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# Quantification of a cell-mediated immune response against varicella zoster virus by assessing responder CD4$^{high}$ memory cell proliferation in activated whole blood cultures

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ABSTRACT

*Background:* Herpes zoster (HZ) is caused by reactivation of a latent varicella zoster virus (VZV). The potential to develop HZ increases with age due to waning of memory cell-mediated immunity (CMI), mainly the CD4 response. Therefore, VZV-CD4-memory T cells (CD4-M) count in blood could serve as a barometer for HZ protection. However, direct quantification of these cells is known to be difficult because they are few in number in the blood. We thus developed a method to measure the proliferation level of CD4-M cells responding to VZV antigen in whole blood culture.

*Methods:* Blood samples were collected from 32 children (2–15 years old) with or without a history of varicella infection, 18 young adults (28–45 years old), and 80 elderly (50–86 years old) with a history of varicella infection. The elderly group was vaccinated, and blood samples were taken 2 months and 1 year after VZV vaccination. Then, 1 mL of blood was mixed with VZV, diluted 1/10 in medium, and cultured. CD4-M cells were identified and measured by flow cytometry.

*Results:* There was distinct proliferation of $CD3^{+}CD4^{high}CD45RA^{-}RO^{+}$ (CD4$^{high}$-M) cells specific to VZV antigen at day 9. The majority of CD4$^{high}$-M cells had the effector memory phenotype $CCR7^{-}$ and was granzyme B-positive. CD4$^{high}$-M cells were detected in blood culture from varicella-immune but not varicella-non-immune children. Meanwhile, a higher level of CD4$^{high}$-M proliferation was observed in young adults than in the elderly. The CD4$^{high}$-M proliferation level was boosted 2 months after VZV vaccination and maintained for at least 1 year in the elderly.

*Conclusion:* Quantifying VZV responder CD4$^{high}$ -M cell proliferation is a convenient way to measure VZV CMI using small blood volumes. Our method can be applied to measure VZV vaccine-induced CMI in the elderly.

**Clinical study registry numbers:** (www.clinicaltrials.jp) 173532 and 183985.



## 1. Introduction

Varicella zoster virus (VZV) is a human-restricted alphaherpesvirus.

The majority of people have been exposed to varicella in childhood and remain latently infected. Varicella virus reactivation accompanied by low-level VZV cell-mediated immunity (CMI) results in herpes zoster (HZ), which is also known as shingles [1].

Immune responses generated in children during natural VZV infection are known to confer lifelong protection against VZV reinfection. However, many studies have confirmed an increase in HZ incidence with age [2–5]. In addition, these studies suggest that CMI senescence appears to be responsible for HZ development. Multiple lines of evidence support an augmented predominant cytotoxic CD4 T cell response after varicella infection or after HZ

*Abbreviations:* HZ, Herpes Zoster; VZV, varicella zoster virus; CMI, cell mediated immunity; CD4-M, CD4 memory cells, IFN-γ, interferon gamma; ANOVA, analysis of variance; CI, confidence interval; PBMC, peripheral blood mononuclear cell.

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[6–9]. Hayward et al. conducted an *in vitro* experiment showing that CD4 but not CD8 T cells are responsible for the cellular cytotoxic response against VZV-infected cells [6,7]. Milikan et al. demonstrated in another *in vitro* experiment that using CD4 T cells isolated from 65-year-old women with HZ controlled productive VZV infection in retinal pigment epithelial cells in a mechanism partly mediated by cytolysis [8]. Human clinical data showed boosted VZV-specific CD4 T cell responses in adults after re-exposure to VZV via household contact with infected children [9]. Moreover, an association between reduced CD4 T cell responses specific to VZV and a high incidence of herpes zoster in patients with systemic lupus erythematosus was reported [10]. Reduced levels of VZV-specific CD4 T cells were also reported for immunocompromised individuals who are at high risk of herpes zoster [11]. Despite the fact that CMI is important in protecting hosts from HZ, directly measuring the level of this response is still difficult, perhaps because of the low levels of circulating VZV-specific memory cells [12].

Several attempts have been made to assess the level of VZV-specific CMI response *in vitro*, including lymphocyte proliferation or responder cell frequency (RCF) assay [13], interferon gamma (IFN-γ) ELISPOT assay [14], and intracellular cytokine assay (ICA) [12]. These methods have improved our knowledge about responding immune cells. All of the *in vitro* methods have shown a predominant Th-1 CD4 response with Th1 cytokines, mainly IFN-γ [11,15,16]. These methods require peripheral blood mononuclear cell (PBMC) separation, which is technically complex, laborious, requires a high volume of blood, and is a limiting factor in the case of children. Other attempts have been made using whole blood-based assays, including IFN-γ release assay [17] and intracellular cytokine measurement [18]. These methods depend on direct stimulation of whole blood with VZV antigen. In contrast to using PBMCs, using whole blood retains all blood components and maintains cells in their *in vivo* ratios. Whole blood-based assays do not require separations, and are therefore quicker to perform, although cellular portions are difficult to analyze. In addition, data from IFN-γ assays have shown that secreted IFN-γ amounts are highly variable between individuals [19]. Although IFN-γ is an important marker for activated immune cells, it is still an intermediate-acting cytokine secreted by activated macrophages, natural killer cells, B cells, and T cells [20]. Despite being used for decades as a CMI marker in *Mycobacterium tuberculosis* vaccine studies, IFN-γ reliability as the only correlate for protection is limited because of the difficulty correlating different functions of proliferating CD4 T cells and the amount of secreted IFN-γ [21]. Trannoy et al. showed that IFN-γ secretion could be detected in only 70% of VZV-seropositive subjects and that the IFN-γ secretion assay was less sensitive than the lymphocyte proliferation assay [22].

In summary, several attempts have been made to measure VZV-specific CMI, but most of these methods seem to be difficult to perform and might require radioactive materials resulting in safety considerations. As a result, no clinical correlation of protection from HZ exists. Therefore, new attempts to evaluate VZV-specific CMI response are needed.

Here, we developed a simple methodology to quantify VZV CMI by measuring the proportion of VZV responder memory cells in whole blood culture. We then evaluated our method in varicella immune and non-immune children. Finally, we determined the efficiency of our method in quantifying VZV vaccine-induced CMI in the elderly.

## 2. Study subjects and methods

### 2.1. Study subjects

This study enrolled healthy children and adults to obtain a single 2-mL blood sample. In addition, we enrolled elderly from the VZV vaccine clinical study to obtain three 5-mL blood samples: one pre-vaccination, one 2 months post-vaccination, and one 1 year post-vaccination.

The first group consisted of 32 children (2–15 years old) from a pediatric clinic (Baba Clinic, Osaka, Japan) (Supplementary Table 1). This group was divided into 12 children with no history of varicella infection, confirmed by enzyme immunoassay (EIA) to be negative for VZV-specific IgG antibodies; 19 children with a history of varicella infection (1 year or longer), confirmed by a positive EIA for VZV-specific IgG antibodies; and one child with acute varicella confirmed by EIA to have a high titer of VZV-specific IgM antibody and to be negative for VZV-specific IgG antibodies (Supplementary Table 1). The second group consisted of 18 young adults (28–45 years old) and one elder (62 years old) with a history of varicella enrolled from Biken employees. The major exclusion criterion for all participants was a history of HZ. Written informed consent was obtained from the parents of the children or from healthy adults after explaining the aim of the study and before blood sampling by the responsible physician.

Regarding the VZV vaccine clinical study, 80 elderly subjects between 50 and 89 years of age were enrolled from three different clinics (Osaka, Japan) from April 2017 to June 2018. Subjects who previously came to the clinic requesting Oka/Biken live attenuated varicella VZV vaccine (manufacturer BIKEN, lot numbers VZ198, VZ199, and VZ200) were approached about volunteering for this study. After explanation and informed consent, we collected background data (age, sex, varicella/HZ history, history of recent contact with varicella/HZ patients). Major exclusion criteria were history of HZ and a confirmed history of immunodeficiency disease. Five milliliters of heparinized blood was withdrawn before vaccination (on the vaccination day), 8 weeks, and 1 year after vaccination. The responsible physician monitored all subjects for adverse events. This vaccine study was audited by a third-party auditor (Imedix, Osaka, Japan), and the trial information was registered at www.clinicaltrials.jp under nos. 173532 and 183985.

The Biken Institutional Ethical Review Board approved this research under protocol numbers 14-01 (children and adults) and 17-03 (elderly). The ethics committee of Hyogo College of Medicine also approved the study (children and adults only) under protocol number 1801.

### 2.2. Blood samples

The responsible physician collected blood from volunteers in sodium heparin tubes (cat. 31773, Nipro, Japan).

### 2.3. Antigen preparation

A live VZV-infected MRC-5 cell extract and an uninfected MRC-5 cell extract were prepared according to the standard operating procedure of vaccine preparation. Briefly, human diploid MRC-5 cells were cultivated to confluence in suitable culture flasks. The flasks were then divided into 2 groups: the first group was infected with Varicella Oka-strain (VZV) and the other was not infected (Mock). Both groups were then incubated for 2–3 days with virus cultivating media at 37 °C. When the VZV-infected cells demonstrated 70% cytopathogenicity, both VZV and Mock cells were washed with PBS twice and harvested independently using 0.02% EDTA. The VZV and Mock cells were then centrifuged and suspended in a buffer containing sodium glutamate, sucrose, and other suitable stabilizers (gelatin-free). Finally, the cells were sonicated and briefly centrifuged. The protein concentration in the supernatant (cellular extract) was measured using a Pierce BCA Protein Assay Kit (cat. 23227, Thermo Fischer Scientific, USA). The total protein concentration was then adjusted for both groups

to 500 μg/mL. The extracts were aliquoted in 1-mL tubes and stored at −80 °C.

### 2.4. Blood stimulation and cultivation

Two milliliters of blood was divided equally, and each aliquot was mixed with 50 μg of either live VZV antigen ($5 \times 10^4$ PFU) or mock antigen in 50-mL Falcon tubes (cat. 352070, Corning, Mexico). Nine milliliters of RPMI medium 1640-GlutaMAX™ (cat. 61870036, Thermo Fischer Scientific, USA) supplemented with 10% fetal bovine serum (FBS; cat. 10099, Gibco, Australia) was added to each tube and mixed gently. The tubes were then incubated at an incline at 37 °C and 5% $CO_2$ for 9 days (Fig. 1a).

### 2.5. Sample treatment and staining for flow cytometry

For responder total CD4 memory cell (CD4-M) measurement, 1-mL samples were taken from each culture tube (VZV and mock) after pipetting to make a homogenous mixture. The samples were then centrifuged at 180*g* for 5 min. The supernatants were removed, and the cells were stained for 15 min at room temperature (25 °C) with the following cocktail of antibodies in normal saline containing 1.0% bovine serum albumin (BSA); anti-CD3-Allophycocyanin (APC) (cat. IM2467, Beckman Coulter, USA), anti-CD4- Phycoerythrin-Cyanin 7 (PC7) (cat. 6607101, Beckman Coulter, USA), anti-CD45RO-Phycoerythrin (PE) (cat. A07787, Beckman Coulter, USA), and anti-CD45RA-Fluorescein isothiocyanate (FITC) (cat. 304106, BioLegend, USA). The red blood cells were then lysed, and the remaining cells were fixed using Optilyse-C (cat. A11895, Beckman Coulter, USA) according to the manufacturer's protocol. The mixture was then centrifuged at 180*g* for 5 min and washed twice with 1 mL of PBS (–) to remove any unbound antibodies. The final pellet was suspended in 200 μL of PBS (–), transferred to 96-well plates, and the CD4-M proportion was measured on the flow cytometer.

For $CD4^{high}$-M subtype characterization and intracellular granzyme B staining of cells from five randomly selected adults, the following procedure was used. On the morning of day 9, the culture was mixed by gentle pipetting and 1 mL each from the VZV and Mock-stimulated cultures was transferred to other tubes. One microliter of brefeldin-A solution (cat. 420601, BioLegend, USA) was then added to each of the cultures and incubated for 6 h. The samples were centrifuged as described earlier, and the cells were stained for 15 min at room temperature with the following antibody cocktail in normal saline with 1% BSA: anti-CD3-BV785 (cat. 317330, BioLegend, USA), anti-CD4-PC7 (cat. 6607101, Beckman Coulter, USA), anti-CD45RA-BV421 (cat. 304129, BioLegend, USA), anti-CD45RO-PE (cat. A07787, Beckman Coulter, USA), and anti-CD197 (CCR7)-APC (cat. 353214, BioLegend, USA). The red blood cells were then lysed and the remaining cells fixed as described earlier. For intracellular granzyme B staining, the samples were first washed with PBS (–) and treated with intracellular stain and permeabilization solution (cat. 421002, BioLegend, USA) according to the manufacturer's protocol. The treated samples were stained with anti-granzyme B-FITC (cat. 515403, BioLegend, USA) in normal saline containing 1.0% BSA for 30 min on ice. Each sample was then centrifuged at 180*g* for 5 min and washed twice using 1 mL of PBS (–) to remove any unbound antibodies. The final pellet was resuspended in 200 μL of PBS (–), transferred to 96-well plates, and measured on the flow cytometer.

### 2.6. Flow cytometric analysis

Samples were analyzed using a CytoFlex instrument (Beckman Coulter, USA) that had been standardized with CytoFlex beads (Beckman Coulter, USA). Compensation was performed using CytExpert software version 2.3.0.84 (Beckman Coulter, USA) based on non-stained and single-stained samples and/or Veri-cells™ PBMC (cat. 76682, BioLegend, USA). The data obtained were analyzed using Kaluza software ver. 2.1 (Beckman Coulter, USA). Forward and side scatter excluded dead cells and debris.

The responder CD4-M population was identified by gating on $CD3^+$ $CD4^+$ and the classical memory marker, $CD45RA^-CD45RO^+$ [23]. Gating on $CD3^+CD4^{high}$ cells identified VZV antigen-specific CD4 cells, and their memory phenotype was confirmed using the same memory marker, $CD45RA^-CD45RO^+$. The numbers of CD4-M and $CD4^{high}$-M cells were calculated based on gating of more than 10,000 $CD3^+$ events.

The $CD4^{high}$-M population was further characterized based on $CCR7^-$ expression as the effector memory subtype and $CCR7^+$ as the central memory subtype. In addition, the $CD4^{high}$-M population was analyzed for intracellular granzyme B.

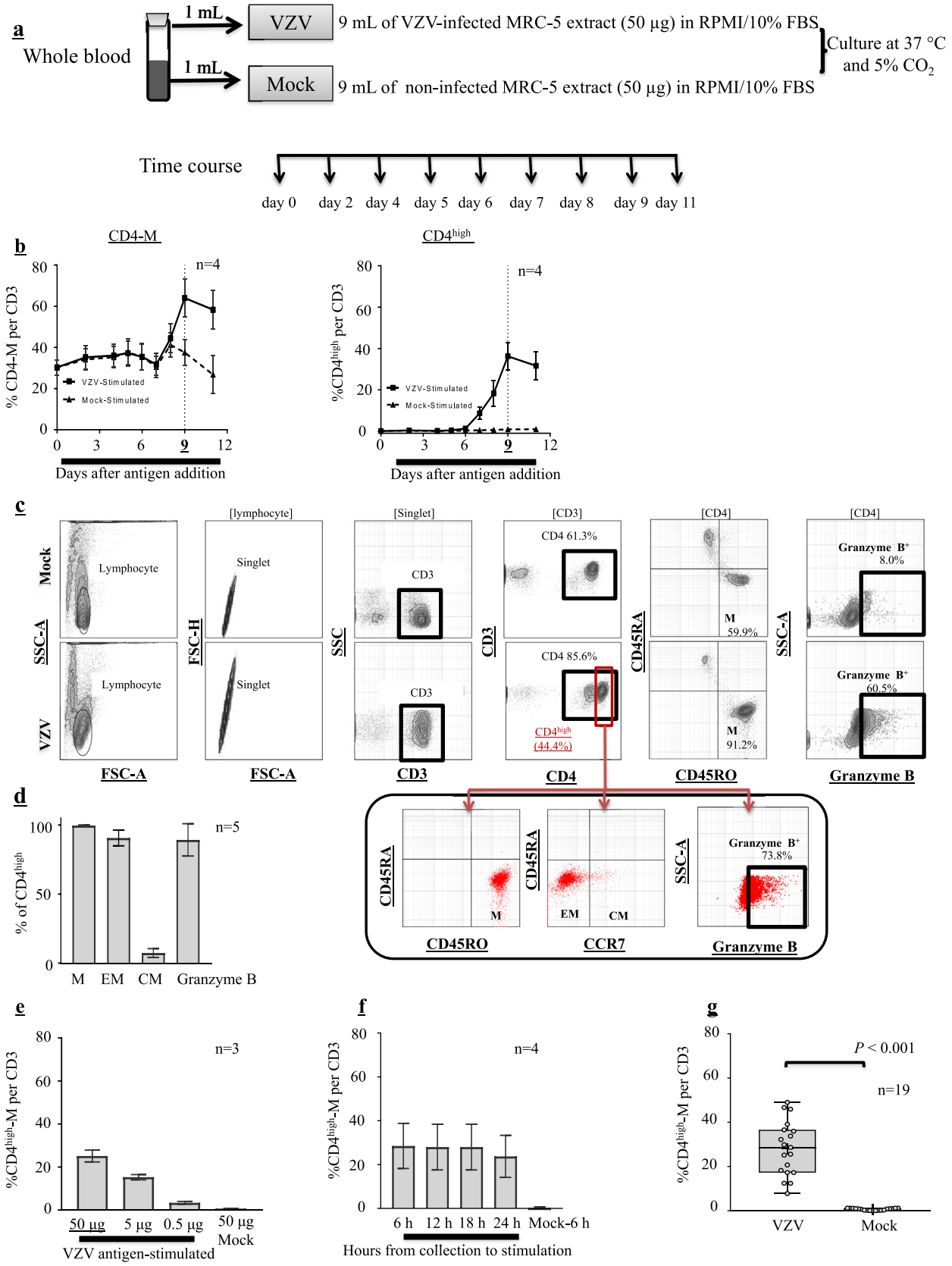
### 2.7. Statistical analysis

Statistical analyses and graphical representations were performed in GraphPad Prism version 7.0.2 for Windows (GraphPad Software, La Jolla, California, USA). Two-tailed parametric tests were used for the statistical analyses after verifying that the $CD4^{high}$-M proportions were normally distributed. To compare VZV-stimulated and mock-stimulated cultures from the same sample, a paired student T-test was used. To compare naïve samples and varicella-immune samples, an ordinary one-way ANOVA and Tukey's multiple comparison tests with a single pooled variance were employed. Finally, to compare pre-vaccine and post-vaccine samples at three different time points, we used repeated measures ANOVA and Tukey's multiple comparison tests, with variances computed for each individual. Significant differences were identified by $P$-values $\leq 0.05$.

## 3. Results

### 3.1. Establishment of the whole blood cell culture condition

VZV-specific memory T cells are infrequent in the circulating blood, and their direct measurement is difficult and requires large blood volumes [12]. We proposed measuring their proliferative response in whole blood instead. This is because whole blood assays have the advantage of being fast to perform, with more natural conditions that retain all blood components and maintain cells in their *in vivo* ratios. In the literature, previous whole blood-based assays showed varying stimulation periods that depended on the antigen, measured components, and culture conditions [18,24–28]. To determine the best time interval to measure the level of responder CD4-M proliferation, we performed a time-course analysis using blood from four donors with a history of varicella, but not HZ. Two milliliters of blood from each donor was equally divided and treated with either VZV antigen (live Oka virus) or mock antigen. The mixtures were then cultivated for 11 days, and the cultures were sampled at 0, 2, 4, 5, 6, 7, 8, 9, and 11 days (Fig. 1b). The responder CD4-M proportion was analyzed for more than 10,000 CD3 (as T cell marker) events in each sample. CD4-M was distinguished using the classical memory marker $CD45RA^-$-$CD45RO^+$. Flow cytometry analyses showed that the proportions of CD4-M in VZV antigen-stimulated samples started to increase on day 7, were the highest on day 9, and then declined on day 11. On the other hand, the mock antigen-stimulated samples showed no apparent change in CD4-M proportion (Fig. 1b, left panel). Additionally, we observed a specific memory population in the VZV-stimulated cultures having a $CD3^+CD4^{high}CD45RA^-$ $CD45RO^+$ phenotype (Fig. 1c). The $CD4^{high}$ population showed a


a
Whole blood
1 mL
VZV
9 mL of VZV-infected MRC-5 extract (50 µg) in RPMI/10% FBS
1 mL
Mock
9 mL of non-infected MRC-5 extract (50 µg) in RPMI/10% FBS
Culture at 37 °C and 5% CO2
Time course
day 0 day 2 day 4 day 5 day 6 day 7 day 8 day 9 day 11
b
CD4-M
CD4high
n=4
% CD4-M per CD3
%CD4high per CD3
VZV-Stimulated
Mock-Stimulated
Days after antigen addition
c
Mock
VZV
SSC-A
FSC-A
Lymphocyte
[lymphocyte]
Singlet
FSC-H
[Singlet]
CD3
SSC
[CD3]
CD4 61.3%
CD4 85.6%
CD4high (44.4%)
CD4
[CD4]
CD45RA
CD45RO
M 59.9%
M 91.2%
Granzyme B+ 8.0%
Granzyme B+ 60.5%
Granzyme B
d
% of CD4high
n=5
M EM CM Granzyme B
CCR7
EM
CM
Granzyme B+ 73.8%
e
%CD4high-M per CD3
n=3
50 µg 5 µg 0.5 µg 50 µg Mock
VZV antigen-stimulated
f
n=4
6 h 12 h 18 h 24 h Mock-6 h
Hours from collection to stimulation
g
P < 0.001
n=19
VZV
Mock

similar trend in growth as CD4-M and was most distinct on day 9 (Fig. 1b right panel). This population was detected in the VZV antigen-stimulated samples, but not in the mock antigen-stimulated correlates. Therefore, we chose to measure the CD4-M proliferation levels on day 9 of culture.

### 3.2. Characterization of $CD4^{high}$ cells

We then characterized this responder $CD4^{high}$ population using anti-CCR7 to determine the memory subtype of the proliferated cells and anti-granzyme B antibodies to determine their direct cytotoxic ability. Blood from another five adult donors was used and analyzed on day 9. As expected, the $CD3^{+}CD4^{high}$ population was also specific to VZV- antigen-stimulated cultures. This population had the $CD45RA^{-}CD45RO^{+}$ phenotype (Fig. 1c and d). The majority of these memory cells also had the CD4 effector memory phenotype $CCR7^{-}$ and stained positive for granzyme B (Fig. 1c and d).

We concluded that the $CD4^{high}$ population had the memory cell phenotype and proliferated in response to VZV antigen ($CD4^{high}$-M). The majority of the $CD4^{high}$-M population belonged to the effector memory subtype. In addition, the majority of $CD4^{high}$-M had cytotoxic attributes. We then proposed that the $CD4^{high}$-M population had the potential to be used to measure VZV CMI.

### 3.3. $CD4^{high}$-M proportion shows VZV antigen dose-dependent proliferation response

To confirm the antigen specificity of the $CD4^{high}$-M cells, we attempted the assay with three different VZV-antigen doses (50, 5, and 0.5 μg) and 1 mock-antigen dose (50 μg) on day 9 of culture using blood from another three adult donors. We then measured the $CD4^{high}$-M cell proliferation level as described earlier.

Flow cytometry analyses revealed VZV antigen dose-dependent proliferation of $CD4^{high}$-M cells (Fig. 1e). The results suggested that the measured $CD4^{high}$-M proportions were VZV antigen-specific.

### 3.4. Evaluating the effect of time from blood sampling to stimulation of VZV responder $CD4^{high}$-M cells on day 9

It is often not feasible to perform the flow cytometry assay immediately after blood sampling. To investigate the functional stability of whole blood, we attempted the assay using blood from four different adult donors. We then allowed the blood to stand for 6, 12, 18, 24, and 48 h at 25 °C before starting the assay. Using repeated measures ANOVA and Tukey's multiple comparison tests, we found that the measured proportions of $CD4^{high}$-M showed no statistical distinction until 24 h (Fig. 1f). In 48-h samples, we could not determine the $CD4^{high}$-M proportion, and many aggregated and dead cells were observed by flow cytometry (data not shown). We then decided to perform the assay within 24 h of blood sampling. Finally, the $CD4^{high}$-M proportions in all adult samples used to optimize the assay were characterized (Fig. 1g). The minimum and maximum values of the $CD4^{high}$-M proportions obtained in the VZV-stimulated samples were 7.7% and 48.9%, respectively. Meanwhile, the minimum and maximum values of the $CD4^{high}$-M proportions of the mock-stimulated samples were 0.05% and 1.2%, respectively.

### 3.5. Analysis of VZV responder $CD4^{high}$-M cells in naïve children, as well as three different age groups (children, young adults, and elderly) with a history of varicella infection

We then evaluated the validity of our assay in children (1–15 years old) who had or did not possess anti-VZV IgG antibodies, including one child with acute varicella. In addition, we assessed the age effect on proliferation of VZV responder $CD4^{high}$-M cells by comparing the children's data to those of young adults (28–45 years old) and the elderly (>50 years old).

The maximum and mean measured proportions of VZV responder $CD4^{high}$-M cells in naïve children (1–11 years old, n = 12) were 1.0% and 0.6% (95% CI [0.4, 0.9]), respectively. Meanwhile, the measured VZV responder $CD4^{high}$-M proportions were significantly higher in all children ($P < 0.0001$), young adults ($P < 0.0001$), and elderly ($P < 0.0001$) with a history of varicella infection (Fig. 2).

Regarding the effect of age on the proliferated VZV responder $CD4^{high}$-M cells, young adults (n = 18) showed a significantly higher ($P = 0.006$) mean (28.1%, 95% CI [22.2, 34.0]) than children with anti-VZV IgG antibody (2–15 years old, n = 19) (16.7%, 95% CI [11.9, 21.9]). The elderly (>50 years old, n = 80), on the other hand, showed a significantly lower mean ($P = 0.001$) $CD4^{high}$-M cell proportion (9.5%, 95% CI [7.5, 12.0]) than young adults (Fig. 2). In addition, the child with acute varicella showed a VZV responder $CD4^{high}$-M proportion equivalent to 0.9% in both mock and VZV-stimulated samples.

These results suggest that VZV responder $CD4^{high}$-M cell proliferation correlates well with a history of varicella in children, adults, and the elderly. We next proposed that VZV responder $CD4^{high}$-M cells are suitable for quantifying VZV-specific CMI.

### 3.6. Proliferation of VZV responder $CD4^{high}$-M cells is boosted in elderly 2 months after VZV vaccination and this increase was maintained for at least 1 year

We next evaluated whether we could detect varicella zoster vaccine-induced CMI based on the level of VZV responder $CD4^{high}$-M cell proliferation. Blood samples were collected from 80 elderly donors (>50 years old) at baseline (vaccination day) and 2 months and 1 year after vaccination. We then measured the proliferation level of VZV responder $CD4^{high}$ -M cells using flow cytometry.

A significant increase ($P = 0.0001$) in the measured proliferation level of VZV responder $CD4^{high}$-M cells was apparent 2 months

**Fig. 1.** Assay setup and optimization. a. Two milliliters of whole blood was divided into two equal portions and treated with either VZV or mock antigen (50 μg). Nine milliliters of RPMI/10% FBS was then added, and the total mixture was cultivated at 37 °C and 5% $CO_2$. b. Line chart with time course data showing CD4-M and $CD4^{high}$ proportions in whole blood from four adult donors after VZV and mock stimulation. Both VZV and mock-stimulated cultures were sampled at days 0, 2, 4, 5, 6, 7, 8, 9, and 11. Lines connecting the mean values and error bars represent standard deviations. c. Example of flow cytometric data of VZV and mock-stimulated cultures at day 9 that shows the gating strategy to identify CD4-M and VZV antigen-specific $CD4^{high}$-M cells. Characterization of the $CD4^{high}$ population in VZV-stimulated cultures at day 9 according to CCR7 and intracellular Granzyme B expression. M = CD4 memory cells ($CD45RA^{-}RO^{+}$), EM = effector memory cells ($CCR7^{-}$), and CM = central memory cells ($CCR7^{+}$). d. Bar plot showing a summary of the characterized $CD4^{high}$-M proportions at day 9 in VZV-stimulated cultures using blood from five adult donors. The mean and error bars represent standard deviations. e. Bar plot showing $CD4^{high}$-M proportions after stimulation with decreasing doses of VZV antigen (50, 5, and 0.5 μg) in comparison to mock antigen (50 μg) at day 9 using blood from three adult donors. The mean and error bars represent standard deviations. f. Bar plot showing day 9 $CD4^{high}$-M proportions in cultures stimulated with VZV antigen 6, 12, 18, and 24 h after collection from four donors compared to their 6-h mock-stimulated correlates. The mean and error bars represent standard deviations. g. Box plot with error bars showing minimum and maximum values of $CD4^{high}$-M cell proportions at day 9 of culture in VZV and mock-stimulated samples from 19 adults, including those from all the 16 adults used to establish the assay. *P*-values were estimated using paired T-test to compare mock and VZV-stimulated samples. Significant differences were identified by *P*-values $\leq 0.05$.

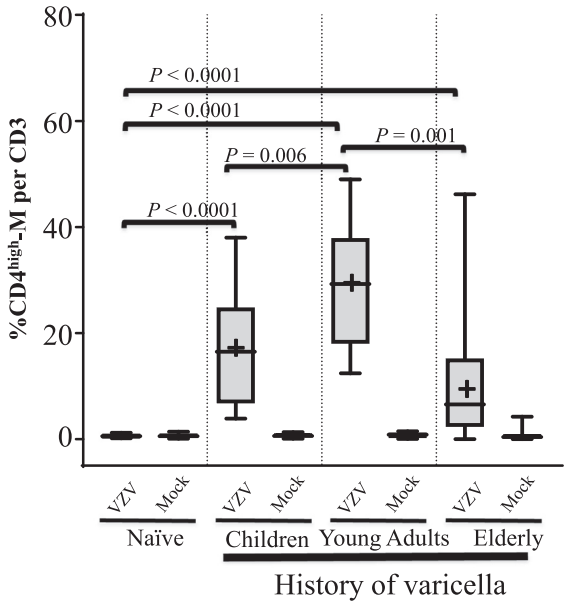



**Fig. 2.** CD4$^{high}$-M proportions in naïve children and in children (2–15 years old), young adults (28–45 years old), and elderly (>50 years old) with a history of VZV infection. Box plot showing CD4$^{high}$-M proportions at day 9 in VZV and mock-stimulated cultures of samples from 12 naïve children (1–11 years old) who were negative for VZV IgG antibody and in 19 children (2–15 years old), 18 young adults (28–45 years old), and 80 elderly with history of VZV infection. Mean values are shown with a '+' sign. Error bars represent the minimum and maximum values measured. *P*-values were estimated using an ordinary one-way ANOVA and Tukey's multiple comparison test with a single pooled variance to compare the difference in means of CD4$^{high}$-M proportions in VZV-stimulated samples belonging to different groups. Significant differences were identified by *P*-values ≤ 0.05.

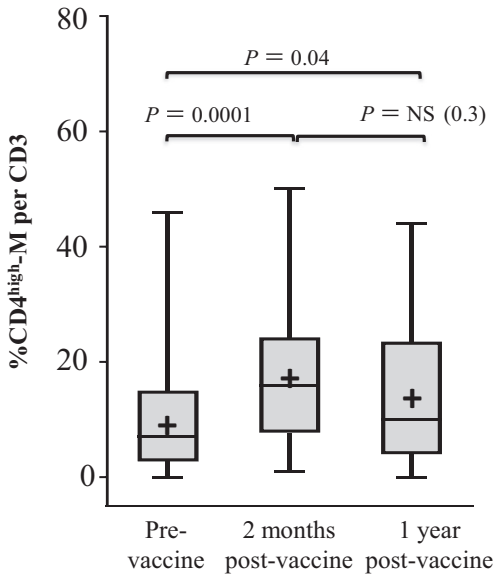



**Fig. 3.** CD4$^{high}$-M proportions in the elderly 2 months (2 M) and 1 year (1Y) after varicella zoster vaccination. Boxplot showing CD4$^{high}$-M proportions at day 9 in VZV-stimulated cultures of samples from 70 elderly participants (>50 years old) at three different time points. Mean values are shown with a '+' sign. Error bars represent the minimum and maximum values measured. *P*-values were estimated using a repeated measures ANOVA and Tukey's multiple comparisons test, with individual variances computed for each individual to compare the difference in means of CD4$^{high}$-M proportions in VZV-stimulated samples from the same participant group. Significant differences were identified by *P*-values ≤ 0.05.

after vaccination (mean 17.1%, 95% CI [13.9, 20.3]) compared to baseline data (mean 9.5%, 95% CI [7.5, 12.0]). This increase in VZV responder CD4$^{high}$-M proliferation was maintained ($P = 0.04$) 1 year later (mean 14.4%, 95% CI [11.5, 17.9]) (Fig. 3).

## 4. Discussion

Anti-VZV CMI is known to be important for protection against HZ. Although there have been many attempts to quantify VZV-specific CMI, correlates of HZ protection have not been established because of difficulties with available assays (blood volume, PBMC separation, storage, experiment establishment, etc.). Here, we introduced a simple method to quantify VZV-specific CMI by measuring the proportion of VZV responder CD4-M using 1 mL of blood, making this assay suitable when blood volume is limited (e.g., from children). This method is easy to interpret and can be used to differentiate between varicella immune and varicella non-immune children.

In the assay setup stage, we scanned the literature and found that stimulation of at least $2 \times 10^5$ PBMCs per antigen for a total of $1 \times 10^6$ cells per test is needed for reproducible results in ELISPOT assays [14,29]. However, to obtain these PBMCs, 10 mL of whole blood is needed [14,29]. Although whole blood is known to contain $10^6$ PBMCs per mL, 10 mL samples were obtained for fear of losing PBMCs during separation, handling, or storage [14,30,31]. We proposed that 1 mL of blood would be sufficient for a whole blood assay, as no such loss of PBMCs is expected. Both live and inactivated VZV antigens have been used for PBMC stimulation [6,14,32]. However, stimulation with live antigen resulted in a better cytotoxic T lymphocyte (CTL) response [15]. Both the Oka strain and low-passage clinical isolates were reported to infect PBMCs at a range as low as 1 in 10,000–100,000 PBMCs [33]. The Oka strain (used here), however, was reported to be less efficient in transmission to melanoma cells compared to other clinical isolates [33]. In addition, isolated dendritic cells and NK cells have been reported to be permissive for low-passage clinical isolates of VZV [34,35]. Moreover, VZV-infected dendritic cells have been reported to transfer VZV to autologous T cells [34]. Furthermore, CD4 CTLs were detected after PBMC stimulation using autologous lymphoblastoid cells infected with live VZV [36]. We thought of using live Oka VZV stimulation of whole blood, which contains many immune cells, to mimic immune responses to VZV that occur *in vivo*. Based on different reports, we chose a 1/10 dilution for long-term culture of whole blood in medium [18,24,37]. Conventional methods to measure CMI depend on calculating the stimulation index (stimulated/mock). However, in our case, CD4$^{high}$-M proliferation was either absent or less than 1% in mock-stimulated samples, thus limiting the need for the stimulation index. A net proportion (VZV-mock) measure was then suggested; however, we preferred using the proportions obtained directly from only VZV-stimulated samples, as no difference was observed, and this approach is direct and simple.

Our results are in agreement with prior observations made using both T cell proliferation and ELISPOT assays highlighting that VZV antigen-responding cells are CD4 cells [6–9] with an RO phenotype [11,16] and cytotoxic ability [8]. Of particular interest is the child with acute varicella infection, whose blood sample showed a CD4$^{high}$-M proportion equivalent to that in naïve children, justifying a cut-off value of 1% and validating the specificity of our method for memory cells. Additionally, our data on the VZV CMI age effect showed that young adults had significantly higher VZV responder CD4$^{high}$-M proportions than children. This difference between young adults and children is logical and consistent with the published hypothesis that adults have experienced more environmental exposures and asymptomatic VZV reactivations during their life span than children [38]. Furthermore, the significantly lower proportions of CD4$^{high}$-M in elderly (>50 years-old) vs. young

adult samples are also consistent with previous reports of PBMC-based assays showing a decline in CMI in the elderly [3,38]. Many studies using PBMC-based methods have shown that VZV vaccination elicits VZV CMI [11,36,39,40]. In contrast to previous publications using ELISPOT assays and showing a decrease in CMI 1 year after vaccination [11,41], the statistical analyses of our data showed that the vaccine boosting effect was still detected 1 year post-vaccination. This result is consistent with a previous report using a long-term-stimulation RCF assay [39]. It has been reported that in long-term assays, there is an expansion of antigen-specific cells that increases the sensitivity of the assay [42]. Moreover, using whole blood culture in our current method gave us the advantage of a lower background compared to other assays, which also increases the assay sensitivity.

We showed that VZV-specific responding memory cells have a $CD4^{high}$ phenotype. Using influenza-specific peptide-stimulated PBMCs, a $CD4^{high}$ phenotype was also observed, indicating antigen-specific cells with cytotoxic attributes [43]. Moreover, we believe that our methodology can be applied to other viruses, in cases where cytotoxic CD4 T-cell proliferation has been reported as crucial for antivirus activity. These viruses might include human immunodeficiency virus type-1, cytomegalovirus, Epstein-Barr virus, hepatitis B virus, human papilloma virus, and others [44]. The cytotoxic effector mechanisms of CD4 are reported to involve Fas ligand, perforins, and granzymes [44]. Evidence in the literature supports the opinion that cytotoxic CD4 T cells are part of the immune response and are not an insignificant laboratory artifact. However, the real function and significance of cytotoxic CD4 cells *in vivo* is still unknown, because their role has always been underestimated when compared to that of CD8 cells [45].

To summarize, we developed a simple method to quantify VZV-specific CMI by measuring the level of VZV-antigen responder $CD4^{high}$-M cell proliferation in whole blood cultures. Our method is specific, easy to interpret, can be applied to children, and correlates well with varicella history. In addition, our method allowed for detection of VZV vaccine-induced CMI. Finally, we believe that the broad applicability of our method might help to define HZ correlates of protection in the future.

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## Conflict of interest

Haredy A.M., Kosaka M., Hirota K., Koketsu R., Ikuta K., Yamanishi K., and Ebina H. are employees of the Research Foundation for Microbial Diseases of Osaka University (BIKEN). Takei M., Iwamoto S., Ohno M., and Okuno T. have no funding or conflicts of interest to disclose.

## Authorship

Haredy A.M. designed and performed the experiments, analyzed the data, and drafted the manuscript. Takei M., Iwamoto S., and Ohno M. explained the experiments to the volunteers, obtained informed consent, and sampled blood from the elderly. Okuno T., in collaboration with Dr. Koichi Baba (deceased), collected blood from children after explaining the experiment and obtained informed consent from their legal guardians. Kosaka M., Hirota K., and Koketsu R. performed the experiments and helped draft the manuscript. Ikuta K., Yamanishi K., and Ebina H. participated in designing the experiments and critically reviewing the manuscript.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.07.068.

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