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Memory CD8⁺ T Cell Responses Expand When Antigen Presentation Overcomes T Cell Self-Regulation

Ian A. Cockburn,* Sumana Chakravarty,* Michael G. Overstreet,* Adolfo García-Sastre,†‡ and Fidel Zavala²*

Antimicrobial memory CD8⁺ T cell responses are not readily expanded by either repeated infections or immunizations. This is a major obstacle to the development of T cell vaccines. Prime-boost immunization with heterologous microbes sharing the same CD8⁺ epitope can induce a large expansion of the CD8⁺ response; however, different vectors vary greatly in their ability to boost for reasons that are poorly understood. To investigate how efficient memory T cell expansion can occur, we evaluated immune regulatory events and Ag presentation after secondary immunization with strong and weak boosting vectors. We found that dendritic cells were essential for T cell boosting and that Ag presentation by these cells was regulated by cognate memory CD8⁺ T cells. When weak boosting vectors were used for secondary immunization, pre-established CD8⁺ T cells were able to effectively curtail Ag presentation, resulting in limited CD8⁺ T cell expansion. In contrast, a strong boosting vector, vaccinia virus, induced highly efficient Ag presentation that overcame regulation by cognate T cells and induced large numbers of memory CD8⁺ T cells to expand. Thus, efficient targeting of Ag to dendritic cells in the face of cognate immunity is an important requirement for T cell expansion.


The generation and expansion of protective T cell responses are the goals of vaccines being developed to fight a variety of diseases including malaria, HIV, and tuberculosis (1–3). The usefulness of generating protective CD8⁺ T cell responses has led to intense interest in understanding the cellular requirements for effective recall and boosting responses. These studies have highlighted requirements for dendritic cells (DCs) and CD4⁺ T cells for CD8⁺ T cell expansion (4–6) and have identified subsets of T cells that play important roles in protection against a variety of pathogens (7–9). Nonetheless, one observation that has not, to our knowledge, been thoroughly addressed in the recent literature is the finding that memory T cell populations, once established, do not readily expand further (10, 11). This is clearly a major obstacle to any vaccine strategy aimed at inducing large numbers of protective T cells. Many of the studies described above have overcome this problem either by using secondary doses of Ag several orders of magnitude larger than the primary immunization or by using adoptive transfer protocols in which small numbers of memory cells, often of a single specificity, are transferred to an otherwise naive mouse before boosting. Although valuable information has come from these studies, neither of these protocols is useful for expanding T cell responses by vaccination.

For the purposes of vaccination, prime-boost regimens (using different vectors for the prime and the boost) have been identified as the best way to expand CD8⁺ T cell responses (12). However, unlike Ab responses, which are generally robustly boosted following re-exposure to Ag, few prime-boost combinations result in strong CD8⁺ boosting (11, 13). This was illustrated in a study using recombinant influenza and vaccinia vectors carrying the murine malaria epitope SYVPSAEQI. It was shown that robust protective CD8⁺ T cell responses could be generated after an influenza-prime/vaccinia-boost combination; however, when the viruses were given in the reverse order a much smaller and non-protective CD8⁺ T cell response was seen (14). We took advantage of this well-established malaria sporozoite model of CD8⁺ T cell immunity to examine the cellular processes involved in expanding memory CD8⁺ T cell responses. By comparing the processes of Ag presentation after priming and boosting with various vectors that all carry the protective SYVPSAEQI epitope, we found that boosting occurred when Ag was targeted efficiently to DCs. If insufficient Ag was made available to DCs, Ag presentation, and consequent T cell boosting, was rapidly limited by the cognate CD8⁺ T cells themselves. Thus, according to these data, T cell expansion can occur when the degree of Ag presentation is sufficient to overcome self-regulation by CD8⁺ T cells.

Materials and Methods

Mice

Female BALB/c mice, 5–8 wk old, were purchased from Taconic. Transgenic mice expressing a TCR specific for the Plasmodium yoelii epitope were derived as previously described (15). Mice from our colony that have previously been backcrossed to the Thy 1⁺ BALB/c background for >20 generations were used. CD11c-DTR mice on a BALB/c background were derived as described and are maintained in our colony as heterozygotes (16). Experiments involving mice were approved by the institutional animal care and use committee of the Johns Hopkins University.

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Repeated exposure to sporozoites: Mice were immunized at weekly intervals with $1 \times 10^7$ γ-spz according to different schedules given on the γ-axes. Ten days after the final immunization, the SYVPSAEQI-specific immune response was measured either by tetramer staining (A) or by ELISPOT analysis (B). Data are the mean and SE based on four mice per group.

**Immunizations**

*Plasmodium yoelii* strain 17X NL sporozoites were obtained and irradiated as previously described (15). The generation of the vaccinia-SYV (VV-SYV), influenza-ME (Flu-ME), and adenovirus-CS (Ad-CS) have been described previously (13, 14, 17): the influenza virus carried the SYVPSAEQI epitope (SYVPSAEQI) integrated into the viral thymidine kinase gene and Ad-CS has the entire CS gene under the control of the E1 gene promoter, meaning that the it will be essentially the only adenovirus-derived gene product produced in infected cells. Doses used were $1 \times 10^7$ γ-spz, $2 \times 10^6$ VV-SYV, $2.5 \times 10^6$ Flu-ME, and $1 \times 10^7$ Ad-CS; all immunizations were given i.v. Ad-CS was kindly provided by Moriya Tsuji (New York University, New York, NY).

**Quantification of SYVPSAEQI-specific T cell number and proliferation**

The Ag-specific response to the SYVPSAEQI epitope was measured by tetramer staining and ELISPOT. PE-labeled SYVPSAEQI-specific H2-Kd tetramers were prepared as previously described (18). Spleen cells were incubated with tetramer and anti-CD8-APC (eBioscience) before FACS. In tetramers were prepared as previously described (18). Spleen cells were separated over a Nycodenz gradient (density, 1.075 g/ml) and the DC-rich low-density fraction was taken. Contaminating non-DC populations were stained with FITC-conjugated anti-CD3, anti-Thy1.2, anti-Gr1, anti-B220, and anti-TER119 Abs (all from eBioscience) and depleted by magnetic bead separation using anti-FITC microbeads according to the manufacturer’s instructions (Miltenyi Biotec). This yielded a 60–80% pure MHC class II$^+$/CD11c$^+$ DC population as determined by FACS.

**Ag presentation assay**

To assess Ag presentation ex vivo, splenic myeloid DCs were purified as described in *Cell isolation* at various times postimmunization. DCs (5 $\times 10^6$) were mixed with an equal number of purified naive CFSE-labeled CDb$^+$-transgenic cells in a single V-bottom well of a 96-well plate. From 8 to 16 cultures were set up for each DC population; 60–65 h later, the cells were harvested and, CFSE dilution in the transgenic cell population was used as a measure of Ag presentation. The amount of Ag presentation was expressed either as the percentage of cells that have divided (% M1 in Fig. 4A) or as the percentage of cells that have entered division. This was calculated by determining the number of cells in each generation peak and dividing by $2^n$ (where $n$ is the generation number) to determine how many naive cells contributed to that peak. The percentage of the original naive population that entered division could then be calculated. From this we subtracted the percentage of cells that entered division when incubated with DCs from a naive animal. Values <0.5% were considered below the limit of detection.

**Figure 1.** CD8$^+$ T cell memory is not substantially increased by repeated exposure to sporozoites: Mice were immunized at weekly intervals with $1 \times 10^7$ γ-spz according to different schedules given on the γ-axes. Ten days after the final immunization, the SYVPSAEQI-specific immune response was measured either by tetramer staining (A) or by ELISPOT analysis (B). Data are the mean and SE based on four mice per group.

**Figure 2.** VV-SYV boosts γ-spz-immune responses. Mice were immunized with $1 \times 10^7$ γ-spz and 21 days later were boosted with one of $1 \times 10^7$ Ad-CS, $2.5 \times 10^5$ Flu-ME, or $2 \times 10^6$ VV-SYV (all given i.v.) or left unboosted. Naive mice were also immunized with the three viral vectors alone. The SYVPSAEQI-specific immune response was measured by tetramer staining at day 10. Data are the mean and SE based on three mice per group.

To assess Ag presentation ex vivo, splenic myeloid DCs were purified as described in *Cell isolation* at various times postimmunization. DCs (5 $\times 10^6$) were mixed with an equal number of purified naive CFSE-labeled CDb$^+$-transgenic cells in a single V-bottom well of a 96-well plate. From 8 to 16 cultures were set up for each DC population; 60–65 h later, the cells were harvested and, CFSE dilution in the transgenic cell population was used as a measure of Ag presentation. The amount of Ag presentation was expressed either as the percentage of cells that have divided (% M1 in Fig. 4A) or as the percentage of cells that have entered division. This was calculated by determining the number of cells in each generation peak and dividing by $2^n$ (where $n$ is the generation number) to determine how many naive cells contributed to that peak. The percentage of the original naive population that entered division could then be calculated. From this we subtracted the percentage of cells that entered division when incubated with DCs from a naive animal. Values <0.5% were considered below the limit of detection.
Cell depletion

DCs were depleted from CD11c-DTR-transgenic mice by 2 i.p. injections of 100 ng of diphtheria toxin 3 days apart. This resulted in ~90% depletion of DCs for ~120 h. BALB/c mice are able to accept this dose without weight loss or the mortality seen in C57BL/6 mice (6). CD8+ cells were depleted by 2 i.p. injections on consecutive days with 200 µg of anti-CD8 Ab (clone 2.43), whereas CD4+ cells were depleted by two i.p. injections on consecutive days of 200 µg of anti-CD4 Ab (clone GK1.5).

Data acquisition and analysis

FACS data were acquired on a FACSCalibur machine and analyzed using FlowJo software (TreeStar). ELISPOT plates were read with an ImmunoSpot plate reader, and the data were analyzed using ImmunoSpot software (Cellular Technology). Graphs were prepared, and statistical analysis was performed using GraphPad Prism (GraphPad Software).

Results

CD8 T cell expansion is not an automatic consequence of re-exposure to Ag

Despite continuous exposure to infectious bites, individuals living in endemic areas develop CD8+ T cell responses of low magnitude to sporozoite Ags (21, 22). This may be because CD8+ T cell responses are hard to expand once they have been established (10). We modeled natural exposure to malaria Ag by giving weekly doses of P. yoelii 17X NL-γ-spz and measuring the Ag-specific CD8+ T cell response by tetramer staining or ELISPOT (Fig. 1). We found that giving four weekly doses of Ag gave an immune response no larger than a single dose of Ag. Even leaving 3 wk between two immunizations did not lead to a large expansion of the CD8+ T cell population. Our data therefore suggest that CD8+ T cell responses are unlikely to significantly expand among individuals in an endemic area who may receive weekly or even daily exposure to parasites.

A vaccinia vector is capable of expanding memory CD8 T cells

One explanation for the previous result is that immunity develops against γ-spz which will clear the parasite on re-exposure and thus limit its ability to further expand immune responses. For this reason, heterologous prime-boost vaccinations are used to expand CD8+ T cells. We therefore tried to boost a single dose of sporozoites with recombinant Ad-CS, VV-SYV, or Flu-ME, all of which carry the protective SYVPSAEQI epitope (13, 14, 23). These vectors have all been extensively described previously; in this system, the vectors were given i.v. for consistency, although all have been shown to elicit protective CD8 T cells regardless of the route of immunization (23, 24). The epitope-specific immune responses to the different prime-boost combinations and the component immunizations alone were measured with H2-Kd tetramer at days 10 and 21 (Fig. 2, A and B). To normalize results between the different vectors, we chose doses of virus that gave approximately equivalent priming responses (Fig. 2, A and B) and defined a simple boosting factor as (prime;boost)/(prime alone + boost alone).

Surprisingly, given that they all induce similar priming responses, Ad-CS and Flu-ME were capable of boosting Ag-specific responses only to ~1–2 × 105 tetramer+ cells/spleen (Fig. 2, A and B) which corresponded to a modest boosting factor of ~2 (Fig. 2C). In experiments in which a 25-fold higher dose of Flu-ME (6.25 × 105) was used to boost sporozoite-immunized mice, we observed only a modest increase in the boosting factor to 2.7 (Fig. 2D). VV-SYV immunization, however, resulted in strong boosting with tetramer+ cells expanding to >5 × 106 cells/spleen, corresponding to a boost factor between 5 and 7 (Fig. 2, A–C). Similar data were obtained when we measured specific responses by ELISPOT (data not shown). We therefore found that in this system not all heterologous prime-boost combinations result in significant CD8+ T cell expansion and that vaccinia vectors have the unusual property of boosting specific CD8+ T cell responses. These observations are consistent with our own previous data and data from various other laboratories working on a variety of systems (3, 25–28).

Boosting by VV-SYV requires DCs but not CD4+ T cells

Given that vaccinia was most capable of inducing memory CD8+ T cell expansion, we investigated the cellular requirements for the T cell expansion induced by this vector. To determine whether VV-SYV boosting required DCs, we took advantage of the CD11c/DTR transgenic mouse system in which the diphtheria toxin (DT) receptor is expressed under the control of the CD11c promoter; therefore, DCs may be depleted by injection with small amounts of DT (16). SYVPSAEQI-specific memory CD8+ T cells, carrying the Thy.1.1 allele, were transferred into naive DTR (Thy.1.2)-transgenic mice. These mice were boosted with VV-SYV with or without concurrent DC depletion. To deplete DCs, DT (100 ng/mouse) was administered twice: 1 day before boosting;

![Image](http://www.jimmunol.org/DownloadedFrom/byguest/April21,2022)

FIGURE 3. Cellular requirements for boosting. A. DCs are required for boosting. 1 × 106 memory Thy.1.1-transgenic cells (purified from the spleens of mice that had received transgenic cells and been primed with 2 × 105 γ-spz) were adoptively transferred into CD11c-DTR transgenic mice on a BALB/c, Thy1.2 background (i), or wild-type mice (ii). The mice were then left unboosted; boosted with VV-SYV 1 day later, or DC depleted and boosted with VV-SYV. Five days after boosting, the spleen cells were harvested, and transgenic cells were identified by staining for Thy1.1 and CD8. Plots show representative FACS data from each group. The values are the mean and SD of the percent of CD8+ Thy1.1+ cells based on three mice per group. B, CD4+ T cells are not required for boosting. Mice were immunized with γ-spz and boosted 21 days later with VV-SYV with or without depletion of CD4+ T cells 2 days before boosting. Other mice received the individual immunizations alone. The SYVP SAEQI-specific immune response was measured either by tetramer staining (i) or ELISPOT (ii) 12 days (■) and 28 days (□) after boosting. Data are means and SEs based on three mice per group; differences between anti-CD4 treated and control mice were not significant in any instance.
Representative data from one of two independent experiments are shown. mune response was determined by tetramer. Data are means and SEs of Ag cross-presentation. Ten days after immunization, the Ag-specific were treated with 10 nmol CpG/mouse 24 h before immunization to block not VV-SYV is limited by CpG treatment 24 h before immunization. Mice to toxicity (Fig. 3Ai). Thus, DCs are required for efficient boosting of served effect in CD11c/DTR mice was not due to any nonspecific or tetramer staining, either 12 or 28 days after boosting (Fig. 3A1). Because boosting by VV-SYV requires DCs, we decided to investigate further the process of Ag presentation by DCs after immunization with Ad-CS-immunized mice compared with VV-SYV-infected mice.

Ag presentation was slower and less efficient in Flu-ME- and Ad-CS-immunized mice compared with VV-SYV-infected mice. Therefore, we decided to investigate further the process of Ag presentation by DCs after immunization with the different vectors. We wished to determine whether there were differences in this respect between the boosting vectors and nonboosting vectors. To measure Ag presentation, splenic DCs were purified from immunized mice and incubated with an equal number of CFSE-labeled transgenic cells; Ag presentation was inferred from the dilution of the label after 60–65 h. Transgenic cells incubated with spleen DCs from naive control mice underwent a small amount of background proliferation (Fig. 4Ai). VV-SYV induced very efficient Ag presentation with >80% of transgenic cells dividing within 24 h of immunization (Fig. 4Bi). Using the CFSE peaks to infer the number of divisions that each cell has undergone, we estimate that ~30% of the transgenic cells entered division. This pattern persisted for at least another 48 h before beginning to wane by 120 h. Ag presentation by DCs after Flu-ME immunization followed a very different course, despite the fact that immunization with this vector results in a immune response of a size similar to that induced by VV-SYV (Fig. 4Bi). Ag presentation was not detectable at 24 h and only began to be seen at 48 h (data not shown) before peaking at 72 h postimmunization. However, the peak response was much more limited with only 10–20% of T cells dividing corresponding to only ~1–3% of the original population having entered division. Similarly, immunization with Ad-CS resulted in Ag presentation which probably peaked at ~72–120 h after immunization, although the overall amount was near the limit of detection (Fig. 4Bi).

FIGURE 5. VV-SYV is able to prime SYVPSAEQI-specific transgenic cells in the presence of γ-sporozoite immunity. Naïve CFSE-labeled SYVPSAEQI-specific transgenic cells (2–3 × 10³) were adoptively transferred into either naïve mice or γ-spz-immune mice. One day later, the mice were immunized with either VV-SYV, Flu-ME, or Ad-CS. Ten days later, spleen cells were harvested and stained with anti-Thy.1.1 and anti-CD8 to identify transgenic cells. A. Numbers of Thy.1.1 CD8⁺ CFSElow cells (expressed as a percent of total CD8⁺ T cells) in the spleen after immunization with each of the vectors in previously naïve mice (□) and preimmune mice (□); values are means and SEs of three mice per group; ns, not significant; *, p < 0.05; **, p < 0.01 by Student’s t test. B. Representative CFSE profiles from each group of mice; plots were gated on Thy.1.1⁺, CD8⁺ cells. Values are the mean and SD of the percent of transgenic cells that have fully retained the CFSE label (M2) based on three mice per group; p values are the differences between the percent of cells retaining the CFSE label in naïve and γ-spz-immune mice by Student’s t test. Data are from one of two experiments that yielded similar results.

and 2 days after boosting; Six days after boosting, the expansion of memory Thy.1.1 T cells was measured by FACS. In the DC-depleted mice, the cells expanded to one-sixth of the level seen in intact mice (Fig. 3Ai). Importantly, DT treatment had no effect on memory T cell expansion in normal mice, showing that the observed effect in CD11c/DTR mice was not due to any nonspecific toxicity (Fig. 3Aii). Thus, DCs are required for efficient boosting of immune responses by VV-SYV. In contrast, depletion of CD4 cells immediately before boosting did not result in a significant reduction in the size of the boost as measured by either ELISPOT or tetramer staining, either 12 or 28 days after boosting (Fig. 3Bi).
We were interested in how VV-SYV could induce such a different pattern of Ag presentation compared with Flu-ME and Ad-CS. Vaccinia virus is known to infect DCs and induce direct presentation of Ag (29–31). In contrast, as indicated by recent studies, normal immune responses to influenza virus appear to develop when viral Ag is available to DCs by cross-presentation (32). The hypothesis that direct infection and Ag presentation plays a role in the induction of CD8\(^+\) T cell responses by VV-SYV was tested by blocking cross-presentation by systemic administration of a TLR ligand (20). CpG treatment 24 h before immunization did not dampen VV-SYV-induced immune responses. In contrast, priming by Flu-ME was strongly abrogated by prior CpG treatment (Fig. 4C). It is possible that CpG may be inducing antiviral mechanisms that affect Flu-ME Ag production; however, recent studies using HSV have showed that CpG treatment blocks viral Ag cross-presentation by systemic administration of a TLR ligand (20). CpG treatment 24 h before immunization did not dampen VV-SYV-induced immune responses. In contrast, priming by Flu-ME was strongly abrogated by prior CpG treatment (Fig. 4C). It is possible that CpG may be inducing antiviral mechanisms that affect Flu-ME Ag production; however, recent studies using HSV have showed that CpG treatment blocks viral Ag cross-presentation by systemic administration of a TLR ligand (20).

VV-SYV is able to target Ag for presentation and prime transgenic cells in the presence of pre-existing CD8\(^+\) sporozoite immunity

Robust early Ag presentation by DCs after VV-SYV immunization could contribute to its property of boosting CD8\(^+\) T cell responses. It has been previously shown that Ag presentation of a given Ag is rapidly limited by feedback regulation by the recently primed CD8\(^+\) T cells (33–35). The effector cells either outcompete naive cells for presented Ag, or simply clear Ag before all naive cells have a chance to be activated; thus, there is a natural limitation on the size of CD8\(^+\) T cell responses. A similar process is likely to occur during secondary immunization to limit T cell expansion. Boosting, however, could occur if there is efficient presentation of Ag, and many memory cells have a chance to see their cognate epitope on DCs before Ag presentation wanes. Because VV-SYV makes large amounts of Ag available on DCs, we hypothesized that it, unlike the other vectors, may be able to overcome feedback regulation. To test this, we compared the ability of the different vectors to drive the expansion of naive CFSE-labeled transgenic cells in sporozoite-immunized mice. In agreement with the hypothesis, VV-SYV was able to prime transgenic cells in both naive and sporozoite-immune mice: in both cases a significant increase in the number of cells was seen (Fig. 5A), and the CFSE label was diluted out completely (Fig. 5B). In contrast, we saw evidence of feedback regulation when we immunized sporozoite-immune mice with Flu-ME and Ad-CS; in both cases, there was significantly less expansion of the transgenic cell population than with immunization in naive control mice (Fig. 5A), and many cells retained the CFSE label (Fig. 5B).

To further investigate the relative ability of VV-SYV and Flu-ME to overcome regulation in immune mice, we compared Ag presentation by DCs after Flu-ME and VV-SYV immunization in naive and sporozoite-immune mice. Because of the limited Ag presentation seen after Ad-CS immunization, it was not possible to include this vector in these experiments. Twenty-four hours after booster immunization by VV-SYV, we saw no reduction in Ag presentation compared with that seen after a priming immunization by this vector (Fig. 6A). Seventy-two hours after...
booster immunization, there is still Ag presentation, although it has declined compared with that seen in primed naive mice by between 20 and 80% in independent experiments (mean, 40%). The level of variation was perhaps due to differences in the immunogenicity of batches of sporozoites used for immunization in independent experiments. In Flu-ME-boosted mice, no Ag presentation was detected 24 h after boosting; whereas 72 h after secondary immunization with Flu-ME, Ag presentation is reduced by ~70% compared with Ag presentation after priming (Fig. 6B). Ag presentation after boosting with a 25-fold higher dose of Flu-ME was also strongly abrogated at 72 h (Fig. 7), although this dose did give some early Ag presentation in immune mice, allowing it to stimulate a modest T cell expansion (Fig. 1D). Taken together, our in vivo and ex vivo Ag presentation data strongly suggest that Ag availability is a crucial limiting factor that may prevent some vectors from being able to boost immune responses.

Boosting responses are regulated by CD8+ T cells

We have shown that Ag presentation by Flu-ME is severely curtailed in γ-spz-immunized mice. There was also a small reduction in Ag presentation after VV-SYV immunization in immune mice compared with naive mice. Given that the only known shared immunological determinant between γ-spz and these viral vectors is the SYVPSAEQI epitope, we have hypothesized that feedback regulation by CD8+ T cells mediated these effects. To test this formally, we tried to recover Ag presentation in immune mice to levels seen in naive mice by removing their CD8+ T cells by Ab depletion. Two weeks after Ab depletion, the mice were boosted with Flu-ME or VV-SYV, and Ag presentation was measured. With both viral vectors, Ag presentation was recovered in sporozoite-immune mice to a level comparable with that seen in naive mice when the mice were depleted of CD8+ T cells (Fig. 6). In contrast, immune mice with depleted CD4+ T cells still showed reduced Ag presentation as in untreated γ-spz-immune mice (Fig. 6).

Finally, the hypothesis that CD8+ T cells limit boosting, either by clearing viral infection from the periphery or by limiting the capacity of DCs to present Ag, was further tested by transferring different numbers of transgenic memory CD8+ T cells into normal mice which were subsequently boosted with VV-SYV. After 10 days, the size of the boost determined and the fold change in the number of cells were measured. In agreement with the hypotheses, the more cells transferred, the smaller was the fold change in the number of cells ($F = 24.1; p < 0.01$ by one-way ANOVA; Fig. 8).

Thus, the more memory cells present before secondary immunization, the greater is their ability to limit their own boosting on a per cell basis. Overall, our data suggest that cognate CD8+ T cells restrict boosting but efficient Ag presentation by DCs may overcome this to permit boosting.

Discussion

In this study, we wished to determine the cellular requirements for T cell expansion and the characteristics required for a vector to efficiently boost T cell responses. We believe this is the first systematic attempt to determine why some recall responses result in T cell expansion and others do not. It was found that DCs are crucial for the expansion of T cells after vaccinia boosting. CD4+ T cells appear not to play a major role at the time of secondary immunization; rather, the most important regulators of CD8+ T cell expansion appear to be the cognate CD8+ T cells themselves. These cells limit Ag presentation after secondary immunization with adeno-virus and influenza virus vectors, resulting in limited CD8+ T cell expansion. CD8+ T cell regulation, however, appears to be overcome by the ability of VV-SYV to efficiently target Ag for presentation by DCs. The resulting Ag presentation can induce large numbers of memory CD8+ T cells to expand. The ability to induce effective Ag presentation by DCs in the face of cognate immunity could be one of the most important determinants of the capacity of a vector to boost CD8+ T cell responses.

To our knowledge, this is the first description of cognate memory CD8+ T cells limiting their own recall responses. The role of early effector CD8+ T cells in limiting priming of naive cells, however, has been described previously by our group and others (10, 33, 34). In these cases, Ag presentation appears to be limited by the first wave of effector T cells, which may inhibit DC presentation of cognate Ag by a variety of means. Effector T cells may directly kill DCs presenting cognate Ag (36–38), strip peptide from surface MHC molecules (39), down-modulate MHC class I-peptide complexes on APCs (40), or outcompete naive cells for Ag on the surface of DCs (41). Potentially, memory cells might also use similar mechanisms to control boosting responses. Recently, it has been shown that effector and effector memory cells, which are normally excluded from resting lymph nodes, can enter reactive (inflamed) lymph nodes in a CXCXR3-dependent manner and kill DCs (42). Additionally, memory CD8+ T cells might clear Ag in the periphery, preventing it from being cross-presented in the lymphoid tissues. Together, these mechanisms allow effector
and memory CD8+ T cells to be powerful regulators of their own priming and boosting.

It is clear from our experiments and those of others (6) that DCs are required for the optimal expansion of CD8+ T cells during recall responses. There is, however, some evidence that DCs are not required for memory CD8+ effector function and pathogen killing. Zammit et al. (6) show that a Listeria monocytogenes infection can be cleared by CD8+ T cells without their expansion. Moreover, experiments from our laboratory have shown that transgenic memory CD8+ T cells can confer protection in the absence of MHC-matched DCs (43). Together with the data presented here, this suggests that CD8+ T cells may respond in a spectrum of ways on re-exposure to Ag. Potentially, effector function may be elicited by Ag presented by any infected cell; however, only DCs appear able to stimulate boosting, perhaps because of the costimulation they provide. The ability of vaccinia virus to infect DCs means that it can target large amounts of Ag for CD8+ T cell boosting, even if this process results simultaneously in viral clearance. In contrast, as suggested above, vectors that provide Ag for cross-presentation might be cleared in the periphery before sufficient Ag can be made available to DCs for boosting.

We have shown that VV-SYV makes ample Ag available on DCs, which allows it to boost immune responses (Figs. 4B and 6A); however, why does this efficient Ag presentation not result in stronger T cell priming? One explanation is based on the genomic size of the vaccinia virus. Compared with Flu-ME, which produces 10 proteins, and Ad-CS, which produces 1, VV-SYV is large with ~200 different protein products and a corresponding abundance of CD8+ epitopes (44, 45). Robust presentation of the SYVPSAEQI epitope after VV-SYV priming takes place alongside similarly strong presentation of many other epitopes. Priming of SYVP SAEQI-specific cells by VV-SYV may be limited by competition for cytokines and Ag by CD8+ T cells induced simultaneously with many different specificities. Crucially, during the boosting phase, this competition might be less important because the larger number of SYVPSAEQI-specific memory T cells will be immunodominant and able to outcompete most naive cells of different specificities (12, 46, 47). If this is true, the relative ability of VV-SYV to boost rather than prime may be helped by its large size and antigenic complexity.

There may be other properties of vaccinia virus, influenza virus, and adenoviruses that may explain their different abilities to boost. Because DCs clearly play an important role in T cell expansion, the relative abilities of different vectors to induce maturation of DCs may be important. Nonetheless, although the viruses used in this study have been reported to induce DC maturation (48–50) there have not been any studies that compare, side by side, the activation status of DCs presenting Ags from different vectors; therefore, we cannot say whether this may have a role in boosting immune responses. Different viral vectors may also vary in their ability to evade the host immune response. Vaccinia virus has within its genome a number of immune evasion genes which may allow it to evade clearance by CD8+ T cells and allow it to persist long enough to target Ag to DCs (51), although, unlike other poxviruses, vaccinia virus does not appear to interfere with MHC class I Ag processing (52). Another alternative explanation for the ability of vaccinia virus to boost would be that VV-SYV readily replicates, whereas the Flu-ME (when given i.v.) and Ad-CS vectors are replication deficient. We do not address this directly here, although a number of previous studies suggest that viral replication may not be crucial for boosting. We have shown that Flu-ME delivered intranasally (allowing it to replicate in the lung epithelium) does not efficiently boost immune responses (14, 24), whereas studies comparing the ability of live and attenuated vaccinia to generate responses against HIV epitopes showed that the attenuated virus was in fact the better boosting vector (26, 27).

Finally, it was surprising to us that Ad-CS did not boost more efficiently in our system, since adenoviruses have previously been suggested to be efficient boosting vectors (53, 54). However, it is also known that adenovirus vectors induce very high primary responses. It may be that if the degree of boosting in previous studies had been normalized to allow for this, as we have done with our boosting factor, it would be found that the intrinsic ability of adenovirus to boost is not so pronounced. It is also compatible with our hypothesis that giving several orders of magnitude more adenovirus or influenza virus might induce more efficient Ag presentation and therefore boosting. However, for vaccination, the immunizing dose is limited by the pathogenicity of the vector. Nonetheless, it is important to emphasize that in our experiments we did not set out to describe novel prime-boost combinations or to suggest the use of one vector over another; rather, we aimed to describe the basic cellular events involved in memory T cell expansion.

There is an urgent need for new vaccines to fight the spread of HIV, malaria, and tuberculosis as well as a host of so-called neglected diseases. To do this, we need a fundamental understanding of how to induce and expand populations of protective memory T cells. Our work shows that the ability of a vector to target Ag early to DCs in the face of cognate immunity is vital for boosting T cell responses. If these properties can be engineered into other vectors, this could be a path to developing better vaccines designed to induce protective CD8+ T cell responses.

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