Hepatocellular Carcinoma Immunosuppression Is Blocked in Mediators When STAT3-Mediated NK Cells Are the Crucial Antitumor

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NK Cells Are the Crucial Antitumor Mediators When STAT3-Mediated Immunosuppression Is Blocked in Hepatocellular Carcinoma

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STAT3 is highly activated in a wide variety of cancers and functions to promote tumor survival. We previously reported that blocking STAT3 activation inhibited human hepatocellular carcinoma (HCC) growth in vitro, but whether this treatment also triggered antitumor immune responses in vivo remained unknown. In this study, we found that blocking the STAT3 pathway in HCC cells dramatically inhibited murine HCC growth in vivo and prolonged survival of tumor-bearing mice. Importantly, the presence of STAT3-blocked HCC augmented NK cell cytotoxicity against HCC and increased expression of molecules associated with NK cell activation and cytotoxicity. In T cell–deficient nude mice, a unique NK cell–mediated antitumor function against STAT3-blocked HCC was suggested. NK cells were shown to be necessary and sufficient in NK or T cell depletion experiments, or by adoptively transferring NK cells. Furthermore, regulatory T cells and immunosuppressive IL-10 and TGF-β cytokines were reduced in mice bearing STAT3-blocked HCC cells, suggesting that these factors may be involved in HCC-induced NK cell suppression. These findings indicate that blocking STAT3 in HCC cells can initiate innate immunity in vivo. The Journal of Immunology, 2014, 193: 2016–2023.

Hepatocellular carcinoma (HCC) is a worldwide health problem, with ~600,000 new cases diagnosed each year (1). In individuals with HCC, host antitumor immunity is dampened by an immunosuppressive environment, which is characterized by functionally impaired T and NK cells, an abnormal increase in regulatory T cells (Tregs), and cytokine imbalance (2, 3). Several previous studies showed that STAT signaling pathways, especially STAT3, were activated in various solid and hematopoietic tumors (4–6). In our previous study, we found that STAT3 was highly activated in human HCCs and that blocking the STAT3 pathway in tumor cells promoted HCC apoptosis in vitro (6). Because tumor cell–expressed STAT3 also functions to regulate host antitumor immunity (7, 8), whether the blockade of STAT3 in HCCs can restore antitumor immune function in HCC-tumor-bearing hosts in vivo remains incompletely understood.

STAT3 is a transcription factor important in inflammation, immunity, and oncogenesis that is activated by many cytokines, such as IL-6, IL-11, and IL-22 (9, 10). Upon stimulation, STAT3 is phosphorylated at residue Tyr^705 or Ser^727 and forms homo- or heterodimers. Once activated, the STAT3 dimer translocates to the nucleus and transactivates target genes (5, 11). STAT3 activity supports tumor cell survival by upregulating expression of the antiapoptotic protein Bcl-xL as well as other proteins essential for tumor cell proliferation and survival, including myeloid cell leukemia sequence 1, survivin, cyclin D1, and MYC (12).

STAT3 also functions as a specific regulator of procarcinogenic inflammation. It induces and maintains a procarcinogenic inflammatory microenvironment during both the initiation of malignant transformation and cancer progression. Tumor-derived factors, such as vascular endothelial growth factor (VEGF) and IL-10, upregulate STAT3 signaling in various immune cell subsets in the tumor microenvironment, which produce more immunosuppressive factors, including IL-6, IL-10, TGF-β, Foxp3, and VEGF, resulting in further suppression of antitumor immunity (13, 14). For example, the capacity of tumor cell–derived STAT3 signaling to impair dendritic cell (DC) maturation was confirmed in a study on PAX3-FKHR–expressing tumor cells; in this case, IL-10 produced by tumor cells under the control of activated STAT3 inhibited DC maturation (15). In human melanoma cells, STAT3 signaling is required for the production of VEGF, IL-10, and IL-6 immunosuppressive factors that, in turn, inhibit the ability of DCs to produce immunostimulating molecules, such as IL-12 and TNF (16). Other studies show that disrupting STAT3 signaling in tumor cells results in the activation of tumor-Ag–specific CD8+ T cells in vivo (14, 17). Furthermore, STAT3 blockade can also activate innate immune cells, including macrophages and neutrophils, leading to the production of proinflammatory mediators and an increase in cytotoxicity against tumor cells (14). However, whether the tumor-impaired function of NK cells—another important innate effector cell population—can be triggered and restored by disrupting STAT3 in tumor cells is still unknown.

Based on its oncogenic and procarcinogenic inflammatory properties, STAT3 may be an ideal potential molecular target for cancer therapy. Our previous studies demonstrated that blocking
STAT3 in tumor cells inhibited their cell growth in several types of cancer cells, including HCC, lung cancer, and human glioma cells (6, 18, 19). In the current study, we analyzed the characteristics of the host immune response to STAT3-blocked HCC tumor cells in vivo, focusing on determining whether STAT3 blockade could convert impaired NK cells into an activated state as well as clarifying the mechanisms involved in this process.

**Materials and Methods**

**Mice**

Six-week-old male NOD/LtScid (developed by crossing NOD/Lt with SCID mice at The Jackson Laboratory, Bar Harbor, ME), T cell–deficient nude (BALB/c-A-nu), BALB/c, and C57BL/6 mice were obtained from Beijing HFK Bioscience (Beijing, People’s Republic of China). NOD/LtScid mice have impaired T and B cell development as well as markedly reduced NK cell number and activity (20). All procedures were performed in accordance with the Institutional Animal Care and Use Committee protocols of Shandong University.

**Cell lines**

H22 cells (BALB/c-derived hepatoma, obtained from the Shandong Academy of Medical Sciences) and Hepal-6 cells (C57BL/6-derived hepatoma, purchased from Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) were cultured in RPMI 1640 medium (Life Technologies BRL, Gaithersburg, MD), supplemented with 10% FBS (Sijiqing, Hangzhou, People’s Republic of China). All ODNs were prepared at a concentration of 100 μM.

**Transfection of ODN**

Cells were transfected with 100 nM ODN by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 6 h in RPMI 1640 (without FBS). Cells were then collected and cultured in RPMI 1640 containing 10% FBS.

**ELISA**

Serum IFN-γ, TGF-β, and IL-10 levels were measured using an ELISA kit (ExCell Bio, Shanghai, People’s Republic of China).

**Cytotoxicity assay**

H22 and Hepal-6 cells were cultured in 96-well plates for 6 h. Then liver or spleen lymphocytes were added at different Eff:Target ratios. Six hours later, 10 μl (5 mg/ml) MTT (Sangon Biotech, Shanghai, People’s Republic of China) was added. After an additional 4-h incubation, 100 μl supernatant was removed from each well, and 100 μl 10% SDS solution was added to the well to dissolve the formazan crystals. The culture plates were then incubated at 37˚C overnight, and the absorbance at 570–630 nm was determined using a Microplate Autoreader (Bio-Rad, Hercules, CA).

**Isolation of primary NK cells**

Lymphocytes were isolated from peripheral blood, liver, and spleen of mice by Ficoll gradient centrifugation. Primary NK cells were then purified using a DX5 positive-selection isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Primary NK cells were cultured in RPMI 1640 supplemented with 100 U/ml human rIL-2 (Changsheng, Changshu, People’s Republic of China).

**Flow cytometry**

To evaluate cell phenotypes, cells were harvested, washed with PBS, stained with Abs, and incubated for 30 min at 4˚C. After an additional wash, flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Abs used in flow cytometry analysis, including FITC-labeled anti-mouse CD49b (DX5) and NK1.1, PE-labeled anti–CD3 (eBioscience, San Diego, CA), were obtained from eBioscience (San Diego, CA). For the Treg cell assay, cells stained with anti-CD4 and anti-CD25 Abs were fixed using FixPerm Buffer (eBioscience, San Diego, CA) for 30 min, incubated with FITC-conjugated anti-mouse Foxp3 Ab (FJK-16; eBioscience) for 30 min at 4˚C, and analyzed by flow cytometry using a FACSCalibur flow cytometer.

**Western blotting**

Cells were solubilized in lysis buffer (1 × 10⁶ cells/ml) and whole-cell extracts were subjected to SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes, blocked with 5% nonfat milk, and incubated overnight with the following Abs: anti–β-actin, anti-STAT3, anti–phospho-specific STAT3 (Ser727) (Cell Signaling Technology, Beverly, MA), and anti–phospho-specific STAT3 (Ser725) (Santa Cruz Bio-technology, Santa Cruz, CA). After washing, the membranes were then incubated with a HRP-conjugated secondary Ab (Millipore, Billerica, MA). The bands were examined by densitometry using AlphaEaseFC software (version 4.0.0; Alpha Innotech, San Leandro, CA).

**RNA isolation and quantitative real-time PCR**

RT-PCR was performed according to the manufacturer’s instructions. Briefly, total RNA was isolated using TRizol (Invitrogen, Carlsbad, CA), and cDNA was synthesized using the Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using the SYBR Green kit (Bio-Rad) on an Opticon Monitor (Bio-Rad). Levels determined for target genes were normalized to that of the β-actin housekeeping gene (for detailed primer sequences, see Supplemental Table 1).

**Depletion of lymphocyte subsets and cytokine neutralization**

For depleting NK cells, T cells, or Treg cells, each BALB/c mouse was injected with 20 μl anti-ASGM1 Ab (Wako, Osaka, Japan), 100 μl anti-CD3 Ab (eBioscience), or 100 μl anti-CD25 Ab (BD Biosciences), respectively, four times within a 2-wk time period. To neutralize TGF-β1 and IL-10 cytokines, 100 μl anti-TGF-β1 Ab (Abcam, Cambridge, MA) and 100 μl anti–IL-10 Ab (PeproTech, Rocky Hill, NJ) were injected four times within a 2-wk time period. All Abs were i.v. injected into the tail vein.

**Apopotosis determination**

Annexin V–FITC kit (BestBio, Shanghai, People’s Republic of China) was used to measure STT3 decoy ODN-induced HCC cell apoptosis at the 100 nM dose. After treatment with decoy or scrambled ODNs for 12 h, cells were harvested, washed with PBS at 4˚C, and resuspended in 100 μl binding buffer (1 × 10⁶ cells/ml) containing 5 μl annexin V–FITC and 10 μl propidium iodide. After cells were incubated away from light for ~10–15 min at room temperature, stained cells were analyzed by flow cytometry.

**Luciferase reporter gene assay**

For the reporter gene assay, murine HCC cells were plated at density of 1 × 10⁵ cells/well in 96-well plates (Costar). Then either the pGL3-STAT3-Luciferase or the pGL3-STAT6-Luciferase reporter gene was cotransfected with the pRL-TK reporter gene (Promega, Madison, WI) in the presence of STAT3 decoy ODN or scrambled ODN using Lipofectamine 2000. The Dual-Glo Luciferase Assay System (Promega) was used to determine luciferase activity (6).

**Statistical analysis**

Statistical analysis was performed using a paired Student’s t test. A p value < 0.05 was considered statistically significant. Statistical significance was determined as **p < 0.01 and *p < 0.05 compared with control.

**Results**

**STAT3 activation supports HCC growth in vitro and in vivo**

STAT3 decoy ODN specifically binds to activated STAT3, effectively blocking STAT3 from binding to its binding element (21, 22). Our previous study demonstrated that STAT3 decoy ODN had an inhibitory effect on human HCC growth in vitro (6). To address whether the presence of STAT3-blocked HCC tumor cells altered the characteristics of the HCC-immunosuppressed host immune response in vivo, an immunocompetent mouse model of HCC was required.
To generate such a model, we first identified whether STAT3 decoy ODN (Decoy) was also functional in murine HCC cells. As shown in Supplemental Fig. 1A, p-STAT3 was constitutively expressed in the murine HCC cell lines, Hepa-1-6 and H22, and Decoy was efficiently transfected into both cell lines in a dose-dependent manner (Supplemental Fig. 1B) and was stable over time in HCC cells (Supplemental Fig. 1C, 1D). Using a luciferase reporter gene assay, Decoy substantially reduced the transcriptional activity of STAT3; this result was in contrast to the scrambled ODN (Scramble) control, which failed to show any inhibitory effect on STAT3 transactivation. These observations indicate that STAT3 decoy ODN can specifically inhibit STAT3 transcriptional activity via competitive DNA binding (Supplemental Fig. 1E).

Using this model, we subsequently analyzed the effect of STAT3 decoy ODN on murine HCC growth in vitro. The results showed that Decoy significantly inhibited murine HCC cell growth in two different cell lines (Hepa-1-6 and H22) as compared with the Scramble or Lipofectamine 2000 (Lipo) controls for ODN (Fig. 1A), similar to our in vitro results in human HCC cells (6). We then tested whether blocking STAT3 in murine HCC cells also exhibited antitumor effects in vivo in a homograft mouse model. Indeed, the growth of Decoy-treated H22 or Hepa-1-6 cells was significantly slower than that of control Scramble- or Lipo-treated cells in BALB/c or C57BL/6 mice, respectively (Fig. 1B). Moreover, mice bearing Decoy-treated H22 cells survived significantly longer (up to 72 d) than mice bearing control-treated H22 cells, which all died within 51 d; similar results were observed in C57BL/6 mice bearing Decoy-treated Hepa-1-6 cells (Fig. 1C). Therefore, these results suggest that blocking STAT3 in HCC cells inhibits their growth both in vitro and in vivo.

Blocking HCC STAT3 triggers the antitumor cytolytic function of NK cells

Our observation that blocking STAT3 in murine HCC cells reduced tumor growth and prolonged survival in vivo could be due to intrinsic effects affecting cell survival as well as extrinsic effects on the host, including on host immunity and on NK cells in particular. To address this possibility, we tested whether the presence of STAT3-blocked H22 or Hepa-1-6 cells affected NK cell activation and function in tumor-bearing mice. First, we excluded the possibility that the in vivo immune effects we measured were from dead or dying cells. To do this, we ensured that the inoculated HCC cells were all in the same state and that dead cells were excluded by treating H22 or Hepa-1-6 cells with 100 nM ODN for 12 h and recounting the cells before inoculation, so that there was no significant difference in apoptosis among the experimental groups (Supplemental Fig. 2A). Furthermore, to avoid the influence of different tumor sizes among the mice in different groups, we performed our experiments when tumor volumes were similar among each group of mice (~2 wk after injection of HCC cells) (Supplemental Fig. 2B).

To evaluate whether blocking STAT3 in HCC cells also affected NK cell function, we isolated lymphocytes from the liver or spleen and determined their cytolytic activity against HCC cells. As shown in Fig. 2A, lymphocytes from BALB/c mice bearing Decoy-treated H22 cells displayed stronger cytolytic ability than that from mice bearing Scramble-treated H22 cells. Both NK cells and T cells are major effectors in the host antitumor immune response. We therefore evaluated their frequency and activation in tumor-bear- ing mice under these conditions and found a significantly increased CD3+DX5+ NK cell population in PBMCs from mice bearing Decoy-treated H22 cells compared with that from mice bearing Scramble-treated H22 cells (Fig. 2B). This increase in NK cells was accompanied by elevated activation- and cytotoxicity-associated molecules on their surface, including CD69, NKG2D, and FasL (Fig. 2C). Meanwhile, more NK cells had infiltrated into Decoy-treated HCC tumor tissues (Fig. 2D), and these NK cells exhibited augmented CD69, NKG2D, and FasL expression on their surface (Fig. 2E). In contrast, no obvious changes in T cell frequency were observed in either PBMCs or tumor tissues. These phenomena were reproduced in C57BL/6 mice bearing Decoy-treated Hepa-1-6 cells (Supplemental Fig. 3). These data indicate that blocking STAT3 in HCC cells can promote NK cell activation and antitumor function.

To rule out the participation of T cells in the observed in vivo antitumor effect, we repeated the above experiment in nude mice, which have normal NK cell number and function, but no functional T cells (23). The data showed that the growth of Decoy-treated H22 cells in nude mice remained significantly slower than that of nude mice bearing control-treated cells (Fig. 3A). Similar to what we observed in wild-type BALB/c mice, NK cell frequency was elevated in mice bearing Decoy-treated H22 cells compared with mice bearing control-treated H22 cells 2 wk after inoculation, which was nearly equal to that of nontumor-bearing mice (Fig. 3B). Additionally, expression of NK cell activation molecules was also significantly higher in mice bearing Decoy-treated H22 cells, including CD69, NKG2D, and FasL (Fig. 3C). There was no significant difference between mice bearing Scramble-treated H22 cells and Lipo-treated H22 cells.

**FIGURE 1.** Blocking STAT3 by decoy ODN inhibited HCC growth in vitro and in vivo. (A) H22 or Hepa-1-6 cells were transfected with 100 nM Decoy, Scramble, or Lipo ODN, and cell growth was assessed in an in vitro assay. (B) H22 and Hepa-1-6 cells (2 × 10⁴) treated with 100 nM Decoy or control ODNs for 12 h were s.c. injected into the right posterior flank of BALB/c and C57BL/6 mice, respectively, and the volume of tumor tissue was measured every week for each mouse. (C) The survival rate was evaluated from mice bearing HCC cells transfected with Decoy, Scramble, or Lipo. Data are expressed as the mean ± SD. Statistical significance for the Decoy group was determined as *p < 0.05 and **p < 0.01 compared with the Lipo or Scramble control groups (n = 6).
We subsequently tested whether the tumor growth reduction in mice bearing STAT3-blocked HCC cells required NK or T cells by treating BALB/c mice with 100 μl anti-ASGM1 or anti-CD3 Ab to deplete NK or T cells, respectively, four times within a 2-wk time period; efficient deletion of both cell populations was confirmed by flow cytometry (data not shown). These mice were then inoculated with H22 cells or Decoy-treated H22 cells, as above. As shown in Fig. 3D, depletion of either NK or T cells attenuated the Decoy-mediated antitumor effect, although depletion of NK cells was significantly more effective than that of T cells. Moreover, the antitumor ability was further inhibited in NOD/Lt-SCID mice, which had T and B deficiency combined with impaired NK cell activity. These findings indicate that NK cells are necessary for anti-HCC immune responses normally inhibited by STAT3 signaling.

FIGURE 2. Blocking STAT3 in HCC triggered the activation and antitumor function of NK cells. Two weeks after inoculation with transfected H22 cells, the frequency and activation status of various lymphocyte populations were analyzed. (A) Lymphocytes from the liver and spleen were isolated from mice bearing Decoy- or Scramble-treated H22 cells, and their cytotoxicity against H22 cells was evaluated by the MTT method. Data are representative of three independent experiments and are expressed as the mean ± SD. Statistical significance for the Decoy group was determined as *p < 0.05 compared with the Lipo or Scramble control groups. (B) The lymphocyte populations in PBMCs from BALB/c mice were analyzed by flow cytometry. The histograms show the statistical analysis of the percentages of the NK (CD3−DX5+) and T cell (CD3+DX5−) populations in tumor- or nontumor-bearing mice (No tumor). (C) Expression levels of CD69, NKG2D, or FasL activation molecules on NK cells were evaluated by flow cytometry. The histograms represent the statistical analysis of the percentage of positive cells expressing each activation marker. (D) The percentage of infiltrated NK cells in tumor tissues. Data are expressed as the mean ± SD. Statistical significance for the Decoy group was determined as *p < 0.05 compared with the Lipo or Scramble control groups (n = 6). (E) Expression levels of CD69, NKG2D, or FasL activation molecules on NK cells isolated from tumor tissues were evaluated by flow cytometry. Data shown are one representative of similar data obtained from six mice.

FIGURE 3. NK cells regained function in T cell–deficient or –depleted mice bearing STAT3-blocked murine HCC cells. Transfected H22 cells (2 × 10^6) were s.c. inoculated into the left posterior flank of BALB/cA-nu nude mice. (A) Tumor size was measured over time. (B) Two weeks after inoculation, CD3−DX5+ NK cells were analyzed by flow cytometry, and the frequency of NK cells in PBMCs of transfected H22 or nontumor-bearing nude mice (No tumor) was calculated. (C) Expression levels of molecules associated with NK cell activation were also evaluated by flow cytometry. (D) BALB/c mice were injected with 20 μl anti-ASGM1 or 100 μl anti-CD3 Abs four times within a 2-wk time period to deplete NK (d-NK) or T (d-T) cells, respectively. These mice, including NOD/Lt-SCID mice, were then inoculated with H22 cells or Decoy-treated H22 cells (2 × 10^6 cells/mouse), and the volume of tumor tissue for each mouse was recorded once per week. Data are expressed as the mean ± SD. Statistical significance was determined as *p < 0.05, **p < 0.01 compared with the controls (n = 6).
To confirm the role of these NK cells with restored function in promoting the in vivo antitumor immune responses against HCC cells with intact STAT3 signaling, we tested whether NK cells activated in the presence of STAT3-blocked HCC cells were sufficient to reduce tumor burden and prolong survival in mice inoculated with fresh H22 tumor cells. As depicted in Fig. 4A, NK cells were sorted by MACS from nude mice inoculated 2 wk prior with Decoy-, Scramble-, or Lipo-treated H22 cells. NK cell purity was determined to be ~87% (Fig. 4B), and these purified NK cells were adoptively transferred into recipient NOD/Lt-SCID mice that harbored dramatically reduced NK cell number and function (20). One week later, the frequency and activation status of NK cells in PBMCs from these recipient mice were analyzed by flow cytometry, and these recipient mice were inoculated with H22 cells. As shown in Fig. 4C, the number of NK cells was similar among the recipient mice (upper panel); however, expression of the CD69, NKG2D, and FasL activation molecules was significantly higher on NK cells from donor mice inoculated with Decoy-treated H22 cells than on NK cells from donor mice inoculated with Scramble- or Lipo-treated H22 cells (lower panel). This activated NK cell phenotype in mice receiving NK cells from donors inoculated with Decoy-treated H22 cells correlated with the following outcomes: significantly smaller tumor size (Fig. 4D); prolonged survival from a mean of 52 d in control recipients to a mean of 86 d in recipients receiving NK cells from mice inoculated with the Decoy-treated H22 cells (Fig. 4E); and lower levels of serum IL-10 and TGF-β (Fig. 4F). Collectively, these findings demonstrate that blocking STAT3 in HCC tumor cells enhances antitumor immune responses, which are greatly dependent on NK cell activation.

**Blocking STAT3 makes HCC cells more sensitive to NK cell cytolysis**

Tumor cells resist NK cell cytolysis by changing their expression of immunosuppressive/immunostimulatory cytokines as well as of ligands for NK cell receptors. Thus, we analyzed whether expression of cytokines or NK cell ligands was directly influenced by blocking STAT3 in HCC cells. To begin addressing this, we first evaluated the sensitivity of Decoy- or control-treated HCC cells to cytolysis by liver or spleen lymphocytes isolated from healthy mice, in which NK cells play an important role in tumor clearance (24, 25). Compared with Lipo- or Scramble-treated HCC cells, Decoy-treated HCC cells were significantly more sensitive to lysis by spleen or liver lymphocytes (Fig. 5A). We then analyzed whether changes in cytokine or NK cell ligand expression occurred in Decoy-treated HCC cells by quantitative real-time PCR. Compared with Lipo-treated HCC cells, the immunosuppressive IL-10 and TGF-β cytokines were significantly reduced, whereas immunostimulating cytokines, such as the type I IFNs, IFN-α and -β, were significantly increased in Decoy-treated HCC cells (Fig. 5B). Additionally, the expression of NKG2D ligands, including H60, MULT1, and RAE-1, was upregulated in Decoy-treated HCC cells (Fig. 5C). These findings indicate that blocking the STAT3 pathway in HCC cells can increase the sensitivity of HCC cells to immune-mediated cytolysis, most likely by NK cells.

**Constitutive activation of HCC STAT3 plays an important role in inhibiting NK cell function**

In the tumor microenvironment, tumor cells secrete various immunosuppressive factors and induce the generation and activation of negative regulatory cells that suppress antitumor immune responses (26–28). Therefore, we further investigated the mechanisms underlying the ability of tumor-derived STAT3 signaling to inhibit immune responses. In accordance with the above observations, serum protein levels of IL-10 and TGF-β were downregulated and IFN-γ was upregulated in mice inoculated with Decoy-treated H22 cells compared with that in control mice bearing Lipo-treated or Scramble-treated H22 cells (Fig. 6A). Next, to determine whether the reduction of IL-10 and TGF-β mediated by STAT3 blockade in H22 cells was required for reversing the suppressed NK cell function, cytolysis was evaluated after incubating liver and spleen lymphocytes were incubated with Decoy- or control-treated H22 cells in the presence or absence of TGF-β and IL-10.

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**FIGURE 4.** The antitumor immune response in mice bearing STAT3-blocked HCC was dependent on NK cell activation. (A) Flow chart of NK cell transfer assay. Briefly, 2 × 10⁶ H22 cells treated with 100 nM Decoy, Scramble, or Lipo ODN for 12 h were s.c. inoculated into the left posterior flank of nude mice. Two weeks later, mice were sacrificed, and CD3⁺DX5⁺ NK cells were purified by MACS. Then 1 × 10⁶ CD3⁺DX5⁺ NK cells/mouse were transferred into NOD/Lt-SCID mice. After 1 wk, 2 × 10⁶ H22 cells were inoculated into the left posterior flank of recipient NOD/Lt-SCID mice. (B) The purity of NK cells sorted by MACS was analyzed. (C) One week after NK cell transfer, flow cytometry was used to analyze NK cell populations in PBMCs from recipient NOD/Lt-SCID mouse and to determine the percentage of CD3⁺DX5⁺ NK cells expressing CD69, NKG2D, or FasL. The volume of tumor tissue (D), survival (E), and serum IL-10 and TGF-β levels (F) of each recipient NOD/Lt-SCID mouse were evaluated. Data are expressed as the mean ± SD. Statistical significance for the Decoy group was determined as *p < 0.05 compared with the Lipo or Scramble control groups (n = 6).
neutralizing Abs in the supernatant. Neutralizing TGF-β and IL-10 significantly increased the cytolysis of the lymphocytes compared with untreated or Lipo-treated H22 cells, suggesting that the STAT3-mediated suppression of cytolytic activity by lymphocytes required TGF-β and/or IL-10. These immunosuppressive cytokines were also sufficient to suppress this cytolytic ability, as cytolysis by lymphocytes incubated with Decoy-treated HCC was inhibited after adding exogenous TGF-β and IL-10 into the supernatant.

**FIGURE 5.** The sensitivity of HCC cells to NK cell cytolysis was increased by blocking STAT3. H22 or Hepa-1-6 cells transfected with Decoy, Scramble, or Lipo for 12 h were used as target cells. (A) The sensitivity of H22 or Hepa-1-6 to cytolysis by liver or spleen lymphocytes from C57BL/6 or BALB/c mice, respectively, was detected by an MTT assay. (B) H22 and Hepa-1-6 cells were transfected with Decoy, Scramble, or Lipo ODN. After 6 h, cells were collected, and the mRNA levels of the indicated cytokines were analyzed by quantitative real-time PCR. (C) Hepa-1-6 cells were transfected with 100 nM Decoy, Scramble, or Lipo ODN. After 6 h, cell surface expression of various NKG2D ligands was detected by flow cytometry. Data are representative of three independent experiments and are expressed as the mean ± SD. Statistical significance for the Decoy group was determined as *p < 0.05, **p < 0.01 compared with the Lipo or Scramble control groups.

**FIGURE 6.** The constitutive activation of HCC STAT3 played an important role in inhibiting NK cell function. (A) Transfected H22 cells (2 × 10⁶) were s.c. inoculated into the left posterior flank of BALB/c mice. Two weeks later, serum IL-10, TGF-β, and IFN-γ levels were measured in tumor- or nontumor-bearing (No tumor) mice by ELISA. Data are expressed as the mean ± SD. Statistical significance for the Decoy group was determined as **p < 0.01 compared with the Lipo or Scramble control groups (n = 6). (B) H22 cells were transfected with or without Decoy ODN, and anti–IL-10 (2.5 μg/ml) and anti–TGF-β (2.5 μg/ml) Abs, or recombinant IL-10 and TGF-β (2 ng/ml), were added into the supernatant. Liver or spleen lymphocytes were then added to the cultures, and cytotoxicity was evaluated. Data are representative of three independent experiments and are expressed as the mean ± SD. Statistical significance was determined as *p < 0.05 and **p < 0.01 compared with the controls. (C) Transfected H22 cells and Hepa-1-6 (2 × 10⁶) were s.c. inoculated into the left posterior flank of BALB/c mice and C57BL/6 mice, respectively. Treg cells in PBMCs were assayed in tumor- or nontumor-bearing (No tumor) mice, as described in Materials and Methods. (D and E) BALB/c mice were injected with 100 μl anti–IL-10 and anti–TGF-β Abs, or with an anti-CD25 Ab, four times within a 2-wk time period. Mice were then inoculated with control H22 cells or Decoy-treated H22 cells (2 × 10⁶ cells/mouse). The volume of tumor tissue for each mouse was measured every week (D), and NK cell frequency and activation status in PBMCs (E) were evaluated by flow cytometry 2 wk after H22 inoculation. Data are expressed as the mean ± SD. Statistical significance was determined as *p < 0.05 and **p < 0.01 compared with the control (n = 6).
(Fig. 6B). Taken together, these data indicate that TGF-β and IL-10 act as important factors in the STAT3-mediated HCC-induced immunosuppressive effect on NK cells.

It is well known that activated Treg cells secrete large amounts of IL-10 and TGF-β, and that Tregs induced by IL-10/TGF-β exhibit suppressive effects on immune responses (28, 29). Because we showed that both IL-10 and TGF-β were reduced in the serum of mice upon blocking STAT3 in HCC cells, we determined whether Treg numbers were affected in mice bearing STAT3-blocked HCC cells. Indeed, as shown in Fig. 6C, the frequency of Tregs in mice bearing Decoy-treated H22 cells was lower than that in control groups and was nearly equal to the nontumor-bearing mice; similar results were also observed in tumor-bearing C57BL/6 mice. These results suggest that the presence of STAT3-blocked HCC cells reduced Treg numbers in tumor-bearing hosts. These STAT3-regulated Tregs and immunosuppressive cytokines were necessary to suppress antitumor immunity, as depleting Tregs or neutralizing IL-10 and TGF-β significantly decreased H22 growth to an extent similar to Decoy-treated H22 cells. Meanwhile, tumor growth in mice with Ab-neutralized IL-10 and TGF-β was similar to that from Treg-depleted mice (Fig. 6D). Furthermore, depleting Tregs or neutralizing IL-10/TGF-β also significantly enhanced NK cell activation, which was even further improved when Tregs were depleted in mice bearing Decoy-treated H22 cells (Fig. 6E). These results indicate that overactive STAT3 signaling in HCC tumor cells normally induces impaired NK cell function by a mechanism involving immunosuppressive Tregs and cytokines, and that blocking STAT3 can convert suppressed NK cells into activated NK cells that can at least partially restore antitumor immunity.

Discussion

HCC is a difficult cancer to treat not only because it induces immunosuppressive mechanisms that function to inhibit any antitumor activity in the host, like many other cancers, but also because it is located in the liver, which is known to be an immunotolerant organ. In this study, we investigated the effect of blocking STAT3 signaling in HCC cells on antitumor responses in vivo in a mouse model of HCC. Our previous study found that STAT3 was highly activated in human HCC and that STAT3 decoy ODN exerted significant antitumor function by promoting apoptosis in vitro (6). In this study, our results confirmed this effect in murine HCC cell lines (Fig. 1) and demonstrated that STAT3 decoy ODN treatment could not only directly inhibit HCC growth both in vitro and in vivo, but also break HCC-mediated immune suppressive and enhance host antitumor immune responses, especially the antitumor function of NK cells. We further determined the underlying molecular mechanisms involved in this process: blocking STAT3 in HCC cells reduced the production of immunosuppressive TGF-β and IL-10 cytokines and attenuated the number of Tregs, thereby relieving their collective inhibitory effect on NK cells. Additionally, our data suggest that production of some NK cell stimulators may also be induced by blocking STAT3, such as IFN-α/β, which can further activate NK cells and promote their antitumor function. Because host immunity in individuals with HCC is characterized by an immunosuppressive environment that includes impaired T and NK cell function, abnormally high Tregs, and cytokine imbalance (2, 3), the exciting increase in the number and activation of NK cells, but not T cells, in mice bearing STAT3-blocked HCC cells (Fig. 2, Supplemental Fig. 3) suggests that targeting STAT3 may recover NK cell function as a therapy for HCC. Therefore, both the direct proapoptotic function and the indirect triggering of the NK cell-mediated antitumor response we find in this study in our in vivo model provide further support for STAT3 as a potential target for anti-HCC therapy.

The NK cell population is a component of innate immunity that plays an important role in eliminating cancer and is responsible for cancer immunosurveillance. Importantly, innate immune cells not only exert direct antitumor effects, but also play supporting roles in CD8+ T cell–mediated elimination of tumor cells (30, 31). To clarify the role and properties of NK cells in the presence of STAT3-blocked HCC, we first investigated the NK cell response in T cell–deficient nude mice. Even in the absence of functional T cells, NK cells could still be activated in mice bearing STAT3-blocked H22 cells, and these NK cells displayed an anti-HCC response (Fig. 3A–C). In a second experimental series, depleting NK cells with an anti-ASGM1 Ab greatly decreased host antitumor effects against Decoy-treated H22 cells; interestingly, these effects were more profound than in T cell–depleted mice (Fig. 3D). Finally, adoptively transferred NK cells from donor mice bearing STAT3-blocked H22 cells into recipient NOD/Lt-scid mice exerted significantly stronger antitumor responses than NK cells from mice bearing control H22 cells, and these recipient mice survived longer (Fig. 4). These findings demonstrate that NK cells are the crucial antitumor mediators when STAT3-mediated immunosuppression is blocked in vivo.

In addition to impaired NK cell function, an abnormal increase in Tregs and myeloid-derived suppressor cells as well as an imbalance of cytokines is found in HCC patients. In the tumor cells themselves, antiapoptotic molecules and cytotoxic determinants are upregulated, whereas the Ag-presentation molecule, MHC, is downregulated; moreover, HCC cells secrete various immunosuppressive cytokines, including VEGF, IL-10, and TGF-β (32, 33). Among these, IL-10 and TGF-β—often produced abundantly in cancer patients—are potent immune suppressors that negatively regulate NK cell function (34, 35). Interestingly, IL-10 and TGF-β expression was downregulated in STAT3-blocked H22 cells as well as in the serum of mice bearing STAT3-blocked H22 cells, which was concomitant with a decrease in Treg cells (Figs. 5, 6); additionally, both Treg depletion and IL-10/TGF-β neutralization significantly restored NK cell activation and promoted their anti-HCC immune response. Furthermore, the expression of NKG2D ligands, including H60, MULT1, and RAE-1, was increased in Decoy-treated HCC cells (Fig. 5C), which enhanced the sensitivity of HCC cells to NK cell–mediated cytolysis. Our data thus suggest a working model in which blocking STAT3 in HCC cells mediates changes in their cytokine profile, which weakens the suppressive activity of Treg cells and enhances NK cell function; this enhanced NK cell function further facilitates the generation of adaptive immune responses, ultimately leading to a break in HCC-mediated immune tolerance. DCs might be involved in this process, and the precise mechanism underlying each of these steps will be further investigated in future studies.

Taken together, these data indicate that blocking STAT3 in HCC cells can initiate innate immunity in vivo. It is worthy to note that, although we have investigated the effect of decoy ODN treatment on STAT3 signaling in HCC therapy in the current study, this strategy may also be useful as a therapy for other tumors.

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Disclosures

The authors have no financial conflicts of interest.
References


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Supporting Figure

Figure S1. STAT3-decoy ODN was efficiently transfected into murine HCC cell lines and specifically blocked STAT3 transactivation in cancer cells

A. Western blot analyzed the levels of STAT3 and p-STAT3 in H22 and Hepa1-6 cells. B. H22 and Hepa1-6 cells were transfected with FITC-labeled decoy ODN at different doses. The efficiency of transfection was detected by FACS. C. H22 cells were transfected with FITC-labeled Decoy ODN, and then these H22 cells were injected into peritoneal cavity of BALB/c mice. FITC-positive H22 cells were analyzed by FACS once a week. D. Hepa1-6 cells were transfected with FITC-labeled Decoy ODN, and then these cells were injected into the right side axillary of C57BL/6 mice. After 6 weeks, the tumor issues were harvested, and FITC-labeled Decoy ODN was observed under fluorescence microscope in the frozen section slices. E. H22 and Hepa1-6 cells were co-transfected with pGL3-STAT3 -Luciferase construct or pGL3-STAT6 -Luciferase construct and STAT3-decoy or scramble ODN in the presence of Lipofectamine™ 2000, while Renilla expression vector pRL-TK was co-transfected to normalize the transfection efficiency. After 24 h, luciferase activity
of lysed cells was measured using a dual-Glo™ Luciferase assay system. Data were representative of three independent experiments.

Figure S2. Apoptosis rate of HCC before inoculation and the tumour size of mice bearing HCC within 2 weeks.

When transfected with 100 nM Decoy for 12h and cultured in medium for 12h, these HCC cells were harvested and re-counted by trypan blue exclusion method and the cell concentration was adjusted to $2 \times 10^7$ Ml. A. The apoptosis rates of H22 or Hepa1-6 cells were detected by Flow cytometry using Annexin V-FITC and PI. Data were representative of three independent experiments. B. $2 \times 10^6$ H22 cells treated as described above were injected into the right posterior flank of BALB/c mice. 2 weeks later, the volume of tumors was measured. The tumor tissues were under the same size. The histogram represented statistical analysis of 6 mice per group. Data are representative of three independent experiments with 6 mice per group, statistical significance was determined as **$p < 0.01$ and *$p < 0.05$ compared to control.
Figure S3 Blocking STAT3 in Hepa1-6 cells triggered the activation and anti-tumour function of NK cells. Two weeks after inoculation with transfected Hepa1-6 cells, the frequency and activation status of various lymphocyte populations were analyzed. **A.** Lymphocytes from liver and spleen were isolated from mice bearing Decoy- or Scramble-treated Hepa1-6 cells, and their cytotoxicity against Hepa1-6 cells was evaluated by the MTT method. Data are representative of 3 independent experiments and are expressed as the mean ± SD. Statistical significance for the Decoy group was determined as *p<0.05 compared to the Lipo or Scramble control groups. **B.** The lymphocyte populations in PBMCs from C57BL/6 mice were analyzed by flow cytometry. The histograms show the statistical analysis of the percentages of the NK (CD3\(^{-}\)NK1.1\(^{+}\)) and T cell (CD3\(^{+}\)NK1.1\(^{-}\)) populations in tumour- or non-tumour–bearing mice (No tumour). **C.** Expression levels of CD69, NKG2D or FasL activation molecules expressed on NK cells were evaluated by flow cytometry. **D.** The percentage of NK cells infiltrated in tumor tissues. The histograms represent the statistical analysis of the percentages of positive cells expressing each activation marker. Data are expressed as the mean ± SD. Statistical
significance for the Decoy group was determined as \( p < 0.05 \) compared to the Lipo or Scramble control groups (n=6). E. Expression levels of CD69, NKG2D or FasL activation molecules expressed on NK cells isolated from tumour issues were evaluated by flow cytometry. Data shown are one representative of similar data obtained from 6 mice.

**Table S1**

Sequences of primers specific for mouse genes used for real-time PCR analysis

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