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

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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 (bgal)–recombinant adenovirus vector. After i.v. administration in C57BL/6 mice, we observed marked memory inflation in the βgal96 epitope, whereas a second epitope, βgal475, 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 insights 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 when 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 (CD27⁻, CD28⁻, CD62L⁻, CD127⁻, and IL-2⁺⁺⁺) (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 naive 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 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, βgal497–504 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 βgal497 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 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 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, France) according to the manufacturer’s specifications. Virus titer was determined in a cytopathic effect assay. In brief, serial dilutions of adenovirus were used to infect HER-911 cells on a 96-well microtitrate plate, and cytopathic effect was determined after 5 d by microscopy. Tissue culture infectious dose of 50% was calculated by the Reed–Muench method. Ad-LacZ aliquots were stored at −80˚C in PBS and injected either i.v. or intradermally (i.d.; earlobe) at a dose of 2 × 10^8 PFU.

Viruses

Recombinant MCMV expressing the βgal protein under the transcriptional control of the HCMV ie1/ie2 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 × 10^8 PFU.

Recombinant Vaccinia expressing the βgal protein (Vacc-LacZ) (33) was used. UV-LacZ was propagated and titrated on BSC-40 cells, and injected i.p. at a dose of 2 × 10^8 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 H2-Kb–restricted βgal497–504 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/6-SJL 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 βgal497–504 (DAPYTNV) (37), the βgal497–504 (ICPMYARV) (38), and the M45 (HGRNASFI) (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 (AppliChem) and 170 U/ml collagenase II (Life Technologies) at 37˚C for 45 min. Cell aggregates were dispersed by passing the digest through a cell strainer (BD).

Intracellular cytokine staining

Splenic, liver, and lungs samples were removed at indicated time points after immunization with 2 × 10^7 PFU Adeno-LacZ. Single-cell suspensions of 2 × 10^5 lymphocytes were incubated for 2 h at 37˚C in 150 µl culture medium (RPMI) containing 5% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin, and stained with mAb against MHC class II (rat anti–mouse MHC II, clone RM427, BD Biosciences) and with the viability dye eFluor 450–labeled anti–CD44, anti–CD62L, anti–NKG2D, and anti–NKG2A, B–PE. Lysing Solution (BD Pharmingen) was used to permeabilize the cells with Fixation/Permeabilization Solution (BD). Staining of samples was performed with an intracellular cytokine staining (ICP) kit (BD) according to the manufacturer’s instructions. In brief, cells were fixed for 1 h at 4˚C with 1% paraformaldehyde and washed in PBS. Cells were then permeabilized for 1 h at 4˚C with 0.5% saponin and washed again in PBS. Intracellular cytokine staining was performed with anti–IFN–γ, anti–TNF–α, anti–IL-4, anti–IL-17, and anti–IL-6 mAbs. Staining reactions were performed at 4˚C for 20 min. Cells were washed and stained with anti–CD8–PerCp-Cy5.5, anti–CD8–eFluor 450, anti–CD3–Alexa700, and anti–NKG2A,B–PE. Cells were analyzed by flow cytometry.
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 tetramer and the other half were stained with CellTrace violet (Invitrogen). A maximum concentration of 5 × 10^6 cells/ml was incubated 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 spleenocytes/ml in each group. Recipient B6 mice were injected i.v. with 10 × 10^6 spleenocytes 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 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 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 mRNA concentration in the unknown samples was calculated using the data from the standard curve. Each sample was measured as a triplicate and the average concentration was used. Final copy numbers were calculated per microgram total RNA.

Adaptive transfer of TCR transgenic T cells

Single-cell suspensions from spleens of Ly5.1^B6 mice were subjected to hypotonic RBC lysis and stained with CFSE (Invitrogen). A maximum concentration of 2.5 × 10^7 cells/ml was incubated 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 × 10^7 spleenocytes/ml. Recipient B6 mice were injected i.v. with 2 × 10^7 B6-Ly5.1^B6 spleenocytes 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 (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 (LNs) were isolated at different time points, and βgal-specific CD8^+ T cells were quantified by staining with MHC-I tetramers. βgal96-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 βgal96-specific CD8^+ T cell population was maintained over time (Fig. 1D).

In contrast with βgal497-specific CD8^+ T cells, tetramer staining for βgal497-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 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 βgal96 and βgal497-specific CD8^+ T cells were found in LNs (inguinal LN; βgal96-specific CD8^+ T cells: day 21 = 0.6 ± 0.1%, day 50 = 0.4 ± 0.1%, day 100 = 0.4 ± 0.1%; βgal497-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).

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). This ratio is used to distinguish responses where...
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 inflammatory 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 βgal96-specific inflammatory 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 inflammatory CD8⁺ T cells barely changed their phenotype over time. They display a comparable effector-memory phenotype (CD62Llo, CD27lo, IL-7Rhi, KLRG1lo, NKG2A/Dhlo) from day 21, which is maintained up to day 200 after immunization.

In contrast to these data, βgal97-specific CD8⁺ T cells acquired a divergent phenotype after contraction. This had features of a central memory pool (CD62Lhi, CD27hi, IL-7Rlo, KLRG1hi, NKG2A/Dhi) 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 βgal90-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 βgal97-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 (βgal97-specific CD8⁺ T cell) population with a central memory T cell (TCM) and βgal-specific CD8⁺ T cell memory inflation is exclusive to the AdV.
phenotype and the inflationary (βgal97-specific CD8+ T cell) population, with progression toward an effector-memory phenotype, identical to that seen in MCMV infection (4, 9, 10, 16, 41) and independent of the route of injection. Interestingly, this is true for lymphocytes from blood, spleen, liver, and lung, but not from LNs. In LNs, the fraction of βgal96-specific cells that displayed a TCM phenotype was much higher, with >30% (data not shown). This impact through the anatomic site was also reported after MCMV infection (10, 41).

βgal97-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 βgal97-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 βgal97-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 βgal97-specific CD8+ T cells after stimulation with βgal97-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. βgal97-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, βgal97-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 4. Inflating CD8+ T cells are strong IFN-γ and TNF-α producers. C57BL/6 mice were immunized with $2 \times 10^9$ 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, gated on live CD3+CD8+ T lymphocytes. (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 peptide on day 100 in spleen, liver, and lung, gated on live CD3+CD8+ T lymphocytes. (D) TNF-α production on days 21, 50, and 100 in spleen, liver, and lung. Mean percentages of IFN-γ+ respectively 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).

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 inflammatory 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 naive 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 post-immunization 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−/− 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 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 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).

FIGURE 5. Inflating CD8+ T cells show progressive differentiation toward polyfunctionality and maintain cytotoxicity. B6 mice were immunized with 2 × 10^9 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+ 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 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 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).

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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.

FIGURE 6. Low-level Ag persistence in Ad-LacZ–immunized B6 mice. (A) Viral genome distribution after immunization with Ad-LacZ. LacZ DNA copy numbers per microgram total DNA were determined with quantitative real-time PCR on different time points after immunization in liver, lung, spleen, and hLNs. Values <10 copies are detectable but not quantifiable. Pooled data from two independent experiments for each time point are shown (mean ± SEM; liver: n = 3–12; spleen: n = 3–12; lung: n = 3–8; hLN: n = 3–6). (B) βgal expression after immunization with Ad-LacZ. LacZ mRNA copy numbers per microgram total mRNA were determined with quantitative real-time PCR at different time points after immunization in liver, lung, spleen, and hLNs. mRNA copy numbers <10 are detectable but not quantifiable. Pooled data from two independent experiments for each time point are shown (mean ± SEM; liver: n = 3–7; spleen: n = 3–7; lung: n = 3–6; hLN: n = 3–4). (C) Low-level Ag persistence after i.v. immunization of B6 mice with Ad-LacZ. CFSE-labeled, βgal96-specific, Ly5.1+ TCR-transgenic CD8+ T cells from Bg1 mice transferred on day 20 after Ad-LacZ immunization proliferated in spleen, hLNs, and lung 3 d after transfer. Numbers indicate the percentage of proliferated Ly5.1+ CD8+ T cells that are donor derived. Pooled data from two independent experiments for each time point are shown (± SEM; spleen: n = 4; liver: n = 4; hLN: n = 4; lung: n = 4). (D) Percentage of proliferated Ly5.1+ TCR-transgenic CD8+ T cells in spleen analyzed 3 d after adoptive transfer in mice previously immunized with Ad-LacZ on different time points (days 4, 8, 14, 20, 50, and 100). Mean percentage (± SEM; n = 3–5) is indicated.
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., 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 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

**FIGURE 7.** βgal96-specific CD8+ T cell memory inflation critically depends on CD4+ T cell help but is immunoproteasome independent. (A and B) No priming and expansion of βgal96-specific CD8+ T cells without CD4+ T cell help. B6 and MHC-II KO mice were immunized i.v. with 2 × 10^9 PFU Ad-LacZ. Tetramer analysis for βgal96-specific CD8+ T cells was performed on days 21, 50, and 100 after immunization. (A) Expansion of βgal96-specific CD8+ T cells in blood 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 lymphocyte compartment is indicated (± SEM). B6: day 21, n = 3; day 50, n = 3; day 100, n = 3; blood MHC-II KO: day 21, n = 4; day 50, n = 4; day 100, n = 4. *p < 0.05, **p < 0.01, ***p < 0.001.

This experiment was performed in the animal facility in St. Gallen. (C) Inflationary potential expressed by the ratio of percentage of tetramer+ CD8+ T cells from day 100 to day 21 in B6 and MHC-II–deficient mice. (D-G) B6 and LMP7 KO mice were immunized i.v. with 2 × 10^9 PFU Ad-LacZ. (D) Tetramer staining for βgal96-specific CD8+ T lymphocytes on day 21 in blood of C57BL/6 (left) and LMP7 KO (right) mice, gated on live lymphocytes. (E) Expansion of βgal96-specific CD8+ T cells in blood of B6 (black circles) and LMP7 KO (black squares, dotted line). (F) Tetramer staining for βgal96-specific CD8+ T lymphocytes on day 21 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 Tem phenotype rather than the Tem phenotype typically seen (data not shown). Interestingly, although CD8T cell expansion was significantly reduced, on day 100 after immunization, βgal-specific CD8T cells were nevertheless still present in blood, liver, lung, and spleen (Fig. 7A, 7B). Thus, CD4T cell help is clearly essential both for the initial priming of βgal-specific CD8T cells and the evolution of an effector-memory phenotype. However, for the maintenance of these cells in the memory phase, CD4T cell help might be dispensable. This theory is supported by findings exploiting other rAdVs (42, 46). CD4T 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, CD4T cell help appears necessary; if viral reactivation is not controlled, CD4T 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 CD4T cell help; however, further experiments are required to determine at what stage such help is critical.

We demonstrated that CD8T 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 CD8T cell responses for several weeks after adenoaviral vector immunization has been described previously, followed by contraction (21, 25, 29–31, 46, 47), although robust CD8T cell memory inflation sustained over many months has not been observed. Previous studies have indicated that prolonged CD8T cell responses depended on low levels of Ag (30, 31). Interestingly, in such models, specific CD8T 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-specific CD8T cell response with that seen in WT mice. In contrast, the noninflating βgal-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 CD8T 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+ 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 CD8T 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 CD8T cell immunity, although potentially such pools may simply decline very slowly.

The observation that βgal-specific CD8T 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 Tem, compared with Tem (52, 53). Specifically, for chronic hepatitis B virus and HCV infection and Mycobacterium tuberculosis, where a high number of functional CD8T 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 CD8T cell populations (CD45RA+CCR7+, 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 CD8T cell populations, which, after the first phase, contract to a low-level memory population of a Tem phenotype. Second, we should further dissect the molecular mechanisms underpinning the evolution of inflationary CD8T 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 CD8T 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 CD8T cell memory inflation is comparable in the two models. Together with findings from studies with other rAdV and from MCMV infection, we suggest that CD8T cell memory inflation is a distinct, evolutionarily conserved, stereotypical memory response, observed in all different routes of exposure to diverse vectors. This response depends at least initially on CD4T 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 adenovirus’ 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.

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