Published OnlineFirst September 19, 2018; DOI: 10.1158/0008-5472.CAN-18-1049

Tumor Biology and Immunology

Cancer Research

# Reduced CD160 Expression Contributes to Impaired NK-cell Function and Poor Clinical Outcomes in Patients with HCC

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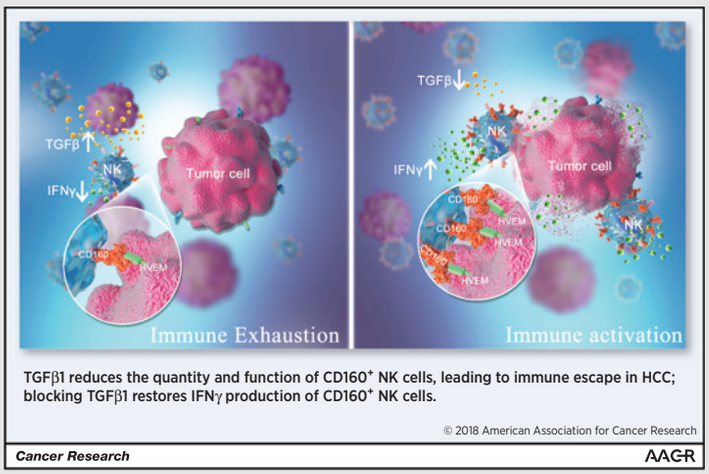
## Abstract

We previously reported that deficiencies in natural killer (NK)–cell number and function play an important role in the progression of hepatocellular carcinoma (HCC). However, the mechanisms underlying this phenomenon remain obscure. In this study, we analyzed the expression of CD160 on intrahepatic NK cells by evaluating peritumoral and intratumoral tissues of 279 patients with HCC and 20 healthy livers. We observed reduced expression of CD160 on intratumoral NK cells, and patients with lower CD160 cell densities within tumors exhibited worse disease and a higher recurrence rate. High-resolution microarray and gene set enrichment analysis of flow cytometry–sorted primary intrahepatic CD160$^+$ and CD160$^-$ NK cells of healthy livers indicated that human CD160$^+$ NK cells exhibited functional activation, high IFNγ production, and NK-mediated immunity. In addition, global transcriptomic analysis of sorted peritumoral and intratumoral CD160$^+$ NK cells revealed that intratumoral CD160$^+$ NK cells are more exhausted than peritumoral CD160$^+$ NK cells and produce less IFNγ. High levels of TGFβ1 interfered with production of IFNγ by CD160$^+$ NK cells, blocking of which specifically restored IFNγ production in CD160$^+$ NK cells to normal levels. These findings indicate that reduced numbers of CD160$^+$ NK cells, together with the functional impairment of CD160$^+$ NK cells by TGFβ1, contribute to tumor immune escape. In addition, restoring the expression of CD160 and blocking TGFβ1 appear a promising therapeutic strategy against liver cancer.




TGFβ1 reduces the quantity and function of CD160$^+$ NK cells, leading to immune escape in HCC; blocking TGFβ1 restores IFNγ production of CD160$^+$ NK cells.

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**Significance:** These findings show that reduced number and function of CD160$^+$ NK cells in the tumor microenvironment contributes to immune escape of HCC; blocking TGFβ1 restores IFNγ production of CD160$^+$ NK cells.

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**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corrected online March 25, 2019.

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**doi:** 10.1158/0008-5472.CAN-18-1049

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## Introduction

Immune checkpoint blockade has become a promising therapeutic approach to reverse immune cell exhaustion. Blocking antibodies targeting cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) and programmed death 1 (PD-1) on T cells have demonstrated great clinical success against an increasing number of human cancers. Recent studies have also revealed the potential roles of natural killer (NK) cells in immune exhaustion and attempted to discover new checkpoint proteins that might be involved in NK-cell exhaustion (1).

CD160 (also known as BY55) was discovered in 1993 by Bensussan and colleagues (2). The major form of CD160 is a glycosylphosphatidylinositol-anchored cell surface molecule with a single IgV-like domain that is weakly homologous to killer-inhibitory receptors (3). In humans, CD160 expresses on NK cells, NKT cells, γδ T cells, $CD8^+$ T cells, most intestinal intraepithelial T cells, and a small subset of $CD4^+$ T cells (2–5). CD160 shows a broad but low affinity to MHC class I molecule in both human and mice (6, 7). Human CD160 also binds to herpes virus entry mediator (HVEM) with a higher affinity than to MHC class I molecule that results in the inhibition of T-cell activation (8, 9). On the other hand, B- and T-lymphocyte attenuator (BTLA), LT-α, and LIGHT also bind to HVEM, in which CD160 needs to compete with BTLA for the binding of HVEM (10). A recent study reveals that human NK cells are specifically costimulated by HVEM-CD160 binding, but not by LIGHT, LT-α, or BTLA (11). HVEM enhances human NK-cell activation, resulting in an increased secretion of IFNγ and TNFα. The binding between CD160 on NK cells and HVEM on tumor cells boosts the cytolysis of target cells, whereas HVEM-BTLA binding reduces the cytolysis of target cells, indicating the role of HVEM as a functional regulator that activates NK cells through binding with CD160 while limits inflammation through binding with BTLA (11).

Engagement of CD160 with HLA-C also enhances the cytotoxicity of circulating NK cells (7, 12). In addition, unlike ubiquitously expressed NK-cell receptors, CD160 specifically expresses on NK cells with the most potent cytotoxic function (2), suggesting an important role of CD160 in NK-cell cytotoxicity and cytokine production. Furthermore, study using *CD160*$^{-/-}$ mice has shown that CD160 is essential for NK-mediated IFNγ production, and $CD160^+$ NK-cell sufficiency is necessary for controlling tumor growth in B16 melanoma and RMA-S lymphoma models (13). Clinical data have shown that tumor HVEM expression significantly correlates to the postoperative recurrence and survival of patients with liver cancer (14). However, little is known about human $CD160^+$ NK cells *in vivo*, especially in the liver, where the proportion of NK cells is more than 5 times higher than those in the peripheral blood or the spleen (15, 16).

The importance of NK cells in cancer immunity, especially in liver cancer, is underrated (17). Previous studies have suggested that NK cells in the intratumoral tissues (IT) of both patients with hepatocellular carcinoma (HCC) and murine models are functionally exhausted, and a positive correlation between NK-cell density and a better prognosis of patients with HCC has been established (18–21). In this study, by investigating 20 healthy livers and 235 paired IT and peritumoral tissue (PT) of patients with HCC, we evaluated the potential of CD160 as a novel indicator of NK-cell exhaustion and cancer prognosis. $CD160^+$ NK cells exhibited an activated phenotype and produced more IFNγ; however, its cumulative percentage, absolute number, and mean fluorescence intensity (MFI) were all significantly reduced in the IT of HCC tissues. Furthermore, global transcriptomic analysis of sorted IT and PT $CD160^+$ NK cells demonstrated that IT $CD160^+$ NK cells are more exhausted and produce less IFNγ when comparing with PT $CD160^+$ NK cells. High level of TGFβ1 in the tumor microenvironment inhibited IFNγ production of $CD160^+$ NK cells, and blocking TGFβ1 may restore IFNγ production of $CD160^+$ NK cells. Finally, patients with reduced intratumoral CD160 expression were accompanied by late tumor–node–metastasis (TNM) stage, tumor metastasis, and poorer outcome. These findings provide the first description of human intrahepatic $CD160^+$ NK cells and indicate the existence of a new immune escape mechanism through the negative regulation of NK cells by manipulating CD160 expression in patients with HCC.

## Patients and Methods

### Patients

Liver tumor tissue specimens from 235 patients with HCC who had undergone curative resection between 2006 and 2010 (Cohort 1) were obtained from the Bank of Tumor Resources at Sun Yat-Sen University. Fresh tumor tissue samples were obtained from 44 patients with HCC during surgery at Department of Hepatobiliary Surgery of The First Affiliated Hospital of University of Science and Technology of China and First Affiliated Hospital of Xinjiang Medical University (Cohort 2). Among these samples, 39 were paired PT (collected 2 cm distal to the tumor site) and IT of the same patient. Peripheral blood samples from healthy controls (HC) and patients with HBV infection, liver cirrhosis (LC), or HCC were obtained from First Affiliated Hospital of Anhui Medical University. Normal liver tissues ($N = 20$) collected distal to liver echinococcosis were obtained from First Affiliated Hospital of Xinjiang Medical University. Pilot studies were conducted to ensure sample sizes are large enough to detect the effects. The clinical characteristics of all tissue samples from patients with HCC are summarized in Supplementary Table S1. Univariate analysis of disease-free survival (DFS) and overall survival (OS) of patients in cohort 1 is shown in Supplementary Table S2. The etiology of all patients with primary HCC includes viral infection, LC, and alcoholic fatty liver. The number of samples used in each experiment and the details of PT/IT availability of each patient are provided in Supplementary Table S3. The details of all patients are provided in Supplementary Table S4. All samples were anonymously coded in accordance with the Helsinki Declaration. Written-informed consent was obtained from each patient included in the study, and the protocols of all study cohorts were approved by the Ethical Board of the Institutional Review Board of the University of Science and Technology of China.

### Immunohistochemistry

Paraffin sections were dewaxed in xylene and rehydrated with distilled water. Following incubation with antibodies against human CD160 (ab202845; Abcam), adjacent sections were stained with DAB Peroxidase Substrate Kit (SK-4100; Vector Laboratories). Positive and negative controls were tested before



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formal staining. Pilot studies were conducted to ensure sample sizes are large enough to detect the effects. The integrated optical density (IOD) was quantified using ImagePro Plus software (Media Cybernetics) in a blinded manner as previously described (20, 22).

### Flow cytometry

Peripheral leukocytes were isolated via Ficoll–Isopaque (Solarbio) gradient centrifugation. Liver tissue–infiltrating lymphocytes were obtained as previously described (20). The peripheral lymphocytes, liver-infiltrating lymphocytes, and sorted NK cells from the *in vitro* cultures were stained with fluorochrome-conjugated Abs and then analyzed through flow cytometry. Antibodies against the following proteins were used for staining: CD3 (SK7), CD56 (B159), CD16 (3G8), CD160 (BY55), CD226 (DX11), NKp30 (p30-15), NKp44 (p44-8), NKp46 (9E2), NKG2D (1D11), CD244 (2–69), CTLA-4 (BNI3), IFNγ (B27), CD107a (H4A3), Granzyme B (GB11), Perforin (δG9; BD PharMingen); BTLA (4D6), LAG3 (17B4; Abcam); TIGIT (MBSA43), CD96 (NK9.39; eBioscience); NKG2A (131411), TIM3 (344823; R&D systems). The stained cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson), and the data were analyzed using FlowJo analysis software 7.6.1 (Treestar).

### Gene expression profiling analysis

Purified NK cells from human liver tissues were first enriched by MACS using the NK Cell Isolation Kit (MiltenyiBiotec), and $CD160^{+/-}$ hepatic NK cells were isolated by FACS Aria cell sorter (BD Biosciences) to attain a purity greater than 95%. For analyzing the molecular signatures of human $CD160^{+/-}$ NK cells or PT/IT $CD160^{+}$ NK cells, purified $CD160^{+/-}$ NK cells or PT/IT $CD160^{+}$ NK cells (three healthy donors were pooled for each cell type) were submitted for microarray analysis using the Whole Human Genome Microarray Kit (G4112F, Agilent Technologies). Transcription profile chip service was provided by Shanghai Biotechnology Cooperation. Microarray image analysis was performed using Agilent's Feature-Extraction V9.1.3 software (Agilent Technologies). Expression values were $\log_2$-transformed, and subsequent analyses were conducted using SAS statistical software online (http://www.ebioservice.com/). The microarray data were deposited into the National Center for Biotechnology Information GEO repository under accession numbers GSE109197 and GSE118114.

### *In vitro* NK-cell culture system

Purified NK cells were enriched from whole blood via negative selection (NK Cell Isolation Kit, MiltenyiBiotec). $CD160^{+/-}$ peripheral NK cells were isolated by FACS Aria cell sorter (BD Biosciences) to attain a purity greater than 95%. The cells were incubated in medium alone or in medium with recombinant TGFβ1 (1 ng/mL; PeproTech) or IL10 (10 ng/mL; PeproTech) combined with IL15 (10 ng/mL; PeproTech) and IL2 (100 U/mL). In another culture model, NK cells were cultured in the presence of 20% HCC patient plasma with or without anti-human TGFβ1-neutralizing antibody (Clone 27235, R&D Systems) or control IgG (BD Biosciences) for 72 hours.

### The Cancer Genome Atlas database

A database (https://cancergenome.nih.gov/), the Cancer Genome Atlas (TCGA), has generated comprehensive, multidimensional maps of the key genomic changes in 33 types of cancer. In our study, 373 HCC samples with detailed CD160 gene expression data were selected from the updated TCGA database (Raw data at the NCI; source mutation data from GDAC Firehose). Patients with fully characterized tumors, intact DFS and OS data, complete RNA-seq information, and those without pretreatment were included. We used this database to explore the prognostic value of CD160 gene in patients with HCC as previous described (23, 24).

### Statistical analysis

Significant differences between two unpaired groups were determined by either the Mann–Whitney test or unpaired *t* test. Significant differences between two paired groups were determined by either the Wilcoxon matched-pairs signed rank test or paired *t* test. Significant differences between three or more groups were determined by Kruskal–Wallis ANOVA followed by Dunn multiple comparisons test. Results are expressed as mean ± SEM. Simple correlations were summarized using the Pearson correlation coefficient or the Spearman correlation coefficient (*r*). The Kaplan–Meier analysis and the Gehan–Breslow–Wilcoxon test were used to analyze the DFS and OS of patients with cancer. Univariate analyses of the prognostic factors for DFS and OS were performed with the Cox proportional hazards model (SPSS statistics software 22.0, IBM). A *P* value of less than or equal to 0.05 was selected as the level of significance in all analyses (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$).

## Results

### Intratumoral CD160 expression reduces in NK cells of patients with HCC, but not in $CD8^{+}$ T cells

Previously, we have reported NK-cell dysfunction in the IT of patients with HCC. To investigate CD160 expression on intrahepatic NK cells, we analyzed liver-infiltrating lymphocytes in HCs as well as IT and PT of patients with HCC. The cumulative percentage of intratumoral $CD160^{+}$ NK cells was significantly reduced comparing with that of peritumoral or healthy NK cells (Fig. 1A and B), which became significantly evident when comparing paired PT and IT of each patient individually (Fig. 1C). The absolute number of $CD160^{+}$ NK cells was gradually reduced along HC, PT, and IT, and the number in IT was significantly lower than either in HC or PT (Fig. 1D). In addition, the MFI of CD160 illustrated as the ratio of IT:PT was smaller than 1, indicating a lower level of IT CD160 comparing with PT CD160 (Fig. 1E). The cumulative percentages of $BTLA^{+}$ NK cells in PT and IT were significantly higher than that in HC; however, no obvious differences were observed between PT and IT NK cells in terms of both absolute number and percentage (Fig. 1A–D). Furthermore, the MFI of BTLA illustrated as the ratio of IT:PT was larger than 1, indicating a relatively similar level of BTLA in IT and PT (Fig. 1E). Based on the integration of CD160 gene expression in the IT of 373 patients with HCC and PT of 50 patients with HCC from TCGA database, we verified significant reduction of CD160 gene expression in IT (Fig. 1F). Given that CD160 and BTLA also express on $CD8^{+}$ T cells, we then analyzed the expression of CD160 and BTLA on intratumoral $CD8^{+}$ T cells of patients with HCC; however, no significant differences were found in terms of cumulative percentage or absolute number between PT and IT (Supplementary Fig. S1A–S1D). These results suggest that reduced CD160 expression is confined to intratumoral NK cells but not to intratumoral $CD8^{+}$ T cells.



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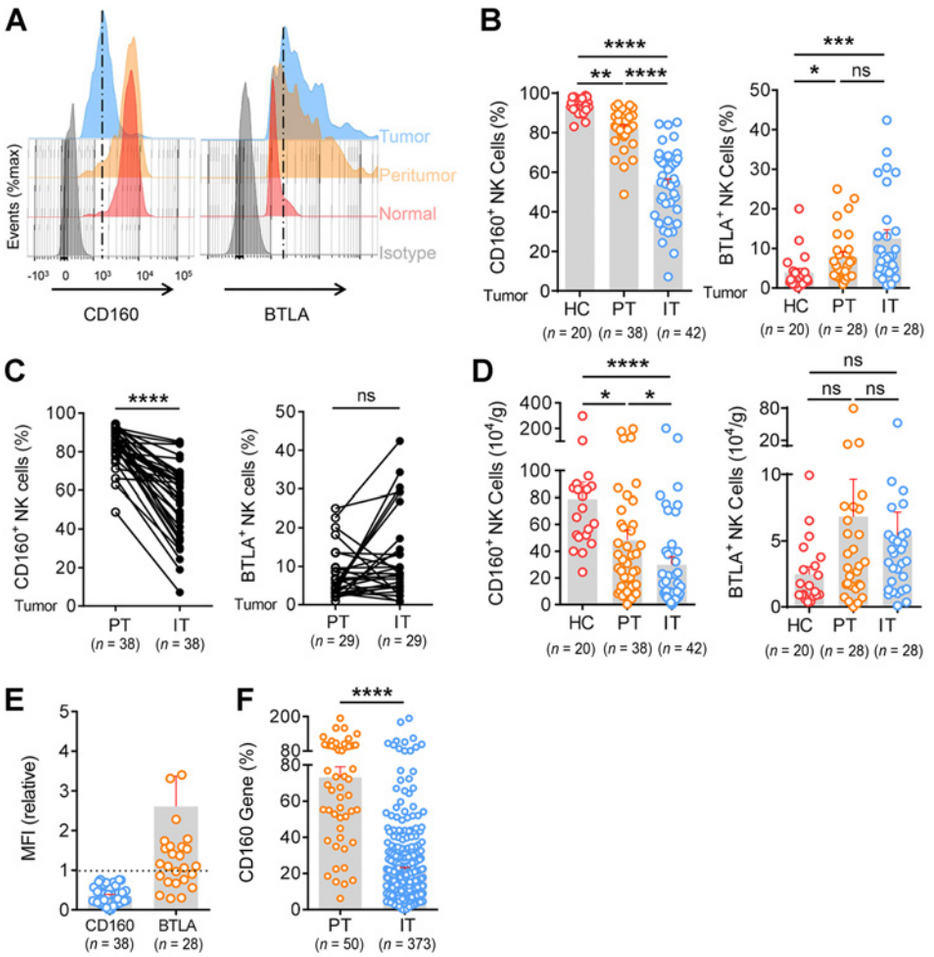


**Figure 1.**
The percentage, absolute number, and MFI of CD160$^+$ NK cells are reduced in the IT of patients with HCC. **A,** The histogram corresponds to cumulative CD160 (left) or BTLA (right) percentage on total CD3$^-$CD56$^+$ NK cells within the lymphocyte gate from a representative normal liver, PT, and IT of patient with HCC. **B,** Cumulative percentage of CD160$^+$ (left) or BTLA$^+$ (right) NK cells in healthy liver, PT, and IT of patients with HCC (Kruskal–Wallis ANOVA, followed by Dunn multiple comparisons test). **C,** Cumulative percentage of CD160$^+$ (left) or BTLA$^+$ (right) NK cells in paired PT and IT of each patient with HCC (Wilcoxon matched-pairs signed rank test). **D,** Absolute count of CD160$^+$ (left) or BTLA$^+$ (right) NK cells in healthy liver, PT, and IT of patients with HCC (Kruskal–Wallis ANOVA, followed by Dunn multiple comparisons test). **E,** The MFI fold change of CD160 (left) or BTLA (right) on intratumoral NK cells is presented relative to that of paired PT from each patient. **F,** CD160 gene expression level in PT and IT of patients with HCC selected from the TCGA database (unpaired *t* test). The results are expressed as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, nonsignificant.

### Comparison between CD160$^+$ and CD160$^-$ NK-cell subsets reveals an activating phenotype and function of CD160$^+$ NK cells

Significant negative correlation was found between the cumulative percentages of CD160$^+$ NK cells and TIM-3$^+$, CD96$^+$, or NKG2A$^+$ NK cells (Supplementary Fig. S2A), whereas significant positive correlation was noted between the percentage of CD160$^+$ NK cells and their intracellular IFNγ, granzyme B, or perforin production (Supplementary Fig. S2B), suggesting a critical role of CD160$^+$ NK cells in the positive regulation of immune responses. In order to investigate the unique properties of CD160$^+$ NK cells and distinguish them from CD160$^-$ NK cells, we purified NK cells from whole blood via negative selection and sorted CD160$^+$ and CD160$^-$ NK cells. Through flow cytometry, we found significant differences between two NK-cell subsets in terms of phenotypic markers and cytokine secretions. CD160$^+$ NK cells expressed significant higher levels of activating receptors such as NKp30, NKp44, NKp46, NKG2D, and CD244 (Fig. 2A), whereas lower levels of inhibitory receptors such as BTLA and CTLA-4 (Fig. 2B). Interestingly, CD160$^+$ NK cells also expressed higher level of TIGIT comparing with CD160$^-$ NK cells (Fig. 2B). Furthermore, significantly higher level of IFNγ was found in CD160$^+$ NK cells, suggesting an activating phenotype and function of peripheral CD160$^+$ NK cells (Fig. 2C; Supplementary Fig. S3A).

To better define primary CD160$^+$ and CD160$^-$ NK cells from the human liver, we subsequently isolated primary hepatic lymphocytes from healthy livers and purified CD160$^+$ and CD160$^-$ NK cells through negative selection and flow cytometry sorting. By using high-resolution microarrays, we identified profound and wide-ranging differences between CD160$^+$ and CD160$^-$ NK cells; a greater than 2-fold change was found in 1,931 expressed genes (Fig. 2D; Supplementary Fig. S3B). These 1,931 genes fell into 8 functional categories: immune response, innate immune response, adaptive immune response, inflammatory response, chemotaxis, apoptotic process, cell adhesion, and signal transduction (Supplementary Fig. S3C). In addition, these genes were analyzed in Cytoscape to create a pathway enrichment network illustrating the overall representation of biological pathways relatively dominated by downregulated (green) or upregulated (red) genes (Supplementary Fig. S3D). A quantitative analysis of gene expression differences revealed that molecules associated with NK-cell activation, such as NKp46 (*Ncr1*), NKp30 (*Ncr3*), NKp80 (*KLRF1*), CD69 (*Cd69*), CD226 (*Cd226*), CD244 (*Cd244*), KLRG1 (*Klrg1*), and NTBA (*SLAMF6*), are upregulated in CD160$^+$ NK cells (Fig. 2E). Inhibitory-related molecules, such as LAG-3 (*Lag3*), PD-1



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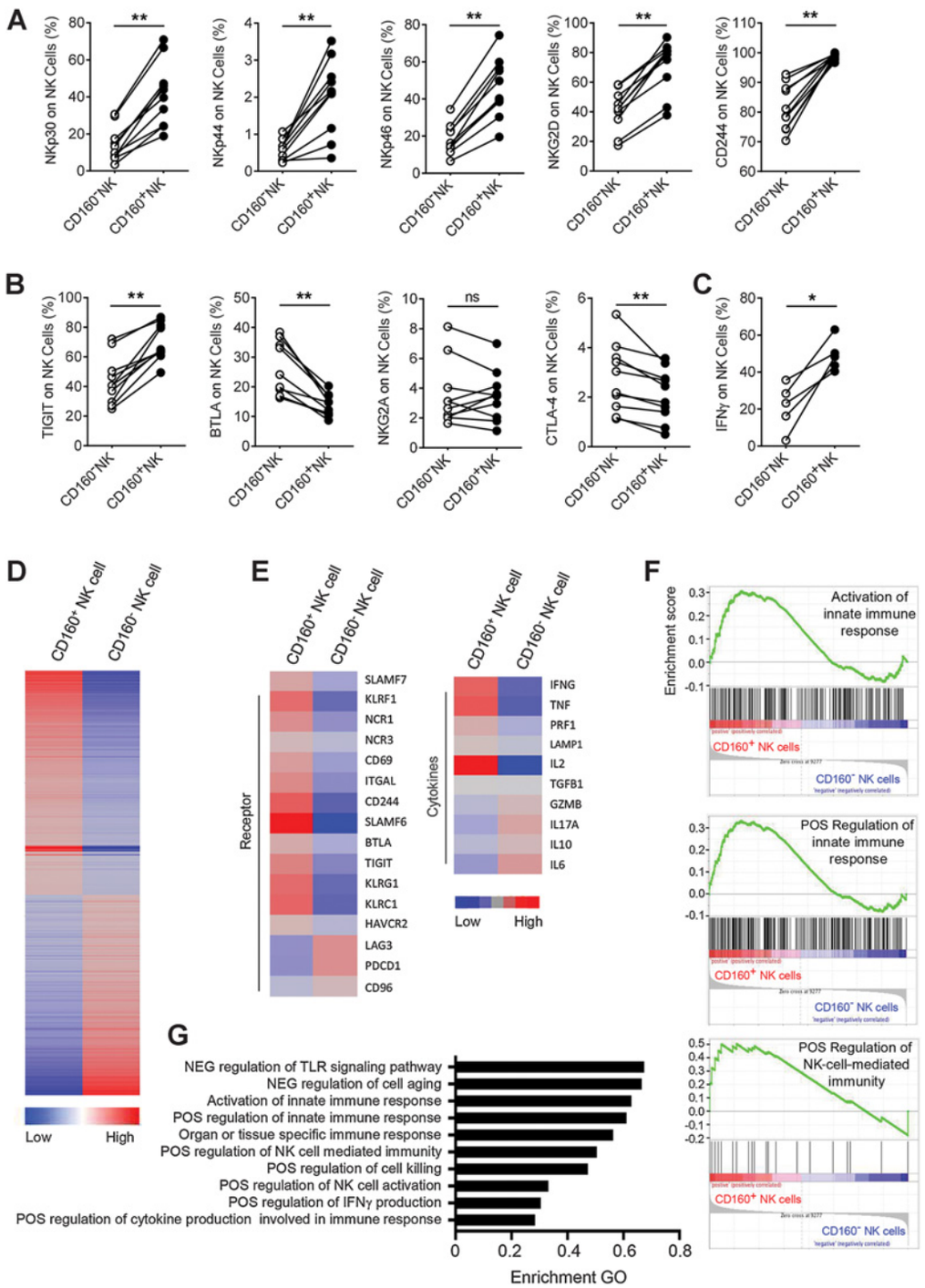


**Figure 2.**

Comparison between CD160$^+$ and CD160$^-$ NK-cell subsets. **A,** Cumulative percentage of activating receptors including NKp30, NKp44, NKp46, NKG2D, and CD244 on sorted peripheral CD160$^+$ and CD160$^-$ NK-cell subsets (Wilcoxon matched-pairs signed rank test). **B,** Cumulative percentage of inhibitory receptors including TIGIT, BTLA, NKG2A, and CTLA-4 on sorted peripheral CD160$^+$ and CD160$^-$ NK-cell subsets (Wilcoxon matched-pairs signed rank test). **C,** Cumulative percentage of IFNγ on sorted peripheral CD160$^+$ and CD160$^-$ NK-cell subsets (Wilcoxon matched-pairs signed rank test). The results are expressed as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ns, nonsignificant. **D,** Heat map of mRNA transcripts that are up- (red) or downregulated (blue) in the intrahepatic CD160$^+$ and CD160$^-$ NK-cell subsets, as determined by MEV 4.9 software. Heat maps show signal values of the listed genes from -1.0 to 1.0 on a $\log_2$ scale. **E,** Representative receptor and cytokine profiles of CD160$^+$ and CD160$^-$ NK cells. Heat maps show signal values of the listed genes from −0.2 to 0.2 on a $\log_2$ scale for receptor profile and from −0.5 to 0.5 on a $\log_2$ scale for cytokine profile. **F,** GSEA plot of the activation of innate immune response (top), positive regulation of innate immune response (middle), or positive regulation of NK-cell–mediated immunity (bottom) gene signatures in CD160$^+$ NK cells relative to CD160$^-$ NK cells. **G,** GSEA plot of hallmark gene sets from the Molecular Signatures Database of the Broad Institute, showing the most significantly enriched gene sets in CD160$^+$ NK cells and their normalized enrichment scores. GO term IDs used in the figure include 0034122, 0090344, 0002218, 0045089, 0002251, 0002717, 0031343, 0032816, 0032729, and 0002720.



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(*Pdcd1*), and CD96 (*Cd96*), were downregulated in CD160$^+$ NK cells (Fig. 2E). Moreover, CD160$^+$ NK cells highly expressed IFNγ (*IFNG*), TNFα (*TNF*), IL2 (*Il2*), and perforin (*Prf1*; Fig. 2E; Supplementary Fig. S3E and S3F). Furthermore, gene set enrichment analysis (GSEA) revealed that the most highly enriched gene sets in CD160$^+$ NK cells overlapped with published gene signatures for activation of the innate immune response, positive regulation of the innate immune response, positive regulation of NK-cell–mediated immunity, IL2-stimulated NK cells, IL15-stimulated NK cells, and CD57$^-$ NK cells, suggesting that CD160$^+$ NK cells belong to a positively regulated NK-cell subset (Fig. 2F; Supplementary Fig. S3G). GSEA of hallmark gene sets from the Molecular Signatures Database of the Broad Institute showed that most significantly enriched gene sets in CD160$^+$ NK cells are related to tissue-specific immune response, negative regulation of TLR signaling pathway and cell aging, positive regulation of NK-cell–mediated immunity, and NK-cell activation (Fig. 2G). These data suggest that CD160 is highly related to the activation and positive regulation of intrahepatic NK cells.

**Intratumoral CD160$^+$ NK cells are more exhausted compared with their PT counterparts**

A thoroughly comparison between CD160$^+$ and CD160$^-$ NK cell subsets has suggested a possible role of CD160 in the activation and positive regulation of NK cells; however, whether the tumor microenvironment has an effect on their expression remains obscured. We isolated primary hepatic lymphocytes from PT and IT of patients with HCC, respectively and purified PT and IT CD160$^+$ NK cells through negative selection and flow cytometry sorting. High-resolution microarrays revealed certain differences between PT and IT CD160$^+$ NK cells (Fig. 3A). A quantitative analysis of gene expression differences showed that genes associated with NK-cell exhaustion, such as LAG-3 (*Lag3*), PD-1 (*Pdcd1*), TIGIT (*Tigit*), and BTLA (*BTLA*), are upregulated in IT CD160$^+$ NK cells whereas downregulated in PT CD160$^+$ NK cells (Fig. 3B). Moreover, IT CD160$^+$ NK cells lowly expressed IFNγ (*IFNG*) while highly expressed TNFα (*TNF*), IL2 (*Il2*), TGFβ (*TGFB1*), and IL10 (*Il10*; Fig. 3B). Level of IFNγ was also significantly reduced in IT CD160$^+$ NK cells comparing with paired PT CD160$^+$ NK

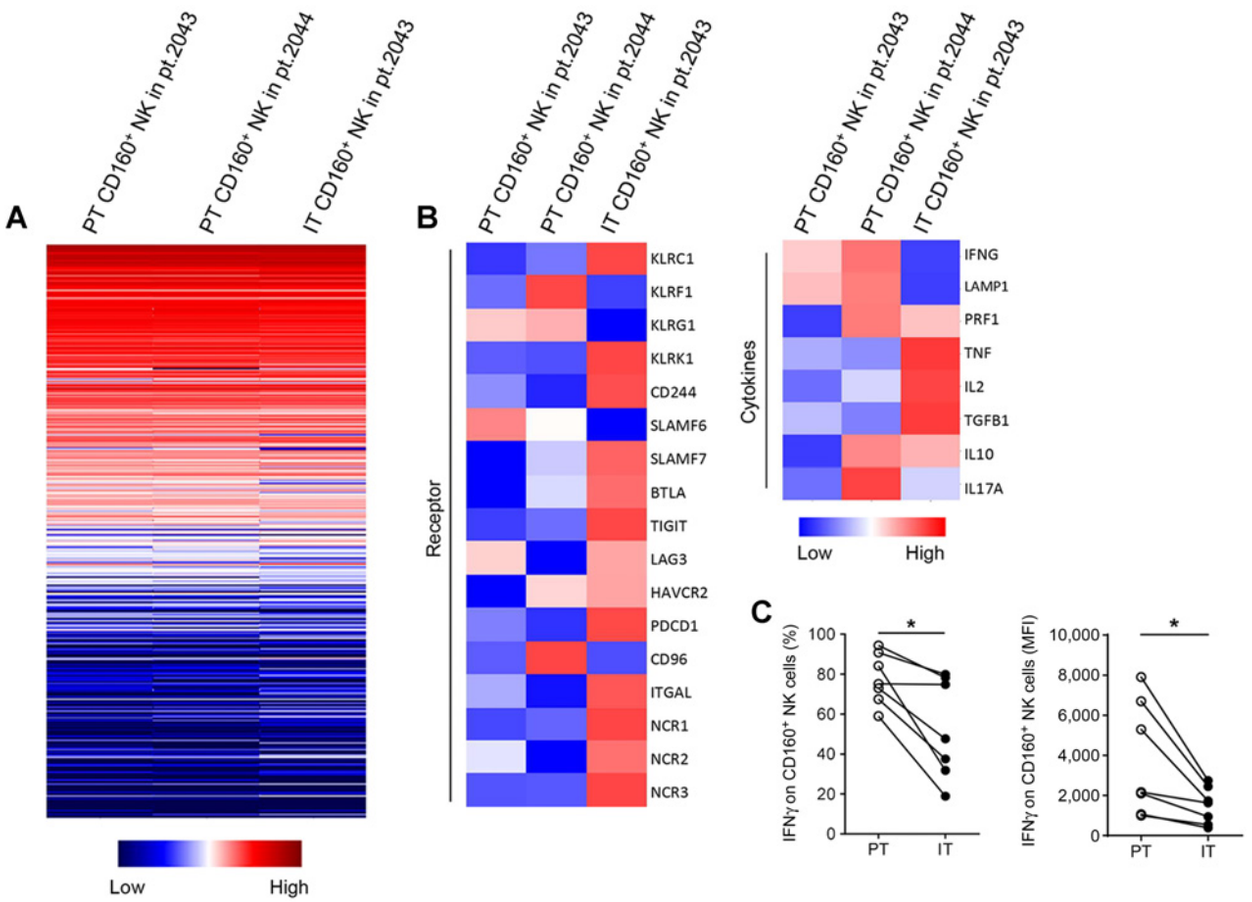


**Figure 3.**

Comparison between peritumoral and intratumoral CD160$^+$ NK cells. **A,** Heat map of mRNA transcripts that are up- (red) or downregulated (blue) in peritumoral and intratumoral CD160$^+$ NK-cell subsets, as determined by MEV 4.9 software. Heat maps show signal values of the listed genes from 0.0 to 4.0 on a $\log_2$ scale. **B,** Representative cytokine and receptor profiles of peritumoral and intratumoral CD160$^+$ NK cells. Heat maps show signal values of the listed genes from −1.5 to 1.5 on a *z*-score scale for cytokine profile and from −1.0 to 1.5 on a *z*-score scale for receptor profile. **C,** Percentage (left) and MFI (right) of IFNγ in CD160$^+$ NK cells from paired PT and IT of each patient with HCC (Wilcoxon matched-pairs signed rank test). *, $P < 0.05$.



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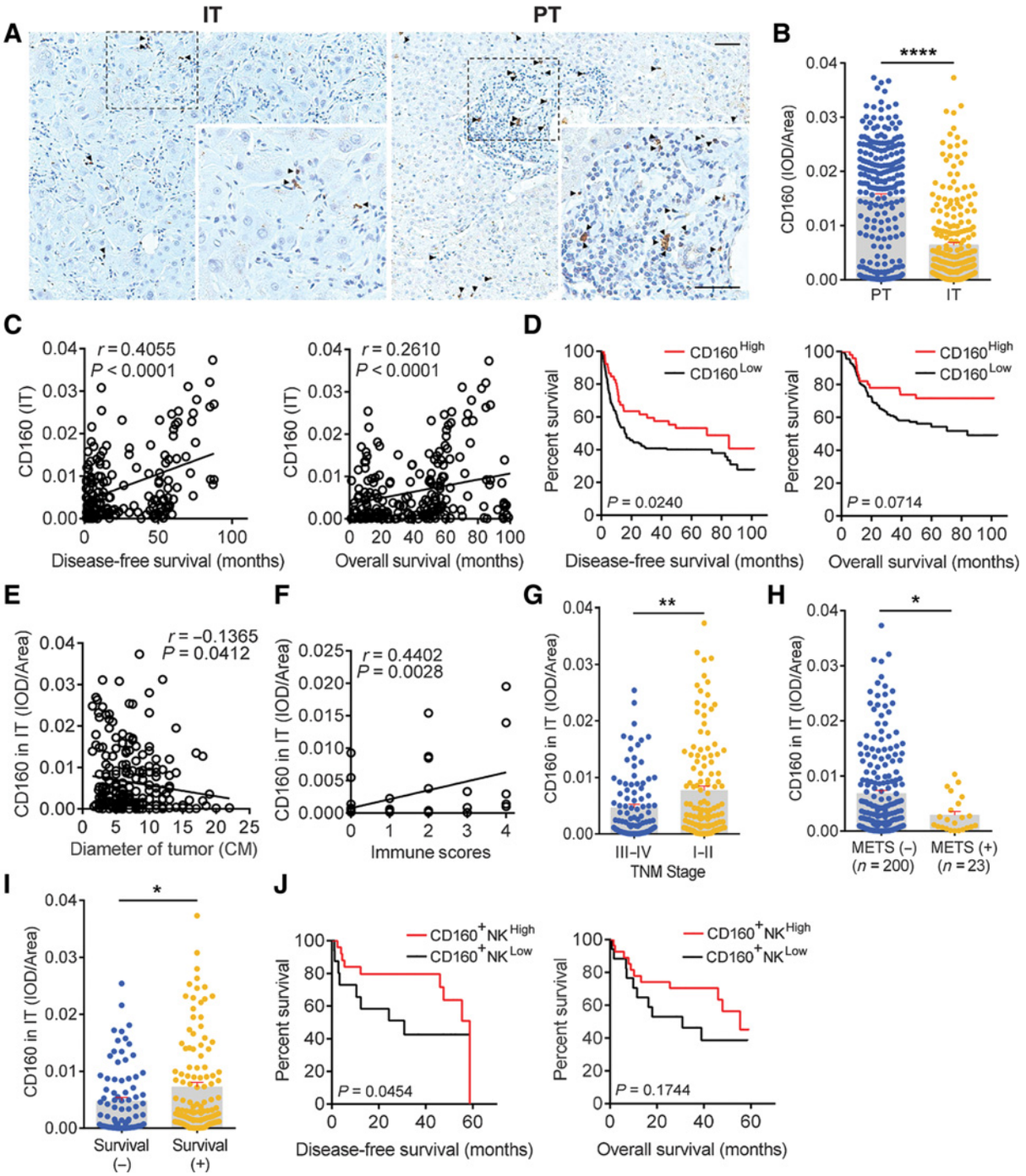


**Figure 4.**

Patients with higher CD160$^+$ cell density within tumor are associated with longer DFS and lower metastasis rate. **A,** Representative micrographs showing CD160$^+$ cells in the IT (left) and PT (right) of patient with HCC. Original magnifications, ×10 and ×40. Bar, 50 μm. **B,** Cumulative IOD/area of CD160 in IT and PT of HCC tissues ($N = 235$; unpaired $t$ test). **C,** Correlation between intratumoral IOD/area of CD160 and DFS (left) or OS (right) of patients with HCC ($N = 235$). Pearson correlation coefficients ($r$) and $P$ values are shown. **D,** Kaplan–Meier survival curve for the duration of DFS (left) or OS (right) in months, according to the density (IOD/area) of CD160 in IT samples (high densities, red line; low densities, black line; Gehan–Breslow–Wilcoxon test). Correlation between intratumoral CD160 density and tumor diameter (**E**) or immune score (**F**). Pearson or Spearman correlation coefficients ($r$) and $P$ values are shown. The IOD/area of CD160 analyzed from IT was used to categorize patients into two groups based on the TNM stage (**G**), the presence/absence of metastases (**H**), or live/death of patients with HCC (Mann–Whitney test; **I**). The results are expressed as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$. **J,** Kaplan–Meier survival curve for the duration of DFS (left) or OS (right) in months, according to the percentage of CD160$^+$ NK cells in IT samples (high densities, red line; low densities, black line; Gehan–Breslow–Wilcoxon test).



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cells on the protein level (Fig. 3C). These data suggest that IT CD160$^+$ NK cells exhibit a more exhausted phenotype when comparing with PT CD160$^+$ NK cells.

### Intratumoral CD160 expression positively associates to a better outcome of patients with HCC

Previous studies have reported that patients with HCC with fewer NK cells are associated to a poorer clinical outcome. To evaluate the potential influence of reduced IT CD160$^+$ NK cells on the outcome of patients with HCC, we analyzed the expression of CD160$^+$ cells in tumor tissues of 235 patients with HCC. As previously described (22, 25), IOD/area has been used to quantitatively analyze the intensity of CD160 through immunohistochemistry. Consistent with results in Fig. 1, CD160 expression was significantly reduced in IT (Fig. 4A and B). Significant positive correlation was observed between the intratumoral intensity of CD160 and DFS ($r = 0.4055$, $P < 0.0001$) or OS ($r = 0.2610$, $P = 0.0003$; Fig. 4C); however, no significant correlation was found between peritumoral CD160 intensity and DFS or OS (Supplementary Fig. S4A). To further assess the predictive potential of intratumoral CD160$^+$ cells, patients were divided into two groups based on the minimum *P* value cutoff value of their densities. The survival curves showed that patients with higher intratumoral CD160 density are correlated to longer DFS ($P = 0.0240$; Fig. 4D) and smaller tumor ($P = 0.0412$; Fig. 4E). In addition, patients with higher immune scores, earlier TNM stages, no metastasis, or OS also exhibited higher intratumoral CD160 density (Fig. 4F–I), suggesting that intratumoral CD160$^+$ cells may play a predictive role in the outcome of HCC. Cox regression and time-to-event outcome analyses indicated that TNM staging, tumor number, and the occurrence of tumor thrombus significantly influence DFS and OS ($P < 0.05$ for all comparisons in Supplementary Table S2). Further analyses showed that intratumoral CD160 density strongly influences DFS and OS ($P = 0.0084$, HR = 0.97 for DFS; $P = 0.0111$, HR = 0.96 for OS; Supplementary Table S2). In addition to intratumoral CD160$^+$ cells, we have also assessed the role of intratumoral CD160$^+$ NK cells in the prediction of HCC outcomes. The survival curves showed that patients with higher cumulative percentage of intratumoral CD160$^+$ NK cells are also correlated to longer DFS ($P = 0.0454$; Fig. 4J). Comparisons between the cumulative percentages of peripheral CD160$^+$ NK cells in HCs and patients with chronic HBV infection (CHB), LC, and HCC demonstrated a significantly higher percentage of CD160$^+$ NK cells in the blood of HCs comparing with that in patients with CHB, LC, or HCC; however, no significant difference in CD160 percentage was observed between patients with CHB, LC, and HCC (Supplementary Fig. S4B and S4C). Together, these results indicate that intratumoral CD160 density as well as the percentage of intratumoral CD160$^+$ NK cells may act as a prognostic marker in predicting DFS of patients with HCC. Patients with higher intratumoral CD160 density are often accompanied by a better disease condition and lower recurrence rate.

### High TGFβ level suppresses IFNγ production by CD160$^+$ NK cells

Previous studies have shown that levels of IL10 and TGFβ are highly elevated in patients with HCC (26–28). In our study, we showed that the expression of TGFβ in IT is significantly higher than that in PT of patients with HCC (Fig. 5A and B). Furthermore, the density of TGFβ in tissues was negatively correlated to the cumulative percentage of CD160$^+$ NK cells (Fig. 5C) or CD160$^+$ IFNγ$^+$ NK cells (Fig. 5D). To evaluate the possibility that altered CD160 expression is caused by IL10 or TGFβ1, we preincubated healthy NK cells with 10 ng/mL IL10, 1 ng/mL TGFβ1, or HCC patient plasma for 72 hours. No significant differences in terms of cumulative percentage and MFI were observed between control NK cells and NK cells preincubated with IL10, TGFβ1, or HCC patient plasma (Supplementary Fig. S5A). On the other hand, the cumulative percentage and MFI of CD160$^+$ IFNγ$^+$ NK cells were both significantly downregulated by the presence of exogenous TGFβ1 or HCC patient plasma (Fig. 5E); however, the presence of exogenous TGFβ1 or HCC patient plasma had no effect on CD160$^-$IFNγ$^+$ NK cells (Fig. 5F). In order to exclude the effect of other cells in this comprehensive microenvironment, we sorted CD160$^+$ and CD160$^-$ NK cells and then preincubated them with 10 ng/mL IL10, 1 ng/mL TGFβ1, or HCC patient plasma for 72 hours. The cumulative percentage and MFI of IFNγ on CD160$^+$ NK cells were both significantly downregulated by the presence of exogenous TGFβ1 or HCC patient plasma (Fig. 5G); however, the presence of exogenous TGFβ1 or HCC patient plasma had no effect on the level of IFNγ of CD160$^-$ NK cells (Fig. 5H). More importantly, treatment with anti-TGFβ1 Abs partially restored IFNγ production of TGFβ1-incubated or HCC plasma–incubated CD160$^+$ NK cells (Fig. 5E and G), indicating a direct effect of TGFβ1, either directly given or indirectly given through HCC plasma, on CD160$^+$ NK cells. One thing to note is that CD160 expression on healthy NK cells reduced gradually during the *in vitro* culturing, indicating that such *in vitro* alterations may mask and weaken the effect of TGFβ1 on CD160 expression in NK cells (Supplementary Fig. S5B).

### The reduction of CD160 expression occurs in both CD56$^{bright}$ and CD56$^{dim}$ NK-cell subsets

NK cells are divided into CD56$^{bright}$ and CD56$^{dim}$ subsets. CD56$^{dim}$ NK cells are primarily responsible for cytotoxicity, whereas CD56$^{bright}$ NK cells are mainly responsible for cytokine secretion. To narrow down the specific NK-cell subset that exhibits reduced CD160 expression, we analyzed the expression of CD160 in different subsets of NK cells of patients with HCC. The cumulative data showed that the percentages of CD160 were significantly decreased in both CD56$^{bright}$ and CD56$^{dim}$ NK cells from IT compared with those from HC and PT (Fig. 6A and B). As expected, the difference became even more evident when comparing paired PT and IT of each patient individually (Fig. 6C). Furthermore, the absolute count of CD160-expressing CD56$^{bright}$ NK cells in IT ($15.3 \pm 30.1 \times 10^3$/g) was significantly lower than that in PT ($28.1 \pm 31.7 \times 10^3$/g), a phenomenon that was not observed in CD56$^{dim}$ NK cells (Fig. 6D). In addition, the intratumoral CD160 MFI was lower than peritumoral CD160 MFI in both NK cell subsets (IT:PT ratio < 1), indicating reduced CD160 levels in both CD56$^{bright}$ and CD56$^{dim}$ NK-cell subsets (Fig. 6E).

## Discussion

Discovery and usage of immune checkpoint blockade have led to a new era of immunotherapy. Previous findings have concentrated more on the checkpoints of T cells and lacked sufficient attention to NK cells; however, accumulating evidence has suggested a positive correlation between NK-cell number and tumor patient outcome, indicating an irreplaceable role of NK cells (29, 30).



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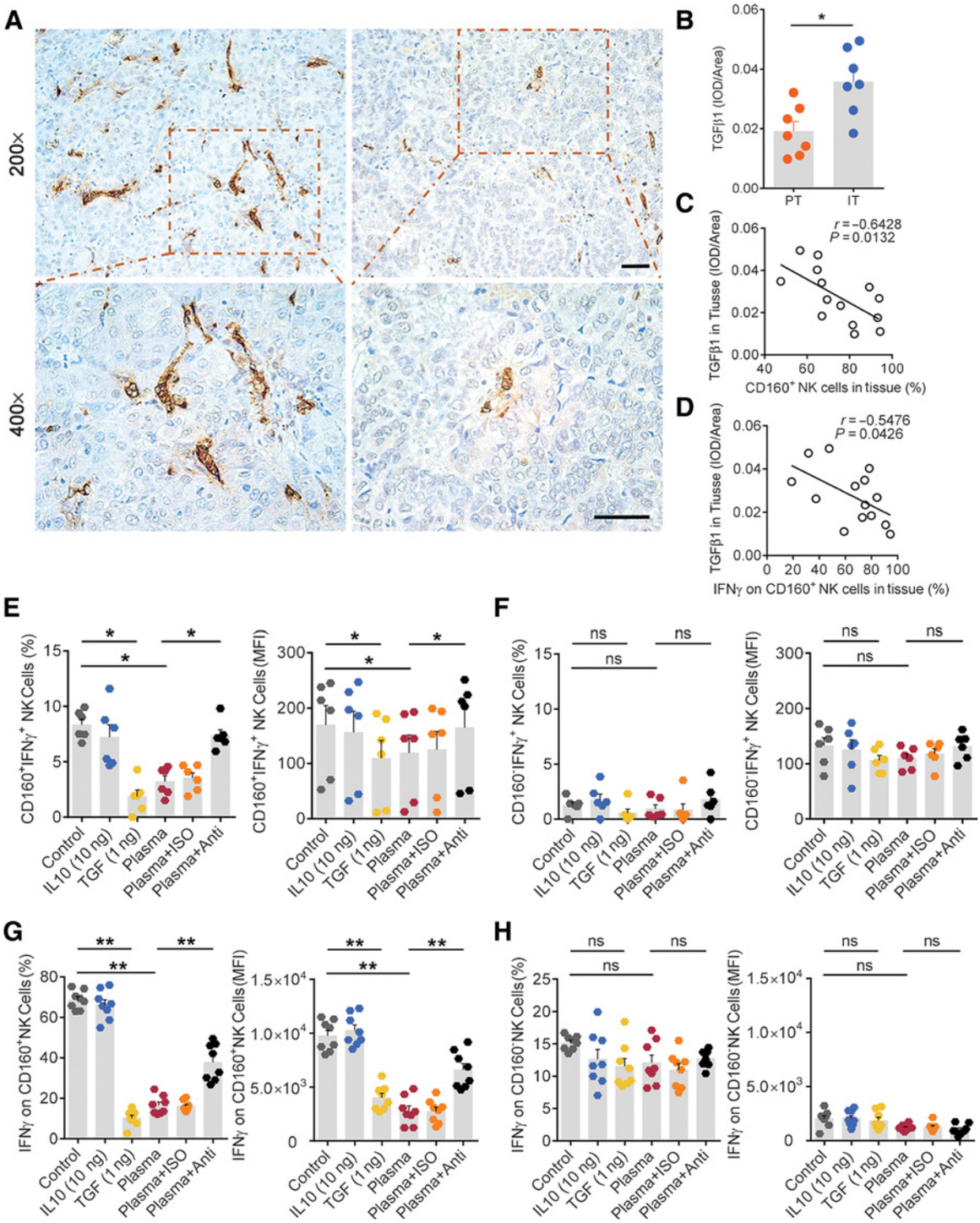


**Figure 5.**

High level of TGFβ1 positively associates with the dysfunction of CD160$^+$ NK cells. **A,** Representative micrographs showing TGFβ$^+$ cells in the IT of patients with HCC under ×200 (top; bar, 50 μm) or ×400 (bottom; bar, 5 μm) magnification. **B,** Cumulative IOD/area of TGFβ in paired PT and IT of HCC tissues ($N = 7$; Wilcoxon matched-pairs signed rank test). **C,** Correlation between cumulative IOD/area of TGFβ in the tissue and the percentage of CD160$^+$ NK cells in the tissue. **D,** Correlation between cumulative IOD/area of TGFβ in the tissue and the percentage of CD160$^+$IFNγ$^+$ NK cells in the tissue. Pearson correlation coefficients ($r$) and $P$ values are shown. **E–H** Peripheral NK cells (**E** and **F**) or sorted peripheral CD160$^+$ and CD160$^-$ NK cells (**G** and **H**) from HCs were cultured with medium alone, TGFβ1 (1 ng/mL), IL10 (10 ng/mL), or HCC patient plasma treated with or without anti-TGFβ1 or isotype control for 3 days. Cumulative percentage (left) and MFI (right) of CD160$^+$IFNγ$^+$ NK cells (**E**), CD160$^-$IFNγ$^+$ NK cells (**F**), IFNγ on sorted CD160$^+$ NK cells (**G**), or IFNγ on sorted CD160$^-$ NK cells (**H**) were analyzed via flow cytometry (Wilcoxon matched-pairs signed rank test). Results are expressed as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ns, nonsignificant.



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Although CD160 receptor is expressed by both NK cells and T-cell subsets, previous studies have been predominantly focused on peripheral $CD8^+$ T cells. Expression of CD160 significantly increases on HCV-specific $CD8^+$ T cells during chronic viral infection and contributes to T-cell exhaustion during persistent infection (31, 32). Similar phenomenon has also been reported in patients with Epstein–Barr virus and cytomegalovirus infections (33). In addition, CD160 is overexpressed on exhausted $CD8^+$ T cells in patients with chronic lymphocytic leukemia (CLL; refs. 34, 35) and is highly related to CLL patient outcome and represents a novel target for diagnosis and therapeutic manipulation (36–38).

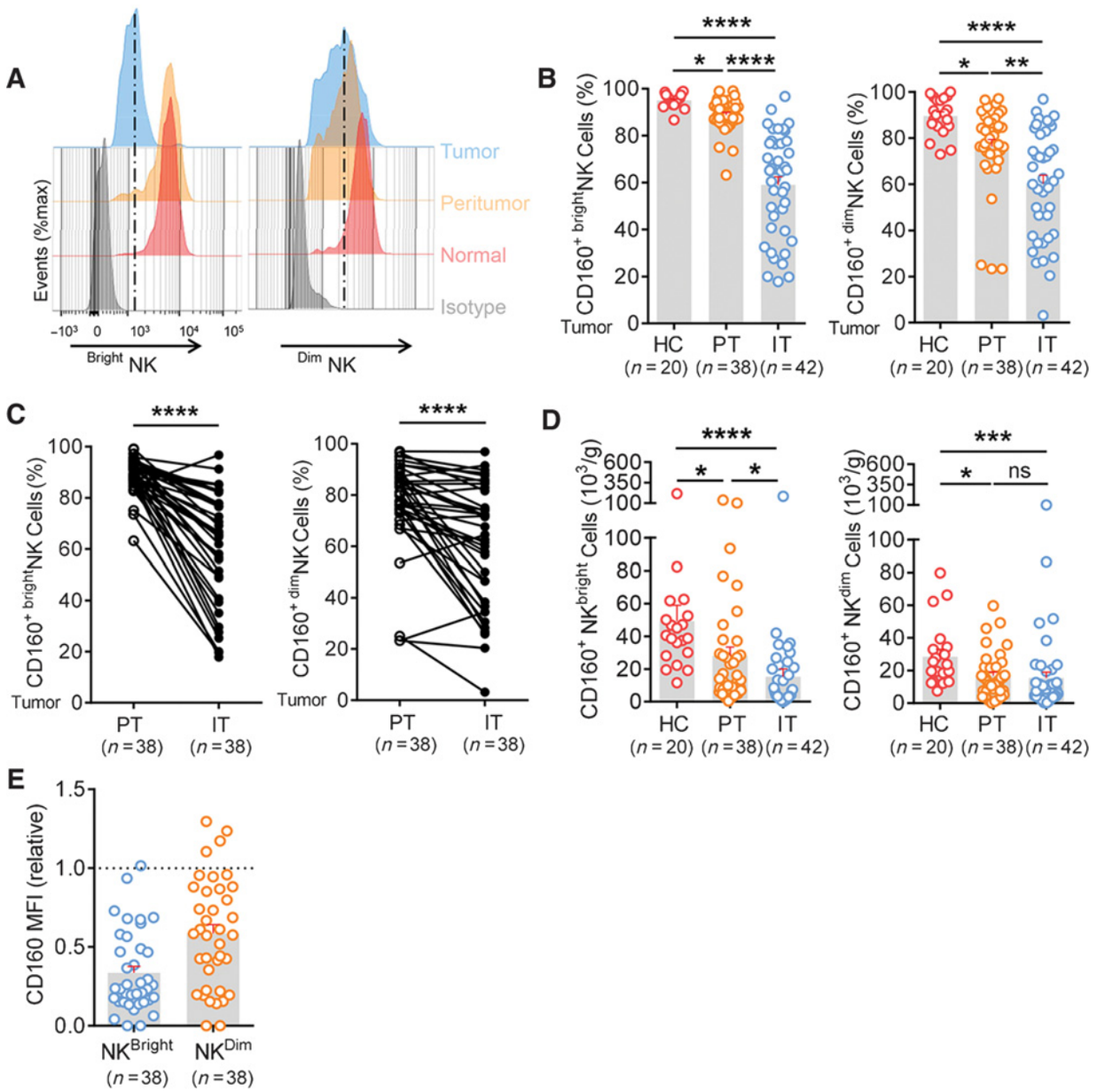


**Figure 6.**

Expression of CD160 reduces on both intratumoral $CD56^{bright}$ and $CD56^{dim}$ NK-cell subsets. **A,** The histogram corresponds to cumulative CD160 percentage on $CD56^{bright}$ (left) or $CD56^{dim}$ (right) NK-cell subsets. **B,** Cumulative percentage of $CD160^+CD56^{bright}$ NK cells (left) or $CD160^+CD56^{dim}$ NK cells (right) in healthy livers, PT, and IT of patients with HCC (Kruskal–Wallis ANOVA, followed by Dunn multiple comparisons test). **C,** Cumulative percentage of $CD160^+CD56^{bright}$ (left) or $CD160^+CD56^{dim}$ (right) NK cells in paired PT and IT of each patient with HCC (Wilcoxon matched-pairs signed rank test). **D,** Absolute count of $CD160^+CD56^{bright}$ (left) or $CD160^+CD56^{dim}$ (right) NK cells in healthy livers, PT, and IT of patients with HCC (Kruskal–Wallis ANOVA, followed by Dunn multiple comparisons test). **E,** The MFI fold change of CD160 on intratumoral $CD56^{bright}$ (left) or $CD56^{dim}$ (right) NK cells presented relative to that of paired PT from each patient. The results are expressed as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, nonsignificant.



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Interestingly, CD160 plays an inhibitory role in T cells, whereas a stimulatory role in NK cells; however, little is known about its role in NK-cell activation and function, as well as its importance in physiologic and pathologic livers. Our study shows significant reduction of CD160 on NK cells from the IT region of HCC tissues comparing with their PT counterparts; however, the difference of CD160 expression between IT and PT has not been observed on $CD8^+$ T cells. Of note, previous study has shown that no significant difference in CD160 expression can be found on $CD8^+$ and $CD4^+$ intrahepatic T cells from healthy and diseased liver tissues compared with T cells from blood (39).

Study using CD160-deficient mice has shown that although cytotoxicity of NK cells is not impaired in $CD160^{-/-}$ mice, IFNγ secretion by NK cells is markedly reduced in $CD160^{-/-}$ mice (13). Functionally targeting CD160 signaling with soluble CD160-Ig also impairs IFNγ production (13). Consistent with the findings in *CD160*$^{-/-}$ mice, comparison between sorted $CD160^+$ and $CD160^-$ intrahepatic NK cells from healthy donors shows that $CD160^+$ NK cells exhibit an activating phenotype with high expression of NKp30, NKp44, NKp46, NKG2D, and CD244, as well as an increased production of IFNγ, which is completely distinct from $CD160^-$ NK cells. Interestingly, comparison between sorted PT and IT $CD160^+$ NK cells shows that IT $CD160^+$ NK cells are more exhausted comparing with PT $CD160^+$ NK cells, and they express high levels of exhaustion markers such as BTLA, LAG-3, and PD-1 and show impaired IFNγ production.

Study using CD160-deficient mice has also pointed out that the control of NK-sensitive tumors is severely compromised in $CD160^{-/-}$ mice (13). $CD160^{-/-}$ mice have developed tumors of significantly larger sizes than wild-type mice in both B16 mouse melanoma and RMA-S lymphoma models (13). Furthermore, functionally targeting CD160 signaling with a soluble CD160-Ig impairs tumor control (13). Consistent with the findings in mice, our study shows those patients with lower intratumoral CD160 expression are accompanied by a worsen disease condition, as well as higher metastasis and recurrence rate. In addition, patients with lower cumulative percentage of $CD160^+$ NK cells in the tissue also exhibit worse DFS, suggesting a promising role of CD160 in predicting metastasis and recurrence of the tumor.

To determine the possible mechanism underlying the impaired production of IFNγ by $CD160^+$ NK cells, we preincubated healthy NK cells with IL10, TGFβ1, or HCC patient plasma for 72 hours. Of note, the cumulative percentage of $CD160^+$ NK cells in HC after 72-hour culturing is only around 30%, which is much lower than the 80% observed in freshly isolated peripheral NK cells. Earlier study has shown that CD160 gene expression in white blood cells is strongly reduced after exercise and training load (40), suggesting that CD160 may be a sensitive indicator of the external environment. Exogenous TGFβ1 or TGFβ1 from HCC plasma reduces the cumulative percentage and MFI of $CD160^+IFNγ^+$ NK cells and the level of IFNγ on sorted $CD160^+$ NK cells; however, they do not affect either the cumulative percentage and MFI of $CD160^-IFNγ^+$ NK cells or the level of IFNγ on sorted $CD160^-$ NK cells. Furthermore, adding TGFβ1 antibody to HCC plasma culturing system may specifically reverse the effect of TGFβ1 on the IFNγ production of $CD160^+$ NK cells. One thing to note is that the cumulative percentage of $CD160^+IFNγ^+$ NK cells is higher than that of $CD160^-IFNγ^+$ NK cells (8% vs. 2%), and IFNγ level of sorted $CD160^+$ NK cells is also higher than that of sorted $CD160^-$ NK cells (70% vs. 15%), suggesting that CD160 is critical in the IFNγ production of NK cells (either under the influence of TGFβ1 or not).

HVEM expression in HCC tumor significantly correlates to postoperative survival and recurrence (14). Our study shows that no significant difference in BTLA expression on NK cells is observed between PT and IT, which helps to elucidate the role $CD160^+$ NK cells in the tumor microenvironment by removing one potential HVEM-binding partner. Three of the potential mechanisms underlying immune escape in HCC include (1) increased level of HVEM expression on tumor cells that enhances inhibitory signals in $CD8^+$ T cells; (2) decreased number of $CD160^+$ NK cells that results in reduced antitumor immunity; and (3) exhaustion of remaining $CD160^+$ NK cells that further reduces their antitumor responses. Along the line, IFNγ produced by $CD160^+$ NK cells plays a critical role because it not only directly offers antitumor immune response but also recruits other immune cells such as T cells, macrophages, dendritic cells, and NK cells to boost the immune response. Reduction and exhaustion of $CD160^+$ NK cells severely impair the production of IFNγ, which in turn results in impaired immune responses.

The detailed mechanistic relationship of CD160 with NK-cell exhaustion and tumor progression still merits further researches. It is difficult to explore the underlying mechanisms in human due to limited number of samples and difficulties in manipulation; however, it is possible to use murine tumor models instead. For example, intratumoral transfer of the $CD160^+$ NK fraction results in tumor regression in $CD160^{-/-}$ tumor-bearing mice, and $CD160^{-/-}$ mice show similar tumor growth kinetics to untreated mice when NK cells are depleted before tumor inoculation, indicating that CD160 is required specifically by NK cells for control of tumor growth (13). Engagement of CD160 induces its polarization and colocalization with PI3K, and pharmacologic inhibitors of PI3K abrogate both CD160-mediated cytotoxicity and IFNγ, TNFα, and IL6 cytokine production (41).

A recent study shows that short incubation of NK cells with IL15 converts membrane-bound CD160 to a soluble form, in which it cannot be detected on the cell surface, but instead can be immunoprecipitated from the culture medium (42). This soluble form of CD160 binds to MHC-I molecule, resulting in the inhibition of cytotoxic $CD8^+$ T-cell activity and CD160-mediated NK-cell cytotoxicity. Due to the limitation of our specimens, we only detected cell surface expression of CD160 in HCC samples, and soluble form of CD160 should be addressed in future studies. Considering our results, we hypothesize that deficiency in the surface CD160 expression not only reduces IFNγ production of NK cells but also increases soluble CD160 expression, which in turn functionally impairs $CD8^+$ T cells and other lymphocytes during immune escape. CD160 was previously described as a marker demarcating ILC1 cells that specialize in IFNγ production (43), whether $CD160^+$ NK-cell subset is part of liver-resident NK cells (ILC1 cells) and if not how they interact with each other in the liver tumor microenvironment have yet to be explored (44, 45).

In summary, $CD160^+$ NK cells exhibit an activated phenotype and produce more IFNγ; however, their cumulative percentage, absolute number, and MFI are significantly reduced in the IT of HCC tissues. In addition, intratumoral $CD160^+$ NK cells are more exhausted and produce less IFNγ when comparing with peritumoral $CD160^+$ NK cells. Patients with HCC with reduced expression of intratumoral CD160 expression are accompanied by a worsen disease condition, higher metastasis rate, and poorer outcome. Furthermore, high plasma level of TGFβ1 results in



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impaired IFNγ production of $CD160^+$ NK cells, and blocking TGFβ1 may restore IFNγ production of $CD160^+$ NK cells to the normal level. Our finding opens new possibilities for understanding the mechanism of NK exhaustion and immune escape in HCC, and it suggests that CD160-related deficiency can be a broad strategy of immune escape by tumors and elucidates the therapeutic potential of CD160 in controlling tumors, particularly the suppression of tumor metastases.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** H. Wei, Z. Tian, C. Sun
**Development of methodology:** H. Sun, H. Wei
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** J. Xu, Q. Huang, M. Huang, H. Wen, R. Lin, M. Zheng, C. Sun
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** H. Sun, M. Huang, K. Qu
**Writing, review, and/or revision of the manuscript:** H. Sun, R. Sun, Z. Tian, C. Sun
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** J. Xu, Q. Huang, M. Huang, K. Li, H. Wei, R. Sun, Z. Tian
**Study supervision:** W. Xiao, Z. Tian, C. Sun

## Acknowledgments

This work was supported by the National Natural Science Foundation of China [81788101 (Z. Tian), 31670908 (C. Sun), 81701631 (H. Sun), 91442112 (C. Sun), 81330071 (H. Wei), 31390433 (Z. Tian), 91542000 (Z. Tian)], Chinese Academy of Sciences (XDB29030000 to Z. Tian), and the Ministry of Science & Technology of China (Major Project 2017ZX10203206003 to R. Sun and 2017ZX10202203-002-001 to Z. Tian).

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Received April 7, 2018; revised August 9, 2018; accepted September 10, 2018; published first September 19, 2018.

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Correction

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# Correction: Reduced CD160 Expression Contributes to Impaired NK-cell Function and Poor Clinical Outcomes in Patients with HCC

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In the original version of this article (1), the affiliations were incorrect. In addition, "tumor cells" in the fourth sentence of the Abstract should have read "tumors," and "paired" in the Fig. 4B legend should have read "unpaired." These errors have been corrected in the latest online HTML and PDF versions of the article. The publisher regrets these errors.

## Reference

1. Sun H, Xu J, Huang Q, Huang M, Li K, Qu K, et al. Reduced CD160 expression contributes to impaired NK-cell function and poor clinical outcomes in patients with HCC. Cancer Res 2018;78:6581–93.

Published online April 1, 2019.
doi: 10.1158/0008-5472.CAN-19-0630
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Published OnlineFirst September 19, 2018; DOI: 10.1158/0008-5472.CAN-18-1049

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

AACR American Association for Cancer Research

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*Cancer Res* 2018;78:6581-6593. Published OnlineFirst September 19, 2018.

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