KLRG1 Impairs CD4⁺ T Cell Responses via p16 𝑖𝑛𝑘4 𝑎 and p27 𝑖𝑛𝑘4 𝑒 Pathways: Role in Hepatitis B Vaccine Failure in Individuals with Hepatitis C Virus Infection

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KLRG1 Impairs CD4$^+$ T Cell Responses via p16$^{\text{ink4a}}$ and p27$^{\text{kip1}}$ Pathways: Role in Hepatitis B Vaccine Failure in Individuals with Hepatitis C Virus Infection


Coinfection of hepatitis B virus (HBV) with hepatitis C virus (HCV) is quite common, leading to an increase in morbidity and mortality. As such, HBV vaccination is recommended in HCV-infected individuals. However, HBV vaccine responses in HCV-infected individuals are often blunted compared with uninfected populations. The mechanism for this failure of vaccine response in HCV-infected subjects remains unclear. In this study, we investigated the expression and function of an inhibitory receptor, killer cell lectin-like receptor subfamily G member 1 (KLRG1), in the regulation of CD4$^+$ T cells and HBV vaccine responses during HCV infection. We demonstrated that KLRG1 was overexpressed on CD4$^+$ T cells from HCV-infected, HBV vaccine nonresponders compared with HBV vaccine responders. The capacity of CD4$^+$ T cells to proliferate and secrete IL-2 cytokine was inversely associated with the level of KLRG1 expression. Importantly, blocking KLRG1 signaling resulted in a significant improvement in CD4$^+$ T cell proliferation and IL-2 production in HCV-infected, HBV vaccine nonresponders in response to TCR stimulation. Moreover, blockade of KLRG1 increased the phosphorylation of Akt (Ser$^{473}$) and decreased the expression of cell cycle inhibitors p16$^{\text{ink4a}}$ and p27$^{\text{kip1}}$, which subsequently enhanced the expression of cyclin-dependent kinase 2 and cyclin E. These results suggest that the KLRG1 pathway impairs CD4$^+$ T cell responses to neoantigen and induces a state of immune senescence in individuals with HCV infection, raising the possibility that blocking this negative-signaling pathway might improve HBV vaccine responses in the setting of chronic viral infection. The Journal of Immunology, 2014, 192: 649–657.

Hepatitis C virus (HCV) infection is a global public health problem, with ~200 million people chronically infected worldwide. HCV-mediated impairment of the host innate to adaptive immune system is imperative for the development of persistent viral infection and poor vaccine responses, although the underlying mechanisms for this failure require further study (1). It is well known that APCs and CD4$^+$ T cells play a pivotal role in the host immune responses to pathogenic infection and vaccination (2, 3). We (2) reported previously that a differential secretion of IL-12/IL-23 by APCs drives Th17 cell development and that the KLRG1 pathway impairs CD4$^+$ T cell responses to neoantigen and induces a state of immune senescence in individuals with HCV infection, raising the possibility that blocking this negative-signaling pathway might improve HBV vaccine responses in the setting of chronic viral infection. The Journal of Immunology, 2014, 192: 649–657.

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Abbreviations used in this article: CDK, cyclin-dependent kinase; HAV, hepatitis A virus; HBsAg, hepatitis B surface Ag; HBV, hepatitis B virus; HBV-NR, hepatitis B vaccine nonresponder; HCV-R, hepatitis B vaccine responder; HCV, hepatitis C virus; HS, healthy subject; KLRG1, killer cell lectin-like receptor subfamily G member 1.

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suggested that HBV vaccine failure may be due to a defect in CD4+ Th cells (11–14), regulatory T cells (15), or in APCs (16, 17); however, this has remained controversial (18, 19). A number of clinical studies (20–24) attempted to correct vaccine nonresponse by adding adjuvants, altering doses, and administrating vaccine through different routes or strategies. These approaches led to varying degrees of improvement in HSs but had limited success in virally infected individuals, in part because of our incomplete understanding of the mechanisms that inhibit vaccine response in this setting.

Recently, it was reported that an inhibitory receptor and marker for cell aging–killer cell lectin-like receptor subfamily G member 1 (KLRG1)–increases substantially on T cells and NK cells during pathogenic infections (25–35). KLRG1 is a transmembrane protein with an ITIM in its cytoplasmic domain and a C-type lectin-like domain in the extracellular region. The expression and function of KLRG1 during chronic viral infection remain elusive; thus, further defining its role in immune responses in a clinically relevant disease model is significant and timely. In this article, we focus on exploring the role of KLRG1 in regulating CD4+ T cell responses to HBV vaccine in HCV-infected individuals. We found that the expression of KLRG1 was significantly upregulated on CD4+ T cells, leading to an overexpression of cell cycle inhibitors (p16ink4a/p27kip1) and impaired cellular functions, which were more prominent in HBV-NRs compared with HBV-vaccine responders (HBV-Rs) during chronic HCV infection.

Materials and Methods

Subjects

The study protocol was approved by a joint institutional review board at East Tennessee State University and James H. Quillen VA Medical Center. A total of 48 HCV-infected subjects and 16 uninfected controls without serologic evidence of prior exposure to HBV or hepatitis A virus (HAV) were recruited into this study to receive either Engerix HBV (if HAV Ab negative) or Twinrix HAV/HBV combination vaccines, as appropriate. The HCV-infected subjects comprised 24 HBV-Rs (defined as hepatitis B surface Ab titers > 10 IU/ml at 1–6 mo following a standard course of HBV vaccination) and 24 HBV-NRs (defined as hepatitis B surface Ab titer < 10 IU/ml at 1–6 mo following a standard course of HBV vaccination). All infected subjects were virologically and serologically positive for HCV, prior to the antiviral treatment, with HCV genotype 1 (70%) and type 2 or 3 (30%) and viral load ranging from 12,300 to 500,000 IU/ml. The subjects not infected with HCV comprised 1 spontaneously resolved individual, 4 sustained virological responders following antiviral treatment, and 11 HSs. Written informed consent was obtained from all participants. The mean age of HCV-infected HBV-NRs was comparable to HBV-Rs and control subjects (p > 0.05).

Cell isolation and culture

Human PBMCs were isolated from the peripheral blood of study subjects by Ficoll-density centrifugation with lympho-H (Atlanta Biological, Lawrenceville, GA) and then viably cryopreserved in freezing medium in liquid nitrogen. If indicated, CD4+ T cells were further purified from isolated PBMCs by negative selection with magnetic beads using a CD4+ T cell Isolation Kit (Miltenyi Biotec, Auburn, CA); cell purity was >95%. Cells were cultured with RPMI 1640 containing 10% FBS (Life Technologies, Gaithersburg, MD), 100 mg/ml penicillin-streptomycin, and 2 mM L-glutamine (both from Thermo Scientific, Logan, UT) at 37°C with 5% CO2 atmosphere for the subsequent experiments.

Flow cytometry

Procedures for intracellular and cytokine staining were performed essentially as described previously (2). Briefly, purified CD4+ T cells or PBMCs were incubated or not with anti-CD3/CD28 Abs (1 μg/ml; eBioscience, San Diego, CA) and then permeabilized with 90% methanol on ice for 30 min. The cells were subsequently incubated with p-Akt (Ser473) (D9E) or rabbit isotype control (DA1E; both from Cell Signaling) for 1 h at room temperature. Fluorescence minus one strategy was used to determine background levels of staining and adjust multicolor compensation for cell gating. The cell analysis was performed on a FACSComp or Accuri C6 flow cytometer (BD, Franklin Lakes, NJ) using CellQuest or FlowJo software (TreeStar, Ashland, OR).

KLRG1 blockade

Purified CD4+ T cells or PBMCs from HCV patients were incubated with anti-human KLRG1 Ab (4 μg/ml; gift from Dr. Hanspeter Pircher) or isotype-control IgG in the presence of various concentrations of anti-CD3/CD28 for different times, as indicated in the Results, and then subject to flow cytometric analysis of intracellular IL-2 and pAkt expression, CFSE assay, and Western blot.

Proliferation assay

PBMCs were labeled with CFSE (2.5 μM; Invitrogen) for 10 min at 37°C, per the manufacturer’s instructions, washed with complete medium, and cultured (5 × 10^6/well) in a 96-well plate in the presence of anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml), and recombinant human IL-2 (100 U/ml; R&D Systems). After culture for 5 d, the cells were immunostained with PE-C4d and Alexa Fluor 488–KLRG1 and analyzed with a FACSComp flow cytometer and FlowJo software.

Western blot

Purified CD4+ T cells from HBV-NRs of HCV patients were incubated with anti-human KLRG1 or control IgG Ab (4 μg/ml in the presence of anti-CD3/CD28) or control (1 μg/ml) for 3 d. The expression of P16INK4A, P27KIP1, cyclin-dependent kinase (CDK)2, and cyclin E in CD4+ T cell was measured by Western blot. Briefly, the cells were lysed in 1× RIPA lysis buffer (Boston BioProducts, Ashland, MA) and supplemented with protease inhibitors/ phosphatase inhibitors (Thermo Scientific) and EDTA on ice. Cell lysates were centrifuged for 15 min at 4°C, and the protein concentrations were measured. Thereafter, protein samples were combined with 4× Laemmli sample buffer (Boston BioProducts), denatured, and separated by SDS-PAGE. Following transfer to an Amersham Hyperion-blot membrane (GE Healthcare, Piscataway, NJ), the membrane was blocked by 3% BSA-TBST and probed with P16INK4A (Bethyl Laboratories, Montgomery, TX), P27KIP1 (BioLegend), cyclin E (BioLegend), CDK2 (BioLegend), or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Finally, the membrane was incubated with an HRP-conjugated secondary Ab (Millipore, Temecula, CA) and developed by Amersham ECL Prime Western blotting Detection Reagents (GE Healthcare Biosciences, Pittsburgh, PA) on Kodak X-OMAT-LS X-ray film (Sigma-Aldrich, St. Louis, MO). Specific bands were quantified by AlphaEaseFC software (Alpha Innotech).

RT-PCR

Purified CD4+ T cells from HSs and HCV-infected HBV-NRs and HBV-Rs were subject to RT-PCR assay to determine the mRNA level of p16INK4A. Total RNA was isolated using a RNeasy Mini Kit (QiAGEN, Valencia, CA). A total of 1 μg/ml RNA was reverse transcribed (Ambion, Austin, TX), and the cDNA was amplified by PCR using the following conditions: 95°C for 10 min, followed by 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s for 30 cycles and then 72°C for 5 min. The primers for p16INK4A gene amplifications were sense: 5′-CCA TCA TCA TGA CCT GGA TGC-3′ antisense: 5′-AGC ATG GAG CCT TCG GCT GA-3′ (Integrated DNA Technologies, Coralville, IA). β-actin gene served as a control for normalization. The amplified products were analyzed by electrophoresis on 2% agarose gels. OD values of the DNA products were determined using a Gal-Pro Analyzer (Version 4.0; Media Cybernetics).

Statistical analysis

Study results were summarized for each group, and the results are expressed as mean ± SD. Comparison between indicated groups in the Results was performed using a variety of tests to demonstrate the least significant
KLRG1 expression is overexpressed on CD4+ T cells in HCV-infected HBV-NRs versus HBV-Rs

We demonstrated previously that impaired APC and T cell functions might play a role in blunting the HBV vaccine response in individuals with HCV infection, although the underlying mechanisms for these cell defects is unclear (2, 6). KLRG1 is an inhibitory receptor expressed on T cells and NK cells and is known marker for cell aging or immune senescence (28–30). As an initial approach to study the role of KLRG1 on CD4+ T cell function and HBV vaccine responses during HCV infection, we first examined KLRG1 expression on CD4+ T cells from HCV-infected versus HCV-uninfected subjects, with or without anti-CD3/CD28 stimulation ex vivo. As shown in Fig. 1A, KLRG1 expression on CD4+ T cells derived from HCV-infected patients was much higher than on cells from HCV-uninfected individuals, but no difference in KLRG1 expression was observed between HCV-resolved subjects and HSs. Also, as we and other investigators (1, 36) reported previously for the relationship between viral load and clinical disease progression or immunological changes, no apparent correlation was found between KLRG1 expression and HCV RNA level. We then examined KLRG1 expression on CD4+ T cells from HCV-infected HBV-NRs and HBV-Rs. As shown in Fig. 1B, after costimulation of TCR with anti-CD3 and anti-CD28, KLRG1 expression on CD4+ T cells increased in both HBV-NRs and HBV-Rs; however, KLRG1 expression on CD4+ T cells in HBV-NRs was significantly higher than that observed in HBV-Rs with HCV infection, either with or without TCR stimulation. This hold true in terms of the percentage of KLRG1+ CD4+ T cell populations (Fig. 1C, p < 0.05), as well as the mean fluorescence intensity of KLRG1 expression levels on CD4+ T cells from HBV-NRs compared with HBV-Rs, regardless of TCR stimulation (Fig. 1D, p < 0.01). These results suggest that HCV infection may induce T cell aging or senescence by upregulation of KLRG1 expression on CD4+ T cells, which may contribute to HBV vaccine nonresponsiveness.

KLRG1 expression is inversely associated with IL-2 expression by CD4+ T cells in HCV-infected HBV-Rs versus HBV-NRs

Previous work revealed that a higher level of KLRG1 expression on CD4+ or CD8+ T cells led to an anergic or senescent status that was characterized by a decrease in IL-2 production or cell proliferation (25, 35). To better understand the effect of KLRG1 expression on human CD4+ T cell function and its role in vaccine responses in the setting of persistent viral infection, we examined IL-2 expression by CD4+ T cells from HBV-NRs and HBV-Rs with chronic HCV infection. As shown in Fig. 2A, HCV-infected HBV-NRs exhibited significantly less IL-2 production compared with HBV-Rs. We then analyzed the relationship between KLRG1 expression and IL-2 production by purified CD4+ T cells in response to TCR stimulation. As shown in Fig. 2B, almost all IL-2–producing cells were KLRG1+ T cells, whereas the majority of KLRG1− T cells failed to produce IL-2. To determine whether IL-2 was produced by Ag-specific CD4+ T cells, we stimulated PBMCs from HCV-infected HBV-Rs ex vivo with HBsAg for 20 h, followed by FACS staining and gating on CD4+KLRG1+ cells, and then analyzed IL-2 expression by CD45RA (naive) and CD45RO (memory) T cells. As shown in Fig. 2C, IL-2 was expressed primarily by memory, rather than naive, CD4+KLRG1+ T cells from HBV-Rs stimulated with HBsAg ex vivo, and the data were reproducible in repeated experiments. Notably, KLRG1 expression was inversely associated with IL-2 production by CD4+ T cells during HCV infection, with HBV-NR CD4+ T cells expressing more KLRG1 and producing less IL-2 compared with those from HBV-Rs (Fig. 2D). Importantly, blockade of KLRG1 signaling with specific anti-KLRG1 significantly increased IL-2 production by CD4+ T cells from HBV-NRs among
HCV-infected individuals compared with subjects treated with IgG control (Fig. 2E). These results indicate that HBV-NRs exhibit poor IL-2 production by their CD4+ T cells, compared with HBV-Rs among HCV-infected individuals; KLRG1 expression is negatively associated with IL-2 production in HCV-infected patients; and KLRG1 blockade can recover the impaired IL-2 production by CD4+ T cells from HCV-infected HBV-NRs.

KLRG1 negatively regulates the proliferative capacity of CD4+ T cells that are more significantly suppressed in HCV-infected HBV-NRs than HBV-Rs.

The ability of KLRG1 to inhibit human T cell proliferative capacity is crucial for T cell aging and immune senescence. Although KLRG1 expression on CD8+ T cells was shown to correlate inversely with their proliferative capacity (25, 35), a role for KLRG1 in the regulation of CD4+ T cell proliferation has not been demonstrated in the setting of HCV infection. In this study, we compared the relationship of KLRG1 expression and IL-2 production by CD4+ T cells from HCV-infected HBV-NRs and HBV-Rs (lower panel). Each symbol represents an individual subject, and the horizontal lines represent median values. (B) Representative dot plots of isotype staining and IL-2 versus KLRG1 staining in purified CD4+ T cells from a patient with HCV infection. (C) Representative dot plots of IL-2 expression by naive versus memory CD4+ KLRG1+ T cells from HCV-infected HBV-Rs stimulated with HBsAg ex vivo. PBMCs from HCV-infected HBV-Rs were stimulated with HBsAg ex vivo for 20 h, followed by FACS staining and gating on CD4+KLRG1+ cells, and were analyzed for IL-2 expression by CD45RA (naive) versus CD45RO (memory) T cells. (D) The relationship between KLRG1 expression and IL-2 production by CD4+ T cells from HCV-infected HBV-NRs (○) and HBV-Rs (●) among HCV-infected individuals. Data were analyzed by Pearson Correlation with two-tailed significance. (E) Purified CD4+ T cells from chronically HCV-infected HBV-NRs (n = 12) were incubated with anti-KLRG1 or control IgG in the presence of TCRs and stimulated for 72 h, immunostained with conjugated Abs to human IL-2, and analyzed by flow cytometry. Representative graph of IL-2 expression by CD4+ T cells treated with anti-KLRG1 versus isotype-IgG control (left panel). Percentages of IL-2–expressing CD4+ T cells treated with IgG and anti-KLRG1 (right panel). Each symbol represents an individual subject, and the horizontal lines represent median values. *p < 0.05.
CD4+ T cells were KLRG12 populations, whereas KLRG1+ T cells proliferated with less cycle following 5 d of TCR stimulation compared with KLRG12 T cells; this was particularly true for HBV-NRs compared with HBV-Rs.

To further investigate the role of KLRG1 in the control of T cell proliferation, we carried out the T cell proliferation assay with CFSE dilution concomitant with KLRG1 blockade in CD4+ T cells from HBV-NRs with HCV infection. In this case, purified CD4+ T cells were used for blocking experiments to avoid the secondary effects from accessory cells in bulk PBMCs. Notably, T cells treated with anti-KLRG1 alone (without TCR stimulation) failed to proliferate, suggesting that the blocking Ab inhibits negative signaling by KLRG1, rather than by directly activating T cells, and its effect requires TCR stimulation to drive cell proliferation. As shown in Fig. 3B, inhibition of KLRG1 signaling in conjunction with TCR stimulation significantly enhanced the proliferative capacity of purified CD4+ T cells, although the cell cycle progression using purified CD4+ T cells was much less than that observed using bulk PBMCs (Fig. 3A), likely as a result of the lack of other cytokine stimulation from accessory cells. Nevertheless, the cell division events were markedly increased in purified CD4+ T cells from six HBV-NRs with anti-KLRG1 blockade compared with those treated with isotype-control IgG (p < 0.001). These results indicate that HBV-NR CD4+ T cells proliferate inadequately, and KLRG1 negatively controls CD4+ T cell proliferation; as such, blocking this inhibitory pathway can rescue T cell proliferation in HCV-infected HBV-NRs.
KLRG1 impairs T cell responses via the p16ink4a pathway in HBV-Rs and HBV-NRs with HCV infection

To determine how KLRG1 inhibits CD4+ T cell proliferation, we further examined the downstream signaling molecules that control T cell cycle progression. p16ink4a is a well-known cell cycle inhibitor and marker of cell aging (37–39). The INK4a pathway regulates cell cycle progression by blocking the CDK4/6–cyclin D complex. This complex increases the phosphorylation of RB, causing it to release the transcription factor E2F. E2F mediates the transcription of several cellular genes that are involved in G1/S progression (39). Given its key role in T cell proliferation, we examined p16ink4a mRNA by RT-PCR and protein expression by Western blot using purified CD4+ T cells from HCV-infected HBV-Rs and HBV-NRs and compared them with HSs. As shown in Fig. 4A, the level of p16ink4a mRNA expression in CD4+ T cells from HBV-NRs was significantly higher than that from HBV-Rs (p < 0.05) and HSs (p < 0.01). Although a relatively higher level of p16ink4a mRNA was detected in CD4+ T cells from HCV-infected HBV-Rs versus HSs, it was not significantly higher. This difference in mRNA expression from three groups of subjects was reproducible in repeated experiments by RT-PCR and was confirmed by its protein-expression levels detected by Western blot. Because both KLRG1 and p16ink4a are regarded as markers for cell aging and immune senescence (28–30, 37–39), we next determined whether these two molecules are functionally linked by blocking KLRG1 signaling in CD4+ T cells and subsequently detecting p16ink4a level by Western blot. As shown in Fig. 4C, p16ink4a expression was inhibited in T cells following TCR stimulation in the presence of anti-KLRG1 compared with those treated with IgG-control Ab. These results indicate that intracellular p16ink4a is involved in the KLRG1-mediated T cell dysfunction and HBV vaccine nonresponsiveness during chronic HCV infection.

KLRG1 inhibits TCR-mediated Akt (Ser473) phosphorylation and downstream signaling pathways in CD4+ T cells during HCV infection

Akt (Thr108 or Ser473) phosphorylation is the initial event of T cell activation upon TCR stimulation (35, 37). Recent evidence suggests an enhancement of Akt (Ser473) phosphorylation in CD8+ CD28−CD27− senescent T cells when PBMCs were stimulated with anti-CD3 in the presence of E-cadherin–blocking Ab (35). To further define the underlying mechanisms involved in the improvement of senescent CD4+ T cell activation and proliferation following blockade of KLRG1 signaling, we assessed the phosphorylation of Akt in CD4+ T cells from HCV-infected HBV-NRs by flow cytometry following TCR stimulation in conjunction with KLRG1 blockade. As shown in Fig. 5A, compared with cells treated with control IgG, blockade of KLRG1 signaling significantly enhanced the phosphorylation of p-Akt (Ser473) in purified CD4+ T cells from HBV-NRs with chronic HCV infection. The data were reproducible using purified CD4+ T cells from eight HCV-infected HBV-NRs and stimulated ex vivo with anti-CD3/CD28 in the presence of anti-KLRG1 versus control IgG (p < 0.01).

**FIGURE 5.** KLRG1 inhibits Akt (Ser473) phosphorylation and downstream signaling pathways in CD4+ T cells during HCV infection. (A) KLRG1 blockade increases p-Akt (Ser473) phosphorylation in purified CD4+ T cells from HCV-infected HBV-NRs. Representative graphs of p-Akt (Ser473) expression in the CD4+ T cells treated with control IgG1 or anti-KLRG1 (left panel). Gray-filled graph represents isotype-control staining. Percentages of p-Akt (Ser473) expression in CD4+ T cells from HBV-NRs following treatment with anti-KLRG1 or control IgG (right panel). The horizontal lines indicate the median values for eight HCV-infected HBV-NRs. (B) T cells of PBMCs from HCV-infected HBV-NRs were stimulated by anti-CD3/CD28 in the presence of anti-KLRG1 or control IgG for 3 d. Total protein was extracted from the cell lysates, followed by Western blot analysis of CDK2, cyclin E, and p27kip1 expression. β-actin served as loading control. Representative images (left panel). Densitometry data of CDK2, cyclin E, and p27kip1 expression, corrected by β-actin level, from three independent experiments (right panel). *p < 0.05, **p < 0.01, ***p < 0.001.
We showed previously that HCV core protein inhibits T cell cycle progression through Akt/p27kip1 pathway (40, 41). Thus, the immune senescence mediated by inhibitory receptors, such as KLRG1, may prevent TCR-mediated PI3K/Akt phosphorylation (25, 35). This, in turn, lifts the block on forkhead box O transcription factors and activates p27kip1, causing G1/S phase growth arrest by blocking the activation of cyclins and CDKs. Therefore, we expect that improved Akt phosphorylation by blocking KLRG1 signaling subsequently will decrease p27kip1 expression and enhance cyclin and CDK activation. To test this hypothesis, T cells from HCV-infected HBV-NRs were stimulated with anti-CD3/CD28 in the presence of anti-KLRG1 or control IgG. p27kip1, as well as cyclin E and CDK2, were detected by Western blot. As shown in Fig. 5B, the expression level of p27kip1 was decreased, whereas cyclin E and CDK2 were increased, in cells treated with anti-KLRG1 versus IgG. The results were reproducible in three independent experiments using purified cells from different HBV-NRs with HCV infection. These results indicate that KLRG1 negatively regulates CD4+ T cell functions by affecting multiple intrinsic regulators, including Akt/p27kip1-related cell cycle proteins (Fig. 6). Thus, manipulating these signaling molecules may provide an alternative approach to improve HBV vaccine responsiveness in HCV-infected individuals.

Discussion

HCV infection is a world-wide infectious disease that can lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. After decades of studies on this immunomodulatory virus, it has become evident that HCV-mediated host immune dysfunction plays a major role in viral persistence, as well as disease progression. Notably, like HIV infection, individuals with HCV infection often do not respond well to HBV vaccinations, and efforts to boost vaccine response have proven to be futile, in part because of our poor understanding of the mechanisms that inhibit vaccine response in this setting. In this study, we used the model of HBV vaccine failure in HCV-infected individuals to explore the role of KLRG1 in regulating CD4+ T cell functions and to examine whether blocking the KLRG1 pathway affects immune responses in HCV patients who have failed HBV vaccinations. Our data show that KLRG1 is overexpressed on CD4+ T cells from HBV-NRs compared with HBV-Rs among HCV-infected individuals. Moreover, HCV-infected HBV-NRs exhibit a more profound dysfunction of CD4+ T cell proliferation and secretion of IL-2 cytokine compared with HBV-Rs, which is inversely associated with the level of KLRG1 expression. Importantly, blocking KLRG1 signaling leads to a significant improvement in CD4+ T cell proliferation and IL-2 production in HCV-infected HBV-NRs in response to TCR stimulation. Additionally, blockade of KLRG1 increases the phosphorylation of Akt (Ser473) and decreases the expression of cell cycle inhibitors p16ink4a and p27kip1, which subsequently enhances the expression of CDK2 and cyclin E. These results suggest that KLRG1 impairs CD4+ T cell responses via p16ink4a and p27kip1 pathways, and the blunted HBV vaccine response during HCV infection might be a result, at least in part, of virus-mediated premature cell aging through the KLRG1-signaling pathway. Based on this study, we propose a model, as depicted in Fig. 6, to illustrate the role of KLRG1 in impairing CD4+ T cell function and HBV vaccine responses in the setting of chronic HCV infection.

Although the role of KLRG1 in immune senescence has been emerging, its link to chronic infection and vaccine response is rather novel. Recently, Lindenstrøm et al. (42) used a bacillus Calmette-Guérin vaccine model to demonstrate that CD4+ KLRG1− IL-2−secreting subsets were central to increased protective efficacy. Their data suggested that the waning of memory immunity that occurred as tuberculous infections became chronic was associated with a loss of IL-2–producing CD4 cells and an increase in KLRG1+ anergic T cells. These findings could be reversed by vaccine boosting, leading to selection induction, expansion, and maintenance of CD4+ KLRG1− memory T cells. Our study supports this concept, in that chronic HCV infection leads to blunted vaccine responses characterized by high KLRG1 expression on CD4+ T cells with impaired ability to proliferate and to produce IL-2.

It is well-recognized that elderly individuals are more susceptible to infections and have decreased responses to vaccinations (43). In general, the immune responses in the elderly are significantly less robust than responses by younger individuals in magnitude, duration, and quality of response, leading to generally poor efficacy in terms of vaccine responses (44–46). Remarkably, vaccine responses, such as HBV, influenza, and pneumococcal vaccine, are also impaired in individuals with chronic viral infection, noted in the setting of HIV, HCV, and CMV infections (47, 48). It is imperative to characterize the mechanisms underlying vaccine nonresponsiveness in chronically viral-infected individuals, because they...
are the individuals most susceptible to superinfection-mediated increases in morbidity and mortality. Most vaccines aim to develop good neutralizing Ab, which involves the activation of APCs, the interaction of the activated CD4+ T cells with their cognate B cells to form germinal centers, and maturation of the B cells to produce specific Abs. Each of these steps can be affected by viral infection and/or cell aging. The mechanisms for the virus-mediated impairment of CD4+ T cell responses, including T cell proliferation and IL-2 production, is a fascinating, yet unclear, research theme because it bridges the innate and adaptive immune (vaccine) responses (46).

To elucidate the mechanisms by which persistent viral infection mediates host immune dysfunction and ultimately leads to blunted vaccine responses, we previously explored the role of HCV-mediated immune exhaustion in HBV vaccine responses during HCV infection. We showed that PD-1 and Tim-3, markers for cell exhaustion, are overexpressed on APCs and T cells of HBV-NRs versus HBV-Rs among HCV-infected individuals (2, 6). In addition to inducing immune exhaustion that impairs essential functional activity, persistent viral infections can lead to immune senescence, with accelerated premature cell aging due to telomere erosion or unrepaired DNA damage (26, 49). In this study, we further demonstrate that KLRG1 and p16ink4a, markers for cell aging, are upregulated in CD4+ T cells in HBV-NRs compared with HBV-Rs with HCV infection. Thus, we believe that HCV may use two critical cell regulatory mechanisms, cell exhaustion and cell aging, through upregulation of two inhibitory pathways, PD-1/Tim-3 and KLRG1/p16ink4a, to dampen the functions of immune cells to respond appropriately to vaccines during chronic infection.

It is possible that modulating these inhibitory receptors, like Tim-3 and KLRG1, which are preferentially expressed in highly differentiated T cells during viral infection, may boost immune responses. Although there has been substantial progress in identifying the mechanisms that regulate both processes separately, it is unclear how these processes interrelate and whether blocking pathways that maintain either the exhausted or the senescent state, or both, can boost vaccine responses, especially in virally infected individuals. A recent study (49) demonstrated that young HIV-infected patients, with a duration of infection < 4 y, have early immune exhaustion leading to premature aging and senescence comparable to the elderly, suggesting that virus induces premature immune senescence associated with high rates of immune exhaustion following short-term infection. We are also exploring the mechanisms underlying how HCV infection induces immune exhaustion and immune senescence that are essential for developing specific strategies to improve vaccine responses in the setting of chronic viral infection. These studies have led to an intersecting field of virus-mediated immune exhaustion and immune senescence with regard to vaccine responses.

In summary, this study delineates the mechanisms of persistent infection-induced immune senescence or cell aging by measuring T cell responses to neoantigen stimulation. Our findings suggest that KLRG1 expression is upregulated and associated with CD4+ T cell dysfunctions that are more prominent in HBV-NRs compared with HBV-Rs among HCV-infected individuals. HCV-induced KLRG1 expression impairs CD4+ T cell responses via p16ink4a and p27kip1 pathways (Fig. 6); thus, inhibition of the KLRG1 pathway and downstream signaling molecules in CD4+ T cells can be therapeutically exploited for improving human immune (vaccine) responses. Our data presented in this article suggest that KLRG1 may be used as a potential predictor for vaccine responses and progression of immune status in chronic HCV infection. Further characterization of KLRG1 expression and functional changes in CD4+ T cells will help us to better understand the pathogenesis of chronic HCV infection, and it may reveal a potential therapeutic target for managing this disease.

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Disclosures

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