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Expression of Killer Cell Lectin-Like Receptor G1 on Antigen-Specific Human CD8+ T Lymphocytes during Active, Latent, and Resolved Infection and its Relation with CD57

Chris C. Ibegbu,* Yong-Xian Xu,* Wayne Harris,* David Maggio,* Joseph D. Miller,* and Athena P. Kourtis†

Killer cell lectin-like receptor G1 (KLRG1) is one of several inhibitory killer cell lectin-like receptors expressed by NK cells and T lymphocytes, mainly CD8+ effector/memory cells that can secrete cytokines but have poor proliferative capacity. Using multiparameter flow cytometry, we studied KLRG1 expression on CD8+ T cells specific for epitopes of CMV, EBV, influenza, and HIV. Over 92% of CD8+ cells specific for CMV or EBV expressed KLRG1 during the latent stage of these chronic infections. CD8+ T cell specific for HIV epitopes were mostly (72–89%) KLRG1+, even though not quite at the level of predominance noted with CMV or EBV. Lower frequency of KLRG1 expression was observed among CD8+ cells specific for influenza (40–73%), a resolved infection without a latent stage. We further observed that CD8+ cells expressing CD57, a marker of replicative senescence, also expressed KLRG1; however, a population of CD57–KLRG1+ cells was also identified. This population may represent a “memory” phenotype, because they also expressed CD27, CD28, CCR7, and CD127. In contrast, CD57+ KLRG1+ cells did not express CD27, CD28, and CCR7, and expressed CD127 at a much lower frequency, indicating that they represent effector cells that are truly terminally differentiated. The combination of KLRG1 and CD57 expression might thus aid in refining functional characterization of CD8+ T cell subsets. The Journal of Immunology, 2005, 174: 6088–6094.

The functions of T lymphocytes and NK cells are regulated by activating and inhibitory cell surface receptors. Killer cell lectin-like receptor G1 (KLRG1) belongs to a superfamily of inhibitory receptors, of which the mouse Ly-49 family and the human killer-cell Ig-like receptor and the killer cell lectin-like receptor family are among the best characterized (1, 2). These receptors bind to MHC class I ligands on target cells and inhibit NK cells from attacking cells expressing class I Ags at normal levels, such as healthy self tissues (as opposed to virally infected or transformed cells) (3).

Although KLRG1 is expressed on 30–60% of murine NK cells, it is also expressed on a fraction of T cells (2, 4). There is evidence for differential regulation of the KLRG1 receptor activation on NK cells and T cells (1, 5). The role of KLRG1 on T lymphocytes has not been fully explored. Expression of KLRG1 identifies T cells in humans that are capable of secreting cytokines but fail to proliferate after stimulation and are thus unable to undergo further clonal expansion (6–9). KLRG1+ cells are preferentially found in the Ag-experienced, CD28−CCR7−“effector” T cell pool. KLRG1 is expressed on T cells that have undergone a large number of cell divisions (10), which is possibly the reason for the increased number of KLRG1+ T cells observed with advanced age and the fact that KLRG1 is expressed on CD4+ T cells at a lower degree (10). Recent findings have suggested that CD8+ T cell clones specific for single epitopes of CMV and EBV are mostly KLRG1+ (8). KLRG1 is expressed on ~44% of human CD8 and 28% of CD4 T cells in the peripheral blood and on ~50% of NK cells (9); these NK cells are of the CD56 “dim” phenotype, a profile of cytotoxicity and reduced proliferative capacity (9). The higher expression of KLRG1 on human, compared with mouse, lymphocytes has also been attributed to the longer human life span resulting in a greater number of cellular proliferations, as a result of multiple infections and other antigenic encounters (9).

An interesting aspect is the correlation of KLRG1 expression with that of CD57, a marker of proliferative inability that can be expressed on CD4+, CD8+ T cells and NK cells (11). CD57 has also been associated with a history of a greater number of cell divisions and short telomeres, markers of senescence (11). CD57 expression is increased in NK cells of patients with HIV infection, probably as a result of chronic antigenic stimulation, and in other conditions associated with immune activation, as well as with increasing age (11–15). How CD57 and KLRG1 interrelate and whether the profiles of expression of different combinations of these markers could offer a more precise functional delineation of these cellular subtypes has not been studied to date.

We set out to explore the functional significance of KLRG1 expression on human CD8+ T cells, by assessing Ag-specific human CD8+ T cell clones that recognize epitopes of viral pathogens causing acute (influenza), chronic/latent (EBV, CMV), and chronic/active (HIV) infections. We used tetramer-staining technology and we simultaneously examined other cell receptor expression on such cells using multicolor flow cytometry. In addition, we wished to determine the relation between expression of KLRG1 and CD57.

Materials and Methods

Study subjects and samples

Blood samples were obtained from three healthy volunteers known to be EBV- and CMV-seropositive by commercially available serologic tests.
The donors had received previous influenza immunizations and were HLA-A2*/H11001, with normal CD4/CD8 ratios. The donors’ age range was 27–40 years; two were men. We also used blood samples from three HIV-infected patients who participated in a HIV vaccine study; they were free of active concurrent infections at the time of testing, and their HIV infection was stable with very low viral loads (860–5820 viral copies/ml) (two of the three were on antiretroviral medications, and one was not on treatment). All subjects were recruited at the Emory University Vaccine Center and gave informed consent for the study. The blood samples were provided in either heparin or sodium citrate anticoagulant tubes. PBMC were isolated from blood samples over lymphocyte separation medium (Cellgro).

### MHC class 1-peptide tetramers

Soluble MHC class 1-peptide tetramers carrying CTL epitopes of CMV, EBV, influenza virus, and HIV-1 proteins were produced as described elsewhere (16). The HLA restriction, peptide sequences, the virus, and the name of the gene products of derived CTL epitopes are presented in Table I. The tetramers were prepared with streptavidin coupled to allophycocyanin (Molecular Probes).

### Flow cytometry

A whole-blood flow cytometry technique was used as previously described (17). Cells were stained with FITC-, PE-, PerCP-, and allophycocyanin-labeled mAb or tetramers. The whole blood samples (200 μl) were stained at room temperature for 20 min; RBC were lysed in FACS lysing solution (BD Biosciences) for 10 min in the dark, washed twice in FACS buffer (PBS containing 2% BSA and 0.1% NaN₃), and fixed in 300 μl of 1% parafomaldehyde. The following mAb (Beckman Coulter) were used: anti-CD3 (clone UCHT1), anti-CD28 (clone CD28.2), anti-CD57 (clone NC1), and anti-CD127 (clone R34.34). The following mAb from BD Pharmingen were used: anti-CD4 (clone SK3), anti-CD8 (clone SK1), anti-CD45RO (clone UCHL-1), perforin, and Ki-67 (clone B56). The CCR7 (clone 150503) was from R&D Systems and the granzyme B was supplied by Caltag. The Alexa-KLRG1 (13A2) was kindly provided by Dr. H. Pircher (University of Freiburg, Freiburg, Germany).

### Intracellular cytokine staining

PBMC (1 × 10⁶) were stimulated with an appropriate peptide (10 μg/ml) (Table I) or staphylococcal enterotoxin B (SEB) (2 μg/ml; Sigma-Aldrich) in 200 μl of RPMI 1640/10% FBS medium containing costimulatory Abs CD28/CD49d (1 μg/ml) and Golgi Plug (BD Pharmingen) for 6 h. After stimulation, PBMC were surface-stained for 20 min at 4°C, and then lysed with 2 ml of FACS Lysse (BD Biosciences) for 10 min at room temperature. The cells were then washed twice with FACS buffer (PBS containing 2% BSA and 0.1% NaN₃), permeabilized for 10 min at room temperature with 500 μl of FACS-Perm (BD Biosciences), washed with FACS buffer, and stored at −20°C until analysis.

![FIGURE 1.](http://www.jimmunol.org/) KLRG1 expression on Ag-specific CD8+ T cells from healthy donors and from HIV-infected subjects. Whole blood was incubated with the respective HLA-A2-restricted epitopes of EBV or CMV (A) and influenza virus (B) of healthy donors with serologic evidence of previous infection with these agents, as shown. Whole blood obtained from HIV-infected patients was incubated with the HLA-A2-restricted HIV gag epitopes RLR and QVP (C).

### Table I. MHC class I peptide tetramers and the respective HLA-restricted and CTL epitopes used in this study

<table>
<thead>
<tr>
<th>MHC/Peptide Tetramers</th>
<th>HLA Restriction</th>
<th>CT Epitope</th>
<th>Viral Agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2/CMV.NLV</td>
<td>A*0201</td>
<td>NLVPKVATV</td>
<td>CMV</td>
</tr>
<tr>
<td>A2/FLU.GIL</td>
<td>A*0201</td>
<td>GILGFVFTL</td>
<td>Influenza virus</td>
</tr>
<tr>
<td>A2/EBV.GLC</td>
<td>A*0201</td>
<td>GLCTLVAML</td>
<td>EBV</td>
</tr>
<tr>
<td>B8/EBV.FLR</td>
<td>B*0801</td>
<td>FLRGRAYGL</td>
<td>EBV</td>
</tr>
<tr>
<td>B8/EBV.RAK</td>
<td>B*0801</td>
<td>RAKFKQGLL</td>
<td>EBV</td>
</tr>
<tr>
<td>A3/HIV.QVP</td>
<td>A*0301</td>
<td>QVPLRPMTYK</td>
<td>HIV</td>
</tr>
<tr>
<td>A3/HIV.RLR</td>
<td>A*0301</td>
<td>RLDPGKKK</td>
<td>HIV</td>
</tr>
</tbody>
</table>

The epitopes belong to Ags of the viral agents shown.
stained with IFN-γ (clone B27) for 20 min at 4°C, washed again, and fixed with 1% paraformaldehyde before acquisition on a FACSCalibur (BD Biosciences). FACS data were analyzed with FlowJo software (Tree Star).

Ag-specific proliferation

PBMC were initially stained with CFSE (Molecular Probes) as previously described (9). They were suspended in RPMI 1640 supplemented with 10% FBS, glutamine, and penicillin/streptomycin (Cellgro) and were stimulated with influenza virus, CMV, or EBV peptides at 10 μg/ml final concentration (Table I). Cells were then incubated for 5 days at 37°C, after which the cells were stained for flow cytometric analysis. In every experiment, a negative control (nonstimulated) was included to control for spontaneous production of IFN-γ, as well as a positive control SEB, 2 μg/ml final concentration (Sigma-Aldrich), to ensure that cells were responsive. In another experiment, the PBMC were not prestained with CFSE but were stimulated with the respective Ags for 5 days and then stained with Ki-67 and other combinations of markers of interest.

Results

Patterns of KLRG1 expression on Ag-specific CD8+ T cells during active, latent, and resolved infection

We examined CD8+ T cells specific for CMV, EBV, and influenza virus epitopes in three healthy donors. CD8+ T lymphocytes specific for the EBV and CMV epitopes were almost exclusively (>92%) KLRG1-expressing in all donors (Fig. 1A). In contrast, only 40–73% of CD8+ cells specific for influenza epitopes were expressing KLRG1 (Fig. 1B).

We also examined CD8+ T cells specific for two HIV gag epitopes on three patients with stable HIV infection. HIV epitope-specific cells were, in their great majority (72–89%), expressing KLRG1, even though at frequencies generally lower than those seen for epitopes of CMV or EBV (Fig. 1C). However, the frequencies were higher than those seen for influenza virus epitope-specific cells. Of interest, in one of the HIV+ patients, who was also CMV- and EBV-seropositive but did not have active disease with either virus at the time of testing, almost all CD8+ T cells specific for CMV and EBV epitopes demonstrated KLRG1 expression, concurrent with the findings in HIV-uninfected patients (data not shown). These data indicate that clones specific for antigenic epitopes of pathogens causing chronic/latent infections during the latent phase, such as herpesviruses, express KLRG1 in their overwhelming majority. This does not hold true for some epitopes of pathogens of resolved infections such as influenza, where the frequency of KLRG1 expression is lower. During HIV infection, which is characterized by chronic viral replication and active infection, rather than distinct “acute” and “latent” stages, KLRG1
expression on CD8^+ effector cells is at frequencies intermediate between the two different stages of infection exemplified above.

**Properties of KLRG1^+ Ag-specific CD8^+ T cells**

To further define the functional properties of KLRG1-expressing CD8^+ T cells, we used four-color flow cytometry for expression of granzyme B, perforin, and CCR7, key markers associated with cytotoxicity and effector function, respectively. We found that granzyme B and perforin were expressed predominantly on KLRG1^+CD28^− cells (89.5 and 55% of KLRG1^+CD28^− cells expressed granzyme B or perforin, respectively). In contrast, KLRG1^−CD28^+ cells expressed CCR7 at a very low frequency (17%); CCR7 was predominantly expressed on KLRG1^−CD28^+ cells (94.2%) (Fig. 2). CCR7 expression is thought to differentiate central memory (CCR7^+^) from effector memory (CCR7^−^) phenotype (18). These characteristics conform to the hypothesis that KLRG1^+CD28^− cells represent an effector phenotype (expressing perforin and granzyme B but low levels of CCR7).

KLRG1^+ lymphocytes were able to produce IFN-γ upon antigenic stimulation with CMV, EBV, and influenza virus peptides, respectively (0.2–0.5% of KLRG1^+ cells produced cytokine, representing a 2.5- to 6.25-fold expansion from unstimulated state), as well as upon superantigen stimulation with SEB (up to 9% of KLRG1^+ cells secreted cytokine, a 112-fold expansion from unstimulated state) (Fig. 3A). Furthermore, upon 5-day stimulation with the respective Ag (CMV, EBV, or influenza epitopes), a

**FIGURE 3.** Cytokine production and capability of expansion of KLRG1^+ CD8 cells. KLRG1^+ cells produce IFN-γ upon stimulation with the respective CMV, EBV, or influenza virus peptides and the superantigen SEB (A). A portion of KLRG1^+ cells express Ki-67, a marker of proliferation, upon in vitro stimulation with the appropriate Ag (B).
CD8 T cell subpopulations defined by their CD57 and CD45RO expression patterns. KLRG1 is expressed on CD57\(^{+}\)CD45RO\(^{+}\), CD57\(^{+}\)CD45RO\(^{-}\) cells, and CD57\(^{-}\)CD45RO\(^{-}\) cells (quadrants 2, 4, and 1). The first two populations (populations in quadrants 2 and 4) did not express CD27, CD28, or CCR7 but expressed perforin, a profile characteristic of effector cells. The latter population (quadrant 1), unlike the previous two, expressed CD27, CD28, CCR7, did not express perforin, and also expressed in its overwhelming majority CD127, a marker of long-lived memory cells.

Fig. 4. Division of CD8\(^{+}\) T cell subpopulations defined by their CD57 and CD45RO expression patterns. KLRG1 is expressed on CD57\(^{+}\)CD45RO\(^{+}\), CD57\(^{+}\)CD45RO\(^{-}\) cells, and CD57\(^{-}\)CD45RO\(^{-}\) cells (quadrants 2, 4, and 1). The first two populations (populations in quadrants 2 and 4) did not express CD27, CD28, or CCR7 but expressed perforin, a profile characteristic of effector cells. The latter population (quadrant 1), unlike the previous two, expressed CD27, CD28, CCR7, did not express perforin, and also expressed in its overwhelming majority CD127, a marker of long-lived memory cells.

**Discussion**

Our study aimed to refine the role of KLRG1 expression on human Ag-specific CD8\(^{+}\) T cells. Two main conclusions derive from our findings: First, a pattern of differential expression of KLRG1 on Ag-specific CD8\(^{+}\) T cells recognizing viral epitopes of resolved (influenza), chronic active (HIV), and chronic latent (CMV or EBV) infections was evident. Thus, KLRG1 was expressed in the...
overwhelming majority of cells specific for epitopes of CMV or EBV during the latent stage. A slightly lower level of expression was observed in cells specific for HIV, an infection characterized by chronic active and ongoing immune activation and continuous viral replication. Even lower levels of KLRG1 expression were seen in cells recognizing influenza epitopes when the infection had resolved. Given that KLRG1 is an inhibitory marker, such high-level expression among herpesvirus- and, to a lesser extent, HIV-specific cells, may serve to keep the immune activation in check during the latent stages following acute infection and in the intervals between recurrences, and, to a lesser degree, during chronic infection that is well controlled. With pathogens such as influenza virus, which become “cleared” after disease resolution, no ongoing immune stimulation ensues and thus less need for inhibitory signaling exists. It would be interesting to study KLRG1 expression during the acute stage of a self-limited or the reactivated stage of a persistent infection. It might be expected that KLRG1 expression would be lower on Ag-specific cells in the acute, compared with the latent stage. Indeed, there is some evidence that other NK cell inhibitory receptors are not expressed by a large fraction of activated effector CD8⁺ T cells during acute primary infection with EBV or hepatitis B in humans (20) or lymphocytic choriomeningitis virus in mice (21). It is interesting to add that a recent report has identified preserved or increased expression of inhibitory receptors on NK cells of HIV-infected viremic individuals, in contrast to activating receptors, which were down-regulated in these patients (22). An alternative hypothesis has been presented (23), whereby inhibitory receptor signaling may actually aid in the formation of long-term memory, by avoidance of apoptosis of inhibitory receptor-expressing cells during an immune response. This hypothesis, which could equally well explain our findings, needs to be further evaluated.

The second conclusion derived from our findings is that the KLRG1⁺ CD8⁺ cell population is heterogeneous in its functional roles, because it contains both terminally differentiated effector cells as well as effector cells destined to become long-lived memory cells. The combination of two markers, KLRG1 and CD57, might help in this differentiation. It has been recently described in a mouse model that expression of the IL-7R (CD127) identifies effector CD8⁺ T cells that give rise to long-lived memory cells (19).
Our finding of CD127 expression on some KLRG1+ cells might suggest that these KLRG1+ cells are indeed destined to become long-term memory cells. Of interest, only CD57 KLRG1+ cells expressed this marker, indicating that CD57 KLRG1+ expression denotes an effector destined to become memory phenotype. In contrast, CD57 KLRG1+ cells (which represent the majority of KLRG1+ cells) are probably terminally differentiated effector cells, because they are capable of cytokine expression and perforin and granzyme B production but do not express CD127, CD27, CD28, or CCR7. We observed, contrary to previous reports (9), that a small portion of KLRG1+ CD8+ T cells expressed Ki-67 upon appropriate Ag stimulation, indicating proliferative ability. It is possible that this capacity is retained by the subset of Ag-specific KLRG1+ cells destined to become memory cells, whereas the majority of KLRG1+ cells, being also CD57+, are terminally differentiated cells, unable to proliferate.

Further studies will hopefully shed more light on the role of expression of this novel marker on subsets of Ag-specific T lymphocytes during different stages of an infection, and with that on key immunologic questions such as control of immune activation and formation of immune memory. Such studies could have important implications for the control of infections, as well as aberrances such as autoimmunity and hematologic malignancies.

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Disclosures
The authors have no financial conflict of interest.

References