUN) (mg/dl), creatinine (Cr) (mg/dl), blood sugar (BS) (mg/dL), sodium (Na) (mEq/L), pH, partial pressure of carbon dioxide in arterial blood (PaCO<sub>2</sub>) (mmHg), bicarbonate ion (HCO<sub>3</sub><sup>-</sup>) (mEq/L), and base excess (BE), prothrombin time (PT) (%), fibrinogen (Fib) (mg/dl), fibrin/fibrinogen degradation products (FDP) ( $\mu$ g/ml); (2) cerebrospinal fluid (CSF) findings including cell numbers (/ $\mu$ l) and protein level (mg/dl); (3) radiological findings including computed tomography (CT) and magnetic resonance imaging (MRI); and (4) details of the prognosis including the type of sequelae.

In November 2011, the first questionnaire was mailed to the chiefs of the pediatric departments at the 1365 hospitals with pediatric wards located throughout Japan. In December 2011, the second part of questionnaire was sent to the 53 hospitals that reported rotavirus-associated encephalitis/encephalopathy (53 hospitals) or sudden unexpected death (7 hospitals) cases on the first questionnaire.

### 2.2. Data analysis for the prediction of the rotavirus-associated encephalopathy/encephalitis prognosis

In order to elucidate the predictive factors for the poor prognosis of patients with encephalitis/encephalopathy, an association between patient prognosis and neuroimaging analysis or laboratory examinations was examined. Each factor was compared between the patients with poor prognosis, such as neurological sequelae and fatal outcome, and the patients without sequelae. An association between abnormal findings of neuroimaging analysis and patient prognosis was tested using a Chi-square test. The presence or absence of abnormal findings on the neuroimaging studies was determined by local neuro-radiologists. Meanwhile, an association between laboratory examinations (WBC, Hb, Plt, AST, ALT, LDH, BUN, Cr, pH, PaCO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup>, BE, PT, Fib, FDP, cell counts in CSF, and protein levels in CSF) and patient prognosis was examined using a Mann–Whitney *U* test. A receiver operating characteristic (ROC) analysis was used to identify

optimal cut-off values for the prognostic factors that had significant differences ( $P < 0.05$ ) between the patients with poor prognosis and without sequelae; the corresponding maximized Youden index (sensitivity plus specificity – 1) was determined. The selected prognostic factors were refined based on these cut-off values using a backward elimination method and ROC analysis in a logistic regression [17]. The statistical analysis was performed using JMP<sup>®</sup> 7 (SAS Institute Inc., Cary, NC, USA).

### 3. Results

#### 3.1. Results of the first questionnaire

The questionnaires were sent to 1365 hospitals and 963 (70.5%) questionnaires were completed. Fifty-eight cases (32 male and 26 female) of encephalitis/encephalopathy and 7 cases (5 male and 2 female) of sudden unexpected death were reported by 53 (5.5%) hospitals; neither encephalitis/encephalopathy nor sudden unexpected death patients were reported by the remaining 910 hospitals (94.5%). The median age of the encephalitis/encephalopathy patients was 2.1 years (range: 0–12 years) and that of sudden unexpected death patients was 1.0 year (range: 0–4 years). Thirty-six of the fifty-eight (62.1%) patients had no sequelae and 15/58 (25.9%) patients had neurological sequelae. Additionally, remaining 7 (12.1%) cases demonstrated fatal outcome cases. Details of the neurological sequelae in the encephalitis/encephalopathy patients were examined in the second questionnaire. No postmortem analysis was carried out in the sudden unexpected death cases.

#### 3.2. Results of second questionnaire

The second questionnaire was used to collect detailed information on 45 of the 58 encephalitis/encephalopathy patients; however, no answer was returned from the hospitals that reported the sudden unexpected death cases. Therefore, the following analyses were performed on the data collected from the 45 patients with rotavirus-associated encephalitis/encephalopathy.

To determine whether the type of dehydration is associated with complication of encephalitis/encephalopathy, serum Na levels were examined in the patients. Interestingly, the serum Na levels varied in these patients, with 18 (40.0%) patients having normal Na level, 8 (17.8%) patients being hypernatremia ( $>145$  mEq/L), and 19 (42.2%) patients having hyponatremia ( $<135$  mEq/L). The CSF data were available in 40 patients. Pleocytosis ( $>10$  cells/ $\mu$ l) was observed in 9/40 (22.5%) patients and the cell count was normal in the remaining 31 (77.5%) patients. An elevation of the CSF protein level (20 mg/dl or more) was observed in 4/40 (10%) patients and no increase was observed in the remaining 36 (90%)

patients. All patients received a brain CT or brain MRI; 34 patients received a brain CT and 34 patients received a brain MRI. The radiological findings are summarized in Table 1. Brain edema was the only abnormal CT finding and it was observed in 9/34 (26.5%) patients. Abnormal MRI findings were observed in 22/34 (64.7%) patients, including 17 patients with hyper-intensity on a diffusion-weighted image.

As described in previously, neurological sequelae were observed in 15/58 (25.9%) patients in the first questionnaire. As shown in Table 2, several cases with severe neurological sequelae were reported in the second questionnaire, including 3 patients with paralysis, 2 patients with psychomotor retardation, 1 patient with epilepsy and psychomotor retardation, 1 patient with ataxia and psychomotor retardation, 1 patient with dysmnnesia and 1 patient with brain death. Seven fatal cases (12.1%) were reported in the 58 encephalitis/encephalopathy patients.

#### 3.3. Identification of predictive factors for the prognosis of encephalitis/encephalopathy patients

As shown in Table 3, an association between 23 clinical factors and neurological sequelae including 7 cases with fatal outcome was analyzed based on the data collected in the second questionnaire ( $n = 45$ ). Although no gender difference was observed between patients with and without neurological sequelae, patients with neurological sequelae were significantly younger than those without the sequelae ( $P = 0.019$ ). Numbers of the subjects were slightly different among the variables due to the different number of the eligible data returned from each hospital. No statistical difference was observed between the patients with and without sequelae for the 12 variables including Hb, Plt, Cr, BS, Na, PT, Fib, FDP, PaCO<sub>2</sub>, CSF cell counts, brain CT findings, and brain MRI findings. In contrast, levels of the 6 variables including WBC ( $P = 0.032$ ), BUN ( $P = 0.044$ ), protein levels in CSF ( $P = 0.011$ ), AST ( $P = 0.003$ ), ALT ( $P = 0.036$ ), and LDH ( $P = 0.007$ ) were significantly higher in patients with poor prognosis than those without sequelae. Moreover, pH ( $P = 0.002$ ), HCO<sub>3</sub><sup>-</sup> ( $P = 0.018$ ), and BE ( $P = 0.011$ ) were significantly lower in the patients with poor prognosis than those without sequelae, which was indicative of higher metabolic acidosis in the patients with poor prognosis. ROC analysis was carried out to elucidate appropriate cutoff values for the 9 variables that were found to be statistically different between the groups (Fig. 1). The cut-off value for the 9 variables were: WBC, 11,000/ $\mu$ l; BUN, 20 mg/dl; CSF protein level, 20 mg/dl; AST, 100 IU/L; ALT, 60 IU/L; LDH, 500 IU/L; pH, 7.15; HCO<sub>3</sub><sup>-</sup>, 10 mEq/L; and BE, –14. In order to identify the optimal prognostic factors from these 9 variables, a backward elimination method in logistic regression was carried out. Based on this

Table 1  
Summary of CT and MRI findings for RV-associated encephalitis/encephalopathy patients.

| Examination | Abnormal findings               | Lesions                         | Number of patients |
|-------------|---------------------------------|---------------------------------|--------------------|
| CT          | Parenchymal swelling            | Diffuse                         | 9                  |
| MRI         | High intensity on DWI           | Splenium of corpus callosum     | 9                  |
|             |                                 | Diffuse subcortex               | 3                  |
|             |                                 | Occipital–temporal white matter | 3                  |
|             |                                 | Frontal subcortex               | 2                  |
|             | High intensity on T2WI or FLAIR | Diffuse white matter area       | 2                  |
|             |                                 | Frontal–temporal cortex         | 1                  |
|             |                                 | Thalamus                        | 1                  |
|             | Parenchymal swelling            | Diffuse                         | 1                  |

Abbreviations: RV, rotavirus; CT, computed tomography; MRI, magnetic resonance imaging; DWI, diffusion-weighted image; T2WI, T2-weighted image; FLAIR, Fluid attenuated inversion recovery.

Table 2  
Summary of neurological sequelae for RV-associated encephalitis/encephalopathy patients.

| Neurological sequelae                | Number of patients |
|--------------------------------------|--------------------|
| Paralysis                            | 3                  |
| Psychomotor retardation              | 2                  |
| Epilepsy and psychomotor retardation | 1                  |
| Ataxia and psychomotor retardation   | 1                  |
| Dysmnnesia                           | 1                  |
| Brain death                          | 1                  |
| Unknown                              | 6                  |

analysis, LDH (>500 IU/L) and pH (<7.15) were selected. The sensitivity and specificity of these criteria for the prediction of poor prognosis were 0.80 and 0.85, respectively (Fig. 2).

#### 4. Discussion

During two-year observation period, 58 encephalitis/encephalopathy cases and 7 sudden unexpected death cases were reported from the 70.5% of the subjected hospitals. Therefore, if we assume that the response from

Table 3  
Clinical factors in RV-associated encephalitis/encephalopathy patients.

| Clinical factors                      | RV-associated encephalitis/encephalopathy patients |  | P-value |
|---------------------------------------|--|--|---------|
|                                       | Without sequelae ( <i>n</i> <sup>*</sup> )         | With sequelae or fatal ( <i>n</i> <sup>*</sup> ) |         |
| Age (years old)                       | 2.1 (1.2–11.6) (29)                                | 1.6 (0.8–5.3) (16)                               | 0.019   |
| Sex (male/female)                     | 17/12  | 7/9  | 0.369   |
| <i>Blood findings</i>                 |  |  |         |
| WBC (/μl)                             | 9550 (4720–30480) (29)                             | 15220 (3100–37900) (16)                          | 0.032   |
| Hb (g/dl)                             | 12.7 (8.4–15.7) (29)                               | 12.4 (6.8–15.7) (16)                             | 0.165   |
| Plt ( $\times 10^4/\mu\text{l}$ )     | 26.8 (65.3–13.9) (29)                              | 24.5 (0.8–113.1) (16)                            | 0.343   |
| AST (U/L)                             | 45 (21–148) (29)                                   | 116 (26–1112) (16)                               | 0.003   |
| ALT (U/L)                             | 24 (12–83) (29)                                    | 65 (9–582) (16)                                  | 0.037   |
| LDH (U/L)                             | 312 (203–532) (29)                                 | 541 (218–1689) (16)                              | 0.007   |
| BUN (mg/dl)                           | 14.3 (7.5–42.0) (29)                               | 23.8 (7.5–68.1) (16)                             | 0.044   |
| Cr (mg/dl)                            | 0.3 (0.8–0.2) (29)                                 | 0.4 (0.2–2.7) (16)                               | 0.196   |
| BS (mg/dl)                            | 99 (10–182) (27)                                   | 71 (8–456) (16)                                  | 0.187   |
| Na (mEq/L)                            | 135 (122–143) (28)                                 | 137 (122–159) (15)                               | 0.080   |
| pH                                    | 7.37 (7.02–7.49) (22)                              | 7.13 (6.84–7.422) (14)                           | 0.002   |
| PaCO <sub>2</sub> (mmHg)              | 35.1 (17.5–59.6) (22)                              | 29.5 (6.9–128.0) (14)                            | 0.948   |
| HCO <sub>3</sub> <sup>-</sup> (mEq/L) | 18.9 (8.7–28.1) (23)                               | 14.1 (4.9–20.5) (13)                             | 0.018   |
| BE                                    | -5.1 (-18.5–4.0) (22)                              | -15.8 (-27.6–4.8) (14)                           | 0.011   |
| PT (%)                                | 77 (15–100) (15)                                   | 56 (13–116) (14)                                 | 0.070   |
| Fib (mg/dl)                           | 233 (117–459) (12)                                 | 237 (107–303) (15)                               | 0.903   |
| FDP (μg/ml)                           | 3.6 (1.3–6.3) (8)                                  | 5.0 (0.9–40.8) (14)                              | 0.278   |
| <i>CSF findings</i>                   |  |  |         |
| CSF cell counts (/μl)                 | 2 (0–77) (27)                                      | 3 (0–226) (13)                                   | 0.531   |
| Protein levels in CSF (mg/dl)         | 19 (10–42) (28)                                    | 27 (10–151) (12)                                 | 0.011   |
| <i>Neuro-image findings</i>           |  |  |         |
| Brain CT findings (normal/abnormal)   | 14/5   | 11/4   | 0.640   |
| Brain MRI findings (normal/abnormal)  | 7/16   | 4/7  | 0.771   |

Each value indicates median (range).

<sup>\*</sup> *n*; Numbers of subjects.