

# Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8<sup>+</sup> T cell responses

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**Vaccinia virus immunization provides lifelong protection against smallpox, but the mechanisms of this exquisite protection are unknown. We used polychromatic flow cytometry to characterize the functional and phenotypic profile of CD8<sup>+</sup> T cells induced by vaccinia virus immunization in a comparative vaccine trial of modified vaccinia virus Ankara (MVA) versus Dryvax immunization in which protection was assessed against subsequent Dryvax challenge. Vaccinia virus–specific CD8<sup>+</sup> T cells induced by both MVA and Dryvax were highly polyfunctional; they degranulated and produced interferon  $\gamma$ , interleukin 2, macrophage inflammatory protein 1 $\beta$ , and tumor necrosis factor  $\alpha$  after antigenic stimulation. Responding CD8<sup>+</sup> T cells exhibited an unusual phenotype (CD45RO<sup>+</sup>CD27<sup>intermediate</sup>). The unique phenotype and high degree of polyfunctionality induced by vaccinia virus also extended to inserted HIV gene products of recombinant NYVAC. This quality of the CD8<sup>+</sup> T cell response may be at least partially responsible for the profound efficacy of these vaccines in protection against smallpox and serves as a benchmark against which other vaccines can be evaluated.**

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Abbreviations used: APC, allophycocyanin; CCR, CC chemokine receptor; MFI, median fluorescence intensity; MIP, macrophage inflammatory protein; MVA, modified vaccinia virus Ankara.

Vaccinia virus immunization provides protection against variola virus, the causative agent of smallpox, and stands as the classic example of a successful vaccine. However, the mechanisms by which this profound protection is mediated are not completely understood. For instance, although vaccinia virus–induced antibody responses have been shown to be necessary and sufficient for protection against monkeypox virus (1), virus-specific T cell responses generated at the time of vaccination may still contribute to effective protection. In addition, monkeypox infection in macaques may not completely recapitulate variola infection in humans and may serve as an inadequate model for protective immunity against smallpox.

It is known that CD8<sup>+</sup> T cells play an important role in immunity to multiple viruses. This has been shown both in pathogens that

are controlled by the host immune response, such as CMV and EBV (2), and pathogens that ultimately defeat it, such as HIV/simian immunodeficiency virus (3–11). Virus-specific CD8<sup>+</sup> T cells contribute to viral control by directly killing virus-infected cells, secreting antiviral factors, and secreting factors that recruit other cells of the immune system (12). Although virus-specific CD8<sup>+</sup> T cells are often measured by a limited number of parameters, such as IFN- $\gamma$  and/or IL-2 secretion, the functional profile of T cells is certainly more diverse, and the combination of functions that confer protection from infection are uncertain.

Recent advances in the number of parameters that can be simultaneously detected by flow cytometry allow more extensive characterization of T cell effector functions at the single-cell level (13). Using this technology, we recently examined five functions of CD8<sup>+</sup> T cells (surface CD107a mobilization for degranulation and

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IFN- $\gamma$ , IL-2, macrophage inflammatory protein [MIP] 1 $\beta$ , and TNF- $\alpha$  production) in HIV-infected progressors and nonprogressors and found that nonprogressors preferentially maintain HIV-specific CD8<sup>+</sup> T cells that effect all five functions on a cell-by-cell basis (14). The association between maintenance of this “5+” population of effector CD8<sup>+</sup> T cells and nonprogression suggests that polyfunctional CD8<sup>+</sup> T cells are an important component of a protective immune response. However, whether a vaccine can induce sustained levels of polyfunctional T cells and whether such responses are associated with protection against viral challenge are unknown. In this context, we studied vaccinia virus-specific T cell immune responses in a randomized, placebo-controlled study of modified vaccinia virus Ankara (MVA) versus Dryvax vaccination (15). The protocol was designed to test whether preimmunization with MVA results in protection from subsequent challenge with the vaccine strain Dryvax, a surrogate for variola infection (15).

We used polychromatic flow cytometry to characterize the functional and phenotypic profile of MVA and Dryvax-induced, antigen-specific CD8<sup>+</sup> T cells in subjects enrolled in this clinical trial. As a subsequent step, we also examined the functional profile of HIV-specific CD8<sup>+</sup> T cells induced by immunization with a recombinant NYVAC vaccine engineered to express HIV-1 clade C Env, Gag, Pol, and Nef. We show that immunization with replication-competent or attenuated vaccinia virus induces CD8<sup>+</sup> T cells that are highly polyfunctional but express an unusual memory phenotype. This polyfunctionality is associated with high levels of IFN- $\gamma$  production, which likely indicates that these CD8<sup>+</sup> T cells are potent effectors.

## RESULTS

### MVA and Dryvax induce a similar CD8<sup>+</sup> T cell functional profile

Vaccinia virus-naïve individuals were challenged with Dryvax after preimmunization with one or more doses of MVA or placebo (Table I). To measure CD8<sup>+</sup> T cell responses induced by MVA immunization and Dryvax challenge, we used polychromatic flow cytometry to measure five T cell functions independently and simultaneously: surface CD107a mobilization (degranulation) and IFN- $\gamma$ , IL-2, MIP-1 $\beta$ , and TNF- $\alpha$  induction after *in vitro* stimulation. Vaccinia virus-specific CD8<sup>+</sup> T cell frequencies were similar whether vaccinia virus or MVA was used for *in vitro* stimulation (Fig. S1, available

at <http://www.jem.org/cgi/content/full/jem.20062363/DC1>). Therefore, we used vaccinia virus infection of PBMCs to measure CD8<sup>+</sup> T cell responses in all donors. Our antibody panel and gating strategy enable identification of CD8<sup>+</sup> T cells exhibiting each and every combination of these five functions (Fig. 1 A). IFN- $\gamma$ , MIP-1 $\beta$ , and TNF- $\alpha$  production dominated the response to vaccinia virus (Fig. 1 B), as nearly all of the responding CD8<sup>+</sup> T cells produced these cytokines. In contrast, only about half of the responding CD8<sup>+</sup> T cells mobilized CD107a and even fewer produced IL-2 after vaccinia virus stimulation. This response pattern was observed after MVA immunization and after Dryvax challenge in both the MVA and placebo groups.

Total frequencies of vaccinia virus-specific CD8<sup>+</sup> T cells were calculated by summing the frequencies of CD8<sup>+</sup> T cells within each unique combination of functions (Fig. 1 C). Thus, each responding cell was counted only once. Before Dryvax challenge (green bars), MVA-induced CD8<sup>+</sup> T cell responses were detected only in individuals who received multiple MVA immunizations. Importantly, responses were not detected in the placebo (0 $\times$  MVA) group before Dryvax challenge. This serves as a control for nonspecific stimulation by vaccinia virus infection *in vitro*. Although there was no difference between the frequencies of total vaccinia virus-specific CD8<sup>+</sup> T cells before Dryvax challenge in individuals who received placebo or one dose of MVA ( $P = 0.085$ ), two doses of MVA induced higher frequencies of vaccinia virus-specific CD8<sup>+</sup> T cells than one dose ( $P = 0.013$ ). After Dryvax challenge (red and blue bars), CD8<sup>+</sup> T cell responses were detected in all individuals; however, frequencies were highest in individuals who were preimmunized with two or three doses of MVA, and their responses persisted through 3 mo after challenge. Specifically, three preimmunizations with MVA resulted in much higher frequency responses after Dryvax challenge than the placebo ( $P = 0.011$ ) or one MVA preimmunization ( $P = 0.013$ ).

Simultaneous measurement of five functions allows detailed assessment of response quality. The vaccinia virus-specific CD8<sup>+</sup> T cell response revealed a polyfunctional response pattern predominantly consisting of cells exhibiting three or more functions (Fig. 1 D). This functional profile was induced by both MVA and Dryvax regardless of preimmunization with MVA. Notably,  $\sim 25\%$  of the total response consisted of cells positive for all five functions. The major populations of polyfunctional cells included those positive for IFN- $\gamma$ , MIP-1 $\beta$ , and TNF- $\alpha$ . CD107a expression and/or IL-2 production were also detected in combination with these cytokines, but populations with these functions contributed less to the overall response.

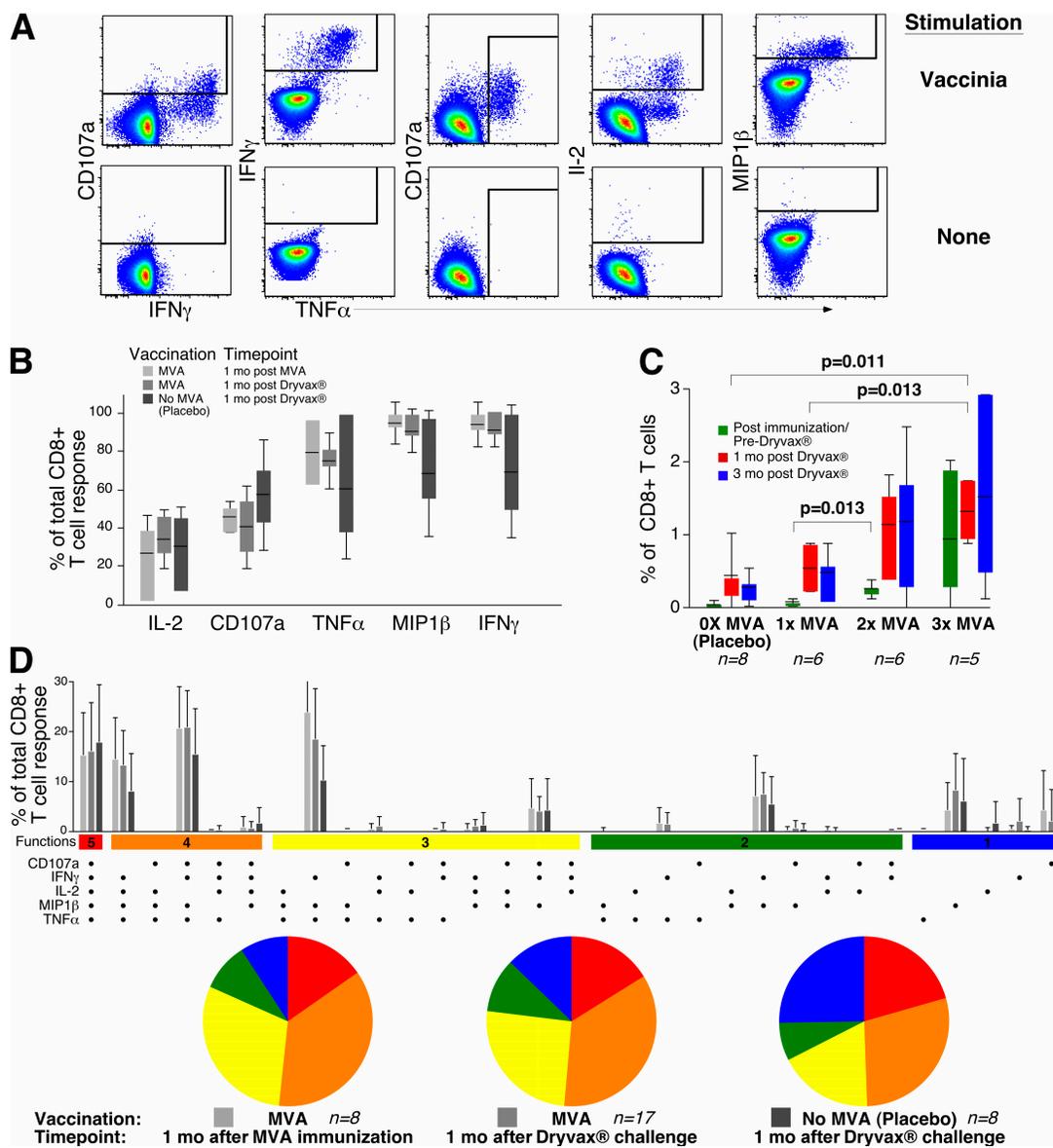
### Polyfunctional CD8<sup>+</sup> T cells produce more IFN- $\gamma$ per cell than cells that make only IFN- $\gamma$

Within a given sample, the median fluorescence intensity (MFI) of each functional parameter is related to the quantitative expression of that function on a per-cell basis. Thus, the relative amount of each cytokine and chemokine

**Table I.** VRC 201 study protocol

Group	<i>n</i>	MVA/sham immunization schedule (mo)	Dryvax challenge (mo)
1	6	0	3
2	8	0, 1	4
3	5	0, 1, 3	6
Placebo	8	Various	Various <sup>a</sup>

<sup>a</sup>Dryvax given 3 mo after last sham immunization.



**Figure 1. Vaccinia virus–specific CD8<sup>+</sup> T cells are highly polyfunctional.** (A) Representative example of the gating strategy used to define 31 functional response combinations of CD8<sup>+</sup> T cells. Single function gates were set based on negative control (unstimulated) samples and were placed consistently across samples. (B) The percentage of the total CD8<sup>+</sup> T cell response is shown. The frequency of CD8<sup>+</sup> T cells expressing each function was divided by the frequency of the total response. All MVA dose groups were combined into one group for analysis. (C) Magnitude of CD8<sup>+</sup> T cell responses before and after Dryvax challenge in each preimmunization

group. (D) Functional composition of the CD8<sup>+</sup> T cell response. Every possible combination of responses is shown on the x axis. Responses are grouped and color coded according to the number of functions. The data are summarized by the pie charts, in which each slice of the pie represents the fraction of the total response that consists of CD8<sup>+</sup> T cells positive for a given number of functions. All MVA dose groups were combined into one group for analysis (as in B). Boxes represent interquartile ranges; mean and SD lines are shown. Bars indicate the percentage of the total response contributed by CD8<sup>+</sup> T cells with a given functional response.

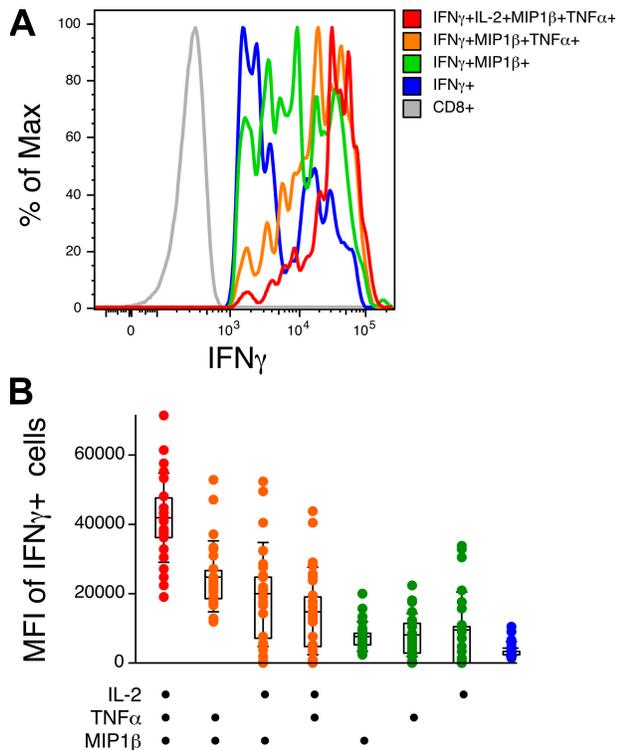
per cell can be measured for each functional population. Previous studies have shown that vaccine-induced, polyfunctional CD4<sup>+</sup> T cell responses in mice produce more IFN- $\gamma$  on a per-cell basis than mono- or bifunctional cells (16). Importantly, these studies showed that MFI measured by intracellular cytokine staining directly correlated with secretion of the cytokines measured by ELISA. Using MFI analysis,

we also found an association between IFN- $\gamma$  MFI and the degree of polyfunctionality (Fig. 2). Specifically, cells with increasing numbers of functions had higher IFN- $\gamma$  MFIs and, thus, produced more cytokine per cell. Surface mobilization of CD107a appeared to be irrelevant for this association; cells producing all cytokines had the highest IFN- $\gamma$  MFI regardless of CD107a expression (unpublished data).

These data demonstrate that a single polyfunctional CD8<sup>+</sup> T cell makes 5–10 times more IFN- $\gamma$  than a single monofunctional CD8<sup>+</sup> T cell. MFI analysis of other cytokines did not reveal such an association.

### Vaccinia virus-specific CD8<sup>+</sup> T cells have an unusual phenotype

We costained with cell surface markers to determine the surface phenotype of vaccinia virus-specific CD8<sup>+</sup> T cells (Fig. 3). Costaining revealed an unusual surface phenotype for memory cells: CD27<sup>(dull)</sup>, CD45RO<sup>(-)</sup>, CD57<sup>(-)</sup>, CD11a<sup>(dull)</sup>, and CC chemokine receptor (CCR) 7<sup>(-)</sup>. Further staining revealed that CD45RO<sup>-</sup> cells were CD45RA<sup>+</sup> (unpublished data). Although vaccinia virus-specific CD8<sup>+</sup> T cells coexpressed CD45RA and CD27, they were clearly not naive; CD27 staining was dimmer than that of naive cells. This phenotype was maintained through several months after Dryvax challenge (Fig. 3 A) and was exhibited by vaccinia virus-specific CD8<sup>+</sup> T cells regardless of their functional profile (Fig. 3 B). Additionally, despite the unusual phenotype, these cells appear to be long lived; we have detected polyfunctional, vaccinia virus-specific CD8<sup>+</sup> T cells exhibiting this unusual phenotype in an individual 5 yr after Dryvax immunization (unpublished data).



**Figure 2. Polyfunctional cells make more IFN- $\gamma$  per cell than monofunctional cells.** (A) IFN- $\gamma$  fluorescence of CD8<sup>+</sup> T cells with different functional properties. Representative example from one individual. (B) Compiled IFN- $\gamma$  MFI of CD8<sup>+</sup> T cells of all cytokine combinations ( $n = 24$ ). Boxes represent interquartile ranges; means and SDs are indicated (note that the average MFI for the IFN- $\gamma$ <sup>+</sup> populations is 446).

### Induction of polyfunctionality and unique phenotype is not a consequence of vaccinia virus stimulation in vitro

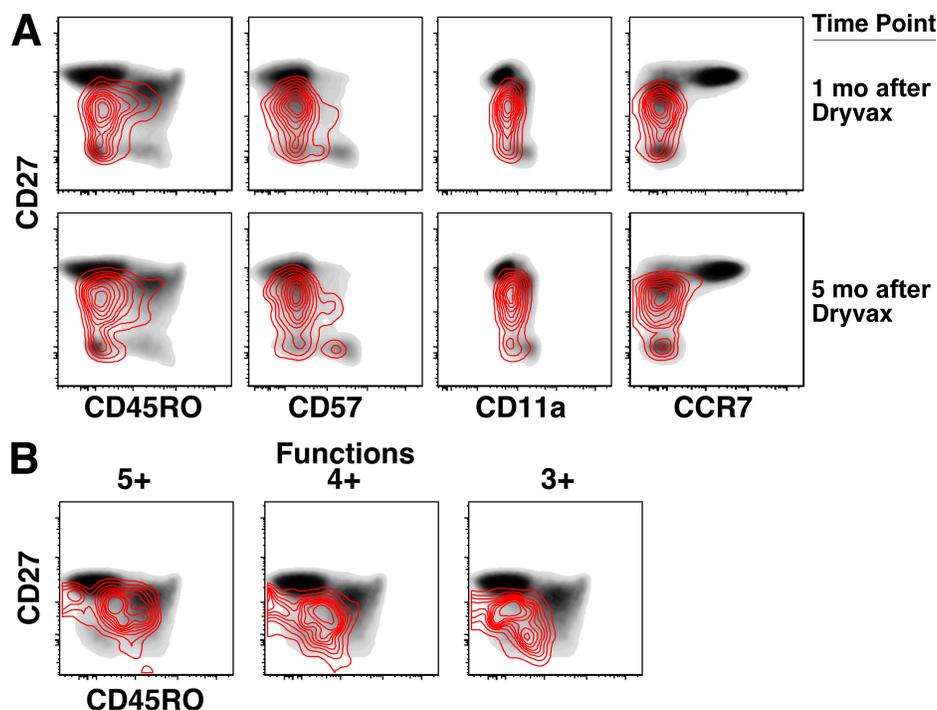
In previous experiments, we examined the function and phenotype of CD8<sup>+</sup> T cells specific for HIV, CMV, EBV, and influenza and found them to be less polyfunctional than the responses to vaccinia virus we report in this paper (14). In addition, the phenotype of these virus-specific CD8<sup>+</sup> T cells was distributed mainly between CD45RO<sup>+</sup>CD27<sup>+</sup> and CD45RO<sup>+</sup>CD27<sup>-</sup> populations. We opined that the phenotype and function of vaccinia virus-specific CD8<sup>+</sup> T cells observed in this study could be caused by use of a live virus stimulation (compared with the peptides used in previous studies). We therefore performed several experiments to address this possibility. HLA-A2 tetramers loaded with three different vaccinia virus peptides were used to identify vaccinia virus-specific CD8<sup>+</sup> T cells. Unstimulated, tetramer-positive CD8<sup>+</sup> T cells displayed the same phenotype as multifunctional cells detected after vaccinia virus stimulation (Fig. 4 A). In addition, peptide was used to stimulate CD8<sup>+</sup> T cells from HLA-A2<sup>+</sup> donors after Dryvax challenge. In both phenotype and function, peptide-specific CD8<sup>+</sup> T cell responses were similar to those detected after live vaccinia virus infection (Fig. 4 B).

An additional experiment was performed to confirm that the functional profile and phenotype of virus-specific CD8<sup>+</sup> T cells detected by virus infection in vitro was similar to that detected after peptide stimulation. Vaccinia virus recombinants expressing HIV-1 clade B Gag were used to stimulate HIV Gag-specific CD8<sup>+</sup> T cell responses from HIV-1-infected individuals. Gag-specific CD8<sup>+</sup> T cells had the same phenotype and function whether stimulated with the vaccinia virus recombinant or overlapping Gag peptides, and differed markedly from the phenotype and function of vaccinia virus-specific CD8<sup>+</sup> T cells (Fig. 4 C).

Collectively, these results show that vaccinia virus-specific CD8<sup>+</sup> T cells express a polyfunctional, CD45RO<sup>-</sup>CD27<sup>intermediate</sup> phenotype that reflects the nature of the specific response generated to the vaccination in vivo. A similar surface marker phenotype was also recently reported in a melanoma tumor antigen-recombinant MVA vaccine trial (17).

### Persistence of vaccinia virus-specific CD8<sup>+</sup> T cell clonotypes through 3 mo after Dryvax

Given the unique surface phenotype of vaccinia virus-specific CD8<sup>+</sup> T cells, we wanted to confirm that these cells persist through 3 mo after Dryvax and exclude the possibility that cells were being recruited from a preexisting pool of memory T cells. Therefore, we performed TCR clonotyping on sorted, tetramer-positive CD8<sup>+</sup> T cells at 1 and 3 mo after Dryvax. The same clonotypes were identified at both 1 and 3 mo after Dryvax (Fig. 5). Importantly, clonotypes with identical CDR3 amino acid sequences were also identical at the nucleotide level (unpublished data). Additionally, the hierarchy of clonotypes was similar at both time points. This strongly suggests that vaccinia virus-specific memory CD8<sup>+</sup> T cells that persist through 3 mo after immunization are derived from the same pool of cells that existed at 1 mo after immunization.



**Figure 3. Vaccinia virus-specific CD8<sup>+</sup> T cells have an unusual phenotype.** (A) Several surface markers were included in the multicolor flow cytometry panel to define the phenotypes of vaccinia virus-specific CD8<sup>+</sup> T cells. To incorporate these markers into the panel, responding cells were identified by staining with Alexa Fluor 680-CD107a and IFN- $\gamma$ , TNF- $\alpha$ , or IL-2 (each conjugated to APC). A CD27-PE antibody conjugate was used to allow for the best discrimination of expression. The phenotype of CD107a<sup>+</sup>cytokine<sup>+</sup> CD8<sup>+</sup> T cells is shown as a red contour plot overlaid

on a density plot of the total CD8<sup>+</sup> T cell population at 1 and 5 mo after Dryvax challenge in a representative individual from the MVA immunization group. (B) The vaccinia virus-specific CD8<sup>+</sup> T cell phenotype is similar among cells of different functional properties. 5+ (CD107a<sup>+</sup>IFN- $\gamma$ <sup>+</sup>IL-2<sup>+</sup>MIP-1 $\beta$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup>), 4+ (CD107a<sup>+</sup>IFN- $\gamma$ <sup>+</sup>IL-2<sup>+</sup>MIP-1 $\beta$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup>), and 3+ (CD107a<sup>+</sup>IFN- $\gamma$ <sup>+</sup>IL-2<sup>+</sup>MIP-1 $\beta$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup>) CD8<sup>+</sup> T cells are overlaid as contour plots on density plots of the total CD8<sup>+</sup> T cell population.

### Vaccinia virus-specific CD8<sup>+</sup> T cells contain granzymes and perforin

About half of vaccinia virus-specific CD8<sup>+</sup> T cells exhibited surface mobilization of CD107a in response to virus stimulation. Therefore, we combined tetramer and intracellular staining to examine the granzyme and perforin content of vaccinia virus-specific CD8<sup>+</sup> T cells. Nearly all tetramer-positive cells contained granzyme A, most contained granzyme B, and up to half were perforin positive (Fig. 6). This suggests that vaccinia virus-specific CD8<sup>+</sup> T cells have the ability to kill virus-infected cells.

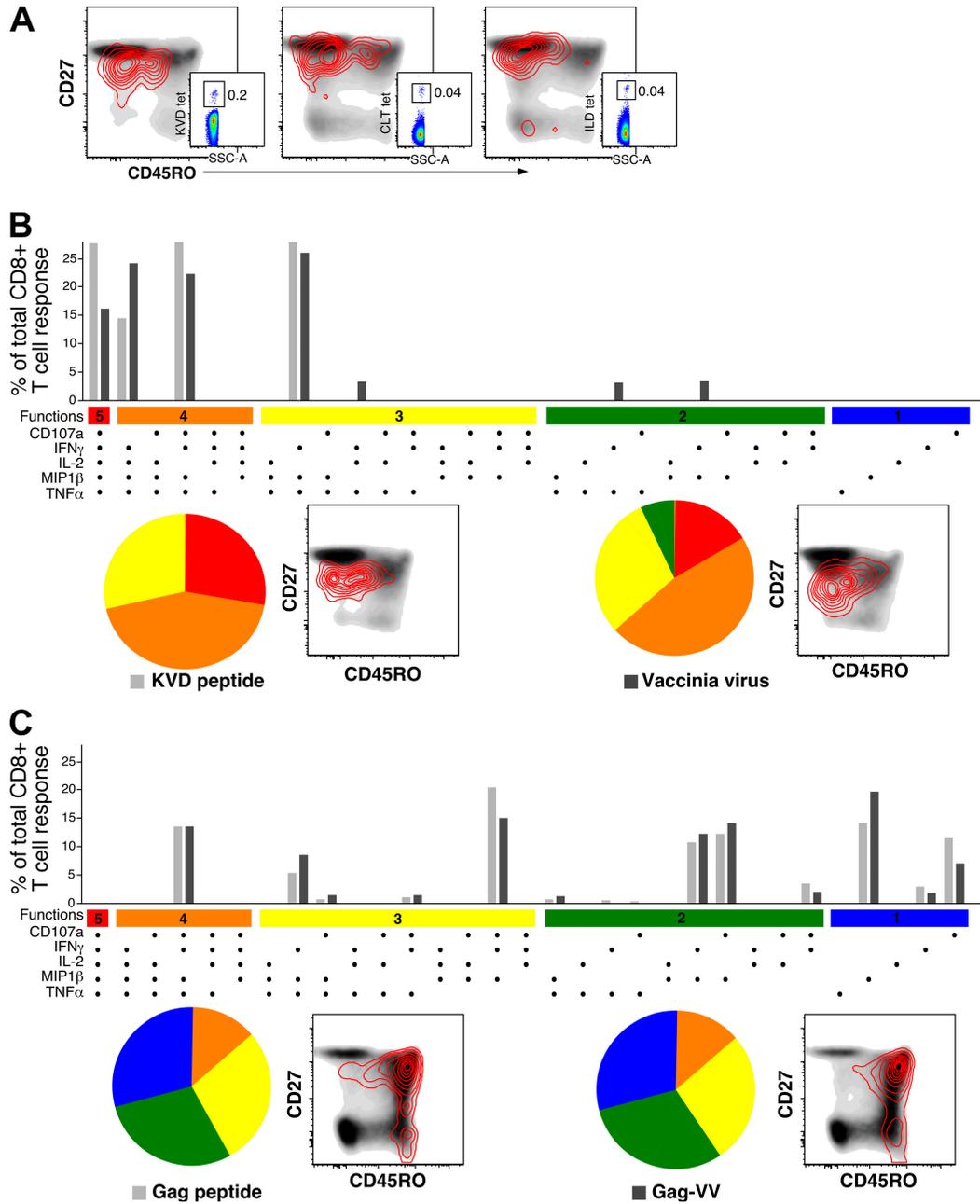
### HIV-1-specific CD8<sup>+</sup> T cells induced by an HIV recombinant vaccinia virus are highly polyfunctional

We posited that HIV-specific CD8<sup>+</sup> T cells induced by an HIV recombinant vaccinia virus vaccine should express the function and phenotype of vaccinia virus-specific CD8<sup>+</sup> T cells. We therefore measured T cell responses in five individuals immunized as part of the EuroVacc vaccine protocol with DNA prime/NYVAC HIV-1 clade C recombinant boost (unpublished data). An HLA-A1-restricted CD8<sup>+</sup> T cell response to the Env peptide YSENSEYY was detected in all five donors (unpublished data). We found that the functional profile and phenotype of Env-specific CD8<sup>+</sup> T cells induced

by vaccination was similar to the vaccinia virus vector-specific CD8<sup>+</sup> T cell response (Fig. 7). Env-specific CD8<sup>+</sup> T cells were highly polyfunctional and exhibited a similar phenotype as vaccinia virus-specific CD8<sup>+</sup> T cells. These data show that the function and phenotype of vaccinia virus vector-specific CD8<sup>+</sup> T cell responses extend to inserts expressed within the vector.

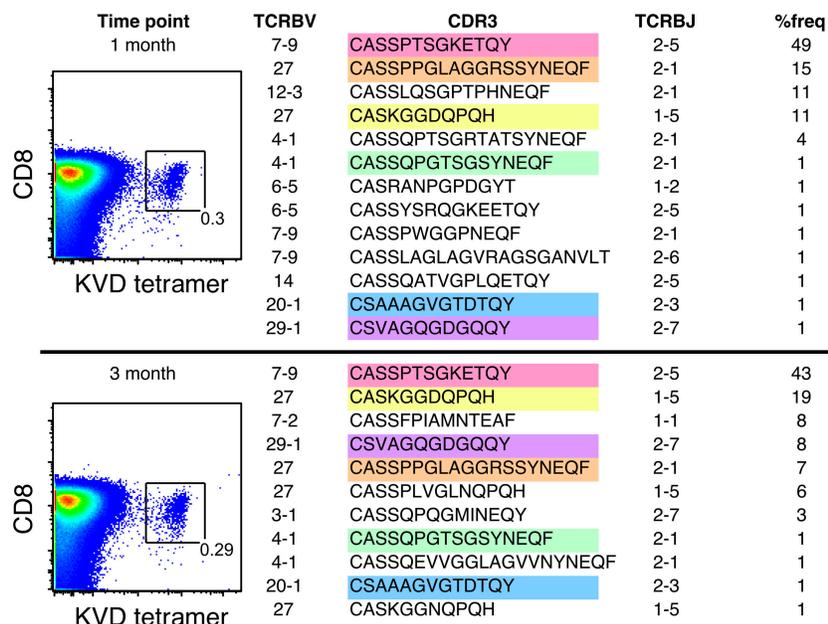
### DISCUSSION

Immunization with vaccinia virus induces lifelong protection from smallpox and can serve as a benchmark for the type of immunity that other vaccines should induce. Vaccinia virus-specific CD8<sup>+</sup> T cells are induced after immunization (18–20), and some HLA-restricted peptides have been identified (21, 22). Vaccination strategies using recombinant vaccinia viruses are currently being tested in clinical trials. In this paper, we report that (a) attenuated vaccinia virus strains such as MVA and NYVAC induce polyfunctional CD8<sup>+</sup> T cell responses similar to those of the completely replication-competent Dryvax strain, (b) polyfunctional CD8<sup>+</sup> T cells make much more IFN- $\gamma$  than cells with fewer functions, (c) vaccinia virus-induced CD8<sup>+</sup> T cells express an unusual phenotype (CD45RO<sup>-</sup>CD27<sup>intermediate</sup>), and (d) DNA prime/HIV recombinant NYVAC boost vaccinations induce polyfunctional CD8<sup>+</sup> T cells specific for the inserted HIV genes.



**Figure 4. The unusual phenotype of vaccinia virus–specific CD8<sup>+</sup> T cells is not a consequence of in vitro stimulation.** (A) Representative KVD, CLT, and ILD tetramer staining of HLA-A2<sup>+</sup> vaccinees after Dryvax challenge plotted as tetramer versus side scatter-area (SSC-A). Numbers indicate the percentage of CD8<sup>+</sup> T cells that are tetramer positive. CD27 and CD45RO expression on tetramer-binding cells is shown as red contour plots overlaid on density plots of the total CD8<sup>+</sup> T cell population. (B) PBMCs from HLA-A2<sup>+</sup> vaccinees were stimulated with peptide for 6 h or vaccinia virus for 9 h. Data from a representative stimulation with KVD is displayed (as in Fig. 1 D). The phenotype of vaccinia virus–specific CD8<sup>+</sup> T cells is shown as a red contour plot overlaid on a density plot of the

total CD8<sup>+</sup> T cell population. To best separate the CD27 high, intermediate, and low populations, a PE-conjugated antibody was used for staining. To accommodate this change from the standard antibody panel, the anti-MIP-1 $\beta$  antibody was omitted. Therefore, the contour plot shows the phenotype of CD107a<sup>+</sup>IFN- $\gamma$ <sup>+</sup>IL-2<sup>+</sup>TNF- $\alpha$ <sup>+</sup> CD8<sup>+</sup> T cells, which (as shown by the bar graph) also produce MIP-1 $\beta$ . (C) PBMCs from HIV-1–infected individuals were stimulated for 6 h with overlapping Gag peptides or for 9 h with Gag recombinant vaccinia virus. Data from a representative individual is shown. The contour plots show the phenotype of CD107a<sup>+</sup>IFN- $\gamma$ <sup>+</sup>IL-2<sup>+</sup>TNF- $\alpha$ <sup>+</sup> CD8<sup>+</sup> T cells.



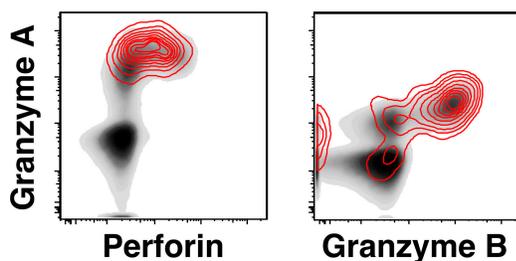
**Figure 5. Clonal analysis of sorted CD8<sup>+</sup> T cells specific for KVD at 1 and 3 mo after Dryvax.** CDR3 amino acid sequence and percent frequency and TCRBJ usage are shown for each clonotype defined by its

CDR3. Sequences with identical amino acid residues are shown in color. Tetramer frequencies are shown as a percentage of total CD8<sup>+</sup> T cells. Data are from one individual.

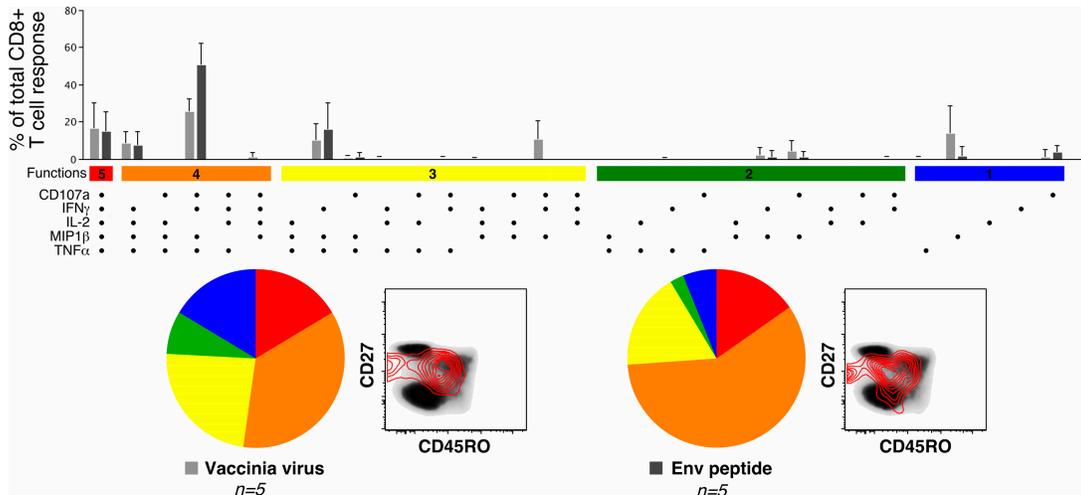
A safer alternative to Dryvax is sought because of the high rate of serious complications in the general population, the increased risk in specific populations (including the immunocompromised and those afflicted with eczematous skin disease), and the potential need for global orthopoxvirus immunity for protection against a bioterrorist threat. MVA is an attenuated strain that exhibits very limited replication in mammalian cells and does not disseminate in the host (23, 24). It may therefore represent a safer alternative than Dryvax. NYVAC was derived from the VACV strain Copenhagen, from which 18 genes, encoding proteins involved in host range and virulence, were deleted (25). Two doses of MVA have been shown to protect macaques from lethal monkeypox challenge (26); however, its immunogenicity and ability to provide protection against smallpox in humans has not been tested.

In this paper, we report that both MVA and Dryvax induce highly polyfunctional CD8<sup>+</sup> T cells that can respond by degranulating and producing IFN- $\gamma$ , TNF- $\alpha$ , and MIP-1 $\beta$ , with or without IL-2. This suggests that extensive replication of the virus in vivo is not required to induce polyfunctional CD8<sup>+</sup> T cell responses in that the same functions were induced by replication-competent Dryvax and replication-defective MVA. However, it is important to note that, whereas one immunization with Dryvax induced measurable responses in vaccinia virus-naïve individuals, more than one dose of MVA was required for detection of vaccinia virus-specific CD8<sup>+</sup> T cells before Dryvax challenge.

The induction and maintenance of highly polyfunctional, virus-specific CD8<sup>+</sup> T cells after immunization with vaccinia virus is consistent with our observation that polyfunctional CD8<sup>+</sup> T cell responses are present in subjects with well-controlled virus infections such as EBV, CMV, influenza, and nonprogressive HIV-1 (14, 27, 28). This suggests that maintenance of highly polyfunctional, virus-specific CD8<sup>+</sup> T cells is beneficial and contributes to effective antiviral immunity. Although polyfunctional CD8<sup>+</sup> T cells characterize the response to vaccinia virus and nonprogressive HIV infection, the specific functional combinations differ between the two responses. For example, vaccinia virus induces a population of CD8<sup>+</sup> T cells effecting four functions (IFN- $\gamma$ , IL-2, MIP-1 $\beta$ , and TNF- $\alpha$ ) that is very low in HIV nonprogressors and not detected in HIV progressors (14). Additionally, the dominant three-functional CD8<sup>+</sup> T cell populations induced also differ between vaccinia virus (IFN- $\gamma$ , MIP-1 $\beta$ , and TNF- $\alpha$ ) and nonprogressive HIV infection (CD107a, IFN- $\gamma$ , and MIP-1 $\beta$ ). TNF- $\alpha$  production differs between



**Figure 6. Vaccinia virus-specific CD8<sup>+</sup> T cells express intracellular granzymes and perforin.** Perforin and granzyme expression on KVD tetramer-binding cells is shown as red contour plots overlaid on density plots of the total CD8<sup>+</sup> T cell population. An APC-conjugated tetramer was used in A; a PE-conjugated tetramer was used in B. Representative staining from one of three donors.



**Figure 7. Env-specific CD8<sup>+</sup> T cells induced by an HIV recombinant vaccinia virus are highly polyfunctional.** Functional composition of the CD8<sup>+</sup> T cell response specific for the vaccinia virus vector and Env insert displayed (as in Fig. 1 D). The phenotype of 5<sup>+</sup> (CD107a<sup>+</sup>IFN- $\gamma$ <sup>+</sup>IL-

2<sup>+</sup>TNF- $\alpha$ <sup>+</sup>MIP-1 $\beta$ <sup>+</sup>) CD8<sup>+</sup> T cells from one representative individual is shown as red contour plots overlaid on density plots of the total CD8<sup>+</sup> T cell population. Mean bars and SD lines are shown.

the vaccinia virus– and HIV–specific response; more than half of vaccinia virus–specific CD8<sup>+</sup> T cells produce this cytokine, whereas less than half of HIV–specific CD8<sup>+</sup> T cells make TNF- $\alpha$ . Thus, the functional profiles of virus–specific CD8<sup>+</sup> T cells are dependent on the infecting virus and may reflect differences in cell tropism or activation through different antigen–processing pathways.

Polyfunctional CD4<sup>+</sup> T cells correlate with protection from *Leishmania major* and *Mycobacterium tuberculosis* in a mouse model (16). Detailed analysis of the *L. major*–specific CD4<sup>+</sup> T cell response revealed that not only was the quality of the response more polyfunctional in protected mice but that polyfunctional cells produced more cytokine per cell than monofunctional CD4<sup>+</sup> T cells. Our data are consistent with these observations in that vaccinia virus–specific, polyfunctional CD8<sup>+</sup> T cells produce larger amounts of IFN- $\gamma$  per cell than cells with fewer functions. This suggests that these polyfunctional CD8<sup>+</sup> T cells are potent effectors and may explain, in part, why they are associated with viral control.

Virus–specific CD8<sup>+</sup> T cells induced by MVA, NYVAC, and Dryvax express CD45RA and CD27. CD45RA is the high molecular mass isoform of CD45, a phosphatase involved in regulating T cell signal transduction by activation of Src family protein tyrosine kinases such as Lck and inhibition of Janus kinases (for review see reference 29). CD27 is a member of the TNF receptor family and functions as a co–stimulatory molecule (30). Although coexpression of CD45RA and CD27 is often used to define naive T cells (31), the intensity of CD27 staining on vaccinia virus–specific CD8<sup>+</sup> T cells is intermediate between CD27–high and –low populations and does not coincide with the naive T cell population. Intermediate CD27 expression has been previously reported on CMV–specific CD8<sup>+</sup> T cells and was associated with rapid cytokine production; expression of granzyme B, perforin,

and CD57; and lack of CD28 expression (31, 32). Vaccinia virus–specific CD8<sup>+</sup> T cells differ in that they lack CD57 but are otherwise similar in their expression of effector molecules. Thus, they are likely good effectors that are maintained as long–term memory cells. Although it is now well–established that some memory CD8<sup>+</sup> T cells express CD45RA, it is unusual that vaccinia virus–specific CD8<sup>+</sup> T cells express this isoform as early as 1 mo after vaccination and through 3 mo after vaccination. Phenotypic analyses of primary and persistent EBV– and CMV–specific CD8<sup>+</sup> T cells have shown predominant CD45RO expression during acute infection with the acquisition of CD45RA expression on some virus–specific CD8<sup>+</sup> T cells months thereafter (33–35). A small proportion of vaccinia virus–specific CD8<sup>+</sup> T cells do express CD45RO at 1 and 3 mo after vaccination, which may indicate that CD45RO expression was more prevalent within the first month after vaccination, before sampling. CD45RA versus RO expression can also be associated with epitope–specificity (34, 35); however, we found a similar distribution of CD45RA<sup>+</sup> and CD45RO<sup>+</sup> cells specific for three different vaccinia virus–derived peptides as for whole virus (Fig. 4).

Expression of CCR7 marks long–lived, central memory cells that home to lymph nodes and can differentiate into effectors upon reexposure to antigen (36). Although vaccinia virus–specific CD8<sup>+</sup> T cells did not express CCR7 through 5 mo after vaccination, long–term T cell immunity after Dryvax immunization has been documented (37–39). Therefore, it is possible that CCR7 expression is gained at later time points after vaccination, that CCR7<sup>+</sup> vaccinia virus–specific CD8<sup>+</sup> T cells reside in lymph nodes and do not recirculate through the peripheral blood early after vaccination, or that expression of CCR7 is not a requisite marker for maintenance of long–lived vaccinia virus–specific CD8<sup>+</sup> T cells.

The ability of MVA to induce polyfunctional CD8<sup>+</sup> T cell responses suggests that safer, attenuated vaccinia virus strains, such as MVA and NYVAC, may be useful vaccine platforms for other viruses, such as HIV. However, several reports in mice and humans (17, 40), as well as our preliminary studies, have found that recognition of recombinant gene inserts is poor. Vaccination strategies using a DNA prime followed by recombinant vaccinia virus boost increase immunogenicity of the recombinant antigen expressed by the viral vector (41). After two DNA primes and two recombinant NYVAC boosts, highly polyfunctional CD8<sup>+</sup> T cells specific for inserted HIV gene products are induced.

In summary, virus-specific CD8<sup>+</sup> T cells induced by vaccinia virus immunization are highly polyfunctional and phenotypically unusual. This polyfunctional CD8<sup>+</sup> T cell profile is induced to both the vector and inserts and is consistent with virus-specific CD8<sup>+</sup> T cell responses observed in well-controlled, persistent infections such as CMV, EBV, and non-progressive HIV-1 infection. Vaccine-induced polyfunctional, but not IFN- $\gamma$  monofunctional, T cells have been shown to correlate with protection of mice against *L. major* challenge. Collectively, these data indicate that vaccines able to elicit polyfunctional T cells are desirable, and suggest that the reason that Dryvax offers exquisite protection against smallpox infection is its inherent ability to induce highly polyfunctional T cell responses.

## MATERIALS AND METHODS

**Vaccine research center (VRC) 201 study protocol.** VRC 201 was a randomized, double-blind, placebo-controlled study that enrolled 76 healthy, vaccinia virus-naïve adults from 18 to 33 yr of age to receive (a) one dose of vaccinia virus (Dryvax); (b) 1, 2, or 3 intramuscular doses of MVA (Therion Biologics Corporation); or (c) placebo followed by a Dryvax challenge 12 wk after the last dose of MVA/placebo. The primary objective of the study was to evaluate the safety of MVA and to identify a schedule of MVA that provides evidence of clinical protection from vaccinia virus challenge. The secondary objective was to examine the immunogenicity of MVA and Dryvax, as determined by vaccinia virus-specific neutralizing antibody, binding antibody, and T cell responses measured by flow cytometric detection of intracellular cytokines. A complete analysis of the primary objective, as well as local and systemic reactogenicity of vaccination, is presented elsewhere (15).

A subset (27 subjects) of the study participants was chosen for more extensive evaluation of their vaccinia virus-specific T cell responses based on the number of cells available and the magnitude of their response. Results obtained using this subset of donors do not appear to be skewed by these selection criteria; within this group, donors with high frequency CD8<sup>+</sup> T cell responses had similar functional profiles as those with low frequency responses. All study participants gave informed consent, and the study was approved by the NIAID Institutional Review Board (NIH 02-I-0316).

**EuroVacc study protocol.** HIV-negative volunteers received two DNA primes (weeks 0 and 4), followed by two recombinant NYVAC boosts (weeks 20 and 24). The NYVAC was engineered to express HIV-1 clade C Env, Gag, Pol, and Nef. A complete analysis of the EuroVacc study is presented elsewhere (unpublished data). For our experiments, T cell responses were analyzed from five vaccinees from a sample obtained 2 wk after the final NYVAC boost. This study was approved by the institutional review boards of St Mary's Hospital and Centre Hospitalier Universitaire Vaudois.

**Polychromatic flow cytometric antibody panels.** The following antibody panel was used to independently quantify five separate T cell functions:

CD3-Cy7 allophycocyanin (APC), IFN- $\gamma$ -FITC, MIP-1 $\beta$ -PE, IL-2-APC, TNF- $\alpha$ -Cy7PE (all obtained from BD Biosciences); CD4-Cy5.5PE (Caltag); CD45RO-ECD (Beckman Coulter); CD107a-Alexa Fluor 680, CD14-Cy5PE, CD19-Cy5PE, CD27-Cascade Blue (conjugated according to standard protocols available at <http://drmr.com/abcon/index.html>); and CD8-QD655 and CD57-QD545 (conjugated as previously described; reference 42). Unconjugated antibodies were obtained from BD Biosciences; Q-Dots, Alexa Fluor 680, and Cascade Blue were obtained from Invitrogen; Cy5 was obtained from GE Healthcare; and PE was obtained from Pro-Zyme. As new reagents became available, and to address additional questions regarding T cell responses, the following reagents were also used in various combinations: CD27-PE, CD11a-FITC, CCR7-Cy7PE (all obtained from BD Biosciences); CD8-QD705, CD27-Cy5PE, CD14-Cascade Blue, and CD19-Cascade Blue (conjugated as described); and a violet fluorescent reactive dye (ViViD; Invitrogen) as a viability marker to exclude dead cells from the analysis (43).

**Peptides.** The vaccinia virus-derived peptides KVDDTFYV (KVD; protein 018L<sub>74-82</sub>) (21), CLTEYLWV (CLT; protein 189R<sub>79-87</sub>) (21), and ILDDNLYKV (ILD; protein 074R<sub>18-26</sub>) (17) were synthesized by New England Peptide, Inc. The HIV-1 peptide YSENSEYY (clade C Env<sub>173-177</sub>) was synthesized by the Centre Hospitalier Universitaire Vaudois peptide facility. Peptides (15-mers overlapping by 11) spanning the HIV-1 clade B Gag protein were synthesized as previously described (44). Peptides were reconstituted in DMSO. Gag peptides were pooled, and all peptides were used to stimulate cells at a final concentration of 2  $\mu$ g of each peptide per milliliter.

**In vitro stimulation and staining of cells.** PBMCs were thawed, resuspended at  $\sim 2 \times 10^6$  cells/ml in R10 media (RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 100 U/ml penicillin G, 100  $\mu$ g/ml streptomycin sulfate, and 1.7 mM sodium glutamate) containing 10 U/ml DNase I (Roche Diagnostics) and rested overnight at 37°C. Restimulation with vaccinia virus in vitro was performed using a modified version of the method first described by Speller et al. (45). In brief, cells were infected with vaccinia virus (multiplicity of infection = 1; Therion Biologics Corporation) in 200  $\mu$ l R10 for 1 h at 37°C. R10 was added to adjust the total volume to 1 ml, and the cells were incubated for an additional 3 h. At that time, 10  $\mu$ g/ml brefeldin A (Sigma-Aldrich), 0.7  $\mu$ l/ml Golgistop (BD Biosciences), and anti-CD107a-Alexa Fluor 680 were added as previously described (46), and the cells were mixed and further incubated at 37°C for 5 h. For some experiments, recombinant vaccinia virus expressing HIV-1 Gag (provided by K. Luzuriaga, University of Massachusetts Medical Center, Worcester, MA) was used for stimulation. Negative control tubes without addition of vaccinia virus were included for all samples. Stimulation of cells from the placebo group before Dryvax challenge served as an additional negative control.

In some experiments, cells were stimulated with 2  $\mu$ g/ml peptide for 5–6 h in the presence of brefeldin A, Golgistop, anti-CD107a-Alexa Fluor 680, and 1  $\mu$ g/ml each of anti-CD28 and anti-CD49d. Negative control tubes without peptide were also included.

After stimulation, cells were washed in PBS containing 1% bovine serum albumin and 0.1% sodium azide and stained with pretitrated surface antibodies. Cells were washed and permeabilized according to the manufacturer's instructions (Cytofix/Cytoperm Kit; BD Biosciences). After washing twice in the supplied buffer, cells were intracellularly stained with pretitrated antibodies against CD3, cytokines, and chemokines. Cells were subsequently washed in the supplied buffer and fixed in PBS containing 1% paraformaldehyde. Fixed cells were stored at 4°C in the dark until flow cytometric analysis (performed within 24 h).

**Flow cytometric analysis.** Cells were collected on an LSR II instrument (BD Biosciences) configured to detect 18 fluorochromes. 350,000–1,000,000 events were collected per sample. Analysis was performed using FlowJo software (version 6.3; TreeStar, Inc.). Cell doublets were excluded using forward scatter (FSC)-area versus FSC-height parameters. To minimize background levels of staining caused by nonspecific binding of antibodies, Cascade Blue

aggregates, CD14<sup>+</sup> cells, CD19<sup>+</sup> cells, and dead cells were excluded using a dump channel. A small lymphocyte gate was created using FSC and side scatter properties. Non-T cells were excluded by gating on CD3<sup>+</sup> cells. CD8<sup>+</sup> T cells were selected by first gating out CD4<sup>+</sup> cells and subsequently gating on CD8<sup>+</sup> cells. Alternatively, CD4<sup>+</sup> T cells were selected by first gating out CD8<sup>+</sup> cells and subsequently gating on CD4<sup>+</sup> cells. All T cell markers were plotted versus IFN- $\gamma$  to observe and include stimulated cells that had down-regulated surface marker expression.

According to this strict selection of CD8<sup>+</sup> T cells, functional analysis was performed by plotting the expression of each functional marker against another, as shown in Fig. 1 A. Boolean combinations of single functional gates were created using FlowJo software to determine the frequency of each response based on all possible combinations of cytokine, chemokine, and CD107a expression. Background responses detected in negative control tubes were subtracted from those detected in stimulated samples for every specific functional combination. The MFI of each functional parameter was also determined for all Boolean gate combinations using FlowJo software. Frequency and MFI data were output from the table editor of FlowJo for formatting in Pestle software (version 1.3; provided by M. Roederer, NIH, Bethesda, MD).

**Data analysis and statistics.** The data analysis program Simplified Presentation of Incredibly Complex Evaluations (version 2.9; provided by M. Roederer, NIH, Bethesda, MD) was used to analyze and generate graphical representations of T cell responses detected by polychromatic flow cytometry. All values used for analyzing proportionate representation of responses are background subtracted. Hence, these values can be less than zero in cases where the background sample had more events in a particular functional gate than the positive. Because negative values cannot be easily considered for representational analysis (i.e., to graphically represent pie charts), these values are set to zero. To avoid a systematic bias introduced by zeroing only negative values, we set a threshold of 0.013% for this dataset, below which all values were set to zero. This value was chosen based on the distribution of negative values in the background-subtracted dataset.

Total frequencies of vaccinia virus-specific CD8<sup>+</sup> T cells were compared between vaccination groups using a two-tailed Student's *t* test assuming unequal variances using JMP software (version 5.1; SAS Institute).

**HLA-A2 tetramers.** Tetrameric recombinant pMHC1 antigens were produced as previously described (47). Biotinylated HLA-A\*0201 heavy chains refolded with  $\beta_2$  microglobulin and vaccinia virus peptides (BioSynthesis) were conjugated by addition of APC-conjugated streptavidin or PE-conjugated streptavidin (both obtained from Prozyme) at a 4:1 molar ratio, respectively, to produce tetrameric pMHC1 complexes. Once prepared, tetramers were stored in the dark at 4°C for no longer than 3 mo.

PBMCs were stained with tetramers at 37°C for 15 min. Cells were washed in PBS containing 1% bovine serum albumin and 0.1% sodium azide and stained with a cocktail of antibodies against the following surface molecules: CD3-Cy7APC, CD8-QD655, CD27-PE, CD45RO-ECD, CD57-FITC (BD Biosciences), CD14-Cascade Blue, and CD19-Cascade Blue. ViViD was also included to exclude dead cells from the analysis.

For granzyme and perforin expression analysis, tetramer staining was performed as described followed by surface staining with antibodies against CD3-Cy7APC, CD8-QD705, CD27-Cy5PE, CD45RO-ECD, CD57-QD565, CD14-Cascade Blue, CD19-Cascade Blue, and ViViD. Cells were permeabilized using the Foxp3 Staining Buffer Set (eBioscience), according to manufacturer's instructions, and intracellularly stained with antibodies against granzyme A-PE or -FITC (BD Biosciences), perforin-FITC (BD Biosciences), and granzyme B-APC (Caltag).

**Flow cytometric sorting.** Sorting was performed on stained cells using a modified FACS DIVA (Becton Dickinson). Instrument setup was performed according to the manufacturer's instructions. All sorts were performed at 25 PSI. Electronic compensation was conducted with antibody-capture beads (BD Biosciences) stained separately with individual mAbs used in the stained samples. Small, live CD3<sup>+</sup>CD14<sup>-</sup>CD19<sup>-</sup>CD4<sup>-</sup>CD8<sup>+</sup> KVD tetramer-positive

cells were sorted directly into 1.5-ml microtubes containing 100  $\mu$ l RNAlater (Ambion) for clonotype analysis. 4,500 and 5,000 cells were collected for 1 and 3 mo after Dryvax challenge, respectively.

**TCR clonotype analysis.** After cell lysis, mRNA was extracted (Oligotex Kit; QIAGEN) and subjected to a nonnested, template switch-anchored RT-PCR using a 3' TCRB constant region primer (5'-GCTTCTGATGGCTCAAACACAGCGACCTC-3'), as described previously (48). Amplified products were ligated into pGEM-T Easy Vector (Promega) and cloned by transformation of competent DH5 $\alpha$  *Escherichia coli*. Selected colonies were amplified by PCR using standard M13 primers and sequenced from an insert-specific primer using fluorescent dye terminator chemistry (Applied Biosystems). 72 and 75 clones were generated and analyzed at 1 and 3 mo after Dryvax challenge, respectively. Pseudogenes and "nonfunctional" sequences that could not be resolved after inspection of the individual chromatograms were discarded from the analysis. Nucleotide comparisons were used to establish clonal identity. Data analysis was performed using Sequencher software (version 4.2; Gene Codes Corporation). The international ImMunoGeneTics nomenclature system was used for TCRBV and TCRBJ (49).

**Online supplemental material.** In Fig. S1, PBMCs were infected overnight with Dryvax or MVA. Intracellular IFN- $\gamma$ /IL-2 staining was used to measure the frequency of responding CD8<sup>+</sup> T cells. Response frequencies were similar whether Dryvax or MVA was used for stimulation after MVA immunization alone or MVA plus Dryvax. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20062363/DC1>.

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