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Development of Virus-Specific CD4⁺ T Cells on Reexposure to Varicella-Zoster Virus

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Immunity to childhood diseases is maintained for decades by mechanisms that, at present, are still unclear. We longitudinally studied immune responses in 16 adults exposed to children experiencing varicella (chicken pox). None of the individuals showed clinical signs of infection, and varicella-zoster virus (VZV) DNA could not be detected in peripheral blood or cultured from nasopharyngeal swabs. Exposure to VZV, however, induced expansion of antigen-specific CD4⁺ T cells in peripheral blood, with concomitant changes in cytotoxic CD8⁺ T cells and natural killer cells. VZV-specific memory CD4⁺ T cells were uniformly CD45RA⁻ and enriched for CD27⁻ cells. The virus-specific cells produced interferon- γ , tumor necrosis factor- α , and interleukin-2. These memory responses to VZV were compared with the primary immune responses of children experiencing varicella. VZV-specific memory CD4⁺ T cell responses largely resemble the primary immune response to VZV.

Immunological memory is the key feature of the adaptive immune system and provides improved protection on reinfection. This is not due to only an increased frequency of antigen-specific T and B lymphocytes, but also the adaptation of these cells to particular pathogens [1, 2]. Maintenance of immunological memory is the basis for the existence of typical childhood diseases. The viruses that cause these diseases are generally highly infectious, and the majority of the population is infected at a young age. Protection against these viruses is usually maintained throughout life.

During acute infection, CD4⁺ T cells expand and differentiate from naive to effector and memory cells [3, 4]. The differentiation of virus-specific CD4⁺ T helper cells can be defined in 2 ways. First, on the basis of the expression of isoforms of CD45, effector cells (CD45RA⁺CD45R0⁺) and memory cells

(CD45RA⁻CD45R0⁺) are defined [3]. Second, production of cytokines distinguishes Th1 cells (synthesizing interferon [IFN]- γ and tumor necrosis factor [TNF]- α) from Th2 cells (synthesizing interleukin [IL]-4, IL-5, IL-10, and IL-13) [5]. During primary infection, virus-specific CD8⁺ T cells differentiate from naive (CD45RA⁺CD27⁺CCR7⁺) to effector (CD27⁺CCR7⁻) cells [6–8], with a concomitant massive expansion [9, 10]. During the latency stage, virus-specific T cells may have distinct phenotypes, ranging from CD27⁺CCR7⁻ to CD27⁻CCR7⁻ [11].

Current hypotheses on the requirements for maintenance of immunological memory are largely based on experimental mouse models and postulate homeostatic proliferation, boosting by antigens, or cross-reactivity as key factors [12–16]. Data from mouse models showed that the frequency of virus-specific memory cells is proportional to the initial clonal burst size [17, 18]. The numbers of memory CD4⁺ T cells seem to decline, whereas numbers of memory CD8⁺ T cells remain constant over time [19]. Virus-specific memory T cells appear to be partially maintained through cytokine-driven homeostatic proliferation. Memory CD8⁺ T cells require IL-15 to persist, whereas the cytokines required for persistence of memory CD4⁺ T cells are still not defined [20–24]. In addition, efficient memory

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responses depend on production of immunoglobulin by long-lived plasma cells (located in the bone marrow) and memory B cells [25, 26]. Virus-specific IgG may persist because of regular contact of memory B cells with immune complexes on the surface of follicular dendritic cells in germinal centers [15].

Little is known about maintenance of long-term immunological memory in humans. Memory responses studied in mice as early as 30 days after primary infection are, at best, only partially comparable to memory responses in humans, which last for decades. The classic example of individuals on the Faeroe Islands, who were protected from measles virus infection >65 years after primary infection [27], led to the hypothesis that memory can be maintained in an antigen-independent manner. Nonetheless, recent data suggest that measles virus may exist in a crippled form in the host and, thus, may restimulate measles-specific T cells [28].

Varicella-zoster virus (VZV) is a highly contagious α -herpesvirus that causes varicella (chicken pox) during primary infection. After primary infection, the virus develops latency in sensory ganglia, from where it can reactivate later in life, causing herpes zoster (shingles). Healthy individuals are protected from clinical symptoms of varicella on reinfection, for a lifetime. We studied the VZV-specific memory response in latently infected adults on subclinical exposure to antigen through contact with children with varicella. Comparison of secondary VZV-specific CD4⁺ T cell responses in adults with primary immune responses in children to VZV revealed, remarkably, great similarities in the kinetics of virus-specific cells.

SUBJECTS, MATERIALS, AND METHODS

Donors. To study secondary immune responses to VZV, 16 VZV-IgG-seropositive adult donors were selected from healthy laboratory workers and from parents of patients of the Emma Children's Hospital of the Academic Medical Center. All donors were selected on the basis of household exposure to their child (and, in 1 case, grandchild) with acute-stage varicella. Donor characteristics are shown in table 1. All donors had experienced varicella at least 20 years before enrollment in the study. Heparinized peripheral blood samples were longitudinally obtained from these donors. The first blood sample was obtained within 3 weeks after the appearance of the exanthem in their child (index case). A single heparinized blood sample was obtained from 10 healthy adult control donors, to compare levels of VZV-specific CD4⁺ T cells on reexposure with the levels observed during latency.

To study primary immune responses to VZV, we selected children experiencing varicella who were admitted to the hospital. They were admitted because of suspected dehydration or prolonged fever. Since children experiencing varicella without underlying immunodeficiencies are seldom hospitalized, only 4 children experiencing primary VZV infection could be se-

Table 1. Donor characteristics.

Donor	No.	Sex, F:M	Age at study entry, mean \pm SD, years
Parents	15	9:6	35 \pm 4
Grandparent	1	1:0	71
Children with primary infection	4	2:2	3 \pm 1

lected. At least 3 blood samples were obtained from each donor, up to 1 year after the clinical course of varicella in the index case. Peripheral blood mononuclear cells (PBMCs) were isolated by use of standard density-gradient centrifugation techniques with Lymphoprep (Nycomed; Pharma). PBMCs were cryopreserved until use and thawed according to standard procedures. All longitudinal samples from each donor were analyzed simultaneously. The study was approved by the local medical ethical committee, and written, informed consent was obtained from the children or their parents.

Determination of VZV-specific CD4⁺ T cells by intracellular cytokine staining. VZV-specific and tetanus-specific CD4⁺ T cell frequencies were determined as described elsewhere [29]. In brief, PBMCs were stimulated for 6 h with VZV antigen (20 μ L/mL; Microbix Biosystems) or tetanus toxoid (TT) (17.6 Lf/mL; RIVM), the final 5 h in presence of brefeldin-A (10 μ g/mL). PBMCs were costimulated by CD28 (2 μ g/mL; CLB 15E8) and CD49d (1 μ g/mL; BD Biosciences). Next, cells were stained for CD4-peridinin chlorophyll protein (PerCP) Cy5.5, in combination with either CD27-phosphatidylethanolamine (PE) or CD45RA-PE (all BD Biosciences), permeabilized by use of the BD-FACS intracellular cytokine staining kit, and stained for IFN- γ -fluorescein isothiocyanate (FITC; BD Biosciences) and CD69-allophycocyanin (APC) (Caltag Laboratories). The CD4⁺CD69⁺IFN- γ ⁺ T cells were designated "antigen-specific" CD4⁺ T cells. The production of TNF- α and IL-2 by VZV-specific CD4⁺ T cells derived from reexposed individuals was determined in the donors after reexposure, by intracellular staining for IL-2-FITC or TNF- α -FITC (BD Biosciences), as described above for IFN- γ -FITC. Production of IL-2 and TNF- α by VZV-specific CD4⁺ T cells could not be determined in the children experiencing primary infection, because of the limited amount of material that could be obtained. Negative controls consisted of stimulation with medium, and positive controls consisted of stimulation with *Staphylococcus aureus* enterotoxin B (Sigma). To quantify the VZV-specific CD4⁺ T cells, background levels of staining were subtracted from the VZV-stimulated samples.

Determination of VZV-specific CD4⁺ T cells by use of IFN- γ ELISPOT analysis. VZV-specific CD4⁺ T cells, as defined by production of IFN- γ , were determined by use of ELISPOT (Mabtech). PBMCs were resuspended in RPMI 1640 medium containing 10% fetal calf serum (FCS) and antibiotics. For the

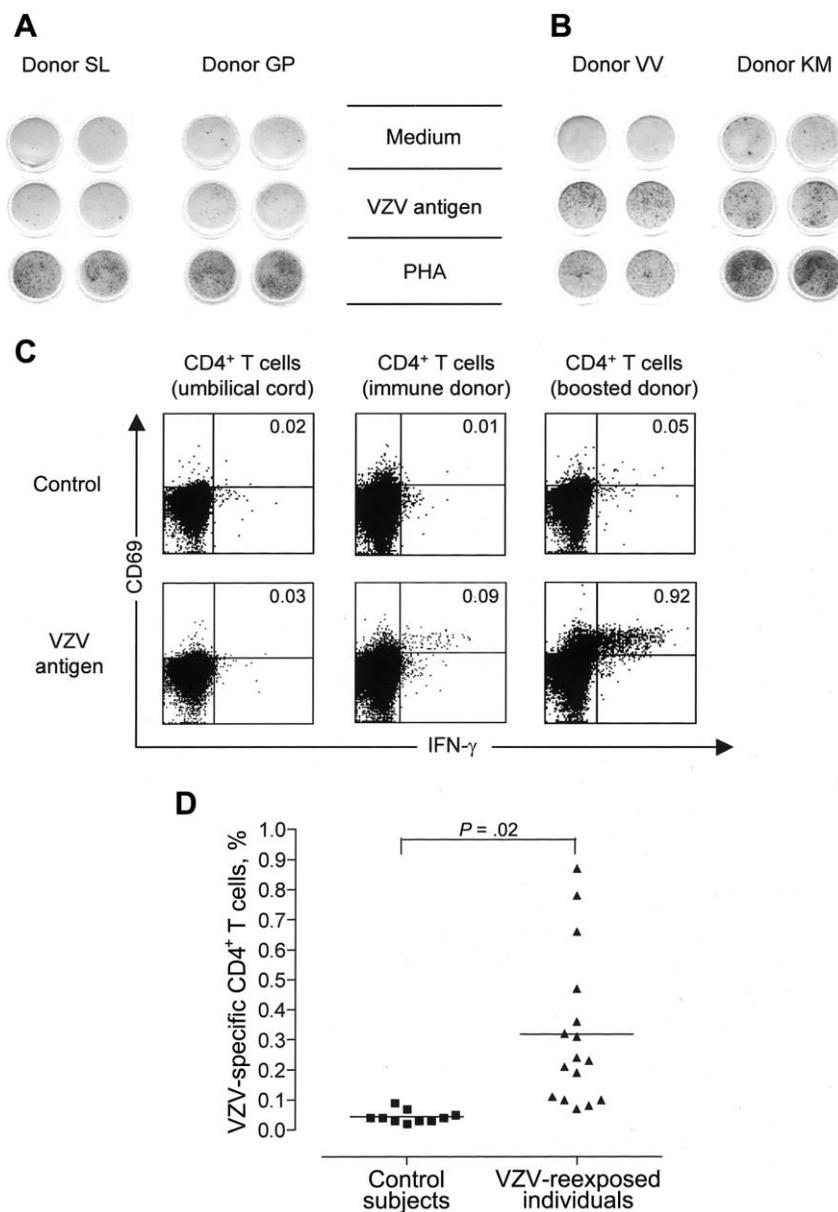


Figure 1. Visualization of peripheral blood varicella-zoster virus (VZV)-specific T cells. Interferon (IFN)- γ -producing T cells could be detected by ELISPOT analysis after restimulation by VZV antigen *in vitro*, at relatively high frequencies in peripheral blood samples from donors who had contact with varicella (*B*), whereas they were not detectable in peripheral blood samples from donors who did not have contact with varicella (*A*). Donor VV was the mother of a child with varicella. Donor KM was a pediatrician who had regular contact with varicella-infected children. *C*, VZV-specific CD4⁺ T cells (i.e., CD69⁺IFN- γ ⁺) could be visualized by intracellular cytokine staining, after restimulation with VZV antigen *in vitro*, in blood samples from immune donors but not in umbilical-cord blood ($n = 3$). Nos. indicate the percentage of CD69⁺IFN- γ ⁺ cells within the CD4⁺ T cell gate. Control, *in vitro* stimulation with medium; VZV antigen, *in vitro* stimulation with VZV antigen. *D*, Increased percentages of virus-specific cells were detected during the early phase after reexposure in boosted individuals, compared with the levels observed in control individuals without documented reexposure ($n = 10$) (0.32 ± 0.25 vs. 0.04 ± 0.02 ; $P = .02$). PHA, phytohemagglutinin.

VZV-specific ELISPOT, PBMCs were stimulated with VZV antigen ($20 \mu\text{L}/\text{mL}$), medium (negative control), or phytohemagglutinin (positive control; $20 \mu\text{g}/\text{mL}$; Murex Diagnostics), at 37°C and 5% CO_2 , in α -human IFN- γ -precoated silent-screen 96-wells plates (Nalge Nunc). PBMCs were cultured in final amounts of 250,000, 125,000, and 62,500 PBMCs/well. After

overnight stimulation, cells were removed by washing with PBS, and wells were incubated with biotin-labeled α -human IFN- γ (Mabtech) for 3 h at room temperature. Next, the wells were incubated with streptavidin-conjugated alkaline phosphatase (ALP) for 2 h at room temperature. Finally, spots were visualized by incubation with 5-bromo-4-chloro-3-indolyl phos-

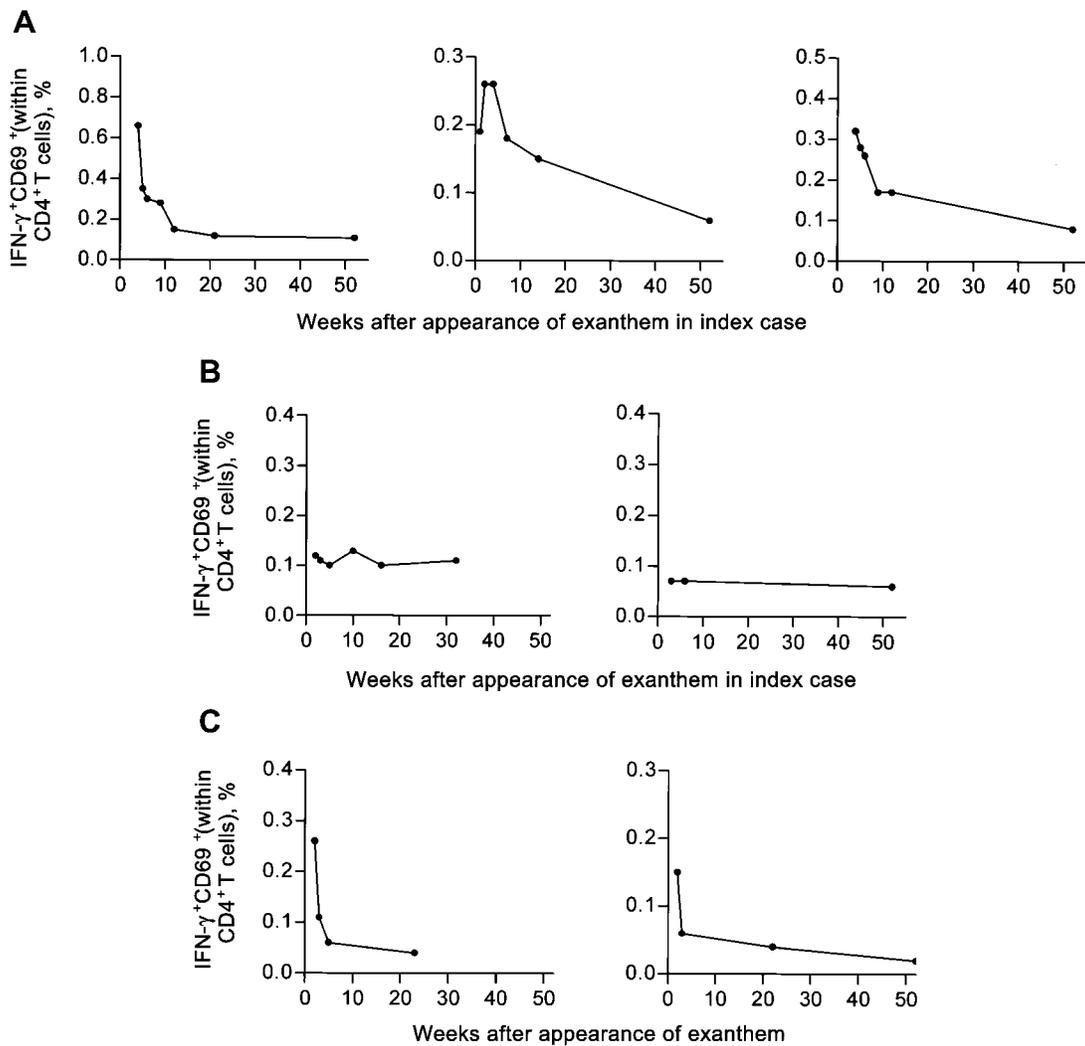


Figure 2. Kinetics of boosting of a human CD4⁺ T cell memory response. Varicella-zoster virus (VZV)-specific CD4⁺ T cells (CD69⁺interferon [IFN]- γ ⁺) could be visualized in peripheral blood by intracellular cytokine staining, after *in vitro* restimulation with VZV antigen. *A*, From the first time point studied, these cells were detected at relatively high frequencies in 11 of 16 donors, reaching peak levels within 4 weeks after the appearance of the exanthem in the index case. Thereafter, frequencies declined and slowly decreased further during the next months. Data shown are representative of 11 donors. The first graph represents the grandparent included in the present study. *B*, In 5 of 16 donors, boosting of VZV-specific CD4⁺ T cells could not be observed by the assay measuring production of IFN- γ . Data shown are representative of 5 donors. *C*, Similar kinetics of VZV-specific CD4⁺ T cells were observed in 2 children experiencing a typical primary VZV infection. Longitudinal analyses could not be performed for the other 2 children experiencing primary infection.

phate/nitro blue tetrazolium (Sigma). Spots were quantified by use of the AELVIS ELISPOT reader and software.

Immunofluorescent staining and flow cytometry. PBMCs were resuspended in RPMI medium containing 10% FCS and antibiotics. A total of 200,000 PBMCs were incubated with fluorescent label-conjugated monoclonal antibodies (MAbs) (concentrations in accordance with the manufacturer's instructions). NK cells were detected by staining with CD3-FITC (BD Biosciences), CD16-PE, and CD56-APC MAbs (Beckman Coulter). CD4⁺ T cell and CD8⁺ T cell subsets were defined by combinations of CD4-PerCP or CD8-APC with CD45RA-FITC and CD27-PE (all BD Biosciences).

Intracellular granzyme B (GrB) and perforin stainings were performed by incubating 500,000 PBMCs with fluorescent label-conjugated MAbs to CD8 and CD27 (BD Biosciences), washing them once, fixing them with 50 μ L of buffered formaldehyde-acetone solution, and subsequently permeabilizing them by washing with 0.1% saponin and 50 mmol/L D-glucose. Cells were then incubated with anti-GrB (Sanquin) and anti-perforin antibodies (Hözel Diagnostika), in accordance with the manufacturer's instructions. Analysis of cells was performed by use of a FACSCalibur flow cytometer and CellQuest software (both from BD Biosciences).

VZV-polymerase chain reaction (PCR). Quantitative PCR

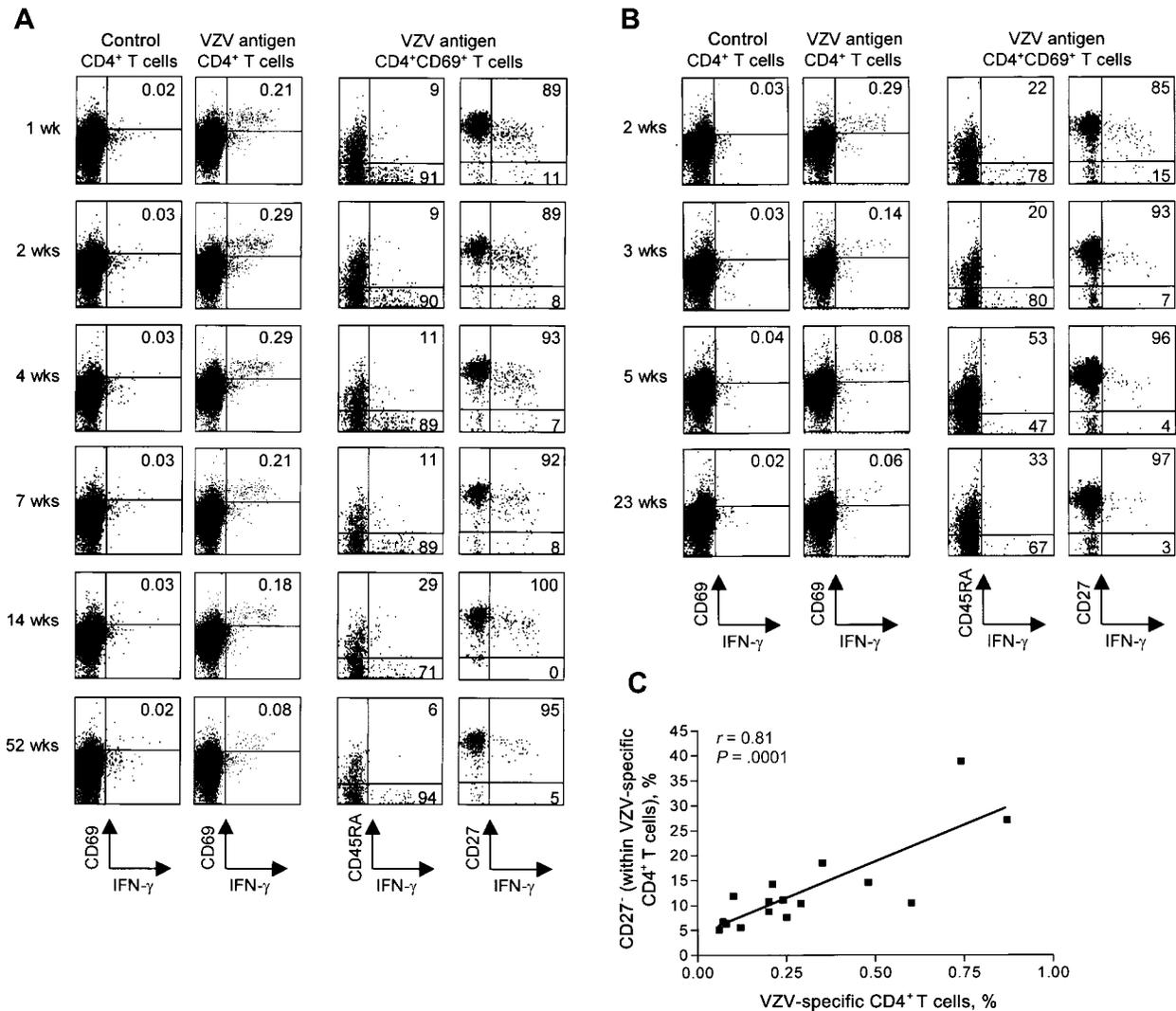


Figure 3. Phenotypic characterization of varicella-zoster virus (VZV)-specific CD4⁺ T cells during a memory and primary response. *A*, Boosting of VZV-specific CD4⁺ T cells (CD69⁺interferon [IFN]- γ ⁺) could be detected by use of the intracellular cytokine staining assay. Nos. in the first 2 columns of panels indicate percentages of CD69⁺IFN- γ ⁺ cells within the CD4⁺ T cell gate. The third column of panels shows the profile of CD45RA on CD4⁺CD69⁺ T cells on stimulation with VZV antigen; nos. indicate the proportion of CD45RA⁺ and CD45RA⁻ cells within the VZV-specific (i.e., IFN- γ ⁺) CD4⁺ T cells. The majority of VZV-specific cells during the memory response were CD45RA⁻, compared with bulk CD4⁺ T cells (data not shown). The fourth column of panels shows the profile of CD27 on CD4⁺CD69⁺ T cells on stimulation with VZV antigen; nos. indicate the proportion of CD27⁺ and CD27⁻ cells within the VZV-specific (i.e., IFN- γ ⁺) CD4⁺ T cells. The VZV-specific CD4⁺ T cells were enriched for a CD27⁻ phenotype during the early phase after reexposure, compared with bulk CD4⁺ T cells (data not shown). Graphs are longitudinal samples from 1 donor, representing 11 donors. Weeks (wks) after appearance of the exanthem in the index case are indicated. *B*, During acute VZV infection, virus-specific CD4⁺ T cells were enriched for a CD27⁻ phenotype and CD45RA⁻, whereas a larger proportion were enriched for CD45RA⁺. Graphs are longitudinal samples from 1 child experiencing primary VZV infection. Insufficient material was available to perform this analysis on the other children experiencing a primary infection. *C*, For each reexposed donor, the peak level of VZV-specific CD4⁺ T cells on reexposure was plotted against the proportion of CD27⁻ cells within this VZV-specific population. Peak levels of VZV-specific CD4⁺ T cells correlated to the percentage of CD27⁻ cells within this virus-specific population. Control, in vitro stimulation with medium; VZV antigen, in vitro stimulation with VZV antigen.

was performed in plasma samples, as described elsewhere [30]. The electrochemiluminescence signal was measured by use of an M-8 analyzer (IGEN).

Anti-VZV IgG. Anti-VZV IgG titers were determined in plasma, as described elsewhere [30]. VZV antigen was obtained

from Virion. Results are expressed as arbitrary units per milliliter of serum.

Statistics. The fraction of VZV-specific CD4⁺ T cells of the total CD4⁺ T cell count was plotted against the fraction of CD27⁻ cells within the VZV-specific CD4⁺ T cell compartment

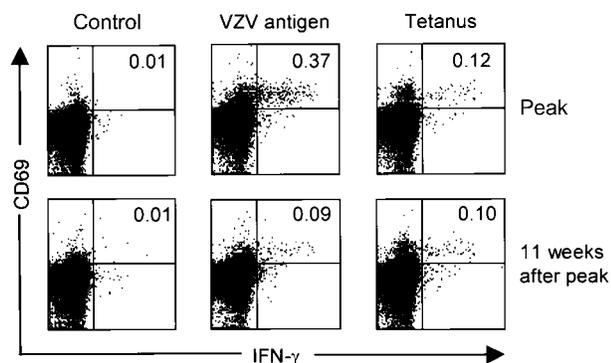


Figure 4. Antigen-specific boosting of varicella-zoster virus (VZV)-specific CD4⁺ T cells on reexposure. Reexposure to VZV may result in boosting of VZV-specific CD4⁺ T cells by antigen-specific mechanisms, since boosting of tetanus toxoid-specific CD4⁺ T cells could not be observed on reexposure to VZV. Data shown are from 1 donor, representing 9 donors in whom tetanus-specific CD4⁺ T cells could be detected. Dot-plots are gated on CD4⁺ T cells. Nos. indicate percentages of CD69⁺interferon- γ ⁺ cells within the CD4⁺ T cell gate. Peak, time point of highest percentage of VZV-specific CD4⁺ T cells observed.

and was analyzed by use of linear regression. The Student's *t* test was used to determine the significance of differences between study groups. *P* < .05 was considered to be statistically significant. All analyses were performed using SPSS (version 11.5; SPSS).

RESULTS

VZV-specific T cells are clearly detectable in reexposed healthy subjects. On validation of a method analyzing VZV-specific CD4⁺ T cell frequencies after natural VZV infection, PBMCs derived from 10 healthy laboratory workers were tested. In 8 of these donors, after stimulation with VZV antigen, VZV-specific T cells were detectable at very low frequencies by IFN- γ ELISPOT analysis, which is in accordance with the results of a study showing that VZV-specific memory T cells were hardly detectable in healthy VZV-seropositive donors [31] (figure 1A). In contrast, VZV-specific T cells were clearly detectable in 2 donors: a mother of a child experiencing acute varicella and a pediatrician who had regular contact with patients with varicella (figure 1B). We therefore postulated that (intimate) contact with a VZV-infected child results in boosting of the VZV-specific response and could, therefore, be used to study childhood disease-specific memory responses in humans, taking household contact as the most certain way of viral contraction.

Kinetics of VZV-specific CD4⁺ T cells. Infected children are already highly contagious (by nasopharyngeal shedding) during the last 4 days of the incubation period, before the appearance of the vesicular exanthem. None of the donors showed any clinical symptoms of varicella during the course

of the study, in contrast to the children experiencing varicella. VZV load was undetectable in the plasma of these donors at all time points studied, indicating that the virus is cleared before dissemination to the periphery, as is seen in primary infection, can take place. To standardize observations in the present study, the appearance of the exanthem in the index case was chosen as time 0. Since it is impossible to predict which individuals will be reinfected with VZV, donors were selected after household contact with a child experiencing varicella; thus, time points before reinfection were not available. After household contact with a child experiencing varicella, all donors tested (*n* = 16) had VZV-specific CD4⁺ T cells detectable, as a clear population of CD69⁺IFN- γ ⁺ cells, within the CD4⁺ T cell population in peripheral blood, after 6 h of *in vitro* restimulation with viral antigen (figure 1C). In contrast, this population could not be observed in naive T cells (i.e., umbilical-cord blood-derived CD4⁺ T cells). Levels of VZV-specific CD4⁺ T cells were significantly increased in the group of individuals who were reexposed to VZV (*n* = 16), compared with the levels in donors who were not reexposed (*n* = 10) (mean \pm SD, 0.34 \pm 0.26 vs. 0.04 \pm 0.02; *P* = .02; figure 1D).

At early time points after the appearance of the exanthem in the index case, VZV-specific CD4⁺ T cells were present at relatively high frequencies (0.21%–0.87%) in 11 of the 16 donors (figures 2A and 3A). Peak levels were reached within 4 weeks, followed by an early, steep contraction, up to week 6, and a slow decrease during the next months. One year later, VZV-specific CD4⁺ T cells were detectable in all donors tested, at levels of ~0.08% of the total number of CD4⁺ T cells. In contrast, percentages of tetanus-specific CD4⁺ T cells remained stable at all time points studied (figure 4). The kinetics of these memory responses resembled those of VZV-specific CD4⁺ T cell responses, as studied longitudinally in 2 of the children with a primary infection (figure 2C). High frequencies of VZV-specific CD4⁺ T cells were observed in all 4 children with a primary VZV infection, within 4 weeks after the appearance of the exanthem, which ranged from 0.10 to 0.23.

In 5 (31%) of the 16 donors, boosting of VZV-specific CD4⁺ T cell responses could not be detected by use of production of IFN- γ as a readout (figure 2B). Although a clearly distinct population of VZV-specific CD4⁺ T cells was present in peripheral blood samples from these individuals, the frequencies of this population remained stable at all time points studied (<0.13% of the total number of CD4⁺ T cells).

Boosted VZV-specific CD4⁺ T cells have a memory T cell phenotype. During a memory response, the VZV-specific CD4⁺ T cells were highly enriched for CD45RA⁻ cells (mean \pm SD, 84% \pm 10%; range, 67%–92%) (figure 3A) and for CD27⁻ cells (mean \pm SD, 13% \pm 9%; range, 5%–39%) (figure 3A), compared with bulk CD4⁺ T cells (data not shown). Dur-

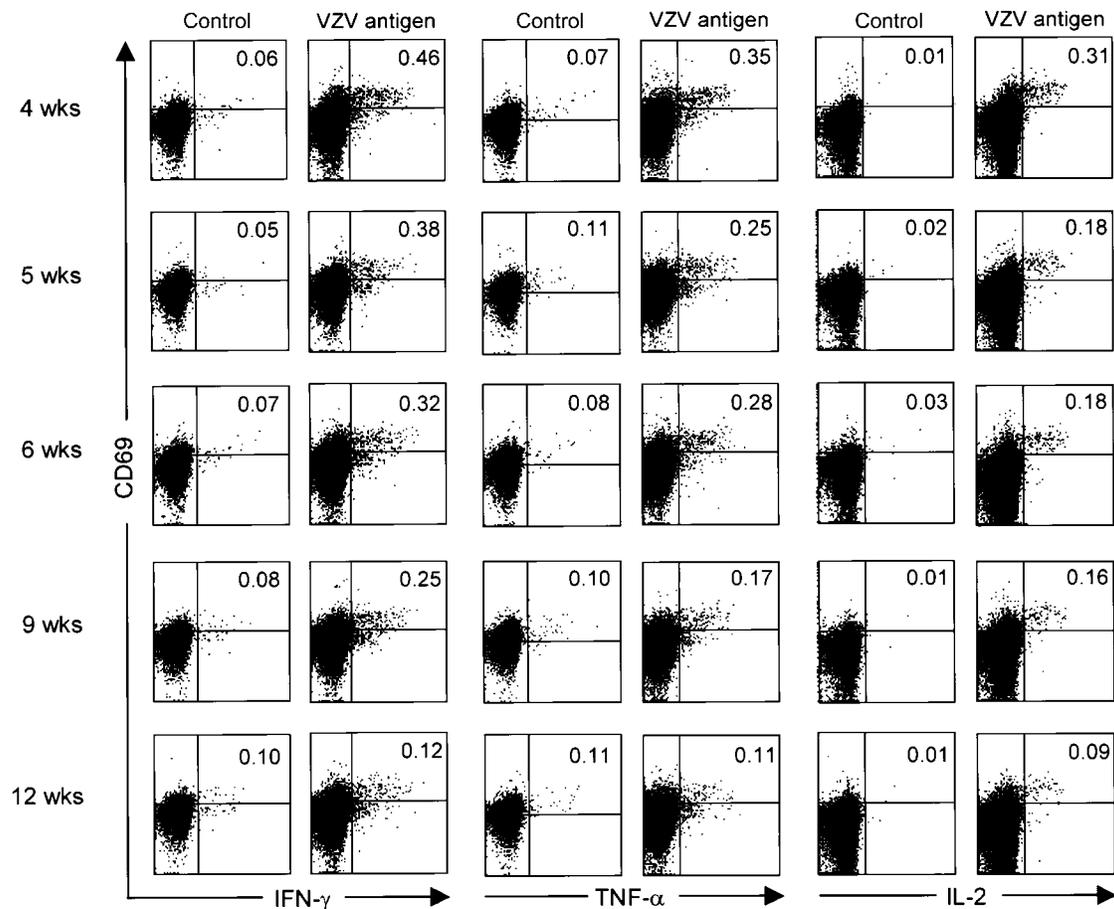


Figure 5. Cytokine production of varicella-zoster virus (VZV)-specific CD4⁺ T cells. VZV-specific CD4⁺ T cells were detected longitudinally in donors on reexposure to VZV. Weeks (wks) after appearance of the exanthem in the index case are indicated. Virus-specific CD4⁺ T cells could be detected by expression of interferon (IFN)- γ , tumor necrosis factor (TNF)- α , and interleukin (IL)-2. Data shown are from 1 donor, representing all reexposed donors. Summarized data from all donors are shown in table 2. Nos. indicate percentages of CD69⁺ cytokine⁺ cells within the CD4⁺ T cell gate. Control, in vitro stimulation with medium; VZV antigen, in vitro stimulation with VZV antigen.

ing acute VZV infection, a larger proportion of VZV-specific CD4⁺ T cells express CD45RA (figure 3B), whereas these VZV-specific CD4⁺ T cells have a similar expression of CD27 (figure 3C). Data could be obtained from only 1 child experiencing varicella. Because of limited availability of material, analysis of the phenotype of VZV-specific CD4⁺ T cells could only be performed for 2 of the children with a primary VZV infection.

Peak levels of VZV-specific CD4⁺ T cells for the reexposed donors (or the levels of these cells at the first time point for those donors who did not show boosting) were plotted against the fraction of CD27⁻ cells within this population. The higher the percentages of VZV-specific CD4⁺ T cells, the higher the proportion of CD27⁻ T cells within this population ($r = 0.81$; $P = .0001$) (figure 3C). Furthermore, mean fluorescence intensity of CD27 on the virus-specific cells was decreased in 7 (44%) of 16 donors. As suggested by previous studies of our group, the down-modulation of CD27 on the virus-specific CD4⁺ T cells is a result of recent interaction with the cellular

ligand CD70 [32, 33]. During latency (1 year after reexposure or primary infection), the VZV-specific CD4⁺ T cells returned to a CD27⁺ phenotype (i.e., they showed levels of expression of CD27 similar to those of the bulk of CD4⁺ T cells; figure 3A and 3B).

VZV-specific T cells produce IFN- γ , TNF- α , and IL-2. During antiviral immune responses, CD4⁺ T cells produce a range of cytokines. We analyzed the cytokine profile of VZV-specific memory CD4⁺ T cells, by measuring IFN- γ , TNF- α , and IL-2. VZV-specific production of IFN- γ , TNF- α , and IL-2 could be detected in CD4⁺ T cells from all donors (figure 5). The production of the different cytokines showed similar kinetics on reexposure (i.e., relatively high shortly after reexposure and declining thereafter). Data from peak responses of all reexposed donors are summarized in table 2. Because of limited availability of material, TNF- α and IL-2 staining of VZV-specific CD4⁺ T cells could not be performed on PBMCs from children experiencing primary VZV infection.

Table 2. Boosted varicella-zoster virus (VZV)-specific CD4⁺ T cells detectable by expression of interferon (IFN)- γ , tumor necrosis factor (TNF)- α , and interleukin (IL)-2.

Donor ^a	IFN- γ	TNF- α	IL-2
1	0.87	0.77	0.69
2	0.78	0.80	0.64
3	0.66	0.61	0.53
4	0.47	0.39	0.27
5	0.37	0.42	0.24
6	0.32	0.31	0.21
7	0.31	0.29	0.26
8	0.26	0.21	0.28
9	0.24	0.26	0.16
10	0.23	0.19	0.17
11	0.21	0.15	0.14
12	0.13	0.17	0.15
13	0.10	0.08	0.07
14	0.10	0.11	0.07
15	0.08	0.07	0.11
16	0.07	0.05	0.09

NOTE. Data are percentages of VZV-specific CD69⁺cytokine⁺ cells within the CD4⁺ T cell fraction and are derived from intracellular cytokine staining experiments.

^a Donors 1–11 showed boosting of VZV-specific CD4⁺ T cells.

CD8⁺ T cells and NK cells show kinetics similar to those of virus-specific CD4⁺ T cells. A comprehensive analysis of VZV-specific CD8⁺ T cell responses is hampered by the unavailability of valid VZV-derived immunodominant peptides for major histocompatibility complex class I. However, when markers for cytotoxicity of CD8⁺ T cells (i.e., expression of perforin and GrB) were studied longitudinally in our cohort, these cells showed similar kinetics to those of the VZV-specific CD4⁺ T cells, on reexposure, in the donors with VZV-specific CD4⁺ T cell boosting (figure 6A), which also held true for NK cells. Peak levels of GrB-expressing cells within the CD8⁺ T cell fraction ranged from 10% to 65%, whereas peak levels of perforin-expressing cells within this fraction ranged from 17% to 48%. Peak levels of NK cells (expressed as the proportion of CD16⁺ or CD56⁺ cells within the CD3⁺ cell fraction) ranged from 21% to 77%. In addition, the same results were obtained when cells from the children with a primary infection were studied (figure 6C). Peak levels of GrB-expressing cells within the CD8⁺ T cell fraction ranged from 20% to 79%, whereas peak levels of perforin-expressing cells ranged from 23% to 65%. Peak levels of NK cells ranged from 10% to 48%. Boosting of cytotoxic CD8⁺ T cells and NK cells could not be observed in the donors without VZV-specific CD4⁺ T cell boosting (figure 6B). The similar kinetics during the early phase after reexposure strongly suggests a role for these cytotoxic cells in the VZV-specific primary and memory responses.

Antibody titers. All donors in the present study were VZV-IgG positive at enrollment. As shown elsewhere, ~64% of individuals show a ≥ 4 -fold increase or decrease in VZV-specific IgG titers on reexposure to VZV [34]. In our cohort, in 8 (50%) of 16 donors, increases or decreases of at least 1.0 AU in VZV-IgG levels (boosting) were observed on reexposure (table 3). VZV-specific IgG levels measured in these donors ranged from 0.65 to 5.52 AU. Peak levels of VZV-IgG did not correlate to peak responses of VZV-specific CD4⁺ T cell responses (data not shown). However, boosting of VZV-IgG tended to be more common in the donors who also showed boosting of VZV-specific CD4⁺ T cells (donors 1–11; table 3). VZV-specific IgG levels measured in the children experiencing a primary VZV infection ranged from 1.34 to 4.60 AU.

DISCUSSION

The present study has provided a detailed analysis of a human virus-specific memory response. Boosting of VZV-specific CD4⁺ T cells was observed after reexposure of immune adults through household contact with children experiencing varicella. The detection of high levels of these cells on reexposure is probably a combination of proliferation and redistribution from regional lymph nodes at the site of infection to the blood. This phenomenon is antigen-specific and is not simply due to bystander-driven proliferation.

The majority of the VZV-specific CD4⁺ T cells in the secondary response, as detected by production of IFN- γ , had a memory T cell phenotype. During acute infection, a larger proportion of these cells express CD45RA, which has also been described to occur in cytomegalovirus (CMV) infection [3]. It is believed that the CD45RA⁺ cells are the first cells to appear in peripheral blood after primary infection, reflecting early migration from lymph nodes to target sites. The enrichment of CD27⁻ cells within the VZV-specific CD4⁺ T cell population and the concomitant down-modulation of CD27 on the CD27⁺ cells indicate recent interaction of this TNF receptor with its ligand, CD70, acting as a costimulus and providing signals for expansion and differentiation [35]. After the peak response, the proportion of virus-specific CD27⁻CD4⁺ T cells diminished. This could be a result of migration of these cells from the blood to target organs in the periphery or, alternatively, may reflect apoptosis of these cells after control of the virus. Finally, the VZV-specific CD4⁺ T cells may have reexpressed CD27 as a consequence of CD70 down-regulation on clearance of the antigen. Phenotypic characteristics of the VZV-specific cells, the majority being CD45RA⁻CD27⁺, resembled those of Epstein-Barr virus- and hepatitis C virus-specific CD4⁺ memory T cells [36, 37].

Similarities in the kinetics of GrB⁺ and perforin⁺ CD8⁺ T cells and NK cells to VZV-specific CD4⁺ T cells suggest a role for these cells in controlling the virus. Questionnaires were used to exclude subsequent contact with an individual experiencing

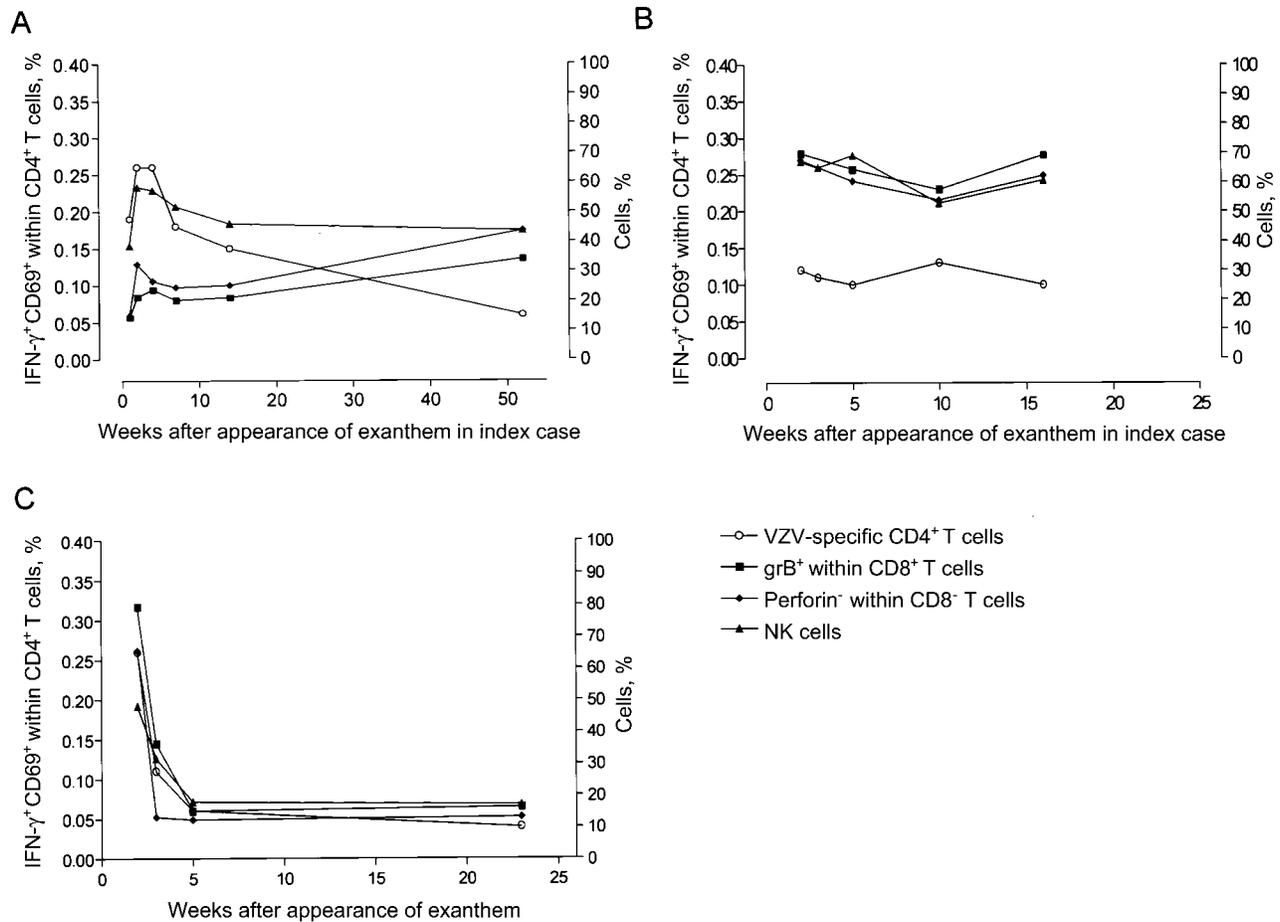


Figure 6. Kinetics of cytotoxic cells on reexposure to varicella-zoster virus (VZV). Data shown are from 1 donor, representing all donors. *A*, Frequencies of cytotoxic CD8⁺ T cells (perforin⁺/granzyme B [GrB]⁺; right Y-axis) showed similar kinetics to VZV-specific CD4⁺ T cells (left Y-axis) in boosted individuals, which also held true for NK cells (right Y-axis). *B*, Cytotoxic CD8⁺ T cells and NK cells remained at unchanged levels in the donors showing no boosting of VZV-specific CD4⁺ T cells. *C*, Comparable results were obtained with cells derived from a child experiencing a typical primary immune response to VZV.

varicella during follow-up, as well as contact with the most obvious intercurrent viral diseases, although previous asymptomatic contacts cannot be excluded.

In 5 of the reexposed donors studied, boosting of VZV-specific CD4⁺ T cells could not be detected by our assays. These assays are limited to the production of cytokines, which is only 1 functional aspect of virus-specific CD4⁺ T cells and, hence, may lead to an underestimation of the true frequencies of these cells. Factors such as recent reexposures to VZV outside of the household cannot be absolutely excluded. Thus, definite conclusions cannot be drawn.

In contrast to blood samples from the children experiencing varicella, VZV DNA was undetectable in blood samples from the reexposed adults at all time points studied, and the virus could not be cultured from nasopharyngeal swabs. During the memory response, the concerted action of neutralizing antibodies, primed virus-specific T cells, and NK cells may clear

the virus in the respiratory mucosa and regional lymph nodes before it can disseminate to the liver and spleen. This memory response is still effective at old age, since, in the present study, no differences in the responses of the grandparent were observed (figure 2A).

In contrast to experimental mice kept under pathogen-free conditions and infected and reinfected within 30 days with a particular virus, humans are infected with viruses early in life and are often reexposed >2 decades later. Furthermore, humans are subjected to sequential infections with a variety of viral pathogens. The clearest indication that maintenance of memory T cells may be antigen independent is their survival after adoptive transfer in mouse models [13, 14]. However, these mice are immunodeficient and, hence, provide indefinite space without competition for resources [15]. In contrast, when memory cells were transferred, at physiological frequencies, to hosts capable of repopulation from primary lymphoid tissue, in the

Table 3. Correlation of varicella-zoster virus (VZV)-specific antibody responses and virus-specific CD4⁺ T cells.

Donor ^a	Maximum VZV-specific CD4 ⁺ T cells, % ^b	Maximum VZV-IgG titer, AU	VZV-IgG boosting ^c
1	0.87	3.66	–
2	0.78	2.12	–
3	0.66	5.26	+
4	0.47	5.07	+
5	0.37	2.45	+
6	0.32	3.29	–
7	0.31	3.89	+
8	0.26	4.86	+
9	0.24	1.74	+
10	0.23	5.52	+
11	0.21	2.54	–
12	0.13	1.39	–
13	0.10	3.06	+
14	0.10	1.85	–
15	0.08	4.45	–
16	0.07	0.88	–

NOTE. AU, arbitrary units.

^a Donors 1–11 showed boosting of VZV-specific CD4⁺ T cells.

^b Percentages are derived from intracellular cytokine staining experiments.

^c VZV-IgG boosting is defined as an increase or decrease of at least 1.0 AU.

absence of antigen, these cells became undetectable within a few weeks [15]. This latter mouse model may be more representative of humans, in whom survival factors are limited and competition between memory cells of different specificities will occur.

Boosting of memory responses to VZV, which is valuable on reexposure to the virus, may also control reactivating virus from latency. Reactivation of VZV from sensory ganglia (herpes zoster) occurs, in particular, in individuals with waning immunity, such as elderly or immunosuppressed individuals [38]. During latency, virus-specific memory CD4⁺ T cells are detectable at stable frequencies in peripheral blood. Frequencies of memory CD4⁺ T cells vary between viruses and individuals. The mean frequencies of memory CD4⁺ T cells specific for VZV and herpes simplex virus (HSV), as determined by production of IFN- γ in healthy immune donors, are low (<0.1% and 0.2%, respectively) ([31] and the present study), compared with the mean frequencies of CMV-specific memory CD4⁺ T cells (0.5%) [39]. The site of latency and episodes of reactivation may determine the differences between the usual frequencies of virus-specific memory T cells in peripheral blood. CMV develops latency in the monocyte/macrophage lineage and endothelium [40], whereas VZV (or HSV) persists in immunoprivileged sites, resulting in low frequencies of memory T cells against VZV, which probably depend more on exogenous boosting. The importance of reg-

ular boosting by specific antigens to maintain the memory pool and protect the individual from reactivation, has been suggested, in an epidemiological study, by Thomas et al. [41], which is in accordance with a hypothesis postulated by Hope-Simpson in 1965 [42]. The importance of exogenous reexposure is of prime importance to the debate whether VZV vaccination should be included in national vaccination programs. By vaccinating children with live attenuated VZV, exogenous reexposures will become limited, since natural infections will be rare. Increased numbers of herpes zoster cases will probably develop in unvaccinated individuals who experienced a natural infection in the past without subsequent reexposure. Further studies of the role that reexposure to exogenous antigen plays in the maintenance of immunological memory are crucial for the development of vaccination strategies and the timing of booster responses.

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