Visualization, Characterization, and Turnover of CD8\(^+\) Memory T Cells in Virus-infected Hosts

By Christine Zimmermann,* Karin Brduscha-Riem,† Claudine Blaser,* Rolf M. Zinkernagel,† and Hanspeter Pircher*

From the *Institute for Medical Microbiology and Hygiene, Department of Immunology, University of Freiburg, D-79104 Freiburg, Germany; and †Institute of Experimental Immunology, University of Zürich, CH-8091 Zürich, Switzerland

Summary

The cellular basis of T cell memory is a controversial issue and progress has been hampered by the inability to induce and to trace long-term memory T cells specific for a defined antigen in vivo. By using the murine model of lymphocytic choriomeningitis virus (LCMV) infection and an adoptive transfer system with CD8\(^+\) T cells from transgenic mice expressing an LCMV-specific T cell receptor, a population of authentic memory T cells specific for LCMV was generated and analyzed in vivo. The transferred transgenic T cells that have expanded (1,000-fold) and then decreased (10-fold) in LCMV-infected C57BL/6 recipient mice exhibited the following characteristics: they were (a) of larger average cell size than their naive counterparts but smaller than day 8 effector cells; (b) heterogeneous with respect to expression of cell surface “memory” markers; and (c) directly cytolytic when isolated from recipient spleens. The time-dependent proliferative activity of these LCMV-specific memory T cells was analyzed in the recipients by bromodeoxyuridine labeling experiments in vivo. The experiments revealed that LCMV-specific CD8\(^+\) memory T cells can persist in LCMV-immune mice for extended periods of time (>2 mo) in the absence of cell division; the memory population as a whole survived beyond 11 mo.

Immunized individuals are able to mount more rapid and more effective B and T cell responses, a phenomenon ascribed to immunological memory. Operationally, T cells can be subdivided into naive, effector, and memory subsets. Naive T cells are resting cells that have not encountered antigen after their release from the thymus, whereas effector T cells are activated cells able to perform specialized functions with high efficiency and without further differentiation. Although it is well established that immunological T cell memory exists, memory T cells are poorly defined. In functional terms, T cell memory can be characterized by an increased frequency of antigen-reactive cells, produced by antigen-driven clonal expansion (1–3). Memory T cells appear to be qualitatively different from naive T cells (4–10). Memory CD4\(^+\) T cells have less stringent requirements for activation than naive T cells (11–15), secrete more complex patterns of cytokines (16–19), and are more effective at helping B cells switch antibody isotype (20). However, most of these studies have been performed with T cells activated in vitro as substitutes for true memory cells. Memory T cells have also been defined with cell surface markers that are induced or lost upon activation. However, many of these changes may reflect activation rather than memory (21); moreover, at least some of these changes are reversible (22, 23).

Many examples have demonstrated that immunological memory persists for many years (24). The mechanisms, however, that are responsible for maintenance of memory T cell function are not understood. Memory T cells may either be specialized, long-lived cells (25–27) or, alternatively, their production is continuously stimulated by persistent (28, 29) or cross-reactive (30) antigen. This focuses interest on the proliferative activity of memory T cells of a defined antigen specificity in immunized hosts. Such experiments have been performed with memory-phenotype T cells with undefined antigen specificity (31), but have not yet been done with authentic memory T cells with defined antigen specificity. Studies in the B cell compartment have revealed that memory B cells specific for the protein PE can persist in PE-primed mice in the spleen for extended periods without cell division (32).

Here, we have used an adoptive transfer system with CD8\(^+\) T cells from TCR transgenic mice to generate a population of authentic memory T cells specific for a defined antigen. The transferred transgenic CD8\(^+\) T cells expressed a TCR specific for the glycoprotein peptide aa33–41 derived
from lymphocytic choriomeningitis virus (LCMV) and that is presented by H-2Db MHC molecules. Without LCMV infection, adoptively transferred transgenic T cells were virtually undetectable in the recipient mice, however, upon activation with LCMV, the transgenic T cells expanded vigorously during the acute phase of the infection and then gave rise to a long-lived population of antigen-experienced T cells, which could be identified with antibodies specific for the transgenic TCR. Thus, this approach provided the means to examine surface markers, functional activity, and turnover of the LCMV-specific memory T cells in LCMV-immune mice.

Materials and Methods

**Mice.** C57BL/6 (B6) mice were obtained from the Institut für Zuchthygiene, University of Zürich or from Harlan Winkelmann (Börchen, Germany) and were backcrossed to B6. The P14 TCR transgenic mice (line 318) have been described previously (33) and were backcrossed onto the B6 background to prevent rejection of the TCR transgenic T cells after adoptive transfer into B6 mice.

**Virus.** The LCMV-WE used in this study was originally obtained from Dr. Lehmann-Grube (Heinrich-Pette-Institut, Hamburg, Germany). It was propagated on L929 fibroblast cells and quantified in a virus plaque assay as described (34). Mice were infected intravenously with 200 PFU of LCMV-WE.

**Adoptive Transfer of TCR Transgenic T Cells.** Sex-matched spleen cells (10^7) from naive transgenic mice containing 5–10% CD8^+ TCR^+ Vc~2+/Vα38^+ cells were injected intravenously in a volume of 0.5 ml medium without FCS into normal nonirradiated B6 mice. 3 d after transfer, mice were infected with LCMV. For continuous bromodeoxyuridine (BrdU)-labeling experiments, transgenic T cells were injected into thymectomized B6 mice 3 wk after thymectomy (35).

**Flow Cytometry.** For detection of transgenic T cells in B6 recipient mice, spleen cells or PBL were incubated on ice with FITC-labeled anti-CD8 (Becton Dickinson & Co., Mountain View, CA), PE-labeled anti-TCR Vα2, and biotinylated anti-TCR Vβ8 (both from PharMingen, San Diego, CA) mAb followed by Tricolor-streptavidin (Caltag, South San Francisco, CA). For the detection of memory markers, cells were stained with FITC-labeled anti-CD8, PE-labeled anti-TCR Vα2 and biotinylated anti-CD62L, CD44, CD45RB (16A), CD49d or CD11b mAb (PharMingen), followed by Tricolor-streptavidin.

**Staining of PBL was performed in PBS containing 2% FCS, 0.1% NaN₃, and the number of transgenic T cells in the spleen was decreased by newly generated naive T cells.** To assess the actual number of cells involved in this expansion and decline, mice transferred with the transgenic T cells were killed both in the acute and the memory phases, and the number of transgenic T cells in the spleen was determined (Fig. 2 c). The percentage of Vα2^+ Vβ8^+ cells in the CD8 subset of splenocytes and PBL was equivalent in the same individual (not shown). The transferred transgenic T cells (5–10 × 10^5) expanded about 1,000-fold during the acute phase of the infection, yielding 3–6 × 10^7 transgenic T cells in the spleen. In the memory phase (6–13 wk after infection)
Figure 1. Flow cytometric detection of TCR transgenic T cells. Spleen cells of the mice indicated were stained with antibodies specific for CD8, TCR Vα2, and TCR Vβ8. The dot plots show expression of TCR Vα2 and Vβ8 gated for CD8+ T cells. Cells were taken from TCR transgenic mice (a), normal B6 mice (b), B6 mice that had received 10^5 TCR transgenic T cells (c), and from B6 recipients of TCR transgenic T cells 8 (d) or 50 d (e) after LCMV infection. Without LCMV infection, transgenic T cells were undetectable in the recipient mice by flow cytometry when tested immediately (c) or 50 d (not shown) after cell transfer. The large numbers in the quadrants indicate the percentage of TCR Vα2+/Vβ8+ cells of CD8+ T cells; the small numbers indicate the percentage of transgenic T cells of total splenocytes.

Figure 2. Kinetics of transgenic T cells in vivo after LCMV infection. B6 mice that received 10^9 TCR transgenic T cells on day −3 were infected with LCMV on day 0. Flow cytometric analysis, as described in infection), the numbers of transgenic T cells (2–6 × 10^6 per spleen) were decreased ~10-fold, when compared to the acute phase, and were increased ~100-fold when compared to the initial input of naive transgenic T cells.

Characterization of Transgenic Memory T Cells. The preceding data demonstrated that the transgenic T cells in LCMV-infected recipient mice were produced by antigen-driven clonal expansion from a few naive transgenic T cells that were undetectable in the recipient mice without LCMV infection. It is therefore proper to refer to this transgenic population in LCMV-immune mice as authentic antigen-experienced memory T cells. The cell size of transgenic naive, effector, and memory T cells was compared by flow cytometry using forward light scatter (FSC). Electronic gates were used to display the FSC of transgenic (R2 = Vα2+/Vβ8+) versus nontransgenic (R3 = Vα2−/Vβ8−) CD8+ T cells analyzed in the same FACS® sample. In uninfected transgenic mice, the FSC of CD8+ T cells expressing the transgenic TCR did not differ from CD8+ T cells expressing endogenous TCR (Fig. 3 a). During the acute LCMV infection, CD8+ T cells were blast-sized with an increased FSC (Fig. 3 b). In LCMV memory mice, CD8+ T cells expressing the transgenic TCR exhibited an increased FSC when compared to CD8+ cells expressing endogenous TCR (Fig. 3 c, mean FSC, 506 versus 468). Thus, the average cell size of LCMV memory CD8+ T cells in LCMV-immune mice was larger than that of naive T cells (mean FSC, 506 versus 468).
versus 465), but smaller than that of effector T cells during the acute phase of the infection (mean FSC, 506 versus 557). It is important to stress, however, that the FSC histograms of the different cell populations overlapped, indicating that cells of similar size can be found in the naive, effector, and memory T cell pool.

The adoptive transfer model described here provided an excellent opportunity to examine cell surface markers associated with T cell memory on authentic, in vivo-generated memory T cells. PBL from noninfected TCR transgenic mice (naive) and from recipients of transgenic T cells at 8 (effector) and >50 d (memory) after LCMV infection were stained with mAbs specific for CD62L (L-selectin), CD44 (Pgp-1), CD11b (Mac-1), CD49d (very late antigen [VLA-4]), and CD45RB. The dot plots shown in Fig. 4 are of gated CD8+ cells and display the expression of these markers both on transgenic (Vα2+) and on host-derived (Vα2−) CD8+ T cells for comparison. The lymphocyte homing receptor L-selectin (CD62L) was expressed on most naive transgenic T cells but was absent on effector T cells in acute

Figure 3. Cell size of transgenic naive, effector, and memory T cells measured by forward light scatter (FSC). B6 recipients of TCR transgenic T cells were infected with LCMV. PBL from naive transgenic mice (a) and from recipient mice 8 (b) and 50 d (c) after infection were stained with CD8-, TCR, Vα2-, and TCR VB8-specific antibodies. The FSC histograms (right) are of R3-gated endogenous CD8+Vα2+/VB8− and of R2-gated transgenic CD8+Vα2+/VB8+ cells. The mean FSC of the gated population is shown. The R2/R3 gates are indicated on the Vo~2/Vb~ dot plots (left) which were gated for CD8+ cells. Identical results were obtained when splenocytes were analyzed (data not shown).
naive effector memory
(day 8) (> day 50)

CD62L
3.3 % 62 %
2.0 % 2.9 %
7.2 % 3.8 %
1.8 % 4.2 %
6.4 % 1.0 %
3.6 % 3.6 %
4.5 % 7.3 %

CD44
35 % 54 %
35 % 49 %
18 % 24 %
6.4 % 1.0 %
36 % 51 %
37 % 44 %
36 % 42 %

CD11b
21 % 21 %
21 % 21 %
42 % 42 %
42 % 42 %
21 % 21 %
21 % 21 %
21 % 21 %

CD49d
42 % 42 %
42 % 42 %
42 % 42 %
42 % 42 %
42 % 42 %
42 % 42 %
42 % 42 %

CD45RB
37 % 14 %
37 % 14 %
37 % 14 %
37 % 14 %
37 % 14 %
37 % 14 %
37 % 14 %

TCR Vα2

Figure 4. Surface phenotype of transgenic naive, effector, and memory T cells. B6 recipients of TCR transgenic T cells were infected with LCMV. PBL from naive TCR transgenic mice (left) and from infected recipient mice 8 (middle) and >50 d (right) after infection were stained with mAbs specific for CD62L (L-selectin), CD44 (Pgp-1), CD49d (VLA-4), CD11b (Mac-1), CD45RB, CD8, and TCR Vα2. The dot plots shown are of gated CD8+ cells.

expression is frequently used to define memory T cells (37). CD45RB expression was decreased on effector cells compared to naive T cells (mean fluorescence, 586 versus 1,207), whereas most memory cells, like naive T cells, expressed a CD45RB

high phenotype (mean fluorescence, 1,197).

The cell size analysis and the expression pattern of adhesion molecules described above indicated that some of the memory T cells displayed an activated phenotype. We therefore asked whether transgenic CD8+ memory T cells were directly cytolytic when tested on target cells coated with the LCMV glycoprotein peptide 33-41 recognized by the transgenic TCR. Transgenic T cells from naive and from acutely infected animals were included in these assays for comparison. The data showed that in contrast to naive cells, ex vivo-isolated transgenic memory T cells lysed LCMV peptide–coated target cells in a short-term, 5 h 51Cr-release assay (Fig. 5). The direct comparison with day 8 transgenic effector cells revealed a 10–20-fold reduced lytic activity on a cell per cell basis, suggesting that the CD8+ memory T cell pool in LCMV-immune mice is heterogeneous and contains only a few cells that display instant cytolytic activities. Spleen cells from LCMV memory (>day 50) B6 mice that did not receive transgenic T cells were not cytolytic in this type of assay (data not shown), indicating that the LCMV-specific lysis was due to the transgenic memory T cell population.

Turnover of Memory T Cells in LCMV-immune Mice. The experiments of the preceding sections indicate that transgenic memory T cells persisted in LCMV-immune mice for prolonged periods (Fig. 2). To analyze the proliferation of transgenic T cells in these hosts, the DNA precursor, BrdU was administered in the drinking water. Incorporation and decay of this label was analyzed in transgenic and nontransgenic CD8+ T cells.

In the first set of experiments, B6 recipients of TCR transgenic T cells were infected with LCMV and given BrdU water for the first week after infection. Then, BrdU was chased by transferring the mice to normal water; the rate of decay of labeled cells was monitored (Fig. 6 a). On day 1 after chase, 88–95% of transgenic (Vα2+) and 66–
77% of the nontransgenic (Vα2−) CD8+ T cells in the spleen were labeled with BrdU. The host-derived nontransgenic CD8+ T cells have receptors of unknown specificity and most of them were stimulated unspecifically by the inflammatory process, since the frequency of LCMV-specific CTL precursors within this population was below 1/20 (data not shown). The percentage of BrdU+ cells in this nontransgenic CD8+ subset declined rapidly during the first 2 wk and remained constant thereafter (11–15%). In marked contrast, the proportion of labeled transgenic CD8+ T cells decayed very slowly during the 8-wk period of observation. 8 wk after chase, 65% of the transgenic memory T cells still carried the BrdU label. The plots displaying TCR Vα2 and BrdU staining intensities gated on CD8+ T cells showed that the BrdU+ and the BrdU− cells appeared as discrete populations within the transgenic Vα2+ subset, and that the mean fluorescence intensity of the BrdU label of the transgenic memory cells did not substantially decrease within the observation period (Fig. 6 b). These data suggest that the transgenic memory T cell pool is heterogeneous, consisting of dividing (BrdU+) and of virtually nondividing (BrdU−) cells.

In the second set of experiments, the kinetics of BrdU incorporation upon continuous labeling was examined. For these experiments, thymectomized B6 recipients of TCR transgenic T cells were infected with LCMV and given BrdU water at 4 (Fig. 7 a) and 17 wk (Fig. 7 b) after infection. Three points emerged from these data. First, the experiments with short-term (4-wk) and long-term (17-wk) LCMV-immune mice yielded similar results. Second, trans-
genic (Vα2+) memory T cells incorporate BrdU with a kinetic similar to the entire pool of host-derived (Vα2−) CD8+ T cells. Third, 30–40% of transgenic memory T cells excluded BrdU over a 7-wk period, implying that these cells were nondividing.

**Discussion**

The present report examines, for the first time, a population containing 95–99% of memory CD8+ T cells induced by a defined antigen in vivo with respect to surface markers and cell cycling. This was made possible by LCMV-induced clonal expansion of the TCR transgenic T cells in an adoptive transfer system. The overall kinetics of >1,000-fold expansion of transgenic T cells followed by a 10-fold contraction agrees well with the kinetics of LCMV-specific CD8+ T cells in normal B6 mice after LCMV infection, as determined by CTL precursor frequency analysis (3). It is noteworthy that spleen cells from day 8 LCMV-immune recipients of TCR transgenic T cells also exhibited cytolytic activity specific for the LCMV nucleoprotein peptide NP 394-408, indicating that the introduction of the transgenic T cells did not preclude the normal host response (data not shown). The frequency of transgenic TCR+ cells per CD8+ cell dropped from the peak of the response to the memory phase only by a factor of two, and afterwards remained remarkably stable. This result fits well with the idea that the clonal burst of virus-specific T cells during the acute phase of the infection primarily determines the size of the memory T cell compartment (26). It further implies that the T cell pool, after an infection, is heavily biased with cells specific for the most recent pathogen, with little change in the T cell repertoire between the acute infection and the memory state.

TCR transgenic CD4+ T cells specific for a chicken ovalbumin peptide have been previously used in similar adoptive transfer experiments by Kearney et al. (38). In these latter experiments, the transferred ovalbumin-specific T cells expanded upon immunization with the antigenic peptide, and at the peak of the response 3% of CD4+ T cells expressed the transgenic TCR. The extent of clonal expansion observed in our transfer model is far greater since 70% of CD8+ T cells were of transgenic origin during the acute phase of LCMV infection. It is conceivable that the different types of antigen challenge—synthetic peptide in CFA versus infectious virus—were responsible for the different degrees of clonal expansion observed in these two transgenic transfer models. However, it is noteworthy that the extent of clonal expansion observed in the LCMV model was similar to the level of expansion of antigen-specific CD4+ T cell “clonotypes” in draining lymph nodes of normal mice primed with pigeon cytochrome c (39).

The adoptive transfer model described here allowed a critical evaluation of the suitability of cell surface markers widely used to define memory T cells. The expression of these markers appears to be highly complex (Fig. 4). Three major points emerged from this data. First, 20–30% of transgenic memory T cells expressed, like naive T cells, a CD62Lhigh phenotype, indicating that expression of the lymph node-homing receptor CD62L (L-selectin) on T cells is reversible in vivo. This observation agrees well with two recent studies demonstrating CTL precursors specific for LCMV, influenza A and Sendai virus in the pool of CD62Lhigh CD8+ T cells (40, 41). Second, CD44 was increased in transgenic effector and memory T cells to the same extent. In contrast, expression of CD44int (VLA-4) was high on transgenic effector T cells, intermediate on memory T cells, and low on naive T cells. Thus, expression of CD44int correlates well with the three different T cell populations in our system. CD49 binds to vascular cell adhesion molecule 1 and is important for recruitment of lymphocytes to sites of inflammation (42–45). Third, CD11b and CD45 isoform expression has been used to discriminate naive and memory T cells (37, 46). Our results revealed that most CD8+ memory T cells generated in our model were CD11b− and CD45RBhigh, like naive T cells. Thus, expression of these markers on CD8+ T cells appears to correlate more closely with cell activation than with memory. It is possible that the few CD11b+ and CD45RBlow cells in the memory T cell pool correspond to memory T cells recently reactivated by persistent or cross-reactive antigen. Our data on CD11b expression on memory CD8+ cells are in contrast to the results reported by McFarland et al. (46) which showed that elimination of CD11b+ cells from responder spleen cells of LCMV-immune mice abolished a secondary LCMV-specific CTL response in vitro. The reason for this discrepancy is not clear. In our system, the proliferative in vitro response of the transgenic memory T cell population containing CD11+ cells was not enhanced when compared to CD11− naive TCR transgenic T cells (data not shown).

There is much controversy as to whether the more rapid and more effective secondary immune response in an antigen-primed host is simply due to the increased frequency of antigen-reactive T cells or to a distinct characteristic of memory T cells (21, 47). The finding that the transgenic memory T cell population exhibited cytolytic activities demonstrates that, in contrast to naive T cells, certain CD8+ memory T cells are rapidly capable of interfering with a second viral challenge by lysing virus-infected cells. The frequency of these “cytolytic” memory T cells within the total memory population is not known. The comparison of the lytic activities on a cell per cell basis revealed that the frequency of these directly cytolytic CD8+ T cells drops 10–20-fold when passing from the acute to the memory phase (Fig. 5). It will be important to determine whether these cytolytic memory T cells are dependent on continuous TCR-mediated stimulation by persistent antigen.

The main conclusion to be drawn from BrdU labeling experiments is that CD8+ LCMV-specific memory T cells turn over at a low rate in LCMV-immune mice. The much more rapid decline of the BrdU label in the bystander-activated CD8+ T cell pool suggests that the average life span of these cells is considerably shorter than that of the antigen-induced transgenic T cells. Tough and Sprent (31) have recently examined, by BrdU labeling experiments, the
turnover of memory T cells defined by surface markers and have reported no or only a slow decay in the percent labeling of memory-phenotype CD8+ T cells in the chase period. Our results using bona fide memory T cells confirm this finding. We did not, however, observe a similar marked switch from BrdUhigh to BrdULow cells during the 8-wk chase period, indicating that, in contrast to memory-phenotype CD8+ T cells, a sizable portion of LCMV-specific memory T cells was not cycling in LCMV-immune mice.

In the continuous labeling experiments, BrdU incorporation by the transgenic memory T cells was similar to the BrdU labeling data of CD62L−CD8+ T cells in the experiments of Tough and Sprent (31), but differed from their results obtained with memory-phenotype CD4+ T cells and CD44high and CD45RBint CD8+ T cells, which exhibited more rapid BrdU incorporation kinetics. These discrepancies may be due to the fact that the memory markers available to date correlate primarily with cell activation and do not discriminate between recently activated T cells and memory T cells.

Our data demonstrate that about half of the transgenic, LCMV-specific memory T cells persisted in LCMV-immune mice for 5–7 wk in the absence of cell division, whereas the other half of the memory T cells incorporated BrdU, thus indicating cell division. The present report does not address the issue of whether persistence of viral antigen is responsible for this low level of proliferation; this question remains to be answered in this system. It is, however, noteworthy that the kinetics of the BrdU labeling of transgenic memory T cells were virtually identical when started either 4 or 17 wk after priming with LCMV (Fig. 7, a and b). This result is different from that obtained in similar experiments analyzing the proliferative activity of memory B cells specific for PE (32). When BrdU was given to mice 4 wk after immunization with PE and continued for 5 wk, 62% of PE-specific memory B cells were labeled. In contrast, when BrdU was given to mice 10 wk after priming, only 12–18% of PE-specific memory B cells incorporated BrdU. It is conceivable that the time-dependent decrease of proliferation of PE-specific memory B cells in PE-primed mice, which is in contrast to the result obtained here with memory T cells in LCMV-infected mice, reflects persistence of antigen; this is more likely to occur after priming with an infectious virus than with protein antigens.

The present analysis of clonally expanded transgenic T cells in recipient mice permitted the definition of cell surface markers and turnover of virus-specific CD8+ memory T cells in vivo. Because of massive expansion of the transgenic T cells seen, the system will simplify further cellular and molecular analysis of effector and memory T cells in immune hosts.

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Address correspondence to Dr. Hanspeter Pircher, Institute for Medical Microbiology and Hygiene, Department of Immunology, Hermann-Herder-Str. 11, University of Freiburg, D-79104 Freiburg, Germany.

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