Liver-infiltrating CD11b−CD27− NK subsets account for NK-cell dysfunction in patients with hepatocellular carcinoma and are associated with tumor progression

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Natural killer (NK) cells have a vital role in killing hepatocellular carcinoma (HCC) cells; however, the mechanism underlying tumor-infiltrating NK (TINK)-cell dysfunction remains poorly understood. Using flow cytometry staining, we precisely characterized the frequency, phenotype and function of NK subsets distinguished by CD27 and CD11b in 30 patients with HCC in comparison to 30 healthy controls. Interestingly, we found a substantial proportion of liver-infiltrating CD11b−CD27− (DN) NK subsets in tumor tissue from HCC patients. Remarkably, these relatively expanded DN NK subsets exhibited an inactive and immature phenotype. By detecting the expression of CD107a and interferon-gamma (IFN-γ) on NK subsets and NK cells, we demonstrated that DN NK subsets exhibited a poor cytotoxic capacity and deficient potential to produce IFN-γ in comparison to the other three subsets, which contributed to the dysfunction of TINK cells in HCC patients. In addition, we found that the presence of DN NK cells was closely associated with the clinical outcomes of HCC patients, as the frequency of DN NK cells among TINK cells was positively correlated with tumor stage and size. A large percentage of DN NK cells among TINK cells was an independent prognostic factor for lower survival in the 60-month follow-up period. In conclusion, a substantial proportion of CD11b−CD27− NK subsets among TINK cells accounts for NK-cell dysfunction in patients with HCC and is associated with tumor progression. Our study may provide a novel therapeutic target for the treatment of patients with HCC.

INTRODUCTION

Patients infected with hepatitis B (HBV) or hepatitis C virus have a higher risk of developing hepatocellular carcinoma (HCC), and chronic HBV infection accounts for over 85% of HCC cases in China.¹ HCC is currently the leading cause of cancer-related deaths worldwide.²,³ Although surgery that includes liver resection and transplantation is the most effective treatment for HCC patients, the recurrence and exacerbation of HCC after surgery remain serious issues.⁴,⁵ To apply novel and efficacious therapies, including immunotherapies, a comprehensive understanding of anti-tumor immune responses is essential. Natural killer (NK) cells display at least two effector functions against neoplastic cells: they can mediate direct cytotoxic activity through degranulation and they are also able to produce a variety of anti-tumor active and immunoregulatory cytokines, such as interferon-gamma (IFN-γ).⁶ Studies have reported a key role for NK cells in the destruction of multiple tumor cell lines derived from human tumors.⁷–¹⁰ However, clinical evidence that NK cells can control tumors in humans remains limited because tumor-infiltrating NK (TINK) cells display functional impairments that contribute to the escape of tumor cells from NK-cell attack.¹¹,¹² It is likely
that the molecular and cellular mechanisms underlying NK-cell dysfunction in HCC have not been defined.

As the first defense of the host immune system against tumors, NK cells account for a large proportion of cells in the liver, with a percentage nearly five times greater than the percentage observed in the blood or spleen. Human NK cells undergo four differentiation stages with respect to the cell-surface density of CD34, CD117 and CD94. NK cells are typically defined as CD56\(^{+}\)CD3\(^{-}\) large granular lymphocytes, and the CD56 marker is highly expressed only during stage 4 (CD34\(^{-}\)CD117\(^{-}\)CD94\(^{-}/+\)). Therefore, the heterogeneity of NK cells during stage 4 has attracted significant attention. On the basis of the expression levels of CD56, human NK cells can traditionally be divided into two main subsets: CD56\(^{bright}\) NK cells in the lymphoid organs and CD56\(^{dim}\) NK cells, found predominantly in the peripheral blood. CD56\(^{bright}\) NK cells perform predominantly immunoregulatory functions that are mediated by their potent cytokine secretion ability, whereas CD56\(^{dim}\) NK cells have a marked cytotoxic function. CD57 was reported to distinguish functionally mature NK subsets of human CD56\(^{dim}\)CD16\(^{+}\) NK cells. Another study demonstrated that the expression of CD94 can differentiate between functional intermediary subsets of CD56\(^{bright}\) and CD56\(^{dim}\) human NK subpopulations. Furthermore, the Tim-3 protein has a negative role in NK-cell maturation and suppresses cell-mediated cytotoxicity. Despite our knowledge of these two subsets, little evidence is available concerning the discrete stages that represent human NK subsets with respect to both maturation and functional divisions. Thus, much effort has recently been devoted to subdividing human NK cells into functionally distinct subpopulations. On the basis of global gene profiles and adoptive transfers of NK subsets, murine NK cells are reported to undergo a four-stage developmental process. Resembling the subsets in mice, human NK cells have been further divided into four functionally distinct subsets based on the surface density of CD27 and CD11b: CD11b\(^{-}\)CD27\(^{-}\) (DN), CD11b\(^{+}\)CD27\(^{-}\) (CD27\(^{+}\) SP), CD11b\(^{+}\)CD27\(^{+}\) (DP) and CD11b\(^{+}\)CD27\(^{-}\) (CD11b\(^{+}\) SP). The specific micro-environment and complex cellular interactions provide crucial signals for modifying the features of NK cells. To investigate whether the NK subsets distinguished by CD27 and CD11b were shaped in the HCC microenvironment and contributed to tumor progression, we precisely characterized the frequency, phenotypes and functions of NK subsets in HCC patients. We provide evidence that DN NK cells preferentially accumulate in tumor tissue. Consistent with an inactive and immature phenotype, these DN NK subsets display poor cytotoxic capacity and an impaired ability to produce cytokines. Moreover, the substantial presence of DN NK cells contributes to NK-cell dysfunction in patients with HCC and is associated with tumor progression. Importantly, we demonstrate an innovative mechanism that underlies the escape of tumor cells from NK-cell attack and is mediated by DN NK cells during anti-tumor immune responses.

### MATERIALS AND METHODS

**Patients**

Peripheral blood and tumor tissue were collected from the Department of Hepatobiliary Surgery at the First Affiliated Hospital of Chongqing Medical University. HCC patients were diagnosed according to the diagnostic guidelines of the European Association for the Study of the Liver. Thirty patients with HBV-related HCC were enrolled in this study, none of whom received chemotherapy or radiotherapy. On the basis of the Barcelona Clinic Liver Cancer (BCLC) staging classification, 10 patients were classified as stage A, 10 as stage B and 10 as stage C. Detailed patient characteristics are shown in Table 1. Fresh tumor tissues, paired adjacent non-tumor liver tissues (at least 5 cm from the tumor margin) and peripheral blood samples from these 30 patients were used for further determinations. As controls, 12 liver tissue samples were obtained from patients with benign hepatic hemangioma, and peripheral blood samples were obtained from 30 healthy individuals. For survival analysis, 30 HCC patients who had undergone surgical resection were studied from 2010 to 2015. All samples were collected after obtaining written informed consent from the donors and following approval by the Hospital Ethic Review Committee of the First Affiliated Hospital of Chongqing Medical University.

**Isolation of lymphocytes from samples**

Freshly dissected tumor and non-tumor tissues were disrupted mechanically and then digested with 0.1% collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA) in serum-free RPMI 1640 for 1 h under agitation. After enzymatic digestion, the single-cell

### Table 1 Basic clinical features of 30 HCC patients

| Clinical factors | Stage A | Stage B | Stage C |
|------------------|---------|---------|---------|
| Cases            | 10      | 10      | 10      |
| Ages (years)     | 26.5 (33–51) | 45 (30–55) | 48 (35–55) |
| Male/female      | 7/3     | 8/2     | 8/2     |
| Tumor diameter (mm) | 25 (21–30) | 40 (34–45) | 64 (47–78) |
| HBV-DNA (–/–<10^5/>10^5 IU/ml) | 5/2/3 | 5/3/2 | 6/3/1 |
| AFP (ng/ml)      | 368 (0–574) | 789 (508–1213) | 696 (475–819) |
| Child-Pugh (A/B) | 7/3     | 7/3     | 6/4     |

Abbreviation: AFP, α-fetoprotein.
suspensions were filtered and washed, and mononuclear cells (MNCs) were purified. Using Ficoll-Hypaque density-gradient centrifugation, tumor-infiltrating lymphocytes (TILs), non-tumor-infiltrating lymphocytes (NILs), and peripheral blood mononuclear cells (PBMCs) were isolated.

**Flow cytometric analysis**

Lymphocytes from human blood/tumor samples were incubated for 30 min with the following mouse anti-human monoclonal antibodies: anti-CD45, anti-CD11b and anti-NKp80 (Biolegend, San Diego, CA, USA); anti-CD3, anti-CD56, anti-CD27, anti-CD7, anti-CD2, anti-CD57, anti-CD11c, anti-CD117 and anti-CD127 (eBioscience, San Diego, CA, USA); anti-NKG2D, anti-NKp30, anti-CD226 and anti-NKp46 (BD Bioscience, San Jose, CA, USA); anti-NKG2A (R&D Systems Inc, Minneapolis, MN, USA); and KIR2DL1/DS1, KIR2DL2/DS3/DS2, and KIR3DL1/DS1 (Beckman Coulter, Fullerton, CA, USA). Mouse serum was used to block nonspecific Fc receptor binding, and isotype-matched IgGs were used as negative control antibodies. The samples were analyzed using a fluorescence-activated cell sorter Calibur flow cytometer (BD Biosciences). Flow cytometry data were analyzed using the FlowJo software (Tree Star, Inc., Ashland, OR, USA).

**Degranulation assay**

The CD107a degranulation assay was performed as an indirect measure of cytotoxicity, as this assay is now widely used to assess the cytotoxic potential of CD8 T cells and NK cells.\(^{34,35}\) Lymphocytes from human tumor samples were stimulated with PMA (phorbol 12-myristate 13-acetate) (50 ng/ml, Sigma) and ionomycin (Calbiochem, Darmstadt, Germany) for 1 h. Anti-CD107a (eBioscience) and monensin (10 μg/ml; Sigma) were added directly to the medium and incubated for 4 h. The cells were then collected and stained with surface antibodies.

**IFN-γ detection**

After stimulation with 50 ng/ml of PMA (Sigma) plus 1 μg/ml of ionomycin (Calbiochem, Darmstadt, Germany) for 1 h at 37 °C in 5% CO\(_2\), the cells were cultured with 10 μg/ml of monensin (Sigma) for 4 h. After staining with surface antibodies, the cells were fixed and permeabilized, followed by intracellular staining for IFN-γ (eBioscience) in the dark. Finally, the cells were detected by flow cytometry.

**Statistical analysis**

Statistical significance was tested using the Mann–Whitney test and the Kruskall–Wallis ANOVA for non-parametric continuous data. Correlations between variables were analyzed using the Spearman’s correlation coefficient (r). Overall survival (OS) curves were estimated using the Kaplan–Meier method, and differences between groups of patients were evaluated using the log-rank test with a minimal P-value. Calculations were performed using GraphPad Prism version 5.00 (GraphPad Software, Inc., San Diego, CA, USA).

**RESULTS**

**NK cells accumulate in tumor tissue**

We first detected the distribution of NK cells in liver-infiltrating lymphocytes (LILs) form controls and HCC patients via flow cytometry staining. The gating strategy for LILs is described in Figure 1a. A substantial population of NK cells was observed among LILs from HCC patients, particularly among TILs, in comparison to lymphocytes from control livers (Figure 1b). No difference in the frequency of circulating NK cells was observed between HCs and HCC patients (Figure 1c). These results indicate that a large population of NK cells accumulates in the tumor tissue of HCC patients.

**DN NK cells preferentially accumulate among TINK cells**

To further determine the impact of HCC on NK subsets, we compared the frequency of NK subsets distinguished by CD11b and CD27 between controls and HCC patients. In comparison with adjacent non-tumor tissue and control livers, the percentage of liver-infiltrating DN NK cells from tumor tissue was increased significantly, whereas the percentages of liver-infiltrating CD11b* SP and DP NK subsets were markedly decreased (Figures 2a and c). No significant alteration of NK subsets in the peripheral blood was observed between HCs and HCC patients (Figures 2b and d). These data demonstrate for the first time that liver-infiltrating DN NK cells expand among TINK cells from HCC patients.

**DN NK cells exhibit an inactive and immature phenotype**

We further analyzed the phenotypic characteristics of the four NK subsets from tumor tissue. As illustrated in Figures 3a–f, the expression of activating receptors (NKG2D, NKp30 and CD226) and inhibitory receptors (NKG2A, KIR2DL2/DS2 and KIR3DL1/DS1) among DN NK subsets was downregulated compared to that of the other three subsets, especially the CD11b* SP populations. In addition, the downregulation of CD2, CD7, CD57 and CD11c, which were expressed on highly mature NK cells, was observed on DN NK cells compared to the other three subsets (Figures 4a–d). In addition, the upregulation of CD117 and CD127, which are typically expressed on immature NK cells, was observed on DN NK cells compared to the other three subsets (Figures 4e and f). Hence, the DN NK subsets within tumor tissue exhibit an inactive and immature phenotype, suggesting an impaired function of these subsets.

**DN NK cells account for the dysfunction of TINK cells**

To investigate the functional characteristics of NK cells in HCC patients, we analyzed the expression of CD107a and INF-γ in response to PMA/ionomycin stimulation. Notably, NK cells from TILs displayed poor cytotoxic capacity and deficient potential to produce IFN-γ compared to NK cells from control liver-infiltrating lymphocytes or NILs (Figures 5a and b). To determine whether the dysfunction of TINK cells was attributable to the expansion of DN NK subsets among TINK cells, we assessed degranulation and cytokine production in NK subsets from TINK cells. Consistent with the observed
phenotypic attributes, DN NK cells exhibited significantly reduced levels of CD107a expression and IFN-γ production in comparison to the other three subsets (Figures 5c and d). In addition, the percentages of DN NK subsets expressing CD107a and IFN-γ within TINK cells were positively correlated with TINK degranulation and cytokine production, respectively (Figures 5e and f). Collectively, the substantial presence of DN NK subsets among TINK cells with deficient function contributes to the dysfunction of TINK cells in HCC patients.

The appearance of DN NK cells is associated with tumor progression in HCC patients
To determine the functional role of DN NK cells among TINK cells in HCC, we evaluated the influence of DN NK cells on clinical outcomes. In this study, the HCC patients were divided into stage A, stage B and stage C groups based on the BCLC staging classification. The clinical information for the HCC patients in our study is presented in Table 1. Remarkably, the frequency of DN NK cells among TINK cells increased as the tumors progressed, as illustrated in Figure 6a. We next investigated the relationship between the frequency of DN NK cells and the size of the tumors. As expected, a positive correlation was observed between the frequency of DN NK cells among TINK cells and the maximum diameter of the resected tumors (Figure 6b). We also investigated the OS curve using the Kaplan–Meier method. Furthermore, the included patients were divided into three groups (low, median and high DN NK%) with respect to the frequency of DN NK cells. Analysis of the Kaplan–Meier survival curve revealed significant differences among the three groups during the 60-month follow-up period (Figure 6c). Thus, these observations suggest that the substantial presence of DN NK cells is closely associated with tumor progression.

DISCUSSION
NK cells are vital elements in defense against HCC, and NK-cell-based anti-HCC therapeutic strategies are becoming increasingly attractive.36–38 One striking dilemma with anti-tumor immunotherapies is that tumor cells use multiple strategies to evade NK-cell attack, leading to NK-cell...
dysfunction. However, the exact mechanism underlying NK-cell dysfunction in patients with HCC remains incompletely defined.

Some documents have demonstrated that the NK-cell developmental program is not fully fixed. In addition, tumor microenvironments can drive the accumulation of abnormal NK-cell subsets, which account for a small population in healthy controls and a large population in individuals with tumors. Interestingly, we found that DN NK cells accumulate in tumor tissue in HCC patients but account for only a small population in adjacent non-tumor or control liver tissue. An investigation of markers of mature and immature NK subsets revealed that the DN NK subsets showed an immature phenotype. When considering the mechanism that underlies the phenotypic alteration of TINK cells, we hypothesize that IL-10 or transforming growth factor-β (TGF-β) may have an important role in altering the NK phenotype. It is well documented that tumor cells suppress immune surveillance by secreting immunosuppressive cytokines, such as IL-10 and TGF-β, to evade the anti-tumor immune response. IL-10 impairs dendritic cell maturation on PAX3-FKHR–expressing tumor cells, and tumor cells negatively regulate NK-cell function by releasing the immunosuppressive factor TGF-β in non-small cell lung carcinoma patients. Thus, IL-10 or TGF-β may also be a crucial element in the abnormal phenotypic alteration of NK cells in HCC. We will test this hypothesis in future experiments. Here, we provide evidence that the HCC microenvironment induces the accumulation of immature DN NK subsets, suggesting an impaired function of these DN NK subsets.

Figure 2 DN NK cells preferentially accumulate among TINK cells. Percentages of the four NK subsets among LILs (a) and PBMCs (b) isolated from controls (n=12 and 30, respectively) and patients with HCC (n=30 and 30, respectively, median ± range). Pooled percentages of the four NK subsets among LILs (c) and PBMCs (d) isolated from controls and patients with HCC. (The results were analyzed using the Mann–Whitney U and the Kruskall–Wallis ANOVA tests. Significance was denoted as *P<0.05, **P<0.01 and ***P<0.001.) HCC, hepatocellular carcinoma; LILs, liver-infiltrating lymphocytes; NK, natural killer; PBMCs, peripheral blood mononuclear cells; TINK, tumor-infiltrating NK.
Figure 3 DN NK cells display an inactive phenotype. The percentages of the NK-cell activation receptors NKG2D (a), NKp30 (b), and CD226 (e) and the inhibitory receptors NKG2A (d), KIR2DL2/DL3/DS2 (f) and KIR3DL1/DS1 (f) (n=30, median±range) expressed on four NK subsets gated from CD56⁺CD3⁻ TINK cells. (The results were analyzed using the Kruskall–Wallis ANOVA test. Significance was denoted as *P<0.05, **P<0.01 and ***P<0.001). NK, natural killer.
Figure 4 DN NK cells display an immature phenotype. The percentage of the mature NK-cell markers CD2 (a), CD7 (b), CD57 (c) and CD11c (d) and the immature NK-cell markers CD117 (e) and CD127 (f) ($n=30$, median ± range) expressed on four NK subsets gated from CD56$^+$CD3$^-$ TINK cells. (The results were analyzed using the Kruskall–Wallis ANOVA test. Significance was denoted as *$P<0.05$, **$P<0.01$ and ***$P<0.001$). NK, natural killer; TINK, tumor-infiltrating NK.
Figure 5 DN NK cells account for the dysfunction of TINK cells. The percentages of CD107a (a) and IFN-γ (b) expression on liver-infiltrating NK cells from control livers (n=12) and tissues from patients with HCC (n=30, median ± range). The percentages of CD107a (c) and IFN-γ (d) expression on the four NK subsets gated from CD56^CD3^- TINK cells (n=30). (e) The percentage of CD107a^+ DN NK subsets is positively correlated with the expression of CD107a on TINK cells. (f) The percentage of IFN-γ^+ DN NK subsets is positively correlated with the expression of IFN-γ on TINK cells. (The results were analyzed using the Kruskal–Wallis ANOVA test and the Spearman’s rank correlation test. Significance was denoted as *P<0.05, **P<0.01 and ***P<0.001). HCC, hepatocellular carcinoma; IFN-γ, interferon-gamma; NK, natural killer; TINK, tumor-infiltrating NK.
NK cells have the ability to recognize and kill invading pathogens and cancer cells. This ability of NK cells depends on the delicate balance between activating receptors and inhibiting receptors. NK cells have a strong cytotoxic capacity against tumor cells, but tumor cells and the tumor microenvironment have developed several mechanisms to induce the dysfunction of NK cells and protect tumor cells from NK-cell-mediated attack. Affected by various microenvironments and signals, NK cells can be divided into three functional subsets: NK tolerant (NK cells with dominant inhibitory signals), NK cytotoxic (NK cells with dominant activating signals, target cells with strong expression of pressure stimulus-induced ligand) and NK regulatory (NK cells with dominant activating signals, target cells with strong expression of inflammatory molecules). The NK tolerant subsets are primarily DN NK cells. Our results reveal that the expanded DN NK subsets in tumor tissue from HCC patients exhibit an inactive phenotype, which is consistent with the NK tolerant phenotype. To determine whether the accumulation of DN NK cells had a negative influence on NK function, we also detected the degranulation and IFN-γ secretion among the NK subsets and NK cells in HCC patients. We found that DN NK cells possess reduced cytotoxicity and an impaired capacity to produce IFN-γ. A positive correlation exists between the function of DN NK cells and the function of TINK cells. It is likely that the accumulation of DN NK subsets within TINK cells contributes to TINK-cell functional deficiency. Our results provide some clues concerning the alteration of NK subsets, phenotypes and functions in the tumor microenvironment. Taken together, the above observations suggest that the tumor microenvironment negatively regulates the cellular phenotype and function of TINK cells by inducing a substantial accumulation of DN subsets. The large population of DN subsets among TINK cells with an immature and inactive phenotype suggests that TINK cells tend to be skewed toward an immature and inactive state. Moreover, the accumulation of DN subsets with a profoundly defective ability to activate degranulation and produce IFN-γ is responsible for the impaired functions of TINK cells, which suppresses their ability to attack tumor cells.

Many novel predictors related to the immune response have been explored recently for potential use in determining the prognosis of patients with tumors. The prognostic value of LILs and cytokines in cirrhotic parenchyma of resected HCC for time to recurrence and survival have also been reported. A high density of CD8+ cells in cirrhotic areas distant from the tumor may be a potential prognostic factor for tumor recurrence in HCC patients after resection. In our study, we demonstrate that the frequency of DN NK cells among...
TINK cells increases as the tumors progress. Moreover, a positive correlation exists between the frequency of DN NK cells among TINK cells and the maximum diameter of the resected tumors. In addition, the HCC patients in three groups (low, median and high DN NK%) displayed significantly different survival rates during a follow-up period of 60 months. These results indicate that liver-infiltrating DN NK subsets in the tumor tissue may be valuable for predicting tumor progression in HCC patients.

In conclusion, the present study has provided new insights into NK-subset immune profiles within HCC patients. To our knowledge, this is the first report that inactive and immature DN NK subsets accumulate in tumor tissue from HCC patients. More importantly, our data provide evidence that expansion of the DN NK population may render TINK cells less tumoricidal and thereby contribute to tumor progression. Thus, the presence of aberrant DN NK subsets within the liver-specific microenvironment may represent a novel therapeutic target for the treatment of HCC patients.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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**AUTHOR CONTRIBUTIONS**

DZZ designed, analyzed and provided overall guidance for the experiments. QFZ designed, performed and analyzed the experiments, and wrote the manuscript. WYW, YX, XW, QFH and YYY performed the experiments. All authors contributed to the experiments.
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