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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’Hara,* 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.

The induction of potent CD8+ T cell responses is an important goal for vaccine strategies against major pathogens and tumors, and defining the induction and maintenance of CD8+ T cell populations has been the focus of many studies. Many vaccines and natural infections provoke a strong effector-memory response in the early phase where the Ag is present, but once the nonpersistent vector or pathogen is eliminated, CD8+ T cell memory contracts to a “central” memory pool, concentrated in secondary lymphoid organs (1). Much attention has been paid to the situation where Ag is not eliminated and persists at a high level, such as in chronic lymphocytic choriomeningitis virus infection (2, 3). Here CD8+ T cell function is lost over time such that memory is functionally impaired or even lost altogether, a phenomenon known as CD8+ T cell exhaustion (3). However, exhaustion is not the only outcome of repetitive Ag stimulation. Studies of low-level persistent viruses such as CMV have revealed a “mirror image” response to that seen with exhaustion, where T cell responses may be enhanced numerically over time and maintain strong functionality; this has been termed CD8+ T cell memory “inflation” (4). Understanding this phenomenon is relevant not only to disease pathogenesis and the biology of immunologic memory, but it also plays a role in vaccine design, where such populations can be harnessed to provide protection against certain chronic viral infections, such as hepatitis C virus (HCV), HIV, and CMV (5).

CD8+ T cell memory inflation was first observed in murine CMV (MCMV) infection (4, 6), and similar findings are observed in human CMV (HCMV) infection. In CD8+ T cell memory inflation, responses to a single epitope may become very large and are maintained at high levels throughout life (4, 7, 8). CMV-specific inflating CD8+ T cells typically show an extreme of the “effector-memory” phenotype (CD27lo, CD28–, CD69–, CD127–, and IL-2Rα+ (9). Cells remain functional and respond vigorously to viral rechallenge, providing protection (4). They are located in the spleen and the periphery, particularly in organs such as liver and lung. It is unclear yet what drives the selection of these “inflationary” epitopes, but it has been shown that it is independent of initial immunodominance (10) and viral gene-expression patterns (11). In MCMV, for example, only one of two epitopes from the same protein is associated with an “inflationary” response (12, 13). This suggests factors other than the kinetics of the viral gene expression could be involved; in particular, recent data reveal immunoproteasome independence is associated with inflation and suggest a significant role for Ag processing in epitope selection during memory development (14).

However, in the MCMV model, many questions remain unanswered. The location and the nature of the cells that process and present Ag, and eventually sustain CD8+ T cell responses are still elusive. Likewise, it is not known for how long Ag needs to be presented to produce such a sustained CD8+ T cell response. It appears that repetitive Ag exposure is an essential factor driving memory inflation, as suggested by analysis of phenotype and activation status (4, 10), and adoptive transfer into naïve hosts (9).
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 \(\beta\)-galactosidase (\(\beta\)gal)–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, \(\beta\)gal\(_{497}\)–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 \(\beta\)gal\(_{119}\) 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 302235 and 302744), 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 Cantonal and federal legislations, and were approved by the Veterinary Officer of the Kanton of St. Gallen.

Adenoviral vector

Recombinant adenovirus expressing the \(\beta\)gal protein under the control of the HCMV promoter (Ad-LacZ) and lacking E1 and E3 genes was used (28). Ad-LacZ was propagated on permissive HER-911 cells and was purified with the Vivapure AdenoPack 20 (Sartorius; Stedim Biotech, Aubagne, Cedex, France) according to the manufacturer’s specifications. Virus titer was determined with the Vivapure AdenoPack 20 (Sartorius; Stedim Biotech, Aubagne, Cedex, France) according to the manufacturer’s specifications. Virus titer was determined in a cytopathic effect assay. In brief, serial dilutions of the adenovirus were used to infect HER-911 cells on a 96-well microtiter plate, and cytopathic effect was determined after 5 d by microscopy. Tissue culture medium (RPMI) containing 5\% FCS in 96-well, round-bottom plates. The cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) as a positive control or left untreated as a negative control. Peptide-specific responses were analyzed after cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) as a positive control or left untreated as a negative control. Protein expression was monitored by staining of blood cells with anti-\(\beta\)2m 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 \(\beta\)gal\(_{903}\) (DAPYTNV) (37), the \(\beta\)gal\(_{907–904}\) (ICPYMARY) (38), and the MHC class I restriction element for the H2-K\(^b\)-restricted \(\beta\)gal\(_{96–103}\) peptide (both from Mimotopes (Melbourne, VIC, Australia).

Flow cytometry

For flow cytometry, single-cell suspensions were generated from the indicated organs, and 1 \(\times\) 10\(^6\) cells were incubated with the indicated mAb at 4\(^\circ\)C for 20 min. For BPL 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 (AppliChem) and 170 U/ml collagenase II (Life Technologies) at 37\(^\circ\)C for 45 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 \(\times\) 10\(^6\) PFU Adeno-LacZ. Single-cell suspensions of 2 \(\times\) 10\(^8\) lymphocytes were incubated for 2 h at 37\(^\circ\)C in 150 \(\mu\)l culture medium (RPMI) containing 5\% FCS in 96-well, round-bottom plates. The cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) as a positive control or left untreated as a negative control. Protein expression was monitored by staining of blood cells with anti-\(\beta\)2m 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.

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Construction of tetrameric MHC-I–peptide complexes

MHC-I monomers complexed with β2m (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 10^6 M CFSE and half with 10^6 M CellTrace violet (Invitrogen). At the indicated time points postinfection, organs were harvested and single-cell suspensions were prepared. 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 sequences were used as primers for real-time quantitative PCR: 5′-GGTGATGAGAACGACCAG-3′ and 5′-CGAGACGCCTGTAAC-3′. The following oligonucleotides were used as probes: 5′-CAGTCTGGCGGTTTCGCAG-3′ and 5′-TACTGCGGATGTTCGTCAG-3′. Probe 1 carried a 5′FAM reporter and probe 2 was Cy5 labeled at the 5′ end.

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 (βgal497 and βgal96). On day 300, βgal497-specific CD8+ T cells had contracted and were detectable at low levels only, whereas the βgal96-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 (LN) were isolated at different time points, and βgal-specific CD8+ T cells were quantified by staining with MHC-I tetramers. βgal497-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 βgal497-specific CD8+ T cell population was maintained over time (Fig. 1D).

In contrast with βgal497-specific CD8+ T cells, tetramer staining for βgal96-specific CD8+ T cells revealed only low levels of tetramer+ CD8+ T cells on day 100 in spleen, liver, and lung (Fig. 2C). 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 βgal96-specific CD8+ T cells in spleen and liver was >25 times higher than the amount of βgal497-specific CD8+ T cells, whereas in the lung, it was 50 times more, the latter two indicating a major redistribution of βgal96-specific CD8+ T cells to peripheral organs.

Only low frequencies of βgal497 and βgal97-specific CD8+ T cells were found in LN (inguinal LN; βgal497-specific CD8+ T cells: day 21 = 0.6 ± 0.1%, day 50 = 0.4 ± 0.1%, day 100 = 0.4 ± 0.1%; βgal96-specific CD8+ T cells: day 21 = 0.3 ± 0.1%, day 50 = 0.09 ± 0.03%, day 100 = 0.02 ± 0.01%), whereas βgal96-specific CD8+ T cells did not inflate, as observed in MCMV infection (10, 41).
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). This ratio is used to distinguish responses where...
Inflationary potential expressed by the ratio of percentage of tetramer+100, LacZ, and expansion of time points. Mean percentage of tetramer+ cells within the CD8+ compartment is indicated (n = 7, day 28: experiments). (b) Inflationary CD8+ T cells was measured with flow cytometry. Mean percentage of tetramer+ cells (no background subtracted) within the CD8+ compartment is indicated (n = 7; data from two independently performed experiments). (B) Inflationary potential expressed by the ratio of percentage of tetramer+ CD8+ T cells from days 100 to 21 in C57BL/6 mice after i.v. and i.d. immunization for both βgal epitopes. (C) B6 mice were i.v. infected with 2 x 10⁶ PFU MCMV-LacZ, and expansion of βgal497-tetramer+ CD8+ T cells was measured with flow cytometry. Mean percentage of tetramer+ cells within the CD8+ compartment is indicated (SEM; day 7: n = 7–9; day 14: n = 7–10, day 28: n = 7, day 50: n = 7–10, day 100: n = 6; data from two independently performed experiments). (D) B6 mice were infected i.p. with 2 x 10⁶ PFU Vacc-LacZ, and expansion of βgal497 (black square) and βgal96 (white circle)-tetramer+ CD8+ T cells was measured with flow cytometry. Mean percentage of tetramer+ cells within the CD8+ compartment is indicated (SEM; day 7: n = 7; day 14: n = 7, day 28: n = 7, day 50: n = 7; data from two independently performed experiments). (E) Representative FACS plots for βgal-tetramer specific staining in naive (black) B6 mice, and MCMV-LacZ and Vacc-LacZ infected B6 mice on day 7 postinfection in blood. Mean percentages of tetramer+ cells (no background subtracted) within the CD8+ compartment are indicated. *p < 0.05, **p < 0.01, ***p < 0.001.

there is a strong contraction detectable in blood after the day 21 peak from those where there is maintenance and/or expansion over time. In this study, compared with βgal497-specific CD8+ T cells, which do not inflate, βgal96-specific CD8+ T cells, after both i.v. and i.d. immunization, show a ratio >1, indicating that these cells are maintained over time and that CD8+ T cell memory inflation after Ad-LacZ is not restricted to the i.v. route.

We further assessed whether βgal-specific CD8+ T cell memory inflation is confined to Ad-LacZ. Therefore, we infected C57BL/6 mice with a βgal-recombinant MCMV (MCMV-LacZ) or Vacc-LacZ. MCMV-LacZ, and Vacc-LacZ infection induced a very small but significant βgal97-specific CD8+ T cell response in blood, compared with nonimmunized (naive) mice (MCMV-LacZ d7 compared with background in naive mice: p = 0.0238; Vacc-LacZ d7 compared with background in naive mice: p = 0.0392). The βgal96-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). Taken together, these data revealed that if the transgene was expressed in a different vector, although a βgal97-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.

Progressive expansion of effector-memory CD8+ T cells

To further characterize βgal96-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 contained 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 costimulator 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 βgal96-specific inflationary CD8+ T cells, βgal97-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 βgal96-specific inflationary CD8+ T cells barely changed their phenotype over time. They display a comparable effector-memory phenotype (CD62LloCD44hiIL-7Rα, KLRG1hiNKG2Ahi) from day 21, which is maintained up to day 200 after immunization. In contrast with these data, βgal97-specific CD8+ T cells acquired a divergent phenotype after contraction. This had features of a central memory pool (CD62LhiCD44loIL-7Rα, KLRG1loNKG2Alo), especially in the spleen and blood. They were also low in expression on NKG2D and NKG2A (Fig. 3B; Supplemental Fig. 1C–F).

Importantly, βgal96-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 βgal96-specific CD8+ T cells do not inflate numerically after s.c. injection of Ad-LacZ, although a distinction between the kinetics of the βgal96-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 βgal96-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)
LNs. In LNs, the fraction of lymphocytes from blood, spleen, liver, and lung, but not from LNs, the fraction of βgal96-specific cells that displayed a T_{CM} phenotype was much higher, with >30% (data not shown). This impact through the anatomic site was also reported after MCMV infection (10, 41).

βgal96-specific CD8^+ T cells retain functionality and show 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 inflationary 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 βgal96-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 βgal96- and βgal497-specific CD8^+ T cells, calculating the ratio of IFN-γ^+ or TNF-α^+-producing cells to tetramer^+ cells, although both populations are clearly functional, βgal97-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 βgal497-specific CD8^+ T cells after stimulation with βgal497-peptide. We suggest that the βgal97-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. βgal96-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, βgal497-peptide–pulsed target cells were killed in a much lower degree, especially if transferred into day 100 mice.

Overall, although βgal96-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 3. βgal96-specific CD8^+ T cells display an effector-memory phenotype. (A) Peripheral blood from day 100 B6 mice was stained with the βgal96 tetramer and Abs specific for CD8 and the indicated cell surface molecules. The plots shown are gated on tetramer^+ CD8^+ T cells (black) or tetramer^+ CD8^+ T cells (gray) from the same sample. (B) Expression of CD44, CD62L, CD127, KLRG-1, and CD27 in βgal96^+ (black squares), βgal96^+ (white circles), and total CD8^+ T cells (gray triangles) in spleen, liver, and lung on different time points after immunization. Mean percentage or mean fluorescence intensity, respectively, of surface molecule-positive cells within the tetramer^+ or the CD8^+ T cell compartment is indicated (± SEM; blood: day 21, n = 6–13, day 50, n = 6–12, day 100, n = 5–7, day 200, n = 3–6; spleen: day 21, n = 9–16, day 50, n = 6–13, day 100, n = 3–8, day 200, n = 5–6; liver and lung: day 21, n = 6–10, day 50, n = 6–9, day 100, n = 5–8, day 200, n = 3–6; range indicates the different markers assessed; data from at least two independently performed experiments).](http://www.jimmunol.org/)

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LacZ mRNA was performed. LacZ mRNA could only be quantified in liver and draining LNs (hLNs) around day 2 after immunization; thereafter, only low levels of specific mRNA were detected, but below the level of quantification (Fig. 6B).

Although Ag was not detectable on the RNA level, the effector phenotype of inflationary memory T cells strongly suggests continuous transgene exposure, most likely through low-level Ag expression. To test this, we adoptively transferred CFSE-labeled βgal96-specific TCR transgenic CD8^+ T cells from Bg1 mice into naïve C57BL/6 mice and day 20, 50, and 100 immune recipients. Three days later, proliferation and activation of transferred cells was measured in spleen, liver, hLNs, and lung by staining for CD44 and quantifying CFSE dilution by FACS analysis. Although Ag expression could not be readily detected at day 20 postimmunization at the transcriptional level, TCR transgenic CD8^+ T cells vigorously proliferated when transferred into day 20 mice (Fig. 6C, 6D). Even in day 50 and 100 immune mice, adoptively transferred cells became activated and proliferated, although to a lesser extent (Supplemental Fig. 3A). The percentage of proliferated TCR transgenic cells was comparable in day 4, 8, and 14 immune mice. In day 20 immune mice, proliferation was slightly reduced compared with the previous days, and it was further reduced in day 50 and 100 immune mice (Fig. 6D).

Taken together, these results demonstrate that CD8^+ T cell memory inflation in this setting is not dependent on viral reactivation and replication; similar findings have been reported for MCMV infection (15, 16).

To assess whether the absence of a βgal96-specific inflating response after MCMV-LacZ or Vacc-LacZ infection was due to the lack of Ag presentation, we likewise adoptively transferred CFSE-labeled βgal96-specific TCR transgenic CD8^+ T cells from Bg1 mice into day 100 MCMV-LacZ immune and day 21 Vacc-LacZ immune recipients, respectively. In MCMV-LacZ immune recipients, adoptively transferred cells became activated and proliferated compared with naive recipients (Supplemental Fig. 3B). In Vacc-LacZ immune recipients, in contrast, adoptively transferred cells did not proliferate compared with naive recipients (Supplemental Fig. 3C). These results, together with data from Fig. 2C and 2D, suggest that Vacc-LacZ is efficiently cleared on day 21 postinfection; hence, no memory inflation is observed. However, after MCMV-LacZ infection, the βgal96 epitope was still presented on day 100 postinfection, suggesting factors other than pure Ag availability are responsible for the lack of CD8^+ T cell memory inflation after MCMV-LacZ infection.

CD4^+ T cell help facilitates inflation of memory effector T cells

To further explore the critical requirements for CD8^+ T cell memory inflation using this model, we investigated the role of CD4^+ T cell help. CD4^+ T cells are essential for CD8^+ T cell memory induction across a range of immunizations (42) and acute infections (43, 44), although their role in memory inflation after MCMV infection is not fully defined (16, 45).

To establish the role of CD4^+ T cells in memory inflation after i.v. Ad-LacZ immunization, we assessed the βgal96-specific CD8^+ T cell response on different time points in blood of MHC-II–deficient and wild-type (WT) C57BL/6 mice. In this setting, the generation of tetramer^+ CD8^+ T cells was clearly dependent on CD4^+ T cell help. Only low levels of βgal96-specific CD8^+ T cells could be detected on days 21, 50, and 100 after immunization, and the percentage in MHC-II KO mice was significantly reduced at all time points compared with C57BL/6 mice (Fig. 7A). Staining for βgal96-specific CD8^+ T cells in spleen, liver, and lung on day 100 revealed similar results, with the tetramer^+ population sig-
lymphocytes. (bars) within the IFN-γ+CD8+ T cell compartment after stimulation with gal96 peptide, gated on live IFN-γ+CD8+ T cells (gray bars), and IFN-γ+LAMP1+CD8+ T cells (dark gray bars) within the IFN-γ+CD8+ T cell compartment after stimulation with the gal96 peptide in spleen, liver, and lung. Mean percentages are indicated (± 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 (CellTrace violet low = control group; CellTrace violet high = βgal96-pulsed 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, spleen, 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 performed experiments).

βgal96-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 immunoproteasomes. 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 reduced, 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 features 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.
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-elicted 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 or magnitude; likewise, regardless of route or magnitude, the inflationary potential expressed by the ratio of percentage of tetramer⁺ CD8⁺ T cells was performed on days 21, 50, and 100 after immunization. (A) βgal96-specific CD8⁺ T cells in blood of B6 (black circles) and MHC-II KO (black squares, dashed line) mice. (B) Tetramer analysis of CD8⁺ T lymphocytes from spleen, liver, and lung of B6 (black bars) and MHC-II KO (gray bars) mice on day 100 after immunization. Mean percentage of tetramer⁺ cells within the CD8⁺ T cell compartment is indicated (± SEM; blood B6: day 21, n = 3; day 50, n = 3; day 100, n = 3; spleen B6: day 21, n = 4; day 50, n = 4; day 100, n = 4; spleen, liver, and lung B6: day 100, n = 3; spleen, liver, and lung MHC-II KO: day 100, n = 4; data from a single experiment. This experiment was performed in the animal facility in St. Gallen). (C) Expansion of βgal96-specific CD8⁺ T lymphocytes on day 21 in blood of C57BL/6 (left) and LMP7 KO (right) mice, gated on live lymphocytes. (D) βgal96-specific CD8⁺ T cells in blood of B6 (black circles) and LMP7 KO (black squares, dotted line). (E) Tetramer staining for βgal96-specific CD8⁺ T cells in blood of C57BL/6 (left) and LMP7 KO (right) mice, gated on live lymphocytes. (G) Expansion of βgal97-specific CD8⁺ T cells in blood of B6 (solid/continuous line) and LMP7 KO (dotted line). Mean percentage of tetramer⁺ cells within the CD8⁺ T cell compartment is indicated. Pooled data from two independent experiments for each time point are shown (± SEM; blood B6: day 21, n = 10; day 50, n = 10; day 100, n = 13; blood LMP7 KO: day 21, n = 10; day 50, n = 9; day 100, n = 5). *p < 0.05, **p < 0.01, ***p < 0.001.
WT mice; overall, this could be summarized as skewed toward a T_CTM phenotype rather than the T_ECM phenotype typically seen (data not shown). Interestingly, although CD8^+ T cell expansion was significantly reduced, on day 100 after immunization, βgal_og-specific CD8^+ T cells were nevertheless still present in blood, liver, lung, and spleen (Fig. 7A, 7B). Thus, CD4^+ T cell help is clearly essential both for the initial priming of βgal_og-specific CD8^+ T cells and the evolution of an effector-memory phenotype. However, for the maintenance of these cells in the memory phase, CD4^+ T cell help might be dispensable. This theory is supported by findings exploiting other rAdVs (42, 46). CD4^+ 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^+ T cell help appears necessary; if viral reactivation is not controlled, CD4^+ 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^+ T cell help; however, further experiments are required to determine at what stage such help is critical.

We demonstrated that CD8^+ 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^+ T cell responses for several weeks after adenoaviral vector immunization has been described previously, followed by contraction (21, 25, 29–31, 46, 47), although robust CD8^+ T cell memory inflation sustained over many months has not been observed. Previous studies have indicated that prolonged CD8^+ T cell responses depended on low levels of Ag (30, 31). Interestingly, in such models, specific CD8^+ 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_og-specific CD8^+ T cell response with that seen in WT mice. In contrast, the noninflating βgal_og-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 nonhematopoietic APCs. Recent data from studies with MCMV demonstrated that hematopoietic APCs primed MCMV-specific CD8^+ 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 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^+ CX3CR1^+ 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^+ 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^+ T cell immunity, although potentially such pools may simply decline very slowly.

The observation that βgal_og-specific CD8^+ 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_ECM compared with T_CTM (52, 53). Specifically, for chronic hepatitis B virus and HCV infection and Mycobacterium tuberculosis, where a high number of functional CD8^+ 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 adenoaviral vector to induce T cell responses against HCV revealed a distinct phenotype with sustained development of effector-memory CD8^+ T cell populations (CD45RA^−/CD62L^−, CCR7^−) (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^+ T cell populations, which, after the first phase, contract to a low-level memory population of a T_CTM phenotype. Second, we should further dissect the molecular mechanisms underpinning the evolution of inflationary CD8^+ 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^+ 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^+ 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^+ 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^+ 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 need broad relevance in future studies.

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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 $2 \times 10^9$ pfu Ad-LacZ and expansion of βgal$^{96}$-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).