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J Immunol 2003; 171:2393-2401; doi: 10.4049/jimmunol.171.5.2393
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A Transgenic Mouse Model Genetically Tags All Activated CD8 T Cells

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Identifying and characterizing Ag-specific CD8+ T cells are central to the study of immunological memory. Although powerful strategies such as MHC tetramers and peptide-induced cytokine production assays exist for identifying Ag-specific CD8+ T cells, alternate strategies that are not dependent upon a priori knowledge of the immunodominant and subdominant antigenic epitopes, as well as the MHC background of the animal are of obvious utility. In this study, we present a transgenic mouse model that uses Cre-loxP recombination to permanently mark all activated CD8+ T cells with β-galactosidase. We used the lymphocytic choriomeningitis virus infection model to track the dynamics of the antiviral CD8+ T cell responses. We show that in this transgenic mouse model system, all of the antiviral effector and memory CD8+ T cells are contained within the β-gal-marked CD8+ T cell population. The Journal of Immunology, 2003, 171: 2393–2401.

The immune system responds with enhanced vigor to Ags encountered in the past. This exaggerated recall immune response, or immune memory, is a central concept in immunology, and it forms the basis of vaccination against infectious disease. Immune memory is mediated, in part, by memory CD4 and CD8+ T lymphocytes that persist in the host long after resolution of the antigenic insult or infection. Following viral infection, APCs process viral Ags and present peptides to CD8+ T cells in the context of class I MHC. Ag-specific CD8+ T cells that recognize the viral peptides presented by the APC undergo massive proliferation and differentiation, a response that can be categorized into three distinct phases: activation and expansion, death, and memory (1–9). The expansion phase is quite spectacular; recent studies in mice have shown that precursor CD8+ T cells specific for individual viral epitopes are able to expand from below the limits of detection (<1000 cells per spleen) to greater than 10⁷ cells per spleen (10). Following the elimination of viral Ag (6) and due to an inherent genetic program (11), there is an equally dramatic death phase, during which 90–95% of the Ag-specific CD8+ T cells die. Virus-specific CD8+ T cells that survive the death phase persist in the host as a stable pool of memory lymphocytes that provides lifelong immunity (6, 8, 9, 12).

Technical advances such as multimers of viral peptide/MHC and peptide-induced cytokine secretion assays (intracellular cytokine staining and ELISPOT) have allowed the quantitation of antiviral CD8+ T cell responses with great accuracy and precision in a variety of model systems, including viruses, bacteria, and tumors, in both rodents and primates (6, 13–21). The advent of peptide/MHC multimers has revolutionized the study of CD8+ T cell memory; however, the construction of MHC multimers is predicated on the assumption that immunodominant and subdominant viral epitopes have been defined, as well as the MHC restriction of the individual epitopes. Similarly, quantitation of virus-specific CD8+ T cells by intracellular cytokine staining or ELISPOT assays is dependent upon a priori knowledge of immunogenic peptides.

Although tetramers have been pivotal in dissecting Ag-specific CD8+ T cell responses, several recent studies have described novel types of Ag-specific CD8+ T cells that, despite being Ag specific, fail to bind tetramers. Using a TCR transgenic model, Blohm et al. (22) have shown that tumor-infiltrating TCR transgenic lymphocytes that are found in tumors expressing their cognate peptide failed to bind MHC/tetramer, exhibited impaired cytolytic activity, and failed to proliferate or elaborate IFN-γ when stimulated with specific peptide. Interestingly, PBL and splenocytes from these same tumor-bearing mice did bind tetramers, exhibited potent cytolytic activity, produced IFN-γ, and proliferated following peptide stimulation. Spencer and Braciale (23) have described influenza-specific murine CD8+ T cells specific for a subdominant epitope of influenza virus that fails to bind influenza tetramers directly ex vivo. Following repeated rounds of stimulation in vitro, these cells transition from a tetramer-negative to tetramer-positive phenotype. Schneck and colleagues (24) have shown that in vitro activated CD8+ T cells from the 2C TCR transgenic mouse bind peptide/MHC dimers 20- to 50-fold better than naive TCR transgenic T cells from the same mouse. Finally, in both mouse and human chronic viral infections, the appearance and persistence of tetramer-positive CD8+ T cells that fail to elaborate effector functions have been described (25–28). Peptide/MHC multimers may be of limited utility in certain situations. Thus, there exists a need for model systems in which polyclonal populations of activated CD8+ T cells specific for various Ags can be identified and followed independent of tetramer binding and peptide-induced cytokine production.

We have previously described a transgenic mouse model system in which both effector and memory CD8+ T lymphocytes are tagged via Cre-loxP recombination, with the marker protein, human placental alkaline phosphatase (PLAP) (29). The system is...
comprised of two transgenic mouse lines; one has a truncated human granzyme B promoter driving the expression of Cre recombinase (GBC) in activated T cells, and the other has a T cell-specific human CD2 promoter driving the expression of a lox-P-flanked transcriptional truncated yeast His3 gene and polyadenylation sequence (STOP) fragment, followed by PLAP (CD2-STOP-PLAP). This mouse model was originally designed to mark all activated CD8+ T cells, but only a small subset of the activated effector CD8+ T cells is marked with PLAP. For instance, when mice doubly transgenic for CD2-STOP-PLAP and GBC are infected with lymphocytic choriomeningitis virus (LCMV), only a small subset (10%) of the CD8+ T cells express surface PLAP at the peak of the anti-LCMV immune response, even though LCMV peptide/MHC multimers, intracellular cytotoxic flow cytometric assays, and direct ex vivo cytotoxicity studies have unequivocally demonstrated that 50–70% of the splenic CD8+ T cells are responding to LCMV (6, 29).

To complement MHC tetramers for the detection of Ag-specific CD8+ T cells and to overcome the deficit of the previously described transgenic model, we sought to establish a transgenic mouse model system that would permanently mark not just a subset, but all activated CD8+ T cells. In the experiments described in this work, we chose bacterial β-galactosidase (β-gal) as the reporter instead of PLAP by using the Cre reporter transgenic mice, ROSA26R (30–32). In ROSA26R mice, Cre-mediated excisional recombination leads to permanent expression of β-gal from the constitutively active ROSA promoter. We crossed ROSA26R mice with the previously described GBC transgenic mice (29) and used the double-transgenic offspring to probe CD8+ T cell activation using the LCMV infection model. Data presented in this work demonstrate that there is a high degree of efficacy in this transgenic marking method; all activated CD8+ T cells are marked with β-gal. We show that following LCMV infection, the population of β-gal-marked CD8+ T cells generated contains all of the antiviral effector and memory CD8+ T cells.

Materials and Methods

Animals and viral infections

C57BL/6j mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in specific pathogen-free conditions in an American Association of Laboratory Animal Care-accredited facility at Emory University. Granzyme B Cre, CD2-STOP-PLAP, and ROSA26R (29, 32) mice were bred and maintained at Emory University following the guidelines of the Institutional Animal Care and Use Committee. LCMV Armstrong and LCMV clone 13 were triple plaque purified on VERO cells and amplified one round on BHK-21 cells (American Type Culture Collection (ATCC), Manassas, VA) (33). For viral infections, mice were injected once i.p. with 2 × 105 PFU LCMV Armstrong. For viral rechallenge assays, mice were injected with 2 × 105 PFU LCMV clone 13 i.v. LCMV Armstrong and LCMV clone 13 were kind gifts from R. Ahmed, Emory University.

Stimulations

Single cell suspensions from naive GBC × ROSA26R double-transgenic mice were cleared of RBCs using RBC lysis buffer (Sigma-Aldrich, St. Louis, MO), A total of 5 × 106 cells was plated in a volume of 1 ml of complete RPMI (10% FCS, 2 mM L-glutamine, 1000 U penicillin G, 1000 U streptomycin, 0.25 μg/ml penicillin B, and 10 mM HEPES (Cellgro, Herndon, VA)). Cells were stimulated with either PMA and ionomycin or PHA and IL-2. PMA and ionomycin were added at a final concentration of 1 μg/ml and 2 μg/ml, respectively. Cells were incubated for 48 h at 37°C in a humidified incubator with 5% CO2. PHA and IL-2 were added to a final concentration of 10 μg/ml and 25 U/ml, respectively. Cells were periodically assayed by FACS after hypotonic loading with fluorescein di-β-D-galactopyranoside (FDG; Molecular Probes, Eugene, OR) and cell surface staining for CD4, CD8, and CD69 (anti-CD8 from BD Biosciences, San Diego, CA; anti-CD4 and anti-CD69 from Caltag, Burlingame, CA). Poly(ÉC) and anti-CD3/CD28 stimulations were conducted on sorted populations of β-gal+ and β-gal+ CD8+ T cells. Sorted cells were cultured with plate-bound Abs to CD3ε (clone 145-2C11; 5 μg/ml) and CD28 (clone 37.51; 2 μg/ml), added >24 h earlier, or with 10 μg/ml poly(I:C). Following stimulation, live cells were assayed by flow cytometry for expression of β-gal, CD8, and CD69.

Peptides

LCMV and control peptides were synthesized by B. Evavold at the Emory University Department of Microbiology and Immunology Peptide Core Facility using F-moc chemistry on a Symphony/Multiplex Peptide Synthesizer. The peptides were purified by HPLC (Rainin Instruments, Woburn, MA).

Abs and staining for flow cytometry

At various time points following LCMV infection, GBC × ROSA26R mice were euthanized by cervical dislocation, spleens were harvested and RBC lysis buffer (Sigma-Aldrich, St. Louis, MO) were kind gifts from R. Ahmed, Emory University. RBC lysis buffer (Sigma-Aldrich, St. Louis, MO). A total of 5 bacteria were retro-orbitally bled into 4% sodium citrate, and lymphocytes were enriched using lymphocyte separation medium (Cellgro). For detection of β-gal, cells were hypotonically loaded with FDG, as previously described (32). Following FDG loading, the cells were surface stained with fluorochrome-conjugated or biotylated Abs to CD8 (clone 53-6.7 from BD Biosciences), CD4, NK1.1, CD44, CD62L, CD43, CD45, CD122, CD69, or CD1a (clones RM4-5, IM7.8.1, MEL-14, 1B11, PC61 5.3, TM-b1, H1.2F1, and 2D7 from Caltag). Streptavidin-coupled PE and allophycocyanin were purchased from Caltag. Abs to Thy-1.1 and Thy-1.2 were purchased from BD Biosciences. Allophycocyanin-conjugated MHC tetramers of LCMV gp33–41, NP396–404, or Qa-1b tetramers folded around the Qdm peptide were generated, as described (15, 34). Stained cells were kept on ice at all times in FACS buffer (1.5% BSA and 0.02% sodium azide) and analyzed on a FACS caliber (BD Biosciences) flow cytometer running CellQuest software. Data were analyzed with FlowJo (Treestar, San Carlos, CA) or CellQuest software.

Cell sorting

RBC-lysed, single cell suspensions of splenocytes from LCMV-infected or naive mice were hypotonically loaded with FDG and stained with anti-CD8 PE and anti-CD4 allophycocyanin (Caltag and BD Biosciences) in PBS containing 3% BSA (Sigma-Aldrich). β-gal+CD8+CD4− and β-gal−CD8+CD4− populations were sorted on a high-speed Cytomation MoFlo cell sorter to 95–98% purity (data not shown).

Cytotoxicity assay

CTL assays were performed, as previously described (6), with the following modifications. MC57G fibroblast cells (ATCC) were pulsed with LCMV gp33–41, LCMV NP396–404, or Qa SIINFEKL peptides at 10 μg/ml for 90 min at 37°C, followed by labeling with 51Cr (PerkinElmer, Boston, MA). Sorted β-gal+ and β-gal− CD8+ T cells were plated in triplicate in 96-well plates at various dilutions; 1 × 104 cells and peptide-pulsed targets were added to achieve the desired E/T ratios, and incubated for 6 h at 37°C. In control wells, medium without effectors were added to target cells to calculate the spontaneous 51Cr release; 1% Triton X-100 was added to targets, followed by a 6-h incubation to obtain maximal 51Cr release. Following incubation, supernatants were collected and transferred to Wallace cell sorters (Wallace/PerkinElmer) and air dried overnight in a laminar flow chemical hood. The plates were then read on a 1450 Microbeta liquid scintillation counter (Wallace/PerkinElmer). Data are expressed as percentage of specific 51Cr release (experimental release – spontaneous release) ×100 (maximum release – spontaneous release) ×100.

Adaptive transfers

β-gal+CD8+CD4− and β-gal−CD8+CD4− T lymphocytes were sorted on a Cytomation MoFlo, as described above. A total of 8 × 103 sorted cells from GBC × ROSA26R double-transgenic mice that had received 2 × 105 PFU LCMV Armstrong >30 days previously was injected into congenic Thy-1.1 mice. As a control, 8 × 105 unsorted cells were injected into separate Thy-1.1 recipient mice. Twelve hours later, recipients were challenged with 2 × 105 PFU LCMV clone 13 i.v. Recipient mice were sacrificed 7 days later, and their spleens were harvested for FACs analysis, and serum viral titers were determined by plaque assay.

Plaque assay

Plaque assays to determine viral titers were performed exactly as described (35). Briefly, serum samples were serially diluted in 1% RPMI and overlaid on VERO (ATCC) cell monolayers in six-well plates (Invitrogen, Carlsbad, CA) and incubated for 90 min. After 90 min, a 1:1 mixture of 2×199 medium (Life Technologies/Invitrogen) and 1% agarose was gently overlaid on the VERO cells, and the plates were incubated in a humidified
incubator with 5% CO2 for 5 days. On the fourth day, a 1:1 mixture of 2×199 medium and 1% Neutral Red (Sigma-Aldrich) was added to visualize plaques.

**Results**

*Rationale for choosing β-gal as a reporter to mark activated CD8+ T cells*

To better understand the population dynamics, kinetics, and ultimate fate of CD8 T cells responding to a viral infection, it is important to track activated CD8 T cells throughout the course of an immune response. To this end, we have previously described a transgenic mouse model system to mark activated CD8 T cells by generating mice doubly transgenic for GBC and CD2-STOP-PLAP (29). These double-transgenic (GBC × CD2-STOP-PLAP) mice, upon infection with LCMV, marked only a subset (10%) of activated CD8+ T cells; the frequency of PLAP-marked CD8+ T cells was considerably less than what others had reported using MHC multimers and cytokine expression-based assays (6, 9, 29).

**FIGURE 1.** Schematic diagram of the GBC and ROSA26R transgenes. *a*, In the GBC transgene, the truncated human granzyme B promoter drives the expression of Cre recombinase in activated T cells. The human growth hormone (hGH) polyadenylation signal sequence was included to enhance mRNA stability. ROSA26R is a Cre-reporter strain in which a loxP-flanked, phosphoglycerate kinase (pgk) promoter-driven neomycin phosphotransferase expression cassette and β-gal-encoding LacZ gene have been engineered downstream of the ROSA promoter using homologous recombination (30). *b*, Cre-expressing cells recombine to excise the neomycin cassette, leaving behind β-gal under the control of the ubiquitous and constitutive ROSA promoter (31).

We analyzed activation-induced CD8+ T cell marking in double-transgenic offspring of the GBC mouse strain crossed to ROSA26R. By examining double-transgenic GBC × ROSA26R mice, we tested the fidelity of the granzyme B Cre transgene to induce Cre recombination-mediated genetic tagging of all activated CD8+ T cells, and were thus able to characterize the size and temporal dynamics of the entire pool of responding CD8+ T cells in an LCMV infection model.

**T cell marking in naive doubly transgenic mice**

It is known that ~8–10% of the CD8+ T cells in naive mice exhibit an activated phenotype. These activated memory-phenotype CD8+ T cells, which are CD44high, arise due to endogenous immune activation presumably by gut flora or food Ags (37–39). We analyzed the spleen and peripheral blood CD8+ T cells of naive GBC × ROSA26R double-transgenic mice for the presence of β-gal-marked CD8+ T cells. As anticipated, 5–10% of CD8+ T cells in naive mice expressed β-gal (Fig. 2). The surface phenotype of the β-gal-marked CD8 T cells in the naive mice was further examined to determine their activation status. β-gal+ CD8+ T cells were CD25+, Fas+, Fas ligand+, TCRαβ+, CD71+, CD69+, and bihapt for CD44 expression (Fig. 2, and data not shown). Of the β-gal+ CD8+ T cells, 82% were CD62Lhigh (Fig. 2). The activation markers expressed by β-gal+ CD8+ T cells are consistent with a memory phenotype (40). In the naive animals, β-gal does not appear to be a surrogate marker for CD44; as many as 50% of the β-gal+ CD8+ T cells are CD44low (Fig. 2), indicating that the rules governing the expression of these markers are different in transgenic and endogenous contexts.

**FIGURE 2.** The endogenously activated memory-phenotype CD8+ T cells in GBC × ROSA26R double-transgenic mice express β-gal. Splenocytes from naive double-transgenic mice were hypotonically loaded with the tracker dye FDG that fluoresces after cleavage by β-gal and stained with fluorochrome-labeled Abs to CD8, CD25, CD44, and CD62L. *a*, Approximately 5–7% of CD8+ T cells in naive double-transgenic mice are β-gal+. *b*, Flow cytometry plots of β-gal+ CD8+ T cells from naive double-transgenic mice stained for the activation markers CD25, CD44, and CD62L are shown in *a*, c. β-gal expression in NK and CD4+ T cells of naive double-transgenic mice is shown as histogram plots. More than 10 naive double-transgenic mice were analyzed. Representative plots are shown.
governing CD44 and β-gal expression in naive mice are not the same. In naive double-transgenic mice, β-gal expression was not restricted to CD8+ T cells. Fifty percent of splenic NK cells and 12% of CD4+ T cells expressed β-gal (Fig. 2c).

**TCR-dependent, TCR-independent, and cytokine stimuli lead to β-gal expression in CD8+ T cells**

It was first necessary to determine the rules that govern the induction of Cre-mediated recombination and de novo expression of β-gal in CD8+ T cells from GBC × ROSA26R mice. Splenocytes from naive GBC × ROSA26R double-transgenic mice, as well as a single-transgenic littermate control mice, were stimulated with TCR-dependent and TCR-independent stimuli in vitro. PMA/Ionomycin was used as TCR-independent stimuli, and PHA or anti-CD3/CD28 was used as the TCR-dependent stimuli. Splenocytes were stimulated for 48 h (Fig. 3a); all types of in vitro stimulation resulted in increased β-gal expression. The frequency of β-gal+ CD8+ T cells after stimulation was 67, 51, and 70% for anti-CD3/CD28, PHA, and PMA/Ionomycin stimulations, respectively. Thus, both TCR-dependent and TCR-independent stimuli lead to *granzyme B* promoter-induced Cre recombination and β-gal expression in double-transgenic CD8+ T cells. Five to seven percent of the input CD8+ T cells are endogenously β-gal+ before in vitro stimulation. We ruled out the possibility that the mitogens were selectively expanding the endogenously activated, pre-existing β-gal+ CD8+ T cells by sorting β-gal+ and β-gal- CD8+ T cells from naive double-transgenic mice and then stimulating the CD8+ T cell populations in culture. For stimulation, we used anti-CD3/CD28 and poly(I:C). Poly(I:C), a potent stimulator of type I IFNs and expressed in double-transgenic CD8+ T cells. Percentages denote the frequency of TCR-dependent, TCR-independent, and cytokine-mediated stimuli.

β-gal marking of CD8+ T cells during an immune response to LCMV

To study T cell activation during the course of an immune response to a viral infection, we infected cohorts of double-transgenic GBC × ROSA26R mice and single-transgenic littermate control mice that are unable to recombine and express β-gal with a single i.p. injection of 2 × 107 PFU of LCMV Armstrong. LCMV is a natural mouse pathogen, and the Armstrong strain is quickly cleared by the antiviral CD8 immune response that peaks 8 days postinfection (35, 43, 44). At various time points following infection, CD8+ T cells from spleen and peripheral blood were analyzed for expression of β-gal in conjunction with markers for lymphocyte activation. Fig. 4a shows that at the peak of the immune response, ~80% of the CD8+ T cells in peripheral blood express β-gal, and expression was equivalent in spleen (79.05% ± 2.76%, Fig. 4b). LCMV infection is quickly eradicated, and the majority of the CD8+ T cells, as tracked by MHC tetramers and viral peptide-elicited IFN-γ expression, undergo apoptosis, such that by day 30 postinfection, only 5–10% of the CD8+ T cells are LCMV specific (6, 9). However, at day 30 postinfection, as many as 74% of peripheral blood CD8+ T cells are β-gal+ (Fig. 4d). The frequency of β-gal+ CD8+ T cells eventually levels off to ~40% by day 100 postinfection (Fig. 4, a and d). The kinetics of β-gal expression in CD8+ T cells of the spleen mirrors that of peripheral blood, except that the overall frequency is proportionally lower at the same time points (Fig. 4b). Overall, the total number of β-gal-marked splenic CD8+ T cells (Fig. 4c) displayed typical immune response kinetics, i.e., expansion, followed by a death phase, followed by a stable memory phase.

The surface phenotype of β-gal+ CD8+ lymphocytes was examined by flow cytometry at the peak (day 8) of the antiviral immune response. At day 8, ~80% of the PBL CD8+ T cells are β-gal+, and we analyzed β-gal+ CD8+ T cells for the coexpression of T cell activation markers (Fig. 5). Of the β-gal+ CD8+ T cells,
99.5% were CD44 high, 94% were CD62L low, 83% were CD43 high, 99% were Ly-6C high, 99% were CD11a high, and 81% bound tetramers of Qa-1b, a reagent that binds to the surface activation marker CD94/NKG2 (34, 45, 46). In contrast, H9252-gal CD8 T cells did not exhibit an activated phenotype (data not shown). Almost all (95%) of the H9252-gal CD8 T cells were negative for the early activation markers CD25, CD69, CD71, and CD43 at day 8 post-LCMV infection (Fig. 5, and data not shown). When splenocytes from immune GBCROSA26R mice were analyzed 30 days following infection (when stable memory CD8 T cells have formed), the H9252-gal-marked CD8 T cells exhibited a memory phenotype. Greater than 80% of CD8 T cells were CD44 high, 68% were CD62L low (32% were CD62L high), 88% were Ly-6C high, and 86% were CD94/NKG2 (Fig. 6), similar to what is seen by other groups in immune C57BL/6J mice (6, 34, 46). H9252-gal-marked memory CD8 T cells were CD25 and CD69 (data not shown).

The frequency and kinetics of H9252-gal-marked CD8 T cells following LCMV infection mirror that shown by others who have used LCMV peptide/MHC multimers to track virus-specific CD8 T cells. However, the data are distinct on two important points. One is that the overall activation of the mouse CD8 T cell repertoire is greater than that predicted solely by reagents that track Ag-specific CD8 T cells. In the primary response, the frequency of H9252-gal CD8 T cells is higher than the frequency (50–70%) of LCMV tetramer-binding CD8 T cells, as reported by Murali-Krishna et al. (6). By summing together all known LCMV epitopes, Murali-Krishna et al. (6) estimated the frequency of H9252-gal-marked CD8 T cells in the spleen die after the peak of the immune response between days 8 and 60. Both the frequency and number of marked CD8 T cells reach a plateau by 50 days postinfection. The kinetics of H9252-gal expression in peripheral blood CD8 T cells of a representative LCMV-infected double-transgenic mouse is shown in d. Plots are gated on CD8 T cells. The same mouse was bled at various time points postinfection, and the frequency of H9252-gal CD8 T cells is shown (d). Data presented are representative of 3–15 mice at each time point. The top row of contour plots represents data from single-transgenic littermate control mice.

FIGURE 4. H9252-gal-marked CD8 T cells exhibit clonal dynamics of expansion, death, and stability in LCMV-infected GBC × ROSA26R mice. The frequency of H9252-gal CD8 T cells in peripheral blood (a) and spleen (b) was monitored by FACS at various time points after LCMV infection. Total number of H9252-gal CD8 T cells in spleen plotted over time is shown in c. Approximately 70% of the H9252-gal-marked cells in the spleen die after the peak of the immune response (Fig. 5, and data not shown).

FIGURE 5. H9252-gal-marked CD8 T cells observed at the peak of the antiviral immune response exhibit an activated phenotype. Splenocytes were harvested from GBC × ROSA26R mice infected i.p. 8 days earlier with a single dose of 2 × 10^6 PFU LCMV Armstrong. The cells were loaded with FDG to detect intracellular H9252-gal expression, and surface stained for activation makers using Abs to CD44, CD62L, CD43, Ly-6C, CD11a, CD69, CD25, or tetramers of Qa-1b to detect CD94/NKG2. The histograms are gated on CD8 T cells.
memory CD8⁺ T cells to be ~10%. In contrast, LCMV-immune GBC × ROSA26R double-transgenic mice have substantially more β-gal⁺CD8⁺ T cells in the memory phase (>day 30) (Fig. 4). Another striking difference is that the decrease or decay of β-gal⁺-expressing CD8⁺ T cells following the peak of the immune response is much more protracted and does not reach the same low frequency, as demonstrated by tetramer studies. As the genetic recombination that takes place in GBC × ROSA26R mice is permanent, these data directly demonstrate that activated CD8⁺ T cells are dying more slowly or non-Ag-specific CD8⁺ T cells are being activated by bystander activation. To better determine the Ag specificity of β-gal⁺ CD8⁺ T cells, we combined β-gal expression with LCMV tetramer staining.

Costains for β-gal and MHC tetramers were performed on CD8⁺ T cells during the effector and memory phases of an anti-LCMV immune response. We were interested to know whether the β-gal-marked CD8⁺ T cells contained the antiviral effectors. GBC × ROSA26R mice are H-2b, and LCMV-specific CD8⁺ T cells can be identified by MHC multimers using viral peptides. When MHC tetramers were used to stain cells in GBC × ROSA26R mice that had been LCMV infected, we observed that all tetramer-binding CD8⁺ T cells were all β-gal⁺. These analyses were performed using LCMV D³gp33–41 and D³NP396–404 tetramers to track the antiviral response using two different LCMV immunodominant peptides (Fig. 7a). Tetramer binding and β-gal expression were compared in CD8⁺ T cells at days 8, 15, 30, and 60 post-LCMV infection. At all time points analyzed, during both the effector and memory phases, LCMV peptide/MHC tetramer binding correlated with β-gal expression. In the memory phase, we wished to determine whether the tetramer-binding LCMV-specific CD8⁺ T cells resided in the β-gal⁺ or the β-gal⁻ fraction. β-gal⁺ and β-gal⁻ CD8⁺ T cells were analyzed for expression of CD44 and tetramer binding (Fig. 7b). All of the LCMV-specific CD8⁺ T cells were in the β-gal⁺CD44⁺ fraction, and not in the β-gal⁻ fraction (Fig. 7b). We also observed that 94% of the β-gal-marked CD8⁺ T cells were CD44⁺, however, ~20% of the β-gal⁻ CD8⁺ T cells were CD44⁺, indicating that the rules that govern CD44 expression and β-gal marking are not the same (Fig. 7b). Overall, these data demonstrate that CD8⁺ T cells responding to LCMV are contained within the β-gal⁺-marked population.

**β-gal-marked CD8⁺ T cells exhibit LCMV-specific cytolytic activity**

Cytolytic killing of virus-infected or viral peptide-pulsed target cells is a hallmark of virus-specific effector CD8⁺ T cells. To determine the cytolytic capability of β-gal-marked CD8⁺ T cells, CD8⁺CD4⁻ β-gal⁺ and CD8⁺CD4⁻ β-gal⁻ T cells were isolated by cell sorting (purity >95%) from the spleens of GBC × ROSA26R mice infected 8 days earlier with LCMV. The cytolytic capability of sorted cells was tested by incubating them with 51Cr-labeled target cells that were pulsed with LCMV gp33–41, NP396–404, or an irrelevant OVA-specific peptide (SIINFEKL). Only the β-gal⁺CD8⁺ T cells (Fig. 8a) contained antiviral effectors capable of killing viral peptide-pulsed targets. Conversely, the purified β-gal⁻CD8⁺ T cells did not contain any cytolytic effectors (Fig. 8b). These data further demonstrate that the antiviral CTL effectors were contained within the β-gal-marked, activated CD8⁺ T cell population at the peak of the immune response.

**β-gal-marked CD8⁺ T cells present in the immune phase contain LCMV-specific memory CD8⁺ T cells**

We have shown that β-gal⁺CD8⁺ T cells at the peak of the infection contain antiviral effectors capable of CTL activity; that β-gal⁻CD8⁺ T cells were incapable of cytolytic activity (Fig. 8b); and that the β-gal⁻ fraction also contained LCMV tetramer-binding CD8⁺ T cells (Fig. 7). Once the Cre-mediated excision has begun, CD8⁺ T cells responding to LCMV are contained within the β-gal⁺-marked fraction. Double-transgenic GBC × ROSA26R and singly transgenic littermate controls were infected with 2 × 10⁸ LCMV Armstrong. a, PBLs were analyzed at various time points by flow cytometry for β-gal expression and binding of LCMV tetramers D³gp33–41 and D³NP396–404. Representative flow cytometry plots from a littermate control and double-transgenic mice are shown. b, In the double-transgenic mice, all of the tetramer⁺ CD8⁺ T cells were β-gal⁺. In memory double-transgenic mice, the D³GP33–41 and D³NP396 tetramer⁺ CD8⁺ T cells are exclusively β-gal⁺CD44⁺ (data for gp33–41 not shown). β-gal⁺ and β-gal⁻ CD8⁺ T cells were analyzed for expression of CD44 and D³NP396–404 tetramer binding (b). All of the D³NP396–404 tetramer-binding CD8⁺ T cells were in the β-gal⁺, and not in the β-gal⁻CD44⁺ fraction. Gating strategy was as depicted.

![FIGURE 6. β-gal⁺ CD8⁺ T cells have a memory phenotype in immune mice. GBC × ROSA26R double-transgenic mice were infected with LCMV Armstrong, and spleen cells were examined 30 days later. Splenocytes were hypotonically loaded with FDG; surface stained with Abs to CD44, CD62L, CD43, Ly-6C, or tetramers of Qa-1b to detect surface CD94/NKG2; and analyzed by flow cytometry. All flow cytometry plots shown are gated on CD8⁺ T cells. Eight to ten mice were analyzed for each marker, and the experiment was repeated at least three times.](http://www.jimmunol.org/)

![FIGURE 7. LCMV-specific CD8⁺ T cells are contained within the β-gal-marked fraction. Double-transgenic GBC × ROSA26R and singly transgenic littermate controls were infected with 2 × 10⁸ LCMV Armstrong. CD8⁺ T cells were isolated from the spleens of infected mice, and stained with Abs to CD44 and tetramers of D³gp33–41 and D³NP396–404. Representative flow cytometry plots from a littermate control and double-transgenic mice are shown.](http://www.jimmunol.org/)
Antiviral cytotoxic effector cells are present exclusively within the β-gal"CD8+ T cell fraction. GBC × ROSA26R mice were infected with 2 × 10⁶ PFU LCMV Armstrong, and splenocytes were harvested 8 days later. CD8"β-gal" and CD8"β-gal" T cells were purified by high speed cell sorting, and the ability of these two fractions to kill LCMV peptide-pulsed target cells was tested in a standard ⁵¹Cr release assay. MHC-matched target cells were pulsed with immunodominant LCMV (gp33–41 or NP396–404) peptides or an irrelevant OVA control peptide (SIINFEKL) and loaded with ⁵¹Cr. Sorted CD4"CD8"β-gal" (a) or CD4"CD8"β-gal" (b) effectors were incubated with targets at various E:T ratios for 6 h, and the amount of released ⁵¹Cr was measured. One percent Triton X-100 and targets only (no effectors) were used to ascertain maximal and spontaneous ⁵¹Cr release, respectively. Percent specific lysis was calculated as (experimental – spontaneous)/(max – spontaneous).

Discussion

In this work, we describe a transgenic mouse model that marks not just a subset, but all activated CD8+ T lymphocytes with β-gal. These mice are healthy and otherwise normal, and are able to clear acute LCMV infection with a kinetics and magnitude indistinguishable from those of C57BL/6 mice. Interestingly, β-gal is expressed in the CD8+ T cells of naive double-transgenic mice, albeit at a low frequency and a relatively low number. This is not unexpected, as there is a basal level of CD8+ T cell activation to endogenous Ags such as food or gut flora, as described by others who have shown that in naive mice, 8–12% of splenic or PBL CD8+ T cells are CD44⁺CD69⁺ (47–50). This also raises the issue as occurred, all lineal descendants of a recombined T cell will propagate the recombination event and continue to express β-gal. Consequently, we hypothesized that the β-gal-marked T cells would contain LCMV-specific memory CD8+ T cells. A primary feature of memory CD8+ T cells is their ability to rapidly expand following re-exposure to their cognate Ag. We used adoptive transfer to determine the fraction (CD8+β-gal+ or CD8+β-gal−) in which the LCMV-specific memory CD8+ T cells exist. We isolated, by cell sorting, purified populations of CD8+β-gal+CD4− and CD8+β-gal−CD4− T cells from the spleens of GBC × ROSA26R mice that had been infected >45 days earlier with LCMV Armstrong. The two cell populations were adoptively transferred into naive recipient congenic Thy-1.1 mice. GBC × ROSA26R mice express Thy-1.2 (CD90.2), and therefore donor T cells can be easily identified in recipient Thy-1.1 (CD90.1) mice. Following adoptive transfer, recipient mice were challenged with virulent LCMV clone 13, and the capacity of the transferred cells to respond was measured by their ability to proliferate and expand in response to LCMV (Fig. 9a). The transferred β-gal+CD8+ T cells responded to the LCMV infection, and they represented ~14% of the recipient mouse’s spleen 7 days postchallenge, and it constituted more than half of the recipient mouse’s total CD8+ T cells. These β-gal+CD8+ T cells were able to bind LCMV tetramers, D⁴gp33–41 and D⁴NP396–404, and continued to express β-gal (Fig. 9a). The presence of the transferred β-gal− memory CD8+ T cells dampened the endogenous response to LCMV (Fig. 9a), a phenomenon observed by others as well (10). In contrast, transferred β-gal−CD8+ T cells failed to expand in response to LCMV infection and did not inhibit the endogenous CD8+ T cells from responding to LCMV infection (Fig. 9a). We then assessed the ability of the transferred β-gal+ and β-gal−CD8+ T cells to control viral replication in the recipient mice. For challenge experiments, we used the virulent, clone 13 strain of LCMV. Recipients that received β-gal+CD8+ T cells were able to limit the replication of LCMV, and three of five mice had serum viral titers below the limit of detection (Fig. 9b). In contrast, mice that received β-gal−CD8+ T cells had high viral titers, typically >10⁶ PFU ml⁻¹ serum (Fig. 9b). These mice had comparable serum viral titers to naive mice that had been infected with LCMV clone 13 (data not shown). Taken together, these data clearly demonstrate that antiviral memory CD8+ T cells were contained within the β-gal-marked CD8+ T cells.

FIGURE 8. Antiviral cytotoxic effector cells are present exclusively within the β-gal"CD8+ T cell fraction. GBC × ROSA26R mice were infected with 2 × 10⁶ PFU LCMV Armstrong, and splenocytes were harvested 8 days later. CD8"β-gal" and CD8"β-gal" T cells were purified by high speed cell sorting, and the ability of these two fractions to kill LCMV peptide-pulsed target cells was tested in a standard ⁵¹Cr release assay. MHC-matched target cells were pulsed with immunodominant LCMV (gp33–41 or NP396–404) peptides or an irrelevant OVA control peptide (SIINFEKL) and loaded with ⁵¹Cr. Sorted CD4"CD8"β-gal" (a) or CD4"CD8"β-gal" (b) effectors were incubated with targets at various E:T ratios for 6 h, and the amount of released ⁵¹Cr was measured. One percent Triton X-100 and targets only (no effectors) were used to ascertain maximal and spontaneous ⁵¹Cr release, respectively. Percent specific lysis was calculated as (experimental – spontaneous)/(max – spontaneous).

FIGURE 9. β-gal-marked CD8+ T cells from immune mice contain memory cells capable of proliferation and conferring protection upon viral challenge. CD4"CD8"β-gal" and CD4−CD8−β-gal" T cells were sorted from Thy-1.2+ immune GBC × ROSA26R mice, adoptively transferred into congenic Thy-1.1+ recipients, and challenged with LCMV clone 13. Seven days later, the recipient mice were sacrificed, and their splenocytes were analyzed for expansion and tetramer binding. a. Mice that received β-gal+CD8+ T cells (top panel) showed proliferation of host NP396- and gp33-specific recipient (Thy-1.1−) CD8+ T cells. No expansion of the transferred Thy-1.2+ β-gal+CD8+ T cells was observed. In contrast, mice that received Thy-1.2− β-gal−CD8+ T cells underwent proliferation and accounted for almost all of the NP396- and gp33-specific CD8+ T cells (bottom panel). Expansion of the transferred β-gal−CD8+ T cells severely dampened the recipient (Thy-1.1− or Thy-1.2−) CD8+ T cell responses. b. Adoptive transfer of β-gal+. And not β-gal− CD8+ T cells reduced viral titers in recipient mouse sera. Seven days post-LCMV clone 13 challenge, the sera of recipient mice were assayed for LCMV by plaque assay. The serum viral titers of mice that received β-gal+ or β-gal− CD8+ T cells are shown. Each point represents an individual Thy-1.1+ recipient.
to whether β-gal is just a surrogate marker for CD44. In the periphery of naive animals, β-gal does not appear to be a surrogate marker for CD44 because only 50% of the β-gal+CD8+ T cells are CD44high (Fig. 2). Following LCMV infection, there is considerable overlap between the CD44high and β-gal+CD8+ T cell populations; 94% of the β-gal+CD8+ T cells are CD44high. Interestingly, the tetramer+ LCMV-specific CD8+ T cells are in the β-gal+CD44+ fraction, and not in the β-gal-CD44+ fraction (Fig. 7b). Thus, although there is considerable overlap, the rules that govern CD44 and β-gal expression are indeed not the same.

During the acute phase (day 8) of the LCMV response, there is a good correlation between the numbers of tetramer+ and β-gal+CD8+ T cells. In the memory phase (>30 days), the best estimates using MHC tetramers and cytokine elaboration assays for all the known immunodominant and subdominant LCMV epitopes place the frequency of LMCV memory CD8+ T cells at 5–10% (6, 9). However, in double-transgenic mice, 100 days post-LCMV infection, ~40% of CD8+ T cells express CD44 and β-gal (Fig. 4). Thus, there is a substantial gap between the number of memory CD8+ T cells accounted by tetramer staining and the memory phenotype CD8+ T cells, as determined by CD44 or β-gal expression. Our data show that in the memory phase, apoptosis of the β-gal+CD8+ T cells is more reduced when compared with primary effectors visualized by the LCMV tetramers. Recently, Grayson et al. (51) demonstrated that there is a differential sensitivity of naive and memory CD8+ T cells to apoptosis in vivo. They showed that upon restimulation, memory CD8+ T cells undergo rapid expansion, and the secondary effector T cells generated from these memory CD8+ T cells underwent much less contraction or apoptosis as compared with effectors generated from naive CD8+ T cells. Similarly, Harty and colleagues (11) have shown that the contraction phase of secondary effector CD8+ T cells is slower and reduced in magnitude than that of primary effectors. Thus, the slowed contraction of the β-gal+CD8+ T cells in the memory phase could be explained, as follows: LCMV infection leads to expansion of LCMV-specific β-gal+ primary effector CD8+ T cells. The infection presumably also induces expansion of the pre-existing (10%) memory-phenotype β-gal+CD44+CD8+ T cells through cytokine-mediated bystander activation (52, 53). As the virus is cleared, the LCMV-specific β-gal+CD8+ T cells undergo apoptosis and contract, but the secondary effectors generated from the pre-existing β-gal+CD44+ memory CD8+ T cells contract at a substantially slower rate. Current studies aim to directly test this hypothesis by elucidating the cellular source and specificity of these apparent bystander CD8+ T cells by introducing LCMV gp33-specific TCR transgene into the ROSA26R × GBC mice.

The current work describes a novel transgenic mouse that permanently marks activated CD8+ T cells. We have shown that β-gal expression can be induced in double-transgenic CD8+ T cells by TCR-dependent and TCR-independent stimuli. Following LCMV infection, large numbers of CD8+ T cells express β-gal. The overall kinetics of the β-gal+CD8+ T cells show remarkable similarity to those seen using MHC tetramers. The contraction phase is delayed and reduced, and a greater number of β-gal+CD8+ T cells are observed in the memory phase when compared with tetramer studies (Fig. 4) (6). Using a variety of activation markers and functional assays (cytotoxic killing, expansion upon rechallenge, limiting viral replication), we have shown that the marked fraction of CD8+ T cells contains all of the antiviral effector and memory CD8+ T cells. A limitation of using β-gal as the reporter is that cell divisions in CD8+ T cells cannot be tracked using CFSE because the β-gal substrate, FDG, and CFSE fluoresce in the same wavelength. We have tried alternative β-gal substrates, but none of them have performed as well as FDG. In addition, FDG staining is cumbersome, and intracellular cytokine staining cannot be done because permeabilization of the cells leads to loss of hypotonically loaded FDG. Wurtz et al. (54) have recently developed an elegant CD2 promoter-driven T cell-specific Cre reporter transgenic line, pLUC, in which T cells before Cre recombination express human CD2 and after recombination express Thy-1.1 on the cell surface. Generating GBC × pLUC double-transgenic mice will enable CFSE labeling and cell division studies, and these breedings are currently in progress. In addition, it will be possible to track Thy-1.1+tagged CD8+ T cells using in situ immunofluorescence. Analysis of spleen sections from naive and LCMV-infected doubly transgenic pLUC × GBC mice will allow tracking of both cell-cell interactions and spacial dynamics of activated CD8+ T cells in situ.

Powerful transgenic strategies such as the one presented in this work will be beneficial in situations in which defined antigenic peptides are unavailable and therefore MHC multimers have not yet been created. Because the strength of the model is that it can irreversibly tag all activated CD8+ T cells, we anticipate that this mouse model system will be useful in monitoring the population dynamics of activated T lymphocytes in infection, transplantation, and autoimmunity models. In addition, the GBC transgenic mice will also be useful in breeding to the various conditional gene knockout mice to dissect the mechanisms involved in lymphocyte activation, expansion, and memory generation.

Acknowledgments
We are grateful to Craig P. Chappell, Melinda Mackereth, and Dr. Guido Silvestri for critically reading the manuscript and helpful suggestions, Karin Smith and Lorra Miller for mouse colony management, Robert Karaffa and Michael Hulsey for cell sorting, and Dr. Rafi Ahmed for encouragement and guidance.

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