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RESEARCH

KLRG1 expression induces functional exhaustion of NK cells in colorectal cancer patients

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Abstract

Background Natural killer (NK) cells are a subset of innate lymphoid cells that possess cytotoxic properties, playing a pivotal role in immune surveillance against tumor cells. However, it remains unclear whether there are any alterations in the quantity and functional status of NK cells in colorectal cancer (CRC).

Methods In this study, we collected peripheral blood samples from both CRC patients and age- and sex-matched healthy controls (HCs). The distribution characteristics, phenotypic changes, functional status, apoptosis susceptibility, and proliferative capacity of circulating NK cells were detected and analyzed by flow cytometry. An in vitro study was performed to investigate the blocking effect of KLRG1 antibody on peripheral blood NK cells in CRC patients.

Results The frequency and absolute number of circulating NK cells were significantly decreased in CRC patients compared to those in HCs. Meanwhile, the function of NK cells from CRC patients was compromised, as shown by the reduced production of IFN-γ, TNF-α, and CD107a, with this impairment becoming increasingly significant as neural invasion progressed and tumor invasion advanced. We further found that the expression of activating receptors NKp30 and NKp46 were reduced, while the expression of inhibitory receptor KLRG1 was remarkably increased. The increased proportion of KLRG1 on NK cells was associated with CRC progression, and KLRG1$^+$ NK cells showed impaired production of IFN-γ, TNF-α, and CD107a and were more susceptible to apoptosis. Importantly, blockade of the KLRG1 pathway could restore the cytokine production and degranulation ability of NK cells from CRC patients.

Conclusions The present study demonstrates that NK cells in CRC patients exhibit functional exhaustion, and KLRG1 blockade restores the effector function of NK cells, indicating that targeting KLRG1 represents a promising strategy for immunotherapy in patients with CRC.

Keywords Colorectal cancer · NK cells · KLRG1 · Dysfunction · Immunotherapy

Abbreviations

AJCC	American Joint Committee on Cancer
CRC	Colorectal cancer
HCs	Health controls
IFN-γ	Interferon-γ
IL-2	Interleukin-2
IL-12	Interleukin-12
IL-15	Interleukin-15
KLRG1	Killer cell lectin-like receptor subfamily G member 1
MHC	Major histocompatibility complex
NK cells	Nature killer cells
PBMC	Peripheral blood mononuclear cells
PMA	Phorbol 12-myristate 13-acetate
TNF-α	Tumor necrosis factor-α
Tim-3	T cell immunoglobulin and mucin domain-containing protein 3
TIGIT	T cell immunoreceptor with Ig and ITIM domains

Introduction

Colorectal cancer (CRC) is emerging as one of the most prevalent malignancies nowadays, accounting for 9.6% of newly diagnosed cases and 9.3% of death caused by cancer worldwide. It ranks third in terms of incidence and second in terms of mortality on a global scale [1]. Despite recent

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declines in overall CRC incidence and mortality rates, the limitations of conventional treatments such as surgery or chemoradiotherapy necessitate the exploration of novel therapeutic approaches to enhance the existing treatment regimen [2, 3]. Over the past decade, immunotherapy has garnered significant attention due to its remarkable efficacy surpassing expectations across various malignancies including CRC [4]. The presence and activation of lymphocytes such as T cells, B cells, and innate immune cells are essential for a successful induction of antitumor immune response. However, circulating immune cells in CRC patients have been reported to undergo significant alterations, particularly in the composition of lymphocyte subsets [5]. The phenotypic landscape of circulating lymphocyte subsets may therefore serve as a reflection of the immune response within the tumor microenvironment, hence offering crucial insights into tumor progression and potential therapeutic targets [6].

Natural killer (NK) cells are innate lymphoid cells with cytotoxic properties. In contrast to T cells, NK cells have the ability to recognize stressed cells, such as tumor cells and specific infected cells, via a major histocompatibility complex (MHC)-independent mechanism, thereby enabling them to respond rapidly to target cells [7, 8]. Besides, the function of NK cells is regulated by a delicate balance between a series of activating receptors including NKG2D, NKp30, NKp46, and inhibitory receptors including killer cell lectin-like receptor subfamily G member 1 (KLRG1), Tim-3, TIGIT, and NKG2A [9]. In tumor patients, inhibitory receptors on NK cells and their ligands on tumor cells are often upregulated, while activating receptors are downregulated. The enhanced inhibitory signal results in an imbalanced integrated signal, thus gradually inducing NK cell exhaustion. Exhausted NK cells exhibit suppressed cytokine secretion and reduced cytotoxicity, which is considered one of the principal mechanisms by which tumor cells evade immune surveillance. Dysfunction of circulating NK cells has been documented in numerous tumor types. However, research on circulating NK cells in CRC patients has primarily focused on analyzing the proportion and quantity of different subtypes [5, 10, 11]. Further comprehensive investigations are warranted to fully understand the impact of CRC on peripheral blood NK cells, as these cells may play an important role in preventing cancer development in humans.

KLRG1 is a conserved C-type lectin inhibitory receptor expressed on diverse subsets of human lymphocytes, including NK cells, $CD4^+$ T cells, and $CD8^+$ T cells [12–14]. The interaction between KLRG1 and its ligand, E-cadherin, impairs proliferation and cytotoxic activity of lymphocytes expressing KLRG1.[15, 16] Furthermore, studies have shown that KLRG1 expression on human circulating NK cells is elevated in several types of cancer and is correlated with functional exhaustion of NK cells [17, 18]. Thus, KLRG1 is suggested to be an immune checkpoint receptor and a potential target for investigating cancer immune therapy.

In this study, we sought to illustrate the impact of CRC on the immunophenotype and effector function of circulating NK cells. We analyzed the distribution and phenotypic characteristics of circulating NK cells from patients with CRC and healthy controls (HCs), as well as their susceptibility to apoptosis and capacity of cytokine production. Our results indicate that susceptibility to apoptosis and impaired function of NK cells in CRC patients are associated with increased expression of KLRG1. This research provides a more comprehensive understanding of the immune status of NK cells in CRC patients and identifies KLRG1 as a potential therapeutic target for immunotherapy.

Materials and methods

Patients and healthy controls

In this study, peripheral blood samples from 144 patients with CRC who were hospitalized at the First Affiliated Hospital of Anhui Medical University and 137 age- and sex-matched healthy controls were collected in the EDTA anticoagulant tube. Clinical characteristics of the enrolled subjects are presented in Table 1. All patients were diagnosed with CRC for the first time based on definitive pathological or histological evidence. Individuals who had (1) received radiotherapy, chemotherapy, or other therapeutic interventions, (2) a history of prior malignancies, persistent

Table 1 Clinical characteristics of the enrolled healthy controls and CRC patients

Variables	Healthy controls $N=137$	CRC patients $N=144$
Age, years	56.12 ± 8.69	61.85 ± 10.31
Sex		
Male	67 (48.91%)	87 (60.42%)
Female	70 (51.09%)	57 (39.58%)
RBC ($\times 10^{12}$/L)	4.66 ± 0.44	4.30 ± 0.60
Hb (g/L)	141.40 ± 13.97	125.20 ± 21.06
Platelet ($\times 10^{9}$/L)	214.00 (185.50–245.00)	234.00 (186.30–277.50)
Albumin (g/L)	45.50 (44.20–47.25)[a]	42.40 (38.90–45.20)
Urea (mmol/L)	4.93 (4.18–5.68)	4.74 (3.81–5.61)
Creatinine (μmol/L)	66.00 (57.00–78.00)	67.00 (57.25–77.00)
Uric acid (μmol/L)	290.00 (247.00–341.00)	290.50 (241.30–331.80)

Data are depicted as *n* (%), mean ± standard deviation (SD) or median (interquartile range, 25 to 75)

RBC, red blood cell; Hb, hemoglobin

[a]Lack of data

infectious diseases, autoimmune disorders, or allergic conditions, and (3) an acute infection within 3 months prior to the recruit were excluded. All HCs enrolled in this study exhibited normal physical parameters, and individuals with autoimmune disorders, a history of cancer, or an acute infection within 3 months prior to the recruit were excluded. All individuals enrolled in this study have signed informed consent to participate in the study. This research was executed under approvals from the Medical Ethics Committee of the First Affiliated Hospital of Anhui Medical University.

Laboratory index detection

One tube of the peripheral blood of CRC patients and HCs were collected in the EDTA anticoagulant tube. After mixing, the detection of red blood cell, hemoglobin, platelet, and lymphocyte were carried out with the XN9000 fully automatic detector (Sysmex, Japan) and its supporting reagents, respectively (all from Sysmex, Japan). Another tube of the peripheral blood of CRC patients and HCs were collected and centrifugated in the procoagulant inert separation tube. The levels of albumin, urea, creatinine, and uric acid were measured using Cobas 8000 modular analyzer for clinical chemistry assays (Hoffmann La Roche, Swiss) and its supporting reagents, respectively (all from Hoffmann La Roche, Swiss).

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly obtained peripheral blood of CRC patients and HCs, using Ficoll-Paque (TBD Science, Tianjin, China) gradient centrifugation. Briefly, fresh blood was diluted with 1 × phosphate buffered saline (1 × PBS), gently layered onto Ficoll, and centrifuged at 380 g for 20 min. The intermediate layer was collected post-centrifugation and subsequently washed with 1 × PBS for twice.

Antibodies and flow cytometry analysis of immune cell phenotype

The flow cytometry staining of PBMCs was performed using the following fluorescently labeled anti-human monoclonal antibodies obtained from BioLegend: CD3 (OKT3, FITC), CD3 (OKT3, PerCP-Cy5.5), CD3 (OKT3, BV421), CD4 (RPA-T4, APC-Cy7), CD8 (SK1, FITC), CD45 (2D1, APC-Cy7), CD56 (HCD56, PE-Cy7), CD56 (HCD56, BV510), KLRG1 (14C2A07, APC), KLRG1 (SA231A2, APC-Cy7), Tim-3 (F38-2E2, PE), Tim-3 (A18087E, APC), NKG2A (S19004C, APC), NKG2A (S19004C, APC-fire750), TIGIT (A15153G, APC), TIGIT (A15153G, BV605), NKG2D (1D11, APC), NKp30 (P30-15, PE-Cy7), NKp46 (9E2, FITC), IFN-γ (4S.B3, APC), TNF-α (MAb11, BV421), CD107a (LAMP-1, PE-Cy7). Antibodies against CD69 (FN50, PE-Cy7) and Ki67 (B56, Alexa-Fluor 647) were purchased from BD Bioscience. An isotype control immunoglobulin G was employed as a control. The PBMCs were incubated with antibodies at 4 °C for 30 min. Cell staining was evaluated using CytoFLEX (Beckman Coulter, USA) and BD FACSCanto flow cytometers. The data were analyzed with the FlowJo software, v10 (Tree Star, USA).

Flow cytometry analysis of immune cell function

For intracellular cytokine analyses, isolated PBMCs were resuspended in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS, ExCell Bio, China), stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA), 1 μg/mL ionomycin, and 10 μg/mL monensin (all from Sigma-Aldrich, USA) for 4 h at 37 °C. CD107a antibody was added at the start of incubation. After staining the cell surface phenotypic molecules, the PBMCs were fixed, permeabilized, and subsequently labeled with antibodies targeting specific cytokines. The cells were then washed to remove excess antibodies, followed by thorough mixing, and finally assessed using flow cytometry. The data were analyzed with the FlowJo software, v10 (Tree Star, USA).

In vitro KLRG1 blocking assay

For KLGR1 blocking assays, isolated PBMCs were resuspended in RPMI-1640 medium containing 10% FBS and seeded into 96-well plates at a density of 5×10^5 cells/well. To assess the effect of KLRG1 blockade on NK cells from CRC patients, PBMCs were then preincubated with 10 μg/mL of anti-human KLRG1 blocking monoclonal antibody (Bio intron) or an equal dose of control IgG antibody for 8 h at 37 °C. These cells were subsequently stimulated with 100 IU/mL IL-2, 100 ng/mL IL-12, plus 100 ng/mL IL-15 for 8 h to evaluate cytokine secretion and granule release.

Apoptosis detection

Apoptosis detection in NK cells was performed using an APC Annexin V Apoptosis Detection Kit (BioLegend). After staining surface phenotypic molecules, cells were washed twice with PBS and resuspended in 1 × binding buffer at a concentration of 1×10^6 cells/mL. Subsequently, a 100 μL sample (1×10^5 cells) was transferred to a 5-mL test tube with 5 μL APC Annexin V and 7-AAD. The cells were then gently vortexed and incubated for 15 min in the dark at room temperature (25 °C). Finally, 400 μL of

1 × binding buffer was added to each tube before analyzed by flow cytometry.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10.2.3. Comparisons between two groups were analyzed using either a paired or unpaired *t* test, Mann–Whitney *U* test, or Wilcoxon matched-pairs signed rank test based on the distribution of group values. Comparisons among multiple groups were analyzed using either a one-way ANOVA, or Kruskal–Wallis test based on the distribution of group values. Data were presented as mean ± standard deviation (SD), unless stated otherwise. $P < 0.05$ was considered significant.

Results

CRC patients exhibit a decreased quantity of circulating NK cells

To investigate potential alterations in NK cell population among CRC patients, the frequency and absolute number of NK cells were analyzed. The gating strategy for NK cells is presented in Fig. 1a. The frequency and absolute number of total NK cells were significantly reduced in CRC patients compared to HCs. (Fig. 1b, c). The distributions of the $CD56^{dim}$ and $CD56^{bright}$ NK cell subpopulations were further investigated. Consistent with the observed changes in total NK cells, CRC patients also exhibited a significant reduction in both $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets (Fig. 1d, e). Additionally, the frequency and absolute number of total lymphocytes were dramatically reduced in CRC

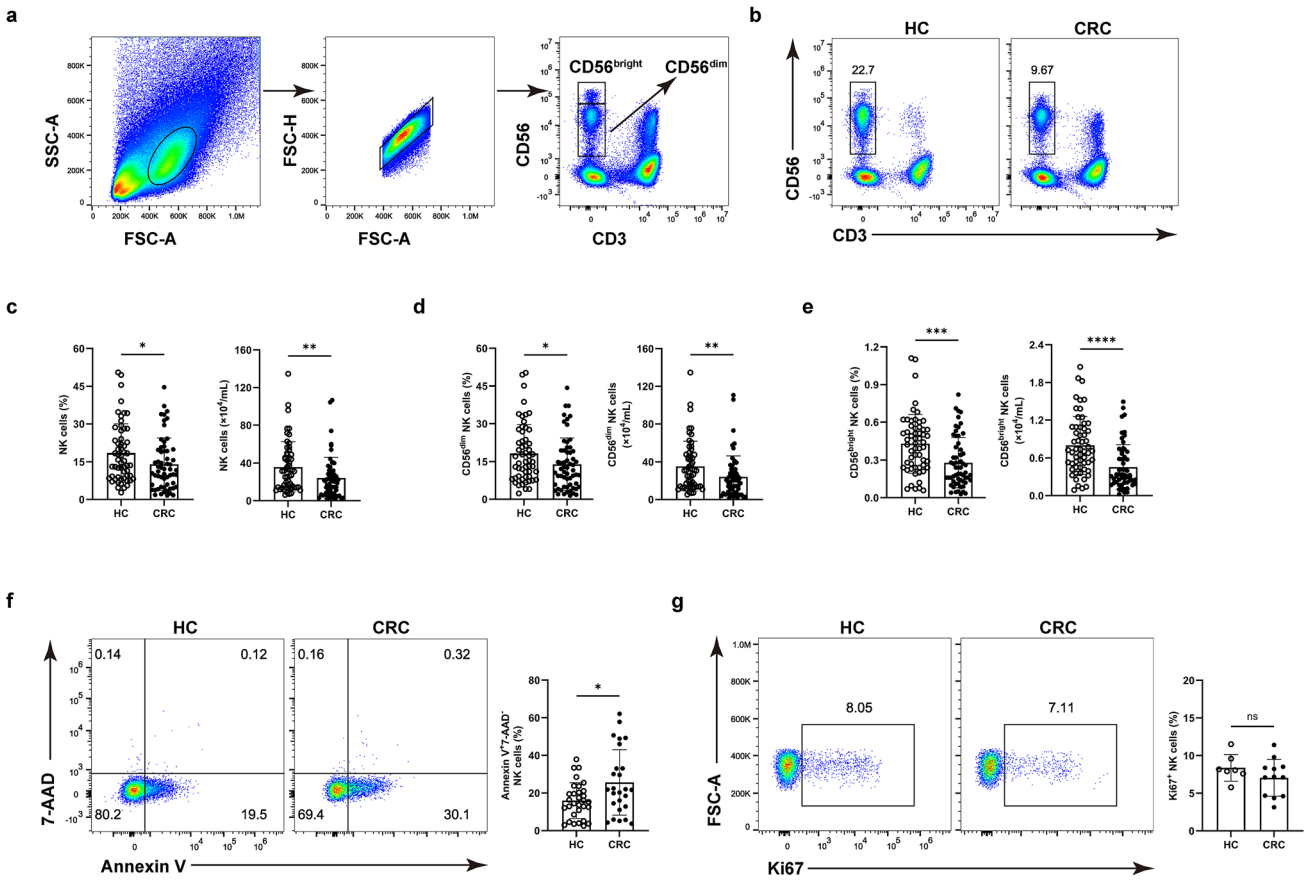


Fig. 1 Frequency and absolute number of circulating NK cells are decreased in CRC patients. **a** Gating strategy for $CD56^{dim}$ and $CD56^{bright}$ NK cells from PBMCs using flow cytometry. **b** Representative flow cytometry plots of NK cells from CRC patients and healthy controls (HCs). Flow cytometry estimations of frequencies and absolute numbers of **c** NK cells, **d** $CD56^{dim}$ NK cells, and **e** $CD56^{bright}$ NK cells in CRC patients and HCs. Inter-group comparisons of **f** early apoptosis through Annexin $V^+7\text{-}AAD^-$ staining and **g** proliferative status by Ki67 labeling in NK cells from CRC patients and HCs. Data are presented as the mean ± standard deviation (SD), with each dot denoting a separate individual. Statistical significance is defined as *P*-values < 0.05. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; ns, not significant

patients (Supplementary Fig. 1a). Due to the decreased number of lymphocytes, the numbers of $CD4^+$ T and $CD8^+$ T cells were significantly reduced in CRC patients, although there were no significant differences in the frequencies of T cells between the two groups. (Supplementary Fig. 1b, c). Furthermore, the mechanisms underlying the decline of NK cells in CRC patients were assessed. Interestingly, we found that there was a significant elevation in the frequency of apoptotic NK cells (Annexin V^+7-AAD^-) in the CRC patients compared to HCs (Fig. 1f). On the contrary, the frequency of proliferative cells ($Ki67^+$) was similar between the two groups (Fig. 1g). Thus, these results indicate that the reduced circulating NK cell count in CRC patients may be attributed to their increased susceptibility to apoptosis.

NK cells are functionally defective in CRC patients

To evaluate whether there is a disparity in NK cell functionality between CRC patients and HCs, cytokine production capacity and CD107a expression were evaluated via flow cytometry following PMA/ionomycin stimulation. The patients diagnosed with CRC exhibited significantly reduced proportions of IFN-γ^+ and TNF-α^+ NK cells, indicating an impaired cytokine secretion status within these cells (Fig. 2a, b). Additionally, in comparison with HCs, CRC patients exhibited a diminished frequency of the lysosome marker CD107a in NK cells upon stimulation, suggesting that the degranulation capability of NK cells was compromised in CRC patients (Fig. 2a, b). In order to investigate the potential impact of CRC on NK cell function, the correlations between the proportion of IFN-γ^+, TNF-α^+, and $CD107a^+$ NK cells, and disease progression were analyzed. The results revealed that as neural invasion occurred and tumor invasion advanced, there was further impairment in the capacity of NK cells to secrete cytokines (demonstrated by lower levels of IFN-γ and TNF-α) (Fig. 2c, d) and in their cytotoxicity (indicated by decreased CD107a) (Fig. 2e). In conclusion, these findings collectively suggest that the function of circulating NK cells in CRC patients is exhausted, and this dysfunction becomes more pronounced as neural or tumor invasion advances.

NK cells from CRC patients exhibit an exhausted phenotype

To investigate the potential impact of CRC on the phenotypic expression of circulating NK cells, we further conducted flow cytometry analysis to examine the expression

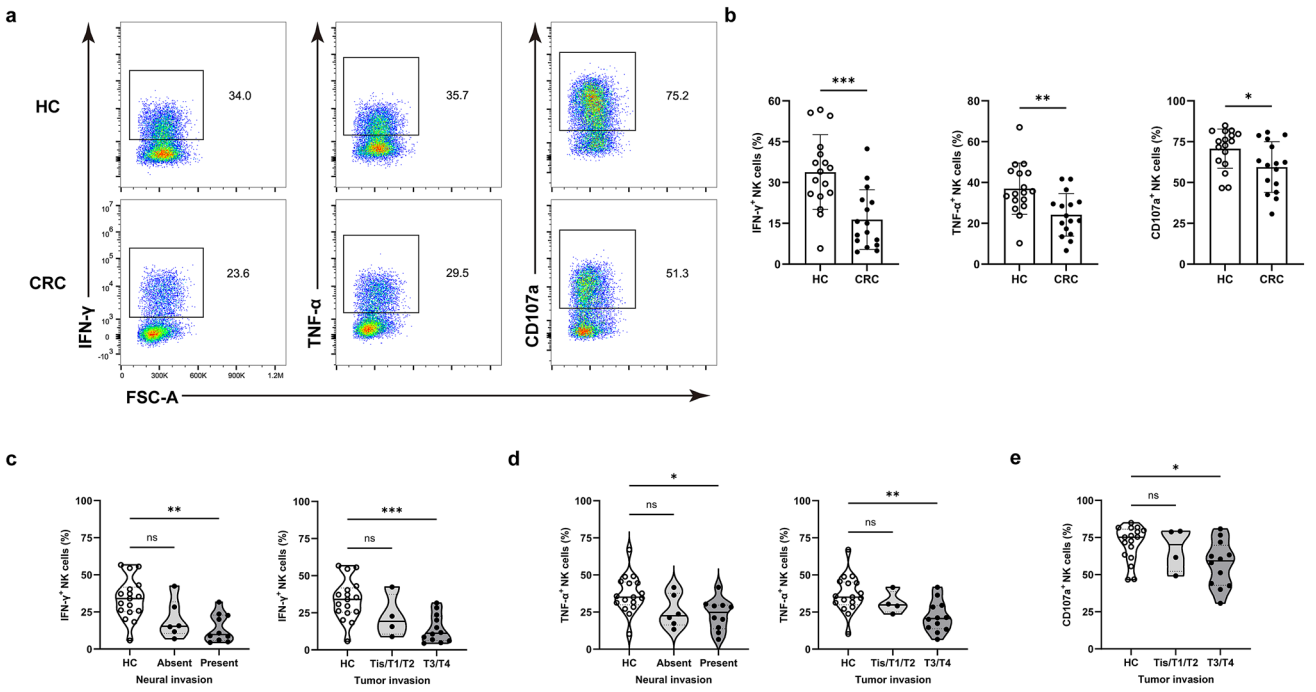


Fig. 2 Circulating NK cells are functionally impaired in CRC patients. **a** Representative flow cytometry plots of IFN-γ, TNF-α, and CD107a levels on NK cells after PMA/ionomycin stimulation of PBMCs from CRC patients and healthy controls (HCs). **b** Analysis of the proportions of IFN-γ^+, TNF-α^+, and $CD107a^+$ NK cells in CRC patients and HCs. Results are shown as the mean ± standard deviation (SD), with each dot denoting a separate individual. Violin plots display the correlations between the proportions of **c** IFN-γ^+, **d** TNF-α^+, and **e** $CD107a^+$ NK cells and CRC progression. Solid and dashed horizontal lines on violin plots depict median and upper and lower quartiles, respectively. Each dot denotes a separate individual. Statistical significance is defined as P-values < 0.05. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant

levels of activating receptors (NKp30, NKp46, NKG2D, and CD69), as well as inhibitory receptors (NKG2A, Tim-3, TIGIT, and KLRG1) on the circulating NK cells derived from CRC patients and HCs. The results showed that the expression of both NKp30 and NKp46 was significantly downregulated in CRC patients (Fig. 3a, b), while no differences were observed in the levels of NKG2D, and CD69 (Fig. 3c, d). The proportions of NKp30 and NKp46 positive cells were also diminished in $CD56^{dim}$ NK cell subset (Supplementary Fig. 2a, b). Among the inhibitory receptors examined, the expression levels of NKG2A, Tim-3, or TIGIT remained unaltered (Fig. 4a–c). However, the expression of KLRG1 was dramatically upregulated in CRC patients compared to HCs (Fig. 4d). Moreover, within the overall NK cell population, KLRG1 was predominantly expressed on $CD56^{dim}$ NK cells, which are recognized for their higher cytotoxicity than $CD56^{bright}$ NK cells, supporting its role as a maturation marker (Fig. 4e). The frequencies of KLRG1 were significantly increased in both $CD56^{dim}$ and $CD56^{bright}$ NK cell subpopulations in CRC patients as well (Fig. 4f). Interestingly, in the $CD4^+$ T and $CD8^+$ T lymphocytes, a higher proportion of KLRG1 was found in CRC patients compared to HCs (Supplementary Fig. 3a). These findings collectively indicate that the phenotype of circulating NK cells is apparently exhausted in CRC patients, as evidenced by the downregulation of the activating receptors NKp30 and NKp46, alongside the upregulation of the inhibitory receptor KLRG1.

KLRG1 upregulation leads to further functional exhaustion in NK cells

In order to explore the potential value of KLRG1 in CRC, the correlations between KLRG1 expression levels and CRC progression were evaluated. As presented in Fig. 5a, an increased proportion of KLRG1 was associated with the presence of neural invasion and vascular invasion, advanced tumor invasion, and advanced cancer grade. These findings suggest that the expression of KLRG1 may exert a significant impact on the development of CRC.

Next, for the sake of exploring the underlying influence of KLRG1 upregulation on circulating NK cell dysfunction and apoptotic susceptibility in CRC patients, we compared the functional differences between $KLRG1^+$ NK cell and their $KLRG1^-$ counterpart in CRC patients. The results demonstrated that $KLRG1^+$ NK cells exhibited a much weaker capacity to produce IFN-γ, TNF-α, and CD107a in response to PMA/ionomycin stimulation compared to $KLRG1^-$ NK cells (Fig. 5b, c). Interestingly, it was observed that $KLRG1^+$ NK cells displayed a much higher propensity to undergo apoptosis than the $KLRG1^-$ subset (Fig. 5d, e). Overall, these results suggest that $KLRG1^+$ NK cells in CRC patients exhibit more impaired function and increased susceptibility to apoptosis, which may contribute to the advanced progression of CRC.

KLRG1 blockade restores NK cell function in CRC patients

The upregulation of KLRG1 on NK cells in CRC patients suggests that targeting KLRG1 could be a promising strategy to enhance the cytotoxicity of NK cells. To further investigate this possibility, PBMCs obtained from CRC patients and HCs were simulated in vitro for 8 h with IL-2, IL-12, and IL-15, either with or without the presence of anti-human KLRG1 blocking monoclonal antibodies. The results revealed that there was a significant reduction in IFN-γ secretion by NK cells in CRC patients compared to HCs following cytokines stimulation (Fig. 6a). Additionally, the expression of CD107a on NK cells was found to be decreased in CRC patients when compared to HCs (Fig. 6b). However, the presence of anti-human KLRG1 blocking monoclonal antibodies significantly enhanced the production of IFN-γ and CD107a in NK cells derived from CRC patients (Fig. 6a, b). Most importantly, the levels of IFN-γ and CD107a in NK cells from CRC patients following KLRG1 blockade were comparable to those observed in HCs (Fig. 6a, b), indicating that KLRG1 blockade not only improved, but also restored NK cell function. These findings suggest that KLRG1 blockade has the potential to restore the impaired function of NK cells, making it a promising immunotherapy strategy for patients with CRC.

Discussion

The most exciting paradigm shift in CRC treatment in recent years has been the utilization of immune check point inhibitors targeting PD-1 and CTLA-4 as a promising therapeutic approach [19–21]. Nevertheless, a considerable proportion of patients still remain unresponsive to the existing therapeutic drugs due to intra- and inter-tumor heterogeneity [22]. Hence, the objective of this study was to investigate the characteristics of NK cells and their phenotypical and functional status in patients with CRC, aiming to identify new predictive biomarkers and potential targets for novel immunotherapies. This study revealed that NK cells in CRC patients had increased expression of KLRG1 but no other inhibitory receptors. The $KLRG1^+$ NK cells exhibited a significant functional deficiency including decreased expression of cytokines and CD107a, which indicated impaired cytotoxicity function. Blocking KLRG1 could restore the capacity of NK cells to produce cytokines, indicating that KLRG1 might represent a promising target for immunotherapy in CRC patients.

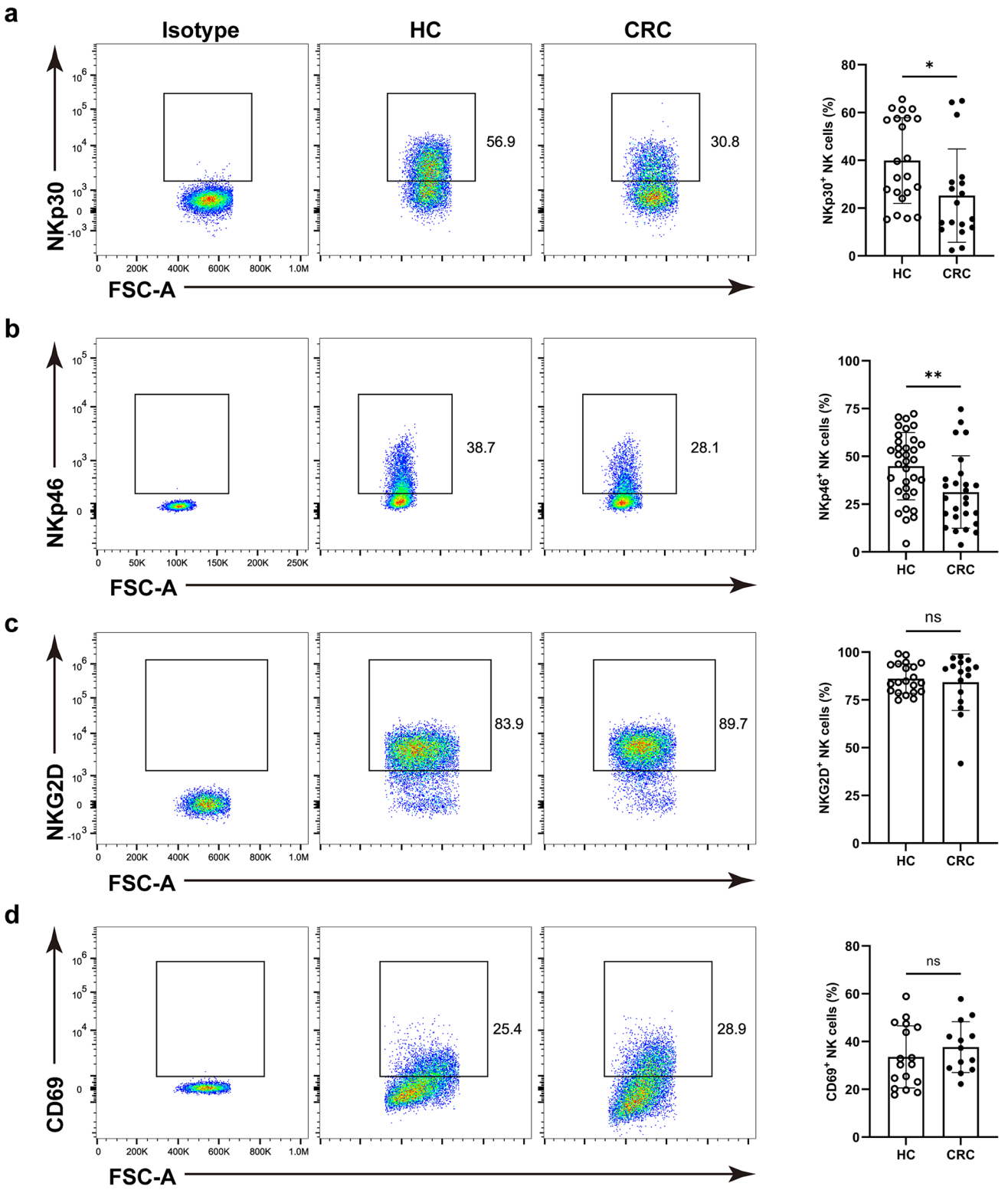
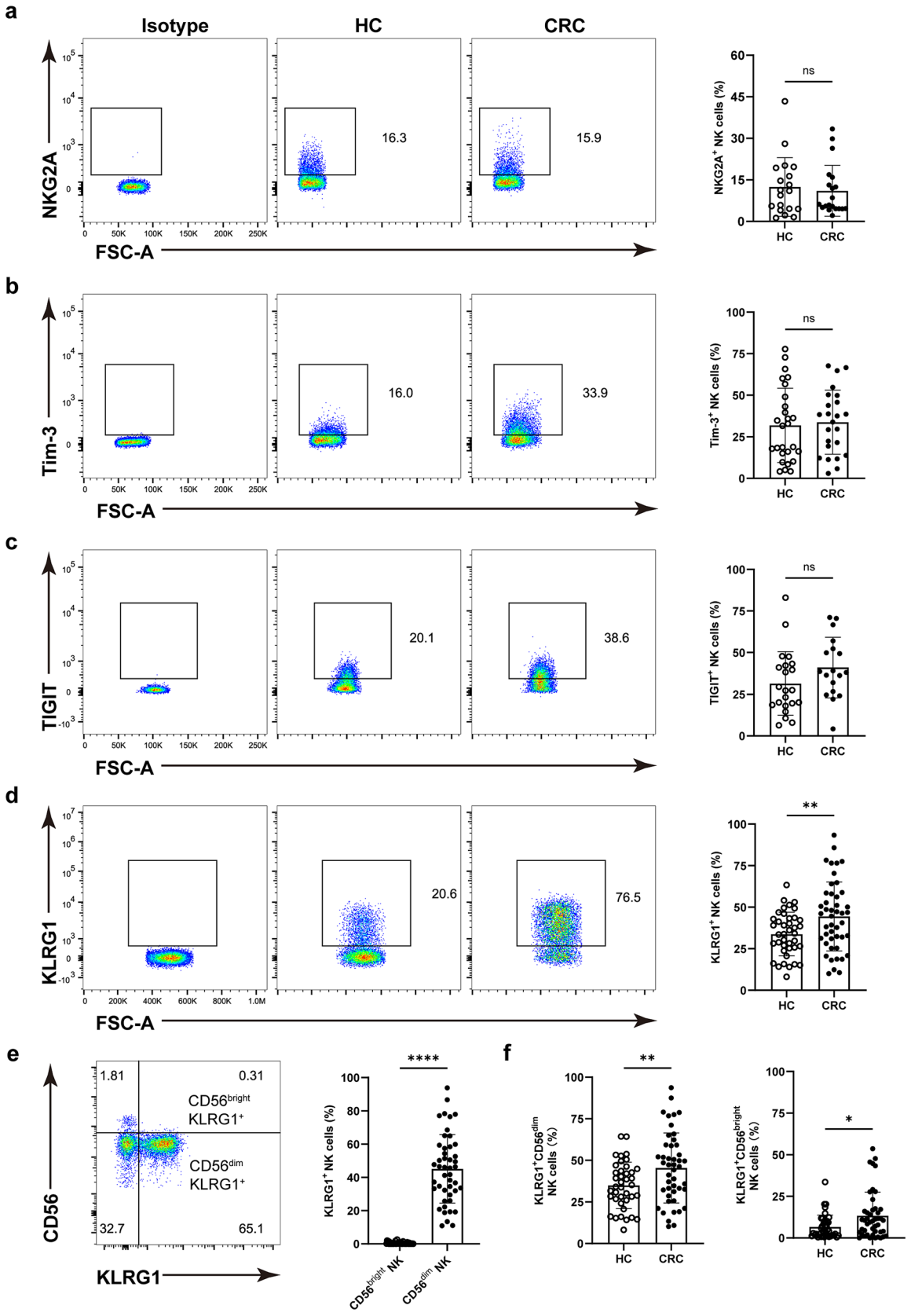


Fig. 3 NK cells in CRC patients have downregulated expression of activating receptors. Representative flow cytometry plots and frequencies of **a** NKp30$^+$, **b** NKp46$^+$, **c** NKG2D$^+$, and **d** CD69$^+$ NK cells in patients with CRC and healthy controls (HCs). Results are shown as the mean ± standard deviation (SD), with each dot denoting a separate individual. Statistical significance is defined as *P*-values < 0.05. *$P < 0.05$; **$P < 0.01$; ns, not significant


a
Isotype
HC
CRC
NKG2A
FSC-A
16.3
15.9
NKG2A+ NK cells (%)
ns
HC
CRC
b
Tim-3
FSC-A
16.0
33.9
Tim-3+ NK cells (%)
ns
HC
CRC
c
TIGIT
FSC-A
20.1
38.6
TIGIT+ NK cells (%)
ns
HC
CRC
d
KLRG1
FSC-A
20.6
76.5
KLRG1+ NK cells (%)
**
HC
CRC
e
CD56
KLRG1
1.81
0.31
CD56bright KLRG1+
CD56dim KLRG1+
32.7
65.1
KLRG1+ NK cells (%)
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CD56bright NK
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HC
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HC
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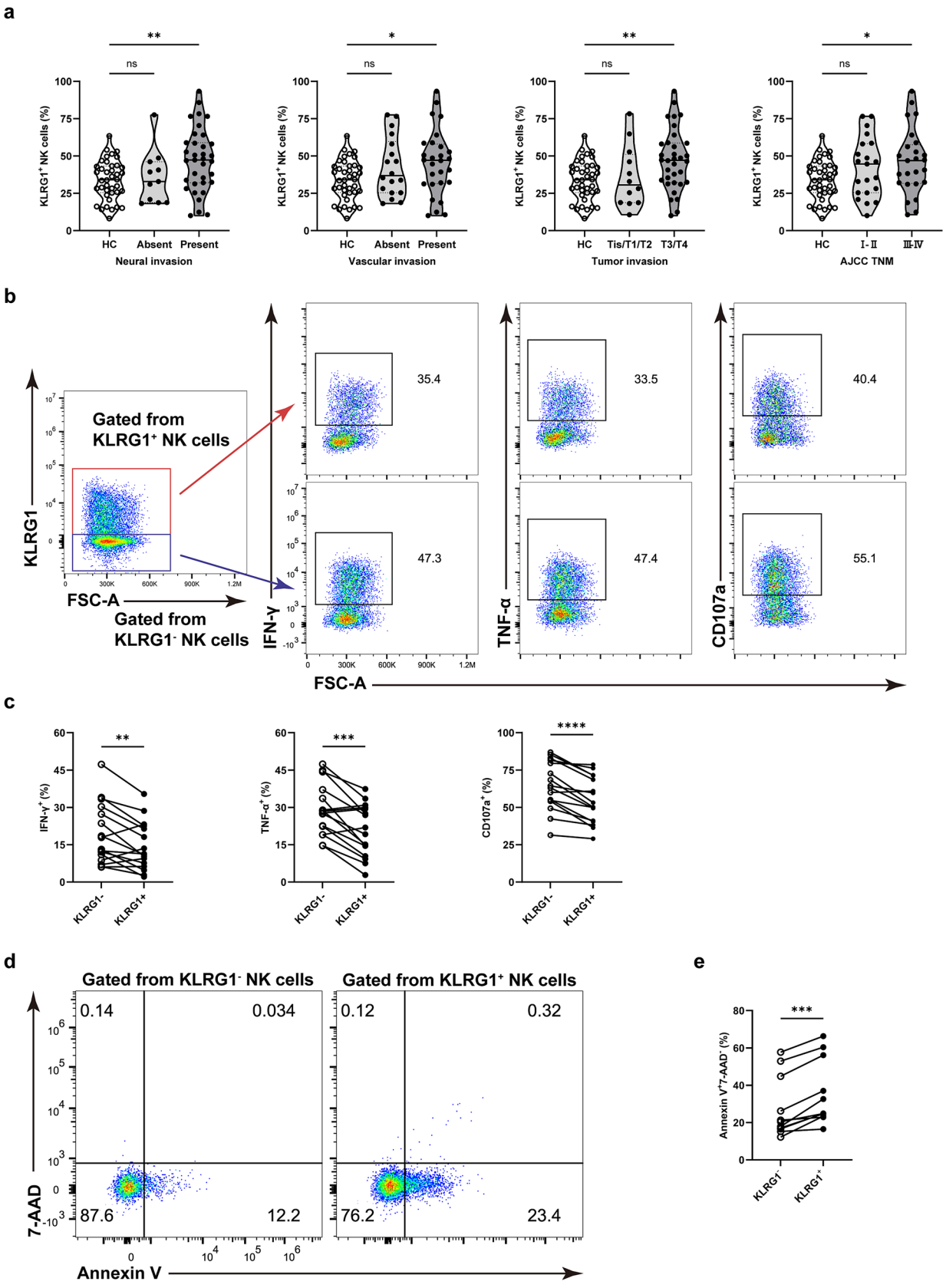
◂**Fig. 4** NK cells in CRC patients have upregulated expression of inhibitory receptors. Representative flow cytometry plots and frequencies of **a** NKG2A$^+$, **b** Tim-3$^+$, **c** TIGIT$^+$, and **d** KLRG1$^+$ NK cells in CRC patients and healthy controls (HCs). **e** Representative flow cytometry plots and frequencies of KLRG1$^+$CD56^{dim} and KLRG1$^+$CD56^{bright} NK cell within the overall NK cell population in CRC patients. **f** Frequencies of KLRG1$^+$CD56^{dim} and KLRG1$^+$CD56^{bright} NK cells in CRC patients and HCs. Results are shown as the mean ± standard deviation (SD), with each dot denoting a separate individual. Statistical significance is defined as *P*-values < 0.05. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$; ns, not significant

As cytotoxic effectors of the innate immune system, NK cells play a pivotal role in immune surveillance against tumor cells. The ability of NK cells to rapidly lyse target cells without prior immunization could have important implications in tumor control, while the release of cytokines by NK cells may exert a profound influence on adaptive immunity [23]. Considering that colorectal tumor-infiltrating NK cells are scarce and severely dysregulated [24, 25], it is plausible that their limited presence may hinder their ability to exert a substantial impact at the tumor site. Additionally, recent research findings have unveiled that a higher proportion of NK cell in the peripheral blood of CRC patients is indicative of a prolonged duration of survival [26]. These studies suggest that circulating NK cells may possess potential efficacy in colorectal tumor surveillance. However, the current understanding of the characteristics of circulating NK cells in CRC patients remains fragmentary. In this study, we revealed that both the frequency and absolute number of NK cells in the peripheral blood were significantly decreased in CRC patients than that of HCs. Nevertheless, the findings of several studies have yielded contradictory conclusions. Krijgsman et al. [5] reported a comparable proportion of circulating NK cells in CRC patients and HCs, whereas Rocca et al. [27] found a significant elevation in the proportion of circulating NK cell in CRC patients. The paradoxical conclusions could potentially be ascribed to differences in inclusion criteria, varied tumor progression degrees among patients, as well as testing methods. Our study also provided a possible interpretation for the observed decline in the frequency and number of NK cells in CRC patients, specifically attributing it to their increased susceptibility to apoptosis.

The activation of NK cells is regulated through the integration of signals from a diverse range of cell surface activating and inhibitory receptors that directly impact their effector function [28, 29]. The expression of multiple activating and inhibitory receptors on NK cells has been found to be significantly dysregulated in various types of cancer [30, 31]. Therefore, the phenotypical and functional status of circulating NK cells in CRC patients was assessed. Activating receptors NKp30 and NKp46 were found to be downregulated on NK cells, while expression of inhibitory receptors NKG2A, Tim-3 and TIGIT did not change. We further found that the inhibitory receptor KLRG1 was remarkably upregulated on NK cells and both CD56^{dim} and CD56^{bright} subtypes. The capability of NK cells from CRC patients to degranulate (as indicated by CD107a expression) and to produce cytokine (as indicated by IFN-γ and TNF-α expression) were compromised following PMA/ionomycin stimulation. It is noteworthy that Rocca et al. [27] showed consistent results with our findings after IL-2 stimulation regarding IFN-γ and CD107a, thereby providing further evidence to support the impairment of NK cells in peripheral blood. In addition, further impairment of NK cell function was observed in conjunction with the presence of neural invasion and advancement of tumor invasion.

Despite the aforementioned findings, the mechanisms underlying NK cell dysfunction in CRC patients remained elusive. As an inhibitory receptor, KLRG1 exerts its suppressive effect on cytotoxicity and cytokine production of NK cells when interacting with three kinds of ligands: E-cadherin, N-cadherin, and R-cadherin [16, 32]. After binding to E-cadherin and N-cadherin on the surfaces of cancer cells or antigen-presenting cells, the immunoreceptor tyrosine-based inhibitory motif (ITIM) of KLRG1 undergoes phosphorylation, which subsequently recruits Src homology-2-containing inositol phosphatase-1 (SHIP-1) and Src homology-2-containing protein tyrosine phosphatase 2 (SHP2) [12, 33, 34]. SHIP-1 and SHP2 dephosphorylate phosphatidylinositol 3,4,5-trisphosphate (PIP3) to phosphatidylinositol 4,5-bisphosphate (PIP2), whereas PI3K activates AKT by phosphorylating PIP2 to produce PIP3 [35, 36]. Consequently, KLRG1 inhibits the PI3K/AKT signaling pathway, resulting in inhibited cytotoxicity and cytokine production of NK cells [37–39]. Additionally, KLRG1 can also impair NK cell function by maintaining the activation of AMP-activated protein kinase (AMPK). Upon ligation, KLRG1 is internalized and directly binds to AMPK, preventing its dephosphorylation by phosphatase-2C (PP2C), leading to reduced cytotoxicity, cytokine secretion, and proliferation of NK cells [40, 41].

An increased percentage of KLRG1$^+$ NK cells was observed in oral cancer patients, and it was shown that the interaction between KLRG1 and its ligand confers protection to tumor cells against NK cytolysis [17]. Serum soluble E-cadherin is known to participate in the progression of various cancer types and its correlation with tumor metastasis are well-established [42, 43]. Recently, it has been reported that E-cadherin plays a significant role in colorectal carcinogenesis [44]. In addition, KLRG1 has been found to be upregulated on CD8$^+$ T cells during persistent antigen stimulation [45]. These indications imply that KLRG1 upregulation might also be involved in the development and progression of CRC. In the present study, we found that KLRG1$^+$ NK cells exhibited significantly greater impairment in cytokine production compared to KLRG1$^-$ NK cells. Moreover, an increased percentage of KLRG1$^+$ NK cells was positively associated with progressive


a
KLRG1+ NK cells (%)
HC
Absent
Present
Neural invasion
Vascular invasion
Tis/T1/T2
T3/T4
Tumor invasion
I-II
III-IV
AJCC TNM
ns
b
Gated from KLRG1+ NK cells
Gated from KLRG1- NK cells
KLRG1
FSC-A
IFN-γ
TNF-α
CD107a
35.4
47.3
33.5
47.4
40.4
55.1
c
IFN-γ+ (%)
TNF-α+ (%)
CD107a+ (%)
KLRG1-
KLRG1+
d
Gated from KLRG1- NK cells
Gated from KLRG1+ NK cells
7-AAD
Annexin V
0.14
0.034
87.6
12.2
0.12
0.32
76.2
23.4
e
Annexin V+7-AAD- (%)

◂**Fig. 5** KLRG1$^+$ NK cells have higher levels of exhaustion than KLRG1$^-$ subgroup in CRC patients. **a** Violin plots display the correlations between KLRG1 expression levels and CRC progression. Solid and dashed horizontal lines on violin plots depict median and upper and lower quartiles, respectively. Each dot denotes a separate individual. **b** Representative flow cytometry plots of IFN-γ, TNF-α, and CD107 expression on KLRG1$^-$ and KLRG1$^+$ NK cells from CRC patients. **c** Proportions of IFN-γ, TNF-α, and CD107a positive KLRG1$^+$ and KLRG1$^-$ NK cells from individual CRC patients. **d** Representative flow cytometry plots of early apoptosis through Annexin V$^+$7-AAD$^-$ staining in KLRG1$^-$ and KLRG1$^+$ NK cells from CRC patients. **e** Proportions of Annexin V$^+$7-AAD$^-$ KLRG1$^-$ and KLRG1$^+$ NK cells from CRC patients. Statistical significance is defined as P-values < 0.05. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; ns, not significant

clinicopathologic characteristics such as the presence of neural and vascular invasion, advanced tumor invasion, and advanced cancer grade. Thus, KLRG1 is very likely to play a crucial role in the functional exhaustion of NK cells in CRC patients. Targeting KLRG1 using specific antibodies has the potential to restore the functional capabilities of NK cells against HIV-infected cells, thereby facilitating the reduction of the viral reservoir [46]. According to Tata et al. [47], in melanoma and breast cancer mouse models, KLRG1 blockade has been shown to synergistically enhance antitumor effects when combined with PD-1 blockade, compared to monotherapy. Furthermore, tumors that are resistant to anti-PD-1 monoclonal antibody treatment can still benefit from combination therapy with KLRG1 blockade. Breast cancer cells can reprogram tumor-exposed NK (teNK) cells and promote metastatic progression. While anti-PD-1 antibody treatment does not suppress the colony-promoting effect of teNK cells, anti-KLRG1 antibody is capable of reducing breast tumor colony formation at concentrations significantly lower than those required for anti-TIGIT antibody [48]. Jones et al. [49] reported that either KLRG1 blockade or knockout can attenuate melanoma tumor growth in tumor-bearing mice. Steven et al. [50] demonstrated that monotherapy with anti-KLRG1 antibody significantly reduces lung metastasis in 4T1 breast cancer mouse models. Furthermore, in MC38 colorectal cancer and B16F10 melanoma mouse models, combination therapy with anti-KLRG1 and anti-PD-1 antibodies resulted in a greater synergistic effect on reducing tumor growth and improving survival compared to anti-PD-1 monotherapy. Therefore, when existing immune checkpoint therapies are ineffective, KLRG1 blockade represents a promising alternative treatment. Both as a monotherapy and in combination with current immune checkpoint

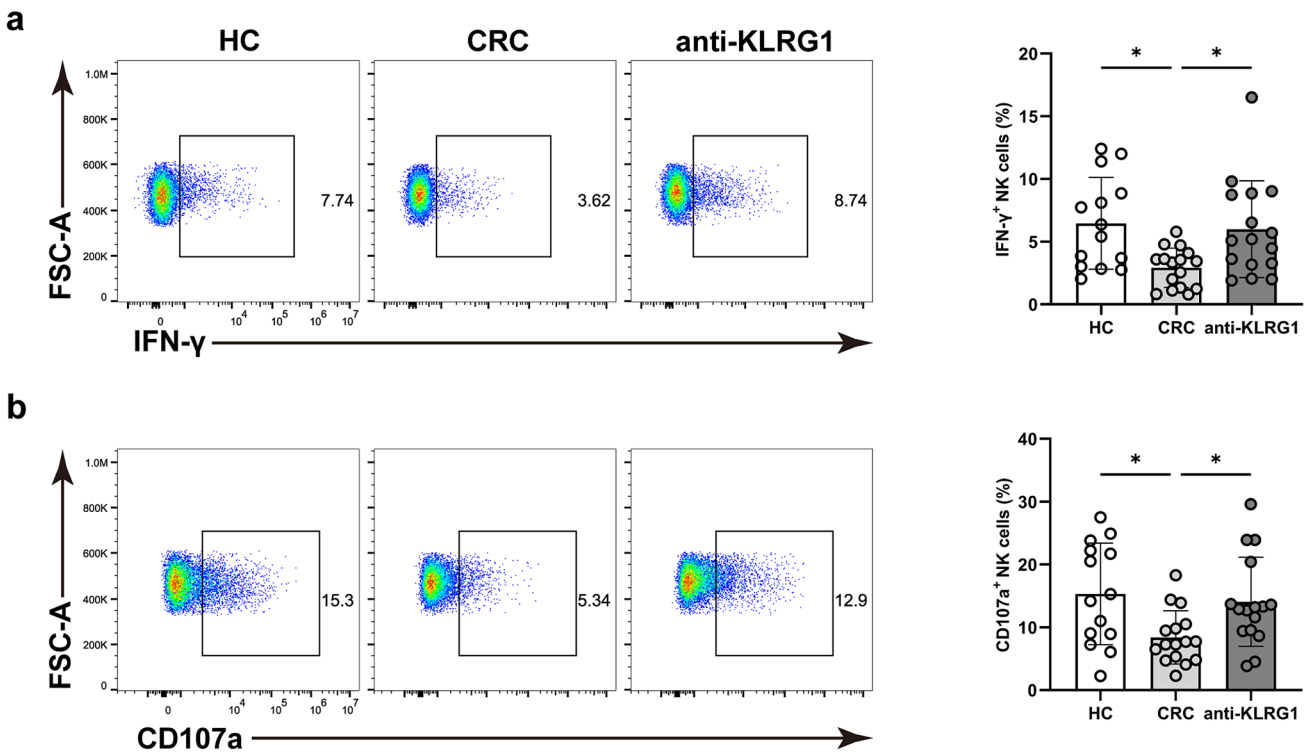


Fig. 6 Blocking KLRG1 restores the effector function of NK cells in CRC patients. Representative flow cytometry plots and frequencies of **a** IFN-γ^+ and **b** CD107a$^+$ NK cells from CRC patients and healthy controls (HCs) in the presence of anti-human KLRG1 blocking monoclonal antibody or control IgG antibody. Isolated PBMCs were stimulated in vitro for 8 h with IL-2/IL-12/IL-15 in the presence of 10 μg/mL anti-human KLRG1 blocking monoclonal antibody or an equal dose of control IgG antibody. Results are shown as the mean ± standard deviation (SD), with each dot denoting a separate individual. Statistical significance is defined as P-values < 0.05. $^*P < 0.05$

inhibitors, KLRG1 blockade has demonstrated potent efficacy in preclinical models. However, whether KLRG1 blockade can exert its effects in human CRC patients remains unexplored. Our data demonstrated that blocking KLRG1 effectively restored the cytokine secretion capacity of NK cells in CRC patients. Altogether, these findings provide further evidence supporting the role of KLRG1 in inducing NK cell exhaustion in CRC patients, consequently prompting tumor immune evasion in CRC. In addition, it is suggested that immunotherapy targeting KLRG1 holds promise for yielding clinical benefits in CRC patients.

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Author contributions Cairui Xu and Ling Tang designed the experiments and wrote the first draft of manuscript. Cairui Xu and Kangli Cao performed the experiments and analyzed the data. Along Ma participated in the collection of clinical samples and data. Meijuan Zheng reviewed and commented on previous versions of the manuscript. Ling Tang and Yuanhong Xu supervised the experiments and revised the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Medical Ethics Committee of the First Affiliated Hospital of Anhui Medical University (PJ 2024-12-16).

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent to publish Not applicable.

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