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Human CD96 Correlates to Natural Killer Cell Exhaustion and Predicts the Prognosis of Human Hepatocellular Carcinoma

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Immune checkpoint blockade has become a promising therapeutic approach to reverse immune cell exhaustion. Coinhibitory CD96 and T-cell immunoglobulin and ITIM domain (TIGIT), together with costimulatory CD226, bind to common ligand CD155. The balancing between three receptors fine-tunes immune responses against tumors. In this study, we investigated the expression of CD96, TIGIT, and CD226 in 55 fresh human hepatocellular carcinoma (HCC) samples, 236 paraffin-embedded HCC samples, and 20 normal human livers. The cumulative percentage, absolute count, and mean fluorescence intensity (MFI) of CD96$^+$ NK cells are significantly increased in the intratumoral tissues of HCC and break the balance between three receptors. Human CD96$^+$ NK cells are functionally exhausted with impaired interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) production, high gene expression of interleukin (IL)-10 and transforming growth factor-beta 1 (TGF-β1), and low gene expression of T-bet, IL-15, perforin, and granzyme B. In addition, blocking CD96-CD155 interaction specifically increases lysis of HepG2 cells by NK cells. HCC patients with a high level of CD96 or CD155 expression within tumor are strongly associated with deteriorating disease condition and shorter disease-free survival (DFS) and overall survival times. Patients with a higher cumulative percentage of CD96$^+$ NK cells within tumor also exhibit shorter DFS. High plasma level of TGF-β1 in HCC patients up-regulates CD96 expression and dynamically shifts the balance between CD96, TIGIT, and CD226 in NK cells. Blocking TGF-β1 specifically restores normal CD96 expression and reverses the dysfunction of NK cells. *Conclusion:* These findings indicate that human intratumoral CD96$^+$ NK cells are functionally exhausted and patients with higher intratumoral CD96 expression exhibit poorer clinical outcomes. Blocking CD96-CD155 interaction or TGF-β1 restores NK cell immunity against tumors by reversing NK cell exhaustion, suggesting a possible therapeutic role of CD96 in fighting liver cancer. (Hepatology 2019;70:168-183).

Compelling evidence has suggested that patients with hepatocellular carcinoma (HCC) show significant reduction in the number of peripheral and intrahepatic natural killer (NK) cells; moreover, NK cells in the tumor are often functionally exhausted with impaired interferon-gamma (IFN-γ) production and cytotoxicity.[1-3] Positive correlation has been established between NK cell density

Abbreviations: CHB, chronic hepatitis B; CRTAM, class I–restricted T-cell–associated molecule; CTLA-4, cytotoxic T-lymphocyte–associated antigen 4; DFS, disease-free survival; GSEA, gene set enrichment analysis; GZMB, *granzyme B; HC, healthy control; HCC, hepatocellular carcinoma; HR, hazard ratio; IFN-γ, interferon-gamma; IL, interleukin; IOD, integral optical density; IT, intratumoral tissue;* KLRC1, *killer cell lectin-like receptor C1; KLRG1, killer cell lectin-like receptor G1; LAG-3, lymphocyte-activation gene 3; MFI, mean fluorescence intensity;* NCR, *natural cytotoxicity receptor; NK cell, natural killer cell; OS, overall survival; PBMC, peripheral blood mononuclear cell;* PDCD1, *programmed cell death 1; PD-1, programmed death-1; PMA, phorbol myristate acetate;* PRF1, *perforin 1; PT, peritumoral tissue; TGF-β1, transforming growth factor-beta 1; TIGIT, T-cell immunoglobulin and ITIM domain; TNF-α, tumor necrosis factor-alpha; TNM, tumor node metastasis.*

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and the prognosis of HCC patients, showing a critical role of NK cells in the control of tumor growth.[3,4] Several immunoglobulin superfamily receptors, including T-cell immunoglobulin and ITIM domain (TIGIT),[5] CD96,[6] CD226,[7] and class I–restricted T-cell–associated molecule (CRTAM),[8] have been reported to play prominent roles in regulating NK and T-cell functions.[9] CD96, TIGIT, and CD226 bind to a common ligand, CD155, and form a pathway in which CD96 and TIGIT deliver inhibitory signals, whereas CD226 delivers activating signals.[10] CD96 was identified more than 20 years ago,[6,9,11] it was originally known to enhance cell adhesion and considered as a potential costimulatory receptor[12]; however, recent studies in *Cd96*$^{-/-}$ mice have revealed its capacity to limit NK cell function through direct inhibition.[13,14] Blocking CD96 suppressed tumor metastasis in different murine models.[10]

Previous studies on CD96 were primarily performed using murine models owing to a limited supply of fresh NK cells from humans. However, differences exist between human and murine CD96; in particular, CD96 expresses at higher level in murine NK cells, whereas basal expression is much lower in human NK cells.[10,15] Expression and function of human CD96 in healthy individuals and cancer patients remain obscured. Because of disputes of previous findings and the limitation of clinical specimens, both academia and industry are interested to know whether CD96 may act as a checkpoint molecule in regulating human NK cells.[9,10,14-18] Furthermore, a careful examination on the balancing between CD96, TIGIT, and CD226 in healthy controls (HCs) and tumor settings merits further research.

Liver is enriched with more than 5 times the NK cells than either blood or spleen[2,19-22]; moreover, CD96, TIGIT, and CD226 have been associated with chronic hepatitis B virus infection in several studies,[2,23,24] and these advantages make the liver an ideal model for investigating the balance between CD96, TIGIT, and CD226 on lymphocytes. In this study, through the use of peritumoral tissues (PTs) and intratumoral tissues (ITs) of 236 HCC patients, we analyzed the expression of CD96, TIGIT, and CD226 on NK cells, respectively. Our study suggests that CD96 may act as a checkpoint molecule in regulating antitumor immune responses of NK cells and predicts the prognosis of HCC patients,

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intervention of which may restore proper innate immunity against tumors. This sheds light on immunotherapy and promotes CD96 as a possible therapeutic target in fighting liver cancer.

Materials and Methods

PATIENTS AND SPECIMENS

Tissue specimens from 236 HCC patients who had undergone curative resection between 2006 and 2010 (Cohort 1) were obtained from the Bank of Tumor Resources at Sun Yat-Sen University (Guangzhou, China). Fresh tumor tissue samples were obtained from 55 HCC patients during surgery at the Department of Hepatobiliary Surgery of The First Affiliated Hospital of the University of Science and Technology of China (Hefei, China) and The First Affiliated Hospital of Xinjiang Medical University (Urumqi, China; Cohort 2). Among these samples, 48 were paired peritumoral (collected 2 cm distal to the tumor site) and intratumoral tissues of the same patient. Peripheral blood samples from HCs and HCC patients were collected from The First Affiliated Hospital of Anhui Medical University (Hefei, China). Normal liver tissues (N = 20) were obtained from liver transplantation at The First Affiliated Hospital of Xinjiang Medical University. Pilot studies were conducted to ensure that sample sizes are large enough to detect the effects. The clinical characteristics of all tissue samples from HCC patients are summarized in Supporting Table S3. The etiology of primary HCC patients includes viral infection, liver cirrhosis, 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 Supporting Table S4. The details of all patients are provided in Supporting Table S5. All samples were anonymously coded in accord with the Helsinki Declaration. Written informed consent was obtained from each patient included in the study, and the protocol of all study cohorts was approved by the Ethical Board of the Institutional Review Board of the University of Science and Technology of China.

Other detailed methods are available in the Supporting Information.

Results

CD96 EXPRESSION IS INCREASED ON INTRATUMORAL NK CELLS OF HCC PATIENTS

Liver-infiltrating lymphocytes from HCs, PT, and IT of HCC patients were analyzed through flow cytometry (Supporting Fig. S1A); the cumulative percentage and absolute count of intrahepatic $CD3^-CD56^+$ NK cells were significantly lower in IT compared to those in PT or HCs (Supporting Fig. S1B). Furthermore, both the cumulative percentage and absolute count of $CD96^+$ NK cells were significantly higher in IT than in PT or HCs, whereas significant reductions were observed in the percentage and number of intratumoral TIGIT-expressing NK cells (Fig. 1A-D). The phenotypic differences became even more significant when comparing paired PT and IT of each patient individually (Fig. 1C and Supporting Fig. S1B). Elevation of CD96 expression (IT:PT ratio >1) and reduction of TIGIT expression (IT:PT ratio <1) on intratumoral NK cells were reconfirmed through mean fluorescence intensity (MFI) analysis (Fig. 1E). Of note, although CD226 expression on NK cells in IT was higher than in PT (IT:PT ratio >1), no significant differences were found in either the proportion or absolute number of $CD226^+$ NK cells (Fig. 1B,D,E). In addition, an *in situ* colocalization image of CD96 with typical NK cell marker CD56 showed that CD96 and CD56 both express on the cell surface and colocalize with each other in the tumor center (Fig. 1F). Considering that CD96, TIGIT, and CD226 also express on $CD8^+$ T cells, we analyzed the expression of these receptors on $CD3^+CD56^-CD8^+$ T cells. No significant difference was found in the absolute count, percentage, or MFI of $CD96^+CD8^+$ T cells between PT and IT (Supporting Fig. S1C-F), suggesting that elevated CD96 expression is only confined to intratumoral NK cells in HCC patients. In addition, the cumulative percentage of NK cells or T cells within $CD96^+$ cells showed no significant difference between groups of HC, PT, and IT (Supporting Fig. S1G).

Human NK cells are classified into either $CD56^{bright}$ or $CD56^{dim}$ NK cells. In HCC, the cumulative percentage of $CD56^{bright}$ NK cells significantly decreased whereas the percentage of $CD56^{dim}$ NK cells significantly increased in IT compared to PT or HCs

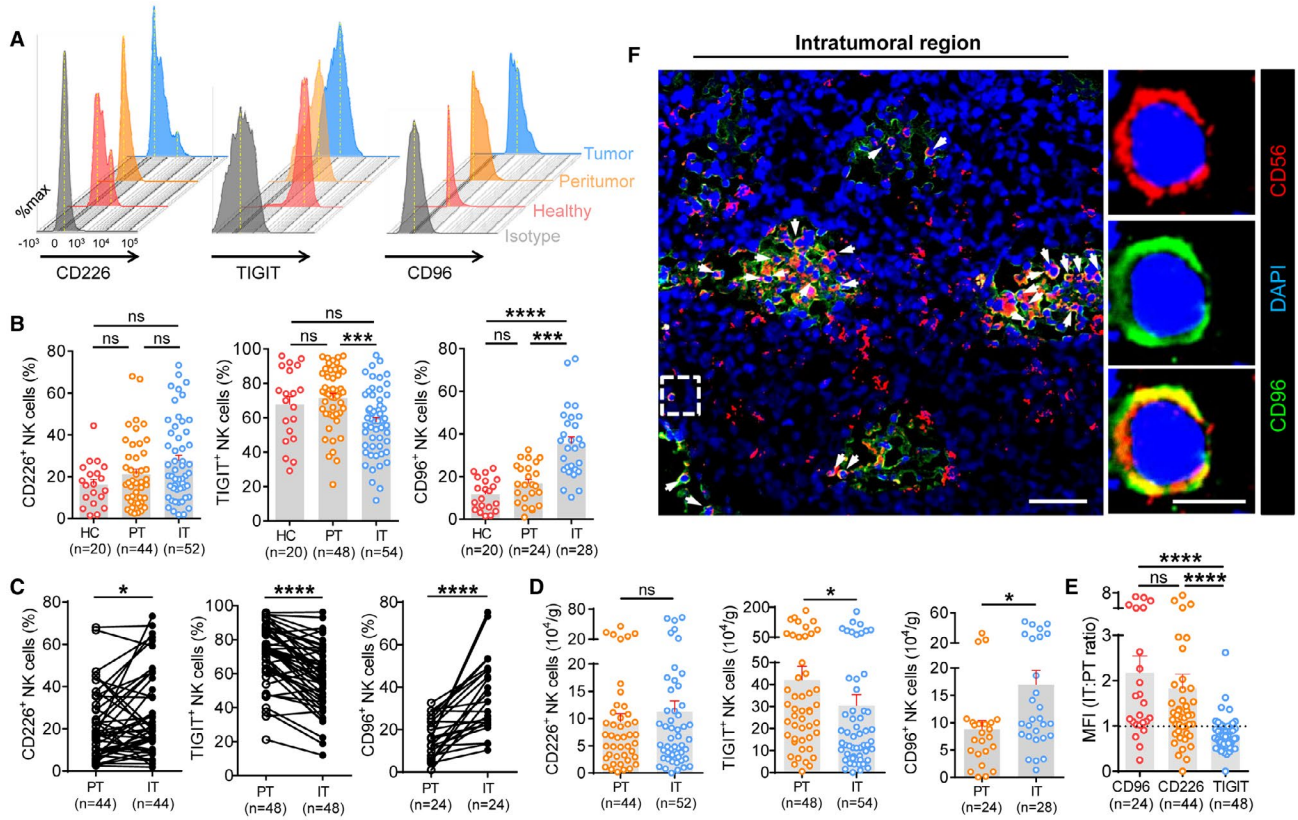

FIG. 1. Expression of intratumoral CD96$^+$ NK cells is elevated in HCC patients. (A) The histogram corresponds to CD226, TIGIT, or CD96 expression on total CD3$^-$CD56$^+$ NK cells within the lymphocyte gate from a representative normal liver, PT, and IT. (B) Cumulative percentage of CD226$^+$, TIGIT$^+$, or CD96$^+$ NK cells in healthy liver, PT, and IT (Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons test). (C) Cumulative percentage of CD226$^+$, TIGIT$^+$, or CD96$^+$ NK cells in paired PT and IT of each HCC patient (Wilcoxon matched-pairs signed-rank test). (D) Absolute count of CD226$^+$, TIGIT$^+$, or CD96$^+$ NK cells in PT and IT of HCC patients (Mann-Whitney U test). (E) MFI fold-change of each receptor on intratumoral NK cells presented relative to that of paired peritumoral NK cells from each patient (Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons test). (F) *In situ* colocalization of CD96 with CD56 in the intratumoral region of HCC specimen. Bar = 5 μm, 50 μm. Results are expressed as means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; ns, not significant.

(Supporting Fig. S2A,B). Furthermore, the percentages of CD96$^+$CD56^{bright} NK cells and CD96$^+$CD56^{dim} NK cells were significantly higher in IT than those in PT or HC (Supporting Fig. S2C-G), implying an important role of CD96 in the cancer nest. On the other hand, the absolute count of CD96$^+$CD56^{dim} NK cells was significantly increased whereas that of CD96$^+$CD56^{bright} NK cells was not (Supporting Fig. S2H,I). Although the cumulative percentage and absolute count of TIGIT$^+$CD56^{bright} NK cells were significantly reduced in IT (Supporting Fig. S2D,H), its expression on CD56^{dim} NK cells did not differ between PT and IT (Supporting Fig. S2F,I). The proportion and absolute count of CD226$^+$CD56^{bright} and CD226$^+$CD56^{dim} NK cells remained relatively stable in different regions (Supporting Fig. S2D,F,H,I). The relative MFI of CD96 and CD226 presented as IT:PT ratio were greater than 1 and the relative MFI of TIGIT was less than 1, indicating elevated level of CD96 and CD226 and reduced level of TIGIT on both ^{bright}NK and ^{dim}NK cells (Supporting Fig. S2J). In addition to hepatic NK cells, the cumulative percentage of CD96$^+$ NK cells in the peripheral blood of HCC patients was also significantly higher than that of HC (Supporting Fig. S3A). Comparison between CD96, TIGIT, and CD226 on NK cells in paired blood, IT, and PT showed that the percentages of CD96$^+$ NK cells, CD96$^+$CD56^{bright} NK cells, and CD96$^+$CD56^{dim} NK cells in IT were more than 3 times higher than the percentages of those in

peripheral blood (Supporting Fig. S3B). These data indicate that the expression of CD96 increases in the tissue and blood of HCC patients and elevation of CD96 is mostly exclusive to NK cells.

BALANCE BETWEEN CD226, TIGIT, AND CD96 EXPRESSION ON INTRATUMORAL NK CELLS IS BROKEN IN HCC PATIENTS

CD226 delivers a positive costimulatory signal, whereas CD96 and TIGIT deliver inhibitory signals through shared ligand CD155. This inevitably raises the hypothesis that an immune balance exist between three receptors and the integrated signaling of CD96, TIGIT, and CD226 decides the net activation or inhibition of lymphocytes. To address this, we analyzed correlations between each of the three receptors respectively. The percentage of $CD226^+$ NK cells was negatively associated with the percentage of $CD96^+$ NK cells without statistical significance in HCs (Fig. 2A). On the contrary, the percentage of $TIGIT^+$ NK cells was positively associated with the percentage of $CD96^+$ NK cells in HCs (Fig. 2B). However, these

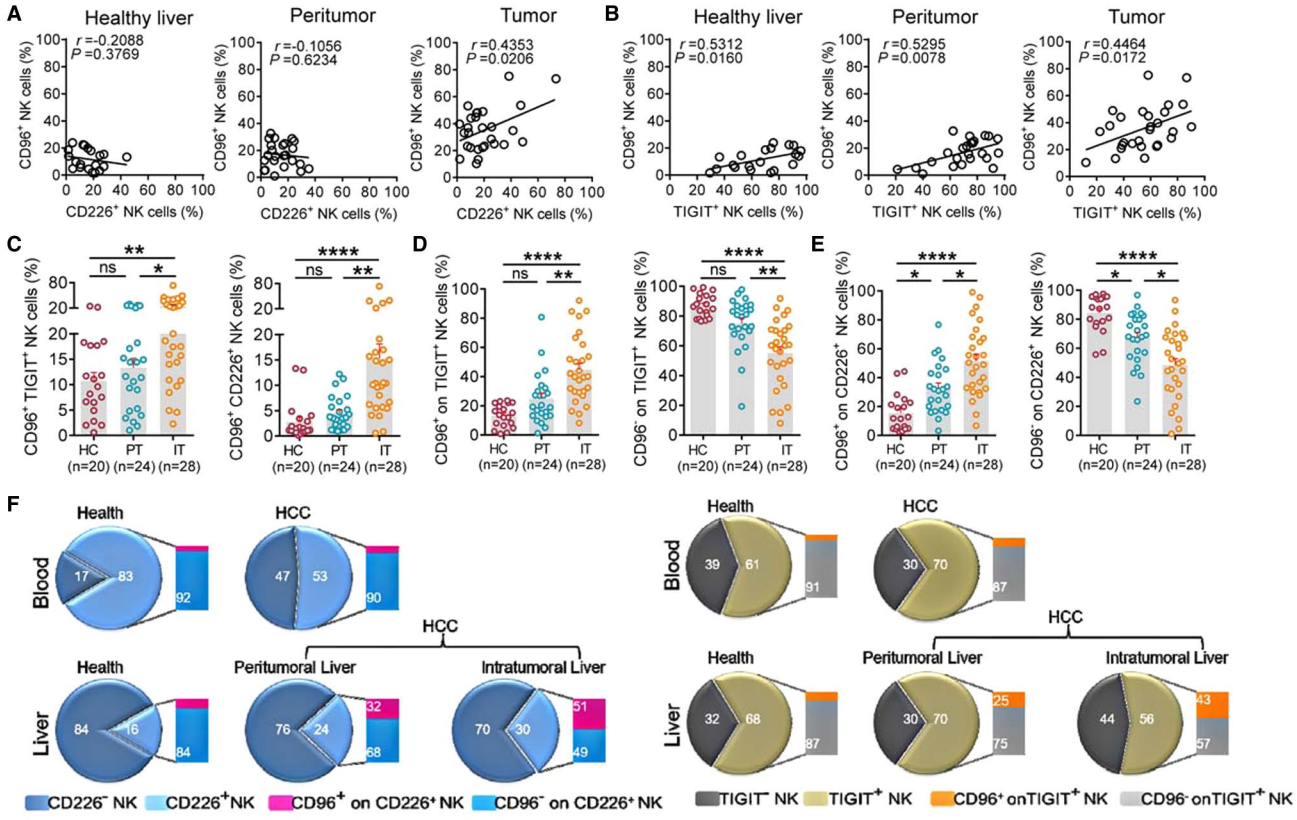

FIG. 2. The balance between $CD226^+$, $TIGIT^+$, and $CD96^+$ NK cells is broken in HCC patients. (A,B) Correlation between the percentage of $CD96^+$ and (A) $CD226^+$ or (B) $TIGIT^+$ NK cells in healthy liver, PT, and IT of HCC patients. (C) Percentage of $TIGIT^+CD96^+$ NK cells (left) or $CD226^+CD96^+$ NK cells (right) in healthy liver, PT, and IT of HCC patients. (D) Percentage of $CD96^+$ or $CD96^-$ cells in $TIGIT^+$ NK cells from healthy liver, PT, and IT. (E) Percentage of $CD96^+$ or $CD96^-$ cells in $CD226^+$ NK cells from healthy liver, PT, and IT. (F, left) Proportion of $CD226^+$ NK cells (light blue) in either peripheral (top) or intrahepatic (bottom) NK cells from HCs and HCC patients. Histogram shows the proportion of double-positive $CD226^+CD96^+$ NK cell (pink) in total $CD226^+$ NK cells (light blue) from the pie chart. (F, right) Proportion of $TIGIT^+$ NK cells (light brown) in either peripheral (top) or intrahepatic (bottom) NK cells from HCs and HCC patients. Histogram shows the proportion of double-positive $TIGIT^+CD96^+$ NK cells (orange) in total $TIGIT^+$ NK cells (light brown) from the pie chart. The percentage of each cell subset is shown in white number. Pearson's correlation coefficients and *P* values are shown in (A,B). Cumulative data in (C-E) were analyzed using the Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons test. Results are expressed as means ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Abbreviation: ns, not significant.

correlations in hepatic NK cells gradually turn over along normal liver, PT, and IT (Fig. 2A,B). The negative correlation between $CD226^+$ and $CD96^+$ NK cells in HCs (r = –0.2088) became less negative in PT (r = –0.1056) and positive in IT (r = 0.4353; Fig. 2A). The positive correlation between $TIGIT^+$ and $CD96^+$ NK cells in HCs (r = 0.5312) became less positive in PT (r = 0.5295) and IT (r = 0.4464; Fig. 2B). These data suggest that the immune balance between three receptors in healthy hepatic NK cells is disrupted in the intratumoral NK cells of HCC patients.

To confirm the imbalance between CD96, TIGIT, and CD226, we further analyzed the percentages of $CD96^+TIGIT^+$ and $CD96^+CD226^+$ NK cells in HCs, PT, and IT. The percentages of $CD96^+TIGIT^+$ and $CD96^+CD226^+$ NK cells were significantly higher in IT than those in PT or HCs (Fig. 2C). Meanwhile, $TIGIT^+$ NK cells can be subdivided into two groups based on CD96 expression; the percentage of intratumoral $CD96^+$ NK cells in $TIGIT^+$ NK cells was significantly higher than those in PT or HCs, whereas the percentage of intratumoral $CD96^-$ NK cells in $TIGIT^+$ NK cells was significantly lower (Fig. 2D). Similar results were found in $CD226^+$ NK cells (Fig. 2E), indicating the possibility that $TIGIT^+$ and $CD226^+$ NK cells are in transition to $CD96^+$ NK cells in the cancer nest. In healthy blood and liver, $CD96^+CD226^+$ subset occupied a small proportion of $CD226^+$ NK cells (8% in blood, 16% in liver) and $CD96^+TIGIT^+$ subset occupied a small proportion of $TIGIT^+$ NK cells (9% in blood, 13% in liver; Fig. 2F). Whereas in tumor, the proportion of $CD96^+CD226^+$ subset was greatly expanded in $CD226^+$ NK cells (10% in blood, 51% in IT) and $CD96^+TIGIT^+$ subset was expanded in $TIGIT^+$ NK cells (13% in blood, 43% in IT; Fig. 2F). These data suggest that the percentage of $CD96^+$ NK cells increases in both $TIGIT^+$ and $CD226^+$ NK cells under the influence of the tumor microenvironment.

HUMAN $CD96^+$ NK CELLS ARE FUNCTIONALLY INHIBITED

Consistent with our previously findings,[25] IFN-γ production by intratumoral NK cells was significantly reduced (Supporting Fig. S3C). Furthermore, intracellular IFN-γ production was negatively correlated to the percentage of tumor-infiltrating $CD96^+$ NK cells (Fig. 3A). To verify the influence of CD96 on cytokine production of NK cells, we stimulated cultured peripheral NK cells from HCs using interleukin (IL)-12 as a moderate stimulant and phorbol myristate acetate (PMA)/ionomycin as a strong stimulant. Significant reduction in CD96 expression was observed after stimulation with IL-12 or PMA/ionomycin compared to medium alone (Fig. 3B). In addition, the ability of $CD96^+$ NK cells to secrete IFN-γ and TNF-α was, surprisingly, significantly impaired (<5%) after activation with PMA/ionomycin *in vitro* (Fig. 3C). In contrast to $CD96^+$ NK cells, $CD96^-$ NK cells produced much higher levels of IFN-γ and TNF-α (Supporting Fig. S3D). These data suggest that CD96 may regulate cytokine production of NK cells.

One of the major advantages of checkpoint blockade is the potential boost of cytotoxicity. Because of a limited number of peripheral NK cells, we evaluated the influence of CD96 blockade on the cytotoxicity of peripheral blood mononuclear cells (PBMCs). Treatment with anti-CD96 antibody showed a mean of 40.0% K562 lysis at a 20:1 E:T ratio compared to 27.7% in controls, indicating the effectiveness of CD96 blockade in strengthening cytotoxicity (Fig. 3D). Meanwhile, combined use of anti-CD96 and anti-TIGIT antibodies resulted in the highest mean of 62.4% K562 lysis (Fig. 3D). To evaluate the potential of the CD96-CD155 signaling pathway, we analyzed expression of CD155 on different target cells and found that hepatoma cell line HepG2 expresses the highest level of CD155 (Fig. 3E). Primary NK cells were purified from HCs through negative selection and incubated with HepG2 cells. Increased HepG2 lysis at 5:1 and 10:1 ratios have been noticed in the presence of anti-CD96 antibody (Supporting Fig. S3E). To further clarify the effect of CD96-CD155 blockade on NK cytotoxicity, primary $CD3^-CD56^+CD96^+$ and $CD3^-CD56^+CD96^-$ NK cells were sorted from purified NK cells. Lysis of HepG2 was remarkably increased in the presence of anti-CD155 antibody (Fig. 3F), suggesting that CD96-CD155 blockade can significantly boost the cytotoxicity of NK cells. Together, these results indicate that CD96-CD155 blockade may enhance NK cytotoxicity against CD155-expressing tumor cells and provide protection against tumors through boosting NK cell immune responses.

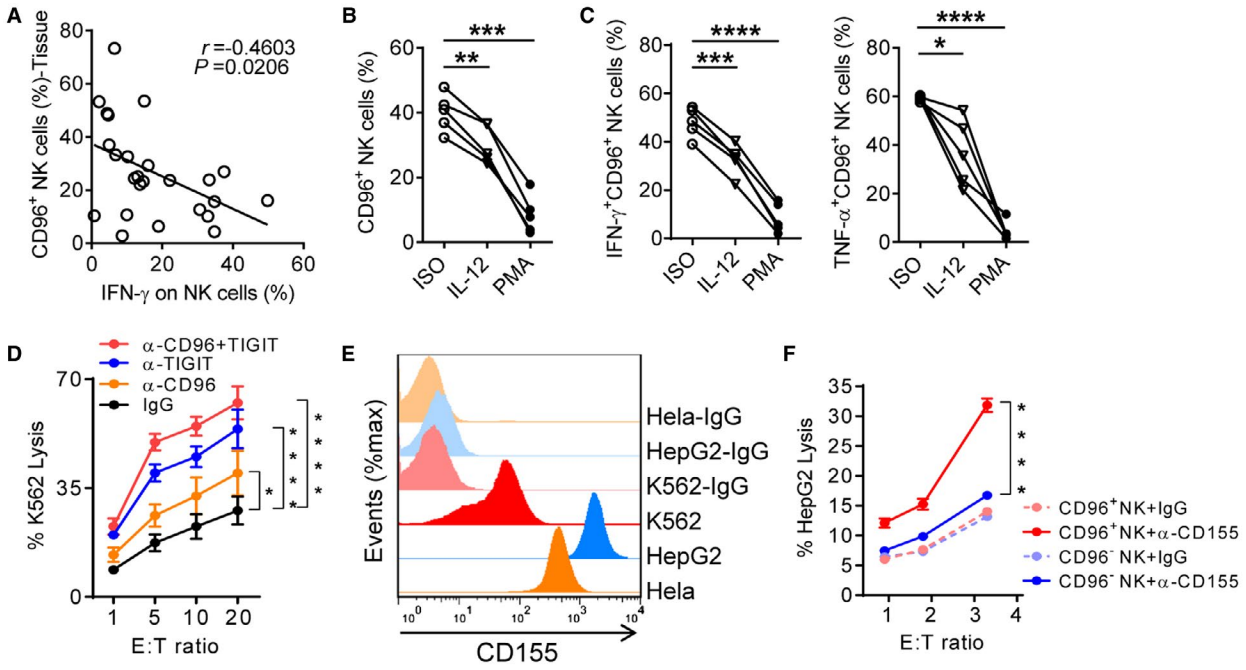

FIG. 3. Human CD96$^+$ NK cells are functionally impaired. (A) Correlation between IFN-γ production of NK cells and percentage of CD96$^+$ NK cells in tissues of HCC patients. Pearson's correlation coefficient and *P* value are shown. Healthy peripheral NK cells were preincubated with medium for 12 hours, followed by PMA/ionomycin stimulation for 4 hours or IL-12 stimulation for 12 hours. Cumulative percentage of (B) CD96$^+$ NK cells or (C) IFN-γ$^+$CD96$^+$ and TNF-α$^+$CD96$^+$ NK cells were monitored by flow cytometry (paired *t* test). (D) Primary healthy PBMCs were cultured with control IgG, anti-CD96, anti-TIGIT, or anti-CD96 combined with anti-TIGIT. Cytotoxicity was assessed by a 7-AAD/CFSE assay using K562 target cells (two-way ANOVA). (E) CD155 expression on Hela, HepG2, and K562 cells were determined by flow cytometry. Isotype controls are shown by the corresponding light color. (F) Lysis of HepG2 cells by healthy primary CD96$^+$ (red) or CD96$^-$ (blue) NK cells in the presence of anti-CD155 antibody (solid line) or control antibody (dotted line; two-way ANOVA). Results are expressed as means ± SEM of at least 3 independent individuals. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Abbreviations: 7-AAD, 7 aminoactinomycin D; ANOVA, analysis of variance; CFSE, carboxyfluorescein succinimidyl ester; IgG, immunoglobulin G; ISO, isoproterenol.

TRANSCRIPTOMIC ANALYSIS REVEALS THAT CD96$^+$ NK CELLS ARE FUNCTIONALLY EXHAUSTED COMPARING TO CD96$^-$ NK CELLS

To better define primary CD96$^+$ and CD96$^-$ NK cells, we subsequently isolated primary hepatic lymphocytes from human liver and purified CD3$^-$CD56$^+$CD96$^+$ and CD3$^-$CD56$^+$D96$^-$ NK cells through negative selection and flow cytometry sorting. By using high-resolution microarrays and verification through real-time PCR, we found profound and wide-ranging differences between CD96$^+$ and CD96$^-$ NK cells. A greater than 2-fold change was found in 701 expressed genes (Fig. 4A and Supporting Fig. S4A), and these 701 genes fell into 11 functional categories as indicated in Fig. 4B. Quantitative analysis of gene expression differences revealed that inhibitory-related molecules, such as TIGIT (*TIGIT*), programmed death-1 (PD-1; programmed cell death 1 [*PDCD1*]), natural killer group 2A (NKG2A; killer cell lectin-like receptor C1 [*KLRC1*]), CD355 (*CRTAM*), and lymphocyte-activation gene-3 (LAG-3; *LAG3*), are overexpressed in CD96$^+$ NK cells (Fig. 4C), whereas activation-related molecules, such as CD226 (*CD226*), killer cell lectin-like receptor G1 (KLRG1; *KLRG1*), CD244 (*CD244*), CD69 (*CD69*), NKp44 (natural cytotoxicity receptor [*NCR*]*2*), and NKp30 (*NCR3*), are down-regulated in CD96$^+$ NK cells (Fig. 4C). Importantly, IL-10 (*IL10*) and transforming growth factor-beta (TGF-β; *TGFB1*) were highly expressed in CD96$^+$ NK cells whereas T-bet (T-box 21; *TBX21*), IL-15 (*IL15*), perforin

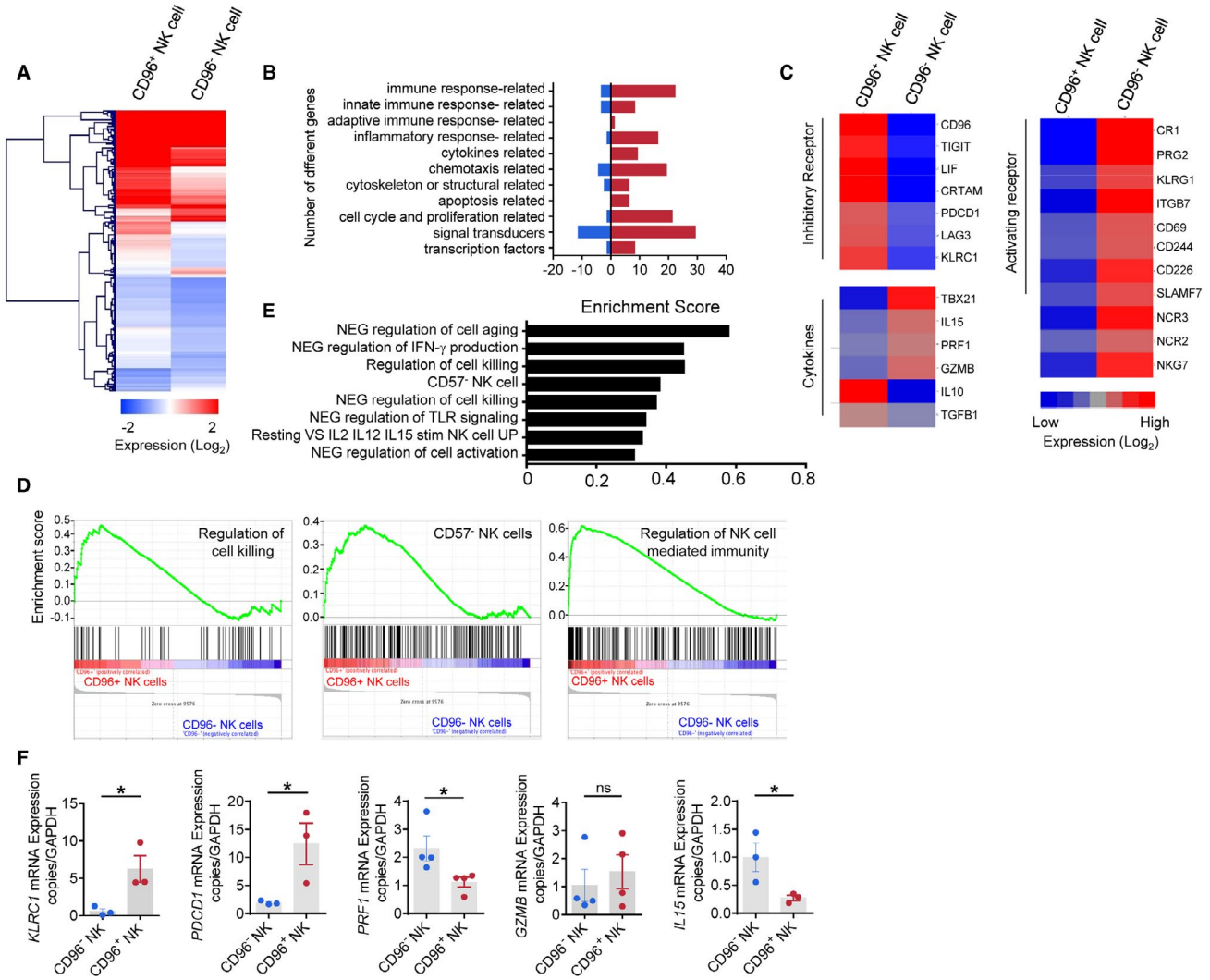

FIG. 4. Comparison between CD96$^+$ and CD96$^-$ NK cell subsets. (A) Heatmap of mRNA transcripts up- or down-regulated in CD96$^+$ and CD96$^-$ NK cell subsets, as reported by MEV 4.9 software. (B) Distribution by functional category of up-regulated (red) or down-regulated (blue) genes in CD96$^+$ and CD96$^-$ NK cells. Genes with greater than 2-fold differences are included. (C) Representative cytokine, inhibitory receptor, or activating receptor profiles of both CD96$^+$ and CD96$^-$ NK cells were shown. Heatmaps showing signal values of the listed genes from −0.5 to 0.5 on a $\log_2$ scale. (D) GSEA plot of the regulation of cell killing (left), CD57-negative NK cells (middle), or the negative regulation of NK-cell–mediated immunity (right) gene signatures in CD96$^+$ NK cells relative to CD96$^-$ NK cells. (E) GSEA plot of genes up-regulated in CD96$^+$ NK cells that overlap with gene sets of known NK-cell–related pathways. GO term IDs used include: GO0090344, GO0032689, GO0031341, GSE23695, GO0031342, GO0034122, GSE22919, and GO0050866. (F) Real-time PCR analysis comparing mRNA levels of selected genes between healthy CD96$^+$ and CD96$^-$ NK cells (unpaired *t* test). Results are expressed as means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Abbreviations: CR1, complement receptor 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GO, Gene Ontology; ITGB7, integrin subunit beta 7; LIF, leukemia inhibitory factor; NEG, negative; NKG7, natural killer cell granule protein 7; ns, not significant; PRG2, proteoglycan 2; SLAMF7, signaling lymphocytic activation molecule family member 7; TLR, Toll-like receptor.

(perforin 1; *PRF1*), and granzyme B (*GZMB*) were lowly expressed (Fig. 4C). Other molecules relating to chemotaxis, apoptosis, immune response, transcription factors, and signal transducers have also been tested (Supporting Fig. S4B,C). Furthermore, gene set enrichment analysis (GSEA) revealed that most highly enriched gene sets in CD96$^+$ NK cells overlap with published gene signatures relating to negative regulation of NK-mediated immunity, IFN-γ production, cell killing, aging, and activation, suggesting a

rather regulatory role of CD96$^+$ NK cells (Fig. 4D,E). Pathway enrichment network illustrates the overall representation of biological pathways relatively dominated by down-regulated (green) or up-regulated (red) genes (Supporting Fig. S4D). Notably, regulation of immune response-involved genes exhibited the most and largest nodes, suggesting the role of CD96 in the regulation of immune responses. Meanwhile, verification through quantitative PCR showed that inhibitory receptors, such as PD-1 (*PDCD1*) and NKG2A (*KLRC1*), were significantly up-regulated, whereas certain secreted factors, such as perforin (*PRF1*) and IL-15 (*IL15*), were down-regulated in CD96$^+$ NK cells (Fig. 4F and Supporting Fig. S4E). Altogether, these data suggest that CD96$^+$ NK cells are functionally exhausted compared to CD96$^-$ NK cells.

CD96$^+$ CELL DENSITY IS ASSOCIATED WITH POOR CLINICAL OUTCOMES OF HCC PATIENTS

To address the predictable potential of CD96, we analyzed the integral optical density (IOD)/area of CD96 in 236 HCC patients. IOD/area of CD96 significantly increased in IT (Fig. 5A,B) and was negatively correlated with immune scores (Fig. 5C). Patients with higher intratumoral CD96 density were associated with later tumor node metastasis (TNM) stage, multiple tumors, presence of tumor thrombus, and higher mortality and recurrence rates (Fig. 5D). Although positive correlation has been observed between intratumoral CD96 density and tumor volume or alpha-fetoprotein level, they were statistically insignificant (Supporting Fig. S5A,B). In addition, a significant negative correlation was found between intratumoral CD96 density and disease-free survival (DFS) or overall survival (OS), but not between peritumoral CD96 density and DFS or OS (Supporting Fig. S5C,D). Patients were further divided into two groups based on the minimum *P*-value cut-off values of their CD96 densities. Patients with lower intratumoral CD96 density had longer OS ($P < 0.0001$) and DFS ($P < 0.0001$; Fig. 5E). A positive correlation was noted between intratumoral CD96 and CD155 densities (Supporting Fig. S5E), and patients with lower CD155 and lower CD96 density within tumor had significantly longer DFS and OS (both $P < 0.0001$; Supporting Fig. S5F). Univariate analysis, Cox regression, and multivariate Cox proportional hazard analysis also confirmed CD96 as an indicator of prognosis (Supporting Tables S1 and S2). HCC patients who had recurrence were accompanied by a higher cumulative percentage of CD96$^+$ NK cells, and consistent with findings on general CD96 density, patients with lower cumulative percentage of CD96$^+$ NK cells exhibited significant better DFS ($P = 0.0484$; Fig. 5F). However, unlike CD96 density, which significantly affects OS, the cumulative percentage of CD96$^+$ NK cells showed no significant effects on the OS of HCC patients (Supporting Fig. S5G). Together, these results indicate that CD96 may act as a prognostic marker in predicting the survival of HCC patients; patients with higher intratumoral CD96 expression are often accompanied by more deteriorating disease condition, higher recurrence rate, and shorter survival time. CD96$^+$ NK cells play a more important role in predicting the recurrence of HCC than in predicting OS of HCC.

HIGHER CD155 EXPRESSION IS CORRELATED TO A POORER PROGNOSIS OF HCC PATIENTS

The imbalance between CD96, TIGIT, and CD226 signaling requires interaction with their common ligand CD155.[14] CD155 expression was significantly increased in IT compared to PT in 236 HCC patients (Supporting Fig. S6A,B). Significant negative correlation was found between intratumoral CD155 density and DFS or OS (Supporting Fig. S4C,D). No significant correlation was observed between peritumoral CD155 density and DFS or OS (data not shown). To further assess the predictive potential of CD155$^+$ cells in the tumor, patients were divided into two groups using the minimum *P*-value cut-off values for CD155 densities.[25-27] The survival curves indicated that lower intratumoral CD155 density correlates with better DFS and OS (Supporting Fig. S6E,F). In addition, patients with lymph node metastases showed significantly higher CD155 expression than those without metastasis (Supporting Fig. S6G). Patients with higher intratumoral CD155 expression exhibited significantly higher incidence (13.59%) of lymphatic metastasis than those with lower CD155 expression (7.69%; Supporting Fig. S6H). Cox regression and time-to-event outcome analysis further indicated that

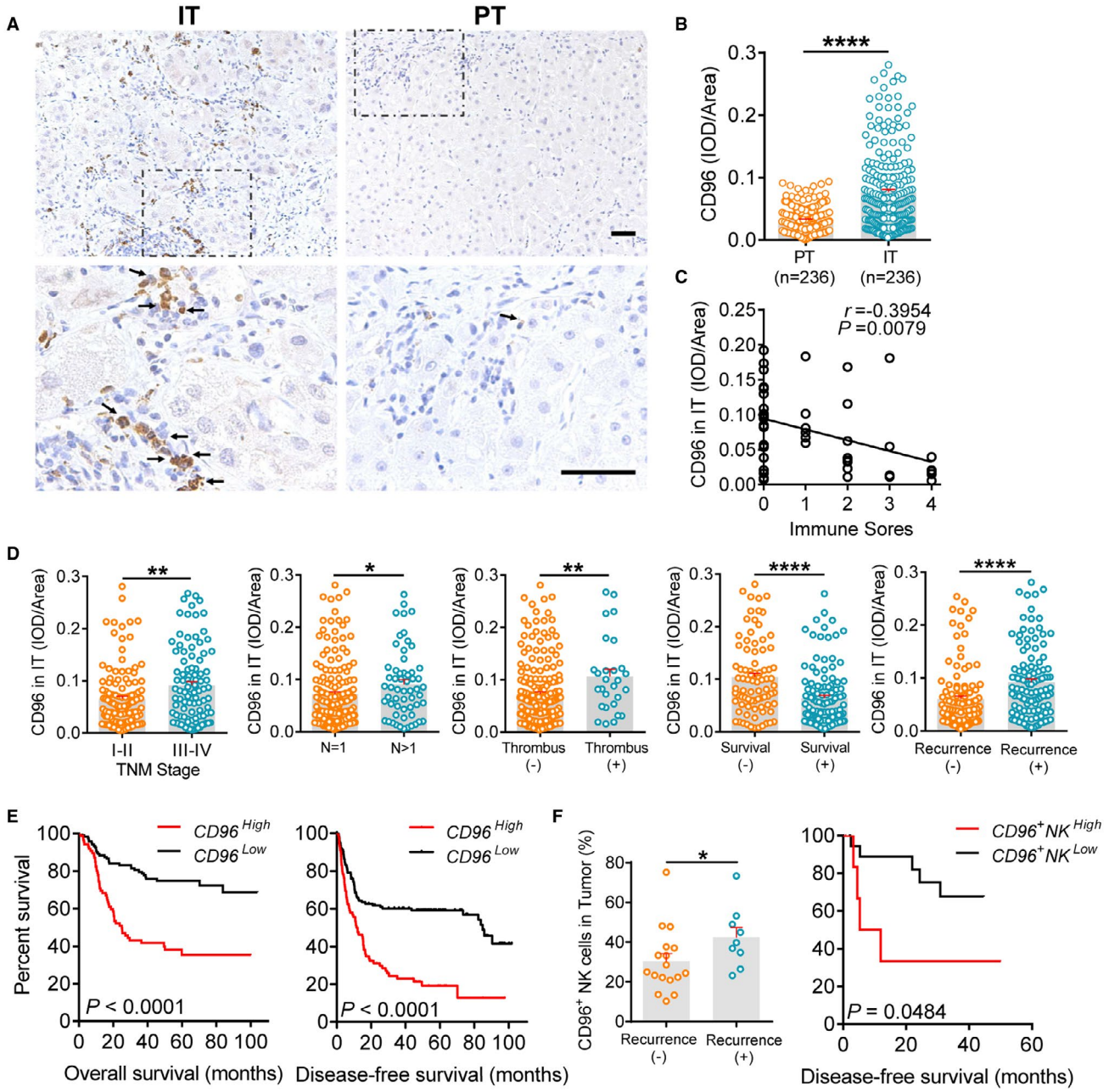

FIG. 5. Higher intratumoral CD96$^+$ cell density is associated with poor disease condition and clinical outcomes in HCC patients. (A) Representative micrographs showing CD96$^+$ cells in IT and PT of HCC patients. Original magnifications: ×20 (top) and ×40 (bottom). Bar = 50 μm. (B) IOD/area of CD96 in PT and IT of HCC patients (N = 236; Mann-Whitney U test). (C) Correlation between intratumoral CD96 expression and immune sores (N = 44). Pearson's correlation coefficient and *P* values are shown. (D) Densities of intratumoral CD96$^+$ cells were compared between patients with different TNM stages, tumor number, presence/absence of tumor thrombus, survival status, or recurrence status (Mann-Whitney U test). (E) Kaplan-Meier survival curves for the duration of OS (left) and DFS (right) in months, according to intratumoral CD96$^+$ cell density (low densities, black; high densities, red; log-rank test). (F, left) Percentage of intratumoral CD96$^+$ NK cells were compared between patients with different recurrence status (Mann-Whitney U test). (F, right) Kaplan-Meier survival curve for the duration of DFS in months, according to the cumulative percentage of intratumoral CD96$^+$ NK cells (low densities, black; high densities, red; log-rank test). Results are expressed as means ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

intratumoral CD155 expression significantly influences DFS of HCC patients ($P = 0.0011$; hazard ratio [HR] = 1.93), as well as TNM staging ($P < 0.0001$; HR = 1.42), diameter of tumor ($P = 0.0013$; HR = 1.99), and the occurrence of tumor thrombus ($P = 0.0028$; HR = 2.33; Supporting Tables S1 and S2). Moreover, CD155 expression was also significantly increased in breast, lung, colon, and pancreatic cancer tissues (Supporting Fig. S6I).

CD96 EXPRESSION ON NK CELLS IS SUSTAINED BY HIGH LEVEL OF TGF-BETA1

Previous studies have demonstrated the role of TGF-β1 in regulating NK cells of HCC-related chronic hepatitis B (CHB) patients and the role of IL-10 in patients with liver cancer.[25,28] Here, we found that the level of TGF-β1 is highly elevated in HCC patients compared to HCs (Fig. 6A). Furthermore, significant positive correlation existed between the cumulative percentage of peripheral $CD96^+$ NK cells in HCC patients and their corresponding plasma TGF-β1 levels (Fig. 6B). To evaluate the possible roles of IL-10 and TGF-β1 in the induction of CD96, we preincubated healthy primary NK cells with different concentrations of IL-10 or TGF-β1 for 72 hours. No significant differences were found in expression of CD96, TIGIT, and CD226 on NK cells in the presence of exogenous IL-10 (Fig. 6C,D and Supporting Fig. S7). Meanwhile, the percentage and MFI of CD96 on NK cells were markedly up-regulated and expression of CD226 and TIGIT on NK cells were down-regulated in the presence of exogenous TGF-β1 (Fig. 6D and Supporting Fig. S7). Furthermore, incubation with HCC plasma significantly increased CD96 expression on NK cells compared to control, and treatment with anti-TGF-β1 antibody partially restored expression of CD96 on HCC plasma-incubated NK cells (Fig. 6D and Supporting Fig. S7). More important, $TIGIT^+$ and $CD226^+$ NK cells transformed into $CD96^+$ NK cells after 72 hours of incubation with HCC plasma, and neutralizing anti-TGF-β1 antibody inhibited the overtransformation of CD96 on HCC plasma-incubated NK cells (Fig. 6E). A similar phenomenon was also found in the case of IFN-γ producing NK cells (Fig. 6F). These data suggest that exogenous TGF-β1 or HCC plasma-derived TGF-β1 increases the expression of CD96 on NK cells, breaks the balance between CD96, TIGIT, and CD226, and decreases IFN-γ production by NK cells. Blocking TGF-β1 restores CD96 expression to normal level and reverses the dysfunction of NK cells.

Discussion

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 PD-1 have shown striking success in the treatment of several cancers.[14] CD96 and TIGIT, together with costimulatory CD226, form a pathway that is very similar to the CD28/CTLA-4 pathway. They bind the common ligand, CD155, and the balancing between three receptors may fine-tune the immune responses against tumors.[14] CD96 has been reported to express on primary human peripheral NK cells and various NK cell lines,[29] and increased expression of CD96 has been identified in patients with acute myeloid leukemia,[30] lung squamous cell carcinoma,[11] CHB, and liver cirrhosis.[23] Consistent with previous findings, our data show increased cumulative percentage, absolute count, and MFI of $CD96^+$ NK cells in HCC patients, which breaks the balance between CD96, TIGIT, and CD226 expression. However, the imbalance only occurs on NK cells, but not on $CD8^+$ T cells.

Several studies have pointed out the exhaustion of NK cells in the HCC microenvironment; NK cells in patients with HCC show impaired cytotoxicity and cytokine production.[1,31-33] Generation of CD96 knockdown mice has demonstrated the role of CD96 as a coinhibitory receptor. Studies using *Cd96*$^{-/-}$ mice demonstrate that CD96 can directly suppress NK cell function[13] and its ability to produce cytokines.[6] Furthermore, *Cd96*$^{-/-}$ mice are more susceptible to lipopolysaccharide-induced inflammation and show resistance to carcinogenesis and experimental lung metastases.[13] Blocking CD96 also suppresses experimental lung metastasis in various tumor models.[10,13] The regulatory role of CD96 has also been established in our study. Comparison between human hepatic $CD96^+$ and $CD96^-$ NK cell subsets through transcriptomic analysis shows that human $CD96^+$ NK

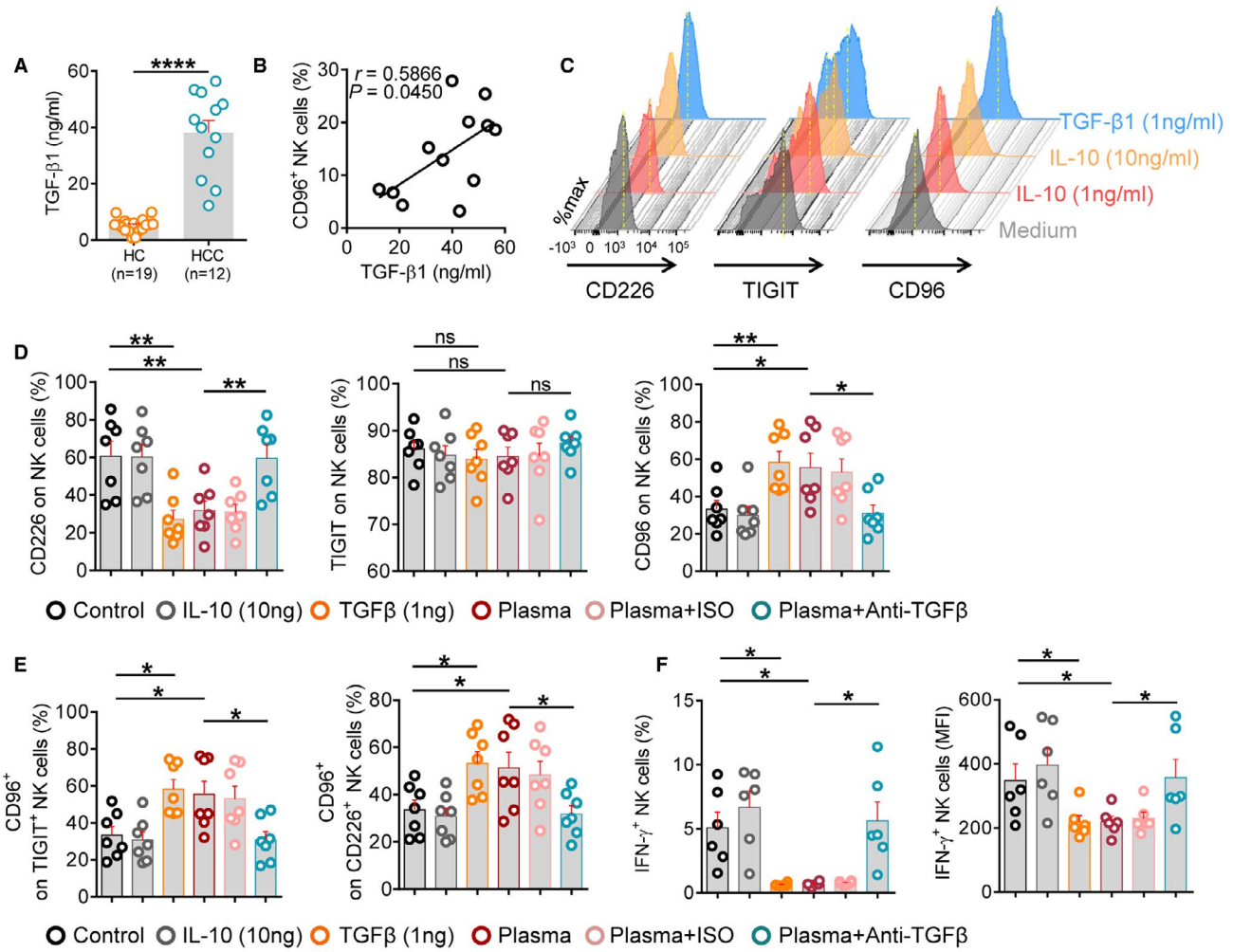

FIG. 6. Increased CD96 expression on NK cells is induced by high level of TGF-β1. (A) Concentration of TGF-β1 detected in plasma of HCs (N = 19) and HCC patients (N = 12; Mann-Whitney U test). (B) Correlation between cumulative percentage of $CD96^+$ NK cells in the blood and their corresponding plasma TGF-β1 level of HCC patients. Pearson's correlation coefficient and *P* value are shown. (C) Peripheral NK cells from HCs were preincubated with TGF-β1 or IL-10 as described in the Materials and Methods. TIGIT, CD226, and CD96 expression were monitored at 72 hours by flow cytometry. 7-aminoactinomycin D has been used to exclude dead/dying cells. Histogram corresponds to NK cells treated with medium alone (gray), TGF-β1 (1 ng/mL, blue), or IL-10 (1 ng/mL, red; 10 ng/mL, orange) in 1 representative donor. Healthy PBMC-derived NK cells were cultured with either medium alone, TGF-β1 (1 ng/mL), IL-10 (10 ng/mL), HCC plasma, HCC plasma combined with anti-TGF-β1 Ab, or HCC plasma combined with control IgG for 3 days. (D) Percentage of $CD226^+$ (left), $TIGIT^+$ (middle), or $CD96^+$ (right) NK cells was monitored by FACS. (E) Percentage of $CD96^+TIGIT^+$ (left) or $CD96^+CD226^+$ (right) NK cells was analyzed by FACS. (F) Percentage and MFI of IFN-γ^+ NK cells are shown. Cumulative data were analyzed by the paired *t* test. Results are expressed as means ± SEM of at least 6 individuals from more than three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Abbreviations: Ab, antibody; FACS, fluorescent-activated cell sorting; IgG, immunoglobulin G; ns, not significant.

cells are enriched with inhibition- and exhaustion-related genes. Furthermore, the cumulative percentage of peripheral $CD96^+$ NK cells dramatically decreases, and their capacity to produce IFN-γ and TNF-α is severely impaired after PMA/ionomycin stimulation. Moreover, lysis of K562 cells by PBMCs is augmented in the presence of anti-CD96 antibody, anti-TIGIT antibody, or combination of anti-CD96 and anti-TIGIT antibodies. The regulatory role of $CD96^+$ γδT cells in cytotoxicity against HCC cell lines has also been reported.[34] The work by Olivier Toutirais et al. shows that lysis of HepG2 by γδT cells is augmented

in the presence of antibody against CD96, which is even more evident in lysis of HuH7 cells.[34]

CD155 is the shared ligand for CD96, TIGIT, and CD226; it is barely or weakly expressed in various normal human tissues and can be induced through cellular stress. CD155 is frequently overexpressed in malignant-transformed cells as well as antigen-presenting cells (macrophages and dendritic cells) within the tumor microenvironment.[16] CD96 and TIGIT can counterbalance CD226-mediated NK cell activation by interacting with CD155.[35] CD96 shows a medium binding affinity to CD155, which is weaker than that of TIGIT, but is stronger than that of CD226. The inter-relationships of CD96, TIGIT, and CD226 on NK cells and the dominance of any one pathway depend on relative receptor expression available to transmit ligand signals, as well as expression of relevant ligands.[14,36] A study by Chan et al. has indicated that CD96 competes with CD226 for CD155 binding and directly suppresses NK cell function by binding with CD155.[13] In CD155-overexpressing liver cancer, increased interaction between $CD96^+$ NK cells and $CD155^+$ tumor cells is likely to bypass the activation signal of CD226, resulting in the exhausted phenotype and impaired function of NK cells. Our study shows that lysis of HepG2 cells by $CD96^+$ NK cells is significantly increased in the presence of anti-CD155 antibody compared to lysis of HepG2 cells by $CD96^-$ NK cells, indicating that blocking CD96-CD155 interaction can effectively reverse CD96-induced exhaustion and restore normal immune responses of NK cells. Of note, patients with higher CD155 and CD96 expression are associated with shorter DFS and OS, suggesting the therapeutic value of blocking CD96-CD155 in treating HCC. Besides combined use of CD96 and CD155, each of them also has the potential to act as a prognostic factor in predicting the clinical outcomes of HCC. In addition, patients with higher cumulative percentage of $CD96^+$ NK cells within tumor exhibit significantly worse DFS; however, it shows no effects on the OS of HCC patients, suggesting that $CD96^+$ NK cells play a more important role in predicting recurrence of HCC than in predicting OS of HCC. On the other hand, follow-up times of HCC patients contributing fresh liver tissues are around 50 months, which are much shorter compared to the 100-month follow-up times in patients contributing paraffin-embedded samples. The shorter follow-up time makes $CD96^+$ NK cells less significant in predicting OS of HCC in terms of statistical analysis.

Recent findings have demonstrated the role of TIGIT as a checkpoint molecule associated with NK cell exhaustion in tumor-bearing mice and patients with colon cancer.[37] Unlike expression of TIGIT in mice, it is constitutively expressed on NK cells in humans. Basal expression of TIGIT is extremely high in normal peripheral NK cells (~90%) and relatively high in normal intrahepatic NK cells (~70%). CD96, on the other hand, is lowly expressed in healthy livers whereas it is highly expressed in HCC. Expression of CD96 increases whereas the expression of TIGIT decreases in HCC, and our *in vitro* experiment shows that $TIGIT^+$ NK cells may transform into $CD96^+$ NK cell under the influence of TGF-β1; these data all suggest that CD96 plays a more important role than TIGIT in the HCC microenvironment.

Exogenous TGF-β1 or TGF-β1 from HCC plasma reduces the cumulative percentage and MFI of CD96 on NK cells; adding TGF-β1-antibody to HCC plasma culturing system may specifically reverse the effect of TGF-β1 on expression of CD96. However, detailed molecular mechanism underlying the CD96-CD155 signaling pathway, and its relationship with NK cell exhaustion and tumor progression still merits further research. Study of underlying mechanisms in humans is difficult because of limited samples and difficulties in manipulation; however, it is possible with murine models. For example, antimouse CD96 monoclonal antibodies inhibit experimental metastases in B16F10 melanoma, 3LL lung carcinoma, and RM-1 prostate carcinoma models, and this antimetastatic activity of anti-CD96 antibody is dependent on NK cells, CD226, and IFN-γ, but independent of activating Fc receptors.[10] Moreover, the reason as to why the cumulative percentage of inhibitory $CD96^+$ and stimulatory $CD226^+$ double-positive NK cells increases in the tumor microenvironment needs to be solved, and the potential of CD96 blockade in regulating other immune cells, such as $CD4^+$, $CD8^+$, γδ T cells, and macrophages, needs to be considered. Because of limited supply and ethical restriction of fresh HCC tissues, our functional experiment was based on $CD96^+$ NK cells collected from healthy peripheral blood; future functional analysis on intratumoral $CD96^+$ NK cells may explain the potential differences between tumor and healthy $CD96^+$ NK cells.

Multiple immunosuppressive pathways coexist in the tumor microenvironment and their cotargeting may provide a more powerful immune response than targeting each one alone. Our study demonstrates that although anti-CD96 or anti-TIGIT antibody alone could increase the effectiveness of PBMC lysis against K562 cells, combined treatment of anti-CD96 and anti-TIGIT antibodies shows a more powerful boost in the lysis of K562 cells. A recent study using *Pdcd1*$^{-/-}$*CD96*$^{-/-}$ and *Tigit*$^{-/-}$*CD96*$^{-/-}$ mice shows increased suppression of subcutaneous tumor growth and complete response in *Pdcd1*$^{-/-}$*CD96*$^{-/-}$ mice compared to *Pdcd1*$^{-/-}$ or *CD96*$^{-/-}$ mice.[38] Neoadjuvant PD-1 blockade followed by adjuvant inhibition of CD96 also significantly prevents relapse of pancreatic ductal adenocarcinoma.[39] Anti-CD96 shows more effectiveness in combination with anti-CTLA-4 or anti-PD-1, coblockade of CD96 and PD-1 potently inhibits lung metastases and increases local NK cell IFN-γ production and infiltration, whereas blocking CD96 in *Tigit*$^{-/-}$ mice significantly reduces metastases.[10]

In summary (Fig. 7), our study reports dynamic imbalance between expression of CD96, TIGIT, and CD226 on NK cells and shows increased expression of CD96 on intratumoral NK cells of HCC patients. CD96^{+} NK cells are functionally exhausted with impaired cytokine production. Primary hepatic CD96^{+} NK cells are enriched with genes associating with negative regulation of immune responses and NK-mediated immunity as revealed by transcriptomic analysis. Furthermore, HCC patients with a high level of CD96 or CD155 expression within tumor are strongly associated with deteriorating disease condition and poorer outcomes. HCC patients with higher cumulative percentage of CD96^{+} NK

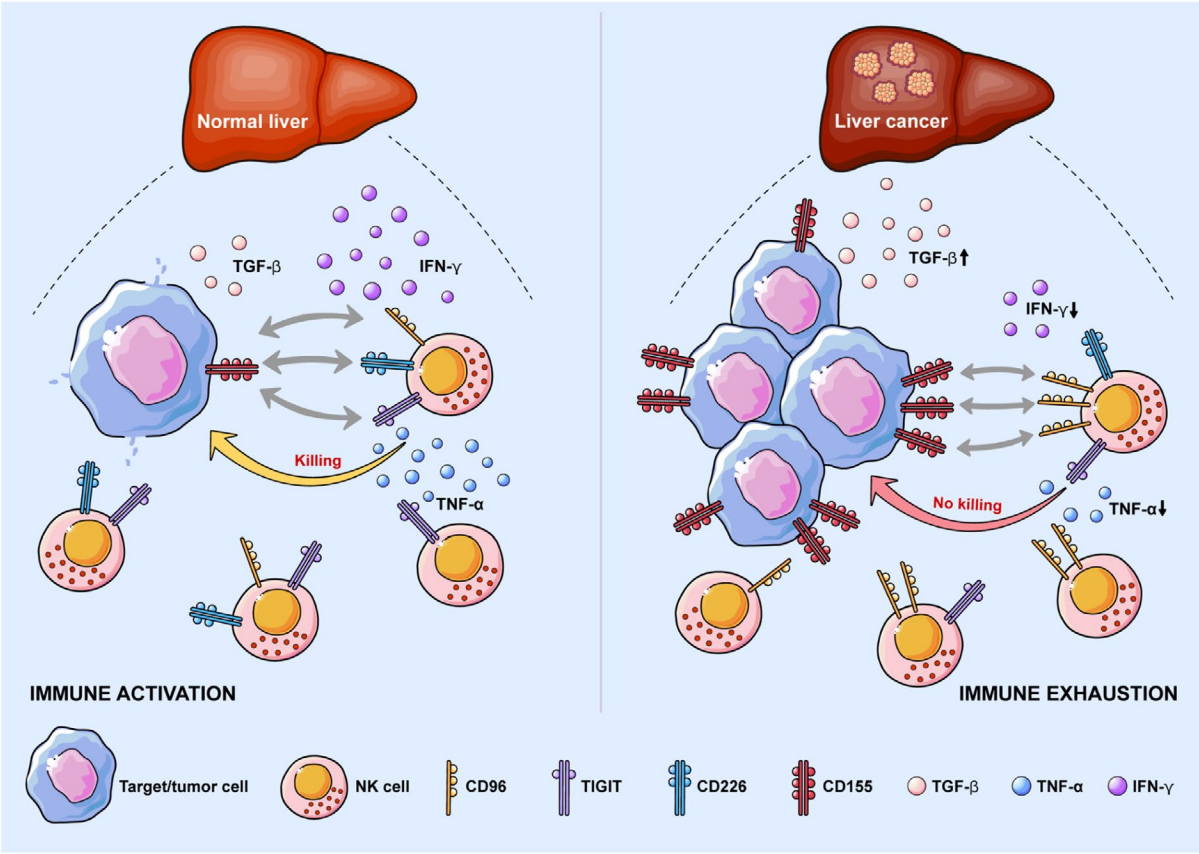

FIG. 7. HCC microenvironment breaks the balance between CD96, TIGIT, and CD226 on NK cells by increasing expression of CD96, which, in turn, impairs the antitumor immune responses of NK cells. Postulated mechanisms showing inhibitory receptor CD96, as well as its ligand CD155, significantly expand in the cancer nest. Induction of CD96 on NK cells and the binding with their ligand CD155 on tumor cells specifically impairs cytotoxicity and cytokine secretion of NK cells, contributing to immune escape in HCC.

cells within tumor also exhibit shorter DFS. A high patient plasma level of TGF-β1 up-regulates CD96 expression and dynamically shifts the balance between CD96, TIGIT, and CD226 in NK cells. Blocking TGF-β1 restores CD96 expression and IFN-γ production of NK cells, indicating a possible intervention approach. Cotargeting of CD96 with other immunosuppressive receptors may provide a more powerful boost in antitumor immune responses and pave the way for future development of therapeutics in fighting liver cancer.

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Supporting Information

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