Original Paper

Human Leukocyte Antigen-G Inhibits the Anti-Tumor Effect of Natural Killer Cells via Immunoglobulin-Like Transcript 2 in Gastric Cancer

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Key Words

HLA-G • ILT2 • Natural killer cell • Gastric cancer • Tumor escape

Abstract

Background/Aims: Human leukocyte antigen-G (HLA-G) plays an important role in inhibiting natural killer (NK) cell function and promoting immune escape. However, the specific mechanism of HLA-G on NK in gastric cancer (GC) remains not well understood. This study investigated the expression of HLA-G in GC and the role of HLA-G-effected NK cells in GC progression. **Methods:** HLA-G expression in GC tissues obtained from 49 patients with GC was analyzed by immunohistochemistry and western blot. The number of tumor-infiltrating NK cells and the expression of their surface receptors were analyzed by immunohistochemistry and flow cytometry, respectively. The effect of HLA-G on NK cell proliferation was examined by Cell Counting Kit-8 (CCK8) assay. LDH release assay was used to evaluate the effect of HLA-G on the cytotoxic activity of NK cells, and the levels of IFN- γ and TNF- α in the co-cultured supernatant were detected by ELISA. Mice bearing a xenograft tumor model were used to examine the effect of HLA-G on the anti-tumor effect of NK cells. **Results:** HLA-G positive expression was detected in most of the GC tissues, and was correlated with the adverse prognosis of the disease. The expression of HLA-G was negatively associated with the number of tumor-infiltrating NK cells. Furthermore, GC cell lines with overexpressed HLA-G revealed their ability to inhibit the cell proliferation and cytotoxic activity of NK-92MI cells, and reduce the secretion of IFN- γ and TNF- α through immunoglobulin-like transcript 2 (ILT2). Finally, this *in vivo* experiment was able to prove that HLA-G can inhibit the anti-tumor effect of NK cells through ILT2. **Conclusion:** The expression of HLA-G was strongly correlated with the adverse prognosis of GC. The reason may be that it inhibits the proliferation and cytotoxic activity of infiltrating NK cells through ILT2.

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Published by S. Karger AG, Basel

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Introduction

Gastric cancer (GC) is considered the fifth most common malignancy worldwide [1]. In China, GC morbidity and mortality rank second among all malignant tumors [2]. Due to limited census measures, GC patients are mainly those with an advanced cancer stage. That is, the efficacy of surgical treatment is limited. In recent years, immunotherapy has become very popular for cancer treatments [3]. Compared to conventional therapies such as chemotherapy, these approach increases tumor specificity, and thereby preserves the healthy cells of the patient

Tumor cells can undergo immunoediting to survive and grow under selective pressure, eventually escaping immune surveillance [4]. Innate immune surveillance plays a key role in controlling tumor progression [5]. Natural killer (NK) cells are an important part of the innate immune system, and have a vital role during the early tumor progression phase, since they are the first defense system activated in the body [6-8]. NK cells may attach malignant cells by perforin, granzyme and IFN- γ secretion, and through antibody-dependent cell-mediated cytotoxicity (ADCC), thereby promoting lysis-mediated tumor cell death. Studies have shown that NK cell dysfunction in malignant tumors may be strongly correlated to disease progression [9, 10]. Furthermore, several data have shown that the re-education of infiltrating NK cells can be correlated to GC biological behaviors [11-13].

Human leukocyte antigen-G (HLA-G) is a non-classical major histocompatibility complex (MHC) class I antigen. Its mRNA was confirmed in 1987 [14]. During normal physiological conditions, HLA-G is mainly expressed in trophoblast cells and immune tolerance organs [15-21]. However, under pathological conditions, an aberrant expression of HLA-G can occur, such as autoimmune diseases, infections, inflammation and tumors [22-25]. At present, HLA-G is considered an important immunosuppressive molecule in the body [26], and plays an important role in fetal-maternal immune tolerance, organ transplantation and the immune escape of tumors [27-31]. HLA-G can inhibit the function of many immune cells such as NK cells, CD4+ and CD8+ T cells, and dendritic cells (DC) by binding to cell surface-expressed receptors, including immunoglobulin-like transcript 2 (ILT2), ILT4 and killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4) [32]. Furthermore, studies have shown that the upregulation of HLA-G expression may be one of the reasons that cause tumors to escape immune surveillance [33, 34]. However, its exact mechanism remains not well-understood, calling for further investigations.

The present study aims to investigate the expression of HLA-G in GC, and investigate the mechanism of interaction between HLA-G and NK cells.

Materials and Methods

Patients and samples

From February 2015 to November 2016, 49 GC samples obtained from 49 GC patients and paired normal tumor-adjacent samples (at least 3 cm from the tumor site) were obtained from the Department of Gastrointestinal Surgery, The First Affiliated Hospital of Chongqing Medical University, China. None of the GC patients were treated with chemotherapy before the operation. Invasion depth, lymph node metastasis and clinical stages were classified according to AJCC (7th edition) guidelines. The clinical characteristics of all patients are shown in Table 1 and Table 2. The last follow-up date was February 10, 2017. The use of human tissue specimens was approved by the Ethical Committee of The First Affiliated Hospital of Chongqing Medical University, and a written informed consent was obtained from each patient (Approval number: 2016-106).

Immunohistochemistry

The formalin-fixed and paraffin-embedded samples were cut into 4- μ m sections, and were processed for immunohistochemistry, as previously described [35]. Incubation with anti-HLA-G (1:50; Abcam, USA; catalog number: ab52455; clone: 4H84) and anti-human CD57 (1:50; Abcam, USA; catalog number:

Table 1. Correlation of clinicopathologic feature with HLA-G expression and tumor-infiltrating NK cells (Part I)

Clinicopathological feature	Total (N=49)	HLA-G expression		χ^2	p	Infiltrating NK cells mean±SD	t/F	p
		Negative (N=19)	Positive (N=30)					
Gender				0.016	0.898		-0.261	0.796
male	33 (67.35%)	13 (68.42%)	20 (66.67%)			29.50±24.96		
female	16 (32.65%)	6 (31.58%)	10 (33.33%)			31.36±19.99		
Age (years)				0.752	0.386		-1.757	0.085
<60	17 (34.69%)	8 (42.11%)	9 (30.00%)			22.26±12.00		
≥60	32 (65.31%)	11 (57.89%)	21 (70.00%)			34.28±26.69		
Tumor size (cm)				0.258	0.612		0.077	0.939
≤3	21 (42.86%)	9 (47.37%)	12 (40.00%)			33.30±25.60		
>3	28 (57.14%)	10 (52.63%)	18 (60.00%)			32.75±23.52		
Tumor location				0.587	0.746		0.460	0.634
Cardia	15 (30.61%)	7 (36.84%)	8 (26.67%)			32.48±19.10		
Body	9 (18.37%)	3 (15.80%)	6 (20.00%)			34.69±31.86		
Antrum	25 (51.02%)	9 (47.36%)	16 (53.33%)			27.03±22.25		
Differentiation				0.077	0.782		1.008	0.319
Well and Moderate	22 (44.9%)	9 (47.37%)	13 (43.33%)			33.82±24.47		
Low	27 (55.1%)	10 (52.63%)	17 (56.67%)			27.08±22.25		
Borrmann-type				2.940	0.401		0.583	0.659
I	20 (40.82%)	10 (52.63%)	10 (33.33%)			32.46±24.38		
II	23 (46.94%)	6 (31.58%)	17 (56.67%)			30.95±24.84		
III	4 (8.16%)	2 (10.53%)	2 (6.67%)			20.80±8.57		
IV	2 (4.08%)	1 (5.26%)	1 (3.33%)			15.50±1.56		
Pathological type				1.057	0.304		-1.284	0.206
Adenocarcinoma	44 (89.80%)	16 (84.21%)	28 (93.33%)			28.68±23.35		
Other	5 (10.20%)	3 (15.79%)	2 (6.67%)			42.68±20.35		

ab187274) antibodies were used. The percentage of HLA-G positive cells was graded according to the following criteria: -, <10%; +, 10%-30%; ++, 30%-50%; +++, >50% [36]. Five of 200 fields were randomly selected, and the mean value was used to account for the number of NK cells.

Western blot

Total protein was extracted after digesting the tissues and cells with lysis buffer. Equal amounts of protein were resolved *via* SDS-PAGE and transferred onto PVDF membranes. Then, the membranes were blocked with 5% non-fat dry milk in TBS for two hours, and incubated overnight at 4°C with primary antibodies against the following proteins: HLA-G (1:500; Abcam, USA; catalog number: ab52455, clone: 4H84), ILT2 (1:1000; Abcam, USA; catalog number: ab170909), KIR2DL4 (1:1000; Abcam, USA; catalog number: ab107609), and GAPDH (1:1000; Abcam, USA; catalog number: ab9485). Next, the membranes were incubated with the secondary antibody for one hour, and specific protein bands on the membranes were detected using an enhanced chemiluminescence kit (Pierce Biotechnology, USA).

Cell cultures

Human GC cell lines MGC-803, BGC-823, MKN-28, MKN-45, SGC-7901 and AZ-521, human choriocarcinoma cell line JEG-3 (HLA-G positive control), human acute monocytic leukemia cell line THP-1 (ILT2 and KIR2DL4 positive control), and human NK cell line NK-92MI were purchased from the cell resources of the Shanghai Center for Life Science Research Institute of the Chinese Academy of Sciences. MGC-803, BGC-823, MKN-28, SGC-7901, AZ-521 and THP-1 cells were cultured in RPMI 1640 (Gibco, USA) medium supplemented with 10% FBS (Gibco, USA). MKN-45 cells were cultured in RPMI 1640 (Gibco, USA) medium supplemented with 20% FBS (Gibco, USA). JEG-3 cells were incubated in DMEM (Gibco, USA) medium with 10% FBS (Gibco, USA). NK-92MI cells were incubated in MEM α (Gibco, USA) medium containing 12.5% FBS (Gibco, USA) and 12.5% horse serum (Gibco, New Zealand). All these media were added with 1% penicillin/streptomycin (Gibco, USA). The culture flasks were placed in a humidified atmosphere containing 95% air and 5% CO₂ at 37°C.

Table 2. Correlation of clinicopathologic feature with HLA-G expression and tumor-infiltrating NK cells (Part II)

Clinicopathological feature	Total (N=49)	HLA-G expression		χ^2/Z	p	Infiltrating NK cells mean $\pm$ SD	t/F	p
		Negative (N=19)	Positive (N=30)					
History of smoking				0.860	0.354		0.894	0.376
Absence	14 (28.57%)	4 (21.05%)	10 (33.33%)			34.81 $\pm$ 27.50		
Presence	35 (71.43%)	15 (78.95%)	20 (66.67%)			28.22 $\pm$ 21.50		
History of drinking				0.587	0.444		0.413	0.681
Absence	25 (51.02%)	11 (57.89%)	14 (46.67%)			31.46 $\pm$ 24.29		
Presence	24 (48.98%)	8 (42.11%)	16 (53.33%)			28.69 $\pm$ 22.59		
Anemia				5.299	0.021*		-1.515	0.136
Absence	26 (53.06%)	14 (73.68%)	12 (40.00%)			25.43 $\pm$ 21.88		
Presence	23 (46.94%)	5 (26.32%)	18 (60.00%)			35.39 $\pm$ 24.14		
Hypoalbuminemia				0.291	0.590		0.02	0.987
Absence	26 (53.06%)	11 (57.89%)	15 (50.00%)			30.17 $\pm$ 27.34		
Presence	23 (46.94%)	8 (42.11%)	15 (50.00%)			30.03 $\pm$ 18.23		
CEA				0.168	0.711		-0.498	0.621
Normal	40 (81.63%)	16 (84.21%)	24 (80.00%)			29.32 $\pm$ 21.73		
Elevated	9 (18.37%)	3 (15.79%)	6 (20.00%)			33.62 $\pm$ 30.46		
CA19-9				0.406	0.524		0.722	0.474
Normal	36 (73.47%)	13 (68.42%)	23 (76.67%)			31.56 $\pm$ 22.66		
Elevated	13 (26.53%)	6 (31.58%)	7 (23.33%)			26.09 $\pm$ 25.38		
Invasion depth				-2.502	0.012*		2.525	0.091
T1 + T2	8 (16.33%)	6 (31.58%)	2 (6.67%)			44.03 $\pm$ 17.76		
T3	8 (16.33%)	4 (21.05%)	4 (13.33%)			35.90 $\pm$ 18.54		
T4	33 (67.34%)	9 (47.37%)	24 (80.00%)			25.33 $\pm$ 24.25		
Lymph Node Metastasis				-2.443	0.015*		17.212	<0.001*
N0	16 (32.65%)	11 (57.89%)	5 (16.67%)			53.19 $\pm$ 24.35		
N1	8 (16.33%)	2 (10.53%)	6 (20.00%)			25.15 $\pm$ 15.12		
N2	6 (12.24%)	1 (5.26%)	5 (16.67%)			27.13 $\pm$ 15.31		
N3	19 (38.78%)	5 (26.32%)	14 (46.66%)			13.69 $\pm$ 5.05		
AJCC stage				-3.288	0.001*		5.434	0.008*
I	6 (12.24%)	5 (26.32%)	1 (3.33%)			49.00 $\pm$ 28.20		
II	7 (14.29%)	5 (26.32%)	2 (6.67%)			44.86 $\pm$ 33.07		
III	36 (73.47%)	9 (47.36%)	27 (90.00%)			24.09 $\pm$ 17.31		

*P<0.01

Cell transfection

The HLA-G (LV5-HLA-G), ILT2 (LV5-ILT2) and non-targeted negative control viruses (LV5) were purchased from Shanghai GenePharma Co. Ltd. (China). Lentivectors were prepared using 293T cells. Cells were transfected with lentiviral plasmids and accessory plasmids (LV5, PG-P1-VSVG, PG-P2-REV, and PG-P3-RRE). MGC-803 cells were transduced with LV5-HLA-G to overexpress HLA-G. NK-92MI cells were transduced with LV5-ILT2 to overexpress ILT2. Moreover, empty lentiviral vectors were added to the control cells. The HLA-G sequence was: forward, 5'-GCGGCCGCTCCCCAGAC-3'; reverse, 5'-GGATCCCCTTTTCAATCTGAGCTCTTCTTTC-3'. The ILT2 sequence was: forward, 5'-GCGGCCGCGCCACCATGACCCCATCCTCACGGTCTCTGAT-3'; reverse, 5'-ATGCATCTAGTGGATGGCCAGAGTGGCGTA-3'.

Cell isolation and flow cytometry

Tumor-infiltrating lymphocytes were obtained from tissue samples, as previously described [37]. The lymphocytes were washed and resuspended in medium supplemented with 1% FBS for flow cytometry. CD3-PE-CF594 and CD56-BV421 were purchased from BD Bioscience. ILT2-PE and KIR2DL4-PE were purchased from R&D System. Flow cytometry (BD Influx, BD Biosciences) was used to detect the expression of surface receptors ILT2 and KIR2DL2 in NK cells (CD3⁺CD56⁺). Flow cytometric analysis was carried out using FlowJo 10.2 software (FLOWJO, USA).

Quantitative RT-PCR

Total RNA from cells was isolated using TRIzol (TaKaRa, Dalian, China). The concentration and purity of the total RNA samples were measured using a UV spectrophotometer (UltroSpec2100 Pro, Amersham, USA). Total RNA was reverse-transcribed using a PrimeScript RT Reagent kit (TaKaRa, Dalian, China).

The primers for HLA-G and β -actin were as follows: HLA-G (forward 5'-GAGGAGACACGGAACACCAAG-3', reverse 5'-GGTCGAGCCAATCATCC-3'); β -actin (forward 5'-TGACGTGGACATCCGCAAAG-3', reverse 5'-CTGGAAGGTGGACAGCGAGG-3'). Quantitative RT-PCR was performed using the CFX96™ Real-Time System (Bio-Rad, USA) and SYBR Premix Ex Taq™II (TaKaRa, Dalian, China). Mean relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method.

Cell proliferation detection

Co-culture proliferation assays were performed using NK-92MI cells (ILT2 overexpression or low expression) (2, 000 cells/well) and γ -irradiated (40 Gy) MGC-803 cells (HLA-G overexpression or low expression) (1, 000 cells/well). In addition, antibody 5A6G7 (Abcam, USA; catalog number: ab76869) (10 μ g/ml) blocking HLA-G was used. Cell proliferation was examined at 1, 2, 3, 4, 5, 6 and 7 days post-radiation using Cell Counting Kit-8 (CCK8; Dojindo, Japan), according to manufacturer's instructions.

Cell-mediated cytotoxic activity assay

Cytotoxic activity assay was performed using NK-92MI cells (ILT2 overexpression or low expression) as effector cells. MGC-803 cells (HLA-G overexpression or low expression) were used as target cells. The effector-to-target cell ratio (E:T) was 2.5:1, 5:1, 10:1 and 20:1, respectively. In addition, antibody 5A6G7 (Abcam, USA; catalog number: ab76869) (10 μ g/ml) blocking HLA-G was used. A colorimetric-based LDH assay (CytoTox 96® non-radioactive cytotoxic activity assay; Promega, USA) was used, and the cytotoxic activity was calculated according to manufacturer's instructions. The percentage of specific lysis was calculated using the formula: [(test release - spontaneous release) / (maximum release - spontaneous release)] $\times$ 100.

ELISA

The co-culture supernatant concentrations of IFN- γ and TNF- α (Abcam, USA) were determined using ELISA kits at 0, 12, 24, 48 and 72 hours post-radiation, according to manufacturer's instructions.

In vivo experiments

A mouse xenograft model was established using 6-8-week-old male non-obese, severe combined immunodeficient/beige (NOD/SCID) mice, which were obtained from the Laboratory Animal Center of Chongqing Medical University. MGC-803 cells (HLA-G overexpression or low expression) (2×10^6) were subcutaneously injected into the right flank of each mouse. Two weeks later, mice were given an intravenous injection of 1×10^7 of either NK-92MI (ILT2 overexpression) or NK-92MI (ILT2 low expression) cells. PBS (100 μ l) was used for the control group. Tumor volumes were calculated using the following formula: V (mm^3) = length $\times$ width $\times$ width / 2. All animal protocols were conducted in accordance with the Institutional Animal Ethics Care and Use Committee of the First Affiliated Hospital of Chongqing Medical University.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software (IBM, USA). Continuous data were expressed as mean $\pm$ standard deviation (SD). Categorical data were expressed as frequency or percentage. Student's *t*-test or ANOVA was used to compare continuous data. The Mann-Whitney U-test was used for continuous variables that did not conform to the normal distribution (by Shapiro-Wilk test) or categorical data. The chi-square test was used to analyze categorical data. The correlation analysis was performed by Spearman's ρ coefficients. Survival curves were calculated using the Kaplan-Meier method, and compared using the Log-rank test. Cox regression analysis was carried out to identify the independent prognostic factors for predicting survival. $P < 0.05$ was considered statistically significant.

Results

Relationship between elevated HLA-G expression and clinical significance indicated a poor prognosis for gastric cancer

Immunohistochemistry data revealed that the overall positive rate of the expression of HLA-G in cancer tissue was 61.22% (30/49), including a weak positive rate of 8.16%

Fig. 1. HLA-G expression in gastric cancer and the correlations between HLA-G expression and tumor-infiltrating NK cells. (A) Immunohistochemical staining of HLA-G (200x): a, positive expression in cancer tissues; b, negative expression in cancer tissues; c, negative expression in normal tissue. (B) Western-blot analysis of HLA-G expression in gastric cancer tissues was significantly higher compared to normal tissues (***) ($P < 0.001$). (C) Cumulative 2-years OS and DFS curves of the patients positive or negative for HLA-G expression. (D) Immunohistochemical staining of HLA-G and CD57 respectively in gastric cancer tissues (400x): a, HLA-G positive; b, CD57 low density; c, HLA-G negative; d, CD57 high density. (E) Statistical analysis of the number of tumor-infiltrating NK cells with different levels of HLA-G expression (***) ($P < 0.001$ vs. HLA-G negative). (F) The negative correlation with the density of NK cells and HLA-G expression.

(4/49), a median positive rate of 36.69% (17/49), and a strong positive rate of 18.37% (9/49). However, no expression was observed in normal tumor-adjacent tissues (Fig. 1A). In addition, western blot data revealed that the expression of HLA-G in cancer tissue was

Table 3. Univariate and Multivariate analysis of prognostic variables

Variables	Univariate analysis			Multivariate analysis		
	p (OS)	p (DFS)	p (OS)	95% CI	p (DFS)	95% CI
Gender	0.678	0.513	NA	NA	NA	NA
Age	0.862	0.718	NA	NA	NA	NA
Tumor size	0.557	0.352	NA	NA	NA	NA
Differentiation	0.879	0.377	NA	NA	NA	NA
Pathological type	0.974	0.587	NA	NA	NA	NA
History of smoking	0.703	0.807	NA	NA	NA	NA
History of drinking	0.486	0.392	NA	NA	NA	NA
Anemia	0.896	0.863	NA	NA	NA	NA
Hypoalbuminemia	0.544	0.666	NA	NA	NA	NA
CEA	0.498	0.551	NA	NA	NA	NA
CA19-9	0.975	0.617	NA	NA	NA	NA
Invasion depth	0.261	0.197	0.322	0.436-12.479	0.238	0.581-8.917
Lymph node metastasis	0.390	0.270	0.755	0.308-2.350	0.917	0.395-2.300
AJCC stage	0.084	0.093	0.147	0.654-17.194	0.153	0.719-8.198
HLA-G expression	0.050	0.057	0.356	0.500-6.886	0.412	0.549-4.307

CI, confidence interval; NA, not assessed

significantly higher when compared to normal tumor-adjacent tissues ($P < 0.001$, Fig. 1B). By comparing the clinical characteristics of GC patients with different HLA-G expression, it was found that the expression of HLA-G was significantly correlated with preoperative anemia ($P = 0.021$), tumor invasion depth ($P = 0.012$), lymph node metastasis ($P = 0.015$) and clinical stages ($P = 0.001$); but not with other clinical characteristics (Table 1 and Table 2). The survival analysis demonstrated that the 2-year overall survival (OS) and disease-free survival (DFS) rate in patients with positive HLA-G expression was significantly lower compared to patients with negative HLA-G expression ($P = 0.036$ and $P = 0.044$, Fig. 1C). Subsequently, the Cox regression multivariate analysis revealed that HLA-G expression was not significant in terms of the independent prognostic factors for OS and DFS (Table 3).

The decrease in tumor-infiltrating NK cells is associated with the expression of HLA-G and involved in tumor development

Infiltrating NK cells in GC tissue were detected by immunohistochemistry. Compared with HLA-G expression results, the result in this study revealed that the number of infiltrating NK cells in the HLA-G positive group was significantly lower compared to that in the HLA-G negative group ($P < 0.001$), but no statistical significance was observed among these three HLA-G positive groups ($P = 0.999$, $P = 0.762$ and $P = 0.591$; Figs. 1D and 1E). The correlation between the expression of HLA-G and infiltrating NK cells indicate a significant negative correlation ($P < 0.001$, Fig. 1F). Meanwhile, by comparing the number of infiltrating NK cells in each clinical characteristic group of GC patients, it was found that there was a statistical significance between the lymph node metastasis group ($P < 0.001$) and clinical staging group ($P = 0.008$); but no statistical significance was observed when compared with the other groups (Table 1 and Table 2).

HLA-G expression in gastric cancer cell lines and transfection in the MGC-803 cell line

Western blot and qPCR data demonstrated that HLA-G expression in MGC-803, BGC-823, MKN-28, MKN-45, SGC-7901 and AZ-521 cell lines were lower compared to positive control cell line JEG-3 ($P < 0.001$). However, no statistical significance was observed in various GC cell lines (Fig. 2A). Then, lentivirus was used to transfect the MGC-803 cell line to overexpress HLA-G. Western blot detection revealed that the expression of HLA-G in transfected MGC-803 cells was higher when compared to the control groups (blank and empty plasmid) ($P < 0.001$, Fig. 2B).

Fig. 2. HLA-G expression in gastric cancer cell lines, phenotype of tumor-infiltrating NK cells and the receptor expression in NK-92MI cells. (A) HLA-G expression in various gastric cancer lines was lower compared to positive control cell line JEG-3 by western-blot and qPCR ($***P < 0.001$ vs. positive control). (B) Western-blot analysis of HLA-G expression after transfection ($***P < 0.001$ vs. positive): Blank, MGC-803 alone; Negative, MGC-803 transfected with empty vector; Positive, MGC-803 transfected with HLA-G. (C) The percentage of tumor-infiltrating NK cells expressing ILT2 is higher compared to tumor-infiltrating NK cells expressing KIR2DL4 by flow cytometry assessment. (D, E) Expression of HLA-G receptors on NK-92MI cells analyzed by western-blot ($***P < 0.001$ vs. THP-1). (F) Western-blot analysis of ILT2 expression after transfection ($***P < 0.001$ vs. positive): Blank, NK-92MI alone; Negative, NK-92MI transfected with empty vector; Positive, NK-92MI transfected with ILT2.

The expression of receptors ILT2 and KIR2DL4 in tumor-infiltrating NK cells in gastric cancer patients and its transfection in the NK-92MI cell line

Flow cytometry was used to detect HLA-G inhibitory receptor ILT2 and KIR2DL4 on the surface of infiltrating NK cells in GC tissues. The results of the present study revealed that the positive rate of ILT2 on the cell surface was 89%, while the positive rate of KIR2DL4 was 8% (Fig. 2C). However, western blot results revealed that the expression of ILT2 and KIR2DL4 in the NK-92MI cell line was lower when compared to positive control cell line THP-1 ($P < 0.001$, Figs. 2D and 2E). In order to detect the function of ILT2 in NK cells, lentivirus was transfected into the NK-92MI cell line to overexpress ILT2. The expression of ILT2 in NK-92MI cells was higher compared to the control groups (blank and empty plasmid) after transfection ($P < 0.001$, Fig. 2F).

Fig. 3. HLA-G suppresses NK cell proliferation, cytotoxic activity and cytokine production in vitro and suppresses the anti-tumor effect of NK cells in vivo via ILT2 receptor. (A) Cell proliferation was measured by CCK8 assays (** $P < 0.01$, *** $P < 0.001$). (B) Cell cytotoxic activity was measured by lactate dehydrogenase (LDH)-release method (* $P < 0.05$, *** $P < 0.001$). (C, D) Concentration of IFN- γ and TNF- α were measured by ELISA kits (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (E, F) Tumor volume after NK cells injection in cancer xenograft model (** $P < 0.01$, *** $P < 0.001$). ILT2+, NK-92MI cell of overexpress ILT2; ILT2-, NK-92MI cell of low express ILT2; ILT2+ & anti G, NK-92MI cell of overexpress ILT2 plus HLA-G antibody; PBS, negative control.

HLA-G suppresses NK cell proliferation, cytotoxic activity and cytokine production via ILT2

These results show that with overexpression of HLA-G in the GC cell group, the proliferation rate, cytotoxic activity and cytokine production of NK cells with ILT2 overexpression were significantly lower than those of NK cells with low ILT2 expression. In the subgroup plus HLA-G antibody, the proliferation rate, cytotoxic activity and cytokine production of NK cells with overexpressed ILT2 were recovered ($P < 0.05$). In addition, in the GC cell group with HLA-G low expression, the difference in effect in each NK cell subgroup was not statistically significant (Figs. 3A, 3B, 3C and 3D).

HLA-G suppresses the anti-tumor effect of NK cells in vivo

The results of the cancer xenograft model revealed the size of tumors in the GC cell group with overexpressed HLA-G, which were as follows: small, in the subgroup injected with NK cells with low ILT2 expression ($P < 0.01$); medium, in the subgroup injected with NK cells with overexpressed ILT2 ($P < 0.01$); large, in the control subgroup (Fig. 3E). There was no significant difference in tumor size observed in the GC cell group with low HLA-G expression, when compared with the NK cell subgroup. In addition, both groups had significantly smaller tumor sizes, compared to the control subgroup ($P < 0.01$, Fig. 3F).

Discussion

In the present study, our results suggest that the expression of HLA-G was negatively correlated with the number of tumor-infiltrating NK cells in GC tissues for the first time. Our *in vitro* experiments demonstrate and confirm that HLA-G can inhibit NK cell proliferation and cytotoxic activity by binding with the ILT2 receptor, and reduce the production of IFN- γ and TNF- α . In addition, by using a mice animal model bearing a GC xenograft, we were able to prove that HLA-G can inhibit the anti-tumor effect of NK cells *via* the ILT2 receptor.

HLA-G plays an important role in tumor immune escape, and is an important immunosuppressive molecule. It was found that HLA-G is expressed in gastrointestinal tumors, and is closely correlated to poor cancer prognosis [38, 39]. In the present study, we observed that HLA-G was expressed in most GC tissues, and were closely correlated with the depth of tumor invasion, lymph node metastasis and clinical staging; which were consequently involved in GC development [40-42]. Moreover, several other studies have shown that HLA-G may be involved in the development of liver cancer, ovarian cancer and breast cancer [43-46]. In addition, we also found that the proportion of pre-operative anemia patients with HLA-G positive expression was higher compared to patients with HLA-G negative expression. Since pre-operative anemia was associated with tumor cachexia, this might suggest that HLA-G expression was closely related to the adverse prognosis of tumors. Furthermore, the OS and DFS data were similar to the results published in previous studies [40-42]. We also found that HLA-G expression was negatively correlated with the number of tumor-infiltrating NK cells in GC tissues for the first time, suggesting that HLA-G may inhibit the proliferation of NK cells. We further compared the number of infiltrating NK cells with different clinical characteristics, and confirmed that the reduction in tumor-infiltrating NK cells was associated with the poor prognosis of the tumor [10, 11, 13, 47]. In addition, in our initial hypothesis, we confirmed that HLA-G may inhibit the function of infiltrating NK cells to promote the development of GC.

HLA-G is considered as an important factor in inhibiting NK cell function [48-50]. At present, there are two types of HLA-G inhibitory receptors associated with NK cells: immunoglobulin-like transcripts 2, ILT2 (CD85j/LILRB1); killer cell immunoglobulin-like receptors 2DL4, KIR2DL4 (CD158d). ILT2 is mainly expressed in monocytes, macrophages, dendritic cells, B cells, and in some T cells and NK cells; while KIR2DL4 is mainly expressed in uterine NK cells [50, 51]. The intracellular domain of ILT2 contains four immunoreceptor tyrosine-type inhibition motifs (ITIMs), which can transmit inhibitory signals into cells and inhibit the activity of immune cells. Moreover, the affinity of ILT2 and HLA-G is significantly higher compared to KIR2DL4 [52, 53]. The intracellular fraction of KIR2DL4 contains only

one ITIM, but it also has an arginine residue that interacts with FcεRIγ, allowing it to possess both the inhibitory and structural features of the activated receptor, thereby making cell regulation more complex [54-56].

In order to further study the mechanism of HLA-G on NK cells, we identified the expression of HLA-G in six GC cell lines. We found that HLA-G was lower in all cell lines, which was consistent with previous studies [40]. Previous researches have demonstrated that HLA-G expression in ovarian cancer and melanoma decreased or even completely disappeared after long-term culture of fresh HLA-G positive tumor cells *in vitro* [57-60], suggesting that the expression of HLA-G in tumor cells is regulated by the tumor microenvironment [61]. In addition, by using flow cytometry, we found that tumor-infiltrating NK cells were mainly expressed in ILT2. Furthermore, western blot data revealed that ILT2 and KIR2DL4 were low in NK-92MI cells [62]. In order to simulate these *in vivo* conditions, we used lentivirus to transfect NK-92MI cells to overexpress ILT2, and the lentivirus transfection of MGC-803 cells to overexpress HLA-G. The results of the cell proliferation and cytotoxic activity suggest that HLA-G can inhibit the function of NK cells through ILT2 receptors, and the inhibitory role of HLA-G can be blocked by the HLA-G antibody, which was consistent with studies that investigated lymphoma and liver cancer [63, 64]. Similarly, we also observed that the overexpression of HLA-G can reduce the production of IFN-γ and TNF-α *via* ILT2, and that its role can also be blocked by the HLA-G antibody. TNF-α is important in inhibiting tumor growth in most tumors [65]. IFN-γ is mainly secreted by NK cells, which is relevant for inhibiting tumor growth in non-inflammatory-induced tumors [66]. Consequently, the reduction of cytokines with tumor killing may represent another mechanism by which HLA-G can exert its immunosuppression.

During the animal experiments, NOD/SCID mice with extremely low activity of self NK cells were used to establish a cancer xenograft model *in vivo*. We made this choice, because NOD/SCID mice have very low NK cell activity compared to nude mice, which is the ideal animal model for this kind of experiment. Briefly, our results revealed that in the GC group with overexpressed HLA-G, NK cells with low expression of ILT2 revealed an anti-tumor effect; and tumor size was significantly smaller compared to the control group. In addition, the anti-tumor effect of NK cells with high expression of ILT2 was partially inhibited, and the tumor size increased; but this was still smaller compared to the control group. Although, these *in vitro* experiments revealed that HLA-G could inhibit the function of NK cells by ILT2, the *in vivo* experiments revealed that NK cell function remained partially retained. The reason might be that these NK cells were also subjected to the regulation of complex environmental factors *in vivo*, and affected by other immune cells in mice. On the contrary, in the GC group with low HLA-G expression, NK cells exhibited an anti-tumor activity, regardless of the expression level of ILT2. This further suggests that HLA-G can inhibit the anti-tumor effect of NK cells by ILT2.

Conclusion

HLA-G plays an important role in the development, progression and adverse prognosis of GC; and its mechanism may be that it inhibits the function of infiltrating NK cells in GC. Our data demonstrates and re-confirms that HLA-G inhibits NK cell proliferation and cytotoxic activity through ILT2. Recent studies have considered HLA-G/ILT was an important immunological target, and that this is as important as B7/CTLA-4 and PD-L1/PD-1 in tumor immunotherapy [51, 67]. This study provides a theoretical basis for the participation of HLA-G in the immune escape of GC, and provides a new strategy for the immunotherapy of GC using HLA-G as the main target. However, further studies are required to clarify the specific molecular mechanism underlying the role of HLA-G in GC.

Disclosure Statement

The authors declare that they have no competing interests.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No.81272753) and National Key Clinical Specialties Construction Program of China (No [2012].649).

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