Stimulation History Dictates Memory CD8 T Cell Phenotype: Implications for Prime-Boost Vaccination

David Masopust, Sang-Jun Ha, Vaiva Vezys and Rafi Ahmed

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Vaccination is the most successful and cost-effective method to prevent infectious diseases. Despite many successes, we have failed to develop preventative vaccines against important pathogens that may require cellular immunity for control such as HIV, Mycobacterium tuberculosis, and malaria. The development of new vaccine regimens that more successfully establish protective CD8 T cell memory has proven challenging, but heterologous prime-boost schemes hold promise (3, 4). This strategy involves sequential immunization with a common Ag incorporated into different vectors, such as DNA, viral stomaticis virus, poxviruses, adenoviruses, and Listeria monocytogenes (LM) (3–7). Heterologous prime boosting results in larger numbers of Ag-specific memory CD8 T cells than achieved by a single vaccine administration or homologous boosting. However, the impact of this strategy on memory CD8 T cell quality has not been studied.

A single infection or immunization results in expanded frequencies of Ag-specific memory CD8 T cells that differ from naive cells in gene expression, signaling molecules, cell surface phenotype, and trafficking (8–13). These properties allow memory CD8 T cells to more effectively respond to and protect against reinfection (14). It has recently become apparent that memory CD8 T cells exhibit an array of phenotypes, and different populations may serve specialized roles in protection (15–18). Based on this work, memory T cells are often segregated into two general subsets, central (T_{CM}) and effector (T_{EM}) memory (15). In brief, T_{CM} express lymph node homing receptors, such as CD62L or CCR7. T_{EM} lack lymph node homing receptors and are typically defined based on these criteria (19–22). Several additional qualities putatively distinguish these two subsets. T_{CM} have high proliferative potential, express CD27, recirculate preferentially through lymph nodes, require a relatively longer period of reactivation to express cytolytic function, and produce IL-2 upon Ag recognition. In contrast, T_{EM} have less proliferative potential, may not express CD27, recirculate preferentially through nonlymphoid tissues, are immediately cytolytic upon Ag reexposure, and are poor producers of IL-2 (15, 20, 23–28). Although many studies support aspects of this paradigm, there is still little consensus on 1) what factors promote differentiation into each lineage, 2) whether T_{CM} and T_{EM} comprise stable long-lived populations or represent transient states of CD8 T cell differentiation, and 3) whether the complexity of memory CD8 T cell phenotypes can be adequately defined by this nomenclature (12, 17, 29). Diverse conclusions on these issues may be due to analyses of different immunological challenges (that include variables in the history of Ag exposure), analyses of cells isolated from different anatomic locations, and the continued emphasis on lymph node homing receptor expression to connote functional qualities that are not always tested.

Either memory T cell subset may contribute more effectively to protection, depending on the route, dose, replication rate, and tropism of the infectious challenge (20, 30–32). For instance, T_{CM} may be more effective at protecting against systemic infections that require significant CD8 T cell proliferation for adequate control. In contrast, T_{EM} may be more effective at contributing to protection immediately upon re-infection because of their location at body surfaces and constitutive lytic activity. There is a growing belief that the generation of large numbers of T_{EM} positioned at portals of viral entry, such as mucosal tissues, might comprise an essential component to successful vaccination against pathogens such as HIV (18, 33, 34).

In this study, we compared memory differentiation following primary (1°), secondary (2°), or tertiary (3°) immunizations. We found that the number of immunizations had a major effect on...
memory CD8 T cell phenotype, cytolytic potential, cytokine production, proliferative capacity, and anatomic location. Thus, heterologous prime-boost vaccination generated functionally distinct memory CD8 T cells than those induced following a single immunization. In addition, these data demonstrate that memory CD8 T cell differentiation into TCM and TEM is dictated by the cumulative history of Ag exposure, even when interrupted by an extensive rest period.

Materials and Methods
Mice and infections
C57BL/6J mice were purchased from The Jackson Laboratory. All mice were used in accordance with National Institutes of Health and the Emory University Institutional Animal Care and Use Committee guidelines. For the analysis of 1° CD8 T cell responses to vesicular stomatitis virus (VSV), mice were infected by i.v. injection of 1 × 10^6 PFU VSV of the Indiana (Ind) serotype. For the analysis of 2° CD8 T cell responses, mice were primed by i.v. injection of 5 × 10^6 PFU VSV of the New Jersey serotype (NJ), rested for 150 days, and then rechallenged with VSV-Ind. For analysis of 3° CD8 T cell responses, mice were primed with VSV-NJ, rested 150 days, infected by i.v. injection of 2 × 10^6 PFU recombinant vaccinia-N (35), rested an additional 150 days, and then challenged with VSV-Ind. For analysis of 3° CD8 T cell responses, mice were primed with VSV-NJ, rested 150 days, infected by i.v. injection of 2 × 10^6 PFU recombinant vaccinia-N (35), rested an additional 150 days, and then challenged with VSV-Ind.

Thy1.1+ P14 mice bearing the H-2D^b-gp33-specific TCR were fully backcrossed to C57BL/6J mice and maintained in our animal colony (36). For analysis of 1° P14 CD8 T cell responses, we generated P14 chimeras by transferring 2 × 10^5 naive transgenic Thy1.1+ P14 T cells into naive C57BL/6J mice. The following day, mice were infected with VSV-Ind. For analysis of 3° CD8 T cell responses, mice were primed with VSV-NJ, rested 150 days, infected by i.v. injection of 2 × 10^6 PFU recombinant vaccinia-N (35), rested an additional 150 days, and then challenged with VSV-Ind.

Isolation of lymphocytes
Lymphocytes were isolated as described (38). In brief, animals were perfused with PBS, and livers were homogenized through a 100-μm filter (Falcon) in 5% RPMI 1640 medium. Lungs were treated with 1.3 mM EDTA in HBSS (30 min/37°C, shaking at 200 rpm) followed by treatment with 100 U/ml collagenase (Invitrogen Life Technologies) in 5% RPMI 1640 medium/2 mM MgCl2/2 mM CaCl2 (45 min/37°C, shaking at 200 rpm). Intraepithelial lymphocytes (IELs) were isolated as follows: small intestine was removed, Peyer’s patches were dissected, and the intestines were cut longitudinally and then into 1-cm pieces. Gut pieces were incubated with 15.4 mg/ml dithioerythritol in HBSS/HEPES bicarbonate buffer containing 10% FCS (30 min/37°C, shaking at 200 rpm) to remove IELs. Lymphocytes from liver, lung, and gut were purified on a 44–67% Percoll gradient (800 g at 20°C for 20 min).

FIGURE 1. Generating 1°, 2°, or 3° CD8 T cell responses. A. 1°, 2°, or 3° responses to VSV-Ind were generated in C57BL/6J mice. B and C. At various times postinfection, PBL were stained with anti-CD8, CD11a, CD44, and H-2K^b-N tetramers. Representative plots shown are gated on CD8^+ (B) or total (C) lymphocytes. D. Longitudinal analysis of H-2K^b-N-specific response in blood. E. Total number of H-2K^b-N-specific CD8 T cells/spleen 100 days after 1°, 2°, or 3° infections (n = 3–5 mice per time point). The experiment was repeated once with similar results.
Fluorescence flow cytometry

Single-cell suspensions were surface-stained with anti-CD8, CD62L, CD11a, CD44, CD69, Ly56, 1B11, CD27, IL-7Ra, β7, PD-1, KLRG1, Thy1.1, and Thy1.2 Abs (directly conjugated to FITC, PE, PerCP, or allophycocyanin). Cells also were stained with H-2Kb tetramers containing the VSV N protein-derived peptide RGYVYQGL. Intracellular staining for granzyme B and Bcl2 was performed using the Cytofix/Cytoperm kit (BD Pharmingen) in accordance with manufacturer’s directions. Staining for CD107a combined with CD107b was performed as described (31). All staining reagents were purchased from BD Pharmingen with the exception of anti-human granzyme B (Caltag Laboratories) and anti-KLRG1 (clone 2F1; Southern Biotechnology Associates). Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Results

Generating 1°, 2°, or 3° CD8 T cell responses

We wished to determine the impact of the number of immunizations on memory CD8 T cell qualities. Therefore, we generated 1°, 2°, and 3° CD8 T cell responses by infecting naive, immune, or secondary immune mice with VSV-Ind (Fig. 1A). Our experimental design allowed comparison of 1°, 2°, and 3° CD8 T cell responses in the absence of preexisting neutralizing Abs. Rechallenged mice were rested exactly 150 days between infections, and analyses of 1°, 2°, and 3° responses were performed on the same day (see Fig. 1A). Consistent with our previous findings (39, 40), 2° infection resulted in less pronounced contraction of the Ag-specific response, and higher levels of memory than did a single immunization (Fig. 1, B–E). Death of effectors was even less after 3° infection, which resulted in virus-specific memory levels that comprised ~60% of all circulating CD8 T cells and 25% of total lymphocytes (Fig. 1, B–D). It is important to emphasize that this large response was comprised of endogenous CD8 T cells; transgenic CD8 T cells were not used in this experiment. These data illustrate the remarkable potential of the heterologous prime-boost strategy to induce very high levels of cellular immunity.

Secondary (2°) and tertiary (3°) immunizations preferentially increase TEM frequency

CD62L expression is often used to delineate TCM and TEM (19–22). To compare memory differentiation following 1°, 2°, or 3° immunizations, we performed a longitudinal analysis of CD62L expression among H-2Kb/N-specific CD8 T cells in blood. Six days following infection, >95% of effector CD8 T cells were CD62L−, regardless of the history of antigenic stimulation. Consistent with previous publications, the percentage of CD62L− CD8 T cells gradually increased following resolution of 1° infection (20, 41). Surprisingly, we found that 2° and 3° infections resulted in the maintenance of substantially larger frequencies (70–80%) of CD62L− memory CD8 T cells even 150 days later (Fig. 2A). These data prompted a more thorough characterization of memory CD8 T cell phenotype. Within spleen, anamnestic (2° and 3°) memory CD8 T cells expressed more effector-like qualities than did 1° memory CD8 T cells (Fig. 2B). At 100 days postinfection, a large fraction of anamnestic H-2Kb/N-specific CD8 T cells expressed granzyme B (an indication of cytolytic potential) and KLRG1 (a marker of replicative senescence) (42, 43). Likewise, many 2° and 3° memory CD8 T cells remained CD62L− and CD27 low. Thus, anamnestic responses remained in memory CD8 T cells that displayed more effector-like qualities. However, 1°, 2°, and 3° memory CD8 T cells expressed equivalent levels of CD127, a cytokine receptor associated with memory differentiation (44, 45).

We also examined the phenotype of H-2Kb/N-specific 1°, 2°, and 3° memory CD8 T cells within other anatomic locations. Reexposure to Ag resulted in more effector-like memory CD8 T cells in many nonlymphoid tissues (Fig. 2C). For example, memory CD8 T cells in liver, lung, the IEL compartment of the small intestine, and PBL contained higher proportions of CD62L− and granzyme B+ memory CD8 T cells following anamnestic responses. Thus, Ag reexposure resulted in increased frequencies of CD62L−/granzyme B+ memory CD8 T cells throughout the organism, although phenotype did vary among different tissues (Fig. 2C).

It is worth noting that lack of CD62L expression did not provide a perfect surrogate for identifying TEM that expressed granzyme B. Although CD62L− memory CD8 T cells did not express granzyme B, lack of CD62L expression defined a heterogeneous population of memory CD8 T cells. To illustrate this point, we examined the relationship between CD62L and granzyme B expression among 2° memory CD8 T cells. As shown in Fig. 2D, CD62L− CD8 T cells expressed especially high levels of granzyme B in certain nonlymphoid tissues and did not express granzyme B within lymph nodes. Thus, granzyme B expression by CD62L− memory CD8 T cells depends on both their history of antigenic stimulation and their anatomic location (Fig. 2, B–D).
Phenotype of transgenic memory CD8 T cells varies following 1°, 2°, or 3° infection

As shown above, reimmunization of C57BL/6J mice led to the preferential accumulation of T_{EM}. This suggests that memory CD8 T cell differentiation is influenced by the cumulative history of Ag experience. However, it has been reported that a narrowing of the TCR repertoire occurs during 2° responses (46, 47). Thus, it was possible that the observed accumulation of T_{EM} was due to the selective expansion of high-affinity T cells rather than to differences in stimulation history. To address this issue, we studied 1°, 2°, or 3° responses among a monoclonal population of TCR-transgenic CD8 T cells. In addition, differences in Ag-specific CD8 T cell precursor frequencies between naive and previously immunized mice could have accounted for the observation that 2° and 3° responses preferentially augment the proportion of T_{EM}. To address this issue, we normalized the number of naive, 1° memory, and 2° memory CD8 T cells before each infection in an adoptive transfer model. This approach allowed us to perform a more stringent comparison of the impact of stimulation history on memory CD8 T cell phenotype.
We transferred either naive, 1° memory, or 2° memory Thy1.1+/H11001 P14 TCR transgenic CD8 T cells (specific for the gp33 epitope from LCMV) into naive C57BL/6J mice (Fig. 3A), and a day later the recipient mice were challenged with rLM-gp33. A longitudinal analysis of the P14 CD8 T cell response in blood was performed (Fig. 3, B and C). Regardless of the history of Ag experience, CD8 T cells underwent equivalent clonal expansion upon challenge (Fig. 3B). Consistent with previous reports, the contraction of the 2° response was considerably slower than the 1° response (39, 40, 48). This delayed contraction was even more pronounced upon 3° challenge (Fig. 3B). The rapidity of CD62L, CD27, CD127, and Bcl-2 reexpression among P14 in blood was increasingly delayed with additional Ag experience (Fig. 3C). Conversely, cells that had undergone two or three episodes of antigenic stimulation retained significantly greater expression of KLRG1 and granzyme B than did 1° memory CD8 T cells. The progressive increase in granzyme B expression among 2° and 3° memory CD8 T cells correlated with increased lytic activity on a per-cell basis, as measured by direct ex vivo 51Cr-labeled release assays (data not shown). By these criteria, the ratio of TEM to TCM increased proportionally with additional Ag experience among a monoclonal CD8 T cell population, and this was due to neither differences in precursor frequencies between responses nor selection of higher-affinity memory T cells. TEM have an enhanced capacity to produce IL-2 upon antigenic restimulation, and putatively, a reduced capacity to secrete inflammatory cytokines (15). Hence, the ability of 1°, 2°, or 3° memory splenocytes to produce IFN-γ, TNF-α, and IL-2 was compared. Increasing stimulation history was associated with a slightly enhanced ability to produce TNF-α and a decreased ability to produce IL-2, whereas IFN-γ expression was unaffected (Fig. 4, A and B). The membranes of lytic granules contain CD107a (LAMP-1) and CD107b (LAMP-2). Upon T cell activation and release of granule contents, CD107a and CD107b can be detected on the surface of CD8 T cells (31, 49). Interestingly, increasing stimulation history may also be associated with increased degranulation potential, as indicated by CD107a/b surface staining (Fig. 4, A and B).

$T_{EM}$ and $T_{CM}$ differ in anatomic localization. For example, TCM preferentially traffic through lymph nodes, whereas TEM are enriched within nonlymphoid tissues (19, 38). We examined the relative anatomic distribution of 1°, 2°, or 3° memory CD8 T cells by comparing the proportion of P14 within spleen, lung, or inguinal lymph nodes to the total number of P14 present in all three tissues. As shown in Fig. 4C, increasing Ag experience led to a redistribution of memory CD8 T cells from lymph nodes to lung. Thus, by the criteria of anatomic location, additional Ag experience also led to an increase in the ratio of TEM to TCM CD8 T cells.

Accumulation of $T_{EM}$ after DNA heterologous prime-boost immunization

DNA provides a useful vector for prime-boost vaccination and has been extensively used in many different systems (3). Heterologous prime-boost vaccination regimens are often comprised of a DNA vector prime, followed by a viral or bacterial...
vector boost. Therefore, we wished to determine whether boosting of DNA-primed memory CD8 T cells also induced the preferential accumulation of TEM.

CD8 T cell differentiation was compared following a primary DNA immunization or following one or two boosts with viral and/or bacterial vectors (as indicated in Fig. 5A). As shown in Fig. 5, B and C, in vivo restimulation of DNA-primed memory CD8 T cells also led to the preferential accumulation of granzyme B+/CD62L−CD27low/KLRG1+ memory CD8 T cells. Moreover, increasing Ag experience led to a redistribution of memory CD8 T cells from lymph nodes to nonlymphoid tissues such as lung and liver (Fig. 5D). By these criteria, DNA priming followed by boosting with live vectors also led to a selective increase in TEM.

**Proliferative potential of 1°, 2°, or 3° memory CD8 T cells**

After ~50 divisions in vitro, somatic cells reach the “Hayflick limit,” and become senescent to further proliferation. As CD8 T cells exhibit remarkable expansion and contraction in vivo, they potentially undergo very large numbers of divisions upon multiple prime-boost vaccinations (50). In fact, 2° and 3° responses resulted in KLRG1+ TEM (Figs. 2 and 3), characteristics associated with decreased division potential (20, 23, 43). For these reasons, we directly tested the division potential among 1°, 2°, and 3° memory CD8 T cells. Memory CD8 T cells were generated by immunizing the same mouse one, two, or three times (see Fig. 1A). The 1°, 2°, and 3° memory CD8 T cells generated in this system were transferred to naïve congenic Thy1.1+ mice, which allowed us to distinguish host from donor cells. Recipients were challenged with VSV-Ind. Interestingly, 1°, 2°, and 3° memory CD8 T cells exhibited similar proliferative potential upon infection (Fig. 6A). We also addressed proliferative potential among 1°, 2°, and 3° memory P14 CD8 T cells generated in the adoptive transfer model (see Fig. 3A). When equal numbers of naïve or 1°, 2°, or 3° memory P14 were transferred to naïve mice before challenge, we found that 3° memory CD8 T cells had very poor proliferative potential upon 4° challenge (Fig. 6B). Thus, 3° memory CD8 T cells had low proliferative potential in the adoptive transfer model (as in Fig. 3A), but not when the same mouse was repeatedly challenged (as in Fig. 1A). Differences in senescence observed between the two 3° memory CD8 T cell populations may reflect differences in cumulative division history. For instance, 3° infection of 2° immune mice results in only a ~40-fold increase in the Ag-specific CD8 T cell population, owing to the large precursor frequencies before immunization (Fig. 1). In contrast, transfer of small numbers of 2° memory CD8 T cells to naïve mice results in a ~4000-fold increase in the Ag-specific CD8 T cell population upon 3° challenge (Fig. 3B). Thus, we induced an unusually large number of cumulative divisions in our adoptive transfer model. There may be a Hayflick limit on the number of potential CD8 T cell divisions in vivo (51), after which they become refractory to further expansion. It will be important to test whether this limit can be reached upon repetitive boosting of immune individuals.

**FIGURE 5.** Accumulation of TEM after DNA prime-heterologous boost immunizations. A, Naive P14 were transferred to naïve mice, which were subsequently challenged with 200 μg DNA-gp33 i.m. Eighty days later, 1° memory P14 were transferred to naïve mice, which were then challenged with VV-gp33. Eighty days later, 2° memory P14 were transferred to naïve mice, which were then challenged with LM-gp33. B and C, Phenotype of P14 in blood following each immunization. D, Six months following 1° or 3° immunization, the proportion of 1° or 3° memory P14 CD8 T cells in spleen, lung, liver, or lymph nodes, compared with the total number of 1° or 3° memory P14 CD8 T cells in all four tissues (expressed as a percentage).
FIGURE 6. Proliferative potential of 1°, 2°, or 3° memory CD8 T cells. A, H-2Kb/N-specific 1°, 2°, or 3° memory CD8 T cells were generated as in Fig. 1A. One hundred days later, 1.5 × 10⁴ 1°, 2°, or 3° memory cells were transferred to naive Thy1.1 mice. Recipients were infected with VSV-Ind and the donor CD8 T cell response was visualized with H-2Kb/N tetramers and the congenic marker Thy1.2. Representative FACS plots are shown at the peak of the response, 7 days postinfection. B, The 1°, 2°, or 3° memory P14 CD8 T cells were generated as in Fig. 3A. Six months later, 1 × 10⁴ naive or 1°, 2°, or 3° memory P14 were transferred to naive mice. Recipients were infected with LCMV, and representative FACS plots are shown at the peak of the response, 7 days postinfection. All plots gated on CD8⁺ lymphocytes.

Discussion

Memory CD8 T cell differentiation has mostly been studied following a single immunization. This study provides a comparison of memory CD8 T cells following 1°, 2°, and 3° challenges. Upon repeated immunization of the same mouse, three heterologous prime-boost vaccinations elicited enormous endogenous CD8 T cell responses resulting in virus-specific memory levels that comprised ~60% of all circulating CD8 T cells (Fig. 1). Although this frequency of CD8 T cell memory may not be achievable, or even desirable, in humans, it illustrates the potential of this strategy to induce very high levels of cellular immunity.

Prime-boost vaccination not only affected memory CD8 T cell quantity, but also increased the ratio of T_{EM} to T_{CM}. Understanding the developmental cues that influence commitment into the T_{CM} vs T_{EM} lineage are of major interest. It has been demonstrated that high precursor frequencies of naive CD8 T cells leads to rapid reexpression of CD62L following stimulation (22, 52). This suggests that a brief period of antigenic stimulation during priming favors commitment to the T_{CM} lineage. In contrast, prolonged or excessive antigenic stimulation during priming favors commitment to the T_{EM} lineage (20, 52–54). Our data extend this hypothesis by showing that successive rounds of antigenic stimulation, even when interrupted by an extensive rest period, also drive commitment to the T_{EM} lineage (Figs. 2–5). In other words, memory CD8 T cell differentiation is dictated by the cumulative history of Ag exposure. T_{EM} appear to be stably maintained in humans but convert to T_{CM} in mice, suggesting that memory CD8 T cell differentiation is fundamentally different between species (55).

We demonstrate in mice that the T_{EM} population is relatively transient in blood following a single immunization but persists following 2° and 3° challenges. Thus, discrepancies between the stability of T_{EM} observed between humans and mice may reflect differences in the history of Ag exposure among the CD8 T cells studied, rather than unique memory differentiation programs between species.

With regard to CD62L and CD27 expression, cytolytic potential, cytokine production, proliferative capacity, and anatomic distribution, increasing the number of Ag challenges preferentially promoted the generation and maintenance of T_{EM} (Figs. 2–5). Effector functions and location of memory CD8 T cells may have important implications for protective immunity. Increasing cytolytic T_{EM} within nonlymphoid tissues may be a desirable product of prime-boost vaccinations against pathogens that are naturally encountered through mucosal surfaces, such as HIV and M. tuberculosis (18, 33, 34). Moreover, the accumulation of T_{EM} within nonlymphoid tissues may be an important feature of cellular immunity upon repetitive reexposure to Ag. For instance, many viruses that naturally infect via mucosal surfaces, such as influenza virus, evolve unique serotypes that avoid preexisting Ab-dependent sterilizing immunity. However, due to MHC polymorphism, viruses cannot avoid preexisting cellular immunity. In cases such as influenza, when new serological variants cross-react with preexisting cellular immunity, memory CD8 T cells can contribute to protective immunity by decreasing the severity of infection (56–61).

KLRG1 is an inhibitory receptor expressed by a fraction of NK cells and T cells that recognizes cadherins (62–64). It has recently been proposed that KLRG1 is a marker of CD8 T cell senescence. The maintenance of KLRG1 expression by 2° and 3° memory CD8 T cells was striking (Figs. 2, 4, and 5) but not necessarily indicative of senescence (Fig. 6). However, 3° memory CD8 T cells that were driven to senescence upon repeated transfers and boosts were indeed KLRG1⁺. Dissecting the relationship between KLRG1 expression and senescence remains an interesting issue. CD8 T cells that are repetitively stimulated by persistent viral infections express another inhibitory receptor, programmed death 1 (PD-1) (65). It should be noted that memory CD8 T cells did not express PD-1 following heterologous prime-boost vaccination. For instance, following immunization with VSV-Ind (as in Fig. 1), the geometric mean fluorescence intensity (GMFI) of PD-1 was 4.03 ± 0.10 on 1° memory CD8 T cells, 2.45 ± 0.14 on 2° memory CD8 T cells, and 2.63 ± 0.10 on 3° memory CD8 T cells. Following immunization with VV-gp33 (as in Fig. 3), the GMFI of PD-1 was 2.07 ± 0.02 on 1° memory P14 CD8 T cells, 1.99 ± 0.02 on 2° memory P14 CD8 T cells, and 1.72 ± 0.03 on 3° memory P14 CD8 T cells. Thus, PD-1 expression may depend on recent antigenic stimulation. In this regard, 3° T_{EM} generated by heterologous prime-boost vaccination were not identical with exhausted CD8 T cells specific for certain chronic infections (66).

The effect of heterologous prime boosting on memory CD8 T cell frequency and phenotype likely depends on both the combination of vectors and the route of immunization. The qualities of primary memory CD8 T cells generated by a particular vaccine and the degree of CD8 T cell reactivation upon boosting might be particularly important variables. For instance, a single immunization with live replicating pathogens vs DNA-gp33 results in memory CD8 T cells with very different phenotypes and functional
properties (data not shown). It appeared that primary DNA immu-
nization had long-term consequences on CD27 expression, even 
following boosts with live vectors (Fig. 5). Thus, the nature of the 
primary immunization may impart particular qualities on memory 
CD8 T cells regardless of the boosting strategy. The degree of CD8 
T cell reactivation also may have important consequences on the 
quantity and quality of memory CD8 T cells following boosting. 
For instance, if preexisting immunity limits reinfection, boosting 
may have little effect on memory CD8 T cell quantity (57) and 
quality. However, in all models of prime-boost vaccination that we 
used, including combinations of DNA, viral, and bacterial vectors, 
we observed that boosting resulted in the preferential accumulation 
of memory CD8 T cells that retain effector qualities.

A major finding of our study is that heterologous prime-boost 
vaccination with replicating vectors is capable of eliciting very 
large numbers of effector-like memory CD8 T cells positioned 
within nonlymphoid tissues that serve as potential points of patho-
gen entry. Although these qualities may be particularly desirable 
for protective immunity against certain pathogens, it is important 
to note that the prime-boost strategy may have one important 
drawback. The data in Fig. 6B suggest that memory CD8 T cells 
can undergo a finite number of divisions, even if rested between en-
vironmental exposures.

Our study demonstrates that TEM differentiation is coupled to 
the history of Ag encounter, even if stimulation events are inter-
rupted by an extensive rest period. Analyses of 2′ and 3′ responses 
should be incorporated into existing models of memory T cell 
lineage differentiation. Most importantly, the accumulation of TEM 
is an important consequence of the heterologous prime-boost vac-
cination strategy. These observations have implications for estab-
lishing protective immunity at sites of pathogen entry.

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