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Differential Regulation of Killer Cell Lectin-Like Receptor G1 Expression on T Cells

Scott H. Robbins,*† Stephanie C. Terrizzi,* Beate C. Sydora,‡ Toshifumi Mikayama,§ and Laurent Brossay*‡

The killer cell lectin-like receptor G1 (KLRG1) is the mouse homologue of the rat mast cell function-associated Ag and contains a tyrosine-based inhibitory motif in its cytoplasmic domain. It has been demonstrated that KLRG1 is induced on activated NK cells and that KLRG1 can inhibit NK cell effector functions. In this study, we show that in naive C57BL/6 mice KLRG1 is expressed on a subset of CD44highCD62Llow T cells. KLRG1 expression can be detected on a small number of Vα14 NK T cells but not on CD8αα+ intraepithelial T cells that are either TCRγδ+ or TCRαβ+. We also show that KLRG1 expression is dramatically induced on ~50% of the CD8+ T cells during both a viral and a parasitic infection. Interestingly, during Toxoplasma gondii infection, KLRG1 is up-regulated on CD4+ T cells. Although KLRG1 expression can be induced on both NK cells and T cells, the molecular mechanism leading to the induction of KLRG1 differs in these two subsets of cells. Indeed, the up-regulation of KLRG1 on NK cells can be driven in vivo by cytokines, whereas KLRG1 cannot be induced on CD8+ T cells by cytokines. In addition, although induction of KLRG1 on T cells appears to require TCR engagement in vivo, TCR engagement is not sufficient for KLRG1 induction in vitro. Taken together, these data suggest that the expression and induction of KLRG1 on T cells are tightly regulated. This could have important biological consequences on T cell activation and homeostasis. The Journal of Immunology, 2003, 170: 5876–5885.

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3 Abbreviations used in this paper: KLRG1, killer cell lectin-like receptor G1; MCMV, murine CMV; LCMV, lymphocytic choriomeningitis virus; α-GalCer, α-galactosylceramide; IEL, intraepithelial lymphocyte.

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cytokines in vivo or in vitro and that in vitro TCR engagement is not sufficient for the induction of KLRG1 on T cells.

Materials and Methods

Abs and reagents

TCR-β-FITC and TCR-β-αllophycocyanin (clone H57), CD4-αllophycocyanin (clone RM4.5), CD8-α-PE (clone 53-6.7), NK1.1-PE (clone PK1.36), Ly49C/I/F/H-FITC (clone 14B11), and isotype controls were purchased from eBioscience (San Diego, CA) and were used for FACS analysis in this study. CD3 (clone 145-2C11) and CD28 (clone 37.51) were purchased from eBioscience and used for in vitro cross-linking. In addition, TCR-γ-δ-PE (clone GL3), CD4-PerCP (clone RM4.5), CD5-α-PerCP (clone 53-6.7), CD8-β-PE (clone 53-5.8), CD44-PE (clone IM7), CD20-PE (clone MEL-14), KLRG1-PerCP (clone 2A1), IFN-γ-αllophycocyanin (clone XMG1.2), biotinylated KLRG1 (clone 2F1), NK2CA/CE, and Ly49A (clone A1), isotype controls, and Streptavidin-PerCP were purchased from BD Pharmingen (San Diego, CA). Mouse IL-7, IL-12, and IL-18 PerCP were purchased from BD PharMingen (San Diego, CA). Mouse NKG2A/C/E, and Ly49A (clone A1), isotype controls, and Streptavidin-PerCP were purchased from BD Pharmingen (San Diego, CA). Mouse IL-2 was purchased from BD Pharmingen. Mouse IL-7, IL-12, and IL-18 were purchased from R&D Systems (Minneapolis, MN). IFN-γ and IL-15 were provided by Dr. C. Biron. α-Galactosylceramide (α-GalCer) CD1d tetramers, kindly provided by Drs. S. Sidobre and M. Kronenberg, were generated as described (25). The anti-KLRG1 rat mAb (clone 1F10) was produced in our laboratory (see below).

Mice

Male C57BL/6 and C57BL/10SgSnAi-Tyr mice (Taconic Laboratory Animals and Services, Germantown, NY) were purchased for these studies. All mice were maintained in pathogen-free breeding facilities at Brown University (Providence, RI). All mice were males between 5 and 9 wk of age except where indicated. Experiments were conducted in accordance with institutional guidelines for animal care.

Generation of anti-KLRG1 1F10 mAb

The generation and characterization of the rat IgG2b/κ anti-KLRG1 mAb 1F10 will be described elsewhere (T. Mikayama, manuscript in preparation). Fisher rats were immunized i.p. with 100 µg of soluble KLRG1 in CFA, boosted twice with soluble KLRG1 in IFA and boosted one additional time with KLRG1 transfected cells. Ab-producing cells were obtained from the fusion of spleen cells from rats and myeloma cell lines (SP2/0). Following purification on protein G-Sepharose 4B affinity column (Pharmacia, Piscataway, Nj), the 1F10 mAb was subsequently biotinylated or FITC-labeled using kits from Pierce (Rockford, IL). 1F10 specificity was determined by reactivity to KLRG1 transfected cell lines (Ref. 21 and data not shown).

Infection protocols

Stocks of Smith strain MCMV salivary gland extracts were prepared as previously described (26). Infections were initiated on day 0 with 5 × 10⁴ PFU of MCMV delivered i.p. For LCMV infections, animals were injected i.p. with 2 × 10⁶ PFU of LCMV-Armstrong clone E350 as described (27). The ME49 strain of T. gondii was passaged as cysts in C57BL/6 mice. Experimental animals were infected with 20 ME49 cysts by i.p. injection as described (28).

In vivo BrdU treatments

In vivo BrdU pulsing was performed by injecting 1 mg BrdU per MCMV-infected mouse i.p. 1 h before sacrifice. Analysis for BrdU incorporation was performed using the BrdU Flow kit (BD Biosciences, San Diego, CA).

Isolation of lymphocytes

To obtain splenic lymphocytes, spleens were minced, passed through nylon mesh (Tetko, Kansas City, MO), washed once in 3% PBS-serum, and cell suspensions were layered on a two-step discontinuous Percoll gradient (Pharmacia Fine Chemicals, Piscataway, NJ). Intraperitoneal lymphocytes (IELs) were prepared by digestion of the small intestines in DTT/HBSS solution followed by passage through nylon mesh (Small Parts, Miami Lakes, FL). After washing, cell suspensions were layered on a two-step discontinuous Percoll gradient for density separation (29). Splenocytes, hepatic lymphocytes, and IELs were collected after centrifugation for 30 min at 900 × g. Lymphocytes were isolated from lymph nodes by mechanical grinding. For the staining of naive memory T cells, splenocytes were isolated as previously described followed by the depletion of CD19-positive cells using magnetic beads (Miltenyi Biotec, Germany).

In vitro anti-CD3 cross-linking for CD8⁺ T cell IFN-γ production

Splenic lymphocytes were isolated and incubated with 5 µg/ml plate-bound anti-CD3 for 6 h, with brefeldin A (Sigma-Aldrich, St. Louis, MO) added for the last 3 h of culture. Cells were then harvested and analyzed for NK2CA/CE, KLRG1, CD8α, and intracellular IFN-γ protein by four-color staining followed by flow cytometry.

In vitro anti-CD3/CD28 cross-linking and cytokine treatment of naive T cells

Splenic leukocytes were isolated, in some cases enriched for CD8⁺ cells using magnetic beads, and incubated with 1 µg/ml plate-bound anti-CD3 and 1 µg/ml plate-bound anti-CD28 for 2, 3, 4, 5, 7, or 9 days with or without IFN-α (1000 U/ml), IL-2 (50 U/ml), IL-7 (10 ng/ml), IL-12 (10 ng/ml), IL-15 (10 ng/ml), and IL-18 (20 ng/ml) were indicated. Cultured cells were then harvested and analyzed for TCRα, CD8α, and KLRG1 by four-color staining followed by flow cytometry.

Flow cytometric analysis

Cells were suspended in buffer comprised of PBS containing 3% FCS and 0.02% NaN₃. Cells were first incubated with 2.4G2 mAb for 20 min. Cells were then stained with mAbs specific for cell surface markers or isotype controls for 30 min at 4°C. After two washes, Streptavidin-PerCP was added for another 30 min when appropriate. Cells were then washed and fixed in 2% paraformaldehyde in PBS. Intracellular staining for IFN-γ protein was performed using the Cytofix/Cytoperm kit (BD Pharmingen). Depending on the experiments and the tissue, 1 × 10⁵-1 × 10⁶ events were collected on a FACS caliber. The data were acquired and analyzed using CellQuest software (BD Biosciences).

Results

KLRG1 is expressed on a limited number of T cells from naive C57BL/6 mice

Using a specific mAb (1F10) that we recently developed to the KLRG1 molecule, we demonstrate that KLRG1 is expressed on a small percentage of splenic CD8⁺ and CD4⁺ T cells. A representative three-color flow cytometry experiment shows that ~2.0% of the total conventional CD8⁺ T cells, defined as TCRβ⁺ and CD8α+, and ~1.5% of the total conventional CD4⁺ T cells, defined as TCRβ⁺ and CD4⁺, is isolated from the spleen of naive C57BL/6 mice express the KLRG1 molecule (Fig. 1). KLRG1 expression can also be detected on T cells from other tissues such as from the liver or lymph node (Fig. 1). A similar percentage of KLRG1-positive CD8⁺ and CD4⁺ T cells can be detected using the commercially available anti-KLRG1 mAb 2F1 (data not shown).

KLRG1 is expressed on a subset of CD44highCD62LlowCD8α⁺ and CD44highCD62LlowCD4⁺ T cells isolated from naive C57BL/6 mice

Previous reports describe the expression of NK cell inhibitory receptors such as CD94/NKG2 and members of the Ly49 family on memory T cell populations (30). To determine whether KLRG1 is expressed on naive T cells isolated from naive mice have a memory phenotype, we first enriched total splenocytes for CD19-negative cells by negative selection using anti-CD19-labeled magnetic beads. We then stained the T cell-enriched splenocytes with mAbs directed against the memory cell markers CD44 and CD62L. The cells were simultaneously stained with anti-KLRG1 mAb and anti-CD8α or anti-CD4 mAbs. We show that KLRG1 is expressed on 11.6% ± 4.5% of CD44highCD62LlowCD8α⁺ T cells as well as on 6.6% ± 1.7% of the CD44highCD62LlowCD4⁺ T cells isolated from the spleens of 12-wk-old naive mice (Fig. 2 and A). We also analyzed naive mice at various ages ranging from 4 to 66 wk and compared the
expression of KLRG1 on the total CD8⁺ and CD4⁺ T cells and on the CD44hi CD62Llo CD8⁺ and CD44hi CD62Llo CD4⁺ T cell populations (Fig. 2B). No appreciable difference was found in the expression of the KLRG1 molecule on T cells when young vs old mice were compared. As we could not detect the KLRG1 molecule on non-memory T cells, we conclude that in naive mice the KLRG1 molecule is mainly expressed on CD8⁺ and CD4⁺ T cells that express memory markers.

Expression of KLRG1 on Vα14i NK T cells and IELs from naive C57BL/6 mice

Our findings that the KLRG1⁺ T cells have an activated memory phenotype lead us to investigate whether nonconventional T cells, such as NK T cells and IELs, which are known to express memory cell markers express the KLRG1 molecule on their cell surface. Vα14i NK T cells have an invariant TCR, Vα14 1α18 (formerly 1α281), and recognize the synthetic glycolipid α-GalCer in the context of the nonclassical MHC class I molecule CD1d (31, 32). The majority of Vα14i NK T cells also express the C57BL/6 NK cell Ag NK1.1 (33). We examined KLRG1 expression on Vα14i NK T cells, defined as TCRβ⁺ and α-GalCer loaded CD1d tetramer-positive, in both the spleen and the liver and found that ~1.5% of Vα14i NK T cells isolated from the spleen and ~5.0% of the Vα14i NK T cells isolated from the liver of naive C57BL/6 express the KLRG1 molecule (Fig. 3A). Previous studies have described the preferential expression of Ly49G2 and Ly49C/I NK cell inhibitory receptor expression on the CD4⁺ NK1.1 T cell subset (34). In contrast to Ly49 expression, we find in this study that both the CD4⁺ and CD4⁺ Vα14i NK T cell populations express the KLRG1 molecule in a similar manner (data not shown).

IELs are a subset of T lymphocytes that reside among epithelial cells of the small intestine. Within the IEL population there are conventional TCRαβ⁺ CD8αβ⁺ and TCRαβ⁺ CD4⁺ cells (type A) as well as CD8αα⁺ cells that are either TCRγδ⁺ or TCRαβ⁺ (type B) (35). We analyzed each T cell subset of the IEL population for the expression of the KLRG1 molecule and found that in each subpopulation, ≤0.4% exhibit KLRG1 cell surface expression (Fig. 3B).

KLRG1 expression is induced on T cells following MCMV and T. gondii infection

Previous reports have described the induction of NK cell inhibitory receptors on T cells during infectious challenges (9). During the course of our recent work on the induction of KLRG1 on NK cells during MCMV infection, we observed that a significant proportion of T cells, isolated from infected mice, were also positive for the cell surface expression of the KLRG1 molecule. In this study, we demonstrate that KLRG1 is induced on CD8⁺ T cells following MCMV infection (Fig. 4, A and B). The up-regulation peaks at day 7 postinfection with 59.4 ± 8.1% of CD8⁺ T cells isolated from the spleen and 51.2 ± 7.0% of CD8⁺ T cells isolated from the liver expressing the KLRG1 molecule. After day 7, the percentage of KLRG1⁺ CD8⁺ T cells decreases and by day 28 post-MCMV

![FIGURE 1. Expression of KLRG1 on T cells from naive C57BL/6 mice.](image1)

![FIGURE 2. KLRG1 is expressed on a subset of CD44hi CD62Llo CD8⁺ and CD44hi CD62Llo CD4⁺ T cells isolated from naive C57BL/6 mice.](image2)
infection ~30–35% of the CD8+ T cells express KLRG1. Although Kb or Db class I tetramers loaded with MCMV-specific peptides are currently unavailable, recent studies examining NK cell inhibitory receptor expression on CD8+ T cells using LCMV as a model (13, 23) suggest that the majority of the CD8+ T cells up-regulating KLRG1 are responding to MCMV infection in an Ag-specific manner.

We extended our studies on the induction of the KLRG1 molecule on CD8+ T cells using the intracellular parasite *T. gondii*. We found that during *T. gondii* infection KLRG1 is induced on CD8+ T cells isolated from the spleen and liver in a manner similar to that which we observed during MCMV infection (Fig. 4, A and B). During *T. gondii* infection, KLRG1 induction plateaus at day 9 postinfection with 40.9 ± 3.6% of CD8+ T cells isolated from the spleen and 41.2 ± 1.5% of CD8+ T cells isolated from the liver expressing the KLRG1 molecule. As with MCMV infection, a significant percentage of CD8+ T cells retain expression of the KLRG1 molecule on day 28 after *T. gondii* infection. Thus, the up-regulation and expression of the KLRG1 molecule on CD8+ T cells is not pathogen specific as its induction is initiated on CD8+ T cells responding to both viral and parasitic challenges. As it is unlikely that the Ag-specific T cells for both MCMV and *T. gondii* are confined within the KLRG1+ T cell population of naive mice, these data suggest that KLRG1 expression is induced on KLRG1-negative T cells. In support of this, the in vivo mAb depletion of KLRG1+ cells before MCMV infection did not alter the percentage of the KLRG1+ CD8+ T cells at day 7 post-MCMV infection (data not shown).

Reports of NK cell inhibitory receptor expression on T cells have been largely concentrated on those describing CD8+ T cells (12–15). In this study we demonstrate that during *T. gondii* infection the up-regulation of the KLRG1 molecule is initiated on CD4+ T cells as well as CD8+ T cells (Fig. 4, A and B). The induction peaks at day 9 postinfection with 47.8 ± 3.0% of the CD4+ T cells isolated from the spleen and with 51.2 ± 6.0% of the CD4+ T cells isolated from the liver expressing KLRG1. The

![Figure 3](image3.png)

**FIGURE 3.** Expression of KLRG1 on Vα14i NK T cells and IELs from naive C57BL/6 mice. A. Lymphocytes were isolated from the spleen and liver of uninfected C57BL/6 mice. KLRG1 expression on Vα14i NK T cells, defined as TCRβ+ and CD1 tetramer-positive (Tet)+, is shown. B. IELs were isolated from the small intestines of uninfected C57BL/6 mice. KLRG1 expression on CD8+ T cells, defined as TCRβ+ and CD8β+, on CD4+ T cells, defined as TCRβ+ and CD4+, on CD8α+ TCRβ+ cells, defined as TCRβ+, CD8α+, and CD8β+, and on CD8α+ TCRγδ+ cells, defined as TCRγδ+, CD8α+, and CD8β+ is shown. One animal from a group of four mice representative of two independent experiments is shown.

![Figure 4](image4.png)

**FIGURE 4.** Induction of KLRG1 on T cells following MCMV and *T. gondii* infection. A. Splenic lymphocytes were isolated from MCMV or *T. gondii*-infected C57BL/6 mice on days 1.5, 5, 7, 9, 14, 21, and 28 postinfection and analyzed as in Fig. 1. KLRG1 expression on CD8+ and CD4+ T cells is shown for each day. B. Expression of KLRG1 on splenic and hepatic CD8+ and CD4+ T cells from the experiment described in A. Results are expressed as mean and error bars represent ± SD of three mice per group. One experiment representative of three is shown.
induction of KLRG1 on CD4+ and CD8+ T cells during T. gondii infection follows similar kinetics as demonstrated in both the spleen and the liver (Fig. 4B). These findings indicate that the inducible expression of the KLRG1 molecule during infectious challenge is not restricted to the CD8+ T cell population and that CD4+ T cells are also capable of KLRG1 up-regulation.

The induction of KLRG1 expression on CD8+ T cells during acute MCMV infection is associated with selective proliferation

The kinetics of the expansion and contraction of the KLRG1+ CD8+ T cell population in response to MCMV infection mimics the prototypical CD8+ T cell response that occurs following viral challenges. One hallmark of the CD8+ T cell response to viruses is extensive cellular proliferation during the days preceding the peak of T cell expansion (36). We were interested in determining whether the CD8+ T cells that had acquired KLRG1 expression in response to MCMV infection were in the process of active proliferation. To address this issue we analyzed KLRG1+ CD8+ T cells for acute BrdU incorporation at various times post-MCMV infection. C57BL/6 mice that had been previously infected with MCMV were pulsed in vivo with BrdU via i.p. injection 1 h before sacrifice. The rapid BrdU pulse allowed us to identify cells that were currently undergoing cell division.

A comparison of the KLRG1+ CD8+ T cells and KLRG1− CD8+ T cells shows a profound difference (54.7 ± 2.4% and 14.3 ± 1.4%, respectively) in the percentage of splenic CD8+ T cells that are positive for BrdU incorporation at day 4.5 post-MCMV infection (Fig. 5, A and B). A similar difference in BrdU incorporation can be seen between the KLRG1+ and KLRG1− CD8+ T cells isolated from the liver (Fig. 5, A and B). An analysis of KLRG1+ and KLRG1− CD8+ T cells within the BrdU+ population yielded similar results (data not shown). These data demonstrate that the CD8+ T cells that have acquired expression of the KLRG1 molecule are rapidly proliferating in response to MCMV infection and that this proliferation is likely a contributing factor to the dramatic expansion of the KLRG1+ CD8+ T cell population.

KLRG1+ and KLRG1− CD8+ T cells express other NK receptors on day 7 post-MCMV infection

To determine whether other NK receptors are induced on KLRG1+ CD8+ T cells during MCMV infection, we analyzed the total CD8+ T cell populations of naive C57BL/6 animals for the expression of NKG2A/C/E, 2B4, Ly49C/I/F/H, and NK1.1. Only a limited number of the CD8+ T cells isolated from the spleen of naive animals express these receptors (data not shown). We next analyzed the expression of NKG2A/C/E, 2B4, Ly49C/I/F/H, and NK1.1 at day 7 post-MCMV infection (the peak of CD8+ T cell KLRG1 expression). Of these receptors, only NKG2A/C/E molecules are induced on CD8+ T cells (58.5 ± 4.8%) on day 7 post-MCMV infection (data not shown). Although 59.4 ± 8.1% of the CD8+ T cells express KLRG1 at the same time point, the expression of KLRG1 and NKG2A/C/E is not concurrent (Fig. 6). Indeed, NKG2A/C/E is expressed on 70.4 ± 6.0% of the KLRG1+ CD8+ T cells and 38.0 ± 4.0% of the KLRG1+ CD8+ T cells on day 7 post-MCMV infection (Fig. 6). Similar results were obtained on day 9 post-MCMV infection and on CD8+ T cells isolated from the liver (data not shown).

Both KLRG1+ CD8+ T cells and KLRG1− CD8+ T cells isolated from the spleen on day 7 post-MCMV infection are capable of producing IFN-γ

To investigate the functional characteristics of both the KLRG1+ and KLRG1− CD8+ T cell populations during MCMV infection, we analyzed these populations for their ability to produce IFN-γ. To do so, we isolated total splenocytes on day 7 post-MCMV infection and measured IFN-γ protein by intracellular staining in the total, the KLRG1+, and in the KLRG1− CD8+ T cell populations upon anti-CD3 recall stimulation. We demonstrate that both the KLRG1+ and the KLRG1− CD8+ T cell populations from infected animals have the ability to produce IFN-γ under these conditions. As shown in Fig. 7, A and B, 53.5 ± 5.7% of the total CD8+ T cells, 73.58 ± 2.0% of the KLRG1+ CD8+ T cells, and 31.9 ± 4.9% of the KLRG1− CD8+ T cells isolated on day 7 post-MCMV infection are positive for IFN-γ expression. The presence of naive CD8+ T cells in the KLRG1− population (naive CD8+ T cells do not make IFN-γ under these experimental conditions) (data not shown) is likely to be a contributing factor to the differences in the percentage of KLRG1+ vs KLRG1− CD8+ T cells that are IFN-γ-positive.

Our finding that NKG2A/C/E is expressed by some but not all KLRG1+ CD8+ T cells and that some KLRG1− CD8+ T cells are positive for NKG2A/C/E expression lead us to investigate the possibility that these different CD8+ T cell populations may have varying abilities to produce IFN-γ. As shown in Fig. 7B, the phenotypic heterogeneity of the CD8+ T cell population, based on
KLRG1 and NKG2A/C/E expression, does not necessarily correlate with a functional heterogeneity within the responding CD8\(^+\) T cell population during the acute phase of MCMV infection.

**KLRG1 expression is maintained on CD8\(^+\) T cells on day 70, 130, and 190 post-MCMV infection**

MCMV infection of an immunocompetent host results in chronic infection with viral persistence within the salivary glands (37). Our observations that KLRG1 is expressed on T cells with a memory phenotype in naive animals and that KLRG1 expression is maintained on CD8\(^+\) T cells on day 28 post-MCMV infection lead us to investigate whether MCMV infection results in the long-term expression of the KLRG1 molecule on CD8\(^+\) T cells. We find that on day 7 post-MCMV infection, KLRG1 is expressed on 24.4 ± 0.5% of the total CD8\(^+\) T cells isolated from the spleen and 48.9 ± 3.1% of the total CD8\(^+\) T cells isolated from the liver (Fig. 8). Within the CD44\(^{high}\)CD62L\(^{low}\) T cell population KLRG1 expression is maintained on 45.5 ± 12.8% of the CD44\(^{high}\)CD62L\(^{low}\)CD8\(^+\) T cells isolated from the spleen and on 49.6 ± 7.0% of the CD44\(^{high}\)CD62L\(^{low}\)CD8\(^+\) T cell isolated from the liver (Fig. 8). Similar results were observed on days 130 and 190 post-MCMV infection (Fig. 8). The well-documented long-term persistence of MCMV within the host (37) likely contributes to the maintenance of the significant population of KLRG1\(^+\) CD8\(^+\) T cells detected on days 70, 130, and 190 post-MCMV infection.

**In vivo cytokines alone and TCR engagement alone are not sufficient for the induction of KLRG1 on naive T cells**

The mechanism for the induction of the KLRG1 molecule on T cells is currently unknown. To determine whether T cell stimulation through in vivo cytokine signaling could drive the expression of KLRG1 on T cells we took advantage of a TCR transgenic mouse (C57BL/10SgSnAi-TgN(TCR P14 LCMV)-Rag2\(^{tm1}110\)) whose CD8\(^+\) T cells are specific for the LCMV peptide P14. We hypothesized that if infected with LCMV both the cytokine environment induced by the viral infection and the Ag-specific engagement of the transgenic TCR would be provided. In contrast, during MCMV infection the transgenic TCR would not be engaged in an Ag-specific manner but the viral-induced cytokine environment would be present. Thus we were able to utilize the LCMV P14 CD8\(^+\) T cell transgenic mice infected with either LCMV or MCMV to determine whether KLRG1 induction could be driven in vivo by the cytokine environment produced during an infectious challenge in the absence of Ag-specific TCR signaling.

As shown in Fig. 9, the induction of KLRG1 on NK cells in the LCMV P14 mice during both LCMV and MCMV infections was similar to that which we previously demonstrated in C57BL/6 mice (Fig. 9A and Ref. 21). KLRG1 expression is also induced on the transgenic CD8\(^+\) T cells during LCMV infection in a manner comparable with previous studies (Fig. 9, A and B, and Refs. 13,
Increasing evidence indicates that CD8\(^+\) T cells can exhibit the expression of both activating and inhibitory NK cell receptors (6–10) and that when engaged under some conditions, these receptors are capable of modulating T cell functions (12, 14, 16, 17, 39–42). In this report, we demonstrate that in naive C57BL/6 mice the NK cell inhibitory receptor KLRG1 is expressed on subsets of both CD8\(^+\) and CD4\(^+\) conventional T cells. We find that the expression of KLRG1 on CD8\(^+\) and CD4\(^+\) T cell subsets isolated from naive animals appears to be largely restricted to T cells that exhibit a memory phenotype. Our observation that KLRG1 is expressed by a subset of phenotypically memory T cells in naive animals is consistent with reports that describe the expression of other NK cell inhibitory receptors on CD8\(^+\) memory T cells (10, 11, 30). It has been hypothesized that NK cell inhibitory receptor expression

Discussion

In contrast, KLRG1 was not induced on the P14 LCMV specific transgenic CD8\(^+\) T cells during infection with MCMV (Fig. 9, A and B). These results indicate that the in vivo immune environment produced in response to MCMV infection that is capable of inducing KLRG1 expression on NK cells is not sufficient to drive the induction of the KLRG1 molecule on CD8\(^+\) T cells. Collectively, these data suggest that TCR signaling may be a requirement for the in vivo induction of KLRG1 on T cells in response to pathogen encounter and that it is unlikely that T cells activated in vivo via bystander mechanisms during the course of an infectious challenge will acquire KLRG1.

Previous studies have shown that in vitro TCR stimulation is sufficient for the induction of CD94/NKG2 receptors on T cells (13, 38). To determine whether KLRG1 could be induced on CD8\(^+\) T cells under conditions that are sufficient for the induction of CD94/NKG2 molecules, we enriched splenocytes isolated from naive C57BL/6 animals for CD8\(^+\) cells using magnetic beads. Following enrichment, the cells were >96% CD8\(^+\) (data not shown). We then stimulated the CD8 enriched subset with anti-CD3 and anti-CD28 mAbs. In contrast to CD94/NKG2, KLRG1 expression is not induced on CD8\(^+\) T cells on day 4 after in vitro CD3 cross-linking (Fig. 9C). As described in Table I, KLRG1 was not up-regulated on CD8\(^+\) or CD4\(^+\) T cells in vitro using total splenocytes on days 2, 3, 4, 5, 7, and 9 in the presence or absence of exogenous cytokines with or without anti-CD3 and anti-CD28 stimulation. Although the mechanism controlling KLRG1 induction on T cells remains elusive, collectively these results demonstrate that the expression of the KLRG1 molecule is differentially regulated on NK cells and T cells during infectious challenge and that the requirements for the induction of KLRG1 on T cells are different from those governing the expression of CD94/NKG2 molecules.


FIGURE 8. KLRG1 expression is maintained on CD8\(^+\) T cells on days 70, 130, and 190 post-MCMV infection. Splenic and hepatic lymphocytes were isolated from C57BL/6 mice on day 70, 130, and 190 post-MCMV infection and analyzed as in Fig. 1 and Fig. 2. The percentage of KLRG1\(^+\) T cells from the total number of CD8\(^+\) T cells as well as the percentage of KLRG1\(^+\) T cells from the CD44\(^{hi}\)CD62L\(^{lo}\)CD8\(^+\) T cells is shown. Results are expressed as mean and error bars represent ± SD of three mice per group.


FIGURE 9. In vivo cytokines and TCR engagement alone are not sufficient for the induction of KLRG1 on naive T cells. A. Splenic lymphocytes were isolated from LCMV and MCMV-infected P14 TCR transgenic mice on day 9 postinfection and analyzed for KLRG1 expression on both NK cells, defined as TCR\(\beta^+\) and NK1.1\(^+\), and on CD8\(^+\) T cells, defined as TCR\(\beta^+\) and CD8\(^+\). B. Expression of KLRG1 on CD8\(^+\) T cells is shown from the experiment described in A. Results are expressed as mean and error bars represent ± SD of three mice per group. One experiment representative of three is shown. C. Splenic lymphocytes were isolated from naive C57BL/6 mice, enriched for CD8\(^+\) cells, and cultured at a concentration of 5 × 10\(^3\) cells per ml in the presence of 1 μg/ml plate-bound anti-CD3 and 1 μg/ml plate-bound anti-CD28 mAbs for 4 days. Expression of NK2A/C/E, Ly49A, and KLRG1 on CD8\(^+\) T cells (shaded histogram) vs isotype control (open histogram) is shown. One experiment representative of three is shown.
on memory T cell populations provides a mechanism that raises the activation threshold of memory T cells (11). The role for these receptors on memory T cells would therefore be one of contributing to memory cell homeostasis by preventing the inappropriate activation of the memory T cell pool by self or nontypical mechanisms (11). The origin of T cells that exhibit a memory phenotype in naive mice is most likely the result of specific activation from environmental stimuli. In humans, KLRG1 is expressed on 40% of the total CD8+ and 20% of the total CD4+ T cell populations (43). Voehringer et al. (43) attributed this higher percentage of KLRG1+ cells to multiple infections experienced by humans during their longer lifetime.

In addition to memory T cell subsets, the expression of a variety of NK cell receptors has been described on nonconventional T cell populations (6, 34). For example, the majority of NK cell receptors has been described on nonconventional T cell subsets, linking and/or cytokine treatment

\[ \text{KLRG1 induction was not observed by cytokine treatment alone. Cytokine concentrations: IFN-}\alpha (1000 U/ml), IL-2 (50 U/ml), IL-7 (10 ng/ml), IL-12 (10 ng/ml), IL-15 (10 ng/ml), and IL-18 (20 ng/ml). } \]

\[ \text{Anti-CD3 (1 µg/ml), anti-CD28 (1 µg/ml). } \]

\[ \text{Similar results were observed for CD4+ T cells. } \]

\[ \text{Similar results were observed on days 2, 3, 4, 7, and 9. } \]

The role of CD4+ T cells in host resistance to both MCMV and *T. gondii* infections has been previously demonstrated (45–48). Interestingly, the acquisition of KLRG1 expression by CD4+ T cells occurs in the host response to *T. gondii* but not in the response to MCMV. In light of previous findings that NK markers, specifically NK1.1, DX5, and ASGM1, are up-regulated on CD4+ T cells during viral infections (7), it is somewhat surprising that KLRG1 in not induced on CD4+ T cells in response to MCMV infection. The discrepancy in the induction of the KLRG1 molecule on CD8+ vs CD4+ T cells during MCMV infection is consistent, however, with our findings that KLRG1 is expressed by subsets of T cells isolated from naive mice that exhibit an activated or memory phenotype and not by the entire memory T cell populations. These findings are also consistent with the idea that the specific criteria required for the induction of KLRG1 on T cells is not always met during the process of T cell activation (see below).

The dynamics of the up-regulation of KLRG1 on CD8+ T cells during both the viral and the parasitic challenges is similar to the induction of KLRG1 seen on NK cells during their in vivo activation (21). However, we find that the mechanism underlying the induction of KLRG1 on T cells vs NK cells is strikingly different. Previously, we demonstrated that the induction of KLRG1 on NK cells was independent of a specific activation signal as it occurred during both the NK cell response to viral infections as well as during the in vivo activation of NK cells via pathogen-free mechanisms (21). In this study, we demonstrate that an immune environment sufficient to induce KLRG1 on NK cells is insufficient to induce the expression of the KLRG1 molecule on T cells in the absence of appropriate TCR engagement. This suggests that pathogen-specific TCR engagement may be a requirement for the induction of KLRG1 in T cells in the context of infectious challenge and demonstrates that the molecular mechanism leading to the induction of the KLRG1 molecule differs between NK and T cells. Interestingly, however, the induction of KLRG1 on CD8+ T cells could not be obtained in vitro under conditions that drive the up-regulation of CD94/NKG2 molecules. These data demonstrate that not only is KLRG1 expression differentially regulated on NK cells vs T cells but that the mechanism governing the expression of KLRG1 on T cells during the process of their activation is different from the mechanism that controls the expression of other known NK cell inhibitory receptors.

A recent report documents the induction of KLRG1 on CD8+ T cells when they were transferred into a B6-Rag-1–/– lymphopenic host but not upon their transfer into sublethally irradiated lymphopenic hosts (13). As the authors point out, this induction is likely due to the extensive cellular proliferation T cells undergo upon transfer into a B6-Rag-1–/– lymphopenic host that is a result of low affinity self-peptide presentation to the donor T cells. Collectively, the data presented in this study, as well as the data from others (13, 23, 49), suggest that a strength independent TCR engagement is required but is not sufficient for the induction of KLRG1 on CD8+ T cells.

The function of KLRG1 on T cells is not clear. Although our and other studies (23) demonstrate that both KLRG1+ and KLRG1− CD8+ T cells have the ability to produce IFN-γ, their ability to make cytokines in the presence of the KLRG1 ligand is unknown. It has been recently demonstrated that KLRG1+ memory T cells proliferated poorly when they are compared with KLRG1− memory T cells, suggesting that the KLRG1 expression correlates with senescence (23, 43). Our data presented in this study, which demonstrates the selective proliferation of KLRG1+ T cells during the acute phase of MCMV infection, in combination with the data from other studies, just previously mentioned (23, 43), supports a model where the initial activation of CD8+ T cells

### Table 1. KLRG1 is not induced on T cells upon in vitro TCR crosslinking and/or cytokine treatment

<table>
<thead>
<tr>
<th>KLRG1 on CD8+ T Cells’ Day 5 Posttreatment</th>
<th>Isotype (mean fluorescence intensity)</th>
<th>oKLRG1 (mean fluorescence intensity)</th>
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<tr>
<td>none</td>
<td>4.99</td>
<td>6.84</td>
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<tr>
<td>IFN-α</td>
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<td>11.05</td>
</tr>
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<td>IL-18</td>
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<tr>
<td>IFN-α, IL-2, IL-7, IL-12, IL-15</td>
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<td>11.60</td>
</tr>
</tbody>
</table>

* KLRG1 induction was not observed by cytokine treatment alone. Cytokine concentrations: IFN-α (1000 U/ml), IL-2 (50 U/ml), IL-7 (10 ng/ml), IL-12 (10 ng/ml), IL-15 (10 ng/ml), and IL-18 (20 ng/ml).
* Anti-CD3 (1 µg/ml), anti-CD28 (1 µg/ml).
* Similar results were observed for CD4+ T cells.
* Similar results were observed on days 2, 3, 4, 7, and 9.
induces both their proliferation and the induction of KLRG1 on their cell surface. Once KLRG1 is expressed by T cells, an arrest in their proliferation is initiated via, perhaps, engagement of the KLRG1 molecule with its natural ligand. Currently, however, the ligand for the KLRG1 molecule is unknown and the ability of KLRG1 to block T cell proliferation once engaged has yet to be reported. A recent report, however, which utilizes T cells isolated from KLRG1 transgenic mice demonstrated that KLRG1 engagement effectively blocks TCR-induced CD8+ T cell calcium mobilization and is also capable of producing a modest inhibitory effect on CD8+ T cell killing (49).

Taken together, the data presented in this study, from previous work from our laboratory (21), and from other groups (20, 23, 49, 50) strongly suggests that the KLRG1 molecule may play a common role in regulating cells of both the innate and adaptive immune system. A better understanding of the role inhibitory receptors such as KLRG1 have in the regulation of T cell functions will undoubtedly have important implications on future therapeutic strategies aimed at modulating T cell responses.

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References


