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Cutting Edge: Memory CD8 T Cell Compartment Grows in Size with Immunological Experience but Nevertheless Can Lose Function

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The size of the adaptive immune system is considered to be kept constant by the attrition of pre-existing memory. However, recently it was shown that the CD8 memory compartment can grow in size and the number of pre-existing memory is largely preserved, predicting that pre-existing immunity should be maintained (Vezys et al.; Nature 457: 196–199). Experimental proof for this assumption is still lacking. We address this question in the Listeria monocytogenes (L.m.) infection model and confirm the growth of size of the memory compartment by subsequent vaccination with modified vaccinia virus Ankara. We also find only modest attrition of pre-existing L.m.-specific memory CD8 T cells. However, pre-existing protective immunity toward L.m. is not preserved. Pre-existing L.m.-specific effector-memory cells, in contrast to central memory cells, become altered, and this results in a significant loss of pre-existing protective immunity. Our findings are clinically relevant for vaccines introducing new CD8 memory cells in high numbers, as this might influence pre-existing immunity. The Journal of Immunology, 2009, 183: 6898–6902.

An important characteristic of the adaptive immune system is a faster and more efficient response to a second infection with the same pathogen mediated by memory cells (1, 2). Multiple diverse Ag-specific memory CD8 T cells accumulate over time due to multiple diverse infections. This pool is not invariable; any new infection leads to new Ag-specific CD8 T cell populations that interact and compete with the pre-existing diverse memory T cell pool, altering the composition of and being formed by the existing repertoire (3). Therefore, a balance is needed between de novo generation of memory and maintenance of pre-existing memory. In addition to competition on the basis of different specificities, the pool of memory cells is composed of different subtypes such as central memory (CM)4 cells, with a high proliferation capacity but reduced immediate effector function, and effector-memory (EM) cells, with low proliferation capacity but profound immediate effector function (4–7). Both subtypes might have different requirements for maintenance as well as competition with other immune cells.

CD8 T cell frequencies for one given epitope can reach high numbers (8), and memory frequencies can remain remarkably stable for the lifetime of an individual mouse (9). Humans, in contrast to mice under experimental conditions, are exposed to many different infections and vaccinations during a lifetime, and it is considered unlikely for them to maintain high frequencies of Ag-specific memory cells for any previously encountered Ag. Instead, non-cross-reactive, pre-existing memory cells should partly disappear, and this could be demonstrated in different experimental viral and bacterial infection models in mice (3, 10–12). A human study confirmed that vaccinia virus-specific memory CD8 T cells can persist for >10 years, but in 50% of observed individuals, virus-specific CD8 T cells vanished (13). By the implied attrition of pre-existing memory cells, the size of the adaptive immune system should be kept constant.

These observations raise concerns about vaccines that introduce high numbers of CD8 memory cells, as might be needed against virus infections in which Abs are not sufficient, e.g., HIV (14). This conventional interpretation of published data has recently been challenged by the finding that the memory CD8 T cell compartment can grow in size with immunological experience and that memory CD8 T cells specific for a previously encountered Ag. Instead, non-cross-reactive, pre-existing memory cells should partly disappear, and this could be demonstrated in different experimental viral and bacterial infection models in mice (3, 10–12). A human study confirmed that vaccinia virus-specific memory CD8 T cells can persist for >10 years, but in 50% of observed individuals, virus-specific CD8 T cells vanished (13). By the implied attrition of pre-existing memory cells, the size of the adaptive immune system should be kept constant.

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4 Abbreviations used in this paper: CM, central memory; BCG, bacillus Calmette-Guérin; EM, effector memory; L.m., Listeria monocytogenes; MVA, modified vaccinia virus Ankara.

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To test this important aspect, we used a prime-boost vaccination protocol with a relevant viral vector vaccine modified vaccinia virus Ankara (MVA) in Listeria monocytogenes (L.m.)-immune mice (vaccinia virus had also been used in the original study demonstrating memory compartment expansion; Ref. 15). MVA vaccination of previously L.m.-infected mice induced high numbers of memory CD8 cells specific for a single MVA Ag that were, in fact, almost as high as the total number of CD8 T cells in unvaccinated mice. Thus, we confirm that the overall number of memory cells can increase by immunological experience. The number of pre-existing CD8 memory cells specific for L.m., that is, L.m.-specific cells generated before vaccination with MVA, showed only modest attrition, and L.m.-specific CD8 cells could be effectively expanded by a second infection with L.m. to levels as high as those in mice without intermittent MVA vaccination. However, on the functional level we found a severe impairment. After challenge with a lethal dose of L.m., mice with previous MVA vaccination succumbed to infection, had a Listeria-burden as high as that of L.m.-naive mice, and were unable to lyse Ag-loaded target cells in in vivo cytotoxicity assays. All of these outcomes were observed despite the preserved recall expansion of L.m.-specific memory T cells, a hallmark characteristic of CM cells.

Based on these surprising finding, we propose a model in which CM cells are less impaired by subsequent infections or vaccinations and are able to expand faster after re-encounter. In contrast, EM cells, which are necessary for an immediate resistance against fast replicating pathogens, might get severely altered during competition with additional memory cells despite an overall enlargement of the effector-memory T cell compartment.

Materials and Methods

Mice, bacterium, and virus

C57BL/6J mice (Harlan Winkelmann) were i.v. infected with L.m. expressing OVA (H. Shen, University of Pennsylvania School of Medicine, Philadelphia, PA). Five and 10 wk after L.m. infection, MVA vaccination was performed by i.v. injection of 10^6 PFU of MVA (16). All experiments were performed at least twice with at least three mice per group.

Staining of cells and in vivo cytotoxicity assay

Cells were incubated with ethidium monazide (Molecular Probes) and Fc block (BD Pharmingen) followed by Abs (CD8 and CD62L; BD Pharmingen) and MHC multimer (IBA). For intracellular cytokine staining, cells were incubated with OVA257–264 or DMSO (Sigma-Aldrich) and stained as recommended by BD Pharmingen.

For in vivo cytotoxicity assays, splenocytes were either loaded with OVA257–264 and labeled with CFSE (Molecular Probes) at a high concentration or not loaded and labeled at a low concentration. Equal numbers of either cell type were injected i.v. Blood was analyzed for CFSE-positive cells.

Statistical analysis

All statistical analyses were performed using GraphPad Prism4 software. Results are expressed as mean ± SD. Differences between groups were analyzed for statistical significance using an parametric unpaired two-tailed Student t test.

Results

The overall memory pool increases with vaccination

To establish a pool of preexisting CD8 memory T cells derived from polyclonal naive precursors, we infected C57BL/6J mice with L.m. expressing OVA. L.m.-immune mice are resistant to subsequent infection with high-dose L.m. in a CD8 memory T cell-dependent manner (17–19). After L.m.-specific memory was established (35 days), mice received a prime-boost vaccination with MVA. MVA is a highly attenuated strain of vaccinia virus that has lost the ability to replicate efficiently in mammalian cells and, thus, is cleared early after application and does not persist as reported for other viruses such as, e.g., influenza or vesicular stomatitis virus. Therefore, MVA is widely considered the vaccinia virus strain of choice for clinical applications (20).

Using this protocol, we observed a massive overall increase in CD8 T cells in the vaccinated group, still remaining late in the memory phase (100 days after second MVA injection; Fig. 1). In fact, the number of CD8 cells specific for one given immunodominant epitope of MVA (B8R20–27) was almost equal to the overall numbers of CD8 T cells in an unvaccinated host. These results are almost identical with and confirm recent data derived in a different system using adoptively transferred TCR-transgenic CD8 T cells and different virus models, e.g., vaccinia virus (15). Thus, in this experimental setting the memory compartment can grow in size with immunological experience.

CD8 memory T cells are phenotypically heterogeneous and CD62L is referred to as a marker that allows discrimination between EM (CD62Llow) and CM (CD62Lhigh) cells (7, 21, 22). These results are almost identical with and confirm recent data derived in a different system using adoptively transferred TCR-transgenic CD8 T cells and different virus models, e.g., vaccinia virus (15). Thus, in this experimental setting the memory compartment can grow in size with immunological experience.

Attrition of the preexisting memory pool is only modest

Consistent with previous reports (11, 12, 23), the percentage of pre-existing L.m.-specific CD8 T cells strongly declined 100 days after the last MVA injection in the spleen (Fig. 2A) and peripheral organs (lung and liver; data not shown). However, due to the overall increase in CD8 T cells, the attrition seemed less severe in terms of total numbers. The reduction was only 29% compared with nonvaccinated mice, which again is in line with a recent publication (15).

Recall responses are not functionally impaired by subsequent vaccination

To test whether the moderate reduction in the numbers of pre-existing memory cells has functional consequences, L.m.-immune mice were prime-boost vaccinated with MVA or kept
unvaccinated. Three months later, mice were challenged with \(2 \times 10^5\) CFU of L. m. In vaccinated mice, L. m.-specific cells expanded as well as or even better as compared with unvaccinated mice. This was the case for spleen (Fig. 2B), lung, and lymph node tissue (data not shown). CM cells are a subgroup within the memory compartment with high proliferation capacity. The unimpaired expansion of pre-existing CD8 T cells indicates that CM cells are preserved and react quickly to secondary encounter with L. m., providing evidence that the CM population is unaltered both in numbers and function. However, when mice were challenged with a higher dose of L. m. (\(2 \times 10^6\) CFU) we observed that 50% of the mice in the MVA-vaccinated group died, whereas none died in the unvaccinated group. This was despite an equal or greater cell expansion in surviving vaccinated mice compared with unvaccinated mice (data not shown). We, therefore, hypothesized that the functionality of CM cells might be affected by the prime-boost vaccination with MVA.

Pre-existing EM CD8 T cells are functionally impaired by subsequent vaccination

A subgroup of Ag-specific CD8 T cells that shows immediate effector function, the so-called EM cells, is more potent for protecting against a secondary infection with rapidly replicating pathogens (4, 6). The susceptibility of vaccinated mice to a high-dose L. m. infection suggests a reduction or impairment of pre-existing L. m.-specific EM cells within the overall effector memory pool that is being increased by vaccination. To determine how many L. m.-specific EM remain after MVA vaccination, we tested the production of IFN-γ as well as TNF-α and IL-2 (supplemental Fig. S1) after L. m.-specific in vitro restimulation. In contrast to multimer staining (Fig. 2A), we detected a reduction in relative frequencies as well as in absolute numbers in the spleen (Fig. 3A), lung and liver tissue (data not shown). Thus, although we find an overall increase of EM cells after prime-boost vaccination, pre-existing L. m.-specific EM cells seem to be reduced and functionally altered.

To test more directly for effector function, L. m.-immune mice were challenged with \(2 \times 10^6\) CFU of L. m. and subsequently monitored for survival. As shown in Fig. 3B, although L. m.-immune mice fully resisted a high-dose challenge, L. m.-immune mice that received a prime-boost vaccination with MVA succumbed to infection. In line with this observation, 2 days after L. m. rechallenge the Listeria burdens in the spleen (Fig. 3C) and livers (not shown) of vaccinated mice were as high as those in naive mice that had not previously encountered L. m. The relatively high dose of L. m. used for these recall infections was necessary to demonstrate the extreme consequences of our findings and to stress out the relevance of our results for effective protective immunity; it is important to mention that L. m.-immune unvaccinated mice survived this relatively high infection dose without significant signs of disease and that similar results were obtained with lower dose infection (\(2 \times 10^5\) CFU). In addition, using in vivo cytolysis assays to circumvent the
possible limitations of high-dose infections, we found that preexisting CD8 T cells from vaccinated mice were unable to lyse Ag-loaded splenocytes as quickly and potently as those from unvaccinated mice (Fig. 3D), clearly indicating the impairment of memory, exerting immediate effector function, i.e., EM. This alteration did not generally affect the immune status of such mice. MVA prime-boost-challenged mice effectively controlled primary infection with *Listeria* like unvaccinated mice (supplemental Fig. S2) and mounted similar *L.m.*-specific T cell responses (supplemental Fig. S3). Furthermore, we and others have previously confirmed that MVA-specific T cells after MVA prime-boost vaccination are highly functional and protective (16).

Phenotypical analyses of *L.m.* (OVA) and MVA-specific CD8⁺ memory T cells for CD62L and CD127 expression demonstrated similarities within the CM compartment (supplemental Fig. S4), which match with the finding of similar recall expansion rates. Also, differences within the "phenotypical" effector memory compartment were only minor. Although these data indicate some limitations of phenotypical memory T cell characterization for the prediction of in vivo T cell quality, they confirm that T cells with an EM phenotype are not generally deleted during MVA prime-boost vaccination.

**Discussion**

We demonstrate that although the attrition of pre-existing memory CD8 T cells can be modest in quantity and the memory compartment can grow in size with subsequent vaccination, the quality of pre-existing memory T cells might not necessarily be preserved by this mechanism. Indeed, EM cells, in contrast to CM cells, can become ablated or impaired after applying a commonly used prime-boost vaccination scheme that results in a significant loss of pre-existing protective immunity. EM cells are needed for the clearance of *L.m.* and other fast-replicating pathogens like the vaccinia virus (4, 6). In other settings such as the lymphocytic choriomeningitis virus, for example, CM cells have been demonstrated to be sufficient and no loss in protection might be seen if EM cells are selectively altered (4).

We conclude from the data presented in this report that CM cells are preserved differently and are more resistant to heterologous infections/vaccinations compared with EM cells, as we see an expansion of *L.m.*-specific cells that is independent of the vaccination status of mice. We cannot assert that preexisting CM cells are not affected at all by subsequent prime-boost vaccination with MVA, because small differences may be camouflaged simply by the tremendous proliferation capacity of these cells. However, we never detected any impairment of recall expansion, and in most cases we even observed greater cell numbers of Ag-specific T cells after rechallenge of vaccinated mice. This greater expansion could be explained by the loss of EM cells, which have been suggested to kill APCs, thereby restricting the expansion of proliferation competent (CM) cells (24).

CM cells reside in the lymph nodes and, due to their fast expansion capacity, very few cells are likely to be enough to replenish the organs with newly generated effector cells if low infection doses are used and no immediate effector function is needed. In contrast, EM cells are at the frontline to fight invading pathogens but proliferate poorly and, thus, a significant minimal number is required to ensure a fast and effective response toward rapidly replicating infectious agents. In our experimental system, the total number of EM cells is increased by prime-boost vaccination with MVA, but pre-existing *L.m.*-specific EM cells vanish or loose function. Such a reduction in *L.m.*-specific CD8 T cells producing IFN-γ is in line with findings in a different experimental setting using *L.m.*-infection followed by bacillus Calmette-Guérin (BCG) (10). Tumor (OVA)-specific protection induced by OVA-expressing *Listeria* was abolished after intermittent BCG immunization, but *L.m.*-specific protection not. BCG is present for months after infection and might induce a general activation status of APCs. Pre-activated innate immunity by itself can confer some protection to *L.m.* and might compensate for reduced *L.m.*-specific T cells; this mechanism might be less effective for protection against a locally implanted tumor. MVA, in contrast to BCG, is rapidly cleared by the immune system and does not lead to long-term activation of APCs.

Preservation of EM cells might especially be a matter of space; EM cells reside in nonlymphatic organs (9), and although we and others (15) observed expansion of the EM compartment in peripheral organs, this expansion cannot be infinite. Therefore, at some point decisions have to be made as to which cell to keep and which one to eliminate. Multiple mechanisms might operate to induce the attrition of pre-existing CD8 memory T cells. For example, early production of type I IFNs during infection has been suggested to contribute to the erosion of memory T cells (23, 25). In this regard, it is interesting that i.v. infection with MVA, but not wild-type vaccinia virus (as used by Vezys et al.; Ref. 15) is a strong inducer of IFN production that is independent of gene expression or viral replication (26). However, in contrast to the lymphocytic choriomeningitis virus model, our data argue against a dominant effect of type I IFNs in the MVA prime-boost regimens; we observed a comparably mild attrition in the numbers of pre-existing memory cells, as did Vezys et al. (15), apart from the strong type I IFN induction due to MVA. In line with this, first experiments showed no changes in the vaccination-induced impairment of EM cells in type I IFN receptor-deficient (IFNAR⁻/⁻) mice (data not shown). Another candidate cytokine is IFN-γ. IFN-γ has also been demonstrated to be involved in the erosion of pre-existing memory targeting different intracellular bacteria. Pathogen virulence was claimed to be responsible for the extent of erosion in this setting (27). Although MVA as a vaccination vector is nonpathogenic, prime-boost vaccination with MVA still induces CD8 T cells to produce large amounts of IFN-γ, and this might contribute to the attrition of pre-existing EM. Why CM cells are more resistant to erosion than EM cells needs to be determined; perhaps they have access to specific sites where they receive other and/or additional survival factors (bone marrow or lymph nodes). Very low numbers of CM cells, which do not need very much space, can already give rise to large recall responses; therefore, it might be more feasible to maintain memory diversity primarily within this compartment. EM cells are without any doubt more potent during the early phase of infection, but they can be newly regenerated out of CM cells (6, 28). However, a loss of EM can be fatal if the infection develops faster than the EM cell recruitment out of CM cells, as is obviously the case for *L.m.* and vaccinia virus, and perhaps also for HIV (14).

For all three of the epidemiologically most relevant targets in vaccination, HIV, malaria and tuberculosis, pre-existing Ag-specific effector T cells seem to be the main components in conferring T cell-mediated protection. Therefore, for these very
important diseases the functional attrition of preexisting immunity by subsequent vaccinations also has to be considered.

Disclosures
The authors have no financial conflict of interest.

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