KLRG1 Negatively Regulates Natural Killer Cell Functions through the Akt Pathway in Individuals with Chronic Hepatitis C Virus Infection

Jia M. Wang, Yong Q. Cheng, Lei Shi, Ruo S. Ying, Xiao Y. Wu, Guang Y. Li, Jonathan P. Moorman, Zhi Q. Yao

Department of Internal Medicine, Division of Infectious Diseases, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee, USA; Department of Biochemistry and Molecular Biology, Soochow University School of Medicine, Suzhou, China; International Center for Diagnosis and Treatment of Liver Diseases, 302 Hospital, Beijing, China; Department of Infectious Diseases, Xian Jiaotong University College of Medicine, Xian, China; Department of Hepatology, Guangzhou Number 8 People’s Hospital, Guangzhou, China; Hepatitis (HCV/HIV) Program, James H. Quillen VA Medical Center, Department of Veterans Affairs, Johnson City, Tennessee, USA

In this study, we demonstrate that killer cell lectin-like receptor subfamily G member 1 (KLRG1), a transmembrane protein preferentially expressed on T cells, is highly expressed on CD56\(^+\) NK cells, which are significantly reduced in their numbers and functions in the peripheral blood of patients with chronic hepatitis C virus (HCV) infection compared to subjects without infection. KLRG1 expression is also upregulated on healthy NK cells exposed to Huh-7 hepatocytes infected with HCV \textit{in vitro}. Importantly, the expression levels of KLRG1 are inversely associated with the capacity of NK cells to proliferate and to produce gamma interferon (IFN-\(\gamma\)) but positively associated with apoptosis of NK cells in response to inflammatory cytokine stimulation. KLRG1\(^+\) NK cells, including CD56\(^{bright}\) and CD56\(^{dim}\) subsets, exhibit impaired cell activation and IFN-\(\gamma\) production but increased apoptosis compared to KLRG1\(^-\) NK cells, particularly in HCV-infected individuals. Importantly, blockade of KLRG1 signaling significantly recovered the impaired IFN-\(\gamma\) production by NK cells from HCV-infected subjects. Blockade of KLRG1 also enhanced the impaired phosphorylation of Akt (Ser473) in NK cells from HCV-infected subjects. Taken together, these results indicate that KLRG1 negatively regulates NK cell numbers and functions via the Akt pathway, thus providing a novel marker and therapeutic target for HCV infection.

Hepatitis C virus (HCV) infection is a global health problem, affecting approximately 200 million people worldwide (1). The most remarkable feature of this blood-borne virus is its ability to evade host innate and adaptive immunity, resulting in over 80% of infected individuals developing a chronic infection. The current treatment is not optimal, and no vaccine is available, in part due to our incomplete understanding of HCV-host interactions that lead to viral persistence (2). While a high rate of genetic variability in the HCV E1/E2 envelope proteins (quasispecies) may facilitate viral escape and persistence in the face of antiviral immunity, HCV immune responses appear to be too weak to resolve infection in most humans or to protect against reinfection in chimpanzees (3, 4). It is now clear that HCV-mediated impairment of cellular immune responses is a major mechanism by which persistent infection is established. Efforts to define clinical correlates of HCV persistence have focused primarily on CD4\(^+\) and CD8\(^+\) T cell responses (5); the role of innate immunity, in particular natural killer (NK) cells, in determining the outcome of HCV infection has recently received intense scrutiny (6, 7).

NK cells comprise the first line of host defense against invading pathogens. They generally become activated in an early phase of viral infection and play an essential role in eliminating HCV-infected cells directly by cytolytic killing and indirectly by secreting cytokines (8). They also interact with professional dendritic cells (DC) and virus-specific T cells in implementing antiviral immunity (9). Not surprisingly, HCV has evolved multiple strategies to counter the host’s NK cell response (6). Compromised NK cell functions have been reported in chronically HCV-infected individuals, whereas normalization of depressed NK activity after antiviral therapy is associated with a low frequency of relapse and improved sustained virologic responses (SVR) (10–15). Notably, aberrant NK activities may also contribute toward liver injury (8). Therefore, further studies are required to understand how NK cells are fine-tuned during host defense and in liver injury during HCV infection.

Human NK lymphocytes have been functionally divided into several subpopulations based on the relative expression of CD3, CD16, and CD56 markers (16–18). The CD56\(^{bright}\) and CD56\(^{dim}\) NK cell subsets differ in their maturation status, proliferative capacity, cytotoxic activity, and cytokine production (16–18). The majority (90%) of peripheral blood and spleen NK cells are CD56\(^{dim}\) and are considered to be the mature effectors, with enhanced natural and major histocompatibility complex (MHC)-unrestricted lymphokine-activated killing (LAK) ability, whereas a minority of the NK cells are CD56\(^{bright}\), which function as an important source of immune-regulatory cytokines (16–18). Shifts of these subsets have been reported in patients with acute to chronic HCV infection and following antiviral therapy (10–15). The antiviral activity of NK cells is regulated through surface NK receptors, including the inhibitory killer immunoglobulin-like receptor (KIRs) and the leukocyte immunoglobulin-like receptors (LIRs) in humans and the Ly49 molecules in mice. Natural cytotoxicity receptors NKP30, NKP44, NKP46, and C-type lectin-like receptors CD94/NKG2, comprising inhibitory (NKG2A) or activating (NKG2C/NKG2D) isoforms, represent additional NK cell receptors (16–18). Classical and nonclassical MHC molecules

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Address correspondence to Jonathan P. Moorman, moorman@etsu.edu.

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serve as ligands for these NK receptors. It is noteworthy that inheritance of particular KIR genes or their polymorphisms involved in the control of NK activity predispose to chronic infection or treatment resistance (19–23). Expression levels of these inhibitory and stimulatory receptors on NK cells and their ligands on hepatocytes, cholangiocytes, stellate cells, and Kupffer cells are significantly altered during HCV infection and contribute to the pathogenesis and progression of liver disease (24, 25).

Killer cell lectin-like receptor subfamily G member 1 (KLRG1) is a transmembrane protein with immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic domain and a C-type lectin-like domain in the extracellular region (26). KLRG1 was first identified on the rat mucosal-type mast cell line RBL-2H3, as antibody-mediated ligation of KLRG1 inhibits release of inflammatory mediators from these cells; thus, the protein was originally named mast cell function–associated antigen (MAFA) (27). In humans and mice, KLRG1 is found preferentially expressed on subsets of NK and T cells, but not on monocytes or granulocytes, and it binds three members (E, N, and R) of the cadherin family to regulate lymphocyte activities (28–30). After birth, KLRG1 expression in T cells from peripheral blood decrease rapidly, to reappear in effector/memory T cells in adults (31). Of note, monoclonal antibody (MAb)–mediated cross-linking of KLRG1 inhibits both cytokine production and NK cell–mediated cytotoxicity (32).

Interestingly, pathogenic infections increase the percentage of KLRG1-expressing lymphocytes (32–41). More specifically, on-going antigen triggering in chronic viral infection increases the abundant expression of KLRG1 by virus-specific CD8 T cells infected with human immunodeficiency virus (HIV), cytomegalovirus (CMV), or Epstein-Barr virus (EBV) with impaired proliferative capacity, whereas influenza virus, a typical acute viral infection, elicits a considerably lower frequency of KLRG1+ cells within influenza virus–specific T cells (39). Despite these insights into KLRG1 functions, very little information is currently available about the expression of KLRG1 on NK cells and its role in NK subset function during chronic infection.

In this study, we examined KLRG1 expression on NK cells in individuals with chronic HCV infection and assessed the effects of KLRG1 on NK cell subset (CD56bright and CD56dim) functions and the Akt signaling pathway. We found that KLRG1 expression is upregulated and inversely associated with a reduced NK cell number, impaired NK cell activation and proliferation, suppressed gamma interferon (IFN-γ) production, and defective Akt (Ser473) phosphorylation but positively correlated with apoptosis of NK cells in patients with chronic HCV infection. We also found that antiviral therapy with interferon/ribavirin (IFN/RBV) corrects the NK cell subset discrepancy and KLRG1 expression levels, and blockade of KLRG1 signaling significantly improves NK cell IFN-γ production and Akt phosphorylation. These results suggest that KLRG1 negatively regulates NK cell frequency and function in HCV infection.

MATERIALS AND METHODS

Subjects. The study subjects comprised three populations: 32 subjects with chronic HCV infection prior to antiviral treatment, 10 subjects who had received IFN/RBV therapy with SVR, and 10 healthy subjects (HS). The study protocol was approved by an institutional review board at East Tennessee State University and the James H. Quillen VA Medical Center (ETSU/Va IRB, Johnson City, TN). HCV genotype (70% type 1, 30% type 2 or 3) and viral load (ranging from 12,300 to 500,000 IU/ml) were determined by Lexington VAMC, and all subjects were virologically and serologically positive for HCV. Healthy subjects were negative for HBV, HCV, and HIV infections. Of the study subjects, 85% were male. The mean age of HCV-infected individuals was comparable to that among HS (P > 0.05).

Cell isolation and culture. Human peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of study subjects by Ficoll-density centrifugation with lympho-H (Atlanta Biological, Lawrenceville, GA). Where indicated, CD56+ NK cells were further purified from PBMCs by magnetic beads conjugated to anti-CD56 antibody; CD3+ CD56− NKS were also purified from PBMCs by negative selection according to the manufacturer’s instructions (purity, >95%;Miltenyi Biotec Inc., Auburn, CA). The cells were cultured as previously described (42).

Flow cytometry. Procedures for detection of cell surface markers and intracellular cytokine staining were performed essentially as described previously (42,43). Briefly, PBMCs (0.2 × 106 per well in a 96-well plate) were stimulated with 10 ng/ml recombinant human interleukin-12 (rhIL-12; ebioscience, San Diego, CA), or isotype control IgG for 18 h, followed by 1 μg/ml Brefeldin A (BioLegend, San Diego, CA) 4 h prior to harvesting the cells, thus for-bidding cytokine secretion. Cell surface markers were stained with specific conjugated antibodies that included phycoerythrin (PE)-CD3 and peridinin chlorophyll protein (PerCP)-CD56 (ebioscience, San Diego, CA), PE-Annexin V (BD Biosciences), allophycocyanin (APC)-CD69 (ebioscience, San Diego, CA), Alexa Fluor 488-KLRG1 (H. Pircher), and Alexa Fluor 488–E-cadherin (R&D Systems Inc., Minneapolis, MN) (31). For staining of intracellular IFN-γ (Miltenyi Biotec Inc., Auburn CA) and granzyme B (ebioscience), the cells were fixed and permeabilized by adding Cytofix/Cytoperm (BD Pharmingen). Cells were washed three times and fixed in 100 μl CellFix (BD Pharmingen) per well. The intracellular cytokine staining was carried out using an Inside Stain kit (Miltenyi Biotec) per the manufacturer’s instructions. Isotype-matched control antibodies (ebioscience) and fluorescence minus-one (FMO) controls were used to determine background levels of staining and to adjust multicolor compensation as a gating strategy. The cells were sorted on a FACScan flow cytometer or Accuri C6 flow cytometer (BD, Franklin Lakes, NJ) and analyzed by using CellQuest or FlowJo software (Tree Star, Inc., Ashland, OR).

Proliferation assays. PBMCs were labeled with carboxyfluorescein succinimidyl ester (CFSE; 2.5 μM; Invitrogen) for 10 min at 37°C per the manufacturer’s instructions, washed with complete RPMI medium, and cultured (5 × 104 cells/well) in a 96-well plate in the presence rhIL-12 (10 ng/ml; ebioscience) and rhIL-2 (50 U/ml; R&D Systems). After culture for 6 days, the cells were immunostained with PE-CD3, PerCP-CD56, and Alexa Fluor 488-KLRG1 and analyzed with a FACScan flow cytometer (BD, Franklin Lakes, NJ).

Blocking assay. Purified NK cells from HCV-infected patients were incubated with anti-human KLRG1 (3 μg/ml; obtained from Hanspeter Pircher), anti-human E-cadherin (5 μg/ml; EMD Millipore Corporation, Billerica, MA), or isotype control IgG for 54 h, followed by stimulation with rhIL-12 (10 ng/ml; ebioscience) and rhIL-2 (50 U/ml; R&D Systems). After culture for 6 days, the cells were immunostained with PE-CD3, PerCP-CD56, and Alexa Fluor 488–KLRG1 and analyzed with a FACScan flow cytometer (BD, Franklin Lakes, NJ).

Phosphocytometry. Purified NK cells were incubated with anti-human KLRG1 (3 μg/ml; from H. Pircher) or isotype control IgG in 96-well plate with complete RPMI 1640 medium containing rhIL-12 (10 ng/ml) and rhIL-2 (50 U/ml) (ebioscience) for 72 h, after which the cells were pulsed with rhIL-15 (100 ng/ml; ebioscience) for 1 h. The NK cells were fixed, permeabilized, and sequentially incubated with pAkt (ser473) antibody (D9E; Cell Signaling, Boston, MA) or rabbit isotype control IgG (DA1E; Cell Signaling, Boston, MA) for 1 h at room temperature. The cells were analyzed on a FACScan flow cytometer (BD, Franklin Lakes, NJ) by using FlowJo software (Tree Star, Inc., Ashland, OR).

Coculture of healthy PBMCs with HCV-transfected or untransfected HuH-7 hepatocytes. Transfection of HuH-7 hepatocytes (kindly provided by T. J. Liang, Liver Section, NIH NIDDK) with HCV JFH-1
strain (kindly provided by T. Wakaia) was carried out as described previously (42, 43). Prior to the coculture experiment, HCV-transfected or untransfected Huh-7 hepatocytes were serum starved for 18 h, then activated with rhIFN-γ (0.1 μg/ml; R&D Systems) for 48 h. Activated hepatocytes were removed from plates with 0.05% trypsin–EDTA and then plated at 5 × 10^5 cells/well in a 12-well plate. PBMCs or negatively purified NKs were then added to the adherent hepatocytes in RPMI 1640 medium and cocultured for an additional 48 h, and the expression levels of KLRG1, CD69, CD107a, IFN-γ, and granzyme B in CD56+ NK cells were analyzed by flow cytometry.

Statistical analysis. Study results were summarized for each group, and results are expressed as the means ± standard deviations (SD). Comparisons between two groups were performed by multiple comparisons testing with the least significant difference or Turkey’s procedure, depending on an analysis of variance (ANOVA) F test (Prism software, version 4; GraphPad Software) and a nonparametric Mann-Whitney U test. A pairwise t test was used to compare the significance of changes in KLRG1 blockage experiments. Correlations between KLRG1 expression on NK cells and IFN-γ or Annexin V expression were analyzed by using a Pearson correlation program. P values of <0.05, <0.01, and <0.001 were considered significant or very significant.

RESULTS

NK cells are significantly reduced, with a discrepancy of CD3− CD56bright and CD3− CD56dim subsets in the peripheral blood of patients with chronic HCV infection. Human NK cells are divided into two subsets, CD56bright and CD56dim, according to their expression levels of CD56. About 90% of peripheral blood NK cells belong to the CD56dim subset, which is mainly cytolytic, while the remaining 10% of NK cells are in the CD56bright subset and primarily produce cytokines (16–18). To characterize the effects of HCV infection on NK cells and their subsets, we compared the percentages of total CD56+ NK cells, and the percentages of CD56bright or CD56dim cells within NK cells, in the gated lymphocytes or NK populations in PBMCs from 29 HCV patients, 9 SVR individuals, and 8 HS. As shown in the representative dot plots and summary data in Fig. 1A and B, CD3− CD56+ NK lymphocyte frequencies in patients with chronic HCV infection were significantly reduced compared with those with SVR and HS, while there was no difference between the SVR and HS groups. Interestingly, CD56bright NK subsets were expanded and CD56dim NK subsets contracted in chronic HCV patients compared to SVR and HS; this finding held true with regard to the percentages of cell subsets based on whole lymphocytes as well as the NK populations, although there was no significant difference in the CD56bright NK subset in the whole lymphocyte populations in HCV-infected versus uninfected subjects (Fig. 1B). These results suggest that HCV infection reduces the total NK cell numbers and shifts the NK subsets, whereas successful antiviral therapy recovers this HCV-induced NK cell discrepancy.

KLRG1 expression is upregulated on human NK cells upon HCV infection. KLRG1 is preferentially expressed on subsets of NK cells and antigen-experienced T lymphocytes, as it binds to cadherins and exhibits inhibitory effects on lymphocyte activities (27–29). To determine the effects of KLRG1 on human NK subsets during HCV infection, we examined KLRG1 expression levels on gated NK cells in PBMCs from 24 chronically HCV-infected patients, 9 SVR individuals, and 8 HS. The representative dot plots and summary data for the percentages of KLRG1+ cells on CD3−, CD56+, CD56bright, and CD56dim NK cells are shown in Fig. 2A and B. Of note, KLRG1 expression in chronically HCV-infected individuals was significantly elevated, not only on CD3− CD56+ NK cells but also on CD3− CD56bright and CD3− CD56dim NK subsets compared to SVR subjects and HS. In addition to the percentages of positive cell frequencies, the mean fluorescence intensity (MFI) of KLRG1 expression on NK cells in HCV-infected patients was also significantly higher than for SVR subjects and HS (Fig. 2C). However, there was no significant difference for KLRG1 expression levels on NK cell subsets between SVR and HS (Fig. 2B and C). These results demonstrate that HCV infection upregulates KLRG1 expression, and IFN/RBV therapy in individuals with SVR normalizes the NK cell number as well as KLRG1 expression levels on these cells.

The above-observed KLRG1 expression might be a result, rather than a cause, of NK cell dysregulation during HCV infection. Additionally, the driving force for KLRG1 upregulation during HCV infection remains to be determined. To further elucidate the role of HCV in regulation of KLRG1 expression, we employed a newly established cell culture system by transfecting Huh-7 hepatocytes with the HCV JFH-1 strain in vitro to mimic the in vivo setting of early HCV infection (42, 43). To this end, PBMCs or purified NKs from HS were cocultured with HCV-transfected or untransfected Huh-7 hepatocytes for 48 h, followed by flow cytometric analysis for KLRG1 expression and NK functions. Consistent with the data observed in natural HCV infection, HCV+ Huh-7 cells significantly enhanced KLRG1 expression on cocultured CD3− CD56+ cells (Fig. 2D).

In addition to the KLRG1 receptor, we also examined the expression of the KLRG1 ligand E-cadherin on HCV-transfected or untransfected hepatocytes as well as different populations of PBMCs from HCV-infected and uninfected individuals. As shown in Fig. 2E, E-cadherin was detected at higher levels in HCV-transfected Huh-7 than HCV-untransfected Huh-7 cells. Additionally, E-cadherin was expressed at high levels on CD14+ monocytes, at low levels on CD19+ B cells and CD56+ NKs, but not on CD4+ or CD8+ T lymphocytes. Interestingly, HCV infection seems to upregulate KLRG1 expression on these cells. These accessory cells may provide a ligand for interaction with KLRG1 that is expressed on T cells and NKs but not on monocytes or granulocytes (data not shown).

Since KLRG1 has been recognized as an inhibitory receptor and senescence marker for T cells (31–41), it may also function as a regulator to control NK cell function. To exclude the possible secondary effects from accessory cells in bulk PBMCs, we repeated the coculture assay with purified NK cells. While these NK cells cocultured with HCV-transfected hepatocytes expressed higher levels of KLRG1, they expressed less CD69 and IFN-γ, primarily by KLRG1− NKs, than those cocultured with HCV-untransfected hepatocytes (Fig. 2F), suggesting that HCV may induce KLRG1 expression, as well as its ligand’s expression, to inhibit NK cell activation and function.

Increased KLRG1 expression is associated with decreased IFN-γ production by NKs in HCV infection. NK and T cells produce IFN-γ, a critical cytokine for clearance of infectious pathogens (44). Previous reports have implicated the increased KLRG1 expression on T cells in impairing their capacity for proliferation and IFN-γ production (33, 45). To determine the role of KLRG1 in regulation of NK cell function, here we examined the relationship between KLRG1 expression and IFN-γ production in NK cells and its subsets in individuals with HCV and HS. As shown in Fig. 3A, an impaired production of IFN-γ was observed in CD3− CD56+ NK cells, including CD3− CD56bright and CD3− CD56dim
subpopulations in PBMCs, derived from chronically HCV-infected patients compared with those from HS, and this was very significant in the CD56<sup>bright</sup> populations, the major subsets for cytokine secretion, suggesting that HCV inhibits IFN-γ production by NK cells (Fig. 3B). Notably, the majority of IFN-γ was primarily produced by KLRG1<sup>−</sup> NK cells, and this was also observed in CD56<sup>bright</sup> and CD56<sup>dim</sup> subpopulations, both in HS and HCV-infected individuals (Fig. 3B). To determine whether isolated NKs behave in the same manner as in bulk PBMCs and whether accessory cells are required for their behavior, we examined the relationship between KLRG1 expression and IFN-γ production by using purified NKs from HCV-infected and uninfected subjects in response to cytokine stimulation. As shown in Fig. 3C with isolated NKs, similar to the gated NKs in bulk PBMCs (Fig. 3A), NKs from HCV-infected individuals produced less IFN-γ than those of HS, and the majority of IFN-γ was expressed by KLRG1<sup>−</sup> NK cells, whereas KLRG1<sup>+</sup> NKs produced little IFN-γ, if any, in both populations. Notably, the KLRG1 expression level on purified NK cell was lower than those in bulk PBMCs, which we believe was likely due to secondary effects from accessory cells (such as monocytes or T or B lymphocytes, either directly or indirectly by cytokine secretion) in response to ex vivo stimulation. Moreover, KLRG1 expression on CD3<sup>−</sup> CD56<sup>−</sup> NK cells was inversely correlated with the IFN-γ production by these cells (Fig. 3D), indicating that KLRG1 may negatively control IFN-γ production by NK cells.

We also assayed the proliferative capability of NK cells from HCV-infected patients versus those from HS in a CFSE dilution assay with or without stimulation. As shown in Fig. 3E, the proliferation of NK cells with increased KLRG1 expression from chronically HCV-infection.
fected patients was much lower than in HS. Moreover, in both HCV-infected individuals and HS, the proliferative capacity of KLRG1/NK cells was much lower than that of KLRG1/NK cells. These results clearly illustrated that HCV infection upregulates KLRG1 expression that is linked to a low capacity for proliferation and cytokine production by NK cells, suggesting that KLRG1 is a negative regulator or biomarker for NK cells.

KLRG1 expression is associated with apoptosis of NK cells in HCV infection. KLRG1/NK cells have been reported to exhibit properties of end-stage or senescent cells during the contraction phase of immune responses, including decreased proliferation, increased apoptosis, and reduced effector function in mice (32, 46). To further determine whether the reduced NK cell number and impaired NK cell function was due to apoptosis of NK cells during HCV infection, we analyzed Annexin V expression on total CD3− CD56+ NK cells and CD3+ CD56bright and CD3− CD56dim subsets in patients with chronic HCV infection, individuals after treatment with SVR, and HS. Percent cell frequency in the gated area is shown in the dot plots. (B) Summary data showing the percentages of KLRG1 expression in total CD3− CD56− NK cells and CD3− CD56bright and CD3− CD56dim subsets. Each dot represents one individual, and the horizontal bars represent mean values. *, P < 0.05; NS, no significance. (C) MFI of KLRG1 expression on CD3− CD56+ NK cells. **, P < 0.01; NS, no significance. (D) Upregulation of KLRG1 expression on NK cells incubated with or without HCV-transfected (HCV+) or untransfected (HCV−) Huh-7 hepatocytes. Briefly, healthy PBMCs were cocultured with HCV-transfected or untransfected Huh-7 cells, and KLRG1 expression on CD3− CD56− NK cells was analyzed by flow cytometry as described in Materials and Methods. Summary data (means ± SD) from three independent experiments are shown. *, P < 0.05; NS, no significance. (E) Expression of the KLRG1 ligand E-cadherin on HCV-infected and uninfected Huh-7 hepatocytes as well as on different populations of PBMCs from 3 HCV-infected and 3 uninfected subjects. The percentage of cells expressing E-cadherin was calculated as the percentage in the sample minus that in the isotype control. (F) Representative dot plots of CD69 expression (left panel) and IFN-γ production (right panel) by KLRG1+ or KLRG1− NKs, including total purified CD3− CD56− NKs as well as CD56bright and CD56dim subsets, cocultured with HCV− or HCV+ Huh-7 hepatocytes. The percent cell frequency in the gated area is shown in the dot plots.
correlated with the apoptosis of NK cells from HCV-infected patients (Fig. 4C). Again, we examined the relationship between KLRG1 and Annexin V expression on purified NK cells in response to ex vivo stimulation. As shown in Fig. 4D, HCV patient NKs exhibited more apoptotic cells, which were mainly KLRG1⁺ NKs, than did those from HS. In contrast, NKs derived from HCV-infected subjects expressed less of the degranulation marker CD107 (Fig. 4E) and cytolytic enzyme granzyme B (data not shown), which are primarily produced by KLRG1⁺ NKs, than did NKs from HS. These findings revealed that the apoptotic and cytolytic activities of NK cells clearly correspond to higher surface expression of KLRG1 on NK cells, implying that KLRG1 might also be a hallmark for infection-mediated NK cell senescence or premature cell aging.

KLRG1 blockade recovers the impaired IFN-γ production by NK cells in chronic HCV infection. Given the fact that high levels of KLRG1 expression are inversely associated with the impaired capability of NK cells to produce IFN-γ in chronic HCV infection,
we next sought to determine whether blockade of KLRG1 signaling improved IFN-γ production by NK cells from HCV-infected patients. It has been reported that MAb-mediated cross-linking of KLRG1 inhibits mouse NK functions (32); therefore, to further elucidate the role of KLRG1 in human NK function and exclude the possibility of secondary effects on NK cells from DC and T cells, we investigated IFN-γ production by activated NK cells with or without blockade of KLRG1 signaling. To this end, purified CD56+ NK cells from 6 chronically HCV-infected patients were incubated with anti-KLRG1 or control IgG antibodies for 54 h, followed by rhIL-12 and rhIL-2 stimulation for an additional 18 h. The representative flow cytometric dot plots and summary data of intracellular IFN-γ production by total CD3+CD56+ NK cells, as well as CD3+CD56bright and CD3+CD56dim subsets, from an HCV-infected patient and HS are shown. 

**FIG 4** KLRG1 expression is positively associated with apoptosis of NK cells following HCV infection. (A) PBMCs from HCV-infected patients and HS were stimulated with 10 ng/ml rhIL-12 for 18 h, followed by 1 μg/ml brefeldin A 4 h prior to harvest of the cells (to block cytokine secretion). PBMCs were immunostained and then analyzed for Annexin V expression on total CD3+CD56+ and CD3+CD56bright and CD3+CD56dim NK cells by flow cytometry. Representative dot plots for Annexin V expression on KLRG1+ and KLRG1– NK cells, including CD3+CD56+ and the CD3+CD56bright and CD3+CD56dim subsets from an HCV-infected patient and HS are shown. (B) Average percentage of Annexin V expression from the KLRG1+ versus KLRG1– cell fraction of total CD3+CD56+ NK cells and the CD3+CD56bright and CD3+CD56dim subsets. Results are expressed as means ± SD of the percentages of Annexin V expression by NK cells from 24 HCV-infected patients versus 8 HS. *, P < 0.05; **, P < 0.01. (C) Relationship between KLRG1 expression and Annexin V expression on CD3+CD56+ NK cells from 24 HCV-infected patients, based on Pearson correlation analysis. r = 0.535; **, P < 0.01. (D) Representative dot plots for KLRG1 and Annexin V expression on negatively selected NK cells, including CD3+CD56+ and the CD3+CD56bright and CD3+CD56dim subsets, from an HCV-infected subject and HS. (E) Representative dot plots for KLRG1 and CD107a expression levels on negatively selected NK cells, including CD3+CD56+ and the CD3+CD56bright and CD3+CD56dim subsets, from an HCV-infected subject and HS. The percentage of cells in the gated area is shown in the dot plots.
KLRG1 alone without rhIL-12/rhIL-2 stimulation failed to secrete IFN-γ, suggesting that the blocking antibody inhibited negative signaling by KLRG1 rather than directly activating NK cells, and its effect requires additional positive signals generated by cytokine stimulation to drive cell proliferation. These findings further supported the notion that KLRG1 negatively controls IFN-γ production by NK cells during HCV infection.

The ligands of KLRG1 are E-, N-, and R-cadherins. We have examined E-cadherin expression on different populations in PBMCs and hepatocytes. Interestingly, HCV infection seems to upregulate E-cadherin expression on some of these cells (Fig. 2E). We also attempted to block KLRG1 signaling with anti-E-cadherin antibodies, but only a minimal rescuing effect on IFN-γ production was observed in PBMCs stimulated with rhIL-12/rhIL-2 in the presence of anti-E-cadherin versus the IgG control (data not shown). Because of multiple other KLRG1 ligands existing in the system, we would expect less efficiency in blocking KLRG1 ligands than in blocking the KLRG1 receptor itself.

KLRG1 blockade reinvigorates NK cells by correcting the impaired Akt (Ser473) phosphorylation in HCV infection. Lymphocyte activation is initiated by Akt phosphorylation at Ser473 (45). To determine the underlying mechanism for correction of NK cell function via blockade of KLRG1 signaling, we further investigated the phosphorylation of Akt in NK cells following KLRG1 blockade by flow cytometric analysis. As shown in Fig. 5B, Akt phosphorylation was im-

FIG 5  KLRG1 blockade recovers the impaired IFN-γ production through the Akt pathway by NK cells in chronic HCV infection. (A) Purified NK cells from HCV-infected patients were incubated with anti-human KLRG1 or isotype IgG for 54 h, followed by stimulation with rhIL-12 and rhIL-2 for 18 h, and then subjected to flow cytometric analysis of intracellular IFN-γ expression levels as described Materials and Methods. With the blockade by KLRG1 antibody, IFN-γ production was upregulated. Representative dot plots measuring IFN-γ production by total CD3−CD56− and CD3−CD56dim and CD3−CD56bright NK cell subsets with the KLRG1 blocking antibody or control IgG are shown on the left, and the average percentages of IFN-γ production by different subsets of NK cells are shown on the right. Results are expressed as means ± SD from 8 independent experiments. *, P < 0.05; **, P < 0.01. (B) The frequency of pAkt expression by purified NKs from 8 HCV-infected and 8 uninfected subjects following ex vivo stimulation as described in Materials and Methods. *, P < 0.05. (C) KLRG1 blockade restores the phosphorylation of Akt in NK cells from HCV-infected patients. Purified CD56+ NK cells from HCV-infected patients and HS were incubated with anti-human KLRG1 or isotype control IgG in the presence of rhIL-12 and rhIL-2 for 72 h, then costimulated with rhIL-15 for 1 h, followed by intracellular staining of phosphorylated Akt, and then analyzed by flow cytometry as described in Materials and Methods. The representative flow histogram overlap against isotype control staining (gray-filled area) for pAkt expression in NK cells with IgG (blue line) versus anti-KLRG1 (red line) treatment is shown on the left, and summary data of the percentage and mean fluorescence intensity of pAkt expression in NK cells from 6 HS versus 6 HCV-infected patients, treated with the IgG control versus KLRG1 blocking antibody is shown on the right. *, P < 0.05; **, P < 0.01.
paired in NK cells derived from 8 individuals with chronic HCV infection compared to those from HS following ex vivo stimulation, and blockade of KLRG1 signaling in purified CD56+ NK cells significantly enhanced Akt (Ser473) phosphorylation compared to the cells treated with control IgG. These results were reproducible in independent experiments using purified NK cells from multiple HCV-infected individuals (n = 6), in terms of both the percentage of pAkt-positive cell numbers and the MFI of pAkt expression levels in NK cells, indicating an inhibitory role for KLRG1 in regulation of human NK cell functions through the Akt pathway.

**DISCUSSION**

Chronic HCV infection is a worldwide infectious disease, leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (1). Although the pathogenesis of HCV infection remains only partly elucidated, it has become evident that, in addition to viral mutational escape, the dysregulation of innate to adaptive immune responses plays a major role in viral persistence and disease progression. The mechanisms involved in the HCV chronicity include dysfunction of monocyte and dendritic cells, impairment or depletion of NK cells, suppression of CD4+ and CD8+ effector T cells, and accumulation of Th17 cells and Foxp3+ regulatory T cells (2–10, 42–43, 47–49). Since NK cells comprise the first line of host defense against invading pathogens, reduction of NK cell frequency and cytokine secretion may contribute significantly to the impaired cellular immune response and virus persistence in HCV infection. Despite intensive research on the regulation of NK cell frequency, phenotype, and function during HCV infection (10–15, 47), the exact mechanisms for contraction of NK cell subsets with impaired NK cell function in the setting of HCV infection are not completely understood.

KLRG1 is an inhibitory receptor preferentially expressed by NK and antigen-experienced T cells in mice and humans (28–41). Recent studies have indicated that repetitive antigen stimulation leads to an increase of KLRG1 expression by virus-specific CD8+ T cells in chronic viral infections, such as infections with HIV, CMV, and EBV, but not in resolved infection, such as influenza virus infection (39). Chronic viral infections yield virus-specific, CD127+/KLRG1+ T cells with an impaired proliferative capacity, whereas resolved infections are characterized by the presence of CD127+/KLRG1−/CD8− memory T cells with a potent proliferative ability (40, 41). Therefore, KLRG1 is also regarded as an immune senescence marker reflecting infection-mediated cell aging (35). While the majority of studies have focused on KLRG1 expression and inhibitory effects on CD4+ and CD8+ T lymphocytes in pathogenic infections (32–41), information regarding the expression of KLRG1 and its role in NK cells during chronic viral infections is limited.

In this study, we analyzed the expression of KLRG1 and its role in NK cell number and function in the peripheral blood of individuals with chronic HCV infection. We found that (i) there is a significant difference in the NK cell frequency between patients with chronic HCV infection and individuals after antiviral treatment with SVR or HS without infection. Although the frequency of CD3−CD56bright NK cells in HCV patients was higher than observed in those with SVR and HS (perhaps reflecting the NK cell differentiation and maturation status in the setting of chronic HCV infection), the frequency of CD3−CD56dim NK cells, the major NK cell subset in HCV patients was remarkably lower than in those with SVR and HS, leading to a total CD56+ NK population contraction in chronic HCV infection. However, there was no significant difference in the total NK cell frequency, or in the CD3−CD56bright and CD3−CD56dim NK subsets, between SVR and HS, indicating that successful antiviral treatment can correct the frequency and phenotype of NK cells in HCV infection. (ii) KLRG1 expression levels on CD3+CD56− NK cells, including CD3−CD56bright and CD3−CD56dim subsets, were significantly increased in chronically HCV-infected subjects compared to SVR subjects or HS. This was recapitulated in vitro, as we showed that KLRG1 expression was upregulated on NK cells cocultured with hepatocytes expressing HCV. Notably, KLRG1 was preferentially expressed on the less-responsive subset of CD56−, CD56bright, and CD56dim NK cells with respect to IFN-γ production. Moreover, the level of KLRG1 expression was inversely correlated with the ability to produce IFN-γ in CD3−CD56+ cells, and KLRG1+ NK cells produced less IFN-γ than KLRG1− subsets, suggesting that KLRG1+ NK cells are dysfunctional or senescent. This is consistent with the observation that production of IFN-γ during CMV infection in C57BL/6 mice is primarily by KLRG1− NK cells (32). Intriguingly, KLRG1− NK cells, including the total CD3−CD56− NK population, as well as CD3−CD56bright and CD3−CD56dim subsets, were found to be more sensitive, whereas KLRG1− NKs were resistant, to inflammatory cytokine-mediated cell apoptosis, which was more prominently observed in HCV-infected subjects versus HS. (iii) The elevated KLRG1 expression on NK cells and subsets was corrected by antiviral therapy with IFN/RBV in individuals with SVR. Furthermore, blockade of KLRG1 signaling significantly recovered the impaired IFN-γ production by NK cells from HCV-infected individuals. Additionally, blockade of KLRG1 also enhanced the impaired phosphorylation of Akt (Ser473) in NK cells from chronically HCV-infected patients. Taken together, these results indicate that HCV-induced expression of KLRG1 negatively controls NK cell number and function via the Akt pathway during chronic viral infection.

The CD56dim population is the major subset of NK cells that exhibit cytolytic activity and can destroy virally infected cells, malignant cells, and play regulatory roles in autoimmunity. In this regard, we believe that KLRG1 primarily mediates the loss of CD56dim NK cells, which may abolish the protective role of NK cells against virally infected hepatocytes. Indeed, KLRG1 is highly expressed on the CD56dim subset from HCV-infected patients, leading to very significant cellular apoptosis (P < 0.01) compared to NK cells from HS in response to the inflammatory cytokine stimulation. On the other hand, the CD56bright population is a minor subset of NK cells that primarily produces cytokines to indirectly combat virally infected cells. In this context, KLRG1 is also found to be upregulated in the CD56bright subset from HCV-infected patients, leading to very significant impairment of this subset in its ability to produce IFN-γ (P < 0.001) compared to HS. These results are in agreement with our previous observations that another negative signaling molecule, T cell immunoglobulin and mucin domain protein 3 (Tim-3), is upregulated on NK cells to dampen their functions, with inhibited activation (CD69), proliferation (Ki69), degranulation (CD107a), and killing activity (granzyme B) following HCV infection (data not shown). This in turn interplays with monocytes and T cells (including Th17 and Foxp3+ Tregs) in immune dysregulation that contribute to viral persistence (42–43, 48–49). This also supports the notion that HCV may utilize KLRG1—an intrinsic mercenary of cell feedback
regulation—for the purpose of virus persistence and thus facilitate chronic infection.

In conclusion, our results indicate that KLRG1 plays a pivotal role in control of NK cell number and function during HCV infection. HCV appears to induce KLRG1 expression on NK cells to disrupt NK cell function, and blocking KLRG1 signaling may reverse HCV-induced NK cell dysfunction. It is not clear, however, how KLRG1 is upregulated by HCV infection at this point. Additionally, KLRG1 is an inhibitory receptor, but only when it binds to its ligands (E- N-, or R-cadherin) to transmit an inhibitory signal (28–30). The mechanism of how anti-KLRG1 MAb treatment improves NK cell function is also unclear. We suspect that either KLRG1 ligands are also expressed on NK cells and the anti-KLRG1 MAb blocks the binding sites of cadherins, or antibody ligation of KLRG1 itself delivers a positive signaling and reverses the unresponsiveness of NK cells from virally infected individuals. Since E-cadherin is upregulated on HCV-infected hepatocytes, antigen-presenting cells, and NKs, we believe that interference of the interaction of NK KLRG1 with its ligands on accessory cells, rather than a direct activation of NKs, is likely the mechanism for NK functional recovery after anti-KLRG1 treatment. Nevertheless, given the unique functions of NK cells as the initial defense against intruding pathogens, identification of factors such as KLRG1 that control NK differentiation, maturation, homeostasis, and activation status is critical to understanding innate immune regulation and to improving immunotherapy of chronic viral infection.

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