Research Article

Cancer Immunology Research

Enhanced Cancer Immunotherapy with Smad3-Silenced NK-92 Cells

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Abstract

Natural killer (NK) cells, early effectors in anticancer immunity, are paralyzed by TGFβ1, an immunosuppressive cytokine produced by cancer cells. Development and activity of NK cells are largely inhibited in the Smad3-dependent tumor microenvironment. Here, we used genetic engineering to generate a stable SMAD3-silencing human NK cell line, NK-92-S3KD, whose cancer-killing activity and cytokine production were significantly enhanced under TGFβ1-rich condition compared with the parental cell line. Interestingly, we identified that the IFNG gene is a direct E4BP4 target gene. Thus, silencing of SMAD3 allows upregulation of E4BP4 that subsequently promoting interferon-γ (IFNγ) production in the NK-92-S3KD cells. More importantly, NK-92-S3KD immunotherapy increases the production of not only IFNγ, but also granzyme B and perforin in tumors; therefore, inhibiting cancer progression in two xenograft mouse models with human hepatoma (HepG2) and melanoma (A375). Thus, the NK-92-S3KD cell line may be useful for the clinical immunotherapy of cancer. *Cancer Immunol Res; 6(8); 965–77. ©2018 AACR.*

Introduction

Cancer is still one of the leading causes of death in the world. Surgery, chemotherapy, and radiotherapy have been the mainstays of cancer treatment for decades. However, outcomes are still unsatisfactory due to side effects, drug resistance, recurrence, and metastasis. Cancer cells are heterogeneous, versatile, and adaptable, leading to primary and secondary resistance (1). Side effects induced by systemic administration of cytotoxic anticancer drugs can produce serious clinical problems (2). Therapies that target the tumor microenvironment show promise as cancer, tumor growth, invasion, and metastasis rely on stromal conditions (3).

Indeed, immunotherapies based on cytotoxic T lymphocytes and natural killer (NK) cells have progressed in clinical practice (4, 5). Following encouraging results from clinical studies of NK cell–adoptive therapy on leukemia (6–10), NK cell–based immunotherapy has been suggested as a therapeutic option for solid tumors. Several studies demonstrated that the quantity of intratumoral NK cells is negatively correlated with tumor progression (11, 12). However, application of NK cell–based therapies to solid tumors remains challenging due to immunosuppressive cytokines and reduced expression of activating receptors on NK cells in the microenvironment of solid tumors (13, 14). Various strategies have been explored to enhance anticancer activities of NK cells in the tumor microenvironment including, for example, overexpressing IL2, IL15, and NKG2D in NK cell (15–17), downregulating NKG2A, or delivering high-affinity CD16 (HA-CD16), CCR7 (18–20), as well as chimeric antigen receptors (CAR), such as CD19, CD20, Her2/Neu, ErbB2, CEA, GPA7, and EpCAM (21) into the NK cells.

TGFβ1 produced by cancer cells promotes cancer progression by restricting the function of immune cells against cancer (22). During tumorigenesis, TGFβ1 triggers the malignant progression by inducing epithelial-to-mesenchymal transition and tumor-associated angiogenesis as well as by suppressing anticancer immunity in the tumor microenvironment. In addition, TGFβ signaling can suppress the cytolytic activity of NK cells via downregulating CD16-mediated IFNγ production and interferon responsiveness *in vitro* (23, 24). TGFβ1 also promotes conversion of NK cells into ILC1-like cells in cancer models (25, 26). Thus, targeting TGFβ signaling in the tumor microenvironment with TGFβ-neutralizing antibody, antisense oligonucleotide, or TGFβ receptor inhibitors is a promising strategy for eliminating cancers (27–29). However, complete blockade of TGFβ1 signaling will also affect its anti-inflammatory features and cause autoimmune disease, including systemic inflammation, cardiovascular defects, or autoimmunity in mouse models (30). Thus, identification of a precise and accessible therapeutic target in the downstream pathway of TGFβ signaling could separate the anti-inflammatory actions from the cancer-promoting outcomes.

We have shown that Smad3, a downstream mediator of TGFβ signaling (31), in the tumor microenvironment promotes tumor growth, invasion, and metastasis in mice. Our findings showed the importance of E4BP4 in TGFβ1/Smad3-mediated NK-cell development, but its role in NK-92 cell–mediated cytotoxicity against cancer was unexplored (32). Thus, the present work aims to investigate the role of the TGFβ1/Smad3/E4BP4 axis in the

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

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doi: 10.1158/2326-6066.CIR-17-0491

anticancer activity of NK-92 cells and develop a NK cell–based Smad3-targeted immunotherapy. Here, we showed that E4BP4 mediates production of anticancer effectors in NK-92 cells and that knockdown of SMAD3 blocked TGFβ1-mediated immunosuppression in a genetically engineered SMAD3-silencing human NK-cell line (NK-92-S3KD). We found that *IFNG* is a direct target gene of E4BP4 and that knockdown of *SMAD3* enhances cancer-killing activities of NK-92 cells by blocking the TGFβ1/Smad3/E4BP4 inhibitory axis, thus preserving IFNγ production. Treatment with NK-92-S3KD produced better anticancer effects than NK-92-EV (empty vector control) in NOD/SCID mice bearing human hepatoma (HepG2) or melanoma (A375) *in vivo*. The parental cell line NK-92 has already been investigated in clinical trials (33). This NK-92-S3KD cell line may improve the anticancer efficiency of NK cell–based clinical immunotherapies.

Materials and Methods

Antibodies, cell lines, and mice

Antibodies used were listed in Supplementary Table S1. NK-92, A375 (CRL-1619, an epithelial malignant melanoma from a 54-year-old female), HepG2 (HB-8065, an epithelial hepatocellular carcinoma from a 15-year-old male), and 293T cell lines were obtained from ATCC in 2016. The HepG2-Luc cell line was preserved in our laboratory. The cell lines were not further authenticated in the past year and underwent no mycoplasma testing, but were cultured with antimicrobial reagent normocin (InVivoGen) for 2 weeks prior experiments. NOD/SCID (NOD.CB17-Prkdcscid/J; 6–8 weeks old) mice were purchased from The Jackson Laboratory (Stock No: 001303) and housed under the regulation of the Animal Experimentation Ethics Committee (AEEC) of The Chinese University of Hong Kong.

Cell culture

NK-92 cells were cultured in MEM alpha medium (Life Technologies) according to ATCC guidelines. HepG2-Luc and A375 cells were cultured in DMEM/F12 medium (Life Technologies), supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin in 5% CO_2 at 37°C. 293T cells were maintained in DMEM-High Glucose medium (Life Technologies) supplemented with 10% fetal bovine serum in 5% CO_2 at 37°C.

Generation of recombinant lentiviral particles rLV-hSMAD3

With the vector pLVX-shRNA1-Puro (Biowit Technologies; Supplementary Fig. S1), cDNA sequence coding shRNA against SMAD3 mRNA (Supplementary Table S1) was cloned to generate the recombinant plasmid pLVX-shRNA1-Puro-hSMAD3. Plasmid was packaged (Biowit Technologies) into lentiviral particles (rLV-hSMAD3). NK-92 cells were transduced with rLV-hSMAD3 and transformants selected with puromycin (InvivoGen). The expression level of Smad3 was determined by real-time PCR and Western blot.

Western blot analysis

Protein from cultured cells was extracted using the radio immunoprecipitation assay (RIPA) lysis buffer. Western blot analysis was performed as described earlier (23). In brief, after blocking nonspecific binding with 5% bovine serum albumin, membranes were then incubated overnight at 4°C with the primary antibody listed in Supplementary Table S2, then stained with the IRDye800-conjugated secondary antibody (Rockland Immunochemicals). Signals were detected by LiCor/Odyssey infrared image system (LI-COR Biosciences). Results were quantified by ImageJ program (https://imagej.nih.gov/ij/) and expressed as ratio after normalized against GAPDH expression level.

Real-time RT-PCR

Total RNA from cells was isolated using the PureLinkTM RNA Mini kit (Life Technologies). The relevant primer sets used are listed in Supplementary Table S3.

Cytotoxicity assay

NK-92 cell–mediated cytotoxicity was determined with the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (G1780 Promega). Cytotoxicity against cancer cells was measured at effector/target (E/T) ratios of 5:1, 10:1, and 20:1 at 4 hours.

ELISA

ELISA commercial kits for detection of human IFNγ (BioLegend), Granzyme B (MABTECH), perforin (Abcam), and TGFβ1 (R&D Systems) were used. Briefly, NK-92-EV and NK-92-S3KD cells (1×10^6/mL) were cultured in 6-well plates in the presence or absence of TGFβ1 (240-B/CF, R&D Systems) for 12 hours, and the supernatants were collected for ELISA. For preparing the samples from tumor tissue, we added chilled PBS in tumor tissue samples at a ratio of 100 mg tissue per milliliter, and then homogenized the mixture. We centrifuged the mixture at 14,000 rpm for 10 minutes at 4°C, to collect tumor tissue fluids for ELISA. For preparing the samples of serum, we sacrificed tumor-bearing mice on indicated days and collected mouse serum via centrifuging the blood at 3,000 rpm for 15 minutes at 4°C.

MTT assay

NK cells were seeded (1×10^4 cells/well) in 96-well plates and treated with TGFβ1 for 44 hours. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Invitrogen) was added in a final concentration of 0.5 mg/mL and incubated for 4 hours at 37°C. After disposal of the medium in the wells, DMSO was added and absorbance at 490 nm was recorded using a plate reading spectrophotometer.

Immunofluorescence

Activation of Smad3 and expression of CX3CR1 in tumor infiltrated NK-92 cells were identified using two-color immunofluorescence with human FITC-CD56 and p-Smad3 or CX3CR1, followed by PE or Alexa 555-conjugated anti-rabbit secondary antibodies. Expression of perforin, VEGF, and CD31 within the tumor tissues was detected by immunofluorescence with antibodies of Alexa 594-perforin, FITC-VEGF, and Alexa 488-CD31. Cell nuclei were counterstained with DAPI.

Chromatin immunoprecipitation (ChIP) assay

The assay was performed with the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology). A total of 2×10^7 NK-92 cells were treated with or without TGFβ1 for 1 hour for the Smad3/E4BP4 ChIP assay or 12 hours for the E4BP4/IFNG ChIP assay. Rabbit anti-human antibodies used in the ChIP assay were listed in Supplementary Table S3. Primer sets were designed based on the predicted binding site provided by the ECR browser database and were listed in Supplementary Table S3.

Knockdown of E4BP4 in NK-92-S3KD cells

Briefly, NK-92-S3KD cells were transduced with recombinant lentivirus expressing shRNA targeting human E4BP4 constructed on pLVX-ShRNA2-Neo (Supplementary Fig. S2). The cDNA sequence coding shRNA-E4BP4 is listed in Table S1. G418 (GENETICIN) was used for positive clone selection. The selected colonies were then expanded and analyzed for E4BP4 expression with real-time RT-PCR and Western blot.

Dual-luciferase reporter assay

Briefly, for the Smad3/E4BP4 reporter assay, the protein-coding sequence of human SMAD3 was amplified and cloned into pcDNA3.1+ vector to construct the SMAD3-expressing plasmid pcDNA3.1+SMAD3. A reporter plasmid expressing *E4BP4* 3′UTR was constructed with psi-CHECK2. The predicted binding site TATCTGACT was mutated to obtain plasmid expressing mutant *E4BP4* 3′UTR (Supplementary Fig. S3). Similarly, for the E4BP4/IFNG reporter assay, the coding region of human E4BP4 was cloned into pcDNA3.1+. The *IFNG* promoter was cloned into vector pGL-3basic. The predicted binding site GATTACGTATTT in the *IFNG* promoter was mutagenized (Supplementary Fig. S4). Primer sets used in mutation experiments are listed in Supplementary Table S3. Recombinant plasmids were delivered into 293T cells. Luciferase activity was measured with Dual-Luciferase Reporter Assay System (E1910).

Adoptive transfer of NK-92 cells

Animal experiments were approved by the AEEC of the Chinese University of Hong Kong (protocol No. 13/049/GRF). Mice were subcutaneously inoculated with 5×10^6 HepG2-Luc or A375 cells. Seven days after tumor inoculation, when the tumor volume reached 50 mm^3, the mice were randomly assigned into 3 groups. Saline, 2×10^7 NK-92-EV cells or equivalent number of NK-92-S3KD cells were injected into the mice intravenously at days 7, 10, 14, 17, 21, 24, 28, and 31 after tumor cell inoculation. All mice received rhIL2 (200 ng/mouse) every other day via intraperitoneal injection. Tumor size was measured every 4 days and volume was calculated with the following formula: volume (v) = width (w) × length (l) × height (h) × $\pi/6$. *In vivo* imaging system analysis was performed on HepG2-Luc bearing mice at day 35 after tumor inoculation. Mice were sacrificed at day 35, and tumors were weighed. Tumor tissue and mouse serum were collected for further studies.

As for the tumor rechallenge mice model, 7 days after receiving 5×10^6 A375 cells subcutaneous injection, NOD/SCID mice then received intravenous injection of either saline, 2×10^7 NK-92-EV cells, or NK-92-S3KD cells. All mice received 200 ng rhIL2 every other day via intraperitoneal injection after NK-92 adoptive therapy. Fourteen days after tumor inoculation, primary tumors were resected completely from anesthetized mice. All mice were challenged with 5×10^6 A375 cells 4 weeks after NK-92 adoptive therapy and sacrificed 30 days after tumor rechallenge. Further studies were performed as described previously (32).

Measurement of creatinine, lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels

Commercial kits Stanbio-Creatinine LiquiColor Test, ALT/SGPT Liqui-UV Test, and AST/SGOT Liqui-UV Test from Stanbio Laboratory were used. QuantiChrom Lactate Dehydrogenase Kit (DLDH-100) used for LDH detection was purchased from BioAssay.

Statistical analysis

Statistical analyses were performed by one-way ANOVA, two-way ANOVA or t test using GraphPad Prism 5.0 software (Prism 5.0 GraphPad Software).

Results

Knockdown of SMAD3 enhances cancer-killing activity of NK-92 cells

To examine the function of Smad3 in NK-cell anticancer activity, we first developed a stable SMAD3-knockdown human NK-cell line by transducing NK-92 cells with a lentivirus containing shRNA against human SMAD3 mRNA (shRNA-hSMAD3; Supplementary Fig. S1). Real-time PCR demonstrated that shRNA-hSMAD3 transduction downregulated mRNA expression of Smad3 in NK-92 cells (Fig. 1A). More than 70% decrease in Smad3 protein was detected by Western blot analysis (Fig. 1B). Reduction of Smad3 in the clonally selected shRNA-hSMAD3 transduced NK-92 cells was maintained for more than 6 months, and a stable SMAD3-knockdown NK-92 cell line (NK-92-S3KD) was developed.

We then tested the anticancer effects of NK-92-S3KD against human hepatoma and melanoma cells by LDH release assay *in vitro*. As shown in Fig. 1C and D, knockdown of SMAD3 increased the cancer-killing activities of NK-92 cells. To mimic the tumor microenvironment with high TGFβ1, TGFβ1 at a dose of 5 ng/mL was added into the culture. As expected, addition of TGFβ1 significantly inhibited cancer-killing capacity of NK-92-EV cells against HepG2 and A375 cells in various E/T ratios. Knockdown of SMAD3 enhanced the cytotoxicity of NK-92 cells under high TGFβ1 conditions (Fig. 1C and D). In addition, real-time PCR and ELISA also revealed that TGFβ1-mediated suppression on the production of anticancer effectors (i.e., IFNγ, granzyme B, and perforin) as well as the expression of activation markers (NKp30 and NKp44) were attenuated in NK-92-S3KD cells compared with NK-92-EV cells (Fig. 2; Supplementary Fig. S5). These results demonstrated that knockdown of SMAD3 in the human NK-92 cells attenuated TGFβ1-mediated immunosuppression, which in turn enhanced these cells' cancer-killing effect and cytotoxic effector production. To compare the effects of TGFβ receptor blockade or SMAD3 disruption on the anticancer activity of NK-92 cells, we examined the effect of TGFβ receptor type I and II dual blocker (LY2109761) on IFNγ production by NK-92 cells. As shown in Supplementary Fig. S6, blocking TGFβ signaling with LY2109761 restored the production of IFNγ by NK-92 cells. Blockade of TβRI/II kinase activity also enhanced IFNγ production by NK-92-S3KD cells compared with NK-92-EV cells in response to TGFβ1. This effect on IFNγ production may be associated with either incomplete knockdown of SMAD3 in NK-92 cells or a TGFβ-dependent Smad3-independent mechanism. However, no significant change was observed in NK-92-S3KD cells compared with NK-92-EV cells regarding proliferation rate and expression of NKG2D, NKG2A, and CX3CR1, shown by MTT assay, real-time PCR, and immunofluorescence (Supplementary Figs. S5 and S7–S9).

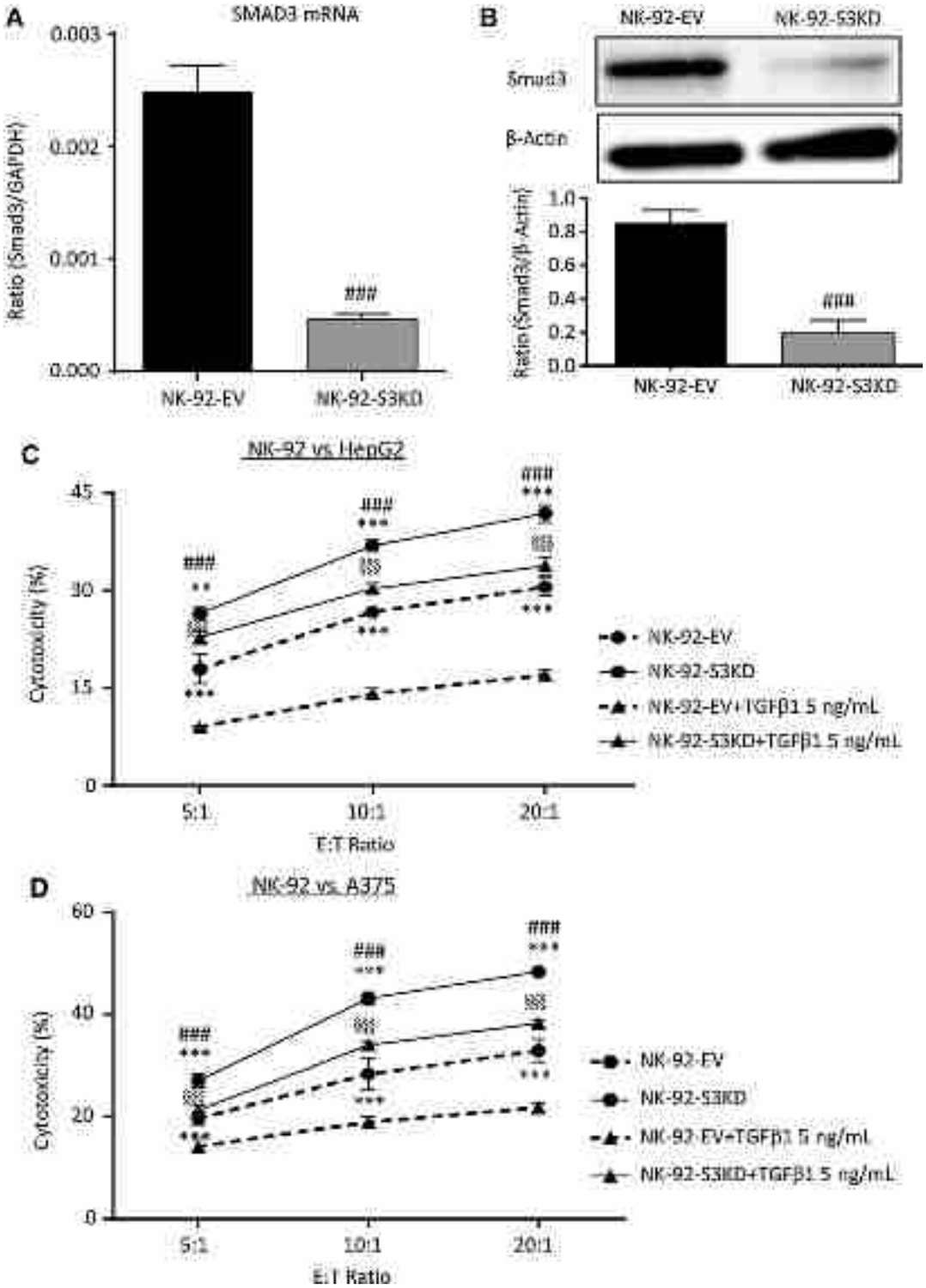

Figure 1.

Silencing SMAD3 largely enhances cancer-killing activities of NK-92 cells. **A** and **B**, Real-time PCR and Western blot analysis showed that transduction of shRNA-SMAD3 significantly downregulates Smad3 mRNA and protein expression in NK-92 cells. **C** and **D**, The cancer-killing activities of NK-92-S3KD cells against HepG2 and A375 cancer cells were significantly higher than the parental cell line NK-92-EV in both presence or absence of TGFβ1 (5 ng/mL). The cytotoxicity was measured at various E:T ratios by cell-mediated cytotoxicity assay kit. Data represent mean ± SD for groups of three independent experiments. ###, $P < 0.001$ versus NK-92-EV cells; **, $P < 0.01$; ***, $P < 0.001$ versus TGFβ1-treated cells; §§§, $P < 0.001$ versus TGFβ1-treated NK-92-EV cells.

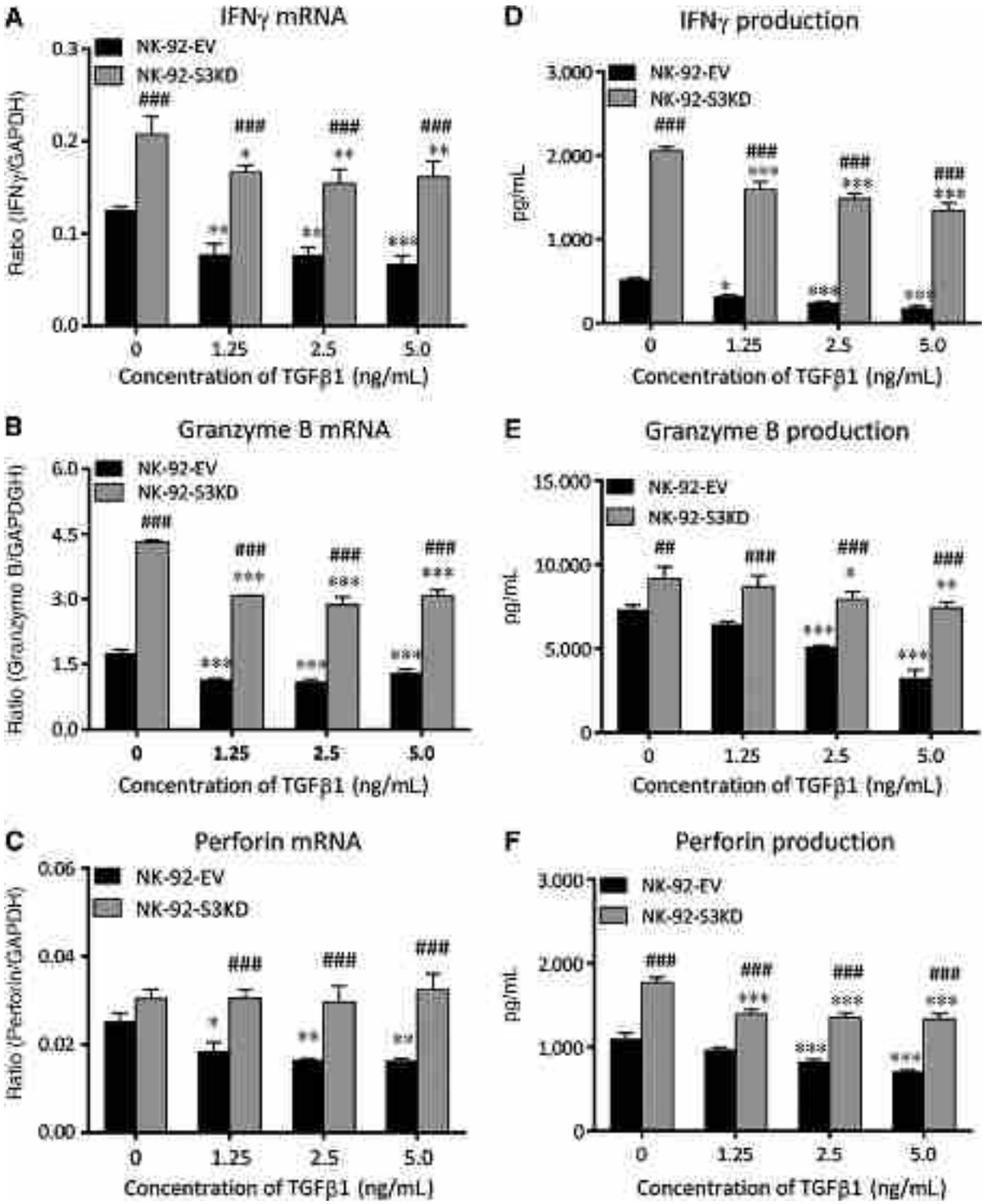

Figure 2.

Disruption of SMAD3 enhances anticancer effector productions in NK-92-S3KD cells. **A–C**, The NK-92-EV or NK-92-S3KD cells treated with indicated concentration of TGFβ1 were collected at 3 hours for real-time PCR. Results showed that silencing SMAD3 significantly increased the mRNA levels of anticancer effectors IFNγ, granzyme B, and perforin. **D–F**, NK cells treated with indicated concentration of TGFβ1 for 12 hours, then their conditional medium was collected for ELISA. Results showed that silencing SMAD3 significantly enhanced the production of anticancer effectors (IFNγ, granzyme B, and perforin) in the NK-92-S3KD cells under TGFβ1-induced immunosuppression. Data represent mean ± SD for groups of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus control; ##, $P < 0.01$; ###, $P < 0.001$ versus NK-92-EV cells.

Smad3 disruption enhances E4BP4-depedent cytokine production

We next examined the mechanism whereby disruption of SMAD3 promoted anticancer activities of NK-92 cells. We showed that TGFβ1 can suppress murine NK-cell differentiation via a Smad3/E4BP4-dependent mechanism (32), but the effect of TGFβ1 on human NK-92 cells is unknown. In this study, we examined the regulatory role of the TGFβ1/Smad3/E4BP4 axis in the activity of NK-92 cells. As shown in Fig. 3A and B, with real-time PCR and Western blots we detected that addition of TGFβ1 induced phosphorylation of Smad3 and inhibition of E4BP4 mRNA expression in a dose-dependent manner. Blockade of Smad3 with the Smad3 inhibitor (SIS3; ref. 34) or viral-mediated knockdown (SMAD3-KD) resulted in an increase in the expression of E4BP4 mRNA and protein as well as the production of IFNγ in the NK-92 cells under the high TGFβ1 condition (Fig. 3C–F). These findings demonstrated that TGFβ1/Smad3/E4BP4 signaling regulated activity of NK-92 cells.

***IFNG* is a direct E4BP4 target gene regulated by Smad3 in NK-92 cells**

The transcription factor E4BP4 was studied in the context of NK-cell differentiation (35); however, its role and regulatory mechanisms in NK-92 cells remain unexplored. In this study, we studied the inhibitory mechanism of Smad3 in E4BP4-dependent anticancer activity of NK-92 cells. We identified a binding site for Smad3 on the 3′UTR of the human *E4BP4* (NFIL3) genomic sequence (Fig. 4A). ChIP assays revealed that TGFβ1 promoted the physical binding of Smad3 to the 3′UTR of the *E4BP4* gene, therefore inhibiting the transcription of *E4BP4* (Fig. 4B). A

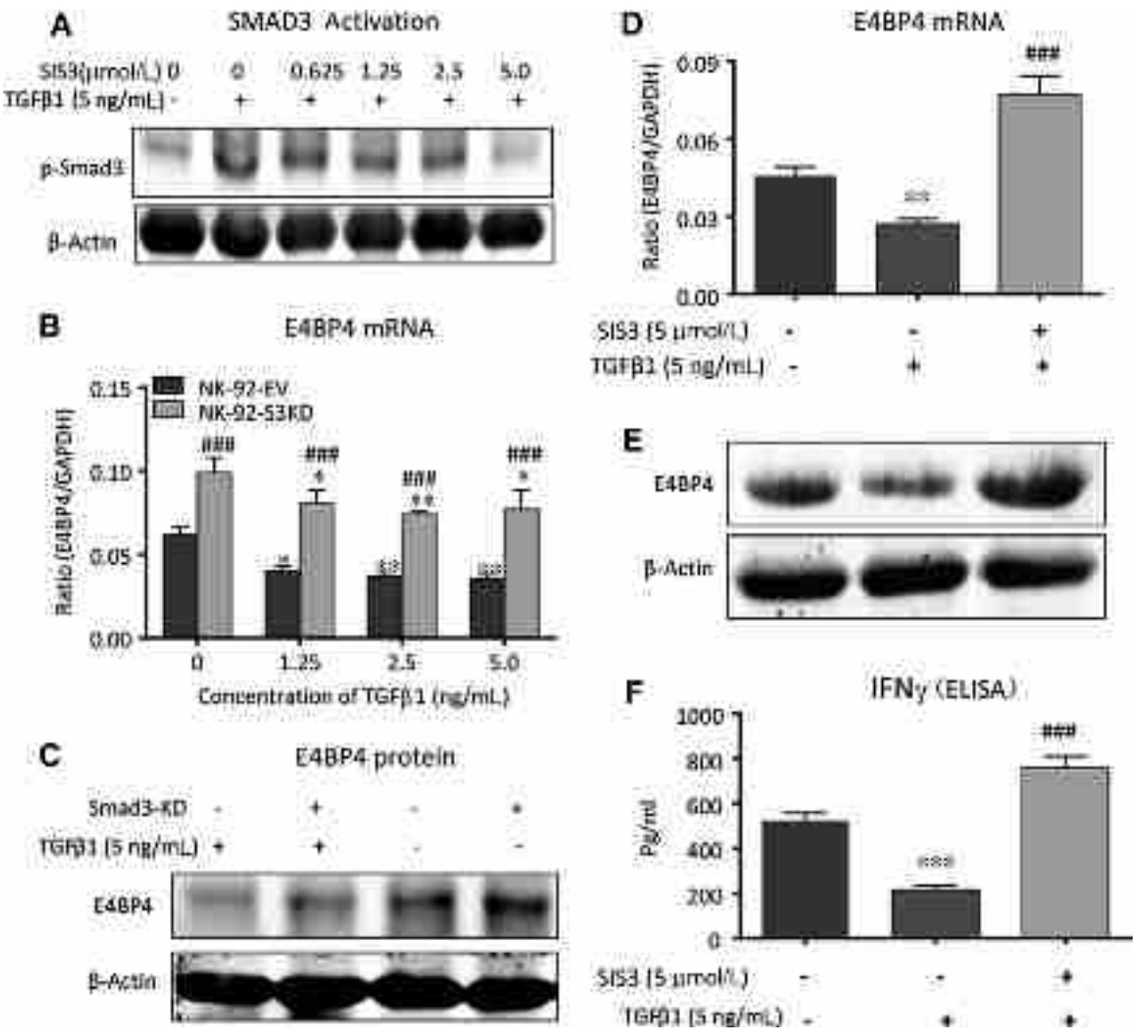

Figure 3.

TGFβ1/Smad3 signaling suppresses IFNγ production via an E4BP4-dependent mechanism in NK-92 cells. **A**, Western blot analysis of Smad3 phosphorylation in NK-92, **B,** Real-time PCR of E4BP4 mRNA expression in NK-92-EV and NK92-S3KD cells with or without addition of TGFβ1. **C,** Western blot analysis of E4BP4 protein expression in NK-92 cells treated with 5 ng/mL of TGFβ1. **D–F,** Real-time PCR, Western blot analysis of E4BP4 and ELISA of IFNγ in NK-92-EV cells treated with Smad3 inhibitor SIS3 (5 μmol/L) and TGFβ1 (5 ng/mL). Data represent mean ± SD for three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus blank control (0 ng/mL of TGFβ1); ###, $P < 0.001$ versus NK-92-EV or TGFβ1-treated only.

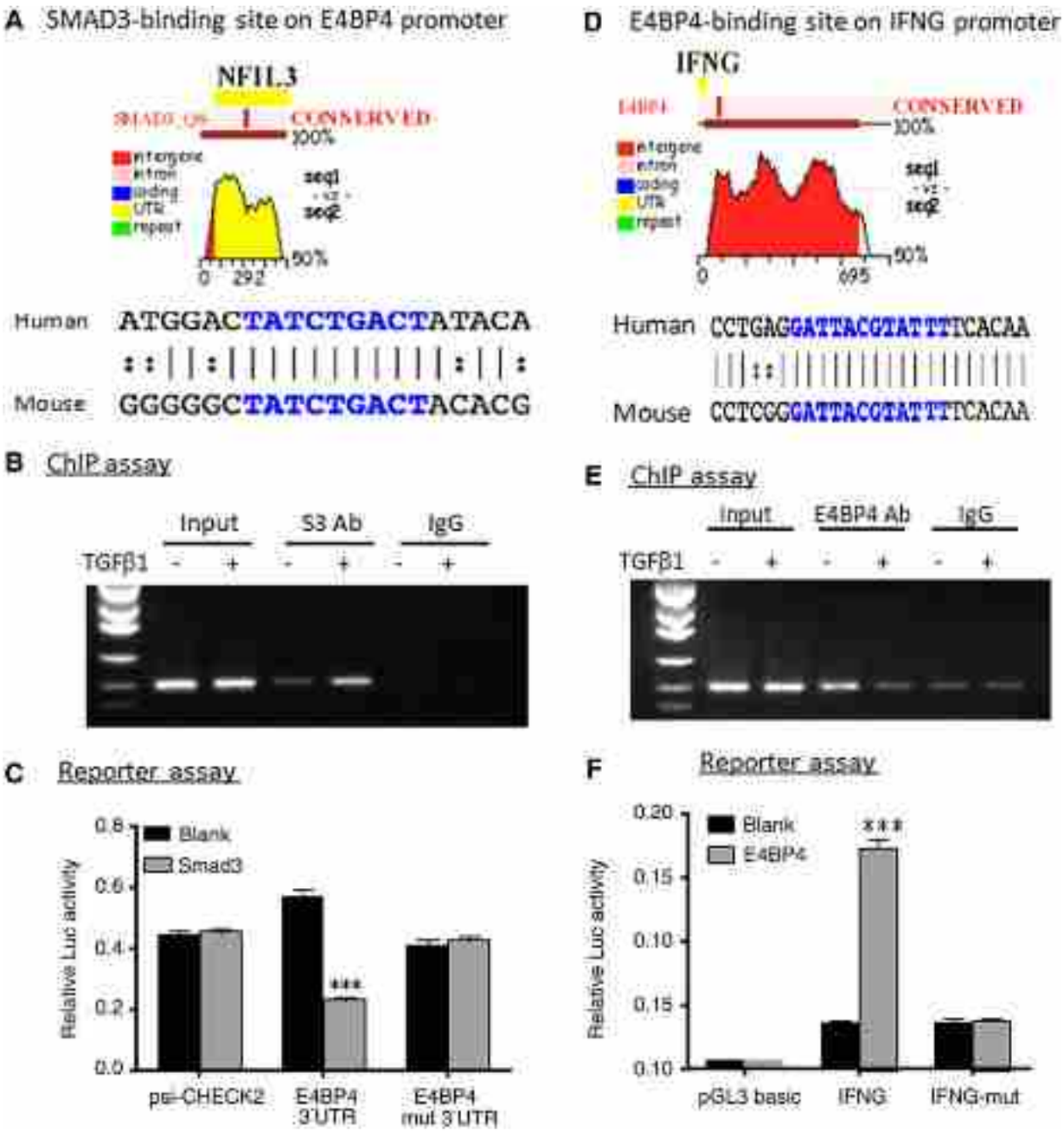

Figure 4.

IFNG is a direct E4BP4 target gene in human NK-92 cells. **A,** A Smad3-binding site on the 3′ UTR of *E4BP4 (NFIL3)* was predicted by the ECR browser. **B,** ChIP assay detected increased Smad3–E4BP4 binding in response to TGFβ1 (5 ng/mL) at 1 hour. **C,** The promoter activity of *E4BP4*. Overexpression of Smad3 protein suppressed promoter activities of *E4BP4*. Suppression was prevented when the predicted Smad3-binding site on the 3′UTR of *E4BP4* genomic sequence was mutated. **D,** An *E4BP4*-binding site on the promoter region of the *IFNG* gene was predicted by the ECR browser. **E,** ChIP assay detected E4BP4 binding on *IFNG* was reduced at 12 hours in response to TGFβ1 (5 ng/mL). **F,** Overexpression of E4BP4 protein enhanced the promoter activity of *IFNG*, which was prevented by mutation of predicted E4BP4-binding site on the *IFNG* promoter sequence. Data represent mean ± SD for three independent experiments. ***, $P < 0.001$ versus empty vector control.

binding site of E4BP4 protein is also predicted in the promoter of the human *IFNG* genomic sequence by ECR browser (ref. 36; Fig. 4). TGFβ1 suppressed the binding of E4BP4 to the *IFNG* promoter as shown in Fig. 4E. Thus, TGFβ1 was capable of inhibiting the promoter activity of *IFNG* by reducing the availability of E4BP4 proteins via the TGFβ1/Smad3/E4BP4 inhibitory axis, thereby blocking the transcription of the *IFNG* gene in NK-92 cells (Fig. 4C and F). The dual-luciferase reporter assays showed

that mutation of the Smad3 or E4BP4 binding sites abrogated their transcriptional regulatory effects on the E4BP4 or *IFNG* promoter activities, respectively (Fig. 4C and F). *In vitro*, silencing of SMAD3 significantly increased expression of E4BP4 and IFNγ mRNA and protein in NK-92 cells (NK-92-S3KD) in an E4BP4-dependent manner shown by the double SMAD3- and E4BP4-knockdown NK-92 cells (NK-92-S3/E4KD; Fig. 5). Therefore, our findings demonstrated knockdown of SMAD3 restores the IFNγ production by NK-92 cells in a TGFβ1-mediated immunosuppressive microenvironment via the Smad3/E4BP4/IFNG axis.

NK-92-S3KD suppresses cancer progression in mice bearing human xenografts

To assess the antitumor effect of NK-92-S3KD *in vivo*, xenograft tumor models of human hepatoma (HepG2) and melanoma (A375) were generated in NOD/SCID mice in which the host NK cells are deficient. On day 7 after subcutaneous tumor inoculation, the HepG2- or A375-tumor-bearing mice were treated with saline, NK-92-EV, or NK-92-S3KD cells (2×10^7 cells/mouse) twice a week with IL2 administration (200 ng/mouse) every other day. Growth of HepG2 and A375 tumors was inhibited by both NK-92-EV and NK-92-S3KD cells, but tumor growth was inhibited to a greater extent by NK-92-S3KD cells (Fig. 6; Supplementary Fig. S10). In line with the *in vitro* findings, as shown in Fig. 7, treatment with NK-92-S3KD cells increased both intratumoral and serum levels of IFNγ, granzyme B, and perforin in the HepG2-tumor-bearing mice compared with the saline-treated controls as well as the NK-92-EV-treated mice. Furthermore, perforin expression of NK-92-S3KD cells was enhanced in the tumor microenvironment of HepG2-bearing NOD mice associated with an increment in intratumoral necrosis compared with the NK-92-EV cells (Supplementary Fig. S11). Though preventing phospho-Smad3 nuclear translocation, knockdown of SMAD3 did not induce significant change in the amount of tumor-infiltrating NK-92 cells (Supplementary Fig. S12), which may be related to the parallel expression of CX3CR1 on NK-92-EV and NK-92-S3KD (Supplementary Fig. S9B). In addition, the serum concentration of TGFβ1 and expression of VEGF and CD31 in HepG2-tumor were not altered by the treatment with NK-92-S3KD (Supplementary Fig. S13). These findings demonstrated that silencing SMAD3 in human NK-92 cells enhances their anticancer activities rather than their tumor-infiltrating capacity *in vivo*.

To evaluate the persistence of injected NK-92 cells and their long-term tumor-killing capability, mice were rechallenged with 5×10^6 A375 cells 4 weeks after primary NK-92 adoptive cell therapy. As shown in Supplementary Fig. S14, 30 days after tumor rechallenge (thus 58 days after NK-92 adoptive cell therapy), both NK-92-EV and NK-92-S3KD immunotherapy still exerted antitumor effects (Supplementary Fig. S14A–S14D). Because

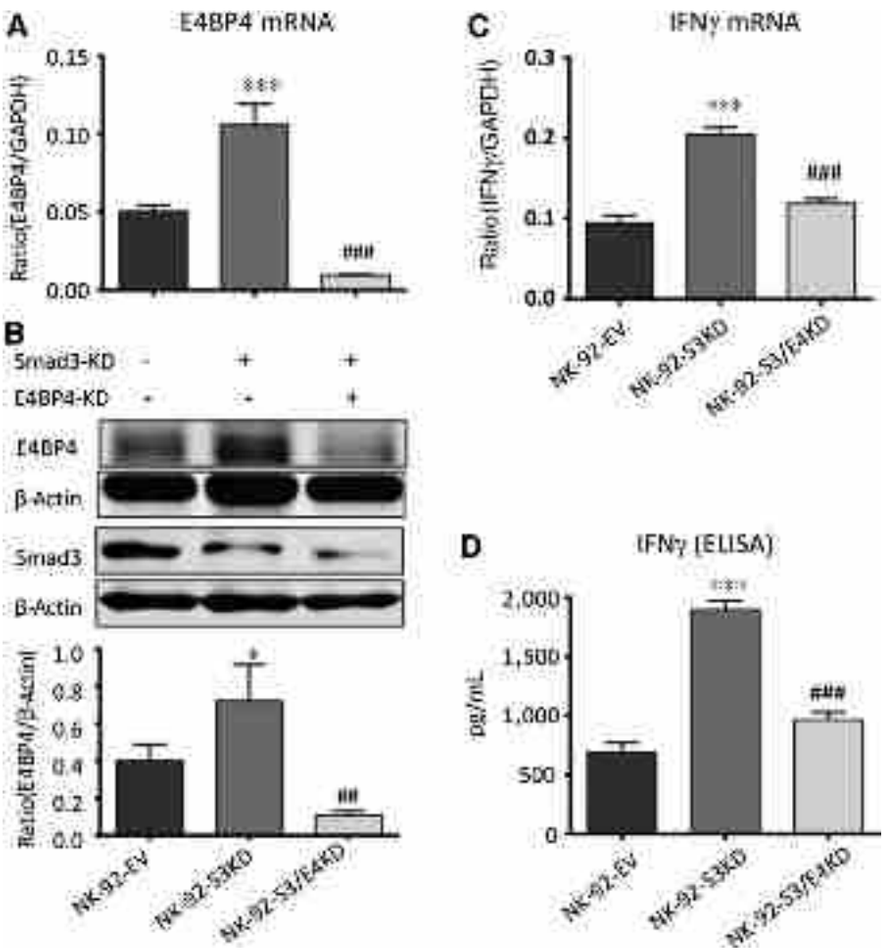

Figure 5.
Silencing E4BP4 abrogates the promoting effect of SMAD3 knockdown on IFNγ production in NK-92 cells. **A,** Real-time PCR result of E4BP4 mRNA expression level in NK-92 cells with or without viral-mediated knockdown of SMAD3 (SMAD3-KD) or E4BP4 (E4BP4-KD). **B,** Western blot analysis of E4BP4 in NK-92 cells. **C,** Real-time PCR result of IFNγ mRNA level in NK-92 cells. **D,** ELISA result of IFNγ production from NK-92 cells. Data represent mean ± SD for three independent experiments. *, $P < 0.05$; ***, $P < 0.001$ versus NK-92-EV; ##, $P < 0.01$; ###, $P < 0.001$ versus NK-92-S3KD cells.

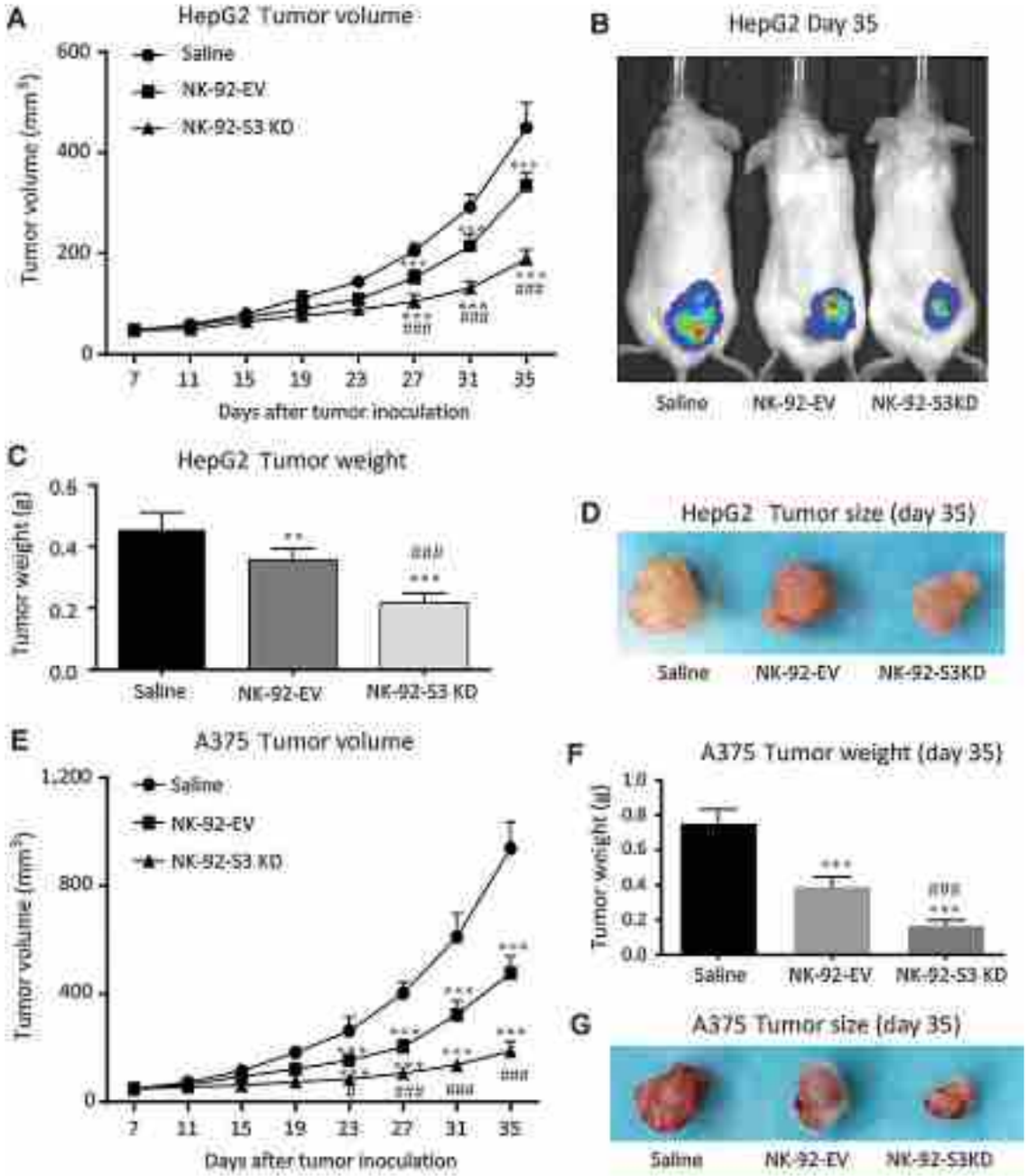

Figure 6.

NK-92-S3KD shows better antitumor effects than NK-92-EV on two xenograft mouse models bearing HepG2 and A375. **A,** Tumor volume of HepG2. **B,** Luciferase intensity imaging of HepG2-Luc tumor-bearing mice. **C,** Tumor weight of HepG2. **D,** Tumor size of HepG2. **E,** Tumor volume of A375. **F,** Tumor weight of A375. **G,** Tumor size of A375. Data represent mean ± SME of three independent experiments for groups of at least 6 mice. **, $P < 0.01$; ***, $P < 0.001$ versus saline group; #, $P < 0.05$; ###, $P < 0.001$ versus NK-92-EV–treated group.

both NK-92-EV and NK-92-S3KD cells persisted and homed to tumors similarly (Supplementary Fig. S14E), the advanced long-term tumor-killing effects of NK-92-S3KD cells as compared with NK-92-EV cells may be attributed to enhanced NK-cell activation and cytokine production.

Treatment with NK-92-S3KD did not cause adverse effects on kidney, heart, and liver. We found no significant changes in serum levels of creatinine, LDH, ALT, and AST in treated mice on day 28 as compared with saline controls (Supplementary Fig. S15).

Discussion

Targeting the tumor microenvironment could be an effective strategy for eliminating cancer. NK cell–based innate

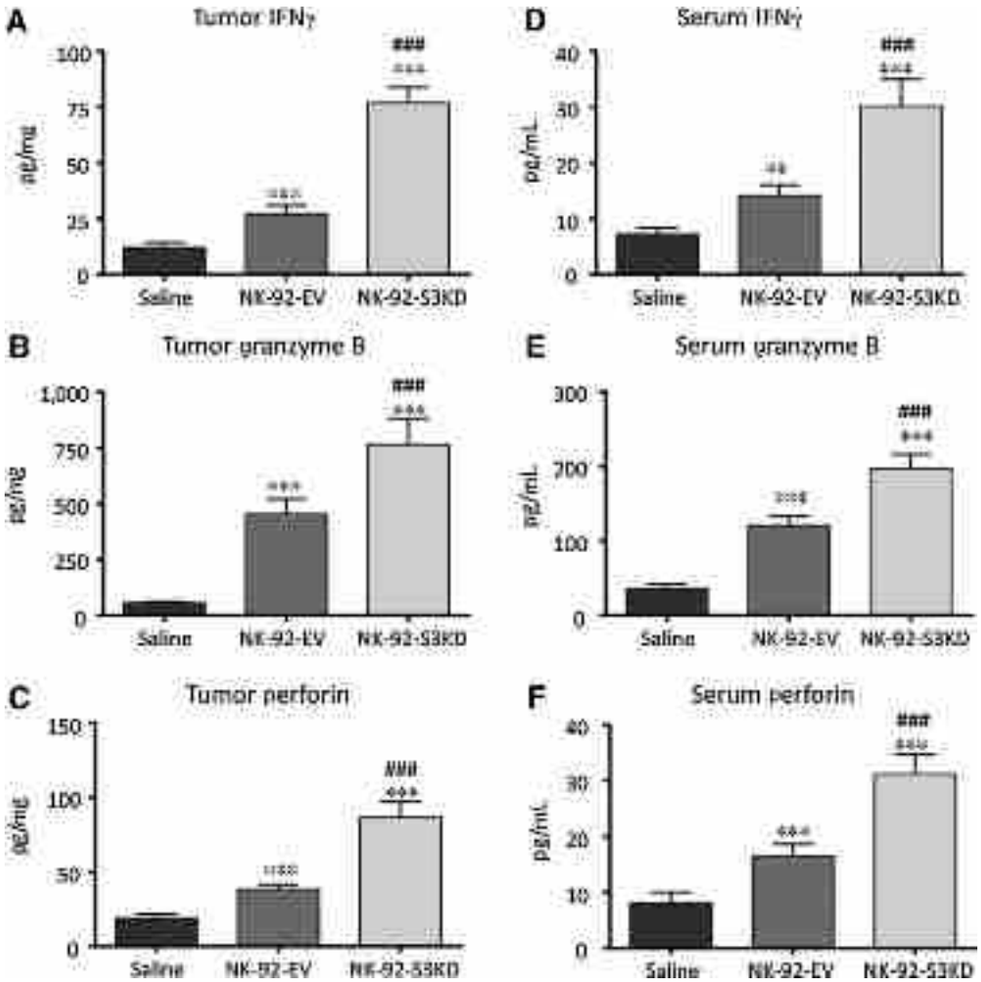

Figure 7.

Immunotherapy of NK-92-S3KD increases anticancer effector productions in HepG2-bearing mice. **A–C**, Tumor tissue–derived human IFNγ, granzyme B, and perforin. **D–F**, Serum levels of human IFNγ, granzyme B, and perforin. Results showed that the levels of IFNγ, granzyme B and perforin in both tumor tissues and serum of HepG2-bearing mice on day 28 after tumor inoculation were enhanced in the tumor-bearing mice treated with NK-92-S3KD cells compared with parental cells. Data represent mean ± SME of three independent experiments for groups of at least 6 mice. **, $P < 0.01$; ***, $P < 0.001$ versus the saline group; ###, $P < 0.001$ versus the NK-92-EV group.

immunotherapy is accessible because it requires no prior immunization and is also antigen independent, not MHC restricted, and less likely to induce GVHD (37, 38). Such problems otherwise limit the value of T cell–based adaptive immunotherapy (39). However, the outcomes of clinical trials using NK cell–based adoptive cell therapy are inconsistent (40, 41). Although NK-92 is the only FDA-approved cell line for use in clinical trials, it has demonstrated minimal efficacy, thus limiting its use and suggesting the need for more suitable NK-cell populations (33, 42). In this work, we improved the cancer-killing effects of NK-92 by knocking down Smad3 production in those cells, to generate NK-92-S3KD cells. Our findings showed that SMAD3 knockdown enhances the anticancer effects of NK cells without inducing significant side effects *in vivo*. Mechanistically, depletion of SMAD3 circumvents the inhibitory effects of TGFβ1 on the E4BP4–IFNG axis in NK-92 cells, thereby promoting anticancer effector production in the tumor microenvironment. Thus, our work developed a strategy to overcome TGFβ1-mediated immunosuppression in the tumor microenvironment. This Smad3-targeted strategy may be clinically effective as a cancer immunotherapy.

Considerable research has been aimed at enhancing the anticancer effects of NK cell–based immunotherapy. Stable IL2 or IL15 overexpression increases the anticancer responses of NK-cell immunotherapy (15, 16). Other strategies for enhancing NK cell–mediated cytotoxicity include overexpressing NK cell–activating receptor NKG2D, downregulating NK cell–inhibitory receptor NKG2A, and delivering the high-affinity CD16 (HA-CD16) gene to NK cells (17–19). Somanshi and colleagues focused on strengthening migration via genetically delivering CCR7 into NK cells (20). Some researchers have focused on improving tumor-recognition and activation capabilities of NK cells by transducing CARs that target tumor antigens such as CD19, CD20, Her2/Neu, ErbB2, CEA, GPA7,

and EpCAM (21). Unfortunately, these various efforts failed to prevent TGFβ1-mediated immunosuppression of NK cells affecting multiple aspects including proliferation, maturation, cytokine production, as well as receptor activation (43). Therefore, the modified NK cells remain paralyzed in the TGFβ1-rich tumor microenvironment. Accumulating evidence demonstrated that TGFβ1 inhibits IFNγ production in NK cells. TGFβ1/Smad3 signaling downregulates IFNγ expression by binding on the promoter region of IFNG as a transcriptional suppressor or by indirectly suppressing T-BET signaling (44). Our present work revealed that TGFβ1/Smad3 suppressed IFNγ by transcriptionally suppressing E4BP4, a master transcription factor previously identified to be responsible for NK cell development (45). Hence, blocking the Smad3/E4BP4/IFNG inhibitory axis by targeting Smad3 in NK-92 may represent an effective immunotherapy for cancer clinically.

One study suggested the development of a TGFβ-tolerant NK cell line (46), in which the TGFβ signaling pathway was blocked in NK-92 cells via genetically overexpressing a dominant-negative TGFβ receptor II. The enhanced anticancer activity of this TGFβ-insensitive cell line was demonstrated on nude mice bearing Calu-1 cells. However, indiscriminately blocking TGFβ at the receptor level may cause unfavorable immune responses by NK cells. T cells isolated from Smad3-deficient mice are resistant to TGFβ1 inhibition (47). Thus, the TGFβ1-tolerant NK cell line, developed by knocking down Smad3, may provide a more specific and effective immunotherapy to circumvent TGFβ1-mediated immunosuppression. However, in our current study, we found that NK-92-S3KD cells are still susceptible to TGFβ1-mediated inhibition, whereas blockade of TGFβRI/II activity also synergically enhances IFNγ production by NK-92-S3KD cells. These may be associated with either incomplete blockage of Smad3 in NK-92-S3KD cells or noncanonical TGFβ signaling pathways, such as MAP kinase pathways, Rho-like GTPase signaling pathways, and PI3K/AKT pathways (48).

Here, we selected the NK cell line NK-92, which is already enrolled for clinical trials, for gene manipulation based on several reasons. First, in comparison with primary NK cells, the NK-92 cell line is more practical for large-scale expansion and quality assurance. Second, NK-92 cells induce less KIR-MHC I–dependent inhibition due to the lack of inhibitory KIRs. Third, the lack of immunogenicity in this cell line results in less chance of being rejected by the immune system of recipients (49). Besides, as an adoptive effector cell widely tested in clinical trials, the safety of NK-92 cells has been verified (50). Genetic modification has been used for improving anticancer effects of T cells (51). However, limited genetic manipulation has been carried out in NK cells due to the technical challenges of gene transfer (21). With variable efficiency of gene delivery in NK cell lines with lentiviral transduction ranging from 2% to 97%, multiple rounds of virus transduction may be required in some cases (52). In order to stably downregulate SMAD3 expression in NK-92 cells, recombinant lentivirus was used in the present study. The sequence encoding shRNA targeting SMAD3 mRNA, delivered into NK-92 cells with recombinant lentivirus, was integrated into the host genome. The expression of SMAD3 protein was knocked down in NK-92 cells, generating the NK-92-S3KD cell line, which exhibits tolerance to TGFβ1 and enhanced anticancer effects *in vitro* and *in vivo*. For clinical application, the safety of using lentiviral vectors in the clinical setting must be considered. Up to present, at least 40 clinical trials using lentiviral vectors have been approved. McGarrity and colleagues, in a study of 263 infusions of lentivirus-transduced cells to assess the safety of the lentivirus vector, followed some of the subjects for over 8 years and observed no adverse events (53). In our study, no organ damage was detected in the tumor-bearing mice receiving NK-92-S3KD cells. Our NK-92-S3KD cell line may facilitate future clinical application of adoptive immunotherapy.

To minimize the influence of the immune system from the tumor-bearing mice, we used NOD/SCID mice, which are deficient in NK and T cells. However, the anticancer effects of NK-92-S3KD cells may be underestimated in this xenograft model, as our data demonstrated that the disruption of SMAD3 enhances production of IFNγ, the anticancer effects of which depend on activation of macrophages (54) and cytotoxic T cells (55). Unfortunately, NOD/SCID mice lack T cells and are deficient in macrophages. Therefore, verifying the anticancer effects and revealing the mechanisms of NK-92-S3KD cells using a humanized mouse tumor model and molecular analysis such as protein array and RNA sequencing may be necessary before our results could move forward to clinical trials.

In conclusion, we have generated a TGFβ1-tolerant NK-92 cell line via genetically targeting Smad3. The enhanced anticancer activity in this NK-92-S3KD cell line is demonstrated *in vitro* and *in vivo*. We identified a TGFβ1-mediated Smad3/E4BP4/IFNG inhibitory axis in human NK-92 cells as well. This TGFβ1-tolerant NK-92 cell line may suggest avenues for clinical cancer immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Q.-M. Wang, P.M.-K. Tang, G.-Y. Lian, K.-F. To, H.-Y. Lan

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Acknowledgments

This study was supported by the Innovation and Technology Fund of Hong Kong (ITS/227/15, ITS-InP/164/16, ITS-InP/242/16; ITS/138/17, InP/347/17, InP/348/17), Direct Grant for Research-CUHK (2016.035), Hong Kong Scholar Program, and the technical supports from Core Utilities of Cancer Genomics and Pathobiology of Department of Anatomical and Cellular Pathology (CUHK).

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Received September 5, 2017; revised March 12, 2018; accepted June 12, 2018; published first June 18, 2018.

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