Cutting Edge: Inhibitory Functions of the Killer Cell Lectin-Like Receptor G1 Molecule During the Activation of Mouse NK Cells

Scott H. Robbins, Khuong B. Nguyen, Nobuaki Takahashi, Toshifumi Mikayama, Christine A. Biron and Laurent Brossay

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The killer cell lectin-like receptor G1 (KLRG1) is the mouse homolog of the rat mast cell function-associated Ag and contains an immunoreceptor tyrosine-based inhibitory motif in its cytoplasmic domain. In this study we demonstrate that both pathogenic and nonpathogenic in vivo activation of NK cells induces the expression of KLRG1 on their cell surface. Upon infection with murine CMV, this induction peaks between days 5 and 7 with ~90% of the NK cells expressing KLRG1. On day 1.5 post-murine CMV infection of C57BL/6 mice, the main producers of IFN-γ are the KLRG1-negative NK cells. This effect has been recapitulated in vitro as we show that engagement of KLRG1 on a transfected NK cell line inhibits both cytokine production and NK cell-mediated cytotoxicity. Taken together, these data illustrate the crucial role played by KLRG1 during the termination of mouse NK cell activation. The Journal of Immunology, 2002, 168: 2585–2589.

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Materials and Methods

Abs and reagents

TCR-β-FITC, NK1.1-PE, NK1.1-biotin, KLRG1-biotin (clone 2F1), IFN-γ-allophycocyanin, CD244 (2B4), isotype controls, and streptavidin-PerCp were purchased from BD PharMingen (San Diego, CA). The following mAbs were used for ELISA: anti-IFN-γ mAb R4-6A2 and XMG1.2-biotin. α-Galactosylceramide (α-GalCer) was synthesized by Kirin Brewery (Gunma, Japan).

Mice and in vivo treatment protocols

Male C57BL/6 (Taconic Farms, Germantown, NY) between 5 and 9 wk of age were used. All mice were maintained at Brown University (Providence, RI) in accordance with institutional guidelines for animal care and use. Smith strain murine CMV (MCMV) salivary gland extracts were prepared as previously described (7). Infections were initiated on day 0 with 5 × 10^3 PFU of MCMV delivered i.p. For α-GalCer experiments, mice were treated i.p. with 2 μg α-GalCer. For lymphocytic choriomeningitis virus (LCMV) infections, animals were injected i.p. with 2 × 10^4 PFU of LCMV-Armstrong clone E350 as described (8). rIFN-α was administered by i.p. injections of 1 × 10^5 U of cytokine daily for 3 days (8).

Isolation of leukocytes and flow cytometric analysis

To obtain splenic leukocytes, spleens were minced and passed through nylon mesh, and cell suspensions were layered on lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) for density separation. Hepatic leukocytes were prepared by mincing and passage through a cell strainer (Falcon, Franklin Lakes, NJ). Cell suspensions were layered on two-step discontinuous Percoll gradients (Pharmacia, Peapack, NJ) for density separation. Cells were labeled with mAbs specific for TCR-β, NK1.1, KLRG1, or isotype controls as described (8). Intracellular staining for IFN-γ protein was performed by using the Cytofix/Cytoperm kit (BD PharMingen).

Cell lines and transfectants

The KY-2 NK cell clone (9) was grown in complete RPMI 1640 (Life Technologies, Rockville, MD), with 200 U/ml IL-2. The P815 tumor cell line was cultured in complete RPMI 1640. The KLRG1 wild-type construct was generated by PCR amplification using KLRG1-specific oligonucleotides. The KLRG1 cDNA was subcloned blunt into the pBabe-puro retroviral vector (PharMingen). Stable cell lines were generated by retroviral transduction as described (10).

Streptavidin-mediated cytokine production assay

Streptavidin-coated plates were purchased from Pierce (Rockford, IL). Plates were washed with PBS followed by the addition of 0.5 μg/ml of the activating NK1.1-biotin mAb to each well. Where indicated, 2 μg/ml IFN-γ was added to the culture medium.
KLRG1-biotin mAb or isotype-biotin control mAb were also added and incubated at 22°C for 1 h. After three washes, 2 × 10⁶ KY-2 or KY-2-KLRG1 cells in complete RPMI 1640 (no IL-2), preincubated with or without 10 μg/ml purified KLRG1 Ab for 1 h at 4°C, were added to appropriate wells. The cytokine levels in cell culture supernatants were detected by ELISA.

Redirected lysis assay

The redirected lysis assay using ⁵¹Cr-labeled P815 target cells was conducted as described (11). E:T ratios of 100:1, 33:1, 10:1, 3:1, and 1:1 were used. mAbs were added at a final concentration of 20 μg/ml and incubated with effector cells for 30 min at 4°C before adding target cells. After 16 h at 37°C, supernatants were harvested and ⁵¹Cr release was measured using the Hewlett-Packard Topcount NXT (Packard Instrument, Meriden, CT). Percentage of specific lysis was determined as follows: (lysis − spontaneous release)/(total release − spontaneous release) × 100 (12).

Results

KLRG1 expression is induced on NK cells following MCMV infection

Using specific rat mAbs we recently developed to the KLRG1 molecule (T. Mikayama, manuscript in preparation), we show that KLRG1 is expressed by ~30% of splenic and hepatic NK cells from naive C57BL/6 animals (Fig. 1A). To study the behavior of KLRG1 molecules on activated NK cells, C57BL/6 mice were infected with MCMV and the expression pattern of the KLRG1 molecule was followed on NK cells from both the spleen and liver at various times postinfection. On day 1.5 postinfection, the height of the NK cell response to MCMV, the percentage of KLRG1⁺ NK cells increases ~2-fold from 30% in naive mice to 60% in infected animals. This increase peaks between days 5 and 7 postinfection with >85% of the liver NK cells and >95% of the splenic NK cells expressing the KLRG1 molecule. The percentage of KLRG1⁺ NK cells then slowly decreases to reach basal levels by day 28 postinfection (Fig. 1, B and C).

The activation of NK cells by NK T cell and cytokine-specific mechanisms induces NK cell KLRG1 expression

To determine the specificity of the induction of KLRG1 on activated NK cells, we stimulated NK cells in vivo via a pathogen-free mechanism by treating them with the glycolipid α-GalCer, a synthetic compound that activates NK cells in an NK T cell-specific manner (13, 14). On day 1.5 following immunization with α-GalCer, the percentage of KLRG1⁺ NK cells increased significantly with 56.5 ± 0.9% of the splenic NK cells and 60.8 ± 3.1% of the liver NK cells expressing KLRG1 (Fig. 2A). The induction of KLRG1 on NK cells after α-GalCer treatment peaked between days 3 and 5 with ~70% of the NK cells from the liver and spleen expressing KLRG1 (Fig. 2B). Thus, both a viral activation and a nonpathogenic activation of NK cells provoke an increase in the frequency of KLRG1⁺ NK cells. Infection with LCMV and treatment with other activators of NK cells such as the cytokine IFN-α provoked a similar induction of KLRG1 on NK cells on day 1.5 post-treatment (Fig. 2A).

IFN-γ production is biased toward KLRG1-negative NK cells on day 1.5 post-MCMV infection

It has been reported that the Ly49H⁺ NK cells selectively produce IFN-γ in response to MCMV (6). Therefore, we hypothesized that the induction of KLRG1 on NK cells may be accompanied by a change in their biological functions. Consistent with previous data, intracellular IFN-γ production by NK cells peaked on day 1.5 post-MCMV infection (data not shown) (15). However, a comparison of the KLRG1⁺ NK cells and KLRG1⁻ NK cells showed a profound difference (6.5 ± 1.7% and 45.5 ± 4%, respectively) in the percentage of liver NK cells that produce IFN-γ (Fig. 3). Similarly, IFN-γ production is biased toward KLRG1⁻ NK cells freshly isolated from the spleen (Fig. 3).

Anti-KLRG1 Ab cross-linking inhibits cytokine production as well as NK cell-mediated cytotoxicity

To determine whether KLRG1 is functionally capable of mediating inhibitory signals, the KLRG1 gene was introduced into the NK cell clone KY-2 using a retroviral gene transfer system. Using these methods we generated three KY-2-KLRG1 clones with high cell surface expression of the KLRG1 molecule (Fig. 4A).

It has been demonstrated that following stimulation with biotinylated anti-NK1.1 Ab bound to plate-immobilized streptavidin
KY-2 cells produce IFN-γ (9). In this work we confirm that KY-2 cells produce IFN-γ upon anti-NK1.1 Ab cross-linking and demonstrate that the KY-2-KLRG1 cells described above also produce the cytokine IFN-γ under identical experimental conditions (Fig. 4B).

To determine the inhibitory functions of the KLRG1 molecule we simultaneously cross-linked the NK1.1 and KLRG1 molecules and found that engagement of KLRG1 inhibits IFN-γ production from the KLRG1-transfected cells but not from the control cells (Fig. 4B). As shown in Fig. 4B, inhibition of 61 ± 5.7% of IFN-γ production was obtained upon KLRG1 cross-linking. The production of TNF-α was simultaneously inhibited in a comparable manner (data not shown). When a biotinylated isotype control Ab is bound to the streptavidin-coated plate along with anti-NK1.1 Ab, KY-2 and KY-2-KLRG1 cells produce IFN-γ at similar levels. Treatment of KY-2-KLRG1 cells with purified KLRG1 Ab, before the addition of the cells to biotinylated anti-NK1.1 and biotinylated anti-KLRG1 Ab-coated wells, blocks the ability of the biotinylated KLRG1 mAb to engage the KLRG1 molecule and subsequently to inhibit the production of IFN-γ (Fig. 4B).

To assess the ability of the KLRG1 molecule to inhibit NK cell-mediated cytotoxicity we performed a redirected lysis assay. KY-2 and KY-2-KLRG1 cells are unable to spontaneously lyse the FcR bearing cell line P815 (data not shown). Upon addition of Ab to the positive signaling receptor 2B4, both the KY-2 and KY-2-KLRG1 cells were stimulated to lyse P815 targets in a similar manner (Fig. 4C). However, the addition of anti-KLRG1 Ab inhibited redirected lysis of the P815 targets by the KY-2-KLRG1 cells by 66.3 ± 3.2% but did not affect the killing mediated by the control cells. Therefore, the engagement of the KLRG1 molecule inhibits both IFN-γ and TNF-α production by NK cells as well as NK cell-mediated cytotoxicity under in vitro conditions.

Discussion

In this report, we demonstrate that the mouse KLRG1 molecule is expressed on a subset of resting NK cells isolated from naïve animals, consistent with recent studies using a different mAb (16, 17). In addition, we show a previously unappreciated dramatic increase in the percentage of NK cells that are positive for the cell surface expression of the KLRG1 molecule following in vivo NK cell activation. This increase in the prevalence of the KLRG1 † NK cells is

**FIGURE 2.** Induction of KLRG1 on NK cells following pathogenic and nonpathogenic NK cell activation. A, Splenic and hepatic leukocytes were isolated from both naïve animals and animals on day 1.5 postinfection with MCMV or LCMV, or on day 1.5 post-treatment with α-GalCer or rIFN-α. Results shown are from one experiment per protocol and are expressed as mean ± SD of three mice per group. One experiment representative of three is shown. B, Splenic and hepatic leukocytes were isolated from α-GalCer-treated C57BL/6 mice at 8 h post-treatment, as well as on days 1, 1.5, 2, 7, 14, and 21 post-treatment, and analyzed as in Fig. 1. Results are expressed as mean ± SD of three mice per group. One experiment representative of two is shown.

**FIGURE 3.** IFN-γ production is biased toward KLRG1 † NK cells on day 1.5 post-MCMV infection. A, Splenic and hepatic leukocytes were isolated from MCMV-infected C57BL/6 mice on day 1.5 postinfection followed by permeabilization and staining for intracellular IFN-γ. IFN-γ production in KLRG1 † and KLRG1 † NK cells isolated from the spleen and liver is shown. B, Production of IFN-γ from KLRG1 † and KLRG1 † NK cells from a group of three mice. Results are expressed as mean ± SD. One experiment representative of at least six experiments is shown.
FIGURE 4. Ectopic expression of KLRG1 inhibits IFN-γ production as well as NK cell-mediated cytotoxicity upon anti-KLRG1 Ab cross-linking. A, KLRG1 expression on KY-2 and KY-2-KLRG1 transfectants is shown by FACS analysis. One KY-2-KLRG1 transfectant of three is shown. B, KY-2 and KY-2-KLRG1 cells were incubated for 5 h with the indicated biotinylated mAbs bound to streptavidin-coated plates. Where indicated, cells were preincubated with purified anti-KLRG1 Ab before being added to the appropriate wells. Supernatants were assayed for IFN-γ concentrations by ELISA. Cytokine levels are expressed as mean ± SD of culture triplicates as percentage of control. Control levels of IFN-γ production was 1.5–2.5 ng/ml for stimulated KY-2 and KY-2-KLRG1 cells, depending on the experiment. ND, Not detected. One experiment representative of four is shown. C, KY-2 and KY-2-KLRG1 cells were used as effectors in a chromium release assay against the FcR⁺ target P815. Effectors were preincubated with the indicated mAbs before addition of target cells. Percentage of redirected lysis is shown. One experiment representative of three is shown.

cell subset appears to be independent of a specific activation signal, as it occurs not only during the NK cell response to viral infection but also during the activation of NK cells via pathogen-free mechanisms.

During the early phase of MCMV infection NK cells play a critical role in protection against the virus by producing high levels of IFN-γ (7). However, the NK cell IFN-γ expression during MCMV is transient and subsides in the spleen by day 2 postinfection (12, 15). Interestingly, we demonstrate that the expression of the inhibitory receptor KLRG1 on NK cells in the early phase of MCMV infection inversely correlates with the ability of NK cells to produce IFN-γ. This dichotomy suggests that inhibition of some of the NK cell effector functions is mediated by the KLRG1 molecule. In addition, there is a temporal relationship between the induction of KLRG1 and the previously reported decline in the serum levels of IFN-γ produced in response to MCMV (15). The almost all-inclusive expression of the KLRG1 molecule on NK cells, in contrast to the slight change in the expression level of the Ly49 family of inhibitory receptors (6, 18), after the peak of the NK cell response strongly suggests an important role for the KLRG1 molecule in terminating NK cell effector functions. KLRG1 may also play a role in terminating T cell functions, as it has been shown that KLRG1 mRNA is induced on effector CD8 T cells during LCMV infection (19). Although the KLRG1 ligand is unknown, its tissue distribution is likely to be broad, as the in vivo NK cell IFN-γ-dichotomy described in this work is not tissue specific.

By introducing the KLRG1 gene into the NK cell clone KY-2 we were able to study the ability of the KLRG1 molecule to initiate inhibitory signals under in vitro conditions. Upon anti-KLRG1 Ab cross-linking, both IFN-γ and TNF-α production are inhibited in KLRG1-transfected KY-2 cells that are simultaneously stimulated by anti-NK1.1 Ab. These data demonstrate that specific engagement of the extracellular domain of the KLRG1 molecule initiates a negative signal capable of blocking cytokine production in activated NK cells. In this study we demonstrate that engagement of KLRG1 not only inhibits cytokine production by NK cells but also initiates a signaling cascade that prevents NK cell-mediated cytotoxicity. The KLRG1 molecule is therefore able to terminate two of the major effector functions associated with NK cells.

In conclusion, we show the induction of the KLRG1 molecule during the in vivo activation of NK cells and demonstrate that KLRG1 is capable of inhibiting NK cell effector functions. Taken together, these data illustrate the role of the KLRG1 inhibitory receptor in the termination of the NK cell response.

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References


