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A possible relationship of natural killer T cells with humoral immune response to 23-valent pneumococcal polysaccharide vaccine in clinical settings

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ABSTRACT

Pneumococcal polysaccharide vaccine (PPV), a type-2 thymus-independent antigen, induces the activation of B cells by directly triggering their antigen receptors. Although this type of antigen generally does not undergo class switching from IgM to IgG, PPV has been known to induce IgG2 in vaccinated subjects, which suggests the possible involvement of certain innate immune lymphocytes supporting the activation of B cells and their class switching. In the present study, we addressed the possibility that natural killer (NK) T cells are involved in Ab production caused by PPV. We measured serum levels of IgG against pneumococcal capsular polysaccharides and the numbers of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ double negative (DN) invariant NKT (iNKT) cells and $CD3^+CD56^+$ NKT cells in the peripheral blood before and after PPV injection. IgG was increased after PPV injection, peaking at 4 weeks after injection in serotypes 6B, 19F and 23F and at 3 months in serotype 14. Low responders, whose serum concentrations of IgG peaked at less than double their original levels, constituted 16%, 13%, 13% and 16% of vaccinated subjects with regard to serotypes 6B, 14, 19F and 23F, respectively. A significant positive correlation was detected between an increase in DN iNKT cells and the elevation of anti-serotype 14 IgG; in serotype 19F, DN iNKT cells were more markedly increased in responders than in low responders. These results suggest that DN iNKT cells may be involved in IgG production caused by vaccination against pneumococcal capsular polysaccharides.

1. Introduction

Streptococcus pneumoniae is a major bacterial agent which causes community-acquired pneumonia as well as other invasive diseases, such as bacteremia and meningitis, which arise as complications of pneumonia in 15–30% of cases [1]. The incidence rate of pneumococcal bacteremia is 18 to 30 per 100,000 in the general population, but can be as high as 56 to 83 per 100,000, especially in people aged 65 years or over in the USA [2–5]. In Japan, pneumonia is the fourth leading cause of death, and *S. pneumoniae* is a leading causative agent of pneumonia, being detected in 23% of community-acquired pneumonia cases [6]. This bacterium is also frequently detected as an etiologic agent in secondary pneumonia arising as a complication of the flu [7–9]. Morens and co-workers have demonstrated that the majority of deaths in the 1918–1919 influenza pandemic resulted directly from secondary bacterial pneumonia caused by common upper respiratory-tract bacteria, among which *S. pneumoniae* was most frequently detected in autopsy lung samples [10].

To prevent these pneumococcal diseases, 23-valent pneumococcal polysaccharide vaccine (PPV) is used for people aged 65 years or older and younger people with certain risk factors such as chronic cardiopulmonary diseases [11]. PPV is a type 2 thymus-independent (TI-2) antigen, which does not require helper T cells for the activation of B cells [12]. While thymus-dependent (TD) antigens activate B cells *via* engagement of CD40 by CD40L

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during cognate interaction with helper T cells, TI-2 antigens directly trigger surface immunoglobulin for the activation of B cells [13]. These different types of antigen produce distinct humoral immune responses: TD-antigens undergo class switching from IgM to IgG, which causes affinity maturation of Ab and induces memory B cell response; TI-2 antigens, on the other hand, do not [14]. Although PPV is a TI-2 antigen, Barrett and Ayoub [15] have found that it induces the restriction of production of IgG2 specific for pneumococcal polysaccharides. Snapper and co-workers [16] have reported that interferon (IFN)-γ contributes to Ab class switching to IgG3 in mice, which corresponds to IgG2 in humans, after PPV administration [15]. These findings suggest that a certain group of innate immune cells may be involved in the activation of B cells and Ab class switching caused by PPV.

Natural killer (NK) T cells, which express both αβ 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 [17]. A major subset of NKT cells is the invariant NKT (iNKT) cells, which possess an extremely limited repertoire with antigen receptors consisting of Vα14-Jα18 in mice and Vα24-Jα18 in humans [18]. These cells recognize glycolipid antigens, such as α-galactosylceramide (α-GalCer), in the context of CD1d molecules on dendritic cells [19], which leads to the rapid production of IFN-γ and IL-4 [20,21]. iNKT cells are concentrated in the thymus, liver and bone marrow in mice [20,22] and occur at a rate of approximately 0.05% in human peripheral blood [23]. In our previous studies using a mouse model [24], iNKT cells were observed to play a critical role in neutrophilic inflammatory responses to and host defense against pneumococcal infection through production of IFN-γ. Interestingly, Kobrynski and co-workers have demonstrated that Ab production after PPV injection was completely abrogated in mice lacking iNKT cells [25]. These earlier observations raised the possibility that iNKT cells may contribute to Ab production and class switching caused by the administration of PPV.

In the present study, to address this possibility in a clinical setting, we analyzed the relationship between serum concentrations of Ab against pneumococcal capsular polysaccharides and the number of $CD4^+$, $CD8^+$ or $CD4^-CD8^-$ double negative (DN) iNKT cells in the peripheral blood of subjects who received PPV administration. We found that DN iNKT cell counts increased, and that this increase was positively correlated with the production of IgG against a certain serotype of *S. pneumoniae*.

2. Materials and methods

2.1. Subjects

Fifty-five outpatients with chronic respiratory diseases were vaccinated with 0.5 ml of PPV (Pneumovax®, Banyu Pharmaceutical Co., Tokyo, Japan), intramuscularly, at the Department of Respiratory Diseases, Katta General Hospital, Shiroishi-shi, Miyagi, Japan between July 2006 and August 2008 after giving informed consent. The PPV23 contained 25 μg each of 23 different types of pneumococcal polysaccharide antigen (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F). Serum samples were collected prior to vaccination and at 2 weeks, 4 weeks, 3 months, 6 months and 1 year after vaccination. The average age of these subjects was 74.4 years (range 61–88 years); 67.3% of subjects were male, 43.6% were smokers, and 10.9% were receiving glucocorticoid therapy. The most common chronic respiratory diseases among these patients were chronic obstructive pulmonary disease, bronchial asthma, bronchiectasis and old pulmonary tuberculosis (Table 1). This study was approved by the institutional ethics committees of Tohoku University, Sendai, Japan (#2005-233) and Katta General Hospital. We also paid the utmost attention to ensure that personal information was handled in compliance with our institutions' guidelines.

Table 1
Clinical characteristics of all subjects ($n = 55$).

	Number (%)
Males	37 (67.3)
Smoking	24 (43.6)
Alcohol abuse	1 (1.8)
Underlying diseases	
COPD	20 (36.4)
Bronchial asthma	13 (23.6)
Bronchiectasis	2 (3.6)
Old pulmonary tuberculosis	6 (10.9)
Chronic cardiovascular diseases	5 (9.1)
Immunosuppressive conditions	1 (1.8)
Chronic renal failure	1 (1.8)
Chronic liver diseases	1 (1.8)
Diabetes mellitus	8 (14.5)
Treatment with glucocorticoids	6 (10.9)
Home oxygen therapy	6 (10.9)

Mean age (yr) ± SD = 74.4 ± 6.6.

2.2. Measurement of anti-pneumococcal capsular polysaccharide Ab

Serotype-specific antibodies against 6B, 14, 19F and 23F (American Type Culture Collection, Manassas, VA, USA) were measured by means of a third-generation Enzyme-Linked Immunosorbent Assay (ELISA) as described previously [26] after absorption of non-specific antigens to cell wall polysaccharide (CWP: Statens Serum Institute, Copenhagen, Denmark) and serotype 22F (American Type Culture Collection). In brief, microtiter plates (MICROLON: Greiner Bio-One, Frickenhausen, Germany) were coated individually with 100 μl of a polysaccharide antigen: either 5 μg/ml of 6B, 2.5 μg/ml of 14, 5 μg/ml of 19F or 2.5 μg/ml of 23F, in PBS. After five hours of incubation at 37 °C, these plates were stored at 4 °C until use, which occurred within 6 months. Prior to testing, the sera from our patients and U.S. anti-pneumococcal reference serum [89-SF: kindly provided by Dr. Milan S. Blake (Food and Drug Administration, Silver Spring, MD, USA)] were also stored at −80 °C. Serum samples and 89-SF were diluted with an absorption buffer of 0.05% Tween-20 PBS to 1:50 and 1:100, respectively, and incubated at room temperature for 30 min. Next, serial two-fold dilution of these sera to 1:51200 were performed arbitrarily; the resulting solutions were added to the wells and incubated at 37 °C for 1 h. After the microtiter plates were washed, a detection antibody, consisting of AP-conjugated goat anti-human IgM or IgG (Southern Biotechnology Associates, Birmingham, AL, USA) diluted to 1:2000, was added to each well. *p*-nitro phenyl phosphate (Sigma–Aldrich, St. Louis, MO, USA) was dissolved with 1 mol/l of diethanolamin (Sigma–Aldrich) to a concentration of 1 mg/ml as a substrate solution. Then, after the plates were washed again, this substrate was added to the wells and incubated at room temperature. Sodium hydroxide was added at 3 M to stop the enzyme reaction, and the absorbance values were detected at 405 nm as well as at 600 nm for reference. The concentrations of IgM and IgG Abs were calculated on the basis of a reference standard based on the 89-SF absorbance and expressed as μg/ml.

2.3. Flow cytometric analysis of PBMCs

Peripheral blood mononuclear cells (PBMCs) were collected from patients before vaccination and at 2 weeks, 4 weeks, 3 months and 6 months after vaccination. After Fc receptors on the cell surface were blocked, PBMCs were stained with FITC-anti CD3 [Clone: UCHT1 (eBioscience, San Diego, CA, USA)] and PE-anti CD56 [Clone:

B159 (BD Biosciences, Franklin Lakes, NJ, USA)] mAbs and PE-α-galactosylceramide (α-GalCer)-conjugated CD1d tetramer. These cells were also stained with APC-anti-CD4 and -CD8 mAbs [Clones: RPA-T4 and RPA-T8 (eBioscience), respectively]. Isotype control IgG (eBioscience) for each Ab and PE-α-GalCer-unconjugated CD1d tetramer were used as references. Flow cytometric analysis was performed using a Cytomics FC500 cytometry system (Beckman Coulter, Fullerton, CA, USA). The number of NKT cells (/μl) was calculated as follows: white blood cell (WBC) counts (100/μl) × % of lymphocytes in WBC/100 × % of NKT cells in lymphocytes. The WBC counts and % of lymphocytes were measured in blood samples collected from the patients during routine examinations.

2.4. Statistical analysis

Ab concentrations in sera, fold increases after vaccination and number of NKT cells in peripheral blood are expressed as geometric means. The concentrations of serum Ab and degrees of change in NKT cell counts during the first 2 weeks after vaccination were compared between responders and low responders using the Mann–Whitney *U*-test. The concentrations of serum Ab between pre- and peak levels were compared using Wilcoxon *t*-test. The correlation between the degree of change from pre-vaccination to peak levels of anti-pneumococcal IgG and the degree of change in NKT cell counts during the first 2 weeks post-vaccination was tested using Spearman's correlation test. A p value less than 0.05 was considered significant.

3. Results

3.1. Serum levels of anti-pneumococcal Ab after vaccination

Initially, we measured the concentrations of IgM anti-pneumococcal Ab against serotypes 6B, 14, 19F and 23F in 15 subjects at various time intervals after pneumococcal vaccination. As shown in Fig. 1A, the pre-vaccination levels of IgM Ab were 0.91, 0.59, 1.04 and 0.26 μg/ml for serotypes 6B, 14, 19F and 23F, respectively, and these levels were not altered during the six months post-vaccination.

Next, we measured the concentrations of IgG anti-pneumococcal Ab against the same serotypes in 55 subjects. As shown in Fig. 1B, in contrast to IgM Ab, IgG Ab began to increase during the second week, reached its peak at the fourth week for serotypes 6B, 19F and 23F and at the third month for serotype 14, then decreased one year after vaccination. For all the serotypes, the peak values were significantly higher than the values measured before vaccination (1.60 *vs.* 4.53, 3.04 *vs.* 12.87, 2.98 *vs.* 7.73 and 1.69 *vs.* 6.32 μg/ml for serotypes 6B, 14, 19F and 23F, respectively). One year post-vaccination, IgG levels had decreased from the peak levels by 12.3%, 37.8%, 25.2% and 41.5% for serotypes 6B, 14, 19F and 23F, respectively.

3.2. Responders and low responders

The individuals who received PPV administration were divided into two groups based on their responsiveness, *i.e.* responders and low responders. Here, we defined responders as individuals whose peak IgG levels were more than twice their IgG levels before vaccination, and low responders as individuals whose serum IgG concentrations were less than 2 μg/ml before vaccination and whose peak IgG levels were less than twice their IgG levels before vaccination. By these definitions, 62%, 62%, 45%, and 65% of the 55 vaccinated individuals were responders, and 16%, 13%, 13%, and 16% were low responders, with regard to the serotypes 6B, 14, 19F and 23F, respectively. As shown in Table 2, for all serotypes, peak IgG levels were significantly higher than IgG levels measured before

Table 2
Serotype-specific antibody levels in responders and low responders.

Serotype	Time point	Geometric mean concentrations (μg/ml) (95% CI)			Geometric mean increase from pre-vaccination to peak concentration (*n*-fold) (range)		
		All subjects ($n=55$)	Responders[a]	Low responders[b]	All subjects ($n=55$)	Responders[a]	Low responders[b]
6B	Pre	1.60 (1.20–2.14)	1.08 (0.76–1.52)	1.33 (1.10–1.60)			
	Peak	4.53 (3.44–5.95)**	4.56 (3.04–6.84)**	2.02 (1.57–2.59)*#	2.83 (0.89–78.89)	4.24 (2.00–78.89)	1.52 (0.11–1.92)
14	Pre	3.04 (1.96–4.70)	2.20 (1.38–3.50)	0.74 (0.35–1.60)			
	Peak	12.87 (8.46–19.59)**	21.14 (13.88–32.19)**	1.11 (0.52–2.37)$	4.24 (0.68–120.18)	9.61 (2.11–120.18)	1.49 (0.86–1.93)
19F	Pre	2.98 (2.20–4.03)	2.04 (1.41–2.95)	1.06 (0.86–1.30)			
	Peak	7.73 (5.70–10.49)**	11.23 (7.55–16.72)**	1.51 (1.18–1.93)$	2.60 (0.81–49.48)	5.51 (2.03–49.48)	1.43 (1.09–1.90)
23F	Pre	1.69 (1.18–2.43)	1.43 (0.95–2.15)	0.61 (0.35–1.06)			
	Peak	6.32 (4.18–9.55)**	8.96 (5.44–14.76)**	0.83 (0.49–1.40)$	3.73 (0.97–60.62)	6.25 (2.03–60.62)	1.36 (0.97–1.67)

*$p<0.05$, **$p<0.01$, compared with pre-vaccination level; #$p<0.05$, $p<0.01$, compared with peak level in responders.
[a] Responders are 34, 33, 26 and 36 subjects for serotypes 6B, 14, 19F and 23F, respectively.
[b] Low responders are 9, 8, 10 and 9 for serotypes 6B, 14, 19F and 23F, respectively.

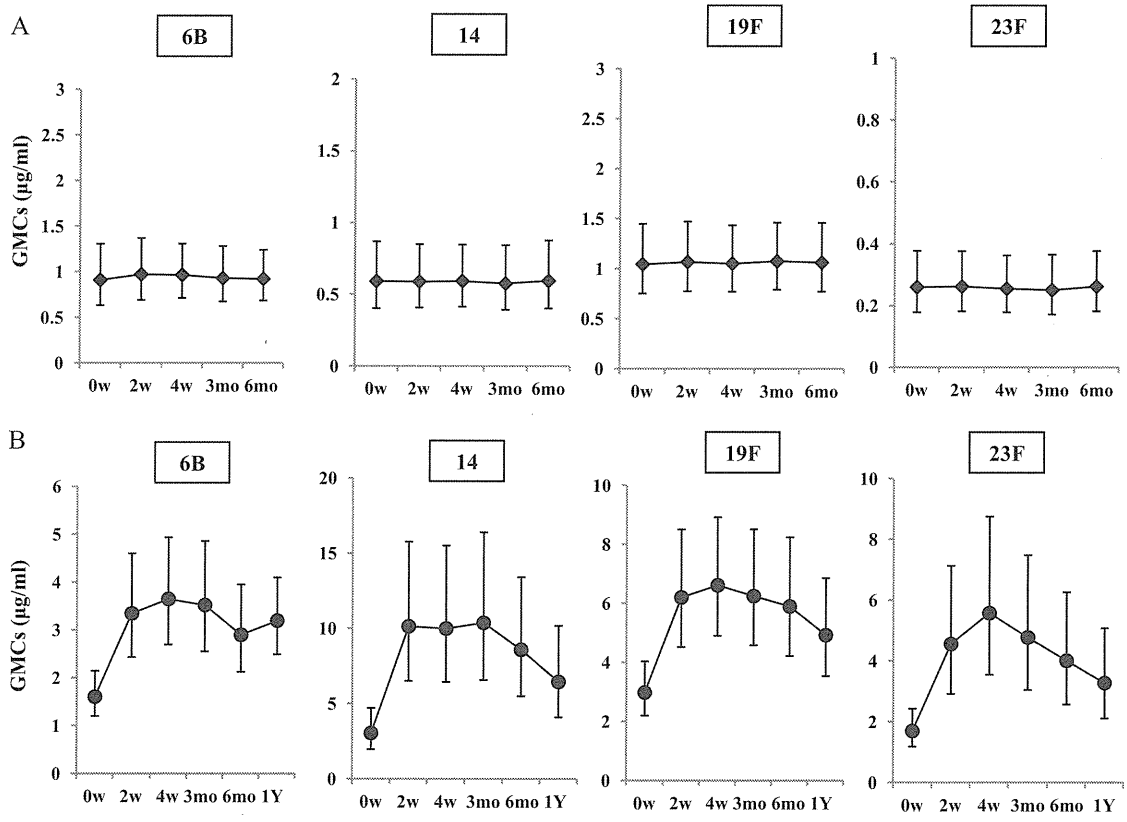

Fig. 1. Serum levels of anti-pneumococcal polysaccharide Abs after PPV injection. Concentrations of IgM (A: $n = 15$) and IgG (B: $n = 55$) Abs against each serotype of pneumococcal capsular polysaccharide in sera were measured at indicated time points after PPV administration. Data are shown as the geometric mean concentrations and 95% confidence intervals. GMCs, geometric mean concentrations; 0w, pre-vaccination; 2w, 2 weeks; 4w, 4 weeks; 3 mo, 3 months; 6 mo, 6 months; 1y, 1 year post-vaccination.

vaccination in the responder group, whereas no such significant increase in IgG concentration was observed in the low responder group, except for serotype 6B [pre-vaccination: 1.33 (95% CI was within 1.10–1.60) *vs.* peak: 2.02 (95% CI was within 1.57–2.59) ($n = 9$, $p < 0.05$)].

3.3. Alteration in the number of NKT cells in the peripheral blood after pneumococcal vaccination

We analyzed the number of NKT cells in the peripheral blood before vaccination and 2 weeks, 4 weeks, 3 months and 6 months after vaccination in 24 individuals, in whom the surface antigens on lymphocytes could be tested. NKT cells were identified as the lymphocytes positively stained with α-GalCer-CD1d tetramer or expressing both CD3 and CD56, and α-GalCer-CD1d tetramer$^+$ lymphocytes were further divided into $CD4^+CD8^-$ ($CD4^+$ iNKT), $CD4^-CD8^+$ ($CD8^+$ iNKT) and $CD4^-CD8^-$ (double negative: DN iNKT) subsets. As shown in Fig. 2, iNKT cell subsets did not show significant elevation in their cell count at any time point after vaccination, although increased iNKT cell counts were observed during the first two weeks in 11 or 12 individuals (data not shown).

3.4. NKT cell counts and serum levels of anti-pneumococcal Ab

In order to address the possible role of NKT cells in the humoral response to the pneumococcal vaccine, we analyzed the relationship between the degree of change in NKT cell counts during the first 2 weeks post-vaccination and the degree of change in serum anti-pneumococcal IgG levels from pre-vaccination to their peak. As shown in Fig. 3, a significant positive correlation was detected between increases in DN iNKT cells and increases in anti-serotype 14 IgG, and there were tendencies toward positive correlations between changes in $CD8^+$ iNKT and DN iNKT cell counts and increases in anti-serotype 19F IgG levels ($p = 0.069$ and 0.067, respectively), and between changes in DN iNKT cell counts and increases in anti-serotype 6B and 23F IgG levels ($p = 0.062$ and 0.082, respectively). By contrast, $CD4^+$ iNKT, $CD8^+$ iNKT and $CD3^+CD56^+$ cells showed neither a positive nor a negative correlation with changes in the serum levels of anti-pneumococcal IgG in all of the serotypes except for 19F in $CD8^+$ iNKT and $CD3^+CD56^+$ cells.

Finally, we compared changes in DN iNKT cell counts between responders and low responders, because these cells showed a tendency toward a positive correlation with Ab responses to PPV. As shown in Fig. 4, in serotype 19F, the increase in DN iNKT cells was significantly more marked in responders than in low responders. This tendency was also observed in serotypes 6B, 14 and 23F, although it was not statistically significant.

4. Discussion

In the present study, serum levels of anti-pneumococcal IgG increased after pneumococcal vaccination, peaking in the fourth week for serotypes 6B, 19F and 23F and in the third month for serotype 14; in 45–65% of vaccinated subjects, these levels increased more than two-fold. There were also low responders, however, producing smaller quantities of anti-pneumococcal Ab; these constituted 16%, 13%, 13% and 16% of our 55 subjects for serotypes 6B, 14, 19F and 23F, respectively. Of the low responders, 15 showed a low response to one of the four serotypes examined, nine showed a low response to two serotypes, and one showed a low response to three serotypes, indicating that 45% of our 55 subjects were low responders for at least one serotype. Although there is no standardized definite on of a low responder, our results appear

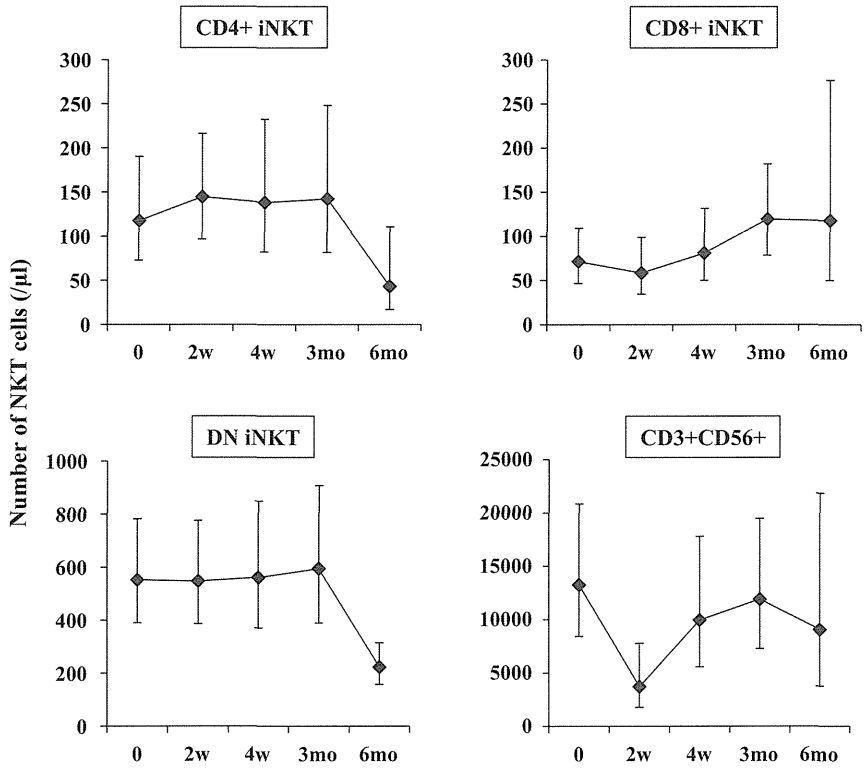

Fig. 2. NKT cells in the peripheral blood after PPV injection. Number of NKT cells in the peripheral blood was examined before PPV administration and 2 weeks, 4 weeks, 3 months and 6 months after PPV administration in 24 individuals. NKT cells were identified as the lymphocytes positively stained with α-GalCer-CD1d tetramer or expressing both CD3 and CD56, and α-GalCer-CD1d tetramer$^+$ lymphocytes were further divided into $CD4^+CD8^-$ ($CD4^+$ iNKT), $CD4^-CD8^+$ ($CD8^+$ iNKT) and $CD4^-CD8^-$ (double negative: DN iNKT) subsets. Data are shown as the geometric means and 95% confidence intervals in each NKT cell subset.

to be in accordance with those of previous investigations, which indicate that 16–31% of vaccinated subjects are low responders, whose anti-pneumococcal Ab levels increase less than two-fold for two among four to seven analyzed serotypes [27–29].

Previous studies have shown NKT cells to be involved in immune responses to TI-2 antigens, as a possible source of the secondary stimulatory signal for B cell activation [25] as well as in protection against pneumococcal infection [24]. These earlier observations suggest that NKT cells may play a certain role in the clinical effects of anti-pneumococcal vaccination. In agreement with this possibility, in the present study, a significant positive correlation was detected between changes in the number of DN iNKT cells, though not of $CD4^+$ iNKT cells, and increases in Ab levels against serotype 14 antigen. Moreover, the increase in DN iNKT cells was more marked in responders than in low responders, and this difference was statistically significant for serotype 19F. However, the positive correlation between DN iNKT cells and Ab levels and the difference in DN iNKT cells between responders and low responders were not significantly detected in other serotypes, although there were such tendencies with lower p values. The increase of study subjects would help in making these differences statistically significant. In addition, there is a possibility that the increase of DN iNKT cell number in responders may be due to overall immune activation of these individuals in response to vaccine, rather than selective effect on NKT cells. This may not apply to our case, because there was no tendency of difference between low responders and responders in other NKT cell subsets (data not shown).

$CD4^+$ and DN iNKT cells are major subsets in humans, both of which secrete large amounts of IFN-γ upon stimulation [21]. Yet these subsets differ in their secretion of such Th2 cytokines as IL-4, IL-5 and IL-13, and in their expression of chemokine receptors, integrins and NK receptors [21,30–32]. Galli and co-workers have demonstrated that iNKT cells promote immunoglobulin production by B cells, an activity that is more potent in $CD4^+$ iNKT cells than in DN iNKT cells [33]. The same group has also reported that activated human iNKT cells directly support the proliferation of and immunoglobulin production by naive and memory B cells. All these experiments were conducted *in vitro*, however, and frequent stimulation of iNKT cells during culture has been reported to cause a shift in their cytokine profile toward a Th2-dominant condition [34], raising the possibility that cultured NKT cells are not always equivalent to those in circulation *in vivo*. In the present clinical study of individuals receiving PPV, the relationship between iNKT cells and Ab production does not seem to be identical between $CD4^+$ and DN iNKT cells. Taken together, the data suggest that these subsets play distinct roles in Ab production by B cells after PPV administration. Further investigation is necessary to define the precise mechanism by which this occurs.

On the other hand, only a limited subset of NKT cells expressing NK cell markers, such as CD56 or CD161, is reactive to α-GalCer-loaded CD1d tetramer [31]. Therefore, $CD3^+CD56^+$ NKT cells, described as NKT-like cells, are distinguished from iNKT cells by certain characteristics, including the differences in their cytokine production profiles and their TCR αβ chains [18]. Our results suggest that iNKT cells rather than NKT-like cells may be particularly involved in IgG production caused by pneumococcal capsular polysaccharides, because no correlation was observed between $CD3^+CD56^+$ NKT cell count and Ab response.

To the best of our knowledge, the current study is the first report presenting clinical data that suggests a possible relationship between the activation of iNKT cells and Ab responses after PPV administration. The increase in DN iNKT cell count seems to be particularly correlated with serotype-specific IgG production, suggesting a higher contribution from DN iNKT cells than from other subsets. The population size in this study was limited, and the

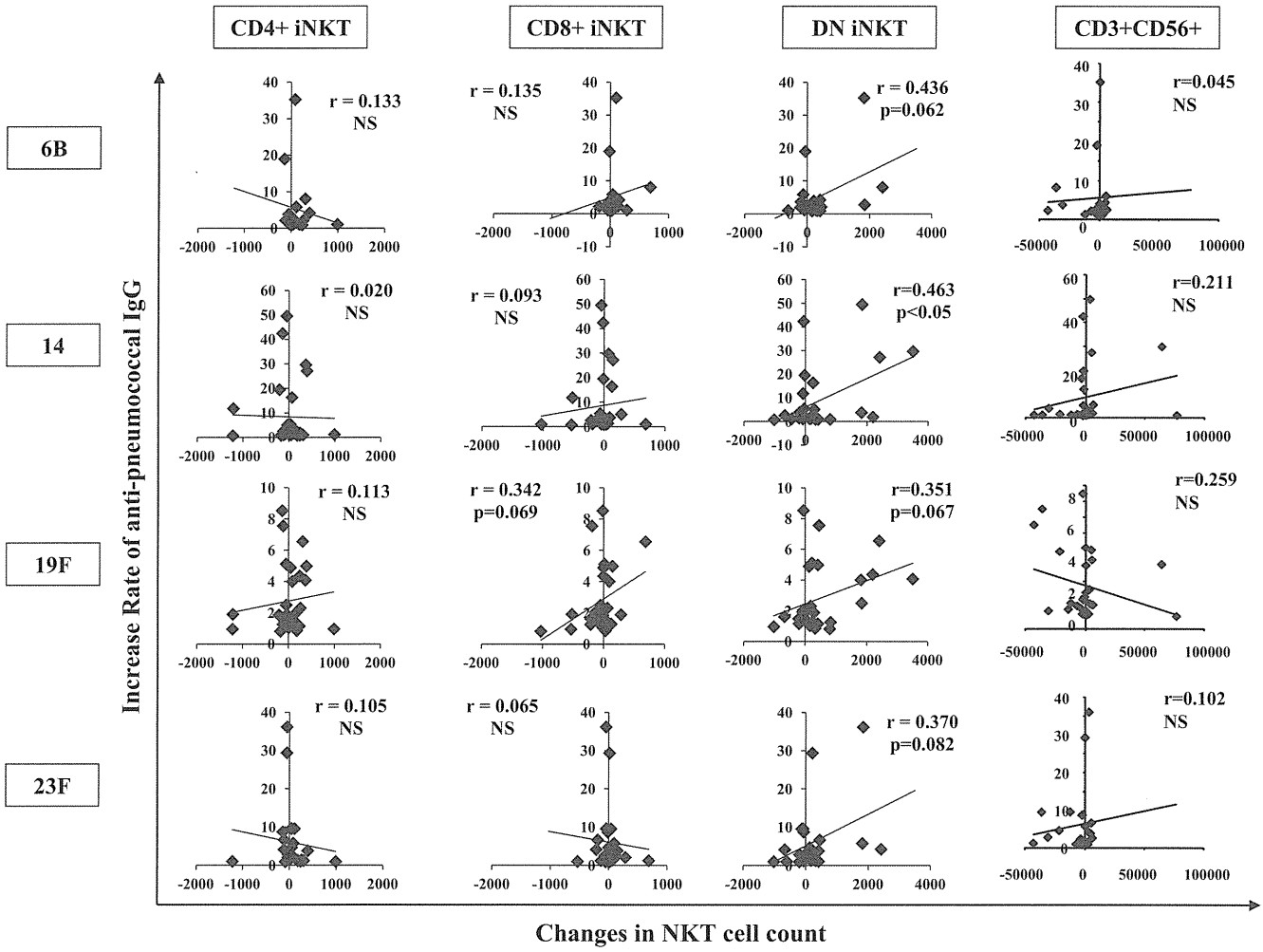

Fig. 3. Relationship between NKT cell counts and anti-pneumococcal IgG. Relationship between changes in NKT cell counts during the first 2 weeks post-vaccination and degree of change in serum anti-pneumococcal IgG levels from pre-vaccination to peak. Each symbol indicates the relationship for one subject. *R* and *P* values and number of subjects in each analysis are shown.

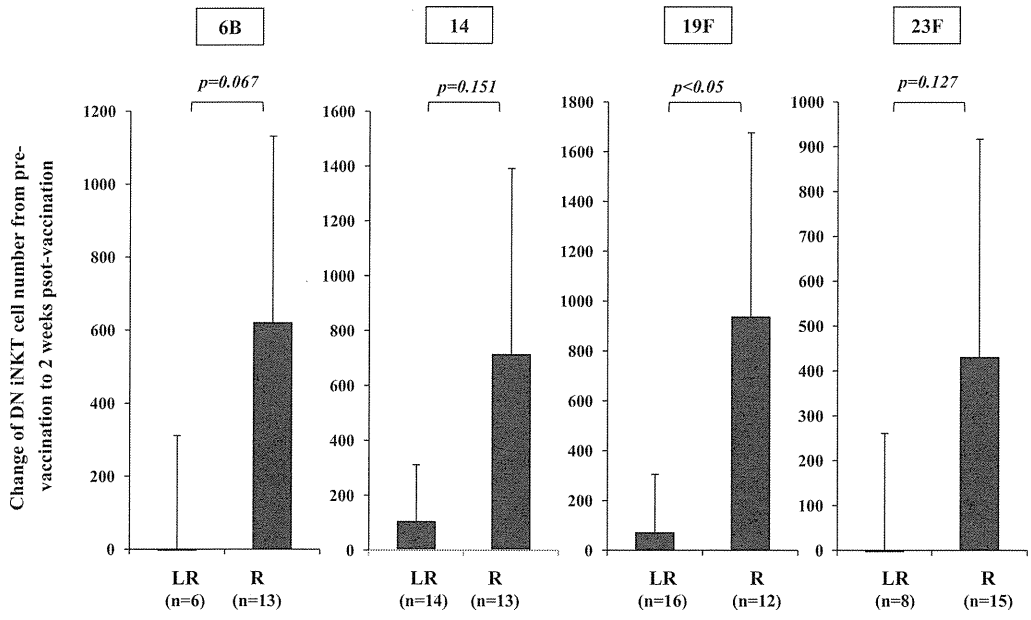

Fig. 4. Changes in DN iNKT cell counts in responders and low responders. Degree of change in DN iNKT cell count during the first 2 weeks after vaccination was compared between responders and low responders for each serotype. Data are expressed as the arithmetic means and 95% confidence intervals of indicated number of subjects. LR, low responders; R, responders.

enrolled subjects were aged (74.4 ± 6.6 years) and had underlying diseases that affected their immune condition. In these respects, there are some limitations in interpreting the results. At present, it remains to be elucidated how iNKT cells are involved in humoral immune responses to pneumococcal capsular polysaccharides in the clinical setting, but further investigations are already under way in our laboratory to define the precise mechanism underlying the relationship between iNKT cells and Ab responses.

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Low opsonic activity to the infecting serotype in pediatric patients with invasive pneumococcal disease

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ABSTRACT

Serotype-specific protective immunity in pediatric patients with invasive pneumococcal disease (IPD) has not been fully investigated. To determine the protective immunity to the infecting serotype, the serotype-specific immunoglobulin G (IgG) levels and opsonization indices (OIs) were examined in 24 Japanese pediatric patients whose serum was collected within one month of an IPD episode between May 2008 and June 2011. The median age (range) of IPD patients was 17 (10–108) months and 63% were boys. In all 17 patients tested, the levels of serotype-specific IgG to the infecting serotype were higher than 0.2 μg/ml, but the OIs to the infecting serotype were <8. The avidities of 19F- or 6B-specific IgG in patients with levels higher than 5.0 μg/ml, but with undetectable OIs, were confirmed to be lower than those in patients with high OIs. Our data demonstrated that although the levels of serotype-specific IgG to the infecting serotype were higher than 0.2 μg/ml in sera of pediatric patients with IPD, the OIs were low one month after the IPD episode. Low opsonic activities in these patients may, in part, be explained by the low avidity of serotype-specific IgG.

1. Introduction

Streptococcus pneumoniae is a leading human pathogen that causes a wide variety of diseases, ranging from otitis media to pneumonia, bacteremia, and meningitis in both children and adults [1]. Antibodies to pneumococcal capsular polysaccharide (CPS) and complement provide protection against pneumococcal strains with homologous or cross-reactive capsular serotypes [2]. Seven-valent pneumococcal conjugate vaccine (PCV7; Prevnar®, Pfizer) has been used for children in the USA since 2000 [3], and the incidence of invasive pneumococcal disease (IPD) caused by the seven vaccine serotypes (VTs) has declined markedly, although the incidence of non-VT infection has not declined [4–6]. A recent study reported that the incidence rate of IPD in children less than 5 years old was 12.6–13.8 per 100,000 in Chiba prefecture, Japan, before the introduction of PCV7 [7]. However, no information is available regarding a possible high-risk population for IPD in Japan, as was reported for Navajo children in the United States [8].

PCV7 was licensed in Japan in October 2009, and a 3 + 1 schedule (three doses for the primary series and one booster) was approved and implemented (http://idsc.nih.go.jp/vaccine/dschedule.html). Further, the Japanese government decided in November 2010 to subsidize PCV7 for children below 5 years of age.

Vaccine-induced protective immunity is currently estimated by measuring the concentrations of serotype-specific immunoglobulin G (IgG) using enzyme-linked immunosorbent assay (ELISA) [9] and the opsonization index (OI) using a multiplex-opsonophagocytic assay (MOPA) [10]. The World Health Organization working group suggested a serotype-specific IgG of

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[1] See Appendix A.

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concentration of 0.35 μg/ml as a putative measure of protection at a population level against invasive disease in infants after immunization with pneumococcal conjugate vaccine [11]. This working group also reported that antibody concentrations of 0.2–0.35 μg/ml measured with the ELISA using serum without serum absorption with 22F polysaccharide correlated best with an OI of 8, which in turn correlates best with protective efficacy. Henckaerts et al. proposed a protective threshold concentration of 0.20 μg/ml assessed with ELISA using serum absorption with 22F polysaccharide as a measure of the serotype-specific IPD efficacy for the pneumococcal conjugate vaccine [12], with the exception of serotype 19F [13]. A recent study also reported that the serological response rate following a three-dose PCV7 primary vaccination as determined using a threshold of ≥0.2 μg/ml IgG and an OI ≥ 8 corresponded well with overall effectiveness against IPD [14]. Although this threshold may not be necessarily applicable to individual patients, it is of interest to determine the protective immunity to the infecting serotype in sera collected during the acute phase in pediatric patients with IPD.

In this study, we therefore examined the IgG levels and OIs to the infecting serotype in sera of pediatric patients within one month of an IPD infectious episode. We report that the opsonic activity to the infecting serotype is low in sera obtained within one month of an episode of IPD.

2. Materials and methods

2.1. Patients

Thirty-two pediatric patients, whose cultures from sterile sites, such as blood or cerebrospinal fluid, were positive for *S. pneumoniae* between May 2008 and January 2012 at 22 hospitals in Japan, were investigated in this study. All patients were enrolled in this study when their attending doctors requested the measurement of the antipneumococcal antibodies in their sera. Sera were obtained from these 32 patients after the episode of IPD. All of the pneumococcal isolates were serotyped using coagglutination tests with rabbit antisera (Statens Serum Institute, Copenhagen, Denmark) at the Department of Bacteriology I, National Institute of Infectious Diseases. Serotype 6C was confirmed by an in-house factor antiserum [15]. All eight patients were excluded from our studies of the protective immunity to the infecting serotype: six patients for whom sera were collected more than one month after the onset of the IPD, one patient who received intravenous immunoglobulin as a treatment of IPD, and one patient with an underlying hypogammaglobulinemia. Consequently, we evaluated antipneumococcal IgG and the OIs to the infecting serotype in 24 pediatric patients with IPD. This study was reviewed and approved by the Ethics Committee of the RIMD, Osaka University, and conducted according to the principles expressed in the Declaration of Helsinki.

2.2. ELISA

Antipneumococcal IgG antibodies were measured with the WHO approved ELISA using a standard reference serum (89-SF) and C-polysaccharide and 22F polysaccharide absorptions as previously described [9,16]. The levels of serotype-specific IgG for the infecting serotypes including 6B, 9V, 14, 19F and 23F were determined according to the WHO protocol [a detailed protocol is available at www.vaccine.uab.edu/ELISAProtocol (89SF)].

2.3. MOPA

A multiplexed opsonophagocytic killing assay (MOPA) for the infecting serotype based on antibiotic-resistant target bacteria was performed at the Research Institute for Microbial Diseases, Osaka University, as previously described [10]. The quality control serum was prepared from pooled sera of adults vaccinated with the 23-valent pneumococcal polysaccharide vaccine (PPV23; Pneumovax®, MSD), and this was used in each assay. The OI was defined as the serum dilution that killed 50% of bacteria, and the OIs were determined using opsotiter3 software according to the WHO protocol (at www.vaccine.uab.edu/UAB-MOPA). Only the OI results for the infecting serotypes including 6B, 6C, 14, 19A, 19F and 23F were used in this study.

2.4. Measurement of protective immunity

Neither the serotype-specific IgG nor the OI was available in one patient with serotype 15B and another with serotype 24F infection. Only the OI was available in three patients with serotype 19A and two patients with serotype 6C infection. The OIs were not determined in another five patients because their sera contained antibiotics. Consequently, the level of serotype-specific IgG or OI to the infecting serotype was measured in 17 patients, and both the levels of serotype-specific IgG and OIs were measured in only 14 patients.

2.5. Avidity of serotype-specific IgG

The avidity of the serotype-specific IgG in sera was evaluated using ELISA by the previously published method with a minor modification [17]. Serum samples that had been preadsorbed C-polysaccharide and 22F CPS were added to the coated microtiter plates, and the plates were incubated for 1 h at 37 °C. After washing the plates, sodium thiocyanate (NaSCN) at concentrations from 0 to 1.0 M was added to each well and the plates were incubated for 15 min at room temperature. After washing of the plates, diluted goat anti-human IgG HRP-conjugate was added to each well. After incubation for 1 h at room temperature, the substrate solution was added to the plates, followed by incubation for 20 min at room temperature. The optical density at 405 nm was measured. The avidity of serotype-specific IgG was expressed as the percentage of absorbance remaining after treatment with different concentrations of NaSCN.

3. Results

The clinical characteristics of the 24 pediatric patients with IPD are shown in Table 1. The diagnosis of these patients included meningitis ($n = 11$), bacteremia ($n = 10$), and bacteremic pneumonia ($n = 2$) and septic arthritis ($n = 1$). The median age (range) was 17 (10–108) months, and 63% were boys. Four patients (17%) had associated comorbid conditions including immune thrombocytopenia and splenectomy, meningoencephalocele, asplenia and single ventricle, and hydrocephalus (V-P shunt). In the 24 examined, the most common infecting serotype was 6B (9 isolates, 38%), followed by 19F (4 isolates, 17%), 19A (3 isolates, 13%), 6C and 14 (2 isolates each 8%) and one isolate each of 9V, 15B, 23F and 24F (4%). The median (range) period from the onset of IPD to the time of serum collection was two (0–23) days.

Three patients received PPV23 due to pre-existing medical conditions (Table 1). Before their episode of IPD, two patients infected with serotype 19F and one patient infected with serotype 9V received PPV23. Because PPV23 contains serotypes 19F and 9V, all three cases were considered PPV23 vaccine failure (VF). Ten patients received one to three doses of PCV7 at various ages as shown in Table 1. Only one patient (Case 18) completed a course of three doses of PCV7 between 2 and 6 months of age. The other nine patients were immunized with PCV7 during the catch-up phase. PCV7 breakthrough infection (BTI) was defined where a patient who received at least one dose of PCV7 had an episode

Table 1
Clinical characteristics of 24 pediatric patients with invasive pneumococcal disease (IPD).

No.	Age (months)	Sex	Diagnosis	Comorbid condition	Infecting serotype	Serum obtained days after IPD	Antibody to the infecting serotype		Vaccination before IPD (doses)	Age at each dose (month)	Category of IPD after PPV23	Category of IPD after PCV7	Outcome
							IgG (μg/ml)	OI					
1	108	M	Meningitis	ITP, splenectomy	19F	10	6.53	2	PPV23(1)	62	Vaccine failure	NA	Alive
2	50	M	Meningitis	Meningoencephalocele	19F	17	5.1	2	PPV23(1)	42	Vaccine failure	NA	Alive
3	75	M	Bacteremia	Asplenia, single ventricle	9V	1	0.57	NT	PPV23(1)	24	Vaccine failure	NA	Dead
4	14	M	Bacteremia	None	6B	11	0.34	2	None	–	NA	NA	Alive
5	38	M	Meningitis	None	19F	4	1.08	2	None	–	NA	NA	Alive
6	14	M	Bacteremia	None	14	5	2.1	5	None	–	NA	NA	Alive
7	13	M	Bacteremia	None	6B	4	2.25	NT	None	–	NA	NA	Alive
8	12	M	Meningitis	None	6B	20	1.81	7	PCV7(1)	10	NA	Breakthrough infection	Alive
9	10	M	Meningitis	None	19F	0	0.85	NT	None	–	NA	NA	Alive
10	17	M	Bacteremic pneumonia	None	19A	2	NA	NT	None	–	NA	NA	Alive
11	30	M	Bacteremic pneumonia	None	6B	0	0.53	2	PCV7(1)	28	NA	Vaccine failure	Alive
12	17	F	Meningitis	None	24F	1	NA	NA	PCV7(1)	16	NA	Non-VT infection	Alive
13	12	F	Meningitis	None	6B	12	0.78	2	None	–	NA	NA	Alive
14	10	M	Meningitis	None	15B	2	NA	NA	None	–	NA	NA	Alive
15	30	F	Bacteremia	None	6B	0	1.18	2	PCV7(1)	26	NA	Vaccine failure	Alive
16	26	F	Bacteremia	None	19A	1	NA	2	None	–	NA	NA	Alive
17	15	F	Bacteremia	None	14	0	1.75	2	None	–	NA	NA	Alive
18	10	M	Bacteremia	None	19A	0	NA	2	PCV7(3)	4, 5, 6	NA	Non-VT infection	Alive
19	30	F	Meningitis	Hydrocephalus (V-P shunt)	6B	23	0.92	2	PCV7(1)	28	NA	Vaccine failure	Alive
20	17	F	Meningitis	None	6B	0	1.38	2	PCV7(2)	9, 11	NA	Breakthrough infection	Alive
21	11	F	Septic arthritis	None	23F	0	0.55	2	PCV7(3)	7, 8, 9	NA	Breakthrough infection	Alive
22	16	F	Bacteremia	None	6B	0	5.62	2	None	–	NA	NA	Alive
23	49	M	Meningitis	None	6C	1	NA	2	PCV7(1)	36	NA	Non-VT infection	Alive
24	14	M	Bacteremia	None	6C	7	NA	NT	PCV7(2)	9, 10	NA	Non-VT infection	Alive

OI, opsonization index; ITP, immune thrombocytopenia; PPV23, 23-valent pneumococcal polysaccharide vaccine; PCV7, 7-valent pneumococcal conjugate vaccine; NA, not applicable; NT, not tested because of antibiotic use; VT, vaccine type.

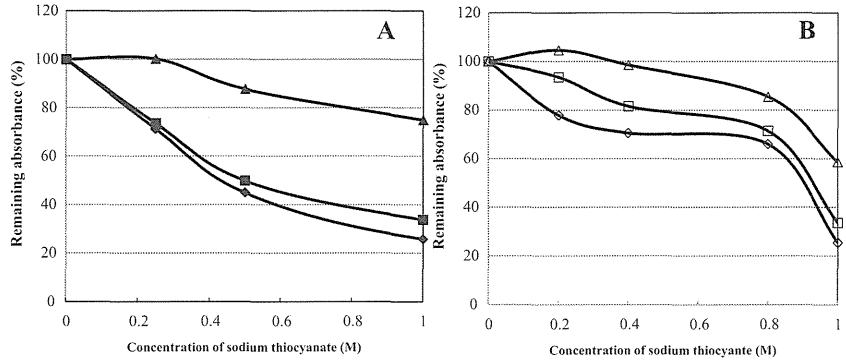

Fig. 1. Avidity of serotype 19F-specific IgG (A) and serotype 6B-specific IgG (B) in sera from pediatric patients with invasive pneumococcal diseases. Two serum samples from Case 1 (closed diamond) and Case 2 (closed square), and the positive control serum (closed triangle) from Case 6 (four months after the episode of IPD and one month after two doses of PCV7 vaccination) were examined for the avidity of serotype 19F-specific IgG. Two serum samples from Case 22 before (open diamond) and after (open square) two doses of PCV7 vaccination, and a positive control serum (open triangle) collected from Case 6 after two doses of PCV7 were used to test the avidity of serotype 6B-specific IgG.

of IPD for which the pneumococcal isolate was a PCV7 serotype, and PCV7 VF was defined as the subset of BTI in which the patient had completed the Advisory Committee on Immunization Practice (ACIP)-recommended PCV7 vaccine schedule at least two weeks before the IPD [18,19]. An instance of an IPD patient who had had at least one dose of PCV7 and for whom the pneumococcal isolate was not a PCV7 serotype was defined as PCV7 non-VT infection. Of 10 patients who received PCV7 previously, three cases (Cases 11, 15 and 19) were classified as PCV7 VF, and three cases (Cases 8, 20 and 21) were classified as PCV7 BTI. The other four cases (Cases 12, 18, 23 and 24) were classified as PCV7 non-VT infection.

The level of serotype-specific IgG or the OI for the infecting serotype was determined for 17 of 24 cases. The levels of specific IgG for the infecting serotype ranged widely from 0.34 to 6.53 μg/ml. In all 17 cases, the level of specific IgG for the infecting serotype was higher than 0.20 μg/ml, the putative threshold for preventing IPD [12,14]. The geometric mean concentration for the 17 cases was 1.35 μg/ml. In contrast, the OI for the infecting serotype was <8 in all of 17 cases. In particular, obvious discrepancies were found in two patients with serotype 19F (Cases 1 and 2) and one patient with serotype 6B (Case 22) who had serotype-specific IgG higher than 5 μg/ml and undetectable OI.

To investigate these discrepancies, we next examined the avidities of serotype 19F-specific IgG in sera from Cases 1 and 2, and the avidities of serotype-6B specific IgG in sera from Case 22. The percentages of remaining absorbance to 19F CPS of the positive control serum (IgG 7.25 μg/ml, OI 2336) collected from a patient (Case 6) after two doses of PCV7 vaccination were 100–75% at concentrations of 0.25–1.0 M of NaSCN (Fig. 1A). In contrast, the percentages of remaining absorbance to 19F CPS of sera from Cases 1 (IgG 6.53 μg/ml, OI 2) and 2 (IgG 5.10 μg/ml, OI 2) to 19F CPS were 74–44% and 71–26% at concentrations of 0.25–1.0 M of NaSCN, respectively.

The percentages of remaining absorbance to 6B CPS of the positive control serum (IgG 4.16 μg/ml, OI 4626) collected from Case 6 after two doses of PCV7 99–59% at concentrations of 0.4–1.0 M of NaSCN (Fig. 1B). In contrast, the percentages of remaining absorbance of serum from Case 22 before PCV7 vaccination (IgG 5.62 μg/ml, OI 2) and after two doses of PCV7 vaccination (IgG 2.37 μg/ml, OI 562) were 71–25% and 81–34% at concentrations of 0.4–1.0 M of NaSCN.

4. Discussion

In pediatric patients with IPD, the serum OIs for the infecting serotype within one month after the infectious episode were <8 in all 17 patients tested for OI, although the levels of IgG for the infecting serotype were higher than 0.2 μg/ml in all 17 patients tested for serotype-specific IgG. Undetectable OIs suggest that the serotype-specific IgG in their sera are largely nonfunctional. Soininen et al. similarly reported that sera from unimmunized children without nasopharyngeal carriage contained serotype-specific IgG, but infrequently had serotype-specific opsonic activity [20].

Three patients received PPV23 before PCV7 was licensed in Japan in 2009 because they were at increased risk for pneumococcal disease. Although the current guideline of the ACIP recommends that children aged 2–18 years with underlying medical conditions should receive PPV23 after completing all recommended doses of PCV13 [21], pediatricians should be aware of the possible induction of nonfunctional IgG by PPV23 in high-risk children aged >2 years. Two patients with PCV7 BTI received one or two doses of PCV7 9–11 months after birth, and two patients with PCV7 VF received only one dose of PCV7 26–28 months after birth. All four of these patients comprised the catch-up cases for PCV7. Interestingly, all cases with BTI or VF were caused by serotype 6B. A recent study from the US reported that 155 of 753 (21%) pediatric IPD cases were PCV7 BTIs caused predominantly by serotypes 6B (32%) and 19F (29%) [18]. The PCV7 BTIs caused by serotype 6B were more likely to have occurred in children who received only one or two PCV7 doses (84%) compared with infections caused by other VTs (61%). Rennels et al. also reported a low immune response to 6B and other serotypes, including 9V and 18C in children who received fewer than three doses of PCV7 [22].

Our data demonstrated that sera collected from Cases 1, 2 and 22 containing 19F- or 6B-specific IgG levels higher than 5.0 μg/ml, but lacking opsonic activity, contained lower avidity of serotype-specific IgG than the positive control sera with high OIs. An improvement of the avidity of 6B-specific IgG was confirmed in the sera with a high OI from Case 22 by two doses of PCV7 vaccination. Two previous studies using sera from healthy adults with or without vaccination with PPV23 demonstrated that higher avidity antibodies were more effective than lower avidity antibodies in in vitro complement-dependent opsonophagocytosis and for in vivo protection against pneumococcal infection in mice [23,24]. These data are, partially, in agreement with our findings of high levels of serotype-specific IgG with low avidity in serum from pediatric patients within one month after IPD. The low avidity of serotype-specific IgG levels may explain the undetectable OIs in sera collected from Cases 1, 2 and 22 within one month of an IPD episode.

O'Brien et al. recently reported the pneumococcal antibody status in a child with of PCV7 vaccine failure caused by serotype 14

[25]. In this patient, the serotype-specific IgG and the OIs in serum were 4.98 μg/ml and 1024, respectively, 35 days after the administration of three doses of PCV7. However, this patient developed occult bacteremia at 9.6 months of age, 53 days after the third dose of PCV7. Because of a slightly decreased serotype-specific IgG (4.25 μg/ml) and a significantly decreased OI of 4 in the serum of this patient after this episode of IPD, the authors suggested that the functional antibodies existing during infection with consumed by binding to the serotype 14 antigen. This finding also suggests that the ELISA assay detected some nonspecific or nonfunctional IgG in the serum of this patient, and is in agreement with the findings in the sera of our pediatric patients with IPD.

The limitations of our study are the small number of IPD cases examined and the variable timing of serum collection, although the sera were all collected within one month after the IPD episode. These limitations meant that we were unable to compare the induction of opsonic activity to the infecting serotype between the acute phase and the convalescence phase in pediatric patients with IPD.

In conclusion, in all of 17 patients tested within one month of an IPD episode, the serum OIs to the infecting serotype were <8, whereas the levels of serotype-specific IgG were higher than 0.2 μg/ml. Low avidity of the serotype-specific IgG were confirmed in three patients associated with the serotype-specific IgG levels higher than 5 μg/ml, but with undetectable OIs.

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Appendix A. The Japanese IPD Study Group

In addition to TO, NI, KM, JN, BC, BC, KT, YA, KO, the members of the Japanese IPD Study Group are Kenji Okada (National Fukuoka Hospital), Takashi Nakano (Kawasaki Medical University), Hideki Akeda (Okinawa Prefectural Nanbu Medical Center), Masako Habu (Tokyo Metropolitan Bokutoh General Hospital), Eri Yamaguchi (Chidoribashi Hospital), Kei Komiya (Nihon University School of Medicine), Shinji Kido (Toyota Memorial Hospital), Takahiro Niizuma (Koshigaya Municipal Hospital), Masato Arao (Saitama Medical University), Fumie Ishiwada (Chiba Kaihin Municipal Hospital), Mai Kubota (Shizuoka Children's Hospital), Kenji Furuno (National Fukuoka-Higashi Medical Center), Yoshio Yamaguchi (National Hospital Organization Tochigi Hospital), Kaoru Obinata (Juntendo University Urayasu Hospital), Mikio Yoshioka (KKR Sapporo Medical Center), and Tomomi Naito (Saiseikai Kawaguchi General Hospital).

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CONCISE REPORT

Impact of tocilizumab therapy on antibody response to influenza vaccine in patients with rheumatoid arthritis

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ABSTRACT

Objectives We assessed the influence of tocilizumab (TCZ), a humanised monoclonal anti-interleukin-6 receptor antibody, on antibody response following influenza vaccination in patients with rheumatoid arthritis (RA).

Methods A total of 194 RA patients received inactive trivalent influenza vaccination (A/H1N1, A/H3N2 and B/B1 strains). All patients were classified into the TCZ (n=62), TCZ+methotrexate (MTX) (n=49), MTX (n=65) and RA control (n=18) groups. Antibody titres were measured before and 4–6 weeks after vaccination using the haemagglutination inhibitory assay.

Results For the A/H1N1 and A/H3N2 strains, the TCZ and TCZ+MTX groups achieved fold increases of 9.9–14.5, postvaccination seroprotection rates greater than 70% and seroresponse rates greater than 40%. For the B/B1 strain, seroresponse rates were approximately 30%, but fold increases and seroprotection rates were 5.0–5.4 and greater than 70%, respectively, in these treatment groups. MTX had a negative impact on vaccination efficacy, but adequate responses for protection were nevertheless demonstrated in the MTX group. Neither severe adverse effects nor RA flares were observed.

Conclusions TCZ does not hamper antibody response to influenza vaccine in RA patients. Influenza vaccination is considered effective in protecting RA patients receiving TCZ therapy with or without MTX.

INTRODUCTION

Influenza vaccination is the most effective method for preventing influenza virus infection and its potentially severe complications. Patients with rheumatoid arthritis (RA) are at an increased risk for infectious diseases due to the nature of RA and its treatment with immunosuppressive agents;[1] therefore, this patient population is a potential candidate for influenza vaccination. Treatment with antitumour necrosis factor α (anti-TNFα) agents may impair antibody response to influenza vaccination in patients with RA and other rheumatic diseases, but the response is large enough to warrant influenza vaccination for such patients.[2–8]

Tocilizumab (TCZ), a humanised monoclonal interleukin-6 (IL-6) receptor antibody, is effective in the treatment of patients with moderate to severe RA who have shown inadequate responses to methotrexate (MTX) and one or more anti-TNFα agents.[9] Our concern is the impact of TCZ on protective antibody response to influenza vaccination because IL-6 was originally identified as a factor that plays an essential role in terminal differentiation of B cells into antibody producing plasma cells.[10] Data regarding the efficacy and safety of influenza vaccination are lacking in RA patients receiving TCZ. Only one attempt at evaluating the efficacy of influenza vaccine has so far been made in a small number of paediatric patients receiving TCZ therapy for systemic onset juvenile idiopathic arthritis.[11]

To address this issue, we determined antibody response to trivalent inactivated influenza vaccine in RA patients being treated with TCZ, MTX or both agents, and compared parameters for efficacy of vaccination among these groups.

METHODS

Patients

RA patients aged 18 or older who had been receiving TCZ (an intravenous infusion of 8 mg/kg every 4 weeks) for at least 4 weeks and/or MTX (6–18 mg per week) for 12 weeks or more at our rheumatology outpatient clinics were invited to participate in this open-label study. RA patients who had been receiving bucillamine or salazosulphapyridine were also included as RA controls. All participants fulfilled the 1987 American College of Rheumatology criteria for diagnosis of RA. Exclusion criteria were current use of 10 mg/day or more of prednisolone, current use of tacrolimus or leflunomide, a recent history (within 3 months) of influenza infection, and a recent history (within 6 months) of influenza vaccination.

Vaccine

We used commercially available inactivated trivalent influenza vaccine (Biken HA, Mitsubishi Tanabe Pharm Corporation, Osaka, Japan) containing 30 μg of purified haemagglutinin of each of the following: A/California/7/2009 (H1N1)-like strain (A/H1N1 strain), A/Victoria/210/2009 (H3N2)-like strain (A/H3N2 strain) and B/Brisbane/60/2008-like strain (B/B1 strain). Patients received a single dose of vaccine (0.5 ml) subcutaneously from October 2011 until January 2012. For RA patients receiving TCZ, the vaccination was done on the same day as TCZ infusion.

HI tests

Sera were collected immediately before and 4–6 weeks after vaccination. For the detection of

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influenza antibodies, haemagglutination inhibition (HI) tests were performed in duplicate at SRL (Tachikawa, Tokyo, Japan), according to WHO standard procedure using haemagglutinin antigens representing all three strains that were included in the vaccine. Geometric mean titres (GMTs) of HI antibodies before and after vaccination, and fold increases relative to prevaccination titres (geometric means of postvaccination to prevaccination antibody titre ratios) were determined. GMTs were calculated from log-transformed values of HI antibody titres. For statistical analysis, a titre of 5 was arbitrarily assigned to sera with undetectable titres of <10. Seroprotection was defined as antibody titres of ≥40. Seroconversion was defined as postvaccination antibody titres of ≥40 in patients whose prevaccination titres were <10. Seroresponse was defined as seroconversion or fold increases in antibody titres of ≥4 in patients whose prevaccination titres were ≥10.

Monitoring adverse effects and disease activity

Systemic adverse events and worsening of RA occurring 4–6 weeks after vaccination were recorded. Systemic adverse effects included fever, tiredness, sweating, myalgia, chills, headache, arthralgia, diarrhoea and common cold-like symptoms. RA activity was monitored using a disease activity score for 28 joints and a clinical disease activity index.

Statistical analysis

In univariate analyses for categorical variables, differences between treatment groups were analysed using the χ^2 test or Fisher's exact probability test. Continuous variables were assessed by the Mann–Whitney U test for comparisons of non-parametric data between the two treatment groups, and analysis of variance with post hoc Tukey's honestly significant difference test for comparisons of parametric data between the four treatment groups. A paired-sample t test was used to compare differences in GMTs between prevaccination and postvaccination.

For all tests, probability values (p values) <0.05 were considered to indicate statistical significance. All calculations were performed using Excel Statistical Analysis 2008 (SSRI Co., Tokyo, Japan) or PASW Statistics V.18 (SPSS Japan Inc., Tokyo, Japan).

RESULTS

Clinical and demographic characteristics of participants

A total of 194 RA patients were classified into four groups according to their ongoing anti-RA therapy. One group of 62 patients was treated with TCZ as a monotherapy (TCZ group); 65 patients were treated with MTX alone (MTX group); 49 patients received a combination therapy consisting of TCZ and MTX (TCZ+MTX group); and 18 patients received bucillamine or salazosulphapyridine monotherapy (RA control group). Clinical and demographic characteristics are shown in table 1.

Antibody titres

After vaccination, GMTs for all strains were increased significantly. Regarding the A/H3N2 strain, a significantly higher post-GMT was obtained in the TCZ group compared with that in the MTX group (p=0.009) (table 2). The TCZ group also showed a higher post-GMT for the B/B1 strain than did the MTX group and the RA control group (p=0.044 and p=0.031,

Table 1 Clinical and demographic characteristics of RA patients prior to influenza vaccination

	MTX group (n=65)	TCZ+MTX group (n=49)	TCZ group (n=62)	RA control (n=18)	p Values between treatment groups
Male/female	11/54	5/44	11/51	3/15	NS
Age, years, mean (95% CI)	67 (65.0 to 68.9)	62.9 (59.8 to 65.9)	65.2 (61.6 to 68.8)	67.3 (62.3 to 72.4)	NS
Prior influenza vaccination, number of patients (%)	47 (72.3)	36 (73.5)	50 (80.6)	12 (66.7)	NS
RA duration, years, mean (95% CI)	9.8 (7.7 to 11.9)	7.5 (5.8 to 9.2)	14.6 (11.5 to 17.7)	11.1 (4.8 to 17.4)	0.029 (M vs T) 0.001 (T/M vs T)
MTX dose, mg/week, median (25th, 75th percentiles)	8 (6, 8)	8 (6, 8)	–	–	NS
MTX duration, months, median (25th, 75th percentiles)	58 (17, 78)	54 (29, 89)	–	–	NS
TCZ duration, weeks, median (25th, 75th percentiles)	–	68 (24, 104)	64 (21, 107)	–	NS
Use of prednisolone, number of patients (%)	13 (20)	12 (24.5)	22 (35.5)	1 (5.6)	0.016 (T vs C)
Prednisolone dose, mg/day, mean (95% CI)	0.87 (0.4 to 1.34)	0.90 (0.33 to 1.47)	1.02 (0.54 to 1.49)	–	NS
Positive RF, number of patients (%)	38 (58.5)	42 (85.7)	46 (74.2)	7 (38.9)	0.002 (M vs T/M) 0.0001 (T/M vs C) 0.005 (T vs C)
Positive anti-CCP Abs, number of patients (%)	46 (70.8)	43 (87.8)	56 (90.3)	6 (33.3)	0.030 (M vs T/M) 0.006 (M vs T) 0.004 (M vs C) <0.0001 (T/M vs C) <0.0001 (T vs C)
CDAI (25th, 75th percentiles)	5.3 (3.7–7.8)	6.2 (4.5–7.8)	9.5 (7.9–11.1)	8.2 (4.8–11.5)	0.001 (M vs T) 0.027 (T/M vs T)
Lymphocytes, /μl, mean (95% CI)	1368 (1237 to 1500)	1395 (1255 to 1535)	1622 (1500 to 1744)	1478 (1098 to 1857)	0.038 (M vs T)

Data were obtained immediately before influenza vaccination. Prior influenza vaccination represents that administered last season (2010/2011). p Values between treatment groups were determined by the Mann–Whitney U test, post hoc ANOVA using Tukey's HSD test, the χ^2 test or Fisher's exact probability test.
ANOVA, analysis of variance; anti-CCP Abs, anti-cyclic citrullinated peptide antibodies; C, RA control group; CDAI, clinical disease activity index; HSD, honestly significant difference; M, MTX group; MTX, methotrexate; NS, not significant; RA, rheumatoid arthritis; RF, rheumatoid factor; T, TCZ group; T/M, TCZ+MTX group; TCZ, tocilizumab.

Table 2 GMTs and fold increases of HI antibodies for three influenza strains in the RA treatment groups prior to and after influenza vaccination

	MTX group (n=65)	TCZ+MTX group (n=49)	TCZ group (n=62)	RA control group (n=18)	p Values between treatment groups
GMTs					
A/H1N1					
Before	31.7 (16.1–47.2)	59.5 (19.9–99.1)	62.0 (25.4–125.4)	15.3 (8.3–22.3)	NS
After	120.5 (75.3–165.6)*	162.1 (86–238.2)**	211.7 (142–281.4)*	169.4 (11.5–327.4)*	NS
A/H3N2					
Before	37.9 (15.5–60.4)	42.6 (25.2–59.9)	55.2 (31.8–78.7)	36.9 (11.9–62.0)	NS
After	120.2 (80.2–160.2)*	140.7 (82–199.4)***	237.8 (169.1–306.5)*	93.9 (54.1–133.6)**	0.009 (M vs T)
B/B1					
Before	45.5 (30.2–60.7)	43.2 (29.8–56.5)	72.1 (53.3–90.9)	23.9 (12.2–35.6)	0.017 (T vs C)
After	103.1 (74.9–131.3)*	105.1 (69.4–140.8)*	161.8 (123.8–144)*	68.9 (45.7–92.1)*	0.044 (M vs T) 0.031 (T vs C)
Fold increase					
A/H1N1	12.6 (5.8–19.5)	14.5 (7.2–21.9)	12.0 (9.8–17.7)	11.2 (3.0–19.4)	NS
A/H3N2	9.6 (5–14.2)	9.9 (5.2–14.6)	12.0 (6.6–17.3)	5.3 (2.7–8.0)	NS
B/B1	3.5 (2.5–4.4)	5.4 (2.4–8.3)	5.0 (3.3–5.7)	5.8 (3.1–8.4)	NS

Data are expressed as the mean (95% CIs). Differences between prevaccination and postvaccination GMTs were assessed using the paired-sample t test. Comparisons between the four treatment groups were performed by post hoc ANOVA using Tukey's HSD test.
*p<0.0001, **p=0.009 and ***p=0.001 based on comparisons with prevaccination titres.
ANOVA, analysis of variance; C, RA control group; GMT, geometric mean titre; HI, haemagglutination inhibition; HSD, honestly significant difference; M, MTX group; MTX, methotrexate; NS, not significant; RA, rheumatoid arthritis; T, TCZ group; TCZ, tocilizumab.

respectively). Fold increases in GMTs for the three strains were ≥3.5-fold in all treatment groups. These groups achieved similar levels of fold increases for each strain and there were no statistically significant differences.

Seroprotection, seroresponse and seroconversion rates

After vaccination, seroprotection rates for the three influenza strains were increased significantly in all treatment groups (figure 1A). The TCZ and TCZ+MTX groups achieved postvaccination protection rates of >70% for all the influenza strains. Regarding the A/H3N2 and B/B1 strains, postvaccination seroprotection rates were significantly higher in the TCZ group compared with those in the other three treatment groups (for A/H3N2, p<0.0005 vs MTX, p=0.001 vs TCZ + MTX p=0.006 vs RA control; for B/B1, p=0.007 vs MTX, p=0.023 vs TCZ +MTX, p=0.007 vs RA control). Seroprotection rates for the A/H1N1 strain were similar among all the groups tested.

For the A/H1N1 and A/H3N2 strains, seroresponse rates were >40% in the MTX, TCZ and TCZ+MTX groups, while the rates for the B/B1 strain in these groups were approximately 30% (figure 1B). The seroresponse rate for the A/H3N2 strain was significantly higher in the TCZ group compared with that in the MTX group (p=0.04). Seroconversion rates for the three influenza strains were greater than 40% in all treatment groups (figure 1C). The TCZ group showed a significantly higher seroconversion rate for the A/H3N2 strain than did the MTX group (p=0.032).

Predictive factors for seroresponse to influenza vaccination

In multivariate logistic regression analysis, TCZ use was not identified as the predictive factor for seroresponse to influenza vaccination (see online supplementary table S1). For the A/H3N2 strain, the negative association of current MTX use with seroresponse was confirmed (p=0.04). Prior influenza vaccination was negatively associated with seroresponse for all the three strains (for A/H1N1, p=0.006; for A/H3N2, p=0.01; for B/B1, p<0.0001). This may have reflected ceiling effects; that is, higher prevaccination protection rates may, at least in part, have influenced the observed seroresponse rates.

Vaccination safety

Neither systemic adverse effects nor exacerbation of RA was experienced by any patients during a follow-up period of 4–6 weeks after vaccination.

DISCUSSION

Antibody response to the A/H1N1 and A/H3N2 strains in the TCZ and TCZ+MTX groups met all three requirements of the European Medicines Agency (EMA) guidance for assessment of influenza vaccines specified by the Committee for Proprietary Medical Products (CPMP).[12] For the B/B1 strain, these treatment groups met two of the EMA/CPMP criteria. The MTX group fulfilled two of the EMA/CPMP criteria for all strains. Multivariate logistic analysis confirmed that TCZ use is not a predictive factor for inadequate antibody response for any influenza strain.

IL-6 works as a B cell differentiation factor, which induces activated B cells to produce immunoglobulin.[10] The blockage of IL-6 activity following TCZ therapy, therefore, would be expected to reduce humoral immune response to influenza vaccination. Kopf *et al*[13] indicated that T cell-dependent antibody response against virus infection is impaired in IL-6-deficient mice. Unlike anti-infliximab or antiadalimumab antibodies, anti-TCZ antibodies rarely developed in RA patients receiving 8 mg/kg of TCZ, even as monotherapy.[14 15] Nevertheless, the present study has clearly indicated that RA patients receiving TCZ therapy can be effectively and safely immunised with influenza vaccine. One possible explanation may be that, unlike rituximab, TCZ is not a B cell-targeting antibody that can induce B cell depletion. Given that a variety of cytokines are released from activated helper T cells, antibody production may not depend simply on IL-6. Costelloe *et al*[16] showed that IL-6 is not required for antigen (influenza virus)-specific antibody responses by non-fractionated tonsillar mononuclear cells or by T cell-depleted B cells in the presence of IL-2. Another explanation may be that IL-6 signalling is not inhibited completely in lymphoid tissue, locations in which vaccination-mediated immune response is initiated, even when maximum saturation of soluble IL-6 receptors in the circulation is achieved with