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Differential Regulation of Granzyme and Perforin in Effector and Memory T Cells following Smallpox Immunization

Michael T. Rock,* Sandra M. Yoder,* Peter F. Wright,*† Thomas R. Talbot,‡ Kathryn M. Edwards,* and James E. Crowe, Jr.2*†

Primary immunization of healthy adults with vaccinia virus induces a local vesicle or “take” in the majority of vaccinees that previously has been shown to correlate with protection against smallpox. However, the immunologic mechanisms underlying this protective response in humans are not well characterized. We have studied human CD8+ T cells for the expression patterns of phenotypic markers and cytolytic effector molecules before and after primary smallpox immunization using nine-color polychromatic flow cytometry. One month after immunization, vaccinees developed vaccinia virus-specific CD8+ T cells with an effector cell phenotype containing both granzyme A and granzyme B. One year after immunization, we found a significant decrease in granzyme B containing cells and an increased memory cell phenotype in virus-specific CD8+ T cells. Perforin was rarely expressed directly ex vivo, but was highly expressed after Ag-specific activation in vitro. Together, these data suggest an important role for effector CD8+ T cells in controlling poxvirus infection, and have implications for our understanding of human CD8+ T cell differentiation. *The Journal of Immunology, 2005, 174: 3757–3764.

The goal of immunization is to induce long-lasting functional cellular and/or humoral responses to a given immunogen and enhanced memory responses that prevent reinfection or greatly reduce the severity of disease following re-infection. The contribution of T cells to antiviral immunity in humans has been well established for many viral pathogens (1). CD8+ T cells follow a program of proliferation and differentiation into CTL armed with effector functions that facilitate pathogen clearance or containment (2, 3). After the early proliferation and expansion phase, most Ag-specific CD8+ T cells undergo programmed cell death; those cells that remain form a population of memory cells. Memory CD8+ T cells, often present for the life of the host, mount rapid, heightened responses to re-infection with the specific pathogen (4). Viral infections also stimulate memory T cells to proliferate, secrete cytokines, and exhibit increased cytolytic activity against virus-infected cells. The dominant mechanism that CTL use to kill virus-infected cells is the granule exocytosis pathway, with perforin and two serine proteases, granzyme A (GrA)3 and granzyme B (GrB), forming the major lines of defense (5).

Experiments in murine models suggested that perforin-mediated cytolysis was not required for resistance to vaccinia virus (VV), vesicular stomatitis virus, or Semiliki Forest virus (6). However, the viruses studied were not highly virulent, natural pathogens of mice. In contrast, perforin-dependent cytolysis was required for resistance to lytic infection with the highly virulent mouse poxvirus ectromelia virus (EV) (7). Furthermore, mice of the highly EV-resistant C57BL/6 background demonstrated an increasing loss of resistance to EV in mutant mice with that background that were deficient in GrA, GrB, or GrA and GrB (8). These studies demonstrated that mice lacking perforin or granzymes were unable to control primary EV infection and suggested an important role for these serine proteases in resistance to poxviruses.

Immunization of healthy adults with VV induces a local vesicle or “take” that has been correlated with protection against natural smallpox infection. This approach successfully eradicated natural smallpox infections worldwide. Despite the astounding success of this vaccine, the precise immune mechanisms associated with protection are poorly understood. There is evidence for both humoral and cellular immunity after immunization. Earlier studies have correlated the level of neutralizing Abs present in serum following immunization with protection against disease. The fact that individuals with T cell deficiency disorders had serious and at times fatal infections following vaccinia immunization, whereas agammaglobulinemic children did not suffer these complications, highlights the role of cellular immunity (9–11). Recently, both CD4+ and CD8+ T cells specific for VV were detected in response to smallpox immunizations, and such responses were detected in vaccinees for 75 years after immunization (12–14). Thus, it is clear that smallpox immunization induces a significant cellular immune response, and that vaccinia-specific CD4+ and CD8+ T cells survive the contraction phase to become established memory T cells. However, a detailed understanding of the human T cell response to smallpox immunization using modern technology is not complete, and the lymphocyte subsets that mediate specific effector functions to poxviruses in humans have not been investigated.

Maintaining a circulating population of memory CD8+ T cells that recognize VV Ags in the circulating pool of memory T cells

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3 Abbreviations used in this paper: GrA, granzyme A; APSV, Aventis Pasteur smallpox vaccine; EV, ectromelia virus; GrB, granzyme B; SEB, Staphylococcus enterotoxin B; TCM, central memory T cell; TEM, effector memory T cell; TEMRA, CD45RA+ effector memory T cell; VV, vaccinia virus.

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may be important in preventing or controlling the severity of poxvirus infections. In the past, expression of a single phenotypic surface marker such as CD45RA, or lack of expression of CD45RO, has been used to differentiate between naive and memory T cell subsets. However, several groups recently have shown that the use of additional markers can provide a more accurate distinction between naive and memory T cells. These studies suggest that CD8\(^+\) T cells can be divided into at least three different populations termed naive, central, and effector memory (15–17). Naive T cells express both CD45RA and CCR7 markers. Central memory T cells (T\(_{CM}\)) express CCR7 and CD45RO, but not CD45RA, and readily proliferate and differentiate to become effector cells in response to antigenic stimulation. Effector memory T cells (T\(_{EM}\)) were defined by the lack of CCR7 expression, and home to inflamed peripheral tissues and display immediate effector function. A proportion of CD8\(^+\) T\(_{EM}\) cells was found to express CD45RA (CD45RA\(^+\) effector memory T cell (T\(_{EMRA}\))) and to contain the largest amounts of perforin. Standard three- or four-color flow cytometric technologies do not permit careful phenotyping of any of these CD8\(^+\) lymphocyte subsets, but the development of flow cytometric technologies capable of detecting eight or more parameters, termed polychromatic flow cytometry (18), is uniquely suited to these studies.

Having available a population of subjects immunized with VV, we sought to define the phenotypic and functional profile of VV-specific CD8\(^+\) T cells induced after smallpox immunization. In the present study, we developed a nine-color cytokine flow cytometry method to quantitate and characterize VV-specific CD8\(^+\) T cells in a cohort of vaccinia-naive individuals receiving the Aventis Pasteur smallpox vaccine (APSV). The APSV is a live virus vaccine containing the New York Board of Health strain of VV. Phenotypic markers CD3, CD4, CD8, CD45RA, and CCR7 were used to identify CD8\(^+\) T cell subsets; intracellular expression of IFN-\(\gamma\), or MHC-peptide tetramer binding, was used to identify VV-specific T cell expression. GrB and GrA and/or perforin was used to identify cytolytic potential, and phenotypic expression of the terminal differentiation marker CD57 was used to identify cells that are replication senescent (19). These studies identified a significant burst of dual-positive GrA/GrB CD8\(^+\) T cells that do not express CD57 1 mo after immunization and a significant decrease of this population in VV-specific T cells maintained as memory cells 1 year after immunization.

Materials and Methods

Subjects

All subjects participating in this study received a primary immunization against smallpox using the APSV in the National Institutes of Health Vaccine and Treatment Evaluation Unit at Vanderbilt University Medical Center. Vaccines, study subjects, and study design were described in detail previously (20). Peripheral blood samples were obtained as part of the vaccine study follow-up; 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 mo) after immunization. In some cases, an additional blood draw was collected 11–12 mo (1 year) after immunization. HLA-A2-expressing subjects were identified by serology (DCI Laboratories) or by flow cytometric analysis with the HLA-A2-specific mAb BB7.2 (BD Pharmingen).

Subjects participating in the main smallpox immunization study at the Vanderbilt University Medical Center were invited to participate in this cell-mediated immune response substudy. This substudy was designed to quantitate T cell responses to the APSV. Of the 148 subjects from the main study, informed consent and successful sample collection before and 1 mo after immunization were obtained from 107 subjects. This cohort was divided into four groups: 1) non-HLA-A2 subjects without a 1-year sample (\(n = 45\)); 2) non-HLA-A2 subjects with a 1-year sample (\(n = 15\)); 3) HLA-A2-positive subjects without a 1-year sample (\(n = 34\)); and 4) HLA-A2-positive subjects with a 1-year sample (\(n = 13\)). We initially tested subjects from group 1 for intracellular IFN-\(\gamma\)-analysis as part of the cell-mediated immune response substudy to quantify T cell responses after immunization. Of the 45 subjects in group 1, 41 subjects had sufficient cell numbers and cell viability for accurate analysis. Of the 47 subjects in groups 3 and 4, 36 subjects had sufficient cell numbers and cell viability for accurate tetramer-binding analysis. Additional subjects from groups 2 and 4 were randomly selected for analysis of VV-specific T cells 1 year after immunization.

Sample processing

Blood samples were collected, labeled for identification, held at room temperature, and processed within 2 h. PBMC were isolated by Ficoll-Hypaque (Sigma-Aldrich) density gradient centrifugation, and resuspended at a concentration of 1 x 10\(^6\) cells/ml in freezing medium containing 90% FBS (Invitrogen Life Technologies) and 10% DMSO. The cells were then transferred to cryogenic vials (Sarstedt), in turn placed in Nalgene Cryo freezing containers (Nalge Nune International), and stored at -80°C. Frozen specimens were transferred to a liquid nitrogen freezer and stored in the vapor phase. At the time of analysis, cryopreserved cells were thawed in a 37°C water bath, incubated with 20 µg/ml DNase (Roche), and washed twice. Viability was determined by trypan blue exclusion. Only samples with viability of ≥80% were used for analysis (mean 90.6%, range 80–100%).

mAbs and reagents

The following anti-human mAbs were obtained from BD Immunocytometry Systems: anti-CD57 FITC, anti-CD3 PerCP, anti-CD45RA Alexa405, and anti-CD8 Cy7-allophycocyanin. Anti-GrA (FITC and PE), anti-GrB Alexa70, anti-perforin FITC, anti-CD107a PE, and anti-IFN-\(\gamma\) Cy7-PE were obtained from BD Pharmingen. Anti-CD4-PE-Texas Red was obtained from Caltag Laboratories, and anti-CCR7 allophycocyanin was purchased from R&D Systems. Tetramers were obtained from Beckman Coulter. The peptide epitopes used in these tetramers, CLTEYLWV and KVDDTFYYV, are conserved among vaccinia and variola (smallpox) viruses and have been described previously (21).

Viral culture

Virus titers from the site of vaccinia inoculation were determined, as described (22). Briefly, samples for viral culture were obtained from each subject at each of the scheduled follow-up visits after immunization. Cotton-tipped swabs were used to obtain specimens from the immunization site lesion, and specimens were directly incoated into 1 ml of standard (Hanks viral transport medium and frozen at -70°C for batch processing. All lesion specimens were inoculated undiluted and at dilutions of 1/10, 1/100, and 1/1000 in triplicate directly onto plates coated with BSC-40 cells (African green monkey kidney) and washed with sterile PBS. After incubation, cells were fixed with formalin and stained with crystal violet to visualize the presence of characteristic vaccinia plaques. Plates without plaque formation at 2 days were considered to be negative for vaccinia. Quantitative titers for positive specimens were expressed as the average of the triplicate counts in log\(_2\) PFU per ml. Specimens with titers that were too high to quantify at initial dilution underwent further dilutions up to 1/100,000.

In vitro stimulation

CD8\(^+\) T cell responses were quantified and characterized using a highly optimized intracellular cytokine-staining protocol that detected VV-specific T cells by their ability to produce IFN-\(\gamma\). Briefly, freshly thawed PBMC were incubated in R10 medium (RPMI 1640 (Sigma-Aldrich), 10% FBS, 10 mM HEPES buffer with 2 mM glutamine and 0.5 mg/ml gentamicin (Invitrogen Life Technologies)) supplemented with anti-CD28 and anti-CD49d Abs (1 µg/ml each; BD Pharmingen). Cells (2 x 10\(^5\)) were cultured with or without a pretreated optimal amount of VV (New York City Board of Health, the strain used to produce APSV) at a multiplicity of infection of 1, or stimulated with Staphylococcus enterotoxin B (SEB; 1 µg/ml; Sigma-Aldrich) at 37°C in 5% CO\(_2\) for 1 h before the addition of brefeldin A (10 µg/ml; Sigma-Aldrich). The cells were incubated for an additional 5 h at a 5-degree incline, and then placed at 4°C overnight before staining. In some experiments, PE-conjugated anti-CD107a was added to the cells during the stimulation period to detect degranulation. All cells were stained on the surface for phenotypic markers of interest and in the intracellular compartment for cytokines and intracellular proteins. For tetramer analysis, freshly thawed PBMC (2 x 10\(^5\)) in R10 medium were incubated with pretreated, optimal amounts of HLA-A2 VV tetramers for 30 min at room temperature. After washing, the cells
were then stained for surface markers, followed by intracellular markers, as described.

**T cell lines**

VSV-specific T cell lines were established, as previously described, with minor modifications (23). Briefly, freshly thawed PBMC from HLA-A2-expressing subjects were diluted up to 2 × 10^6 cells/ml in R10 containing VSV (multiplicity of infection = 0.1) and 25 ng/ml human IL-7 (Pierce). Cells were plated out into 2-m1 wells of 24-well tissue culture plates. Following day 3 in culture at 37°C in 5% CO2, medium changes were performed with R10 containing 10 U/ml IL-2, obtained through the National Cancer Institute Biological Resources Branch Preclinical Repository (Fredrick, MD) from Hoffmann-LaRoche. Cells were analyzed by flow cytometry after 7 days of stimulation, as described below. CTL activity was measured in a standard 51Cr release assay, as previously described (23), using autologous target cells pulsed with peptide CLTEYILWV.

**Flow cytometric analysis**

Surface staining for phenotypic markers was performed with directly conjugated Abs for 30 min at room temperature. After washing, the cells were lysed/permeabilized using BD-FACS Lyse and BD-FACS Permeabilization 2 Solutions (BD Immunocytometry Systems), per manufacturer’s instructions, before intracellular staining. Additional staining was performed with directly conjugated Abs to intracellular proteins (GrA, GrB, perforin, IFN-γ) at room temperature for 30 min. After a final wash, cells were resuspended in 500 μl of PBS containing 1% paraformaldehyde (Poly-science). Multiparametric flow cytometry was performed using a LSRII cytometer (BD Immunocytometry Systems), equipped with three lasers (488 nm solid state, 405 nm solid state, and 633 HeNe). Instrument compensation was performed with Ab capture beads (BD Pharmingen) stained singly with individual Abs used in the test samples. In all cases, 50,000 CD3+CD8+ events were collected. Data analysis was performed using FlowJo version 4.6 (Tree Star). All flow cytometric dot plots are displayed with five-decade logarithmic axes (except forward and side scatter, linear) with a data transformation that allows for improved visualization of events that fall at the lower end of the log scale (24).

**Statistical analysis**

Correlations and statistical significance were determined by Spearman rank correlation analysis. Differences between groups were analyzed using the Mann-Whitney U test for comparison of means. Statistical analyses were performed using Prism 4.0 software (Prism). Values of p < 0.05 were considered statistically significant.

**Results**

**Identification of T cell subsets by polychromatic flow cytometry**

We developed nine-color flow cytometric staining protocols to investigate cellular immune responses following immunization. Phenotypic division of naive and memory cells was used to analyze unique, functionally important subsets of T cells that were readily identified based on surface phenotype and intracellular protein expression (Fig. 1). Gating on cells that did not coexpress CD45RA and CCR7 identified three major CD8+ T cell populations based on expression of GrA and GrB: 1) GrA- and GrB-coexpressing cells; 2) GrA-expressing cells negative for GrB; and 3) cells that were negative for GrA and GrB (Fig. 1, panel 2). Superantigen stimulation, which elicits responses from a wide variety of T cells irrespective of their Ag specificity, showed that CD8+ T cells within each of these populations were capable of producing IFN-γ (Fig. 1, panel 3). The majority of IFN-γ production and CD57 expression was found in CD8+ effector T cells. Further gating on T effector (RA/CCR7), T effector memory (RA/CCR7+), or T effector memory RA/CCR7- showed that the majority of CD8+ T cells expressing GrB and/or CD57 reside in the effector cell populations (Fig. 1, panel 4). The highest percentage of GrA+GrB+ cells was found in the T effector memory RA/CCR7 population, with decreasing amounts in T effector and T CM cells, respectively. This pattern of granzyme expression in memory and effector cells is similar to that of perforin in CD8+ effector T cells (15).

**VSV-specific T cells express high levels of GrA and GrB 1 mo after immunization**

To evaluate the magnitude of VSV-specific cellular immunity following a primary immunization, PBMC were stimulated with live virus in an intracellular cytokine assay that allowed multiparametric evaluation of T cell responses. VSV-specific T cells were measured before immunization and 1 mo postimmunization in 41 volunteers that were immunized with APSV. The frequencies of VSV-specific CD4+ and CD8+ T cells before immunization were below the limit of detection (<0.03%). At 1 mo after immunization, expansion of both CD4+ and CD8+ T cells was observed in all volunteers. At this time, the magnitude of VSV-specific cells ranged from 0.07 to 1.08% of total CD4+ T cells (mean = 0.25%), from 0.04 to 1.57% of total CD8+ T cells (mean = 0.32%), and from 0.17 to 2.65% of total CD4+ and CD8+ T cells (mean = 0.57%). In general, the magnitude of the CD8+ T cell response was ~1.5-fold higher than the CD4+ T cell response 1 mo postvaccination (data not shown).

To evaluate the type of VSV-specific CD8+ T cells induced by primary immunization, cells were mock stimulated, stimulated with VV or with SEB for 6 h, and analyzed by nine-color flow
cytometry (stained for CD3, CD4, CD8, CCR7, CD45RA, GrA, GrB, CD57, and IFN-γ). Each of the three GrA/GrB populations of total CD8⁺ T cells was gated, followed by gating on CD57 vs IFN-γ. Data from two representative subjects, one with relatively high numbers of GrA/GrB-coexpressing CD8⁺ T cells and the other with low numbers of GrA/GrB-coexpressing CD8⁺ T cells, are depicted in Fig. 2A (panels 1 and 2, respectively). A summary of the percentage of GrA⁺/GrB⁺, GrA⁺/GrB⁻, and GrA⁻/GrB⁺ populations from total CD8⁺ T cells, IFN-γ-positive VV-specific CD8⁺ T cells, and IFN-γ-positive CD8⁺ T cells after superantigen stimulation from this cohort of 41 subjects is shown in Fig. 2B. At 1 mo postimmunization, the percentage of granzyme expression in IFN-γ-positive VV-specific populations ranged from 0.0 to 23.0% that were GrA⁺/GrB⁻ (mean = 9.22%), from 8.6 to 61.1% that were GrA⁺/GrB⁺ (mean = 35.1%), and from 27.8 to 82.0% that were GrA⁻/GrB⁺ (mean = 54.5%).

Interestingly, the majority of all CD8⁺ T cells that coexpress GrA and GrB also express CD57. In fact, we observed a direct correlation between CD57 and GrA and GrB. PBMC obtained 1 mo after immunization with APSV indicated in Fig. 1. CD8⁺ T cells were analyzed for IFN-γ expression and each of the three major populations analyzed for IFN-γ and CD57 expression. A. Flow cytometry dot plots from two representative subjects, one with relatively high numbers (panel 1) and the other with low numbers (panel 2) of GrA⁺/GrB⁺ CD8⁺ T cells. The numbers in the corners indicate the percentages of cells within that particular quadrant. B. Scatter plot of the frequencies of GrA⁺/GrB⁻, GrA⁺/GrB⁺, and GrA⁻/GrB⁺ T cells from a cohort of subjects (n = 41). Percentages are shown for total CD8⁺ T cells (●), IFN-γ-positive VV-specific CD8⁺ T cells (○), and IFN-γ-positive CD8⁺ T cells stimulated with SEB (□). Bars indicate mean values.

FIGURE 2. The majority of IFN-γ-positive VV-specific CD8⁺ T cells express GrA and GrB. PBMC obtained 1 mo after immunization with APSV were stimulated with VV and analyzed by nine-color flow cytometry, as indicated in Fig. 1. CD8⁺ T cells were gated based on GrA and GrB expression and each of the three major populations analyzed for IFN-γ and CD57 expression. A. Flow cytometry dot plots from two representative subjects, one with relatively high numbers (panel 1) and the other with low numbers (panel 2) of GrA⁺/GrB⁺ CD8⁺ T cells. The numbers in the corners indicate the percentages of cells within that particular quadrant. B. Scatter plot of the frequencies of GrA⁺/GrB⁻, GrA⁺/GrB⁺, and GrA⁻/GrB⁺ T cells from a cohort of subjects (n = 41). Percentages are shown for total CD8⁺ T cells (●), IFN-γ-positive VV-specific CD8⁺ T cells (○), and IFN-γ-positive CD8⁺ T cells stimulated with SEB (□). Bars indicate mean values.

with GrB, but did not express the terminal differentiation marker CD57 1 mo after immunization against smallpox.

One possible explanation for the large number of GrA⁺/GrB⁺ T cells after immunization is due to the nature of the APSV, which is a live-virus immunization associated with high levels of replicating virus at the site of inoculation. We compared the level of VV-specific CD8⁺ T cells with the peak viral titer found at the site of inoculation. There was no direct correlation between the total burst size of IFN-γ-positive CD8⁺ T cells as a whole or between the number of IFN-γ-positive VV-specific CD8⁺ T cells that coexpress GrA⁺/GrB⁺ and the peak virus titer at the site of inoculation in these 41 subjects (r = 0.076, p = 0.663 and r = −0.132, p = 0.447, respectively).

Immunization does not significantly expand GrA⁺ or GrB⁺ populations at 1 mo postimmunization

Previous reports indicated that GrB can be detected in ~20% of peripheral blood CD8⁺ T cells from healthy adults, and that during CMV infection the number of CD8⁺ T cells that expressed GrB showed temporary expansion (25). To determine whether the in vivo immunization or the 6-h in vitro stimulation with VV significantly altered the percentages of T cells expressing granzymes, we compared the level of GrA and GrB expression in CD8⁺ T cells before or after immunization and in VV-stimulated or mock-stimulated cells. As expected, the majority of CD8⁺ T cells did not express either GrA or GrB (mean = 60.3%). Expression of GrA alone or in combination with GrB in CD8⁺ T cells varied greatly between different subjects. The GrA⁺/GrB⁻ population ranged from 8.03 to 39.8% (mean = 22.2%), and the GrA⁺/GrB⁺ population ranged from 2.03 to 35.9% (mean = 15.0%) of total CD8⁺ T cells before immunization. Statistical differences were not detected in granzyme expression between samples collected before and 1 mo after immunization or between mock-stimulated and VV-stimulated PBMC (Table I). These data indicate that neither smallpox immunization nor in vitro VV stimulation significantly alters the percentage of cells that express granzymes 1 mo after immunization, and that short-term in vivo stimulation with VV does not induce significant degranulation or de novo synthesis of granzymes.

Tetramer-positive VV-specific CD8⁺ T cells express GrA and GrB in the absence of viral stimulation

Although we did not detect any significant expansion or reduction in GrA and GrB expression either after immunization or after in vitro stimulation, we could not rule out that stimulation of VV-specific T cells with live virus may alter the granzyme content of Ag-specific cells during the 6-h incubation. To address this question, we used tetramer binding in the absence of viral stimulation...
to evaluate further the cytolytic potential of VV-specific CD8+ T cells. Recently, two HLA-A2-restricted epitopes that are conserved between vaccinia and variola viruses have been described (21). We used each of these VV-peptide tetramers (VV-CLTEYILWV and VV-KVDDTFYYV) in 36 HLA-A2 subjects vaccinated against smallpox to assess the phenotype of VV-specific cells in the absence of in vitro stimulation.

As expected, tetramer-positive cells were not detected in samples obtained before immunization (≤0.01%; Fig. 4). The frequency of CD8+ T cells reacting with an HLA-A2-restricted VV tetramer (≥0.05%) 1 mo postimmunization was significantly higher for VV-CLTEYILWV compared with VV-KVDDTFYYV. CD8+ T cells from 28 of 36 subjects recognized VV-CLTEYILWV (mean = 0.30%; range 0.05–1.70%), whereas only 12 of 36 subjects recognized VV-KVDDTFYYV (mean = 0.12%; range 0.05–0.24%) 1 mo postimmunization. Significantly, the majority of VV tetramer-positive cells coexpressed GrA and GrB (Fig. 4). Tetramer-positive cells that coexpressed GrA and GrB ranged from 45.6 to 92.1% of VV-CLTEYILWV tetramer-positive cells (mean = 73.8%; n = 21), and from 43.2 to 76.8% of VV-KVDDTFYYV tetramer-positive cells (mean = 58.4%; n = 9). These results are similar to the percentage of VV-specific CD8+ T cells identified by intracellular IFN-γ analysis (mean = 60.2%; Fig. 2). Thus, more than one-half of the VV-specific CD8+ T cells coexpress GrA and GrB 1 mo after immunization, and this finding was confirmed in two separate assay systems to identify VV-specific cells (intracellular IFN-γ analysis or tetramer binding).

**Degranulation of VV-specific CD8+ T cells in response to viral stimulation**

Recently, a flow cytometric assay was developed that identifies CD8+ T cells capable of cytotoxic effector function. This assay relies on measuring the exposure of CD107a and/or CD107b on the cell surface following stimulation (26). These two lysosomal integral membrane proteins are readily detected in cells containing perforin, GrA, and GrB (Fig. 5A). Degranulation is accompanied by the transport of the CD107a and CD107b integral membrane proteins to the cell surface, followed by immediate reinternalization. Cells that degranulate in response to antigenic stimulation can be identified by providing fluorescently conjugated Abs to these proteins in the medium during stimulation. Representative flow cytometry dot plots from three subjects are shown in Fig. 5B. The majority of cells that secrete IFN-γ in response to VV stimulation fail to degranulate, based on expression of CD107a. However, a percentage of VV-specific CD8+ T cells degranulate, and therefore presumably lyse target cells, in response to live virus stimulation.

**VV-specific CD8+ T cells express low levels of perforin 1 mo postimmunization**

The three most prominent components present in cytolytic granules and released by effector cell during degranulation are perforin, GrA, and GrB (5). Perforin is essential for killing by means of the granule exocytosis pathway, and its expression is more restricted than that of serine proteases. Therefore, we investigated perforin expression in VV-specific CD8+ T cells. Gating on each of the three CD8+ T cell populations based on GrA and GrB expression, we found that perforin was predominantly expressed in the GrA+/GrB- population (Fig. 6A). This finding is similar to previous studies suggesting that the majority of CD8+ T cells that express perforin also express GrA (15). Surprisingly, although most VV-specific T cells express one or both granzyme molecules, the majority of VV-specific T cells did not express perforin 1 mo postimmunization. Representative flow cytometry dot plots from two subjects identifying VV-specific T cells by IFN-γ production or by VV-CLTEYILWV tetramer staining are shown in Fig. 6A. The mean percentages of IFN-γ-positive VV-specific and VV tetramer-positive CD8+ T cells that express perforin were 6.31% (range 1.89–16.4%; n = 10) and 7.34% (range 1.98–24.2%; n = 10), respectively. Thus, a clear majority of the VV-specific CD8+ T

### Table 1. Comparison of granzyme expression (mean percentage) of total CD8+ T cells with indicated phenotype before or 1 mo after smallpox immunization (n = 41)

<table>
<thead>
<tr>
<th>Population</th>
<th>Before Immunization</th>
<th>After Immunization</th>
<th>p Value&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control (1) (% )</td>
<td>Vaccinia (2) (%)</td>
<td>Control (3) (%)</td>
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<td>GrA+/GrB-</td>
<td>60.3</td>
<td>60.3</td>
<td>59.7</td>
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<td>GrA+/GrB-</td>
<td>15.0</td>
<td>15.5</td>
<td>15.6</td>
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</tbody>
</table>

<sup>a</sup>Statistics were determined using the Mann-Whitney U test to compare between subjects either before and after smallpox immunization or between control samples and vaccinia virus-stimulated samples.

<sup>b</sup>CD8+ T cells were gated based on GrA and GrB expression.
Viral stimulation. CD8 stained intracellular for CD107a, GrA, GrB, and perforin in the absence of CD8

A

six different experiments are shown.

An overlay dot plot was created to illustrate CD107a and perforin expression for each of three populations (GrA+/GrB+, black; GrA−/GrB−, red; and GrA−/GrB+, blue). B, Assessment of degranulation of VV-specific CD8+ T cells before (Before) and 1 mo postimmunization (After). Cells were stimulated with VV in the presence of directly conjugated CD107a for 6 h. Cells were surface stained, and intracellular content of IFN-γ was analyzed by flow cytometry. Plots are gated on CD8+ T cells and analyzed for CD107a vs IFN-γ. The numbers in the corners indicate the percentages of IFN-γ-positive cells within that particular quadrant. Three representative subjects of at least six different experiments are shown.

FIGURE 5. Degranulation by IFN-γ-positive VV-specific CD8+ T cells. A. Analysis of intracellular expression of CD107a, GrA, GrB, and perforin in CD8+ T cells. Cells were surface stained for CD3, CD4, and CD8 and then stained intracellular for CD107a, GrA, GrB, and perforin in the absence of viral stimulation. CD8+ T cells were gated based on GrA and GrB expression. B, Assessment of degranulation of VV-specific CD8+ T cells before (Before) and 1 mo postimmunization (After). Cells were stimulated with VV in the presence of directly conjugated CD107a for 6 h. Cells were surface stained, and intracellular content of IFN-γ was analyzed by flow cytometry. Plots are gated on CD8+ T cells and analyzed for CD107a vs IFN-γ. The numbers in the corners indicate the percentages of IFN-γ-positive cells within that particular quadrant. Three representative subjects of at least six different experiments are shown.

Viral stimulation. CD8 stained intracellular for CD107a, GrA, GrB, and perforin in the absence of CD8

A

six different experiments are shown.

An overlay dot plot was created to illustrate CD107a and perforin expression for each of three populations (GrA+/GrB+, black; GrA−/GrB−, red; and GrA−/GrB+, blue). B, Assessment of degranulation of VV-specific CD8+ T cells before (Before) and 1 mo postimmunization (After). Cells were stimulated with VV in the presence of directly conjugated CD107a for 6 h. Cells were surface stained, and intracellular content of IFN-γ was analyzed by flow cytometry. Plots are gated on CD8+ T cells and analyzed for CD107a vs IFN-γ. The numbers in the corners indicate the percentages of IFN-γ-positive cells within that particular quadrant. Three representative subjects of at least six different experiments are shown.

FIGURE 6. Differential expression of perforin in circulating vs activated VV-specific CD8+ T cells. A, Flow cytometry dot plots show the expression of perforin and IFN-γ after a 6-h stimulation with VV, or perforin and tetramer binding (VV-CLTEYILWV) in the absence of viral stimulation within the CD8+ T cell population after gating on GrA and GrB expression. Data from two representative HLA-A2 subjects 1 mo after immunization with APSV are shown. B, Flow cytometry dot plots show the expression of perforin and tetramer binding (VV-CLTEYILWV) from one of three representative experiments either directly ex vivo or after 7 days in vitro stimulation with VV. Diagrams show CTL activity against autologous target cells pulsed with specific peptide (CLTEYILWV; ●) or unpulsed (○) in a standard chromium release assay.

Most CD8+ T cells that are expanded in response to primary antigenic challenge display an effector cell phenotype and are lost during the contraction phase, and only a small percentage of stimulated cells become memory cells that persist after the viral infection has been cleared. Therefore, we sought to determine whether the population of VV-specific effector cells that coexpress GrA and GrB is present at 1 year after immunization compared with the frequency of this population 1 mo after immunization. The percentage of IFN-γ-positive VV-specific CD8+ T cells that coexpressed GrA and GrB was significantly reduced 1 year after immunization (mean = 33.2%; range 24.6–53.8%) compared with this population 1 mo after immunization (mean = 60.4%; range 37.0–83.6%), and a corresponding increase in the percentage of cells that expressed GrA singularly or did not express GrA or GrB in cells obtained 1 year after immunization (Fig. 7). Interestingly, a greater percentage of IFN-γ-positive VV-specific cells that coexpressed GrA/GrB expressed higher levels of CD57, and cells that were GrB negative expressed higher levels of CCR7 compared with cells 1 mo after immunization (data not shown). Similar results were observed in three HLA-A2 subjects that had detectable levels of tetramer-positive (VV-CLTEYILWV) cells at 1 mo and 1 year after immunization.

Discussion

This study examined the status of VV-specific CD8+ T cells in healthy adults that received a primary immunization against smallpox. Our results clearly demonstrate that primary immunization with APSV generates a robust effector CD8+ T cell response. This response was strongly biased toward cells with cytolytic potential
based on the coexpression of GrA and GrB in a majority of the Ag-specific cells. The ex vivo IFN-γ assay and MHC-peptide tetramer-binding assays used in our study revealed frequencies of GrA+/GrB+ VV-specific CD8+ T cells of 60.2% (n = 41) and 69.1% (n = 30), respectively. Interestingly, poxviruses have evolved various mechanisms to interfere with the activity of host immune responses and possess large numbers of viral homologues of cytokines, chemokines, and their receptors. Poxviruses also encode proteins related to the serpin family of protease inhibitors, termed SPI-1, -2, and -3 (27). The cytoytic response modifier, SPI-2, has been shown to inhibit serine and cysteine proteases, including in vitro inhibition of GrB (28). However, cell death induced by CTL occurs in the presence of complete caspase blockade, using peptide caspase inhibitors, including crmA (29). This finding indicates that CTL also activate pathways causing caspase-independent cell death. In fact, caspase-independent cell-death pathways induced by GrA and GrB have been described (29, 30).

Thus, although the specific cellular pathways responsible for control of poxvirus infections remain to be elucidated, our data, together with the fact that mice deficient in GrA and GrB were unable to control primary EV infection, suggest an important role for effector CD8+ T cells in resistance to poxviruses.

Cytotoxic granules are specialized secretory lysosomes that are present only in cells with cytolytic capability. However, for maximal antiviral activity, perforin must be secreted together with members of the serine protease family (31). Although the majority of VV-specific CD8+ T cells express granzymes 1 mo after smallpox immunization, they do not express high levels of perforin or GrB and analyzed by flow cytometry. The percentage of IFN-γ-positive CD8+ T cells that were GrA+/GrB+, GrA+/GrB-, or GrA-/GrB+ is indicated for samples obtained 1 mo (○) or 1 year (○) after immunization with APSV. The bars indicate mean values (n = 14). Values of p were determined using the Mann-Whitney U test for comparison of means between groups.

FIGURE 7. Differential expression of GrA and GrB in VV-specific CD8+ T cells 1 mo and 1 year after immunization. PBMC were stimulated with VV for 6 h and stained in a nine-color assay for surface CD3, CD4, CD8, CCR7, CD45RA, and CD57 and for intracellular IFN-γ, GrA, and GrB and analyzed by flow cytometry. The percentage of IFN-γ-positive CD8+ T cells that were GrA+/GrB+, GrA+/GrB-, or GrA-/GrB+ is indicated for samples obtained 1 mo (○) or 1 year (○) after immunization with APSV. The bars indicate mean values (n = 14). Values of p were determined using the Mann-Whitney U test for comparison of means between groups.

in virtually all of these cells. The finding that circulating VV-specific CD8+ T cells express GrA and GrB more frequently than perforin further suggests that these effector molecules are differentially regulated. Tight control of perforin expression is not unexpected given the cytotoxic potential of this molecule. Furthermore, the level of GrA and GrB expression in cells 1 mo postimmunization compared with cells 1 year postimmunization suggests differential regulation of these serine proteases. Taken together, these data suggest that when T cells are not actively recognizing Ag, the majority of CD8+ T cells down-regulate perforin and GrB expression.

This study differs dramatically from the analysis of T cell responses to persisting viruses such as EBV, CMV, or HIV in that immunization with VV more closely resembles an acute infection during which T cell differentiation following infection proceeds in the absence of large amounts of persisting Ag. In chronic infection in which Ag persists at high amounts, one may see a different pattern of effector molecules in Ag-specific T cell subsets. Thus, T cell subsets specific for persisting viruses may contain one population of T cells that has not encountered Ag for several days or weeks and another that recently has been exposed to Ag. This difference in antigenic exposure is an important distinction in the evaluation of immunological responses to immunization. Recently, a murine model of T cell differentiation in the absence of sustained Ag exposure has been proposed (36). The basis of this model is that the TEM is a transitory population representing an intermediate cell type in the effector-to-memory transition. Thus, TCM and TEM cells were not considered distinct subsets, but were part of a direct lineage that ends with the development of TEM cells. The process of T cell differentiation from naive to effectors and subsequently to memory cells continues long after infection has been resolved. Our results support this model, in that a significant burst of VV-specific CD8+ effector T cells is established early after immunization with APSV. These cells lack expression of the lymph node-homing marker CCR7 and display characteristics of effector T cells. VV-specific TEM cells examined 1 mo after immunization rapidly produce IFN-γ and contain high levels of granzymes. In fact, greater than 90% of the T cells at that time point are GrA positive and >50% of those cells also contain GrB. Notably, we observed a significant loss of GrB expression and increased expression of CCR7 in VV-specific cells analyzed 1 year vs 1 mo after vaccination. Most likely this is a result of substantial contraction of the effector T cell population and a transition of a proportion of these cells to the TEM compartment corresponding with the ability to enter the lymph node, a property mainly of TCM.

The results of this study and earlier studies (13, 14) suggest that the replication of substantial amounts of VV within pox lesions at the injection site may be required for the induction of CTL memory responses in recipients of standard smallpox vaccine and optimal induction of virus-specific CD8+ T cells. If CD8+ T cells follow a preprogrammed response of differentiation between TEM to TCM cells, then the type of TEM cells produced early after immunization may be more important than the quantity of the response. Thus, the ability of cells to successfully transit from TEM to TCM may be a fundamental property of protective memory associated with successful immunization. For instance, we noted the fact that the TEM cells present at 1 year postvaccination that maintain expression of GrA and GrB predominantly express CD57, indicative of terminal differentiation and replicative senescence. Thus, the contribution of Ag-specific T cells within this compartment to protective memory may be of questionable value. Of primary importance are the characteristics of TEM cells that allow a proportion of these cells to survive contraction and transition to TCM. Identifying phenotypic properties of those cells would be...
invaluable in evaluating experimental vaccines. Recently, it was proposed that the IL-7Rα chain (CD127) might distinguish effector cells that survive the contraction phase and develop into functional memory cells (37). We are currently investigating the relationship between CD127 and CD8+ and CD4+ T lymphocyte subsets that are induced early and sustained late after immunization with APSV. In light of our findings, it will be important to further characterize the lymphocyte subsets that accompany the immune response to new generation smallpox vaccines, such as further attenuated viruses like modified vaccinia Ankara or the LC16m8 strain, to understand better the role of cell-mediated immunity in protection from smallpox after immunization in VV-naive and VV-experienced individuals.

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Disclosures
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