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*J Immunol* 2013; 190:4162-4174; Prepublished online 18 March 2013;
doi: 10.4049/jimmunol.1202665
http://www.jimmunol.org/content/190/8/4162

Supplementary Material

http://www.jimmunol.org/content/suppl/2013/03/18/jimmunol.1202665.DC1

References

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A New Model for CD8⁺ T Cell Memory Inflation Based upon a Recombinant Adenoviral Vector

Beatrice Bolinger,* Stuart Sims,* Geraldine O’Haras, Catherine de Lara,* Elma Tchilian,* Sonja Firner,† Daniel Engeler, Burkhard Ludewig,† and Paul Klenerman*†

CD8⁺ T cell memory inflation, first described in murine CMV (MCMV) infection, is characterized by the accumulation of high-frequency, functional Ag-specific CD8⁺ T cell pools with an effector-memory phenotype and enrichment in peripheral organs. Although persistence of Ag is considered essential, the rules underpinning memory inflation are still unclear. The MCMV model is, however, complicated by the virus’s low-level persistence and stochastic reactivation. We developed a new model of memory inflation based on a β-galactosidase (βgal)–recombinant adenovirus vector. After i.v. administration in C57BL/6 mice, we observed marked memory inflation in the βgal497 epitope, whereas a second epitope, βgal497, undergoes classical memory formation. The inflationary T cell responses show kinetics, distribution, phenotype, and functions similar to those seen in MCMV and are reproduced using alternative routes of administration. Memory inflation in this model is dependent on MHC class II. As in MCMV, only the inflating epitope showed immunoproteasome independence. These data define a new model for memory inflation, which is fully replication independent, internally controlled, and reproduces the key immunologic features of the CD8⁺ T cell response. This model provides insight into the mechanisms responsible for memory inflation and, because it is based on a vaccine vector, also is relevant to novel T cell–inducing vaccines in humans. The Journal of Immunology, 2013, 190: 4162–4174.

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Recent work has revealed that ongoing production of infectious MCMV is, however, not an absolute requirement (15, 16). Critically, MCMV is a complex model virologically, with a very large genome containing numerous immunoevasins, long-term low-level persistence, and stochastic reactivation at diverse sites. Thus, a simpler and more tractable system to investigate these questions would be desirable.

The phenomenon of memory inflation is not exclusive to CMVs because it is also observed in other viral infections (17–20). However, it has not been described after immunization with nonreplicating vaccine vectors. Recombinant viral vectors for Ag delivery are key to many novel vaccine strategies. In this field, adenovirus vectors (AdVs) have emerged among the most potent of these (21–24). They transduce a variety of cells, but the vector genome does not integrate and their safety is well established (25). Depending on dose, route of immunization, and the transgene used, a spectrum of different T cell responses are elicited after immunization with recombinant AdVs (rAdVs). These responses may range from complete abolition of a functional response to expansion and differentiation of effector T cells (26–31). In this study, we showed for the first time, to our knowledge, that rAdV was capable to induce robust, sustained CD8+ T cell memory inflation, which mimics that seen after MCMV infection.

Using a β-galactosidase (βgal)–recombinant AdV (Ad-LacZ), we found this replication-deficient virus to induce strong memory inflation in vivo, independent of viral reactivation. The system has a number of internal controls, including, critically, a non-inflating epitope generated from the same transgene. Using this model, βgal-specific CD8+ T cells show memory inflation with up to 20% specific cells on day 21, increasing to 30% on day 100 in blood, with further enrichment in tissues such as liver and lung. In contrast, responses against the βgal-specific epitope show classical stable memory with early induction followed by low levels over time. As after MCMV infection, CD8+ T cell memory inflation after Ad-LacZ immunization develops after a single i.v. dose, and the induced CD8+ T cells show an identical effector-memory phenotype.

Overall, immunization with Ad-LacZ provides a unique and robust model for memory inflation. It has broader implications not only for examining the basic biology of sustained effector-memory populations, but also with direct relevance to vaccine development.

### Materials and Methods

#### Ethics statement

Mouse experiments in Oxford were performed according to U.K. Home Office regulations (project license no. PPL 30/2235 and 30/2744), and after review and approval by the local Ethical Review Board at the University of Oxford. Experiments in St. Gallen were performed in accordance with Swiss Kantonal and federal legislations, and were approved by the Veterinary Officer of the Kanton of St. Gallen.

#### Adenoviral vector

Recombinant adenovirus expressing the βgal protein under the control of the HCMV immediate-early IE1/2 promoter-enhancer (MCMV-LacZ RM427) (32) was used. MCMV-LacZ was propagated and titrated on NIH 3T3 cells (European Cell Culture Collection, Porton Down, U.K.) and injected i.v. at a dose of 2 × 10^9 PFU.

Recombinant Vaccinia expressing the βgal protein (Vacc-LacZ) (33) was used. UV-Vacc-LacZ was propagated and titrated on BSC-40 cells, and injected i.p. at a dose of 2 × 10^9 PFU.

#### Mice

Male and female C57BL/6 mice were obtained from Harlan (Blackthorn, U.K.). MHC class II (MHC-II) knockout (KO) (34) mice were on the C57BL/6 background. TCR transgenic Bg1 mice that express a TCR recognizing the H-2 Kb-restricted βgal-specific peptide on >95% of their CD8+ T cells have been described previously (35). Bg1 mice have been generated by Dr. Nicholas P. Restifo (National Cancer Institute, Bethesda, MD) and were kindly provided by Dr. Chris Norbury (Penn State Milton S. Hershey College of Medicine, Department of Microbiology and Immunology). Bg1 mice were crossed with C57BL/6SJL mice expressing the congenic marker Ly5.1 (CD45.1) kindly provided by Dr. Kevin Maloy (Dunn School, University of Oxford, Oxford, U.K.). Transgene expression was monitored by staining of blood cells with anti-Vβ7 by flow cytometry. LMP7 KO mice (36) on the C57BL/6 background were kindly provided by Dr. Marcus Groettrup (Division of Immunology, Department of Biology, University of Constance, Konstanz, Germany). All animals were kept under conventional conditions in individually ventilated cages and fed with normal chow diet. Experiments were carried out with age- and sex-matched animals.

#### Peptides

The βgal<sub>103</sub> (DAPYTNV) (37), the βgal<sub>97</sub>–104 (ICPMYARV) (38), and the M45 (HGIINASF) (39) peptide were purchased from Mimotopes (Melbourne, VIC, Australia).

#### Antibodies


#### Flow cytometry

For flow cytometry, single-cell suspensions were generated from the indicated organs, and 1 × 10^6 cells were incubated with the indicated mAb at 4°C for 20 min. For PBL samples, erythrocytes were lysed with FACS Lysing Solution (BD Pharmingen). Cells were analyzed by flow cytometry using a BD LSR II flow cytometer and FlowJo software, gated on viable leukocytes using the live/dead fixable near-infrared dead cell stain kit from Invitrogen (Paisley, U.K.).

#### Isolation of liver and lung lymphocytes

Perfused livers were smashed through a cell strainer (BD), and lymphocytes were purified by a Percoll (GE Healthcare) gradient centrifugation. Lungs were minced with razor blades and incubated in PBS containing 60 U/ml DNase (ApliChem) and 170 U/ml collagenase II (Life Technologies) at 37°C for 15 min. Cell aggregates were dispersed by passing the digest through a cell strainer (BD).

#### Intracellular cytokine staining

Spleens, livers, and lungs were removed at indicated time points after immunization with 2 × 10^6 PFU Adeno-LacZ. Single-cell suspensions of 2 × 10^6 lymphocytes were incubated for 2 h at 37°C in 150 μl culture medium (RPMI) containing 5% FCS, 2% human heat-inactivated serum, and 2 μg/ml phytohemagglutinin. Cells were fixed with the Cytofix/Cytoperm solution (BD Biosciences) according to the manufacturer’s specifications. Virus titer was determined in a cytopathic effect assay. In brief, serial dilutions of the virus were added to permissive HER-911 cells and were pu

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Construction of tetrameric MHC–I–peptide complexes

MHC-I monomers complexed with βgal (H-2Kb) were produced as previously described (40) and tetramerized by addition of streptavidin-PE (BD Bioscience) or streptavidin-allophycocyanin (Invitrogen). At the indicated time points postinfection, organs were removed and single-cell suspensions were prepared. Aliquots of 1 × 10^6 cells or 100 µl blood were stained using 50 µl of a solution containing tetrameric class I–peptide complexes at 37°C for 20 min followed by staining with mAbs at 4°C for 20 min. Absolute cell counts were determined by counting leukocytes in an improved Neubauer chamber.

In vivo cytotoxicity

Single-cell suspensions from spleens of C57BL/6 mice were subjected to hypotonic RBC lysis. Before injection, half of the cells were loaded with 10^7 M βgal (5′-biotin, Invitrogen) and the other half were stained with CellTrace violet (Invitrogen). A maximum concentration of 5 × 10^6 cells/ml was inoculated in 5 µM CellTrace violet in PBS or 0.5 µM CellTrace violet (negative) for 20 min at 37°C. Cells were washed and resuspended in PBS at a concentration of 5 × 10^6 splenocytes/ml in each group. Recipient B6 mice were injected i.v. with 10 × 10^6 splenocytes in 200 µl PBS. Twelve hours later, blood, spleen, liver, and lungs were harvested and single-cell suspension generated. Cells were analyzed by flow cytometry using a BD LSR II flow cytometer and FlowJo software, and gated on viable leukocytes using the live/dead fixable near-infrared dead cell stain kit from Invitrogen. The ratio of killed cells to control cells was calculated to obtain the percentage of specific killing.

Extraction and quantification of LacZ genome copy numbers in tissue

Tissues were homogenized using a MagNA Lyser instrument (Roche Diagnostics). Whole DNA was isolated using the High Pure PCR Template Preparation Kit (Roche Diagnostics). Real-time quantitative PCR was performed using a LightCycler 480 Real-Time PCR System (Roche Diagnostics) and the LightCycler 480 probes master reaction mix (Roche Diagnostics), according to the manufacturer’s protocol. Data analysis was performed with LightCycler 480 Software (Roche Diagnostics). Oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). The following oligonucleotides from LacZ protein sequences were used as primers for real-time quantitative PCR: 5′-GCGGTGGAAGACCACG-3′ and 5′-CGA AGC CGC CCT GTA AAC-3′. The following oligonucleotides were used as probes: 5′-CAG TCT TGG CGG TTT CGC TAA-3′ and 5′-TAC TGG CAG GCC TTT CGT CAG-3′. Probe 1 carried a 3′ FAM reporter, and probe 2 was Cy5 labeled at the 5′ end. Thermal cycling started with HotStarTaq activation during 15 min at 95°C. Thereafter, 50 cycles of amplification were run consisting of 15 s at 95°C, 30 s at 60°C, and 20 s at 72°C. A negative control, containing reagents only, and serial dilutions of plasmid containing the specific LacZ sequence were included in each run to generate a standard curve. The concentrations of the plasmid dilutions were 280,000, 28,000, 2800, 280, and 28 copies per reaction. LacZ DNA concentration in the unknown samples was calculated using the standard curve and the average concentration was used. Final copy numbers were calculated per microgram total RNA.

Adoptive transfer of TCR transgenic T cells

Single-cell suspensions from spleens of Ly5.1+ B61 mice were subjected to hypotonic RBC lysis and stained with CFSE (Invitrogen). A maximum concentration of 2.5 × 10^6 cells/ml was inoculated in 5 µM CFSE in PBS for 10 min at 37°C. Cells were washed twice with ice-cold PBS and resuspended in PBS at a concentration of 1 × 10^6 splenocytes/ml. Recipient B6 mice were injected i.v. with 2 × 10^6 B61-Ly5.1+ splenocytes in 200 µl PBS.

Statistical analysis

To evaluate statistically significant differences, we used the unpaired two-tailed Student test. The p values <0.05 were considered statistically significant. Statistical data analysis was performed using GraphPad Prism version 5.0a (GraphPad Software, San Diego, CA).

Results

Epitope-specific CD8+ T cell memory inflation after AdV immunization

Immunization of C57BL/6 mice with the replication-deficient Ad-LacZ induced a strong CD8+ T cell response in blood on day 21 against two distinct βgal epitopes (βgal_97 and βgal_90). On day 300, βgal_97-specific CD8+ T cells had contracted and were detectable at low levels only, whereas the βgal_90-specific CD8+ T cell population had increased further (Fig. 1A).

To better define the kinetics of the βgal-specific CD8+ T cell responses induced by Ad-LacZ, we inoculated C57BL/6 mice i.v. with a single dose of Ad-LacZ and tracked longitudinally. Lymphocytes from blood, spleen, liver, lung, and lymph nodes (LNs) were isolated at different time points, and βgal-specific CD8+ T cells were quantified by staining with MHC-I tetramers. βgal_97-specific CD8+ T cells showed memory inflation with up to 20% specific cells on day 21, followed by a continued increase over time to 30% on day 100 in blood (Fig. 1B). These cells were further enriched in tissues such as spleen, liver, and lung on day 100 (Fig. 1C). As in blood, in these organs, the βgal_97-specific CD8+ T cell population was maintained over time (Fig. 1D).

In contrast with βgal_97-specific CD8+ T cells, tetramer staining for βgal_90-specific CD8+ T cells revealed only low levels of tetramer+ CD8+ T cells on day 100 in spleen, liver, and lung (Fig. 1C). The initial expansion phase was followed by contraction and classical stable memory at low but detectable levels over time (Fig. 1D). When data were displayed as absolute numbers of Ag-specific T cells per organs, comparable results were obtained (Fig. 1E). On day 200, the total amount of βgal_90-specific CD8+ T cells in spleen and liver was ~25 times higher than the amount of βgal_97-specific CD8+ T cells, whereas in the lung, it was 50 times more, the latter two indicating a major redistribution of βgal_90-specific CD8+ T cells to peripheral organs.

Only low frequencies of βgal_90-specific CD8+ T cells were found in LNs (inguinal LN; βgal_90-specific CD8+ T cells: day 21 = 0.6 ± 0.1%, day 50 = 0.4 ± 0.1%, day 100 = 0.4 ± 0.1%; βgal_97-specific CD8+ T cells: day 21 = 0.3 ± 0.1%, day 50 = 0.09 ± 0.03%, day 100 = 0.02 ± 0.01%), whereas βgal_90-specific CD8+ T cells did not inflate, as observed in MCMV infection (10, 41).

CD8+ T cell memory inflation is not restricted to the i.v. route, but is confined to the vector

It has been shown previously that the localization of the inoculum of AdVs determines the quality of CD8+ T cell responses (26).
Thus, to identify whether memory inflation in the Ad-LacZ model is restricted to the i.v. route, we evaluated the βgal-specific CD8+ T cell response after i.v. and i.d. Ad-LacZ injection in blood of C57BL/6 mice. We performed tetramer staining for the inflating (βgal96) and the noninflating epitope (βgal497; Fig. 2A). Interestingly, exactly the same pattern for both the βgal96- and βgal497-specific CD8+ T cell response is seen after i.v. and i.d. immunization. Although the βgal96-specific response was significantly reduced after i.d. immunization compared with i.v., CD8+ T cell maintenance was not impaired, and βgal96-specific CD8+ T cells clearly showed memory inflation in this setting. This is even better illustrated looking at the inflationary potential of the CD8+ T cell responses, by considering the day 100 to day 21 ratio of tetramer+ cells (Fig. 2B).

FIGURE 1. A single dose of Ad-LacZ is sufficient to induce CD8+ T cell memory inflation. C57BL/6 (B6) mice were immunized i.v. with 2 × 10⁹ PFU Ad-LacZ. Tetramer analysis for the indicated βgal epitopes was performed on days 21, 50, 100, 200, and 300 after immunization. (A) Tetramer staining for βgal96- and βgal497-specific CD8+ lymphocytes on days 21 and 300 in blood, gated on live lymphocytes. (B) Expansion of βgal96- (black circles) and βgal497- specific (white squares) CD8+ T cells in blood. (C) Tetramer staining for βgal96- and βgal497-specific CD8+ lymphocytes on day 100 in spleen, liver, and lung. (D) Tetramer analysis for lymphocytes from spleen, liver, and lung on days 21, 50, 100, 200, and 300. Mean percentage of tetramer+ cells within the CD8+ T cell compartment are indicated (± SEM; blood: day 21, n = 17; day 50, n = 21; day 100, n = 15; day 200, n = 12; day 300, n = 12; spleen: day 21, n = 16; day 50, n = 15; day 100, n = 8; day 200, n = 9; day 300, n = 10; liver and lung: day 21, n = 13; day 50, n = 17; day 100, n = 8; day 200, n = 9; day 300, n = 9). (E) Total counts of βgal96- and βgal497-specific CD8+ lymphocytes in spleen, liver, and lung on days 21, 50, 100, and 200 after immunization. Mean of total numbers of tetramer+ cells within the CD8+ T cell compartment are indicated (± SEM; spleen: day 21, n = 16; day 50, n = 13; day 100, n = 8; day 200, n = 6; liver and lung: day 21, n = 12; day 50, n = 11; day 100, n = 11; day 200, n = 6). Each experiment was repeated at least once.
Inflationary potential expressed by the ratio of percentage of tetramer+100, which do not inflate, LacZ, and expansion of within the CD8+ compartment is indicated (bartment is indicated (b), LacZ infected B6 mice on day 7 postinfection in blood. Mean percentages staining in naive (= background) B6 mice, and MCMV-LacZ and Vacc-LacZ infection are indicated. *p = 0.0238; Vacc-LacZ d7 compared with background in naive mice: p = 0.0392). The βgal497-specific CD8+ T cell response was detected, but at levels not significantly above background (background in naive mice: 0.17 ± 0.02%; day 7 MCMV-LacZ: 0.4 ± 0.07%; day 7 Vacc-LacZ: 0.31 ± 0.08%; Fig. 2E). Together, these data revealed that if the transgene was expressed in a different vector, although a βgal497-specific CD8+ T cell was primed, no CD8+ T cell memory inflation was induced (Fig. 2C, 2D), indicating that the latter is not solely a property of the insert.

Overall, our data suggest that Ag presentation established after both i.v. and i.d. inoculation is sufficient to maintain inflationary responses, and βgal-specific CD8+ T cell memory inflation is exclusive to the AdV.

To further characterize βgal497-specific CD8+ T cells generated after i.v. inoculation, we compared them with MCMV-specific inflationary CD8+ T cells, using phenotypic markers defined from conventional analyses of MCMV-specific cells (4, 9, 10). Hence, we costained βgal-tetramer+ CD8+ T cells for adhesion and trafficking molecules such as CD44 and CD62L, for the cytokine receptor CD127 (IL-7Rα), the NK cell receptors KLRG-1, NKG2A, and NKG2D, and the costimulatory marker CD27, and compared them with the tetramer+ CD8+ T cell population (Fig. 3A; Supplemental Fig. 1A, 1B). Staining for CD44 and CD62L indicated that inflating cells are a memory pool with a predisposition to accumulate in nonlymphoid organs (CD44hiCD62Llo). The inflating population downregulated IL-7Rα, expressed IL-15Rβ, was high in KLRG1, NKG2A, and NKG2D, and showed reduced expression of the costimulatory molecule CD27 compared with tetramer+ CD8+ T cells. These characteristics are typical for an Ag-experienced effector-memory CD8+ T cell population.

We next examined the expression of these markers on βgal497-specific inflationary CD8+ T cells, βgal497-specific, and total CD8+ T cells in blood, spleen, liver, and lung up to day 200 after immunization (Fig. 3B; Supplemental Fig. 1C–F). We demonstrated that βgal497-specific inflationary CD8+ T cells rarely changed their phenotype over time. They display a comparable effector-memory phenotype (CD62Llo, CD27hi, IL-7Rlo, KLRG-1hi, NKG2A/Dhi) from day 21, which is maintained up to day 200 after immunization. In contrast with these data, βgal497-specific CD8+ T cells acquired a divergent phenotype after contraction. This had features of a central memory pool (CD27lo, IL-7Rlo, KLRG1hi, NKG2A/Dlo), especially in the spleen and blood. They were also low in expression on NKG2D and NKG2A (Fig. 3B; Supplemental Fig. 1C–F).

Importantly, βgal497-specific CD8+ T cells after i.d. injection displayed an identical effector-memory phenotype on days 21, 50, and 100 in blood as that seen after i.v. immunization (Supplemental Fig. 2C).

We also went on to test the impact of inoculation on induction of memory and phenotype via alternative routes such as s.c. and i.m. We noted that βgal497-specific CD8+ T cells do not inflate numerically after s.c. injection of Ad-LacZ, although a distinction between the kinetics of the βgal497-specific CD8+ T cells and βgal497-specific CD8+ T cells could still be observed. However, the generation of the effector-memory phenotype associated with the βgal497-specific CD8+ T cells was clear in all cases and independent of the route of immunization (Supplemental Fig. 2).

Taken together, Ad-LacZ induced two completely distinct CD8+ T cell memory populations: the conventional (βgal497-specific CD8+ T cell) population with a central memory T cell (TCM)
The fraction of CD8+ T cells from blood, spleen, liver, and lung, but not from LNs, retains functionality and shows rapid acquisition and maintenance of cytotoxicity.

We next assessed the functionality of the ‘inflating’ memory population. Intracellular staining for IFN-γ and TNF-α after peptide stimulation revealed that inflammatory βgal-specific CD8+ T cells efficiently secreted effector cytokines (Figs. 4, 5A, 5B). Indeed, both sets of βgal-specific memory cells are strong IFN-γ and TNF-α producers upon stimulation with the cognate peptide. Whereas IFN-γ and TNF-α secretion on day 21 is comparable for both βgal-specific populations, inflationary CD8+ T cells, especially in peripheral organs, dominated cytokine secretion in the memory phase (Figs. 4, 5A, 5B).

Furthermore, staining for IFN-γ, TNF-α, and LAMP-1 revealed that βgal-specific inflationary CD8+ T cells were polyfunctional. On day 100 postimmunization, most of the IFN-γ+ CD8+ T cells in spleen, liver, and lung were also positive for LAMP-1, indicating that they had degranulated and released effector molecules. Many IFN-γ–producing, LAMP-1+ inflationary CD8+ T cells additionally secreted TNF-α. This pattern of functionality was seen at all time points measured (Fig. 5A, 5B). Overall, if we consider the difference in the amount of βgal497- and βgal96-specific CD8+ T cells, calculating the ratio of IFN-γ+ or TNF-α+ producing cells to tetramer+ cells, although both populations are clearly functional, βgal96-specific CD8+ T cells are stronger IFN-γ and TNF-α producers on a per-cell basis (data not shown). Furthermore, this ratio also revealed a higher number of IFN-γ+ and TNF-α–producing CD8+ T cells compared with βgal96-specific CD8+ T cells after stimulation with βgal96-peptide. We suggest that the βgal96 tetramer may bind specific T cells with a relatively low avidity; hence, the frequency of tetramer-specific CD8+ T cells might be underestimated compared with the true frequency. Alternatively, there may be binding to distinct MHC molecules. Both alternatives will be assessed further in future studies.

We next assessed the killing capacity of the βgal-specific CD8+ T cells, using an in vivo cytotoxicity assay. βgal497-peptide–pulsed target cells were efficiently killed in vivo 12 h after adoptive transfer into day 21, 50, and 100 immune mice. This is seen not only in spleen and blood (Fig. 5C), but also in peripheral organs such as liver and lung (Fig. 5D) at all time points, indicating that inflationary CD8+ T cells retain their cytotoxic capacity. In comparison, βgal96-peptide–pulsed target cells were killed in a much lower degree, especially if transferred into day 100 mice.

Overall, although βgal497-specific CD8+ T cells show evidence of repeated Ag exposure, they are not exhausted and remain functional, even at day 100 after immunization.

Low-level Ag persistence enables CD8+ T cell memory inflation

Compared with MCMV infection, the Ad-LacZ model is virologically simple. There is no viral replication, which clearly facilitates tracking the virus, and both epitopes are generated from a single gene product.

To establish whether viral genome persists, we performed quantitative real-time PCR. LacZ DNA copy numbers per microgram total DNA were assessed in spleen, liver, lung, and hepatic LNs (hLNs) at different time points after Ad-LacZ immunization. On day 300, viral genome was still found in spleen, liver, and lung, but not in hLNs (Fig. 6A).

Next, we addressed whether the transgene was still expressed at late time points. To do this, first, quantitative real-time PCR for
**FIGURE 4.** Inflating CD8+ T cells are strong IFN-γ and TNF-α producers. C57BL/6 mice were immunized with 2 x 10^8 PFU Ad-LacZ i.v., and intracellular cytokine production assay for IFN-γ (A, B) and TNF-α (C, D) in the presence of the βgal96 or βgal497 peptide was performed. (A) FACS plots showing IFN-γ production after stimulation with βgal96 or βgal497 peptide on day 100 in spleen, liver, and lung. (B) IFN-γ production on days 21, 50, and 100 in spleen, liver, and lung. (C) FACS plots showing TNF-α production after stimulation with βgal96 or βgal497 on day 100 in spleen, liver, and lung. (D) TNF-α production on days 21, 50, and 100 in spleen, liver, and lung. Mean percentages of IFN-γ+ and TNF-α+ cells within the CD3+CD8+ T cell compartment are indicated (± SEM; spleen: day 21, n = 12, day 50, n = 12, day 100, n = 11, day 200, n = 6; liver and lung: day 21, n = 4, day 50, n = 9, day 100, n = 4; data from at least two independently performed experiments).
lymphocytes. (bars) within the IFN-γ response. B6 mice were im-
munized with 2 × 10⁹ PFU Ad-LacZ i.v. (A) FACS plots show TNF-α and
LAMP-1 expression on day 100 in spleen, liver, and lung after in vitro
stimulation with the βgal₉₆ peptide, gated on live IFN-γ+ CD3⁺CD8⁺ T
lymphocytes. (B) Shows IFN-γ single-positive CD8⁺ T cells (white bar),
IFN-γ/TNF-α+CD8⁺ T cells (light gray bars), IFN-γ/LAMP1+CD8⁺ T
cells (gray bars), and IFN-γ/TNF-α⁻LAMP-1⁻CD8⁺ T cells (dark gray
bars) within the IFN-γ+CD8⁺ T cell compartment after stimulation with
the βgal₉₆ peptide in spleen, liver, and lung. Mean percentages are indi-
cated (± SEM; spleen: day 21, n = 6; day 50, n = 6; day 100, n = 5; liver
and lung: day 21, n = 4; day 50, n = 6; day 100, n = 7; data from at least
two independently performed experiments). (C) βgal₉₆-specific CD8⁺ T
cells kill peptide-loaded target cells. Splenocytes from naive C57BL/6
mice were stained with CellTrace violet, loaded or not with the
βgal₉₆ peptide and transferred i.v. into naive B6 mice or B6 mice previously
immunized (days 21, 50, and 100) with Ad-LacZ. FACS analysis of the
surviving donor cells in blood, spleen, liver, and lung of recipient mice was
performed 12 h later. FACS plots show the transferred splenocytes (Cell-
Trace violet low = control group; CellTrace violet high = βgal₉₆-pulsed
specific CD8⁺ T cell memory inflation is
immunoproteasome independent

To address Ag presentation in this setting, we assessed whether βgal
epitopes are processed via immunoproteasomes or via constitutively
expressed proteasomes using LMP7 KO mice. These mice have a deletion of the gene encoding LMP7, a subunit of the
immunoproteasome encoded in a region of the MHC that is critical
for class I–restricted Ag presentation (36). LMP7 functions as an
integral part of the peptide supply machinery and, consequently,
LMP7 KO mice are not able to process Ag via immunoprotea-
somes. Immunizing LMP7 KO mice with Ad-LacZ induced a strong βgal₉₆-specific CD8⁺ T cell response on day 21 in blood,
which is comparable with the one seen in WT animals. The βgal₉₆-
specific CD8⁺ T cell populations expanded further with up to 30%
of tetramer⁺ cells on day 100, which was again equivalent to the
tetramer⁺ response seen in C57BL/6 mice (Fig. 7D, 7E).

In contrast, βgal₄⁹⁷-specific CD8⁺ T cells showed only 0.4%
tetramer⁺ cells on day 21 in blood compared with 7.7% in WT
mice. Not only was the expansion of these cells significantly re-
duced, but also the memory population, with only very low levels
of specific CD8⁺ T cells on day 50 and 100 (Fig. 7F, 7G).

These findings clearly demonstrate that processing of the βgal₄⁹⁷
epitope is immunoproteasome dependent, whereas that of βgal₉₆
and, therefore, memory inflation in this setting is not. These fea-
tures may contribute to the different outcome of the two βgal-
specific CD8⁺ T cell responses after Ad-LacZ immunization.

Discussion

The main characteristics of CD8⁺ T cell memory inflation, largely
known from studies with MCMV, are: 1) inflating CD8⁺ T cell
populations increase and are maintained at a very high frequency
over time; 2) they show an effector-memory phenotype, and they
are distributed in peripheral organs, such as liver or lung; and 3)
although they show features of repeated exposure to Ag, they are
not exhausted and, indeed, retain their functionality over time.

target cells) 12 h after transfer in blood and spleen. Target cells have been
killed efficiently on days 21, 50, and 100. (D) Percentage specific killing of
βgal₉₆ (black circles) or βgal₄⁹⁷ (white squares) target cells in blood,
spine, liver, and lung was measured 12 h after adoptive transfer by FACS
analysis in day 0, 21, 50, and 100 recipient mice. Mean percentage of
specific killing is indicated (± SEM; spleen, blood, liver, and lung: day 21,
n = 6; day 50, n = 6; day 100, n = 8; data from three independently per-
formed experiments).

The fact that βgal₉₆-specific CD8⁺ T cells developed in MHC-II
KO mice and were maintained up to day 100 suggests a CD8⁺
T cell independency for the CD8⁺ T cell maintenance. This was
further confirmed looking at their inflammatory potential in blood
with a day 100 to day 21 ratio > 1, as seen in the WT mice (Fig.
7C). Staining βgal₉₆-specific CD8⁺ T cells from blood, spleen,
liver, and lung for phenotypic markers (on day 100), βgal₉₆-
specific CD8⁺ memory T cells from MHC-II KO mice showed a TCM
phenotype (IL-7R⁺, CD62L⁺, KLRG-1⁻, CD27⁺) compared with
the effector memory T cell (TEM) phenotype seen in C57BL/6
mice. This TCM phenotype directly developed after day 21 (data
not shown). Overall, these results show that not only CD8⁺ T cell
priming and late expansion, but also CD8⁺ T cell phenotype in this
system is CD4⁺ T cell help dependent. Mechanisms independent
of CD4⁺ T cell help may provide limited signals for maintenance
of the residual memory pools.

βgal₉₆-specific CD8⁺ T cell memory inflation is
immunoproteasome independent

To address Ag presentation in this setting, we assessed whether βgal
epitope processing is via immunoproteasomes or via constitutively
expressed proteasomes using LMP7 KO mice. These mice have a deletion of the gene encoding LMP7, a subunit of the
immunoproteasome encoded in a region of the MHC that is critical
for class I–restricted Ag presentation (36). LMP7 functions as an
integral part of the peptide supply machinery and, consequently,
LMP7 KO mice are not able to process Ag via immunoprotea-
somes. Immunizing LMP7 KO mice with Ad-LacZ induced a strong βgal₉₆-specific CD8⁺ T cell response on day 21 in blood,
which is comparable with the one seen in WT animals. The βgal₉₆-
specific CD8⁺ T cell populations expanded further with up to 30%
of tetramer⁺ cells on day 100, which was again equivalent to the
tetramer⁺ response seen in C57BL/6 mice (Fig. 7D, 7E).

In contrast, βgal₄⁹⁷-specific CD8⁺ T cells showed only 0.4%
tetramer⁺ cells on day 21 in blood compared with 7.7% in WT
mice. Not only was the expansion of these cells significantly re-
duced, but also the memory population, with only very low levels
of specific CD8⁺ T cells on day 50 and 100 (Fig. 7F, 7G).

These findings clearly demonstrate that processing of the βgal₄⁹⁷
epitope is immunoproteasome dependent, whereas that of βgal₉₆
and, therefore, memory inflation in this setting is not. These fea-
tures may contribute to the different outcome of the two βgal-
specific CD8⁺ T cell responses after Ad-LacZ immunization.

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These features are highly relevant to the development of new vaccines eliciting sustained effective T cell memory responses for certain chronic viral infections. Although MCMV is a good model to study CMV infection, there are limitations in examining the mechanisms of memory inflation, because it is a complex system, both virologically with its long-term low-grade persistence, its latency and stochastic reactivation at diverse sites, as well as immunologically, with multiple immunologic mechanisms required to establish and maintain control, and a wide range of CD8+ T cell epitopes. Thus, to further understand the mechanism of CD8+ T cell memory inflation, which is still poorly understood, we developed a simpler and more tractable model using Ad-LacZ. In this study, we showed that Ad-LacZ, a nonreplicating AdV, induced a robust inflating CD8+ T cell population against the βgal96 epitope and a conventional CD8+ memory T cell response to the βgal497 epitope after a single i.v. injection in C57BL/6 mice. CD8+ T cell memory inflation after Ad-LacZ immunization revealed strong resemblance to that seen in MCMV infection, providing us with a novel, robust model for memory inflation that is internally controlled and allows dissection of the mechanism underpinning CD8+ T cell memory inflation. These data further support the idea that memory inflation is a stereotypical pathway of the immunologic memory rather than a unique feature of CMVs, and is consistent with observational data of other viral infections (17–20).

In this study, we demonstrated that βgal96-specific inflating CD8+ T cells were polyfunctional and cytotoxic. Although their effector-memory phenotype is consistent with repeated Ag encounter, they were not exhausted compared with CD8+ T cells in other chronic viral infections (particularly lymphocytic choriomeningitis virus in the mouse, but also HIV, HCV, and hepatitis B virus). We further showed that after i.v. immunization with Ad-LacZ, viral genome was still found at very late time points, and Ag was expressed and presented at very low levels (Fig. 6A–D). These findings imply that long-term maintenance of specific CD8+ T cells after rAdV immunization depends on the very low levels of transcriptionally active AdV genomes. In the context of vaccines, this suggests that persisting low-level Ag might be beneficial, resulting in the maintenance of functional TEM in the periphery, at sites of pathogen entry.

Using different routes of injection, we observed that memory inflation after Ad-LacZ immunization is not restricted to the i.v.
route, but is also seen after i.d. immunization. s.c. immunization induced a variant pattern of a βgal96-specific CD8+ T cell response, which was reduced in magnitude and did not increase, but was nevertheless sustained over time (Fig. 2, Supplemental Fig. 2). Importantly, although only i.v., i.d., and i.m. routes gave strictly defined inflation, in terms of increasing frequencies of βgal96-specific cells (day 100/21 ratio > 1), an identical effector-memory phenotype was observed in all cases, that is, after i.v., i.d., s.c., and i.m. immunization. This illustrates that although the magnitude and kinetics of the βgal96-specific CD8+ T cell memory response are dependent on the route of immunization, the phenotype is not (Fig. 2, Supplemental Fig. 2). Two factors are likely to influence these phenomena: first, Ag dose; and second, Ag distribution, the latter reflected in the APCs involved in priming and in memory maintenance. Previous work by other groups (26, 27) demonstrated that the route and dose of adenovirus-based vaccine delivery impacts on distribution of the virus, and thus expansion and trafficking of vaccine-elicited CD8+ T cells. In such experiments, differences in Ag dissemination would readily account for the reduced overall magnitude of the CD8+ T cell response with the non-i.v. compared with the i.v. routes. The distinctive phenotype of inflating populations is, however, shared between populations regardless of the route or magnitude; likewise, regardless of route, there are marked differences in phenotype between the inflating and noninflating epitopes, which emerge over time. The nature of the APCs involved in Ag presentation during the memory phase is discussed further later, but it therefore appears that this APC must be accessible to the inflating memory pool regardless of the route of immunization.

In this study, we also showed that the βgal96-specific CD8+ T cell response was significantly reduced in MHC-II–deficient mice. However, unlike the situation with different routes, βgal96-specific CD8+ T cells in MHC-II KO mice also displayed an altered phenotype compared with the standard i.v. inoculation in...
WT mice; overall, this could be summarized as skewed toward a T\textsubscript{CM} phenotype rather than the T\textsubscript{EM} phenotype typically seen (data not shown). Interestingly, although CD8\textsuperscript{T} cell expansion was significantly reduced, on day 100 after immunization, βgal\textsubscript{LacZ}-specific CD8\textsuperscript{T} T cells were nevertheless still present in blood, liver, lung, and spleen (Fig. 7A, 7B). Thus, CD4\textsuperscript{+} T cell help is clearly essential both for the initial priming of βgal\textsubscript{LacZ}-specific CD8\textsuperscript{T} T cells and the evolution of an effector-memory phenotype. However, for the maintenance of these cells in the memory phase, CD4\textsuperscript{+} T cell help might be dispensable. This theory is supported by findings exploiting other rAdVs (42, 46). CD4\textsuperscript{+} T cell help is likely required during memory inflation in MCMV infection, although the situation is more complex (16, 45). In a model where MCMV reactivation was controlled by injection of an antiviral drug, CD4\textsuperscript{+} T cell help appears necessary; if viral reactivation is not controlled, CD4\textsuperscript{+} T cell help can partially be compensated and less impact on memory inflation was observed (16). Overall, in a setting without viral replication, memory inflation is dependent on CD4\textsuperscript{+} T cell help; however, further experiments are required to determine at what stage such help is critical.

We demonstrated that CD8\textsuperscript{T} T cell memory inflation in this system was not dependent on viral reactivation and replication (Fig. 6). Similar findings were reported for MCMV infection (15, 16), although the levels of inflation seen in these studies were relatively limited. In our model, substantial memory inflation and conventional memory are both induced with a widely used non-replicating AdV. Prolonged CD8\textsuperscript{T} T cell responses for several weeks after adeno viral vector immunization has been described previously, followed by contraction (21, 25, 29–31, 46, 47), although robust CD8\textsuperscript{T} T cell memory inflation sustained over many months has not been observed. Previous studies have indicated that prolonged CD8\textsuperscript{T} T cell responses depended on low levels of Ag (30, 31). Interestingly, in such models, specific CD8\textsuperscript{T} T cells become Ag independent, as shown using a doxycycline-regulated AdV transgenic for SIINFEKL (30).

We further exploited the model to analyze the dependence of memory inflation after Ad-LacZ immunization on processing via immunoproteasomes. Immunization of LMP7 KO mice revealed a comparable βgal\textsubscript{LacZ}-specific CD8\textsuperscript{T} T cell response with that seen in WT mice. In contrast, the noninflating βgal\textsubscript{LacZ}-specific response was completely abolished in LMP7 KO mice (Fig. 7D–G). Interestingly, identical findings regarding immunoproteasome dependency were observed after MCMV infection (14). Clearly, in both settings, processing of inflating epitopes was not dependent on immunoproteasomes (Fig. 7D, 7E) (14). Because of the non-inflammatory environment in the Ad-LacZ model, as well as in MCMV latency, these combined data strongly suggest that processing of inflating epitopes depends on constitutively expressed proteasomes. Constitutively expressed proteasomes can be found in hematopoietic APCs, as well as in nonhematopoietic APCs. Recent data from studies with MCMV demonstrated that hematopoietic APCs primed MCMV-specific CD8\textsuperscript{T} T cells but were not sufficient for driving memory inflation (48), and that nonhematopoietic cells, likely in the LNs, were responsible for driving memory inflation in MCMV infection (41). Similar data were obtained with an rAdV (29). Thus, our data from LMP7 KO mice support the theory of APCs from a nonhematopoietic origin being responsible for prolonged Ag presentation resulting in memory inflation.

Which nonhematopoietic APCs might be responsible, and where, remains to be defined. It has been shown that the major reservoir of MCMV latent genomes are cells of nonhematopoietic origin such as sinusoidal lining cells of the spleen (49), liver sinusoidal endothelial cells (50), or CD11b\textsuperscript{+} CX3CR1\textsuperscript{+} nonhematopoietic cells in the lung (51). After i.v. Ad-LacZ immunization, most of the viral genome is found in spleen, liver, and lung. We directly tested the role of splenic Ag by repeating the experiments in splenectomized mice, and found no effect of splenectomy (Supplemental Fig. 4). Because memory inflation is not impaired in the absence of a spleen, this suggests the liver and/or the lung are the main sites of Ag presentation. Our quantitative real-time PCR results for LacZ DNA, as well as the proliferation behavior of adoptively transferred TCR transgenic CD8\textsuperscript{T} T cells, further support this idea. In this study, directly infected endothelial cells or stromal cells could be potential APCs because evidence suggests direct presentation during memory inflation (41). However, whether this is true after i.v. Ad-LacZ immunization still requires investigation. Regardless of the cell type, it remains unclear how Ag-expressing cells can persist in the presence of specific CD8\textsuperscript{T} T cell immunity, although potentially such pools may simply decline very slowly.

The observation that βgal\textsubscript{LacZ}-specific CD8\textsuperscript{T} T cells are not exhausted and located at a high frequency in peripheral organs renders them to a very desirable cell population for T cell-inducing vaccines. In this context, effector-memory T cells have been shown to respond rapidly to invasive and proliferating pathogens, and were highly protective in epithelial challenges (5). The very high frequencies of specific effector cells at the site of infection might compensate for the reduced proliferative and functional capacity of T\textsubscript{EM}, compared with T\textsubscript{CM} (52, 53). Specifically, for chronic hepatitis B virus and HCV infection and Mycobacterium tuberculosis, where a high number of functional CD8\textsuperscript{T} T cells in the liver or lung is required to eliminate the pathogen, such a vaccine strategy would be of major importance. Interestingly, recent studies using i.m. vaccination with recombinant adenoviral vector to induce T cell responses against HCV revealed a distinct phenotype with sustained development of effector-memory CD8\textsuperscript{T} T cell populations (CD45RA\textsuperscript{−}, CCR7\textsuperscript{−}) (21), showing some features of this phenomenon may be already embedded in current vaccine programs.

To further exploit this, however, we should first identify and then target the APC population(s) responsible for Ag processing and presentation during the memory phase. Dendritic cell–based strategies may, in theory, run the risk for selecting CD8\textsuperscript{T} T cell populations, which, after the first phase, contract to a low-level memory population of a T\textsubscript{CM} phenotype. Second, we should further dissect the molecular mechanisms underpinning the evolution of inflationary CD8\textsuperscript{T} T cell responses to promote this pathway of memory development. Third, we should optimize methods to identify and/or generate inflating epitopes from longer proteins. Clearly, memory inflation depends not only on the processing of the peptide, but also other features of the vector. But given the data on immunoproteasome independence, defining the rules governing the relevant cleavage of inflating versus noninflating peptides should be a priority for future vaccine design. Inflating/immunoproteasome-independent epitopes and noninflating/immunoproteasome-dependent epitopes could both be included to induce long-lived and functional CD8\textsuperscript{T} T cell effector and central memory populations.

Overall, we propose that although MCMV infection and Ad-LacZ immunization are two completely distinct settings, the mechanism for CD8\textsuperscript{T} T cell memory inflation is comparable in the two models. Together with findings from studies with other rAdV and from MCMV infection, we suggest that CD8\textsuperscript{T} T cell memory inflation is a distinct, evolutionarily conserved, stereotypical memory response, observed after different routes of exposure to diverse vectors. This response depends at least initially on CD4\textsuperscript{T} T cell help and may depend on presentation of Ag during the memory phase by unconventional (non-dendritic cell) APCs lacking immunoproteasomes.

rAdVs are well characterized and generally easy to manipulate. They transduce a variety of cells, but the vector genome does not
integrate, and their safety is well established (25). In contrast, CMV would need substantial further work on its use as a vector regarding the safety and efficiency profile. Therefore, the Ad-LacZ model not only facilitates further studies on CD8+ T cell memory inflation, but also enables direct implications, because of adenoviruses’ wide potential as vaccine vectors. This convergence of a window on an emerging and tractable area of immunobiology with a technology that forms the basis for many major vaccine programs means the model presented in this article should be of general value and the implications of the results obtained of broad relevance in future studies.

Acknowledgments

We thank Drs. Chris Norbury, Kevin Maloy, and Marcus Groothu for providing mice (as mentioned in Materials and Methods), Chris Willberg for technical support, and Peter Beverley for the critical discussion of the results.

Disclosures

The authors have no financial conflicts of interest.

References


A new model for CD8$^+$ T cell memory inflation based upon a recombinant adenoviral vector

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Supporting Information
Figure S1. βgal96-specific CD8⁺ T cells show an effector memory phenotype. (A) Peripheral blood from C57BL/6 (B6) mice immunized i.v. with 2x10⁹ pfu Ad-LacZ was collected at different time points and stained with βgal-specific tetramers and antibodies specific for CD8 and the indicated cell surface molecules. βgal96⁺ CD8⁺ T cells (black squares), βgal497⁺ CD8⁺ T cells (white circles), and total CD8⁺ T cells (grey triangles) are displayed. (B) Peripheral blood from d100 B6 mice was stained with the βgal96 tetramer and antibodies specific for CD8, CD122, NKG2A and NKG2D. The plots shown are gated on tetramer⁺ CD8⁺ T cells (black) or tetramer⁻ CD8⁺ T cells (grey) from the same sample. (C, D, E, F) Staining for CD122, NKG2A and NKG2D on different time points in blood (C), spleen (D), liver (E) and lung (F). Mean percentage or MFI respectively of surface molecule positive cells within the tetramer-positive CD8⁺ T cell compartment is indicated (±SEM; blood d21 n=6-13, d50 n=6-12, d100 n=5-7, d200 n=3-6; spleen d21 n=12-16, d50 n=6-11, d100 n=8, d200 n=6; liver and lung d21 n=6-9, d50 n=5-7, d100 n=6, d200 n=3-6; range
indicates the different markers assessed; Each marker was at least measured in two independently performed experiments).
Figure S2. The effector-memory phenotype of βgal96-specific CD8+ T cells is not restricted to the route of immunization. (A) B6 mice were immunized i.v. (black circles), i.m. (black squares, dashed-dotted line) and s.c. (black triangles, dashed line) with 2x10^9 pfu Ad-LacZ and expansion of βgal96- and βgal497-tetramer+ CD8+ T cells was measured. Significantly reduced expansion of βgal96-specific CD8+ T cells after i.m. (d21*; d50**; d100**) and s.c. (d21**; d50***; d100***) injection, and no memory inflation after s.c. immunization. Mean percentage of tetramer-positive cells within the CD8 compartment is indicated (±SEM; s.c. (data from two independently performed experiments) d21 n=7, d50 n=7, d100 n=7; i.m. (data from a single experiment) d21=3, d50=3, d100=3; ). (B) The inflationary potential expressed by the ratio of percentage of tetramer-positive CD8+ T cells from day 100 to day 21 in B6 mice after i.v., i.m. and s.c. immunization for both βgal epitopes is shown. (C) Expression of CD44, CD62L, CD127, KLRG-1 and CD27 in βgal96-positive CD8+ T cells in blood at different time points after i.v. (black circles), s.c. (black
squares, dashed line), i.d. (black triangles, dotted line) and i.m. (black triangles, dotted-dashed line) immunization. Mean percentage or MFI respectively of surface molecule positive cells within the tetramer-positive CD8$^+$ T cell compartment is indicated (±SEM; d21 n=3, d50 n=3).
Figure S3. Long-term low-level antigen persistence after i.v. immunization with Ad-LacZ.

(A) CFSE-labeled, βgal96-specific, Ly5.1+ TCR-transgenic CD8+ T cells from Bg1 mice transferred on day 50 and day 100 after Ad-LacZ immunization proliferated in spleen, liver, hepatic LNs and lung 3 days after transfer. The numbers indicate the percentage of proliferated Ly5.1+ CD8+ T cells that are donor derived (±SEM, spleen n=4, liver n=4, hLN n=4 lung n=4; two independently performed experiments for day 50 and day 100 immune mice). (B) CFSE-labeled, βgal96-specific, Ly5.1+ TCR-transgenic CD8+ T cells transferred on day 100 after MCMV-LacZ infection proliferated in spleen 3 days after transfer. The numbers indicate the percentage of proliferated Ly5.1+ CD8+ T cells that are donor derived (±SEM, spleen n=3). (C) CFSE-labeled, βgal96-specific, Ly5.1+ TCR-transgenic CD8+ T cells transferred on day 21 after Vacc-LacZ infection did not proliferate (in spleen 3 days after transfer). The numbers indicate the percentage of proliferated Ly5.1+ CD8+ T cells that are donor derived (±SEM, spleen n=3).
Figure S4. CD8\(^+\) T cell memory inflation is spleen-independent. B6 mice (black circles) and splenectomized B6 mice (black squares, dashed line) were immunized intravenously with 2x10\(^9\) pfu Ad-LacZ and expansion of \(\beta\text{gal}_{66}\)-tetramer\(^+\) CD8\(^+\) T cells was measured with flowcytometry. Mean percentages of tetramer-positive cells within the CD8 compartment are indicated (±SEM; d8 n=6, d14 n=6, d21 n=3, d50 n=3, d100 n=3, d440 n=3).