

Viral Infections Induce Abundant Numbers of Senescent CD8 T Cells¹

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Viral infections are often accompanied by extensive proliferation of reactive CD8 T cells. After a defined number of divisions, normal somatic cells enter a nonreplicative stage termed senescence. In the present study we have identified the inhibitory killer cell lectin-like receptor G1 (KLRG1) as a unique marker for replicative senescence of murine CD8 T cells. KLRG1 expression was induced in a substantial portion (30–60%) of CD8 T cells in C57BL/6 mice infected with lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus, or vaccinia virus. Similarly, KLRG1 was found on a large fraction of LCMV gp33 peptide-specific TCR-transgenic (tg) effector and memory cells activated *in vivo* using an adoptive transfer model. Transfer experiments with CFSE-labeled TCR-tg cells into LCMV-infected hosts further indicated that induction of KLRG1 expression required an extensive number of cell divisions. Most importantly, KLRG1⁺ TCR-tg effector/memory cells could efficiently lyse target cells and secrete cytokines, but were severely impaired in their ability to proliferate after Ag stimulation. Thus, this study demonstrates that senescent CD8 T cells are induced in abundant numbers during viral infections *in vivo*. *The Journal of Immunology*, 2001, 167: 4838–4843.

Killer cell lectin-like receptors (KLRs)⁵ expressed on CD8 T cells may function as inhibitory receptors and may thereby modulate the immune response (1–3). Although expression of certain KLR family members can be induced in human T cells by cytokines *in vitro* (4), the role and the regulation of these molecules on T cells *in vivo* is poorly defined. We have recently identified the mouse homolog of the KLR member G1 (KLRG1) and we found that KLRG1 mRNA was expressed in murine NK cells and activated CD8 T cells (5, 6). KLRG1 was originally identified in the rat as a mast cell function-associated Ag (7). It is a type II transmembrane glycoprotein with an extracellular domain homologous to C-type lectins and with a cytoplasmic tail that contains an immunoreceptor tyrosine-based inhibition motif. The physiological role of KLRG1 and its potential ligand(s) *in vivo* is still elusive. Cross-linking of rat KLRG1 by a mAb has been shown to inhibit the secretory response to FcεRI stimulation in a basophilic leukemia cell line (8). The inhibitory property of rat

KLRG1 fits well with its structural similarity to other members of the KLR family that function as inhibitory receptors on NK and CD8 T cells (i.e., Ly49 and CD94/NKG2). However, attempts to demonstrate an inhibitory function of KLRG1 on mouse NK cells have failed so far (6). To analyze expression of KLRG1 on T cells at the protein level, we have used a mAb specific for mouse KLRG1. We demonstrate in this study that KLRG1 defines a subset of CD8 effector/memory T cells, which could lyse target cells and secrete cytokines, but lost the ability to vigorously proliferate after Ag contact. Moreover, our data show that these senescent cells are induced in abundant numbers during viral infection *in vivo*.

Materials and Methods

Mice

C57BL/6 (B6) mice were obtained from our breeding colony or from Harlan Winkelmann (Borchen, Germany). DBA/2 mice were purchased from Harlan Olac (Bicester, U.K.). Thy1.1⁺ lymphocytic choriomeningitis virus (LCMV) gp33 TCR-transgenic (tg) mice (line 318) have been described (9, 10). Animals were kept under conventional conditions and were used, unless otherwise indicated, for experiments 6–16 wk after birth.

Viruses and tumors

The LCMV-WE virus strain was originally obtained from R. Zinkernagel (University Hospital Zurich, Zurich, Switzerland) and was grown on L929 fibroblast cells with a low multiplicity of infection. Vesicular stomatitis virus Indiana (VSV_{IND}, Mudd-Summers isolate) was grown with a low multiplicity of infection on BHK cells. Stocks of vaccinia virus (VV) strain WR were produced by infecting BSC 40 cells with a low multiplicity of infection. Mice were infected (i.v.) with 200 pfu LCMV, 2 × 10⁶ pfu VV, or 2 × 10⁶ pfu VSV_{IND} and were examined on day 6 (VV or VSV_{IND}) or day 8 (LCMV) after infection. Adult DBA/2 mice were injected i.p. with 10⁷ live P815 tumor cells transfected with the HLA-Cw3 gene (11). Two weeks thereafter, animals were bled and PBL were analyzed.

Flow cytometry

The generation of the mAb 2F1 specific for mouse KLRG1 has been described (12) and was used as a biotinylated Ab. The following mAbs were purchased from BD Pharmingen (San Diego, CA): FITC- and CyChrome-conjugated anti-CD8, PE-conjugated anti-CD62L, FITC-conjugated anti-TCR Vβ10, and biotinylated and PE-conjugated anti-Thy 1.1. Biotinylated mAbs were detected by allophycocyanin-streptavidin (BD Pharmingen). PE-conjugated MHC class I (H-2D^b) tetramers complexed with LCMV

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⁵ Abbreviations used in this paper: KLR, killer cell lectin-like receptor; KLR member G1, KLRG1; LCMV, lymphocytic choriomeningitis virus; VSV_{IND}, vesicular stomatitis virus Indiana; VV, vaccinia virus; DC, dendritic cell; tg, transgenic.

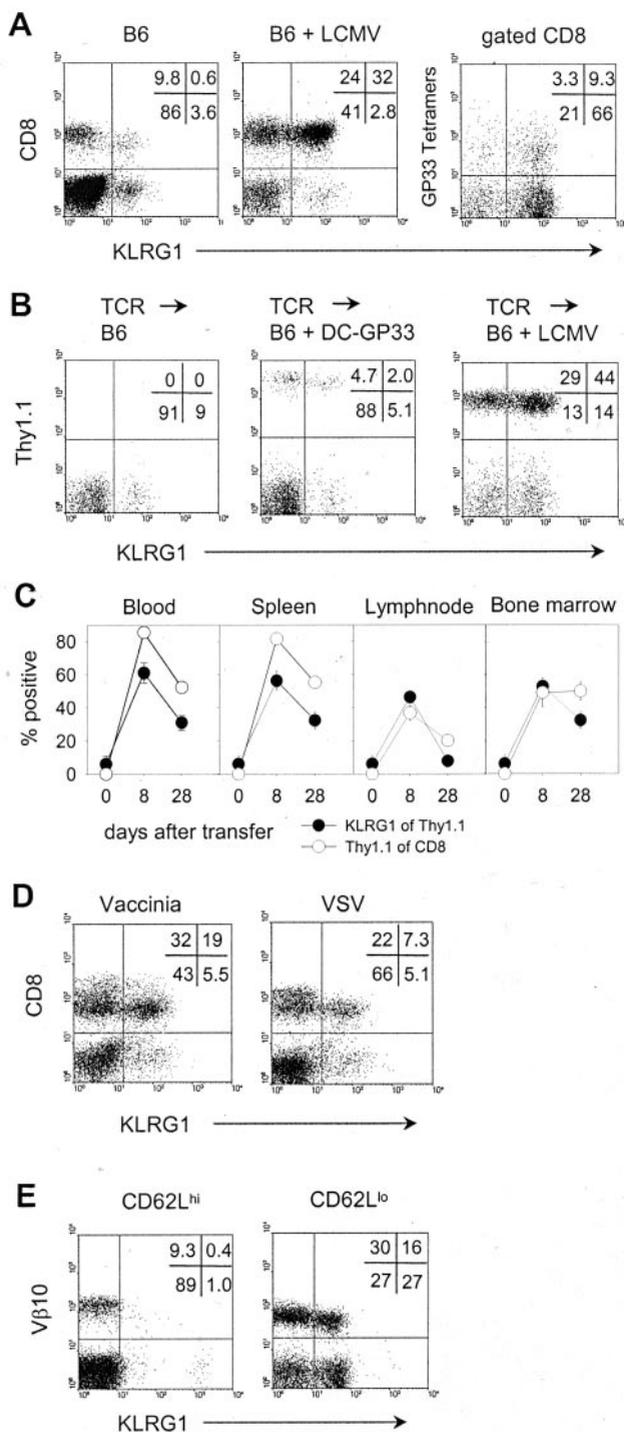


FIGURE 1. KLRG1⁺ CD8 T cells are generated in abundant numbers after Ag stimulation. *A*, KLRG1 expression on LCMV-specific CD8 T cells. PBL from uninfected (*left*) or acutely (day 8) LCMV-infected B6 mice (*middle* and *right*) were stained with anti-CD8 and anti-KLRG1 mAb and with MHC tetramers (D^b-LCMV gp33). *B*, KLRG1 expression on in vivo activated TCR-tg cells specific for LCMV peptide gp33. Thy1.1⁺ TCR-tg cells (10⁵) were transferred into nonirradiated Thy1-congenic B6 mice. After transfer, recipient mice were immunized with LCMV gp33 peptide-loaded DCs (*middle*) or were infected with LCMV (*right*). KLRG1 expression was determined at the peak of expansion of TCR-tg cells on day 4 after DC priming or on day 8 after LCMV infection. The dot plots shown are gated on CD8 T cells of PBL. *C*, Kinetics of KLRG1 expression and expansion of transferred TCR-tg cells after LCMV infection. The percentage of KLRG1⁺ cells of Thy1.1⁺ TCR-tg cells (●) and the percentage of Thy1.1⁺ TCR-tg cells of total CD8 T cells (○) was determined at the indicated time points. *D*, KLRG1 expression on CD8 T cells from B6 mice

gp33 peptide (13) were a kind gift of Dr. T. Dumrese (University Hospital Zurich). Cells were analyzed on a FACSort flow cytometer (BD Biosciences, Mountain View, CA) using CellQuest software.

Adoptive cell transfers

Spleen cells containing 10⁵ naive TCR-tg cells were injected (i.v.) into nonirradiated B6 mice. On the same day, recipient mice were either primed with 10⁶ bone marrow-derived gp33 peptide-loaded dendritic cells (DCs) as described (14) or were infected with LCMV. For the cell cycling experiments, spleen cells containing 10⁷ naive or memory TCR-tg cells were labeled with CFSE (Molecular Probes, Eugene, OR) and were injected (i.v.) into B6 recipient mice that had been infected with LCMV 3 days before. Spleen cells were isolated on day 2 (memory transfer) or day 4 (naive transfer) after transfer. TCR-tg memory cells were generated in vivo by adoptive transfer of 10⁵ naive TCR-tg cells into B6 mice followed by infection with LCMV; they were isolated 4 wk after transfer and infection. For the repetitive transfer experiments, spleen cells containing 10⁵ naive TCR-tg cells were injected into B6 recipient mice, followed by infection with LCMV. Thirty days after transfer and infection, spleen cells of the recipient mice containing 10⁵ TCR-tg cells (primary memory cells) were injected a second time into B6 recipient mice, followed by infection with LCMV. The third transfer of secondary TCR-tg memory cells was performed under the same conditions.

CTL assay and IFN- γ secretion

Spleen cells containing 10⁵ TCR-tg cells were transferred into B6 mice followed by injection of LCMV. After 8 days, spleen cells were stained with biotinylated 2F1/allophycocyanin-streptavidin and PE-conjugated anti-Thy 1.1 and were sorted on a high speed cell sorter (MoFlo; Cytomation, Fort Collins, CO). The cytolytic activity was determined in a 5-h ⁵¹Cr-release assay using EL-4 target cells coated with the LCMV gp33 peptide (KAVYNFATM) or with the control adenovirus peptide E1A₂₃₄₋₂₄₃ (SGPSNTPEI). For intracellular cytokine staining, TCR-tg effector cells were cultured for 5 h together with B6 spleen cells pre-coated (2 h, 37°C, 10⁻⁶ M) with gp33 peptide in medium supplemented with brefeldin A (GolgiStop; BD Pharmingen). Afterward, they were surface-stained for Thy1.1 and KLRG1, fixed, permeabilized, and stained intracellularly using a PE-conjugated anti-IFN- γ mAb (BD Pharmingen).

Stimulation of TCR-tg memory cells

TCR-tg memory cells were generated in vivo by adoptive transfer of 10⁵ TCR-tg cells into B6 mice followed by infection with LCMV; they were isolated 4 wk after transfer and infection. TCR-tg memory cells were stained with biotinylated 2F1 mAb/allophycocyanin-streptavidin and PE-conjugated anti-Thy 1.1 mAb and were sorted on a high-speed cell sorter (MoFlo; Cytomation). For the in vitro experiments, the isolated Thy1.1⁺ KLRG1⁻ and Thy1.1⁺ KLRG1⁺ cells were labeled with CFSE and cultured (1.5 × 10⁵/well) in 24-well plates together with B6 spleen cells (4 × 10⁶/well) pre-coated (2 h, 37°C, 10⁻⁶ M) with LCMV gp33 peptide (KAVYNFATM). After 5 days, the cultures were harvested and the number of Thy1.1⁺ cells of total cultured cells and the CFSE content of Thy1.1⁺ cells was determined by flow cytometry. For the in vivo experiments, Thy1.1⁺ KLRG1⁻ and Thy1.1⁺ KLRG1⁺ cells (10⁵) were injected into B6 recipient mice, followed by infection with LCMV.

Results

Induction of KLRG1 expression on a large portion of CD8 T cells after viral infection

We have used the 2F1 mAb (12) to analyze cell surface expression of KLRG1 on CD8 T cells (Fig. 1). KLRG1 was expressed on a small portion (<10%) of CD8 T cells from naive C57BL/6 (B6) mice, whereas in mice acutely infected with LCMV, more than one-half of the CD8 T cells were KLRG1⁺. Analysis using MHC

acutely infected with VV or VSV. PBL were stained 6 days after infection with anti-CD8 and anti-KLRG1 mAb. *E*, KLRG1 expression on CD8 T cells stimulated by tumor cells in vivo. DBA/2 mice were injected i.p. with live P815 tumor cells transfected with the HLA-Cw3 gene (10). After 2 weeks, KLRG1 expression on peripheral CD8 T cells was analyzed. The dot plots shown are gated on CD8⁺CD62L^{high} (*left*) or CD8⁺CD62L^{low} (*right*) T cells.

class I tetramers (D^b-gp33) revealed that 70–80% of CD8 T cells specific for the LCMV peptide gp33–41 expressed KLRG1 during the acute phase of the infection (Fig. 1A, right). Next, KLRG1 expression was examined on in vivo activated TCR-tg CD8 T cells specific for LCMV peptide gp33–41, using an adoptive transfer system (15). In this system, transferred TCR-tg cells, traced by Thy1.1-specific Abs, remained below detection limit in the recipient mice without Ag stimulation (Fig. 1B, left). Injection of LCMV gp33 peptide-coated DCs into the recipient mice induced clonal expansion of donor TCR-tg cells, and at the peak of expansion (day 4), ~30% of the induced TCR-tg cells expressed KLRG1 (Fig. 1B, middle). Clonal expansion of TCR-tg cells was more vigorous after LCMV infection, resulting in an increased number and percentage of TCR-tg cells that expressed KLRG1 (Fig. 1B, right). After the acute phase of the infection, the relative number of KLRG1⁺ cells in the TCR-tg population slowly declined from ~60% at the peak to ~30% in the memory phase. KLRG1 expression on TCR-tg cells after LCMV infection was also observed in spleen, lymph node, and bone marrow (Fig. 1C). To extend our analysis to other viral systems, B6 mice acutely infected with VV or VSV were examined. As shown in Fig. 1D, KLRG1 was induced in 30–50% of the CD8 T cells in these mice. Finally, KLRG1 expression was determined on CD8 T cells induced by HLA-Cw3-transfected P815 tumor cells in vivo (11). In this system, tumor cell injection leads to expansion of tumor Ag-specific CD8 T cells predominantly expressing TCR V β 10. As illustrated in Fig. 1E, KLRG1 was expressed on ~30% of TCR V β 10⁺ CD8 T cells activated by P815/HLA-Cw3 tumor cells in vivo. Importantly, KLRG1 expression was restricted to activated T cells with down-regulated CD62L. Taken together, these data show that KLRG1 expression was induced in a substantial portion of CD8 T cells after stimulation by viral and tumor Ags in vivo.

Induction of KLRG1 expression on naive CD8 T cells requires a large number of cell divisions

The increased percentage of KLRG1⁺ TCR-tg cells after LCMV infection, when compared with results obtained with weaker DC/gp33 priming (Fig. 1B), suggested a correlation between KLRG1 expression and cell proliferation in vivo. Therefore, KLRG1 expression was compared on activated TCR-tg cells that differed in the number of cell cycles. For this purpose, naive TCR-tg cells were transferred at different time points into preinfected recipient mice. This set-up allowed variation of extent and duration of Ag exposure, i.e., proliferation stimulus for the transferred T cells. Transfer of TCR-tg cells into LCMV-infected recipient mice on day 1 after infection resulted in vigorous proliferation and a high level of KLRG1 expression (60%) on day 6 (Fig. 2A, left). When TCR-tg cells were transferred 2 days after infection, donor cells were already detectable on day 4 after transfer; however, at this early time point, only ~20% of the donor cells were KLRG1⁺. Afterward, the number of TCR-tg cells, and, concomitantly, the frequency of KLRG1⁺ cells in the TCR-tg population increased up to 50%. Finally, KLRG1 expression remained relatively low (~15%) in TCR-tg cells derived from a 30-fold higher inoculum of TCR-tg donor cells that were transferred on day 4 after infection, when viral titers already start to decline (Fig. 2A, right). Taken together, these results indicate that KLRG1 expression on naive CD8 T cells depends on the number of cell divisions. However, it is possible that additional factors besides cell cycling are also required for induction of KLRG1 expression. These environmental factors could be induced by viral infections (i.e., type 1 IFN levels) or tumor cell growth in vivo.

To determine the number of cell cycles required for KLRG1 expression, transfer experiments with CFSE-labeled TCR-tg cells

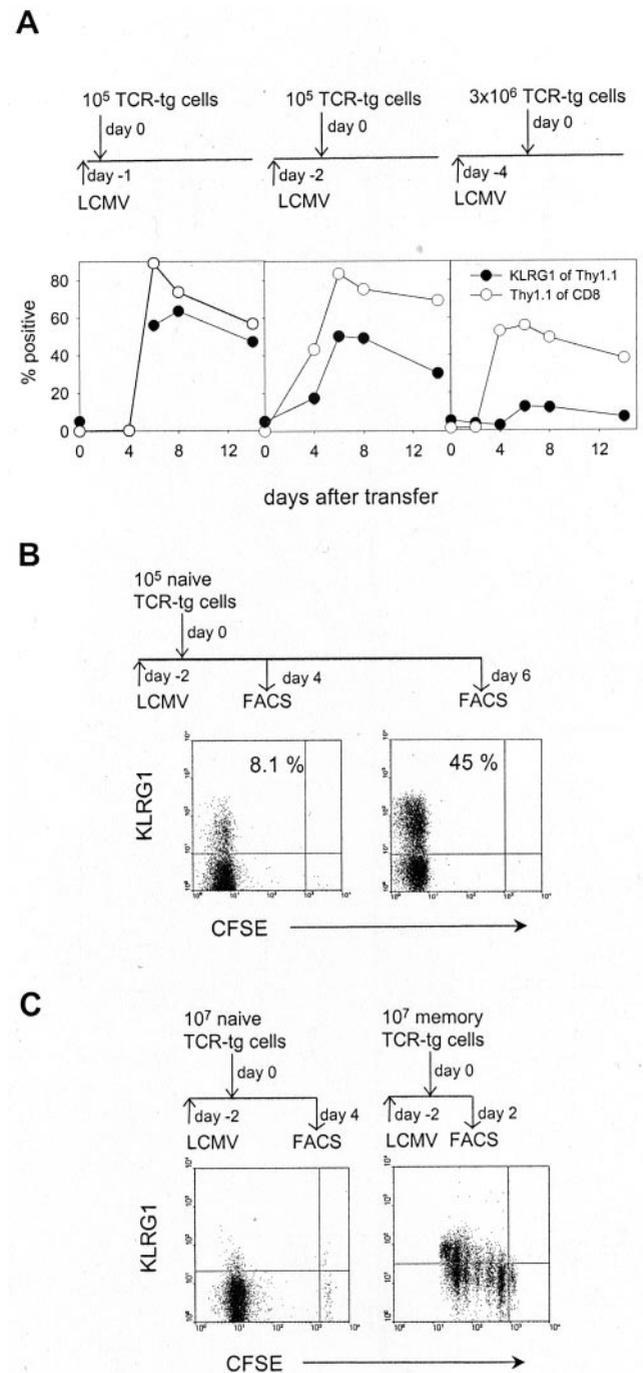


FIGURE 2. Cell cycling-dependent expression of KLRG1. **A**, TCR-tg cells (10^5 or 3×10^6) were transferred into LCMV-infected B6 mice, 1 (left), 2 (middle), or 4 days (right) after infection. At the time points indicated, the percentage of Thy1.1⁺ cells of Thy1.1⁺ TCR-tg cells (●) and the percentage of Thy1.1⁺ TCR-tg cells of total CD8 T cells (○) was determined. **B**, Thy1.1⁺ TCR-tg cells (10^5) were labeled with CFSE and transferred into LCMV-infected B6 recipient mice. PBL of the recipient mice were analyzed 4 (left) and 6 (right) days after transfer. The dot plot shows CFSE vs KLRG1 expression gated on the donor Thy1.1⁺ cells. **C**, Naive (left) or memory (right) Thy1.1⁺ TCR-tg cells (10^7) were labeled with CFSE and transferred into LCMV-infected B6 recipient mice. Spleen cells were analyzed on day 2 (memory transfer) or 4 (naive transfer) after transfer. The dot plot shows CFSE vs KLRG1 expression gated on the donor Thy1.1⁺ cells.

were performed. When TCR-tg cells (10^5) were injected into LCMV-infected mice, the transferred cells cycled rapidly and had completely lost the CFSE label 4 days later (Fig. 2B, left). However, KLRG1 expression was induced on only a few (8–10%) of

these cells. Two days later, the frequency of KLRG1⁺ cells increased to 40–50% (Fig. 2*B*, left). This result indicated that the 7–9 cell divisions required to decrease the CFSE label to background levels were not sufficient to significantly induce KLRG1 expression in naive TCR-tg cells. This experiment was repeated using TCR-tg memory cells, which have a replication history and therefore might be expected to express KLRG1 after less cycles than naive cells. To trace adequate numbers of donor TCR-tg cells early after transfer in the infected recipient mice, higher numbers (10^7) of donor cells were used for these experiments. As shown in Fig. 2*C*, right, KLRG1 expression was induced in TCR-tg memory cells 2 days after transfer into LCMV-infected hosts in a cell cycling-dependent manner. In contrast, naive TCR-tg cells transferred under the same experimental conditions did not significantly express KLRG1 even 4 days after transfer. Thus, memory cells “remembered” the number of cell cycles they had undergone during the initial expansion phase.

To provide further evidence for the cell cycling-dependent expression of KLRG1, TCR-tg cells were repetitively stimulated by LCMV using sequential adoptive transfer protocols. TCR-tg cells stimulated once in vivo (primary memory cells) proliferated less vigorously than naive TCR-tg cells after adoptive transfer and Ag stimulation. However, the percentage of KLRG1⁺ cells in the donor cell population was significantly increased in the secondary and memory phase (Fig. 3*A*, left vs middle). In tertiary transfers, TCR-tg cells that had been stimulated twice in vivo (secondary memory cells) failed to proliferate to detectable levels in four of six recipient mice after the third Ag challenge. In two recipient mice, a low level of expansion of TCR-tg cells was observed. Strikingly, KLRG1 was expressed on almost all (>98%) of these cells, which failed to proliferate vigorously (Fig. 3*A*, right).

To exclude the possibility that the failure of TCR-tg cells to expand in the third transfer was due to a more rapid viral clearance induced by other memory T or B cells, which were also present in the transferred donor cell inoculum, expansion of host CD8 T cells and their KLRG1 expression was determined. As shown in Fig. 3*B*, bottom, the percentage of host CD8 T cells in PBL also increased from ~10% up to 40–60% in the third transfer. Similarly, extent and kinetics of KLRG1 expression on these host CD8 T cells were comparable in all three transfers. Thus, these data strongly argue against the possibility that the failure of TCR-tg cells to expand a third time was due to a more rapid viral clearance.

KLRG1⁺ and KLRG1⁻ T cells do not differ in effector cell function

The adoptive transfer experiments revealed that TCR-tg effector cells induced by LCMV infection could be divided into discrete KLRG1⁺ and KLRG1⁻ populations (Fig. 1*B*, right). To correlate KLRG1 expression with functional activity, KLRG1⁺ and KLRG1⁻ TCR-tg effector cells were purified by fluorescence-activated cell sorting and were then tested for cytolytic activity and cytokine secretion. The two cell populations exhibited similar levels of lytic activity on EL-4 target cells coated with LCMV gp33 peptide, and IFN- γ production after gp33 Ag stimulation did not differ (Fig. 4).

KLRG1 expression is associated with replicative senescence

The repetitive transfer experiments shown in Fig. 3 suggested a correlation between KLRG1 expression and reduced replicative potential. To directly address this issue, KLRG1⁺ and KLRG1⁻ TCR-tg memory cells were purified by fluorescence-activated cell sorting and were stimulated in vitro and in vivo. For the in vitro

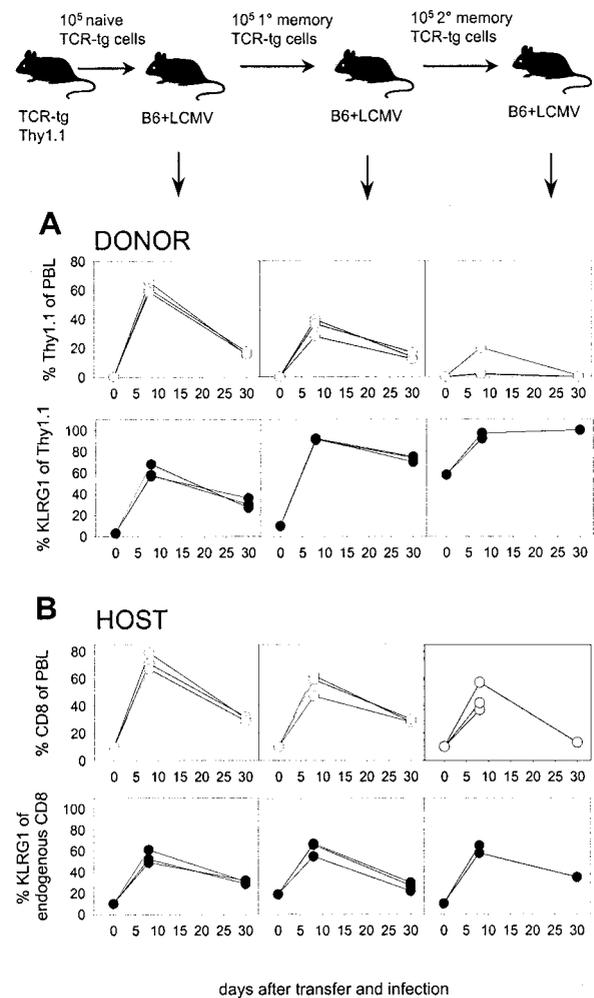


FIGURE 3. Repetitive Ag stimulation of CD8 T cells increases KLRG1 expression and leads to replicative senescence. Thy1.1⁺ TCR-tg memory cells (10^5) from primary transfers (primary memory cells) were transferred into B6 mice followed by LCMV infection. After 30 days, TCR-tg cells (10^5) from these secondary transfers (secondary memory cells) were isolated and transferred for a third time into B6 recipient mice followed by LCMV infection. *A*, In each transfer, the percentage of donor Thy1.1⁺ TCR-tg cells of total CD8 T cells (○) and the percentage of KLRG1⁺ cells of Thy1.1⁺ TCR-tg cells (●) was determined at the indicated time points. Secondary memory cells failed to proliferate at detectable levels in four of six recipient mice after LCMV infection. In *B*, the expansion of host CD8 T cells (% CD8⁺Thy1.1⁻ of PBL) and percentage of KLRG1 expression on these cells in the repetitive transfer experiments shown above are depicted.

experiments, KLRG1⁺ and KLRG1⁻ TCR-tg memory cells were labeled with CFSE and were stimulated in cultures containing LCMV gp33 peptide-loaded spleen cells as APC. KLRG1⁻ TCR-tg memory cells (Thy1.1⁺) proliferated vigorously, as indicated by an increase of Thy1.1⁺ cells in the culture (Fig. 5*A*, left) and a decrease in their CFSE content (Fig. 5*A*, right). The proliferative potential of KLRG1⁺ TCR-tg memory was reduced ~10-fold and the TCR-tg cells showed strikingly less cell divisions when compared with their KLRG1⁻ counterpart. The same result was obtained when the proliferation of KLRG1⁺ and KLRG1⁻ TCR-tg memory cells was compared after adoptive transfer and Ag challenge in vivo (Fig. 5*B*, left). Importantly, the transferred KLRG1⁺ TCR-tg memory cells remained KLRG1⁺, whereas KLRG1 expression was induced in ~60% of the initially KLRG1⁻ TCR-tg memory cells after in vivo proliferation (Fig. 5*B*, right). Taken together, these data demonstrate

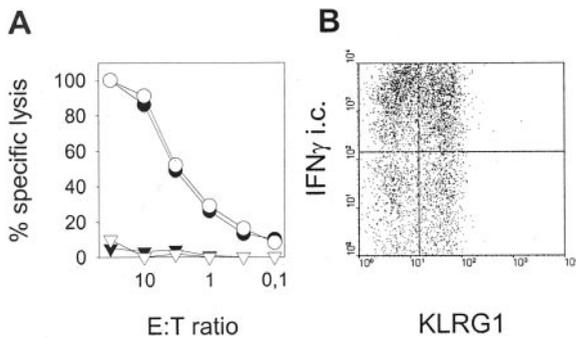


FIGURE 4. KLRG1⁺ TCR-tg cells are cytolytic and secrete IFN- γ . *A*, Cytolytic activity of FACS-purified KLRG1⁺ (filled symbols) and KLRG1⁻ (open symbols) TCR-tg effector cells determined on EL-4 target cells coated with gp33 (circles) or control adenovirus peptide (triangles). *B*, KLRG1 expression vs IFN- γ production of TCR-tg effector cells. TCR-tg effector cells were cultured for 5 h in the presence of the LCMV gp33 peptide. Afterward, they were surface-stained for Thy1.1 and KLRG1, fixed, permeabilized, and stained intracellularly for IFN- γ . The dot plot shown was gated on Thy1.1⁺ TCR-tg cells.

that replicative senescence of Ag-stimulated CD8 T cells in vivo is accompanied by KLRG1 expression.

The numbers of KLRG1⁺ T cells are increased in aged mice both in the CD4 and CD8 subset

The observed link between KLRG1 expression and senescence led us to compare the number of KLRG1⁺ T cells in young and aged

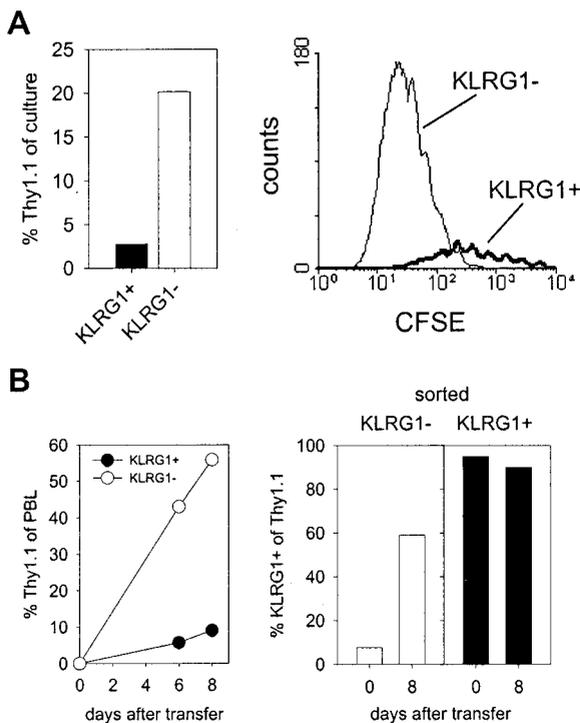


FIGURE 5. KLRG1⁺ TCR-tg cells fail to proliferate in vitro and in vivo. *A*, FACS-purified KLRG1⁺ (filled symbols) and KLRG1⁻ (open symbols) Thy1.1⁺ TCR-tg memory cells were labeled with CFSE and were cultured together with gp33 peptide-loaded B6 spleen cells. After 5 days, the percentage of Thy1.1⁺ cells of total cultured cells (*left*) and their CFSE content (*right*) was determined. *B*, FACS-purified KLRG1⁺ (filled symbols) and KLRG1⁻ (open symbols) Thy1.1⁺ TCR-tg memory cells were transferred into B6 mice followed by LCMV infection. At the indicated time points after transfer and infection, the percentage of Thy1.1⁺ TCR-tg cells of total CD8 T cells in the recipient mice was determined (*left*). Additionally, the percentage of KLRG1 expression on the FACS-purified TCR-tg populations before and after transfer and infection is depicted (*right*).

B6 mice that had not undergone deliberate immunization (Fig. 6). In young adult mice, only a few (<1%) of CD4 or CD8 T cells expressed KLRG1. In mice 18–20 wk of age, KLRG1 was found on 1.2% of CD4 and 11.4% of CD8 T cells, whereas in 1.5-year-old mice, KLRG1 expression was further increased to 2.5% in CD4 and 13.3% in CD8 T cells.

Discussion

The present report demonstrates that senescent CD8 T cells can be identified by expression of KLRG1 and that senescent CD8 T cells are induced in abundant numbers during viral infections in vivo. Recent reports have shown that members of the KLR family such as NK1.1, DX-5, and Ly49 receptors are expressed on subpopulations of murine CD8 T cells with a memory phenotype (16, 17). It is important to stress that these markers were present only at low frequencies (<5%) in the TCR-tg effector and memory populations analyzed in this study (data not shown), indicating that KLRG1 has a unique expression pattern on CD8 T cells. KLRG1 also differs from other known effector (CD43) or memory (CD44) markers because it is found only on a fraction of these cell populations (Fig. 1), which all express CD43 and/or CD44 at a high level (15, 18).

Our data demonstrate that KLRG1 was only expressed on T cells that had undergone a large number of cell divisions. Besides cell cycling, however, a “conditioned environment” in which T cells are stimulated may also be required for KLRG1 expression. Cytokines induced in vivo by viral infection or by tumor cell injection could represent essential factors for induction of KLRG1 expression in vivo. This notion is further supported by our failure to induce KLRG1 expression in long-term T cell cultures from TCR-tg mice stimulated weekly with Ag for 3 mo (data not shown).

The CFSE experiments shown in Fig. 2 indicate that seven to nine cell divisions were not sufficient to induce KLRG1 expression in naive TCR-tg cells and that KLRG1 induction in TCR-tg memory cells required additional six to seven cell cycles. We are not aware of any other gene induced during T cell activation that exhibits a comparable induction kinetics. The precise number of cell cycles required for KLRG1 induction in naive T cells is unknown. After LCMV infection, Ag-specific CD8 T cells expand $\sim 10^3$ - to 10^4 -fold corresponding to 10–15 population doublings (13, 15,

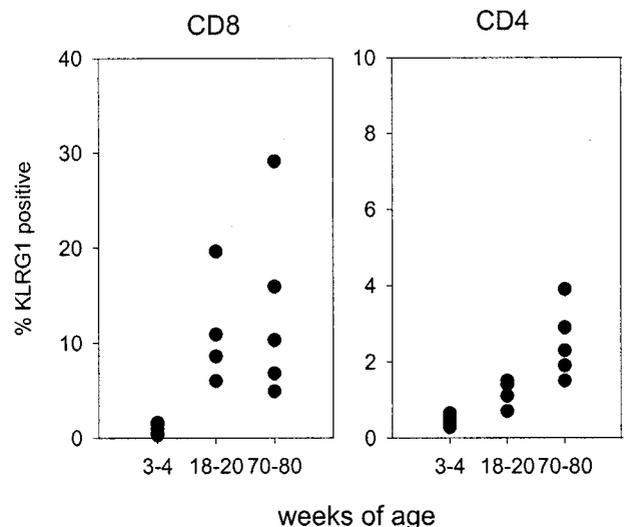


FIGURE 6. The number of KLRG1⁺ T cells increases with age. PBL from B6 mice that had not undergone deliberate immunization were stained with anti-CD4, anti-CD8, and anti-KLRG1 mAb. The percentage of KLRG1⁺ cells among CD8 (*left*) and CD4 (*right*) T cells in individual mice is depicted, and the age of the mice is indicated.

19). However, it is possible that apoptosis also occurs during the expansion phase and therefore, the actual number of population doublings of some LCMV-specific CD8 T cells may even be higher. In addition, it is likely that proliferation of the induced T cells is not synchronous and some T cells may proliferate more than others. The occurrence of discrete KLRG1⁺ and KLRG1⁻ populations among LCMV-specific T cells both in normal B6 mice and in the transfer model supports this notion. Thus, KLRG1⁺ CD8 T cells may have undergone considerably >10–15 population doublings after LCMV stimulation *in vivo*.

The increased number of KLRG1⁺ CD8 T cells in aged B6 mice when compared with young mice agrees well with the postulated cell division-dependent expression of KLRG1. In young adult mice, KLRG1 expression on CD4 T cells was at the detection limit and, in contrast to CD8 T cells, was also not significantly induced in the course of viral infections (data not shown). However, in aged mice a small but significant number of KLRG1⁺ T cells was also found in the CD4 subset. This suggests that KLRG1 can also be expressed in CD4 T cells that have undergone sufficient cell divisions. Indeed, in preliminary experiments we were able to induce KLRG1 expression in LCMV-specific CD4 T cells from SMARTA TCR-tg mice using sequential adoptive transfer protocols (data not shown). The lower frequency of KLRG1⁺ CD4 T cells in aged B6 mice when compared with CD8 can be readily explained by the generally observed lower clonal expansion rate of CD4 vs CD8 T cells after Ag challenge *in vivo* (20–24).

Using fetal human fibroblasts, Hayflick (25) first described that normal somatic cells have a limited replicative potential. The term replicative senescence describes the state in which cells, after having proliferated previously, are unable to further divide. To date, loss of CD28 expression is the only marker known that is associated with replicative senescence of human T cells cultured *in vitro* (26–29). Increased frequencies of CD8⁺CD28⁻ T cells have also been documented in HIV-infected individuals and in centenarians (30, 31). It will be interesting to compare KLRG1 up- vs CD28 down-regulation on human CD8 T cells during aging and also in clinical situations, such as viral infections, which are associated with massive proliferation (i.e., EBV) or exhaustive differentiation (i.e., HIV) of CD8 T cells. In the mouse, loss of CD28 expression cannot be used as a marker for replicative senescence because CD28 is barely detectable even on unstimulated CD8 T cells. Thus, KLRG1 represents the first and so far only marker available to identify senescent murine T cells *in vivo*. The present report raises the intriguing question whether inhibitory signals mediated by KLRG1 are responsible for the impaired proliferation rate of KLRG1⁺ T cells. Further work using KLRG1-transgenic and KLRG1-deficient mice will help to clarify this interesting issue.

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