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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

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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%), 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.

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§Abbreviations used in this paper: KLRG1, killer cell lectin-like receptor G1; SEB, staphylococcal enterotoxin B.
The donors had received previous influenza immunizations and were HLA-A2\(^{1}H11001\), 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 I-peptide tetramers**

Soluble MHC class I-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 \(\mu\)l) 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% NaN\(_3\)), and fixed in 300 \(\mu\)l of 1% paraformaldehyde. 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 \(\times\) 10\(^6\)) were stimulated with an appropriate peptide (10 \(\mu\)g/ml) (Table I) or staphylococcal enterotoxin B (SEB) (2 \(\mu\)g/ml; Sigma-Aldrich) in 200 \(\mu\)l of RPMI 1640/10% FBS medium containing costimulatory Abs CD28/CD49d (1 \(\mu\)g/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 Lyse (BD Biosciences) for 10 min at room temperature. The cells were then washed twice with FACS buffer (PBS containing 2% BSA and 0.1% NaN\(_3\)), permeabilized for 10 min at room temperature with 500 \(\mu\)l of FACS-Perm (BD Biosciences), washed with FACS buffer,
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-\(\gamma\) upon antigen 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
marked expansion of the KLRG1+ cell population expressing the proliferative marker Ki-67 was observed (12.4- to 46.2-fold expansion compared with unstimulated cells) (Fig. 3B), perhaps indicating retained proliferative capacity of Ag-specific cells upon appropriate stimulation.

CD8+ KLRG1+CD57+ cells represent a terminally differentiated effector population, whereas KLRG1+CD57- cells represent central memory cells

An aspect that has not been examined to date is the relation between KLRG1 and CD57, two markers thought to indicate replicative senescence. We examined the expression of several markers on the subpopulations of CD8+ cells defined by patterns of expression of KLRG1 and CD57. CD45RO and CD57 expression on CD8+ cells identified four distinct populations, three of which expressed KLRG1 (Fig. 4). Over 94% of CD57+CD45RO+ cells and CD57+CD45RO− cells, and the majority (74–77%) of CD57−CD45RO+ cells were KLRG1+ (Fig. 4). CD57−CD45RO− cells expressed KLRG1 at a very low frequency (13–17%). We then focused on patterns of expression of CD57 and KLRG1 in relation to other markers. The CD45RO+CD57+ KLRG1+ and the CD45RO+CD57+ KLRG1− populations did not express CD27, CD28, or CCR7, but expressed perforin. This phenotype is consistent with effector memory cells. In contrast, the CD45RO+CD57− KLRG1+ population expressed CD27 and CD28 in their majority, CCR7 at a frequency of ~28–57%, and did not express perforin, a phenotype consistent with central memory cells. Further support for this distinction was offered by the observation that the population of CD57+ KLRG1+ memory cells also expressed CD127 (97%) (Fig. 4), a marker recently identified as an indicator of cells destined to become long-lived memory cells (19). This marker was expressed at much lower frequencies by CD57− KLRG1+ cells (~50%). Taken together, these findings indicate that CD57 expression characterizes effector cells, whereas KLRG1 expression can be present on either memory or effector cells. The combination of CD57 and KLRG1 markers may differentiate between central memory (CD57+ KLRG1+) and effector (CD57− KLRG1+) phenotypes.

Functional characteristics of CD57+CD8+ T lymphocytes

There was very little CD57 expression among CD8+ T cell clones specific for the epitopes of CMV, EBV, and influenza virus that we studied (9–19% of these Ag-specific cells expressed CD57) (Fig. 5A); no IFN-γ expression upon Ag stimulation could be identified on such cells (Fig. 5B). However, some CD57+ cells were able to produce IFN-γ upon stimulation with the superantigen SEB (Fig. 5B). Furthermore, CD57+ cells did not proliferate upon stimulation with SEB, as was seen using CFSE staining (Fig. 5C).

Previous studies have shown that cells expressing KLRG1 or CD57 are unable to proliferate (8, 9). In this study, we confirm that CD57− cells lack proliferative ability. However, we also observed that KLRG1+ Ag-specific cells may retain the ability to expand, as shown by Ki67 expression, upon Ag-specific stimulation. This finding may indicate that simultaneous expression of CD57 and KLRG1 defines a cell phenotype at a more advanced stage of senescence, whereas KLRG1+CD57− Ag-specific cells are destined to become memory cells that may retain the ability to expand upon stimulation.

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

FIGURE 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.
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 aberrations such as autoimmunity and hematologic malignancies.

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Disclosures
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