MAJOR ARTICLE

Cellular Immune Responses to Diluted and Undiluted Aventis Pasteur Smallpox Vaccine

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Background. Recent primary vaccine trials of diluted Aventis Pasteur smallpox vaccine (APSV) demonstrated that immunization "take" rates, defined by the presence of a vesicle or pustule ("take") at the inoculation site 6–11 days after immunization, did not differ between the dilution groups. To our knowledge, there have been no studies that examine the cellular immune response or that distinguish $CD4^+$ T cell responses from $CD8^+$ T cell responses after primary immunization with varying dilutions of APSV.

Methods. In the present study, we examined the cellular immune response in vaccinia-naive healthy adults ($n = 91$) receiving inoculations with an undiluted or diluted (1:5 and 1:10) suspension of the APSV, using an intracellular cytokine staining assay.

Results. The diluted vaccine induced vaccinia virus (VV)–specific $CD4^+$ and $CD8^+$ T cell responses 1 month after primary immunization that were comparable to those induced by undiluted vaccine. The cellular immune responses were correlated with the reactogenicity profile of subjects and did not differ between dilution groups. Furthermore, expression of the interleukin-7 receptor α chain, which has been proposed to distinguish antigen-specific T cells that differentiate into long-lived memory T cells, did not differ among groups, suggesting that dilution of the vaccine does not affect the quantity of VV-specific memory T cells.

Conclusions. APSV is an effective smallpox vaccine inducing strong humoral and cellular immune responses after a primary immunization even at diluted doses.

The general use of the smallpox vaccine in the United States ended in 1972, and the World Health Assembly declared that smallpox had been eradicated globally in 1980 [1]. However, because of concern about bioterrorism, in 2002, smallpox vaccine testing resumed in the United States, by use of stockpiled vaccine prepared in the early 1970s [2]. Two types of vaccinia virus (VV) preparations had been used previously in the United States: a lyophilized form and a frozen preparation (both are live-virus vaccines derived from the New York City Board of Health [NYCBH] VV strain). Approximately 15 million doses of the lyophilized vaccine (Dryvax; Wyeth-Ayerst) had been maintained within the US national stockpile in the event that widespread immunization of the population was needed. In the fall of 2001, Aventis Pasteur reported that multiple lots of Aventis Pasteur smallpox vaccine (APSV) had been in frozen storage for decades. Studies conducted at Vanderbilt University Medical Center and other centers throughout the nation demonstrated that both the lyophilized (Dryvax) and the frozen (APSV) preparations diluted 10-fold retained high immunization "take" rates—defined by the presence of a vesicle or pustule ("take") at the inoculation site 6–11 days after immunization—in vaccinia-naive individuals, thus expanding the US vaccine stockpile [3, 4].

The goal of immunization is to induce long-lasting functional immune responses and enhanced memory responses that prevent reinfection or greatly reduce the

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severity of disease after reinfection. Earlier studies have found a correlation between the level of neutralizing antibodies present in serum after immunization and protection against disease [5, 6]. The fact that individuals with T cell deficiency disorders have had serious and at times fatal infections after VV immunization, whereas agammaglobulinemic children have not been found to have these complications, indicates that cellular immunity is essential in preventing the spread of VV after immunization and, perhaps, in generating protective immunity to variola [7–9]. Both $CD4^+$ and $CD8^+$ T cells specific for VV have been detected after smallpox immunization and have been found to persist for up to 75 years after immunization [10–12]. However, to our knowledge, there have been no studies examining the cellular immune response or distinguishing $CD4^+$ responses from $CD8^+$ responses after primary immunization in a population of subjects immunized with varying dilutions of APSV.

SUBJECTS, MATERIALS, AND METHODS

Subjects. The vaccine trial was performed as part of a multicenter, randomized, double-blind vaccine safety and efficacy trial of the APSV in vaccinia-naive subjects ($n = 340$). The APSV used in this study was manufactured between 1956 and 1957 and maintained at −20°C since manufacture. Potency determinations of the lot revealed chorionic allantoic membrane titer of $10^{7.6}$ pfu/mL. The vaccine, study subjects, study design, and clinical assessments are described in detail elsewhere [4, 13, 14]. Peripheral-blood samples were obtained only from participants at the Vanderbilt University Medical Center study site, after informed consent was obtained under approval from the Vanderbilt University Institutional Review Board. Preimmunization samples were collected during a screening visit or just before immunization, and postimmunization samples were obtained 23–35 days (1 month) after immunization.

All subjects ($n = 148$) participating in the main smallpox immunization study at Vanderbilt were invited to participate in this cell-mediated immune response substudy. Statistical differences were not detected in the clinical outcome ("take"/no "take") between subjects who declined to participate in this substudy ($n = 41$) and those who participated ($n = 107$). Of the 107 subjects enrolled in this substudy, 91 donated samples with sufficient cell numbers and cell viability for accurate analysis before and 1 month after immunization. After the clinical trial was completed but before sample testing was initiated, subjects were contacted for 1 additional blood sample. Of the 28 subjects willing to provide an 11–12-month (1-year) sample, 25 donated samples with sufficient cell numbers and cell viability for accurate analysis at this time point. Sample processing and viability assessment have been described elsewhere [15].

Monoclonal antibodies (MAbs) and reagents. The following anti–human MAbs were obtained from BD Immunocytometry Systems: anti–interferon (IFN)–γ–fluorescein isothiocyanate, anti–CD3-Cy5.5-PerCP, and anti–CD8-Cy7-allophycocyanin. Anti–CD4–Texas Red–phycoerythrin (PE) was obtained from Caltag Laboratories and anti–CD127-PE from Beckman Coulter.

Intracellular cytokine staining. T cell responses were quantified and characterized by use of a highly optimized intracellular cytokine staining (ICS) protocol that detected VV-specific T cells by their ability to produce IFN-γ. Cells (2×10^6) were cultured with or without a pretitered optimal amount of VV (NYCBH strain) at an MOI of 1 for 1 h before the addition of brefeldin-A and were then incubated for an additional 5 h before staining. The ICS assay and flow-cytometric analyses have been described in detail elsewhere [15].

Enzyme-linked immunospot (ELISPOT) assay. A modified ELISPOT assay was performed as described elsewhere [16], except that cells (10^5/well) were cultured with or without VV at an MOI of 1 for 18 h.

Quality control. Control ranges for detection of intracellular IFN-γ were established by a 40-point Levy-Jennings plot, using commercially available human cells that have been stimulated and then frozen for use (BD Pharmingen). High control ranges were established using HiCK-1 cells. Low control ranges were established using HiCK-1 cells diluted 1:10 with HiCK-

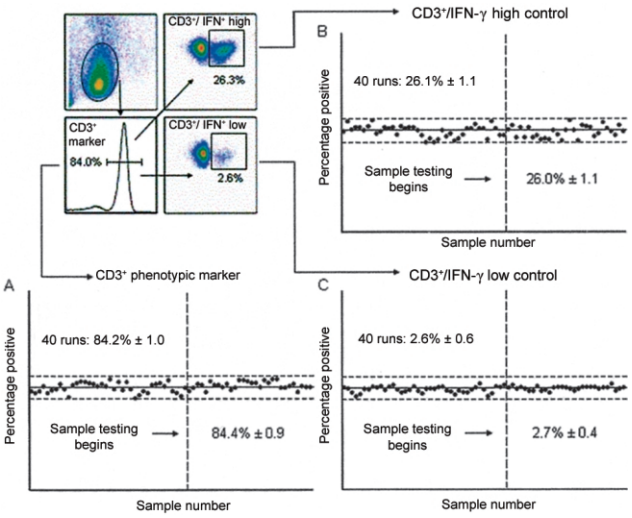

Figure 1. Quality control for detection of intracellular interferon (IFN)–γ. Control ranges for detection of intracellular IFN-γ and phenotypic markers (CD3 is shown) *(A)* were established by a 40-point Levy-Jennings plot, using commercially available human cells that were stimulated and then frozen for use (BD Pharmingen). High control ranges were established using HiCK-1 cells *(B)*. Low control ranges were established using HiCK-1 cells diluted 1:10 with HiCK-3 cells *(C)*. Quality controls were used with each run of clinical samples. Percentages (mean ± SD) are indicated for each of the 40 runs used to establish the Levy-Jennings plots (*left of broken line*) and for each run associated with analysis for clinical trial samples (*right of broken line*).

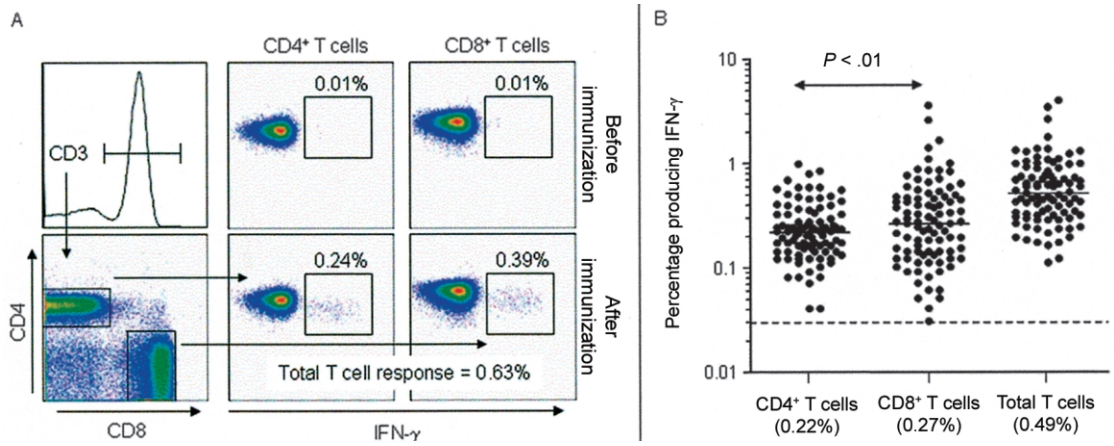

Figure 2. Evaluation of vaccinia virus (VV)–specific T cell responses before and 1 month after primary smallpox immunization. The no. of VV-specific T cells was determined by assessment of interferon (IFN)–γ production, using an intracellular cytokine staining assay. *A,* Gating scheme for assessment of IFN-γ–producing $CD4^+$ and $CD8^+$ T cells, both before and after immunization. *B,* Percentage of $CD4^+$, $CD8^+$, and total T cells (sum of the $CD4^+$ and $CD8^+$ T cell responses) producing IFN-γ 1 month after immunization. The bars and percentages indicate median values ($n = 91$). The *P* value was determined using the Wilcoxon signed rank test to compare VV-specific $CD4^+$ and $CD8^+$ T cell subset responses within subjects.

3 cells. Both "high" and "low" control cells were frozen in single-use aliquots in sufficient numbers to allow use of identical lots across the clinical trial. Data for quality control cells were included in each run of clinical samples.

Statistical analysis. The Wilcoxon signed rank test was used to compare the percentage difference in IFN-γ–producing VV-specific $CD4^+$ and $CD8^+$ T cells within subjects. Differences between dilution groups were analyzed using the Mann-Whitney *U* test for comparison of means. Correlations and statistical significance were determined by Spearman rank correlation analysis. Prism statistical software (version 4.0; Prism) was used for all analyses; $P < .05$ was considered to be statistically significant.

RESULTS

Detection of intracellular IFN-γ from clinical trial samples. Detection of intracellular IFN-γ is a useful parameter for the quantitation of antigen-specific T cells during vaccine trials. To ensure reproducible permeabilization and intracellular detection of IFN-γ, we developed a control assay using commercially available cytokine-positive HiCK-1 cells as the positive control for intracellular IFN-γ. These cells were used undiluted or diluted 1:10 with HiCK-3 cells, a negative control cell line for intracellular IFN-γ, as high and low controls, respectively. Before assaying clinical samples for IFN-γ production, using the control cells, we generated 40-point Levy-Jennings plots and established 95% confidence intervals (CIs) for detection of IFN-γ. Detection of T cell subsets and intracellular IFN-γ was highly reproducible, as evidenced by the interassay variability CIs (figure 1; data not shown). The results of all ICS experiments evaluating clinical samples from this cell-mediated immune response substudy fell within the established ranges for detection of IFN-γ.

T cell responses in subjects who received diluted APSV. Characteristics of the primary vaccine trial evaluating immunization success rates after a primary immunization with APSV in 340 subjects have been described elsewhere [4]. The immunization success rates, defined as the presence of a "take" at the inoculation site 6–11 days after immunization, did not differ among the 3 dilution groups (undiluted, 1:5 dilution, or 1:10 dilution). Importantly, all subjects enrolled in this cell-mediated immune response substudy developed clinically observable pustules at the immunization site.

To study the magnitude of VV-specific cellular immunity after primary immunization, PBMCs were stimulated with live VV in an ICS assay that allowed multiparametric evaluation of T cell responses. VV-specific T cell responses were measured before immunization and 1 month after immunization in 91 subjects who were immunized with APSV and had sufficient cell numbers and cell viability to allow accurate quantitation of the number of VV-specific T cells. Before immunization, all subjects were negative for VV-specific $CD4^+$ and $CD8^+$ T cell responses (<0.03%) (figure 2; data not shown). One month after immunization, all subjects had an increase in VV-specific $CD4^+$ and $CD8^+$ T cell responses. The median frequencies of IFN-γ–producing VV-specific $CD4^+$ T cells and $CD8^+$ T cells were 0.22% and 0.27%, respectively. Interestingly, when the magnitudes of the $CD4^+$ and $CD8^+$ T cell responses 1 month after immunization, measured as the percentage of IFN-γ–producing cells, were compared, the $CD8^+$ T cell response was found to be significantly greater than the $CD4^+$ T cell response (magnitude, ~1.6-fold; $P = .0011$) (figure 2). Overall, the total

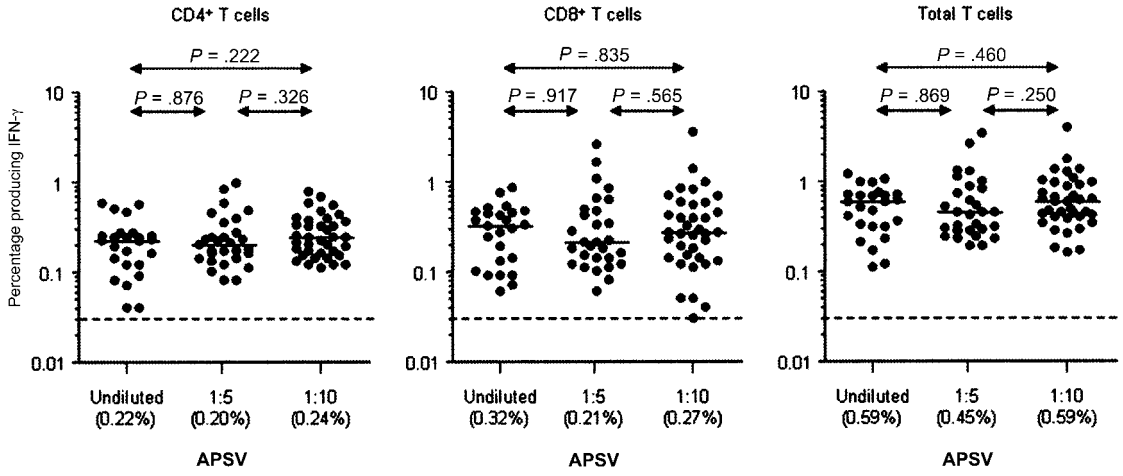

Figure 3. Comparison of vaccinia virus (VV)–specific T cell responses with diluted or undiluted Aventis Pasteur smallpox vaccine (APSV). The no. of VV-specific T cells was determined by assessment of interferon (IFN)–γ production, using an intracellular cytokine staining assay. The data are expressed as the percentage of $CD4^+$, $CD8^+$, and total T cells (sum of the $CD4^+$ and $CD8^+$ T cell responses) producing IFN-γ 1 month after immunization in subjects receiving undiluted APSV ($n = 25$), a 1:5 dilution of APSV ($n = 29$), or a 1:10 dilution) of APSV ($n = 37$). The bars and percentages indicate median values. *P* values were determined using the Mann-Whitney *U* test for comparison of means between dilution groups.

magnitude of the T cell response (sum of the $CD4^+$ and $CD8^+$ responses) ranged from 0.11% to 3.93% (median, 0.49%) of circulating T cells, demonstrating robust VV-specific T cell responsiveness.

Because the parental trial was designed to evaluate the immunization success rate obtained by use of diluted and undiluted APSV, we sought to determine whether there were differences in the magnitude of VV-specific $CD4^+$ and $CD8^+$ T cell reponses between subjects receiving undiluted APSV ($n = 25$), the 1:5 dilution of APSV ($n = 29$), or the 1:10 dilution of APSV ($n = 37$). The elicited VV-specific $CD4^+$ and $CD8^+$ T cell responses in subjects who received the diluted vaccine were similar to those in subjects who received the undiluted vaccine, with no statistical differences found among the dilution groups for either the $CD4^+$ or $CD8^+$ VV-specific T cell respones (figure 3). Thus, APSV induced strong $CD4^+$ and $CD8^+$ T cell responses that were readily detectable 1 month after immunization even at diluted doses.

To further confirm that the VV-specific T cell responses did not differ significantly between dilution groups, we employed a vaccinia-based ELISPOT assay in subjects with sufficient cell numbers available ($n = 89$). On the basis of the number of IFN-γ –producing T cells identified by ICS or by ELISPOT, there were no statistically significant differences among the 3 dilution groups (figure 3 and table 1). These data are further supported by a direct correlation between the percentage of IFN-γ–producing cells detected using the ICS assay and the percentage detected using the ELISPOT assay ($P < .0001$). Thus, comparable frequencies of VV-specific T cells were induced after primary immunization with undiluted APSV, a 1:5 dilution of APSV, or a 1:10 dilution of APSV.

Neutralizing antibody responses from a cohort of 109 subjects (including 84 of the subjects assessed for cellular immunity) have also been evaluated and reported elsewhere [4]. Before immunization, all subjects had baseline reciprocal antibody titers of <40; 1 month after immunization, all subjects, except 1 subject who received the 1:10 dilution of APSV, developed a neutralizing antibody response. In the present study, we summarize the neutralizing antibody data to illustrate that no statistical differences were detected between undiluted APSV and the 1:10 dilution of APSV (table 1) and that the subject without a detectable increase in VV-neutralizing antibody titers displayed substantial VV-specific $CD4^+$ (0.18%) and $CD8^+$ (0.29%) T cell responses 1 month after immunization.

Immune responses and reactogenicity to the APSV. The reaction profiles associated with the parental vaccine trial have been described elsewhere [4]. Overall, differences were not detected in the maximum erythema, induration, or lesion size among the dilution groups or in the 91 subjects evaluated in this cell-mediated substudy (table 1). When the magnitude of the T cell responses in the substudy cohort of subjects was compared with the reaction profile based on maximal lesion, erythema, and induration, we found a correlation between the total T cell response and the $CD4^+$ T cell response and the maximal measured erythema, lesion, and induration sizes (table 2). Similarly, the $CD8^+$ T cell responses correlated with the maximal measured erythema and approached statistical significance when compared with lesion and induration size. The low *r* values may be reflective of assessing cellular immune responses at 1 month rather than at 2 weeks after immunization, because the latter period coincides with the peak T cell response after smallpox immunizations [17]. These data confirm previous reports that

Table 1. Comparison of immunogenicity and reactogenicity with diluted and undiluted Aventis Pasteur smallpox vaccine (APSV) ($n = 91$).

Vaccine dilution	Subjects, no.	ELISPOT result,[a] median (range), SFCs/10^6 cells	IFN-γ ICS result, median (range), %			Reactogenicity, median (range), mm			Antibody titer, geometric mean[b]
			$CD4^+$ T cell response	$CD8^+$ T cell response	Total T cell response	Lesion size	Erythema size	Induration size	
Undiluted	25	460 (53–3627)	0.22 (0.04–0.58)	0.32 (0.06–0.86)	0.59 (0.11–1.21)	14 (11–22)	38 (13–175)	40 (19–105)	626
1:5	29	500 (57–5657)	0.20 (0.08–0.97)	0.21 (0.06–2.57)	0.45 (0.19–3.40)	15 (7–20)	39 (15–150)	36 (17–135)	1125[c]
1:10	37	640 (77–4747)	0.24 (0.11–0.78)	0.27 (0.03–3.53)	0.59 (0.16–3.93)	15 (7–24)	40 (7–100)	40 (10–210)	490
Overall	91	500	0.22	0.27	0.49	15	38	38	686

NOTE. ELISPOT, enzyme-linked immunospot; ICS, intracellular cytokine staining; SFCs, spot-forming cells.

[a] Sufficient cell nos. were not available from 2 subjects receiving the 1:10 dilution for ELISPOT analysis ($n = 35$).

[b] Plaque-reduction neutralization antibody titers from a cohort of 109 subjects including 84 subjects evaluated in this substudy that received undiluted APSV ($n = 34$), a 1:5 dilution of APSV ($n = 36$), or a 1:10 dilution of APSV ($n = 39$) have been reported previously [4]. Statistical differences were not detected between the cohort of 25 subjects evaluated for serological responses only and the 84 subjects evaluated for both serological and cellular immune responses.

[c] Significantly higher neutralizing titers than in volunteers administered the 1:10 dilution ($P = .007$).

Table 2. Summary of Spearman rank correlation analysis comparing percentage of vaccinia virus–specific T cells, skin reactogenicity, and antibody titer.

Reaction measurement	Total T cells		$CD4^+$ T cells		$CD8^+$ T cells	
	r	*P*	*r*	*P*	*r*	*P*
Lesion size	0.250	<.05	0.315	<.01	0.189	.073
Erythema size	0.269	<.01	0.281	<.01	0.228	<.05
Induration size	0.267	<.01	0.365	<.001	0.197	.062
Antibody titer	0.215	<.05	0.193	.078	0.192	.080

the cell-mediated immune response after immunization with live virus correlates with lesion size [17].

Characterization of the memory and CD127 phenotype after smallpox immunization. Samples for evaluation of the VV-specific T cell memory response 1 year after immunization were available from 25 subjects. Not surprisingly, the magnitude of the $CD4^+$ and $CD8^+$ VV-specific T cell responses in samples obtained 1 year after immunization was significantly lower than that in samples obtained 1 month after immunization ($P <$ .0001). One year after immunization, the magnitude of IFN-γ–producing VV-specific $CD4^+$ T cell responses ranged from 0.02% to 0.38% (median, 0.11%), and that of VV-specific $CD8^+$ responses ranged from 0.01% to 0.40% (median, 0.05%). Overall, the total magnitude of the T cell response from this cohort of subjects ranged from 0.03% to 0.57% (median, 0.18%). Interestingly, at 1 year, the $CD4^+$ T cell response was significantly higher than the $CD8^+$ T cell response ($P = .007$; magnitude, ~1.5 fold), in contrast to the results found at 1 month. The preferential persistence of VV-specific $CD4^+$ T cells over VV-specific $CD8^+$ T cells in the memory compartment has been reported elsewhere [18].

Because of insufficient numbers of available samples, we could not evaluate dilution differences in the quantity of VV-specific memory T cells 1 year after immunization, which may be a better indication of protective cellular immunity than the level of response present at 1 month after immunization. To determine whether the quantity of memory T cells differed between dilutions, we sought to evaluate the percentage of VV-specific $CD4^+$ and $CD8^+$ T cells that coexpressed IFN-γ and the interleukin (IL)–7 receptor α chain (CD127). Previous studies have shown that IL-7 promotes the survival and homeostatic proliferation of memory $CD8^+$ T cells [19, 20] and that expression of CD127 distinguishes $CD8^+$ T cells that are more likely to survive and give rise to functional memory T cells [21–25]. In addition, IL-7–dependent signaling pathways also support the emergence and survival of memory $CD4^+$ T cells [26, 27].

The level of CD127 expression on VV-specific IFN-γ–producing T cells was significantly greater in both the $CD4^+$ and $CD8^+$ T cell subsets 1 year after immunization, compared with 1 month after immunization ($P <$ .0001) (figure 4). Similar results were observed in 25 HLA-A2–expressing subjects, using the VV-peptide tetramer (VV-CLTEYILWV) [28] in the absence of viral stimulation (data not shown). Importantly, although there was a wide range of IFN-γ–producing T cells that expressed CD127 at 1 month after immunization (figure 4), there

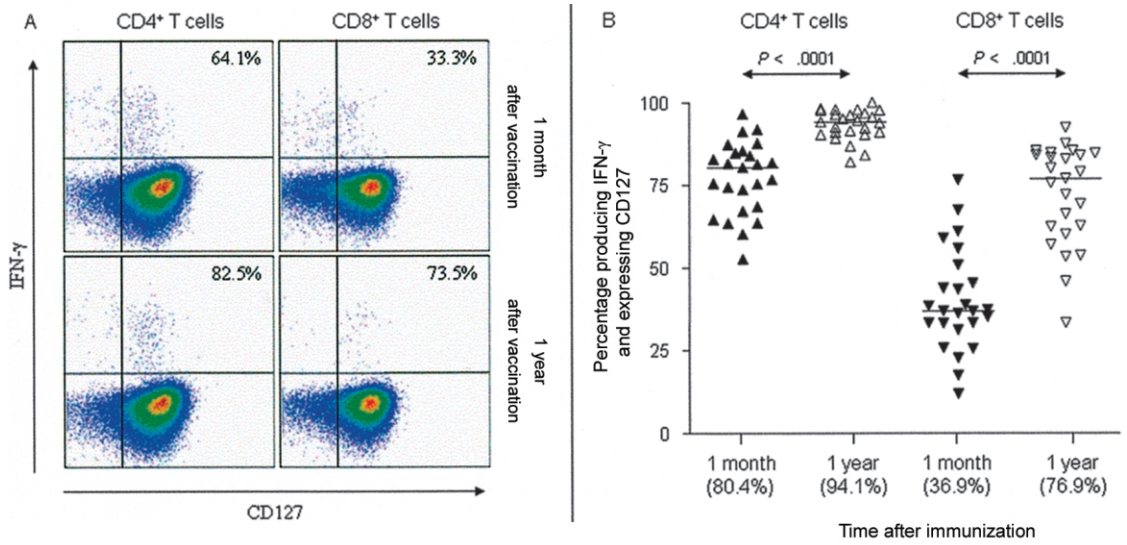

Figure 4. Comparison of interleukin-7 receptor α chain (CD127) expression on vaccinia virus (VV)–specific T cells 1 month and 1 year after primary smallpox immunization. The no. of VV-specific T cells was determined by assessment of interferon (IFN)–γ production, using an intracellular cytokine staining assay. *A,* Gating scheme for evaluating the percentage of IFN-γ–producing VV-specific $CD4^+$ and $CD8^+$ T cells that coexpress CD127 1 month and 1 year after immunization. *B,* Percentage of IFN-γ–producing $CD4^+$ or $CD8^+$ T cells that coexpress CD127 1 month *(black symbols)* and 1 year *(white symbols)* after immunization. The bars and percentages indicate median values ($n = 25$). *P* values were determined using the Wilcoxon signed rank test to compare VV-specific $CD4^+$ and $CD8^+$ T cell subset responses within subjects.

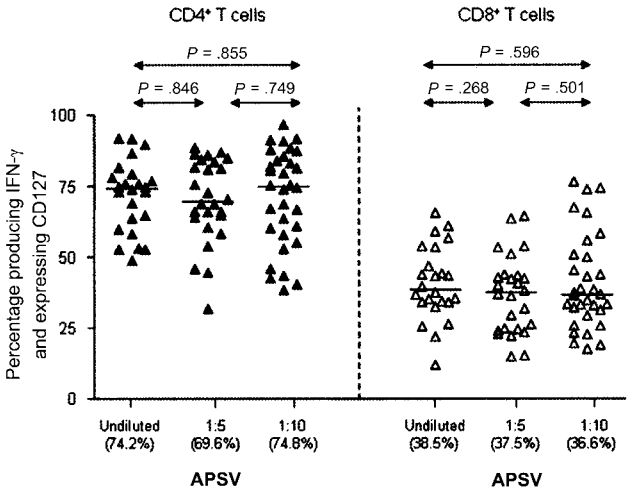

Figure 5. Comparison of CD127 expression by vaccinia virus (VV)–specific T cells in subjects immunized with diluted or undiluted Aventis Pasteur smallpox vaccine (APSV). The no. of VV-specific T cells was determined by assessment of interferon (IFN)γ production, using an intracellular cytokine staining assay. The data are expressed as the percentage of IFN-γ–producing $CD4^+$ *(black symbols)* or $CD8^+$ *(white symbols)* T cells that coexpress CD127 1 month after immunization in subjects receiving undiluted APSV ($n = 25$), a 1:5 dilution of APSV ($n = 29$), or a 1:10 dilution of APSV ($n = 37$). The bars and percentages indicate median values. *P* values were determined using the Mann-Whitney *U* test for comparison of means between dilution groups.

was a strong correlation between the percentage of $CD4^+$ ($r = 0.836$, $P < .0001$) or $CD8^+$ ($r = 0.732$, $P < .0001$) T cells that coexpressed IFN-γ and CD127 1 month after immunization and the percentage of IFN-γ–producing cells that persisted at 1 year. Overall, statistical differences were not detected among dilution groups 1 month after immunization when the percentages of VV-specific IFN-γ–producing $CD4^+$ or $CD8^+$ T cells that coexpressed CD127 were compared (figure 5). Considered together, these data suggest that dilution of APSV does not reduce the quantity or quality of antigen-specific $CD4^+$ or $CD8^+$ T cells induced after primary smallpox immunization.

DISCUSSION

The parental trial to this substudy demonstrated that APSV can be diluted to a 1:10 concentration without a reduction in the "take" rate (100% vesicle formation in the groups receiving undiluted APSV and a 1:10 dilution of APSV) [4]. These observations were mirrored by the immunological success rate observed in the subset of subjects evaluated at the Vanderbilt University Medical Center. Our previous work demonstrated that the humoral immune response did not differ significantly between undiluted APSV and a 1:10 dilution of APSV [4]. In the present study, we have extended these observations by evaluating the VV-specific T cell response. The results of the present study show that a 1:10 dilution of APSV did not alter the quantity of VV-specific $CD4^+$ and $CD8^+$ T cells present in the periphery 1 month after immunization in vaccinia-naive subjects. Considered together, these results clearly demonstrate that primary immunization with APSV induced robust neutralizing antibody and cellular immune responses that did not differ significantly among dilutions.

A pilot study examining cellular immune responses in subjects receiving different dilutions of Dryvax (NYCBH strain) showed a dose-response effect in which cytotoxic T cell and IFN-γ responses were greatest in subjects receiving undiluted vaccine [29]. A subsequent larger trial demonstrated that subjects given undiluted Dryvax had significantly greater local inflammatory responses, fewer satellite lesions, and lower antibody responses than subjects given diluted Dryvax [3, 30]. The authors hypothesized that the differences between subjects receiving diluted and undiluted Dryvax may have been associated with increased cell-mediated immune responses in the undiluted group. The results of this study demonstrated that diluted APSV (NYCBH strain) was not associated with decreased $CD4^+$ or $CD8^+$ T cell IFN-γ responses after primary immunization. We previously reported that there were no significant differences in reactogenicity or antibody responses between undiluted and diluted APSV in vaccinia-naive subjects; although antibody titers were significantly lower in those subjects who received the 1:10 dilution of APSV, compared with those who received the 1:5 dilution of APSV [4]. The differences between these dilution studies may be related to the lyophilization process used in making Dryvax but not APSV, because freeze-drying potentially reduces the relative potency of smallpox vaccines without affecting the plaque-forming unit counts [31]. Other studies evaluating Lister and Lancy-Vaxina vaccines (Lister/Elstree strain) demonstrated that comparable humoral and cellular immune responses were observed in vaccinia-naive subjects receiving diluted (1:10) vaccine [32, 33]. However, although APSV, Dryvax, Lister, and Lancy-Vaxina vaccines are live-virus immunizations and are derived from similar VV strains, such comparisons must be performed with caution, because the present study did not directly compare APSV with other VV vaccines.

Evaluations of the cellular immune response after smallpox immunizations often have focused on VV-specific $CD8^+$ T cells with certain epitope specificity [28] or have used techniques that do not distinguish $CD4^+$ T cell responses from $CD8^+$ T cell responses [11, 29]. In contrast, the results of the present study demonstrate that, at 1 month after immunization, the $CD8^+$ IFN-γ response is dominant and a significant proportion of the cellular immunity generated is also a $CD4^+$ response. At 1 year after immunization, $CD4^+$ T cells showed better persistence than $CD8^+$ T cells. Similar results were observed in a recent study demonstrating that $CD8^+$ T cell responses were 2–

4-fold higher than the $CD4^+$ T cell response, 2 weeks after immunization, but that $CD4^+$ T cell responses were significantly higher 12 weeks after immunization [18]. Considered together, these data suggest that VV-specific $CD4^+$ T cells show preferential persistence over VV-specific $CD8^+$ T cells and that the dynamics of VV-specific $CD4^+$ and $CD8^+$ T cell responses differ.

Previous reports have shown that T cells expressing high levels of CD127 during the effector phase of an immune response acquire the phenotypic and functional characteristics of memory cells [21–27]. These observations indicate that CD127 may be a useful marker for cells destined to become memory cells and may provide a valuable benchmark for evaluating the vaccine-induced protective memory response within 14–28 days after immunization. In this study, we showed that VV-specific $CD8^+$ T cells expressed CD127 at relatively low levels, similar to studies examining $CD8^+$ T cells specific for persistent human viruses such as cytomegalovirus and Epstein-Barr virus [25]. However, once established in the memory pool, the majority of VV-specific $CD8^+$ T cells expressed CD127 at levels similar to those of memory cells specific for acute viruses, such as influenza virus and respiratory syncytial virus [23, 24]. In contrast, the majority of $CD4^+$ T cells were positive for CD127 expression, both at 1 month and 1 year after immunization. The persistence of $CD4^+$ memory T cells may be the result of the increased CD127 expression on this subset, compared with the $CD8^+$ T cell subset. This is not surprising, given the requirement of $CD4^+$ T cell help, during priming and during the memory phases and for the maintenance of memory $CD8^+$ T cells after acute infection [34].

The preferential loss of VV-specific $CD8^+$ T cells may begin early after immunization, between 2 and 4 weeks. We previously have shown that the majority of VV-specific $CD8^+$ T cells express granzyme A and granzyme B 1 month after immunization and that this "effector" cell phenotype is significantly reduced 1 year after immunization [15]. Furthermore, 1 month after immunization, CD127 expression clearly is reduced on $CD8^+$ T cells, compared with $CD4^+$ T cells. Because CD127 and granzyme B trend toward mutually exclusive expression (M.T.R. and J.E.C., unpublished data), it may be that these "effector" cells are selectively removed from the memory compartment but can be recalled after VV stimulation or reimmunization, suggesting an immediate protective response. Clearly, $CD4^+$ T cells are required to generate functional memory $CD8^+$ T cells and B cells. Thus, it is reasonable to suggest that $CD4^+$ VV-specific T cells play a critical role in the generation of $CD8^+$ T cell and B cell memory and contribute significantly to the protective effects that VV-based immunizations provide.

Our work and that of others provide a foundation for additional comparisons of the cellular immunogenicity induced by alternative vaccines, such as further-attenuated viruses like modified vaccinia Ankara (MVA), the LC16 strain, or cell culture–derived VV. Given that a true efficacy trial cannot be conducted using these vaccines, likely correlates of immunity are needed for comparative purposes. For instance, because MVA does not induce "takes" at the inoculation site, the importance of understanding and quantifying both the serological and cell-mediated immune response takes on even greater significance. If these vaccines induce humoral and cellular immune responses that are similar to those induced by the NYCBH- and Lister/Elstree-based vaccines, we may have some confidence that they might be protective. On the other hand, vaccines that poorly induce cellular immunity, compared with the vaccines currently being tested, would be less attractive as alternate vaccines. Such comparisons are needed in the ongoing evaluation of these vaccines.

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