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Induction of Telomerase Activity and Maintenance of Telomere Length in Virus-Specific Effector and Memory CD8+ T Cells

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Acute viral infections induce extensive proliferation and differentiation of virus-specific CD8+ T cells. One mechanism reported to regulate the proliferative capacity of activated lymphocytes is mediated by the effect of telomerase in maintaining the length of telomeres in proliferating cells. We examined the regulation of telomerase activity and telomere length in naive CD8+ T cells and in virus-specific CD8+ T cells isolated from mice infected with lymphocytic choriomeningitis virus. These studies reveal that, compared with naive CD8+ T cells, which express little or no telomerase activity, Ag-specific effector and long-lived memory CD8+ T cells express high levels of telomerase activity. Despite the extensive clonal expansion that occurs during acute lymphocytic choriomeningitis virus infection, telomere length is maintained in both effector and memory CD8+ T cells. These results suggest that induction of telomerase activity in Ag-specific effector and memory CD8+ T cells is important for the extensive clonal expansion of both primary and secondary effector cells and for the maintenance and longevity of the memory CD8+ T cell population. The Journal of Immunology, 2003, 170: 147–152.

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The Journal of Immunology

Elimination of most viral infections depends on the capacity of virus-specific T and B cells to differentiate into anti-viral effector cells and to sustain rapid and prolonged proliferation (1). Acute lymphocytic choriomeningitis virus (LCMV) infection induces profound clonal expansion of Ag-specific CD8+ T cells and is thus an excellent model system for identifying factors that may regulate the proliferative capacity of CD8+ T cells during an immune response. During the primary immune response to LCMV infection, virus-specific CD8+ T cells divide approximately 10–20 times, resulting in up to 50,000-fold increases in the numbers of virus-specific CD8+ T cells (2, 3). In addition to proliferating in response to LCMV infection, Ag-specific CD8+ T cells acquire effector functions, such as cytotoxic potential and production of anti-viral cytokines. This permits rapid and effective elimination of this virus. Following viral clearance, the majority of effector CD8+ T cells die by apoptosis, but those that remain constitute the long-lived population of memory CD8+ T cells that, in response to a secondary viral challenge, can rapidly expand and differentiate into effector cells. The number of memory CD8+ T cells remains stable over long periods of time due to slow, but sustained, cell division (2–8). Thus, the replicative capacity of virus-specific effector and memory CD8+ T cells is a critical factor in successful elimination of virus during a primary infection, main-
from studies of human cells, these studies have mostly been performed in vitro and have been limited in their ability to examine the regulation of these factors in Ag-specific responses. For these reasons, murine systems designed to investigate telomerase expression in response to defined antigenic challenge in vivo have proven valuable (18). In the present study we used LCMV (Armstrong strain) infection as a physiologic model to study the relationship among CD8+ T cell expansion, telomerase activity, and telomere length in Ag-specific cells during an acute viral infection in vivo.

Materials and Methods

**Mice and virus infection**

BALB/c, C57BL/6 (B6), and P14 transgenic (20) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at Emory University (Atlanta, GA) following institutional animal care and use committee protocols. Mice deficient in telomerase RNA template (mTR) and backcrossed to the B6 strain were provided by C. Greider (The Johns Hopkins University, Baltimore, MD) and were crossed with SPRET/Ei as previously described (21). BALB/c, Mus spretus (SPRET/Ei), (BALB/c × SPRET/Ei)F1, and (B6 × SPRET/Ei)F1 mice were bred at Frederick Cancer Research Center (Frederick, MD). P14 chimeric mice were generated as described previously (5, 6, 20). For both primary and secondary viral infections, mice were infected with 2 × 10^6 PFU of LCMV-Armstrong (clone 13) i.p. Effector CD8+ T cells were isolated 8 days postinfection (p.i.), and memory CD8+ T cells were isolated 47–130 days p.i. Secondary challenge was conducted with identical viral infection 32–177 days after primary challenge, and secondary effector cells were isolated 3–5 days later. Virus stocks were grown and quantified as previously described (22).

**Cell surface and intracellular staining**

Production of MHC class I tetramers, cell surface staining, and analysis of intracellular IFN-γ production were performed as previously reported (2). All Abs used were purchased from BD PharMingen (San Diego, CA). CD43 staining was performed using the Ab 1B11, which recognizes the high m.w. isoform of CD43.

**Flow cytometric analysis and sorting of MHC peptide tetramer-positive CD8+ T cells**

Isolation of highly purified (>95% pure) CD8+ T cells from spleens of uninfected and LCMV-infected mice by FACS has been described previously (2). Naive CD8+ T cells were isolated from uninfected BALB/c or (BALB/c × SPRET/Ei)F1 mice based on CD8+, LFA-1int staining or from P14 transgenic mice based on CD8+, CD44hi staining. At the indicated times p.i., primary effector, memory, and secondary effector CD8+ T cells were isolated from infected BALB/c and (BALB/c × SPRET/Ei)F1 mice, based on CD8+ and LNP118–126 MHC class I tetramer-positive staining or from infected P14 chimeric B6 mice based on CD8+ and D7-gp33,4 MHC class I tetramer-positive staining. For some experiments congenic P14 CD8+ T cells that express the Thy1.1 allele were transferred into B6 (Thy1.2) mice. At the indicated times p.i., primary effector, memory, and secondary effector CD8+ T cells were isolated based on CD8 and Thy1.1 staining. Approximately 95% of the Thy1.1+ CD8+ T cells bound D7-gp33,4 MHC class I tetramers (data not shown).

**Telomerase assay**

Telomerase activity of sorted cell populations was detected with the TRAPeze telomerase detection kit (Intergen, Purchase, NY) following the manufacturer’s instructions. Serial dilutions of each cell lysate were assayed. Amplified products were resolved on polyacrylamide gels and stained with SYBR green I (Molecular Probes, Eugene, OR). Telomerase products were detected as a ladder of bands starting from 50 bp, and the internal standard produced a single band of 36 bp. Telomerase bands were measured by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and were analyzed using ImageQuant software (Molecular Dynamics). A sample of each lysate was heated to 85°C to inactivate telomerase and document the telomerase dependence of bands. Each telomerase assay contained a sample of EL4 tumor cell lysate as a normalization control (data not shown). For each sample examined, telomerase activity (units per 5000 cell equivalents) was calculated according to the manufacturer’s instructions. Differences in telomerase activity between naive and effector or memory cells were analyzed by one-tailed Student’s t test.

**Analysis of telomere length**

Telomere length was measured as previously described (23). Briefly, 0.5–5 × 10^5 cells were embedded in agarose gel plugs, treated with protease K, washed, and treated with HindIII and Rsd1 (Roche, Indianapolis, IN) to digest nontelomeric DNA. Telomeric DNA was resolved using pulsed field electrophoresis (Bio-Rad, Hercules, CA), and DNA was hybridized with a 32P end-labeled (CCCTAA)4 telomere-specific probe. Telomere length was measured by PhosphorImager and was analyzed using ImageQuant software.

**Results**

**Characterization of naive and LCMV-specific effector and memory CD8+ T cells**

Previous studies have demonstrated that flow cytometry using dual staining with anti-CD8 mAb and tetrameric MHC class I-peptide complexes can be used to isolate highly purified populations of LCMV-specific CD8+ T cells from LCMV-infected mice (2, 4–6). We used this approach to isolate cells for subsequent analysis of telomerase activity and telomere length in LCMV-specific effector and memory CD8+ T cells in two strains of mice, BALB/c and B6. LCMV infection in both strains of mice generates a robust CD8+ T cell response, and in BALB/c mice this response is predominated by CD8+ T cells that recognize the NP118–126 epitope of LCMV nucleoprotein that is MHC class I D^b restricted. Generation of this large response allows isolation of sufficient numbers of virus-specific CD8+ T cells from these mice for biochemical studies. In B6 mice, however, the CD8+ T cell response is spread across three dominant epitopes. Therefore, to facilitate the recovery of large numbers of LCMV-specific CD8+ T cells in B6 mice, the P14 transgenic strain of mice was used. Nearly 90% of CD8+ T cells in P14 mice express a transgenic TCR that recognizes the NP118–126 epitope of LCMV glycoprotein in association with MHC class I D^b. When P14 cells are adoptively transferred into B6 mice that are subsequently infected with LCMV, the P14 CD8+ T cells dominate the immune response due to their increased frequency over endogenous LCMV-specific precursors (6, 20). Thus, the expression of telomerase was analyzed in independent primary effector, memory, and secondary effector LCMV-specific CD8+ T cell populations derived from LCMV-infected BALB/c or chimeric P14 transgenic B6 mice. LCMV-specific LNP118–126-Positive CD8+ T cells were undetectable in naive BALB/c mice by either MHC class I tetramer staining or IFN-γ production (Fig. 1, A and B, left panels). By 8 days after primary LCMV infection, ~50% of CD8+ T cells are specific for this epitope, and by 40 days p.i., memory CD8+ T cells comprise ~10% of the CD8 T cell population in immune mice (Fig. 1A). To study memory cell recall responses to LCMV, LCMV-immune mice were rechallenged 32–177 days after primary exposure with the virulent strain of LCMV clone 13. This elicited a second wave of rapid proliferation and the generation of secondary effectors. Nearly 100% of primary effector, memory, and secondary effector LCMV-specific CD8+ T cells are functional and can rapidly produce IFN-γ in response to NP118–126 stimulation (Fig. 1B). Furthermore, these cells displayed characteristic patterns of surface marker expression associated with naive, effector, and memory CD8+ T cells (2, 5–7). Naive CD8+ T cells are LFA-1low, CD43hi, and CD62Lhigh, whereas LNP118–126 primary and secondary effector CD8+ T cells are LFA-1hi, CD43hi, and CD62Llow. Tetramer-positive memory CD8+ T cells are LFA-1hi, CD43int, and mostly (60–95%) CD62Lhigh. Acute LCMV (Armstrong strain) infection is resolved by 8 days p.i. LCMV-specific memory CD8+ T cells persist in the absence of Ag (4) and do not exhibit signs of recent TCR activation such as CD69 expression (data not shown).
FIGURE 1. Ag-specific effector and memory CD8^+ T cells are induced in response to LCMV infection. **A**, Spleen cells from naive BALB/c mice or BALB/c mice 8 days p.i. (effector), >40 days p.i. (memory), or 5 days post-secondary infection (2^{o} effector) were stained with CD8 mAb and L^2NP_{118-126} tetramer. Numbers show the percentages of CD8^+ T cells that are tetramer positive. Data for panels A–C are representative of three to six samples analyzed for each group. **B**, Cells were stimulated for 5 h with NP_{118-126} peptide and subsequently double-stained for surface CD8 and intracellular IFN-γ. Numbers show the percentages of CD8^+ T cells that produce IFN-γ. **C**, Expression of LFA-1, L-selectin (CD62L), and the high m.w. isoform of CD43 on naive, primary and secondary effector, and memory CD8^+ T cells isolated from BALB/c mice. **D**, Spleen cells from naive P14 mice or chimeric P14 transgenic mice 8 days p.i. (effector), >40 days p.i. (memory), or 5 days post-secondary infection (2^{o} effector) were stained with CD8 mAb and D^2GP_{33-41} tetramer. Numbers show the percentages of CD8^+ T cells that are tetramer positive. Data for D–F are representative of three samples analyzed for each group. **E**, Cells were stimulated for 5 h with gp_{33-41} peptide and subsequently double-stained for surface CD8 and intracellular IFN-γ. Numbers show the percentages of CD8^+ T cells that produce IFN-γ. **F**, Analysis of LFA-1, L-selectin (CD62L), and the high m.w. isoform of CD43 expression by naive, primary and secondary effector, and memory P14 CD8^+ T cells.
Similar CD8 T cell properties were observed in the naive, effector, and memory CD8+ T cell populations isolated from chimeric P14 transgenic mice. As demonstrated by MHC class I tetramer staining, ~90% of the CD8+ T cells present in uninfected P14 mice are specific for the gp33-41 epitope, but are naive cells, as evidenced by their inability to make IFN-γ in response to peptide stimulation (Fig. 1, D and E, left panels). Naive P14 transgenic cells were transferred into normal B6 mice to generate P14 chimeras that were subsequently infected with LCMV. Eight days after LCMV infection, tetramer-positive cells represented ~50–70% of CD8+ T cells present in these chimeric P14 transgenic mice. By 60 days p.i., D5gp33-41-positive cells constituted 10–20% of the CD8+ T cell populations in immune mice (Fig. 1D). To study memory cell responses to LCMV, LCMV-immune chimeric P14 transgenic mice were rechallenged 60–113 days after primary exposure with LCMV clone 13. As was observed in BALB/c mice, rechallenge results in a second wave of rapid proliferation and generation of secondary effectors. Nearly all the P14 effector and memory CD8+ T cells are functional and can produce IFN-γ in response to peptide stimulation (Fig. 1E). Phenotypic characterization of naive, primary effector, memory, and secondary effector P14 CD8+ T cells (Fig. 1F) revealed that these populations display cell surface molecules characteristically associated with naive, primary effector, memory, and secondary effector P14 CD8+ T cells (Fig. 1F) (2, 5–7).

To avoid the potential stimulatory effects of MHC class I tetramer binding, a second method for isolating P14 CD8+ T cells was also used. Naive congenic Thy-1.1 P14 CD8+ T cells were transferred into B6 (Thy-1.2) mice that were subsequently infected with LCMV. At the indicated times p.i., effector and memory P14 CD8 T cells were isolated based on CD8 and Thy-1.1 staining. The P14 CD8+ T cells isolated by either Thy-1.1 expression or tetramer binding are phenotypically and functionally indistinguishable (data not shown).

Induction of telomerase activity in LCMV-specific effector and memory CD8+ T cells

The isolation of purified naive CD8+ T cells and of LCMV-specific effector and memory CD8+ T cells permitted an analysis of telomerase expression in these populations. Compared with low or undetectable telomerase activity in naive BALB/c CD8+ cells, LNP118–126+ CD8+ primary effector cells harvested on day 8 p.i. have increased telomerase activity (Fig. 2). Moreover, memory cells harvested 60 days post-LCMV infection also expressed higher levels of telomerase activity than those detected in naive CD8+ cells (Fig. 2). In the experiments reported here, telomerase activity was measured in memory cells isolated from 47–130 days after viral infection and was sustained at undiminished levels over this time range (data not shown), indicating that telomerase expression persists in relatively long-lived memory cells. Secondary effector cells induced by a repeated LCMV challenge 32–177 days after initial infection expressed similarly high levels of telomerase, demonstrating that telomerase was expressed during the clonal expansion that occurs in response to viral re-exposure (Fig. 2). A similar pattern of telomerase regulation was observed in purified D5gp33-41 CD8+ T cells isolated from P14 chimeric animals (Fig. 3). Thus, these results demonstrate that in both model systems telomerase activity is induced in both LCMV-specific primary and secondary effector cells and in memory cells. Furthermore, although telomerase activity in primary and secondary effector CD8+ T cells is induced by acute Ag stimulation, these results suggest that since LCMV-specific memory CD8+ T cells persist in the absence of Ag (4), the continued expression of telomerase in memory CD8+ cells occurs independently of further Ag contact.

Regulation of telomere length in LCMV-specific CD8+ T cells

It has been proposed that telomerase can maintain telomere length during cell division and thus maintain replicative capacity (12, 13, 17, 19). Preservation of telomere length might thus be important in supporting the capacity for cell division in both the primary and secondary responses to LCMV and in the maintenance of memory. To investigate telomere length in CD8+ T cells undergoing extensive replication in response to LCMV infection, (BALB/c × SPRET/Ei)F1 mice were analyzed. Previous studies have demonstrated that the lengths of telomeres present in cells from (M. musculus × SPRET/Ei) interspecies cross are bimodal. One population of telomeres is long (>20 kb) and is derived from M. musculus (BALB/c or B6), while the other subset of telomeres is substantially shorter (9.4–11.5 kb) and is derived from the elongation of SPRET/Ei parental telomeres (6.5–9.5 kb). Measurement of the

![FIGURE 2](http://www.jimmunol.org/Downloaded_from http://www.jimmunol.org)
short SPRET/Ei-derived telomeres in F1 lymphocytes has previously been shown to provide a sensitive assay of both telomere shortening and telomere elongation in vivo (21, 23) (Fig. 4A, boxed area). (B6 × SPRET/Ei)F1 mice that are heterozygous knockouts for the telomerase RNA template (17) show a functional haplo-insufficiency in their ability to elongate short Spret/Ei telomeres. That is, heterozygous (BALB/c × SPRET/Ei)F1 mice have SPRET/Ei-derived telomeres that are approximately 2–3 kb shorter than those in wild-type (BALB/c × SPRET/Ei)F1 mice, as illustrated in Fig. 4A. Thus, if telomere length is altered in CD8+ T cells that are undergoing extensive replication in response to LCMV infection, changes of this magnitude should be detectable in the short telomeres of these F1 mice. (BALB/c × SPRET/Ei)F1 mice were challenged with LCMV, and LdNP118–126+ CD8+ T cells were isolated 8 days (primary effector cells) and 60 days (memory cells) later. In addition, a group of LCMV-immune (BALB/c × SPRET/Ei)F1 mice was reinfected with LCMV after the primary infection was resolved, and LdNP118–126+ secondary effector CD8+ T cells were isolated 5 days later. Both primary and secondary LdNP118–126+ effector cells as well as memory CD8+ T cells isolated from LCMV-infected (BALB/c × SPRET/Ei)F1 mice had levels of telomerase activity equivalent to amounts detected in comparable cell populations isolated from BALB/c mice (data not shown). Despite the substantial number of times the cells divided during the generation of primary effector and memory T cells (~10–20 cell divisions) and during the generation of secondary effectors (an additional ~8–10 divisions) (2, 5), effector and memory T cells have telomeres similar in length to those detected in naive CD8+ T cells isolated from uninfected mice (Fig. 4B). This result is consistent with a model in which telomerase activity maintains telomere length in proliferating LCMV-specific effector and memory T cells.

**Discussion**

The purpose of the present study was to address the potential contribution of telomerase to clonal expansion of both primary and secondary LCMV-specific effector CD8+ T cells and to the maintenance of LCMV-specific memory. Previous reports have demonstrated that T cells respond acutely to Ag or mitogen stimulation in vivo and in vitro by proliferating and expressing telomerase (14, 15, 18). The potential role of telomerase in the maintenance of memory is less well characterized. This report demonstrates that LCMV-specific memory T cells express telomerase at levels equivalent to those present in highly activated effector cells generated during the acute phase of LCMV infection. The fact that telomerase remains active in memory T cells >4 mo post-viral infection suggests that this activity can be maintained independently of antigenic stimulation. Thus, this model is uniquely relevant to acute viral infection and, importantly, to strategies for immunization in which nonpersistent antigenic challenge induces long-lasting immunity (i.e., in response to vaccines).
The regulation of telomerase activity and telomere length in T and B lymphocytes has been studied in human and mouse models. Although it has been suggested that mechanisms of in vitro senescence and in vitro telomerase regulation may differ in these two species (24), it is remarkable that in vivo regulation of telomerase activity in lymphocytes is quite concordant in these species (25). During both mouse and human T cell development, telomerase is expressed in developing thymocytes (12–15, 17) (K. Hathcock, unpublished observations), is down-regulated to low or undetectable levels in unstimulated mature peripheral T cell populations, and is induced to high levels in Ag-stimulated populations of mature T cells.

As has been shown for LCMV in mice, the response to EBV infection in humans is marked by extensive replication and differentiation of virus-specific CTL and memory cells. However, in contrast to LCMV (Armstrong strain) where infection is self-limiting and memory CD8+ cells persist in the absence of virus, infection with EBV results in chronic infection, and patients are susceptible to episodic rounds of disease (26). Previous studies (7–28) have reported that EBV-specific effector cells express high levels of telomerase activity, but cells isolated after resolution of the initial infection have substantially lower amounts of telomerase. Since EBV infection is a chronic infection, it is unknown whether the telomerase activity detected in cells that are isolated after resolution of initial infection is expressed by memory cells or by effector cells generated during episodic disease. Thus, telomerase regulation in response to acute and self-limited infection with LCMV may differ from that observed in chronic infections such as EBV and may be a relevant model for long term memory induced by vaccination.

The results presented here demonstrate for the first time that telomerase activity can be maintained in memory CD8+ T cells for extended periods of time in the absence of antigenic stimulation. Moreover, this study demonstrates that effector cells and memory cells, which have undergone extensive replication, express high levels of telomerase and maintain stable telomere length. Taken together, these results are consistent with the hypothesis that proliferating Ag-specific CD8+ T cells maintain their telomeres through the action of telomerase. The most direct evidence that telomerase is necessary for maintenance of telomere length in vivo has been provided by studies of telomerase-deficient mice. Telomeres shorten in these mice with successive generations of telomerase deficiency, leading to infertility and reduced proliferative capacity of somatic cells (17, 29). We are currently breeding late generation telomerase-deficient mice to directly examine the consequences of telomerase deficiency and telomere shortening on virus-specific effector generation and memory maintenance.

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