ANIMAL CELLS AND SYSTEMS
2025, VOL. 29, NO. 1, 46–56
https://doi.org/10.1080/19768354.2024.2442393

# The anti-tumor effect of the IFNγ/Fas chimera expressed on CT26 tumor cells

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**ABSTRACT**
Interferon gamma (IFNγ) is well-known for its ability to stimulate immune cells in response to pathogen infections and cancer. To develop an effective cancer therapeutic vaccine, CT26 colon carcinoma cells were genetically modified to express IFNγ either as a secreted form (sIFNγ) or as a membrane-bound form. For the membrane-bound expression, IFNγ was fused with Fas (mbIFNγ/Fas), incorporating the extracellular cysteine-rich domains, transmembrane, and cytoplasmic domains of Fas. The tumor cells expressing sIFNγ and mbIFNγ/Fas showed slower growth rates compared to the mock-transfected cells. Furthermore, the tumorigenicity of the CT26 cells expressing mbIFNγ/Fas was significantly lower than that of cells expressing sIFNγ or the mock control. Remarkably, about 85% of the mice injected with the mbIFNγ/Fas-expressing tumors remained tumor-free for over two months. Mice that rejected mbIFNγ/Fas-expressing tumors developed systemic anti-tumor immunity against CT26 cells, which was characterized by enhanced levels of $CD4^+$ and $CD8^+$ T cells, as well as natural killer (NK) cells. Interestingly, splenocytes activated with the mbIFNγ/Fas-expressing tumors exhibited higher cytotoxicity than those activated with tumor cells expressing sIFNγ. These findings suggest that expressing the mbIFNγ/Fas chimera in tumor cells could be a promising strategy for developing whole tumor cell vaccines or gene therapies for cancer immunotherapy.

**ARTICLE HISTORY**
Received 6 September 2024
Revised 15 November 2024
Accepted 1 December 2024

**KEYWORDS**
Interferon gamma; Fas; tumor immunotherapy; membrane-bound form; secretory form

## Introduction

In addition to traditional anti-cancer treatments like chemotherapy and radiation therapy, cancer immunotherapy, such as immune checkpoint inhibitors (ICI), adoptive cell therapies (ACTs) and tumor vaccines, has significantly improved survival rates and the quality of life for cancer patients (Esfahani et al. 2020; Jeong et al. 2023). While repeated administration of cytokines can induce a strong anti-tumor immune response, the associated toxicity limits their application in tumor therapy (Rosenberg et al. 1993; Sehan et al. 2023). To avoid side effects of recombinant cytokines, tumor cells have been genetically modified with cytokine genes and tested for their ability to stimulate anti-tumor immune responses (Haas and Hillman 1996; Mackiewicz and Mackiewicz 2010; Diao and Liu 2023). However, many studies have recognized that these approaches can also activate tumor non-specific immune cells (Nahill and Welsh 1993; Tough et al. 1996; Zheng and Liu 1997; Chiang et al. 2015; Abd El-Maksoud et al. 2020). To enhance the efficacy and selectively activate immune cells associated with tumor cells, tumor cells were transfected with cytokine genes engineered to be expressed as membrane-bound forms (Colombo and Forni 1997; Sonn et al. 2005; Li et al. 2006; Choi et al. 2008; Kim et al. 2016; Do Thi et al. 2018; Park et al. 2020).

Interferon gamma (IFNγ) was originally discovered as a substance that interferes with viral replication. IFNs are pleiotropic cytokines with antiviral, anti-tumor and immunomodulatory properties, and they are central mediators of immune responses (Castro et al. 2018a). IFNγ can activate macrophages and cytotoxic T cells, polarize $CD4^+$ T cells to the Th1 type, and suppress Treg development and function (Dunn et al. 2006; Bhat et al. 2017; Castro et al. 2018b). Furthermore, IFNγ plays a role in providing a third signal to activate tumor-reactive T cells (Curtsinger and Mescher 2010). On the other hand, IFNγ exhibits immune modulatory effects by inducing the expression of ligands for inhibitory receptors such as PD-L1 and PD-L2 on stromal and tumor cells (Garcia-Diaz et al. 2017). Additionally, IFNγ induces the expression of

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/19768354.2024.2442393.
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CXCL10 and FAT10 in tumor, with CXCL10 promoting angiogenesis and tumor growth through autocrine action, while FAT10 facilitates tumor metastasis by suppressing p53 and stabilizing ABI3 (Kim et al. 2018; Um et al. 2023; Kim et al. 2024).

Fas (CD95) is a death receptor localized on the surface of various cells and promotes signaling pathways that induce cell death (Aggarwal 2003). The interaction of Fas with its ligand regulates numerous physiological and pathological processes mediated through programmed cell death. When Fas ligand binds, the death domain of Fas forms a death-inducing signaling complex (DISC) and initiates the cell death program (Medema et al. 1997).

In this study, we prepared a tumor cell vaccine expressing engineered IFNγ in a CT26 colon carcinoma model as a chimera with Fas for dual purposes: first, to provide IFNγ to the tumor microenvironment for its immune stimulatory effect, and second, to deliver the Fas signal to tumor cell through the mbIFNγ/Fas chimera and IFNγ receptor interaction in a juxtacrine mode. We provide the first evidence that the CT26 tumor cell vaccine engineered to express chimeric mbIFNγ/Fas molecule effectively stimulates the cytotoxicity of immune cells *in vitro*. Furthermore, mice that rejected the mbIFNγ/Fas tumor cells displayed the acquisition of systemic anti-tumor immunity to CT26 cells involving T cells and NK cells. We propose that expressing mbIFNγ/Fas on tumor cells could be a potential strategy for developing whole tumor cell vaccines or gene therapies for cancer immunotherapy.

## Material and methods

### *Tumor cell line and mice*

The CT26 colon carcinoma cell line, derived from BALB/C mice, was utilized in this study. The cells were cultured in RPMI-1640 medium (Welgene, Korea), supplemented with 10% heat-inactivated fetal bovine serum (FBS; EqualFETAL, Atlas biologicals, USA) and 1% Penicillin-Streptomycin (10,000 U/mL, Gibco, U.S.A.), in a humidified environment with 5% $CO_2$ at 37°C. Female BALB/C mice, purchased at 6 weeks of age from Daehan Biolink (Korea), were used in the experiments. All animal procedures were approved and conducted following the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Chungnam National University (202206A-CNU-115).

### *Antibodies and reagents*

To assess the presence of IFNγ binding on cell membranes, PE-conjugated anti-mouse IFNγ (BD, USA) was used. The proportion of T cells was determined using PE-conjugated anti-mouse CD4 (BD) and APC-conjugated anti-mouse CD8a (BioLegend, USA). The proportion of natural killer cells was assessed using FITC-conjugated anti-mouse CD49b (BioLegend) and PerCP/Cyanine5.5-conjugated anti-mouse CD335 (BioLegend). The amount of MHC I and MHC II expressed on the cell surface was analyzed using PE-conjugated anti-mouse MHC I antibody (Invitrogen) and PerCP/Cyanine5.5-conjugated anti-mouse MHC II antibody (BioLegend).

### *Plasmid construction and transfection*

To obtain murine IFNγ, splenocytes from BALB/C mice were treated with Concanavalin A (0.5 μg/ml) for 48 h, followed by RNA extraction and cDNA synthesis using oligo (dT) primers and AccuPower RT premix (Bioneer, Korea). The cDNA was then used to amplify the DNA sequences for IFNγ and Fas (CD95). For the expression of secreted IFNγ, the entire sequence of IFNγ was used. To express membrane-bound IFNγ, constructs were generated using a portion of the extracellular and transmembrane domains of the TNF family member Fas (CD95). Each DNA insert was cloned into the pcDNA3.1 vector using *HindIII* and *NotI* restriction sites.

For DNA transfection into the CT26 cell line, the Lipofectamine 2000 transfection reagent (Invitrogen, USA) was used. Following transfection, cells were selected using G418 (0.5 mg/ml; AG Scientific, USA). Drug-resistant colonies were screened by extracting RNA, followed by reverse transcription. Cells was subjected to RT-PCR with appropriate primers using Taq polymerase (Genetbio, Korea) to amplify the target genes. The expression of these genes was confirmed by agarose gel electrophoresis.

### *ELISA*

The expression level of IFNγ in the confirmed CT26 cells was quantified using an IFNγ ELISA kit (Invitrogen, USA). Cells were seeded into a 24-well plate at a density of $1 \times 10^6$ cells per well in 1 ml of RPMI medium and incubated for 24 h. The supernatant was collected to measure the levels of secreted IFNγ.

### *MTT assay*

$1 \times 10^4$ cells from wild-type (WT) or stably transfected CT26 cells were plated into a 96-well culture plate, and cell proliferation was evaluated using the MTT (3-(4,5-Dimethylthiazol-2-yl)−2,5-Diphenyltetrazolium Bromide) assay for up to 48 hours (Goldbio, U.S.A.).

### Real-time analysis

For real-time PCR analysis, reverse-transcribed DNA was subjected to SYBR Green Master Mix (BioFACT, Korea) and appropriate primers. 18S rRNA was used for normalization in the real-time PCR analysis. Each PCR amplification was performed in triplicate, and the experiment was repeated three times. Relative mRNA levels were calculated using the $2^{(-\text{delta delta CT})}$ method.

### Tumor challenge

To evaluate the tumorigenic potential of WT or stably transfected CT26 cells *in vivo*, $1 \times 10^5$ cells were subcutaneously injected into the right flank of mice. The mice were monitored every other day for body weight, tumor size, and survival. Tumor size was calculated using the formula $0.52 \times \text{length} \times \text{width}^2$. To determine whether systemic immunogenicity had developed in mice that resisted tumor cells expressing mbIFNγ/Fas, WT CT26 cells ($1 \times 10^5$) were subcutaneously injected into the left flank two months after the initial injection. The mice were monitored every other day for body weight, tumor size, and survival.

### Cell cycle analysis

WT or stably transfected CT26 cells were seeded at a density of $2.5 \times 10^5$ cells per well in a 6-well plate and incubated for 48 h. Subsequently, the cells were harvested and washed once with PBS. The cells were then fixed in 1 ml of 70% ethanol and stored at −30°C for one week. After fixation, the cells were washed twice with PBS. To stain the DNA, 500 µl of PI solution (50 µg/ml propidium iodide with 0.1 mg/ml RNase A) was added, and the cells were resuspended by pipetting. The cells were incubated in the dark at 37°C for 1 h. Cell cycle analysis was performed using flow cytometry (FACS).

### Cytotoxic activity of IFNγ-expressing tumor cells in vitro

To evaluate the cytotoxic effects of IFNγ-expressing tumor cells on immune cells, we co-cultured splenocytes with these cells. At first, WT CT26 cells were treated with 50 µg/ml mitomycin C (MMC, Roche, Switzerland) for 20 minutes at 37°C to inhibit proliferation and then washed with RPMI medium. We immunized mice with the MMC-inactivated WT CT26 cells. After two weeks, spleen cells were isolated, and $1 \times 10^7$ splenocytes were stimulated with $5 \times 10^5$ MMC-inactivated IFNγ-expressing tumor cells (20:1 ratio) in a 24-well plate for three days. Then, the activated splenocytes were harvested and added to $5 \times 10^4$ CT26 WT cells. After another 72 h of co-culture, the splenocytes were carefully removed, and the live cells were counted and photographed under a microscope.

### FACS analysis

To evaluate the cytotoxic effects of IFNγ-expressing cells on immune cells *in vitro*, the proportions of T cells and natural killer (NK) cells in splenocytes were analyzed. Inactivated tumor cells treated with MMC were seeded at a density of $5 \times 10^5$ cells per well into a 24-well plate and incubated for 4 h. Subsequently, splenocytes from immunized mice were added at a 20:1 ratio to the cells for co-culture. After 72 h, the splenocytes were transferred to new wells containing fresh MMC-treated cells and incubated for an additional 72 h. After the second co-culture period, the activated splenocytes were harvested and stained with specific antibodies to determine the proportions of T cells and NK cells.

Mice that rejected mbIFNγ/Fas-expressing cells were subsequently implanted with WT CT26 cells. After 30 days, splenocytes were harvested from these mice and subjected to flow cytometry (FACS) analysis. The spleens were mashed through a 40 µM strainer, and red blood cells were removed using a red blood cell lysis buffer. The cells were then washed three times with FACS buffer, consisting of 1X PBS, 0.02% sodium azide and 2% FBS. FACS antibodies were utilized following the washes to determine T and NK cell proportions.

### Statistical analysis

All data are presented as the mean ± SEM (error bars). Graph-Pad Prism 9 (GraphPad Software, USA) was used to for one-way or two-way ANOVA to indicate significant differences between groups (*$P < 0.05$, **$P < 0.01$, ***$P <$ 0.001). Survival data was analyzed using Kaplan-Meier survival estimates from Origin Pro 8.1 (OriginLab Corporation, USA).

## Results

### Preparation of CT26 cancer cells expressing IFNγ as a secretory or membrane-bound form

Expression vectors were constructed to express IFNγ in CT26 colon cancer cells. For a secretory form of IFNγ, whole cDNA encoding the signal peptide and coding sequences was amplified from mRNA isolated from splenocytes of BALB/c mouse using appropriate primers. For

the membrane-bound form of IFNγ, the IFNγ coding sequence was conjugated with the cDNA of Fas, incorporating the cysteine-rich domains 2 (CRD2) and 3 (CRD3), transmembrane, and cytoplasmic domains of Fas (Figure 1A). Since Fas requires the extracellular CRD2 and CRD3 to induce death signaling, the mbIFNγ/Fas chimera was designed to incorporate these domains.

The expression vectors were transfected into CT26 cells. After 2∼3 weeks, drug-resistant colonies were isolated and analyzed for the expression of the inserted cDNA; mock vector, secretory form of IFNγ (sIFNγ), and membrane-bound form of IFNγ (mbIFNγ/Fas). RT-PCR was performed using appropriate primer sets to identify the expression of the secretory or membrane-bound forms of IFNγ. IFNγ mRNA was detected in most of the cells analyzed, and results for representative cells were presented (Figure 1B). The chimeric forms of IFNγ with Fas were also detected in the mbIFNγ/Fas transfectants. Interestingly, the CT26 cells were found to be IFNγ receptor-positive. Additionally, for mbIFNγ/Fas transfectants, the membrane-bound form of IFNγ was verified to express on the cell surface by FACS analysis (Figure 1C). The stable mbIFNγ/Fas tumor cells clearly represented the enhanced surface expression of IFNγ. ELISA was performed to confirm the protein production and quantify IFNγ in culture supernatants (Figure 1D). A large amount of IFNγ was detected in the culture supernatant from the sIFNγ tumor cells. Smaller amounts of IFNγ were also detected in the culture supernatants of the mbIFNγ/Fas cells. For further characterizing secretory and membrane-bound form IFNγ on CT26, we have used sIFNγ and mbIFNγ/Fas expressing tumor cells.

### *Cell cycle progression is delayed in IFNγ-expressing tumor cells*

In previous reports, IFNγ inhibited cell proliferation by causing cell cycle arrest in H6 liver cancer and L929 fibrosarcoma cells (Prasanna et al. 2007; Rakshit et al. 2014). We, therefore, analyzed the cell proliferation since CT26 cells express IFNγ receptors (Figure 1B). The proliferation rate of IFNγ-expressing tumor cells was delayed compared to that of WT or mock-vector-transfected (Mock) cells, as measured by the MTT assay (Figure 1E). Cell cycle analysis was performed using propidium iodide staining and FACS analysis (Figure 1F). The proportion of cells in the G1 phase was increased in all IFNγ-expressing tumor cells, while their proportions in the S phase decreased. These results suggest that the observed difference in proliferation rate is due to changes in the cell cycle induced by the expression of IFNγ.

### *Expression of mbIFNγ/Fas in CT26 cells reduces tumorigenicity*

To assess tumor-forming ability, BALB/c mice were injected subcutaneously with IFNγ-expressing tumor cells into the right thigh. In short-term observation, as shown in Figure 2A, tumor formation and growth were significantly reduced in mice injected with the tumor cells expressing mbIFNγ/Fas, compared to tumors in WT-, Mock-, and sIFNγ-injected mice. All mice injected with the mbIFNγ/Fas tumor cells remained tumor-free, while tumor growth was found in all mice injected with WT, Mock, or sIFNγ tumor cells. All injected mice were sacrificed on day 20 after tumor cell injection, and the sizes of tumors and spleens were compared. No significant difference in spleen size was observed (Figure 2B). In a repeated experiment of long-term observation, all mice injected with WT, Mock, and sIFNγ tumor cells died of the tumor within two months, whereas 3 out of 4 mice injected with mbIFNγ/Fas tumor cells were tumor-free (Figure 2C–E). Interestingly, sIFNγ-injected mice showed out-growth of tumors at a later time course (Figure 2C). These results indicate that the tumor cells expressing the mbIFNγ/Fas chimera reduces tumorigenicity compared to WT cells, Mock or sIFNγ cells.

### *Both sIFNγ and mbIFNγ/Fas elevate MHC I and PD-L1 expression, but sIFNγ leads to significantly higher PD-L1 expression compared to mbIFNγ/Fas*

IFNγ has been reported to exhibit immune modulatory effects by inducing PD-L1 and PD-L2 expression on stromal and tumor cells (Garcia-Diaz et al. 2017; Mojic et al. 2017). Therefore, we analyzed their expression in tumor cells expressing sIFNγ and mbIFNγ/Fas. The expression level of PD-L1 in sIFNγ- and mbIFNγ/Fas-expressing cells was significantly higher compared to the control groups, although it was lower in mbIFNγ/Fas-expressing cells than in sIFNγ-expressing cells (Figure 2F). The lower PD-L1 expression in mbIFNγ/Fas cells, compared to sIFNγ cells, may contribute, in part, to the retarded tumor growth observed in these cells. Additionally, it has been previously reported that IFNγ induces an increase in MHC I and MHC II expression through IRF-1, which controls CIITA expression, the master transcriptional regulator of MHC II, and targets the ISRE motif in the MHC I region (Schroder et al. 2003); van den Elsen 2011; Jongsma et al. 2019). To explore whether ectopically expressed IFNγ in CT26 cells increases MHC I and MHC II levels through an autocrine or paracrine effect, we analyzed MHC I and MHC II

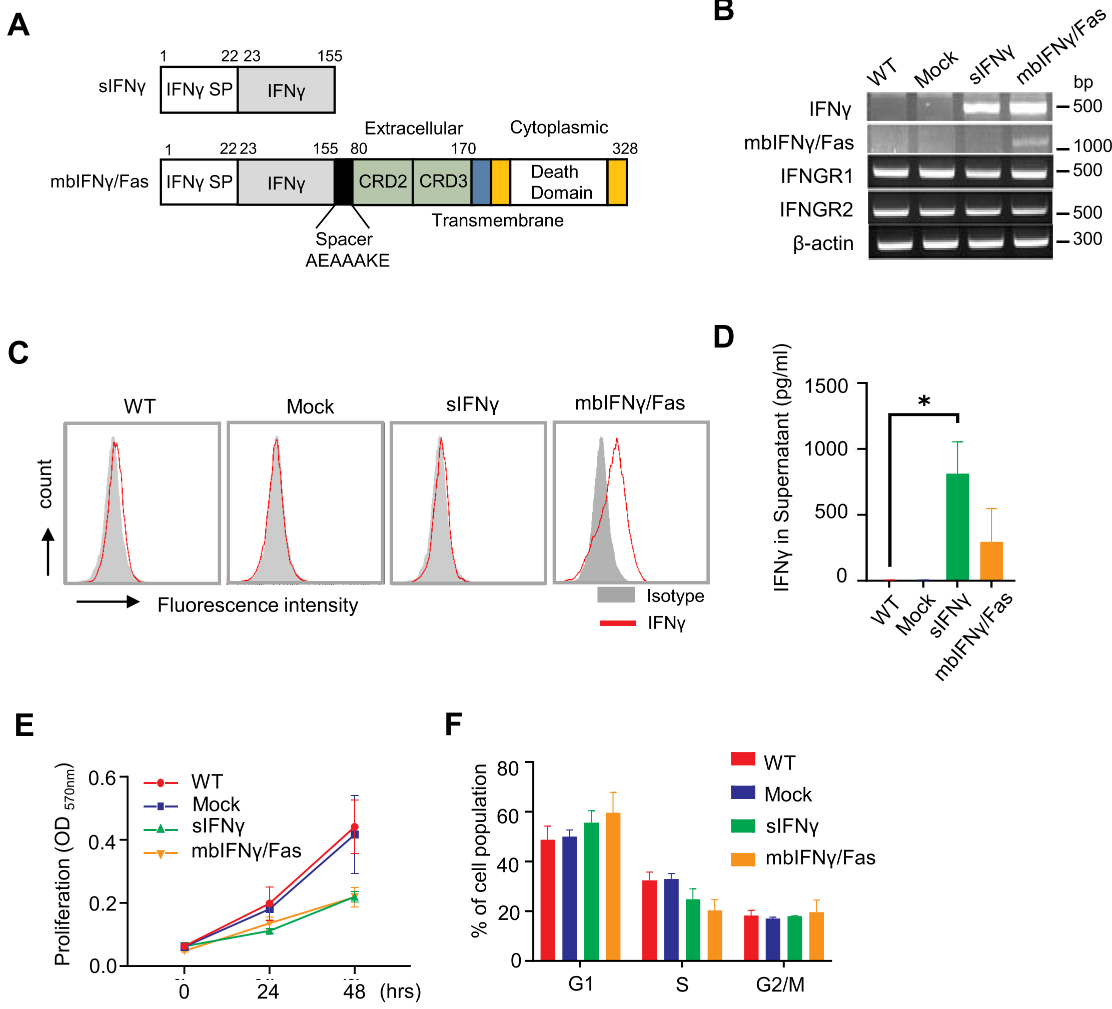


**Figure 1.** Preparation and characterization of IFNγ expressing tumor cells. (A) The design of the expression vectors allows IFNγ to be produced in either a secretory form (sIFNγ) or a membrane-bound form (mbIFNγ). To generate IFNγ in the membrane-bound form, a chimeric cDNA was constructed by incorporating a segment of Fas cDNA, including the CRD2, CRD3, transmembrane domain and cytoplasmic domain. SP, signal peptide. (B) The expression of sIFNγ, mbIFNγ and endogenous IFNγ receptors (IFNGR1 and IFNGR2), in G418-resistant transfected CT26 cells was analyzed by semi-quantitative RT-PCR using the primers listed in Supplementary Table S1 and compared with WT and Mock cells. (C) The selected transfectants were analyzed for the membrane-bound form of IFNγ using flow cytometry with an anti-mouse IFNγ antibody. (D) Supernatants from $1 \times 10^6$ tumor cells cultured for 24 hours were analyzed for secreted IFNγ expression by ELISA. (E) The proliferation of IFNγ-expressing tumor cells was assessed using the MTT assay. (F) Cell cycle analysis of the WT CT26 cells and transfectants was performed using flow cytometry. Data are presented as the mean ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

expression on stably transfected CT26 cells. Flow cytometry analysis showed that both forms of IFNγ similarly enhanced MHC I expression but do not stimulate MHC II expression (Figure 2G).

## *The mbIFNγ/Fas tumor cells effectively establish systemic anti-tumor immunity involving T and NK cells*

As shown in Figure 2E, 6 out of 7 mice injected with the mbIFNγ/Fas tumor cells were tumor-free for over two months. To determine whether these mice had acquired systemic anti-tumor immunity to CT26 cells, we challenged the survived mice with live WT CT26 cells subcutaneously into the contralateral left flank (Figure 3A). Tumors overgrew in all age-matched control mice (Figure 3B). In contrast, mice previously rejected the mbIFNγ/Fas tumor cells showed complete resistance to WT CT26 cells. Although tumor growth was initially observed in one of the three mice that had previously rejected the mbIFNγ/Fas tumor cells, the tumor regressed spontaneously over time. These results indicate that immune memory against CT26 cells was likely established in the mice that rejected the

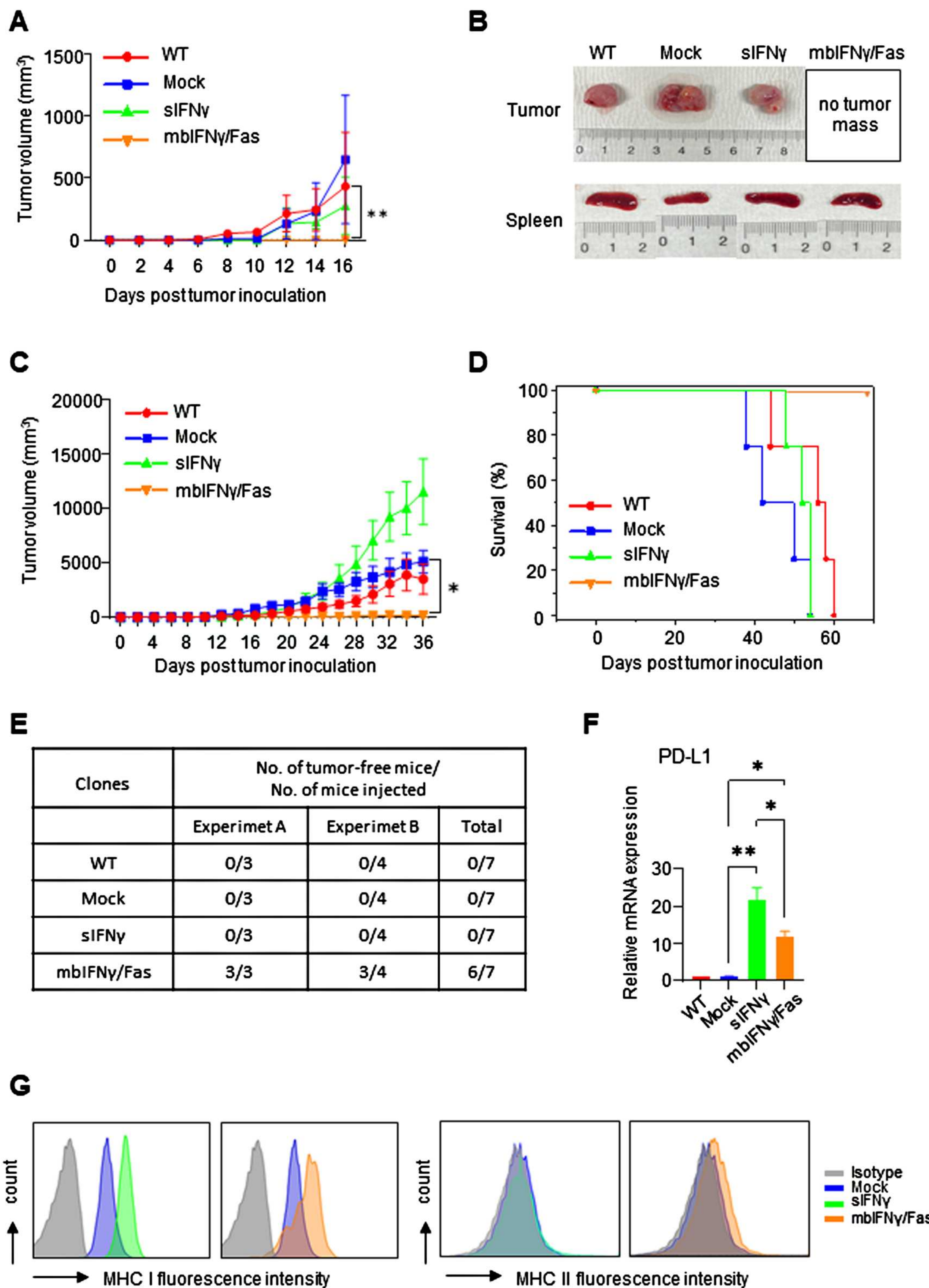


**Figure 2.** Tumorigenicity of IFNγ-expressing tumor cells in mice. (A-B) Each type of tumor cell ($1 \times 10^5$ cells per mouse) was injected subcutaneously into the right flank of mice ($n = 3$). Tumor volumes were monitored every other day for 16 days (A). On day 20 after tumor inoculation, mice with tumors were sacrificed, and the spleens and tumor masses were photographed (B). (C–D) Each type of tumor cell ($1 \times 10^5$ cells per mouse) was injected subcutaneously into the right flank of mice ($n = 4$). Tumor volumes were monitored over a long-term period (C). The survival of tumor-bearing mice is shown (D). (E) The number of tumor-free mice was compared across different tumor cell types in two independent experiments. (F) The relative mRNA expression level of PD-L1 in each type of tumor cell was determined by qPCR analysis. Data are presented as the mean ± SEM of three independent experiments. (G) The expression of MHC I and MHC II on CT26 transfectants was analyzed by flow cytometry. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

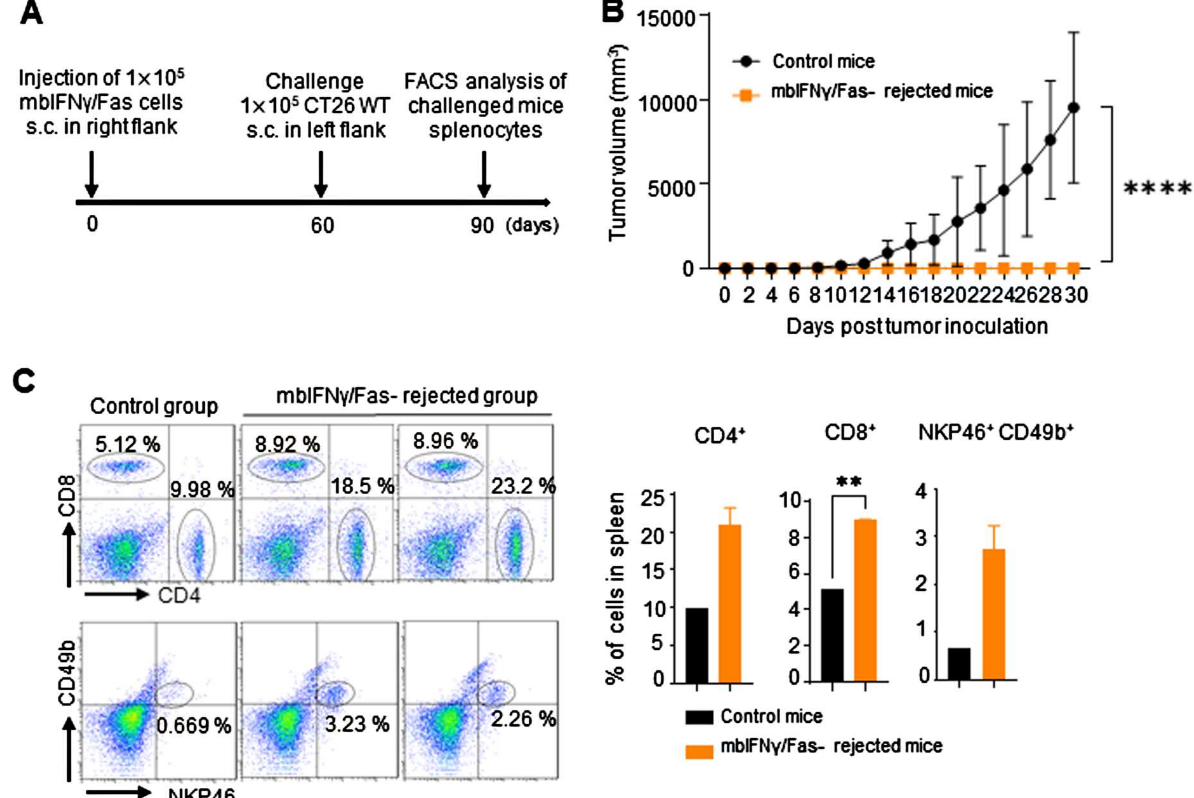


**Figure 3.** Mice that rejected the mbIFNγ/Fas cells developed systemic immunity to WT CT26 cells. (A) A Schematic experimental plan is shown. Tumor-free mice ($n = 3$) previously inoculated with mbIFNγ/Fas cells, were re-challenged with $1 \times 10^5$ WT CT26 cells subcutaneously into the left flank, 60 days after the initial tumor cell injection. At this time, age-matched control mice ($n = 2$) were also challenged with WT CT26 cells as controls. (B) The tumor growth was monitored for 30 days after re-challenge. (C) One month after re-challenge, the mice were sacrificed, and splenocytes were analyzed to determine the proportion of T cells and NK cells (NKP46$^+$CD49b$^+$) (left). The percentages of T cells and NK cells are displayed in a bar graph (right) *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

mbIFNγ/Fas tumor cells. After one month of the WT CT26 challenge, all mice were sacrificed, and T and NK cells in the spleen were analyzed (Figure 3C). We found that CD4$^+$ and CD8$^+$ T cells and NK cells were significantly induced in the mbIFNγ/Fas-rejected mice, suggesting a role for T and NK cells in systemic anti-tumor immunity.

### *The mbIFNγ/Fas-expressing CT26 cells enhance the cytotoxic activity of spleen cells* in vitro

To analyze the effect of IFNγ-expressing tumor cells on immune cells, we immunized mice with MMC-activated WT CT26 cells. After two weeks, spleen cells were isolated and stimulated with MMC-inactivated IFNγ-expressing tumor cells for three days. The activated spleen cells were then added to live WT CT26 cells for an additional 72 hours, and the survival of CT26 cells was assessed. To observe the degree of cell death, we counted live cells and photographed them under a microscope (Figure 4A). Spleen cells activated with the mbIFNγ/Fas cells showed the highest cytotoxic effect against WT CT26 cells, with approximately 40% more dead than the group activated with the Mock cells. In contrast, no significant increase in cytotoxic effect was observed in the group activated with the sIFNγ tumor cells. Interestingly, the NK cell population was prominently increased in the spleen cell population stimulated with the mbIFNγ/Fas cells (Figure 4B). These results suggest that the tumor cells expressing mbIFNγ/Fas enhances the activity of immune cells in splenocytes against CT26, compared to WT cells, Mock or sIFNγ cells *in vitro*.

## Discussion

Whole tumor cell vaccines modified with cytokine genes have been used to stimulate anti-tumor immunity for tumor immunotherapy (Liu et al. 2022; Perez-Banos et al. 2023). In this study, we demonstrate that an enhanced anti-tumor effect is induced when IFNγ is expressed in a membrane-bound form with Fas as a chimera (mbIFNγ/Fas) in CT26 colon carcinoma cells. The tumor cells expressing mbIFNγ/Fas exhibited lower

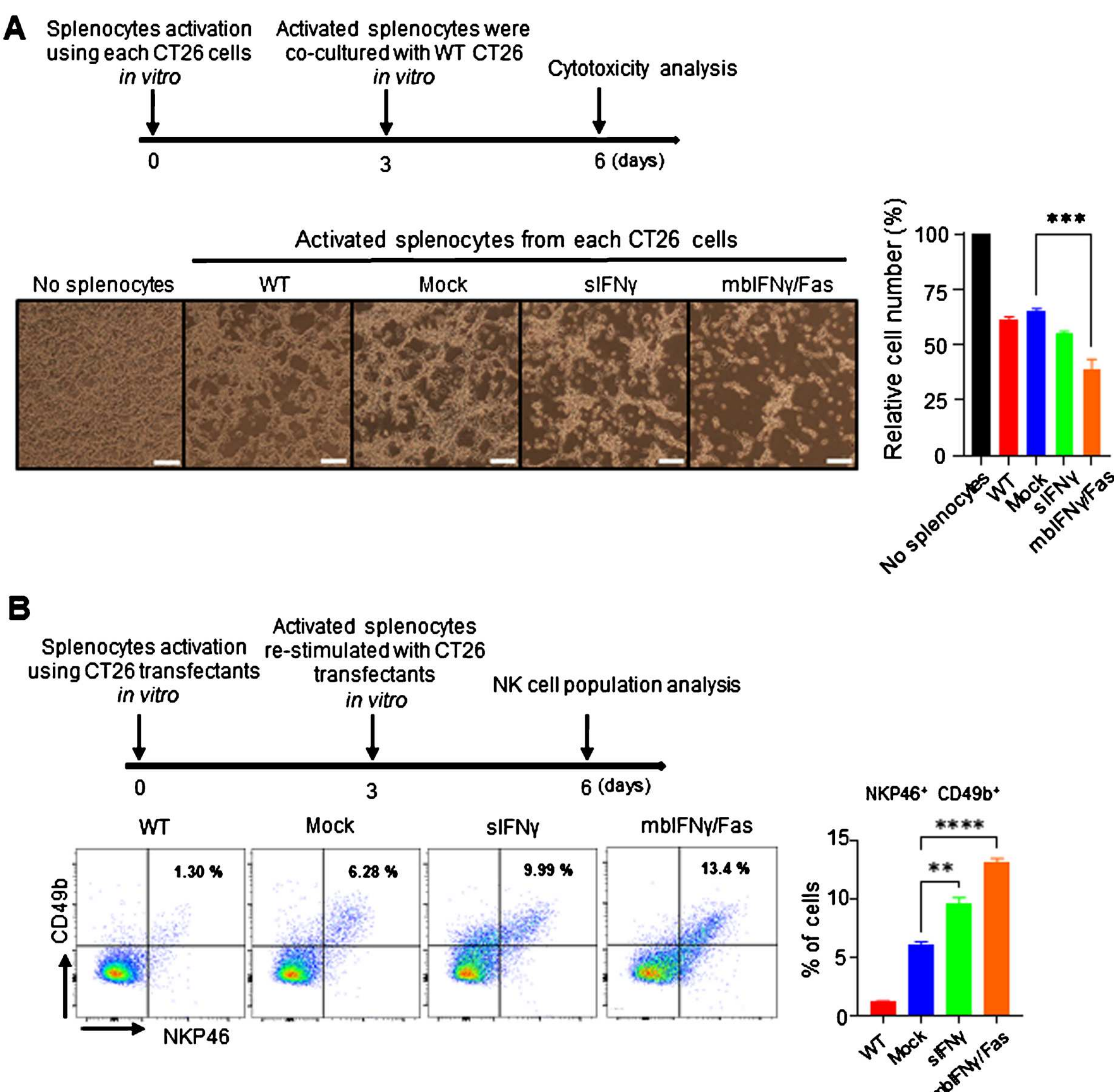


**Figure 4.** Spleen cells immunized with mbIFNγ/Fas tumor cells exhibited enhanced cytotoxic activity against WT CT26 cells *in vitro*. (A) A Schematic experimental plan is shown (Top). Splenocytes were activated with each CT26 transfectants for 3 days. Then, WT CT26 cells were directly co-cultured with these activated splenocytes. After an additional 3 days, the morphology of WT CT26 cells was observed under a microscope to verify cell death (bottom left), and the live WT CT26 cells were counted (bottom right). Scale bar, 100 μm. (B) Splenocytes were primed *in vitro* twice with CT26 transfectants, harvested, and analyzed to determine the percentage of NK cells. Data are presented as the mean ± SEM of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

tumorigenicity, and mice previously rejected the mbIFNγ/Fas tumor cells acquired systemic anti-tumor immunity to CT26 cells.

The tumor cells expressing mbIFNγ/Fas was expected to exert IFNγ effects in the tumor microenvironment. Generally, the growth rate of tumor cells expressing IFNγ was slower than that of WT tumor cells *in vitro* (Figure 1E). Since CT26 cells express receptors for IFNγ, the direct effects of IFNγ expression on cancer cells were analyzed by comparing the proliferation rate and cell cycle progression with control groups. Cell proliferation rate decreased in cells expressing IFNγ, and cell cycle retardation was observed in the G1 phase (Figure 1F). Previous reports have shown that IFNγ inhibits cell proliferation by causing cell cycle arrest in H6 liver cancer and L929 fibrosarcoma cells (Prasanna et al. 2007; Rakshit et al. 2014). Although cell lines differ, these results indicate that IFNγ inhibits cell cycle progression in the G1 phase even in CT26 cancer cells, explaining the slower growth rate.

Another hypothesis is that the interaction of mbIFNγ/Fas with the IFNγ receptor may deliver a death signal to

tumor cells through a juxtacrine interaction, potentially affecting tumor cell viability *in vivo*. Similarly, reports indicate that a chimera fusing the extracellular domain of CD40 with the transmembrane/intracellular domain of Fas maintained the basic signaling properties of Fas when expressed in L929 cells (Rudert et al. 1994). Moreover, TC-1 cells expressing chimeric molecules of MULT1 and FasTI (Kotturi et al. 2008) or IL-12 and FasTI (Yang et al. 2016) effectively activated NK cells and endured Fas-mediated apoptosis. Unfortunately, since both Fas-mediated cell death signaling and the effect of IFNγ on tumor cells converge on caspase-3 activation (Jorgovanovic et al. 2020), it was difficult to differentiate between the Fas-mediated cell death signaling and the effect of IFNγ in the mbIFNγ/Fas tumor cells. If the presence of Fas signaling is configured, the effects of mbIFNγ/Fas tumor cells *in vivo* could be interpreted more clearly.

The sIFNγ tumor cells displayed a decreased cell proliferation rate *in vitro*. However, the tumor growth rate significantly increased in BALB/c mice compared to the control group (Figure 2C). Several studies have reported that when IFNγ is used alone as an immunotherapy agent, it can promote tumor growth through immunosuppressive effects, such as increasing immune checkpoints (Mojic et al. 2017). Analysis of PD-L1 gene expression in the sIFNγ cells used in this study revealed that the level of PD-L1 was significantly higher compared to the control groups and the mbIFNγ/Fas tumor cells (Figure 2F). These results suggest that sIFNγ tumor cells may not exhibit an anti-tumor effect due to increased PD-L1 expression and its associated immunosuppressive effects. However, there was no significant difference in MHC I and MHC II levels between sIFNγ and mbIFNγ/Fas tumor cells. Both forms of IFNγ similarly enhanced MHC I levels and did not alter MHC II levels compared to Mock cells. Interestingly, previous reports have shown that low levels of IFNγ in the tumor microenvironment (TME) promote tumor metastasis via the ICAM1-PI3K-Akt-Notch1 signaling pathway, whereas high levels of IFNγ trigger the JAK1-STAT1 signaling pathway to induce apoptosis in non-small cell lung cancer (Song et al. 2019). In our experiments with IFNγ-expressing tumor cells in mice, sIFNγ may diffuse systematically from the secretion sites, resulting in a low concentration within TME. In contrast, mbIFNγ/Fas does not diffuse, which may be advantageous for maintaining a higher concentration of IFNγ within the TME, potentially contributing to tumor growth inhibition.

When immune cells from the spleen were activated with mbIFNγ/Fas cells *in vitro*, the cytotoxic effect against CT26 cells increased (Figure 4A). This stimulatory effect on immune cells is predicted to result in lower tumorigenicity *in vivo* (Figure 2). Additionally, cancer cells expressing mbIFNγ/Fas may contribute to establishment of systemic anti-tumor immunity, possibly involving $CD4^+$, $CD8^+$ T cells, and NK cells (Figures 3 and 4B). Although the activation of $CD4^+$ subtypes has yet to be identified in our experiment, it is well known that IFNγ promotes T cell differentiation into Th1 cells, enhancing cytotoxic activity and increasing NK cell cytotoxicity. Consequently, IFNγ reinforces antiviral defense, tumor surveillance, and the overall immune response (Castro et al. 2018b). Additionally, IFNγ suppresses Th2 differentiation by inducing T-bet, which disrupts the activity of the Th2-specific transcription factor GATA-3, and it also inhibits Th17 differentiation by preventing the expression of RORγt (Hwang et al. 2005). IFNγ is widely recognized as a pro-inflammatory cytokine that modulates anti-inflammatory responses by preventing Treg cell differentiation and function (Caretto et al. 2010). Regarding the enhanced $CD8^+$ expression by mbIFNγ/Fas (Figure 3), this may lead to a synergic effect when combined with the increased MHC I expression on CT26 cells, resulting in enhanced $CD8^+$ T cell-mediated tumor-killing effects. Furthermore, the higher NK cell population induced by both sIFNγ and mbIFNγ/Fas expression in CT26 cells will enhance the cytotoxic effect. Reports also indicate that IFNγ plays an important role in providing long-term immunity against pathogens by promoting the formation, survival, and function of memory T cells (Whitmire et al. 2007). Although it was anticipated that memory cells would be formed and systemic immunogenicity acquired, direct confirmation was not achieved, highlighting the need for further research.

Numerous clinical trials have explored whole tumor cell vaccines with various adjuvants to enhance immunogenicity (Perez-Banos et al. 2023). Moreover, live tumor cell vaccines are more effective than killed tumor cell vaccines because inactivated tumor cells are limited by their inability to kill tumor cells before eliciting a sufficient immune response (Dondossola et al. 2016; Chen et al. 2023). Live tumor cells have the potential to target tumors and enhance anti-cancer immune responses, especially if a self-killing mechanism is incorporated into the cancer cell vaccine. The results of this study demonstrated a robust anti-tumor effect of mbIFNγ/Fas on cancer cells, suggesting it could represent a new approach for next-generation cancer immunotherapy.

## Author contributions

SYJ and HSS designed the whole research study and performed all assays. In this research, JOL, YSK, and HL

analyzed the data and revised the manuscript. All authors have read and approved the final manuscript.

## Funding

This work was supported by the Technology Innovation Program [grant number 20019707] funded by the Ministry of Trade, Industry and Energy (MOTIE of Korea).

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