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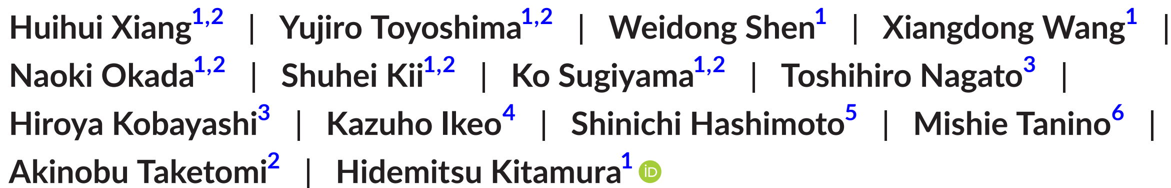
IFN-α/β-mediated NK2R expression is related to the malignancy of colon cancer cells



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Abstract

Neurokinin 2 receptor (NK2R), a G protein-coupled receptor for neurokinin A (NKA), a tachykinin family member, regulates various physiological functions including pain response, relaxation of smooth muscle, dilation of blood vessels, and vascular permeability. However, the precise role and regulation of NK2R expression in cancer cells have not been fully elucidated. In this study, we found that high NK2R gene expression was correlated with the poor survival of colorectal cancer patients, and Interferon (IFN-α/β) stimulation significantly enhanced NK2R gene expression level of colon cancer cells in a Janus kinas 1/2 (JAK 1/2)-dependent manner. NKA stimulation augmented viability/proliferation and phosphorylation of Extracellular-signal-regulated kinase 1/2 (ERK1/2) levels of IFN-α/β-treated colon cancer cells and NK2R blockade by using a selective antagonist reduced the proliferation in vitro. Administration of an NK2R antagonist alone or combined with polyinosinic-polycytidylic acid, a synthetic analog of double-stranded RNA, to CT26-bearing mice significantly suppressed tumorigenesis. NK2R-overexpressing CT26 cells showed enhanced tumorigenesis and metastatic colonization in both lung and liver after the inoculation into mice. These findings indicate that IFN-α/β-mediated NK2R expression is related to the malignancy of colon cancer cells, suggesting that NK2R blockade may be a promising strategy for colon cancers.

KEYWORDS
colon cancer, JAK, malignancy, neurokinin-2 receptor, type I IFN

Abbreviations: CRC, colorectal cancer; DCs, dendritic cells; EMT, epithelial–mesenchymal transition; ERK, Extracellular-signal-regulated Kinase; IFN, Interferon; JAK, Janus kinase; MFI, mean fluorescence intensity; NK1R, neurokinin-1 receptor; NK2R, neurokinin-2 receptor; NKA, neurokinin A; poly I:C, polyinosinic-polycytidylic acid; SP, substance P; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor.

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1 | INTRODUCTION

Tachykinins, such as substance P (SP), neurokinin A (NKA) and B, hemokinin-1, and endokinins, are a family of neuropeptides that act through three types of G-protein coupled receptors (GPCRs), neurokinin-1 receptor (NK1R), neurokinin-2 receptor (NK2R), and neurokinin-3 receptor (NK3R), functioning as neurotransmitters in the peripheral and central nervous systems.[1–3] Neuropeptide signaling through the receptors is well known to regulate various physiological functions such as induction of pain response, relaxation of smooth muscle, dilation of blood vessels, and vascular permeability. It has been demonstrated that tachykinins and their receptors are expressed in several non-neuronal cells, which connect the nervous and peripheral organ systems to control respiratory, cardiovascular, endocrine, and gastrointestinal activity.[2,4] Furthermore, recent studies revealed that neuropeptide receptors are expressed in various cancer cells in the tumor microenvironment, therefore tachykinin signaling through these receptors possibly regulates pathological functions related to the development of cancer and tumor malignancy.[5,6]

Previous research has indicated that SP–NK1R signaling is implicated in the promotion of cancer incidence and facilitates metastatic progression. Various tumor types express NK1R, the main receptor of SP, such as glioblastoma,[7] breast cancer,[8] leukemia,[9] pancreatic ductal adenocarcinoma,[10] hepatoblastoma,[11] and colon cancer,[12,13] which use SP signaling through NK1R to promote the proliferation and survival of cancer cells and to release soluble mediators to promote tumor growth or metastasis. However, the precise role of NK2R expression in cancer cells and the mechanism for tumor malignancy have not been fully demonstrated compared with that of the NK1R-mediated SP signaling cascade.

Colorectal cancer (CRC) is the second leading cause of cancer death worldwide. With increasing mortality observed in recent years, the global burden of CRC is expected to increase by 60% to more than 2.2 million new cases and 1.1 million deaths by 2030.[14] The rise in CRC incidence can be attributed to aging populations, unfavorable daily diet habits, stress, and increased risk factors.[15] Recent studies indicated that the molecular pathobiology of CRC involves viral and/or bacterial infection-induced inflammation, further promoting malignant progression, invasion, and metastasis of the tumor,[16–18] suggesting that patients with inflammatory bowel disease (IBD) have a higher risk of CRC. A recent phase II clinical trial using NK2R inhibitor for irritable bowel syndrome (IBS) with diarrhea reported improved overall symptoms, abdominal pain, and stool patterns in female patients.[19]

Type I IFNs, IFN-α and IFN-β, are the two main IFNs involved in fighting viral and bacterial infections.[20,21] Previous papers revealed that IFN-α/β produced in the tumor microenvironment activate anticancer immunity by targeting dendritic cells (DCs) and noncancerous cells[22] and have the capacity to control cancer cell transformation.[23] When IFN-α/β bind to the receptor, IFN-AR1, Janus kinase (JAK), signal transducer and activator of transcription (STAT) are activated. The IFN-α/β-JAK–STAT signal pathway mediates many aspects of cellular immunity, proliferation, apoptosis, and differentiation. Previous research revealed that activation of IFN-STAT1 signaling triggers apoptosis and cell cycle arrest in various cancer cells.[24,25]

In this study, we first found that high expression levels of NK2R gene in tumor tissues were correlated with poor survival of CRC patients. Next, we revealed that in vitro stimulation with type I interferons, IFN-α/β, or in vivo administration of a synthetic analog of double-stranded RNA, polyinosinic-polycytidylic acid (poly I:C), to tumor-bearing mice significantly augmented NK2R gene expression levels in colon cancer cells. NK2R antagonist treatment combined with IFN-α/β or poly I:C significantly suppressed the proliferation of colon cancer cells in vitro or tumorigenesis of colon cancer-bearing mice, respectively. Furthermore, we confirmed that overexpression of the NK2R gene in murine colon cancer cells promoted tumorigenesis and metastasis in vivo. Here, we report the involvement of IFN-α/β-mediated NK2R expression in the malignancy of colon cancer cells, which may contribute to the development of effective therapy for CRC.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

Fluorescent dye-conjugated anti-CD45 (30-F11) and anti-mouse CD11c (N418) were obtained from BioLegend or BD Biosciences. 7-amino-actinomycin D (7AAD) was purchased from Beckman Coulter. Rabbit polyclonal antibody against NK2R (M-48) was purchased from Santa Cruz Biotechnology, Inc. Recombinant murine IFN-α15 (752802) and human IFN-α2 (592702) were purchased from BioLegend and murine IFN-β (8234-MB) was from R&D systems Inc. Recombinant human IFN-β (092–06061) and neurokinin A agonist (140–07171) were purchased from Wako Pure Chemical Industries. An NK2R-selective antagonist (GR 159897) was purchased from TOCRIS Bioscience. Poly I:C (HMW) was purchased from InvivoGen. A selective JAK1/2 inhibitor (ruxolitinib) was purchased from abcam.

2.2 | Patient specimens

Research protocols involving human subjects were approved by the institutional review board of Hokkaido University Graduate School of Medicine (14–043) and the Institute for Genetic Medicine (14–0004), and Asahikawa Medical University (15143). Written informed consent was obtained from each patient. The study was conducted in accordance with the World Medical Association Declaration of Helsinki.

2.3 | Cell lines and mice

The human colorectal cancer cell line DLD-1 (TKG 0379) was obtained from the Cell Resource Center for Biomedical Research,

Institute of Development, Aging, and Cancer, Tohoku University. The murine colon carcinoma cell line CT26 (CRL-2638) was obtained from the American Type Culture Collection. Wild-type BALB/c mice were obtained from Charles River Japan. All mice were maintained in specific pathogen-free conditions and used at 6–8 weeks of age. All mouse experiments were approved by the Animal Ethics Committee of Hokkaido University (No.14–0062 and No.19–0036) and were conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the University.

2.4 | Knockout of the IFNAR1 gene and overexpression of the NK2R gene in CT26 cells

Murine IFNAR1 CRISPR/Cas9 KO (sc-421,047) and control CRISPR/Cas9 KO plasmids (sc-418,922) were purchased from Santa Cruz Biotechnology. IFNAR1 gene knockout and control mock CT26 cells were established according to the manufacturer's protocol. GFP-expressing CT26 (CT26-mock control) cells and murine NK2R gene-overexpressing CT26 (CT26-NK2R OE) cells were established according to the previous studies.[26,27]

2.5 | Tumor-bearing mouse model

CT26 cells, CT26-NK2R OE cells, CT26-IFNAR1 KO cells, or the corresponding CT26 mock control cells (1×10^6) were injected intradermally into BALB/c mice. NK2R antagonist (2.4 μg/mouse) or DMSO combined with or without control PBS or poly I:C (50 μg/mouse) was intratumorally injected into the CT26-bearing mice at day 5 and then every 4 days thereafter. Tumor size was measured from day 5 and tumor volumes were calculated as described previously.[28] In some experiments, tumor tissues were collected from mice. After collagenase treatment and homogenization, total tumor cells were analyzed by flow cytometry.

2.6 | Metastasis mouse models

GFP-transfected CT26-NK2R OE cells or CT26 mock control cells (1×10^6) were inoculated intravenously into BALB/c mice. Lung tissues were collected from CT26 cell-inoculated mice at day 14 and metastasis were evaluated according to the previous study.[27] In some experiments, GFP-transfected CT26-NK2R OE cells or CT26 mock control cells (2×10^5) were inoculated intrasplenically into BALB/c mice to evaluate metastatic colonization in the liver tissues on day 14.

2.7 | Real-time PCR

Total RNA was extracted from culture cells, tumors, and liver tissues and cDNA prepared as described previously.[26,27] Human NK2R (*TACR2*), human TAC1 (*TAC1*), human GAPDH (*GAPDH*), murine NK2R (*Tacr2*), Tac1, IFN-α (*Ifna*), IFN-β (*Ifnb*), and β-actin (*Actb*) genes were amplified and detected by a real-time PCR detection system (Bio-Rad). The primer sequences and the probe numbers are shown in Table S1. Sample signals were normalized to the housekeeping GAPDH or β-actin gene according to the $\Delta\Delta C_t$ method: $\Delta C_t = \Delta C_{tsample} - \Delta C_{treference}$.

2.8 | Confocal microscopy

DLD-1 cells cultured on 35-mm culture dishes with glass coverslips (Iwaki) were stained with anti-NK2R mAb and DAPI. Fluorescence signals were detected with the Spectral Confocal Scanning system (FV300; Olympus) through a 633/1.4 Oil DIC I lens. The signals were visualized with standard software (Olympus).

2.9 | Flow cytometry

DLD-1 cells were treated with a Fix/Perm Buffer kit (Biolegend) and stained with anti-NK2R mAb. The expression of NK2R was evaluated by FACSCanto II (BD Biosciences) and analyzed with FlowJo software (Tree Star) according to the previous study.[27] In some animal model experiments, $CD45^+CD11c^+7AAD^-$ cells and $CD45^-GFP^+7AAD^-$ CT26 cells were isolated as DCs and colon cancer cells by FACSAria II (BD Biosciences), respectively.

2.10 | Immunohistochemistry

Tumor specimens from patients with CRC were formalin-fixed and paraffin-embedded. After deparaffinization, sections were treated with anti-NK2R antibody (M-48; Santa Cruz Biotechnology, Inc.). Protein expression was visualized as described previously.[27] Tumor tissues obtained from CT26 tumor-bearing mice were fixed in 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries) and then embedded in paraffin. After deparaffinization, sections were stained with HE.

2.11 | Cell proliferation assay

DLD-1 and CT26 cells were plated in 96-well plates and incubated at 37°C for 12, 24, or 48 h. Cell proliferation was determined using a cell counting kit-8 (CCK-8) cellular proliferation assay (Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol.

2.12 | Western blotting

Whole cell lysates prepared from DLD-1 cells or CT26 cells using cold RIPA buffer (Thermo Fisher Scientific) were separated on

SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were probed with anti-phospho-ERK1/2 (Thr202/Tyr204, #4370; Cell Signaling Technology) and α-tubulin (clone DM1A; Sigma-Aldrich) antibodies. The protein signals were detected by Image Quant LAS4000 mini (GE Healthcare). The p-ERK1/2 signals were standardized based on those of α-tubulin by using Image J (National Institutes of Health).

2.13 | Statistical analysis

In vitro experiments were repeated three to five times. In vivo experiments consisting of three to six mice in each group were independently performed two to three times. Single representative experiments are indicated in the figures. All data were expressed as the mean ± standard deviation (SD). Significant differences in the results were determined by one-way or two-way analysis of variance (ANOVA) and Dunnett's post-test. In some experiments, the two-tailed Student's *t*-test was used for the evaluation of differences between two groups. p values of <0.05 were considered statistically significant. Kaplan–Meier survival analysis was performed, and the log-rank test was used to determine statistically significant differences in survival curves among cancer patients.

3 | RESULTS

3.1 | High NK2R expression is correlated with poor prognosis in CRC patients and IFN-α/β augments NK2R expression in human colon cancer cells

To clarify the involvement of NK2R expression in the pathology of patients with CRC, we first evaluated NK2R expression levels in the tumor tissues of 25 CRC patients (Table S2). NK2R was overexpressed in cancerous cells compared with normal mucosal epithelial cells in 22 of 25 CRC patients (Figures 1A and S1). Next, we investigated the relationship between NK2R gene expression levels and survival of patients with CRC through the analysis of existing TCGA RNA-seq data (The Cancer Genome Atlas, TCGA datasets). Kaplan–Meier survival analysis revealed that the higher expression levels of NK2R gene were correlated with poorer survival in CRC patients (Figure 1B).

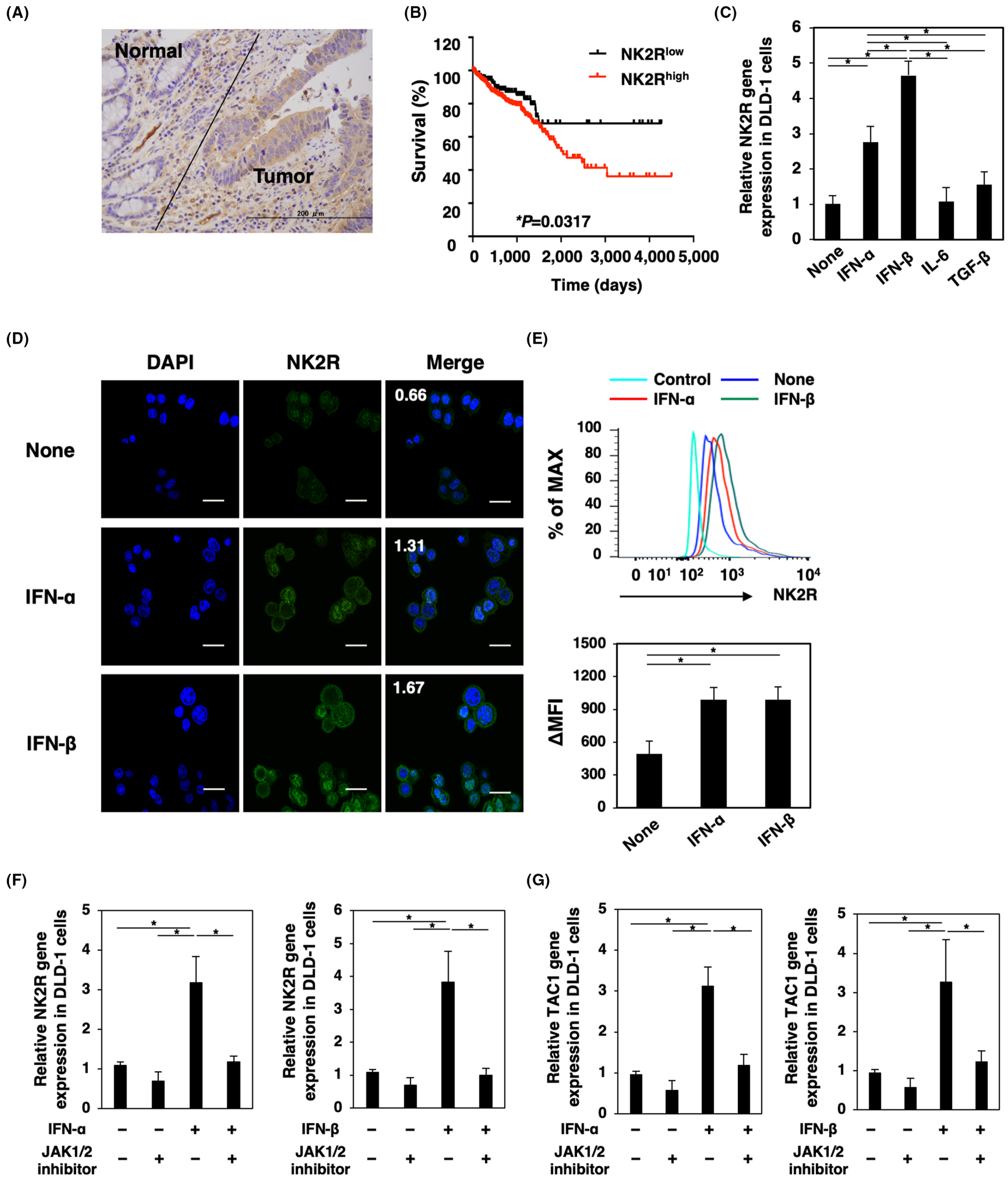
To address the precise regulation of NK2R-mediated neuropeptide signaling in cancer cells, we next investigated the gene expression levels of NK2R and TAC1, which encodes SP and NKA, in human colon cancer cells. Stimulation with IFN-α or IFN-β significantly induced NK2R gene expression in DLD-1 cells, whereas IFN-γ, IFN-λ1, IFN-λ2, IFN-λ3, IL-6, or TGF-β stimulation did not alter the expression levels (Figures 1C and S2). After the IFN-α/β treatment of DLD-1 cells, upregulation of NK2R protein expression was observed by confocal microscopy (Figure 1D) and flow cytometry (Figure 1E). The upregulation of NK2R gene expression levels was significantly suppressed in the presence of a JAK1/2 inhibitor (Figure 1F). Furthermore, we found that TAC1 gene expression levels were significantly enhanced by IFN-α/β stimulation, and this upregulation was reduced by the JAK1/2 inhibitor (Figure 1G). Analysis for ISRE and GAS sequences on the promoters of NK2R and TAC1 genes revealed that STAT1:STAT2 or IRF9 binding motifs existed in the promoter regions of NK2R (*TACR2*, *Tacr2*) and TAC1 (*TAC1*, *Tac1*) genes in both humans and mouse (Figure S3). These findings suggest that IFN-α/β type I interferons may augment neuropeptide signaling in colon cancer cells through the induction of both the NKA ligand and the receptor, NK2R.

3.2 | NK2R-mediated signaling regulates the viability and proliferation of human and murine colon cancer cells in vitro

Next, we investigated the effect of NK2R-mediated neuropeptide signaling on the viability and proliferation of human colon cancer cells. Treatment with NK2R antagonist significantly suppressed the viability and proliferation of DLD-1 cells in the presence of IFN-α or IFN-β (Figure 2A,B). IFN-α/β stimulation reduced the viability and proliferation of DLD-1 cells in vitro and this reduction was recovered by treatment with NKA (Figure 2C,D). We confirmed that stimulation of NKA combined with IFN-α/β enhanced phospho-ERK1/2 expression revel in DLD-1 cells compared to stimulation with IFN-α/β alone (Figure 2E,F). These data suggest that NK2R-mediated neuropeptide signaling may be related to the viability and proliferation of human colon cancer cells.

Furthermore, we found that IFN-α/β stimulation significantly enhanced NK2R and TAC1 gene expression levels in CT26 murine colon cancer cells in a JAK1/2-dependent manner (Figure 3A,B). Treatment

FIGURE 1 Correlation of NK2R expression with prognosis of CRC patients and regulation in human colon cancer cells. (A) NK2R expression was evaluated for sections of lesions and inflammatory areas from 25 patients with CRC. A representative microphotograph of each patient is indicated. Bars represent 200 μm. (B) Kaplan–Meier estimates of survival for 597 CRC patients in TCGA datasets stratified into two groups: high (red, 466 patients) or low (black, 131 patients) NK2R gene expression group. $^*P<0.05$ by a log-rank Mantel-cox test. (C) DLD-1 cells were treated with IFN-α (50 ng/ml), IFN-β (50 ng/ml), IL-6 (50 ng/ml) and TGF-β (50 ng/ml) for 3 h. Mean and SD ($n = 4$) of relative NK2R gene expression levels are indicated. $^*P<0.05$ by Tukey's multiple comparisons test after ANOVA test. (D, E) DLD-1 cells were treated with IFN-α and IFN-β for 24 h. Expression levels of NK2R were evaluated by confocal microscopy and flowcytometry. Representative images of NK2R (green) and DAPI (blue) staining of DLD-1 cells are indicated. Bars represent 20 μm. Representative histograms are indicated. ΔMFIs against each isotype control were calculated. Means and SDs ($n = 4$) are shown. $^*P<0.05$ by Student's t test. (F, G) DLD-1 cells were treated with IFN-α and IFN-β in the absence and presence of JAK1/2 inhibitor (1 μM) for 3 h. Mean and SD ($n = 4$) of relative NK2R and TAC1 gene expression levels are indicated. $^*P<0.05$ by Tukey's multiple comparisons test after ANOVA test



with NK2R antagonist effectively suppressed the viability and proliferation of the IFN-α/β-stimulated CT26 cells (Figure 3C,D). IFN-α/β stimulation reduced the viability and proliferation of CT26 cells in vitro and this reduction was recovered by the treatment with NKA (Figure 3E,F). We confirmed that NKA stimulation combined with IFN-α/β augmented phospho-ERK1/2 expression levels in CT26 cells compared to stimulation with IFN-α/β alone, as in the case of DLD-1 cells (Figure 3G,H). These data suggest that NK2R-mediated neuropeptide signaling is also involved in the viability and proliferation of murine colon cancer cells.

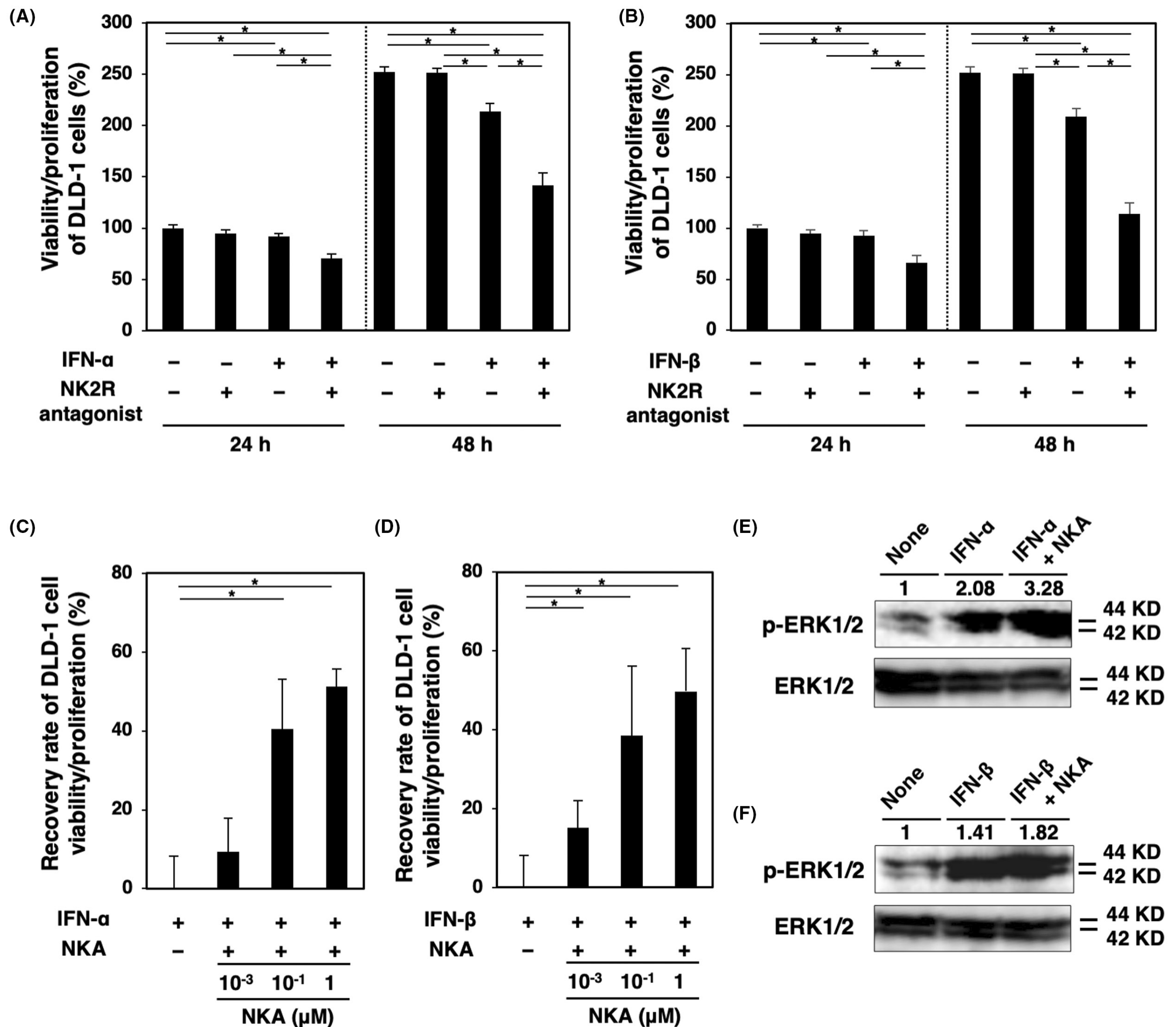
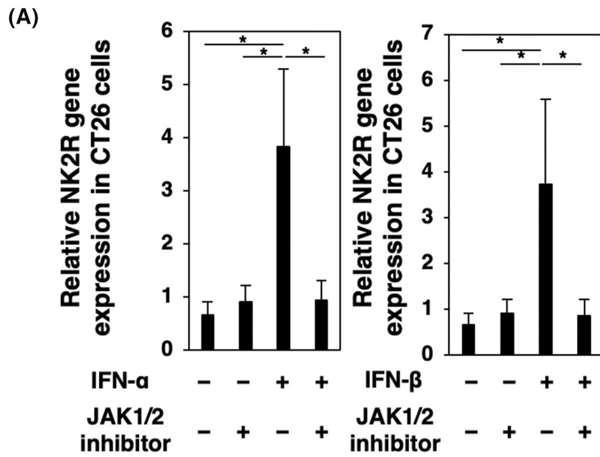
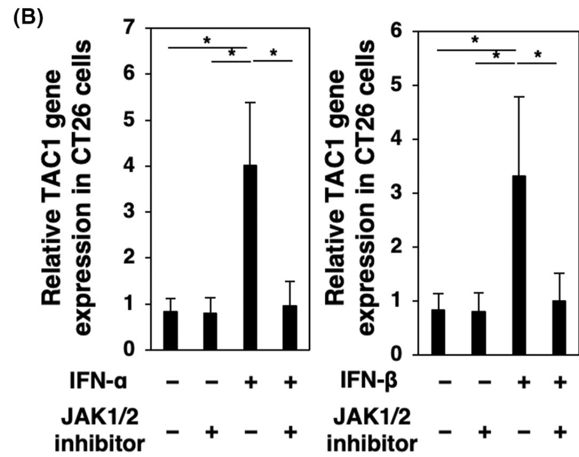
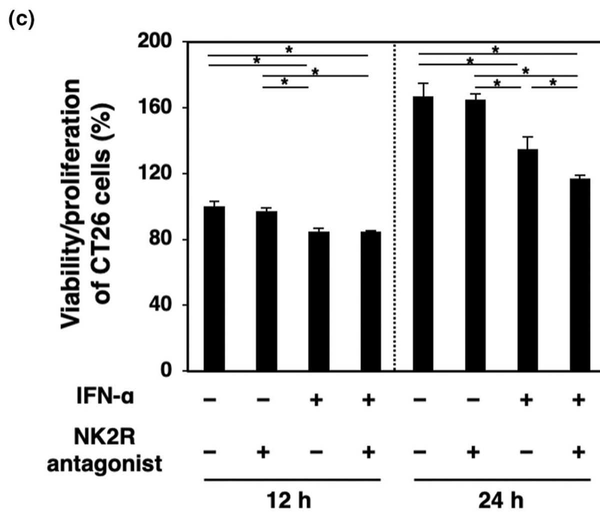


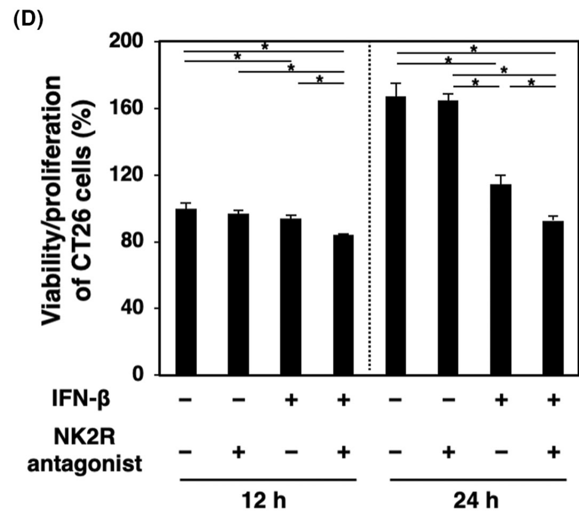
FIGURE 2 NK2R expression and the viability and proliferation of human colon cancer cells in vitro. DLD-1 cells were cultured in the absence and presence of the NK2R antagonist (10 μM) and/or IFN-α (50 ng/ml) and IFN-β (50 ng/ml). (A, B) Viability and proliferation were evaluated at 24 and 48 h. Means and SDs ($n = 4$) of data are indicated. *$P < 0.05$ by Tukey's multiple comparisons test after ANOVA test. (C, D) Recovery rates of cell viability/proliferation were evaluated for the IFN-α/β-treated DLD-1 cells in the presence of NK2R agonist (0, 10^{-3} μM, 10^{-1} μM, 1 μM) for 24 h. Means and SDs ($n = 4$) of data are indicated. *$P < 0.05$ by Student's t test. (E, F) DLD-1 cells were treated with IFN-α/β in the absence or presence of NKA (10 μM) for 24 h. Representative images of p-ERK1/2 and α-tubulin and the relative expression levels of p-ERK1/2 are shown

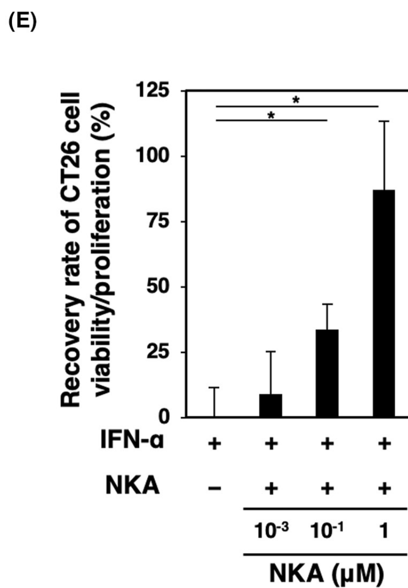
FIGURE 3 The effect of NK2R-mediated neuropeptide signaling on IFN-α/β-stimulated CT26 cells in vitro. CT26 cells were treated with IFN-α (50 ng/ml) and IFN-β (50 ng/ml) in the absence and presence of JAK1/2 inhibitor (5 μM) for 6 h. (A, B) Mean and SD ($n = 3–4$) of relative NK2R and TAC1 gene expression levels are indicated. *$P < 0.05$ by Tukey's multiple comparisons test after ANOVA test. (C, D) Viability and proliferation were evaluated for IFN-α/β-treated CT26 cells in the presence of NK2R antagonist (10 μM) for 12 and 24 h. Means and SDs ($n = 4$) of data are indicated. *$P < 0.05$ by Tukey's multiple comparisons test after ANOVA test. (E, F) Recovery rates of cell viability/proliferation were evaluated for the IFN-α/β-treated CT26 cells in the presence of NK2R agonist (0, 10^{-3} μM, 10^{-1} μM, 1 μM) for 12 h. Means and SDs ($n = 4$) of data are indicated. *$P < 0.05$ by Student's t test. (G, H) CT26 cells were treated with IFN-α/β in the absence or presence of NKA (10 μM) for 24 h. Representative images of p-ERK1/2 and α-tubulin and the relative expression levels of p-ERK1/2 are shown

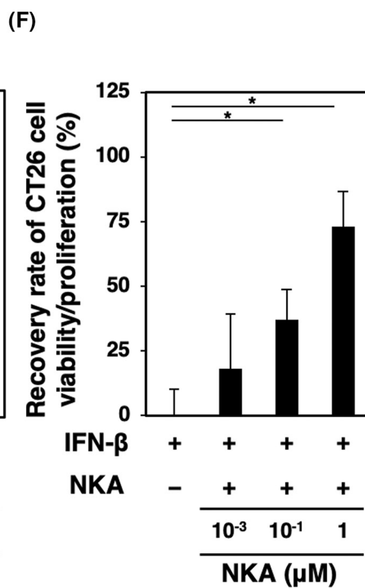

(A)
Relative NK2R gene expression in CT26 cells
IFN-α
IFN-β
JAK1/2 inhibitor

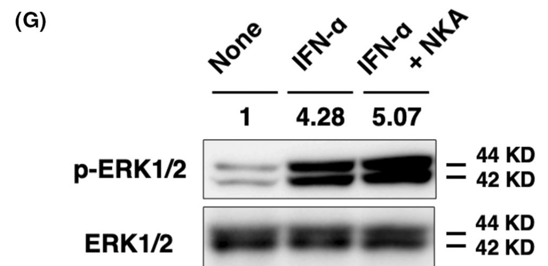

(B)
Relative TAC1 gene expression in CT26 cells
IFN-α
IFN-β
JAK1/2 inhibitor

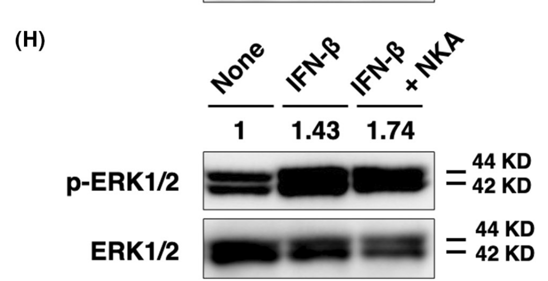

(c)
Viability/proliferation of CT26 cells (%)
IFN-α
NK2R antagonist
12 h
24 h


(D)
Viability/proliferation of CT26 cells (%)
IFN-β
NK2R antagonist
12 h
24 h


(E)
Recovery rate of CT26 cell viability/proliferation (%)
IFN-α
NKA
NKA (μM)


(F)
Recovery rate of CT26 cell viability/proliferation (%)
IFN-β
NKA
NKA (μM)


(G)
None
IFN-α
IFN-α + NKA
1
4.28
5.07
p-ERK1/2
ERK1/2
44 KD
42 KD


(H)
None
IFN-β
IFN-β + NKA
1
1.43
1.74
p-ERK1/2
ERK1/2
44 KD
42 KD

3.3 | Administration of poly I:C augments NK2R expression in murine colon cancer cells and NK2R blockade reduces tumorigenesis in vivo

Next, we investigated the roles of NK2R-mediated neuropeptide signaling in the malignancy of colon cancer cells in vivo. Administration of NK2R antagonist into CT26-bearing mice significantly reduced the tumor growth of CT26 cells (Figure 4A–C). Furthermore, we found that NK2R blockade augmented the reduction of tumorigenesis of CT26 cells by poly I:C treatment in vivo. We attempted to elucidate the in vivo regulation mechanism of NK2R gene expression in the tumor microenvironment. In tumor tissues of the CT26-bearing model, we found

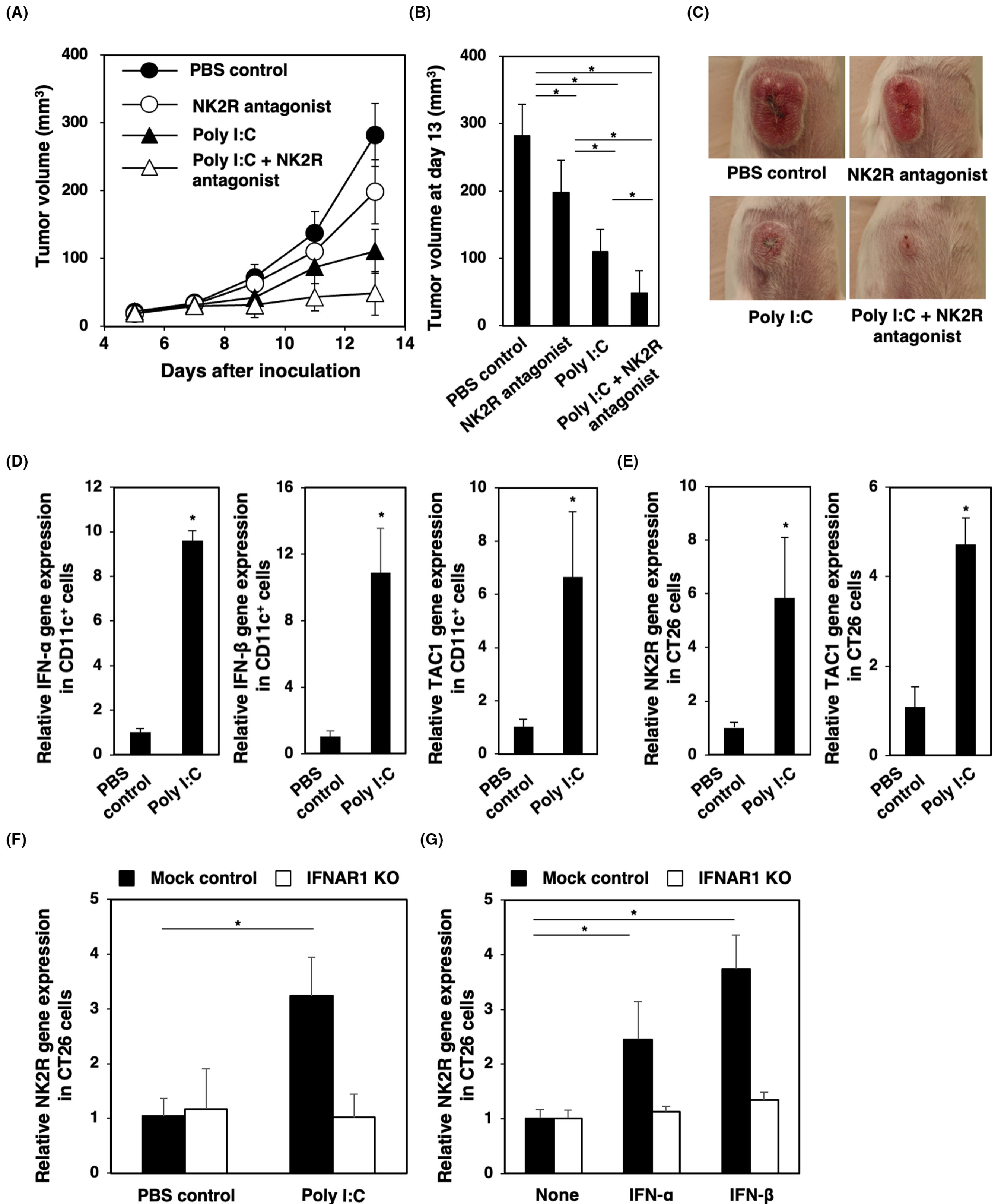


FIGURE 4 The effects of poly I:C and NK2R antagonist on tumorigenesis and NK2R expression levels of CT26 cells in vivo. GFP-transfected CT26 cells (1×10^6) were intradermally injected into BALB/c mice (day 0). Poly I:C (50 μg/mouse) or control PBS combined with or without NK2R antagonist (2.4 μg/mouse) was injected intratumorally into the mice at day 5 and then every 4 days thereafter. (A, B) Tumor growth was monitored for 13 days and tumor size was evaluated at 13 days after injection. Means and SDs ($n = 5$) of the tumor volumes are indicated. $^*P<0.05$ by Tukey's multiple comparisons test after ANOVA test. (C) Representative images of tumors at day 13. (D) Tumor-infiltrating $CD45^+CD11c^+$ dendritic cells were collected from tumor tissues at day 13. Mean and SD ($n = 4$) of the relative IFN-α, IFN-β, and TAC1 gene expression levels are indicated. $^*P<0.05$ by Student's *t* test. (E) $CD45^-GFP^+$ CT26 cancer cells were collected from tumor tissues at day 13. Mean and SD ($n = 4$) of relative NK2R and TAC1 gene expression levels are indicated. $^*P<0.05$ by Student's *t* test. (F) Mock control and IFNAR1 KO CT26 cells (1×10^6) were intradermally injected into BALB/c mice. Poly I:C (50 μg/mouse) or vehicle control (PBS control) were injected intratumorally into the mice at day 5. Mean and SD ($n = 4$) of relative NK2R gene expression levels of colon cancer cells 24 h after the poly I:C treatment are indicated. $^*P<0.05$ by Student's *t* test. (G) Mock control and IFNAR1 KO CT26 cells were treated with IFN-α (50 ng/ml) and IFN-β (50 ng/ml) for 6 h in vitro. Mean and SD ($n = 4$) of relative NK2R gene expression levels are indicated. $^*P<0.05$ by Student's *t* test

IFN-α and IFN-β as well as TAC1 gene expression levels in $CD11c^+$ DCs were enhanced after poly I:C injection into the tumor site (Figure 4D). The gene expression levels of NK2R and TAC1 in CT26 cancer cells were augmented by the administration of poly I:C (Figure 4E). In this study, we confirmed that the enhanced NK2R gene expression in CT26 cells after poly I:C injection was not observed in the case of IFNAR1 KO cells (Figure 4F). Moreover, the induction of NK2R gene expression in CT26 cancer cells after IFN-α/β-stimulation in vitro was abolished in the IFNAR1 KO cells (Figure 4G). These results suggest that neuropeptide signaling through NK2R expression in cancer cells was augmented by IFN-α/β produced by host $CD11c^+$ DCs and NKA by DCs as well as cancer cells in an IFNAR1-dependent manner. Based on these findings, we speculate that IFN-α/β induced by host cells may enhance NK2R-mediated neuropeptide signaling in cancer cells to augment the subsequent tumorigenesis in vivo.

3.4 | Overexpression of the NK2R gene in murine colon cancer cells augments tumorigenesis and metastatic colonization in vivo

To investigate the effects of NK2R expression in cancer cells on tumorigenesis in vivo, we established NK2R gene-overexpressing (NK2R OE) CT26 cell lines. The tumor growth was significantly enhanced by the overexpression of the NK2R gene in CT26 cells compared with the mock control cells (Figure 5A–C). We further evaluated the effect of NK2R expression on metastasis in vivo. The lung metastasis of NK2R OE cells was significantly augmented compared with the mock control cells (Figure 5D–G). NK2R OE cells significantly augmented the metastatic colonization in the liver of the mice (Figure 5H–K). Furthermore, we performed wound healing assay using NK2R OE cells in vitro. As a result, we found that overexpression of NK2R augmented the mobility of cancer cells compared to mock control cells. E-cadherin expression levels were reduced and N-cadherin was increased in NK2R OE cells compared to the mock control cells, suggesting that NK2R overexpressing cells showed epithelial to mesenchymal transition (Figure S4). Based on these data, we speculated that overexpression of the NK2R gene in colon cancer cells was not only involved in the tumorigenesis but also related to the metastatic colonization in vivo.

4 | DISCUSSION

In this study, we revealed that NK2R, a receptor of NKA, was overexpressed in tumor tissues of CRC patients and high expression levels of NK2R gene were associated with poor survival. Furthermore, we confirmed that NK2R and NKA-encoding TAC1 genes were induced by stimulation with IFN-α/β, type I interferons, in colon cancer cells in vitro. The administration of poly I:C, a synthetic analog of double-stranded RNA, to tumor-bearing model mice caused the induction of IFN-α/β and TAC1 genes in the host DCs and both NK2R and TAC1 gene expression in colon cancer cells. Based on these findings, we speculated that augmentation of the NKA–NK2R axis by the host immunity might be involved in the pathological functions related to the malignancy of colon cancers.

A previous study indicated that tachykinins are involved in the different steps of tumor initiation and development, and that neuropeptide signaling through the tachykinin receptors might promote cancer progression by influencing blood flux and neovascularization for tumor formation by neurogenic inflammation caused by severe stress or surgery.[29] It has been reported that the SP–NK1R cascade activates the PI3K/AKT and MAPK signaling pathways, which contribute to the suppression of apoptosis and tumor metastasis of cancer cells.[30,31] Many researchers have reported that activation of GPCR-mediated ERK/MAPK signal pathways in cancer cells caused malignant alteration such as tumorigenesis and metastasis.[32–35] In the present study, we found that NKA stimulation significantly enhanced the phosphorylation levels of ERK and the proliferation of IFN-α/β-treated colon cancer cells. Therefore, we speculated that the augmented NK2R gene expression might contribute to cancer cell malignancy through the activation of the ERK signal pathway.

Previous studies indicated that NK2R expression was increased in the inflammatory cells of intestinal tissue from patients with Crohn's disease and ulcerative colitis, and in the distal ileum of rats with acute necrotizing pancreatitis.[36,37] Chronic inflammation in the tumor microenvironment occasionally promotes cancer development rather than protecting against it.[19,38,39] In our previous study, we found that NK2R expression and NKA production levels of murine DCs were significantly elevated after stimulation with IFN-γ.[27] Furthermore, we revealed that IFN-α/β stimulation augmented NK2R expression levels of human DCs in a STAT1-dependent manner.[40] Generally,

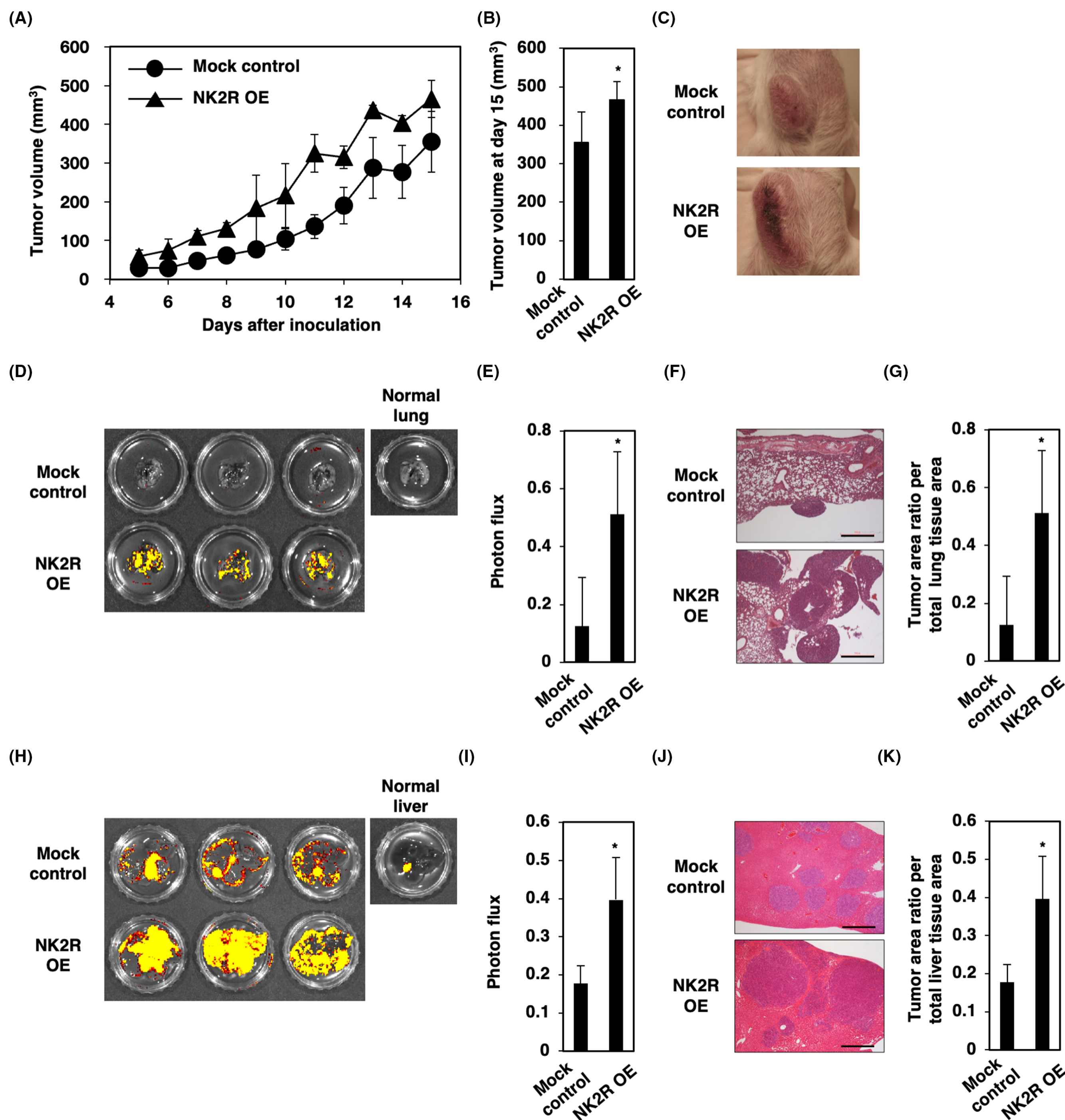


FIGURE 5 Tumorigenesis and metastatic colonization of NK2R-overexpressing CT26 cells in vivo. GFP-transfected NK2R gene-overexpressing (NK2R OE) CT26 cells and mock control cells (1×10^6) were intradermally injected into BALB/c mice (day 0). (A, B) Tumor growth was monitored for 15 days. Means and SDs ($n = 5$) of the tumor volumes are indicated. *$P<0.05$ by Student's t test. (C) Representative images of tumors at day 15. Mock control and NK2R OE CT26 cells (2×10^5) were intravenously or intrasplenically inoculated into BALB/c mice (day 0) and metastatic colonization in the lung or liver was evaluated on day 14, respectively. (D, H) Representative images of CT26-bearing or normal lungs and livers are shown. (E, I) Photon flux was determined from images of lung or liver metastatic colonization model mice ($n = 5$). (F, J) Representative HE-staining images of the lung and liver tissues are indicated. Bars represent 500 μm. (G, K) Ratios of tumor area against total lung or liver tissue area were calculated from four independent sections. Means and SDs ($n = 4$). are indicated. *$P<0.05$ by Student's t test

administration of poly I:C, a TLR 3 agonist, promoted the maturation of DCs, followed by release of type I IFNs.[41] We confirmed that poly I:C treatment induced NK2R and TAC1 gene expressions in DCs and augmented NKA signaling through NK2R expression.[27,40] In this study, we revealed that NK2R overexpressing colon cancer cells augmented metastatic colonization in lung and liver tissues in vivo

(Figure 5D–K). Furthermore, we found that overexpression of NK2R augmented the mobility of cancer cells compared to mock control cells in vitro (Figure S4). E-cadherin expression levels were reduced and N-cadherin was increased in NK2R-overexpressing CT26 cells compared to the mock control cells, suggesting that NK2R overexpressing cells showed epithelial–mesenchymal transition (EMT). Another study demonstrated that not only did NK1R activate the ERK1/2 signaling cascade but also that activation of NK1R promoted proliferation, EMT, and migration of lung cancer cells.[42] Based on these findings, we speculate that activation of NK2R as well as NK1R regulates ERK/MAPK-mediated malignant alteration of colon cancer cells (Figure S5).

Previous research revealed that activation of IFN-STAT1 signaling triggers apoptosis and cell cycle arrest in various cancer cells.[24,25] In this study, we found that type I IFNs not only inhibited the growth of tumor cells but also induced NKA expression of cancer cells. The augmented NK2R-NKA signal reversed the inhibitory effects of the IFN on tumor growth, whereas NKA stimulation enhances further the IFN-induced phosphorylation of ERK1/2 kinase (Figures 2 and 3). Therefore, we speculate that IFN-α/β-mediated induction of NK2R in cancer cells is a kind of feedback activation, suggesting one of the immune escape mechanisms to survive with malignant alteration. Previously our studies revealed that IFN-α/β, or IFN-γ, induced by host immune cells in IL-6-deficient mice augmented PD-L1 expression levels of cancer cells.[27,28] As a result, we confirmed that PD-L1 blockade under the IL-6-deficient condition effectively suppressed tumor growth of cancer cells in vivo. In this study, poly I:C treatment combined with NK2R antagonist showed a more effective antitumor effect (Figure 4). Based on these findings, we speculate that blockade of IFN-α/β-mediated NKA-NK2R signaling may be a promising target for regulation in the tumorigenesis of colon cancer cells in vivo.

A clinical study reported that NKA levels predicted the survival of patients with stage IV well-differentiated small bowel neuroendocrine neoplasms.[43] In midgut carcinoid tumors, patients with persistent plasma NKA levels had significantly worse survival than patients with stabilized or downregulated plasma NKA levels.[44] Moreover, a previous paper indicated that NK2R gene polymorphisms predicted lymph node metastasis in CRC patients.[45] In our study, we found that NK2R was overexpressed in the tumor tissues of patients with CRC and high expression was associated with poor survival from the analysis based on TCGA public dataset.[46,47] Based on these findings, we speculate not only that NKA signaling through NK2R may be related to the development and malignancy of CRC, but also that NKA–NK2R expression levels may be useful biomarkers to evaluate the malignancy of colon cancers.

A recent phase II clinical trial using an NK2R inhibitor for IBS with diarrhea reported improved overall symptoms, abdominal pain, and stool patterns in female patients.[25] In addition, the dual tachykinin NK1/NK2 antagonist DNK333 inhibited NKA-induced bronchoconstriction in asthma patients.[48] An NK2R antagonist suppressed cellular proliferation in metastatic breast carcinoma cells independent of SP effects.[49] In colon cancer, targeting NK1R inhibits the proliferation of human colon cancer cells, as previously described.[50] In this study, we confirmed that the blockade of neuropeptide signaling by in vivo injection of an NK2R antagonist alone or combined with poly I:C significantly suppressed the tumor growth of colon cancer cells, suggesting that NK2R may be a novel target to control the tumorigenesis of colon cancers.

We found that NK2R expression was induced by IFN-α/β-stimulation in colon cancer cells and related to the subsequent malignant alteration, and confirmed that blockade of NK2R-mediated signaling with an antagonist in colon cancer cells significantly reduced proliferation in vitro and tumorigenesis in vivo. Based on these data, we speculate that NK2R expression is related to the malignancy of colon cancer cells, suggesting that regulation of NK2R-mediated signaling will be a promising strategy for the development of more effective therapies for patients with CRC.

AUTHOR CONTRIBUTIONS

H. Kitamura, A.T., and H. Kobayashi designed the study; H.X., Y.T., W.S., X.W., N.O., S.K., K.S., T.N., H. Kobayashi, M.T., A.T., and H. Kitamura performed the experiments. H.X., K.I., S.H., M.T., A.T., and H. Kitamura contributed the reagents/analytic tools used in the study. H.X., K.I., S.H., and M.T. analyzed the data. H.X. and H. Kitamura wrote the paper. All authors read and approved the final manuscript.

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DISCLOSURE

All authors declare no conflict of interest for this article.

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REFERENCES

1. Griebel G, Holsboer F. Neuropeptide receptor ligands as drugs for psychiatric diseases: the end of the beginning? *Nat Rev Drug Discov*. 2012;11:462-478.
2. Steinhoff M, Von Mentzer B, Geppetti P, et al. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. *Physiol Rev*. 2014;94:265-301.

3. Suvas S. Role of Substance P neuropeptide in inflammation, wound healing, and tissue homeostasis. *J Immunol*. 2017;199:1543-1552.
4. González-Santana A, Marrero-Hernández S, Dorta I, et al. Altered expression of the tachykinins substance P/neurokinin A/hemokinin-1 and their preferred neurokinin 1/neurokinin 2 receptors in uterine leiomyomata. *Fertil Steril*. 2016;106:1521-1529.
5. Bar-Shavit R, Maoz M, Kancharla A, et al. G protein-coupled receptors in cancer. *Int J Mol Sci*. 2016;17:1320.
6. Gutierrez AN, McDonald PH. GPCRs: emerging anti-cancer drug targets. *Cell Signal*. 2018;41:65-74.
7. Zhang YX, Li XF, Yuan GQ, et al. β-Arrestin 1 has an essential role in neurokinin-1 receptor-mediated glioblastoma cell proliferation and G2M phase transition. *J Biol Chem*. 2017;292:8933-8947.
8. Nizam E, Köksoy S, Erin N. NK1R antagonist decreases inflammation and metastasis of breast carcinoma cells metastasized to liver but not to brain; phenotype-dependent therapeutic and toxic consequences. *Cancer Immunol Immunother*. 2020;69:1639-1650.
9. Ge C, Huang H, Huang F, et al. Neurokinin-1 receptor is an effective target for treating leukemia by inducing oxidative stress through mitochondrial calcium overload. *Proc Natl Acad Sci USA*. 2019;116:19635-19645.
10. Sinha S, Fu YY, Grimont A, et al. PanIN neuroendocrine cells promote tumorigenesis via neuronal cross-talk. *Cancer Res*. 2017;77:1868-1879.
11. Berger M, Neth O, Ilmer M, et al. Hepatoblastoma cells express truncated neurokinin-1 receptor and can be growth inhibited by aprepitant in vitro and in vivo. *J Hepatol*. 2014;60:985-994.
12. Chen XY, Ru GQ, Ma YY, et al. High expression of substance P and its receptor neurokinin-1 receptor in colorectal cancer is associated with tumor progression and prognosis. *Onco Targets Ther*. 2016;9:3595-3602.
13. Garnier A, Vykoukal J, Hubertus J, et al. Targeting the neurokinin-1 receptor inhibits growth of human colon cancer cells. *Int J Oncol*. 2015;47:151-160.
14. Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global patterns and trends in colorectal cancer incidence and mortality. *Gut*. 2017;66:683-691.
15. Kuipers E, Grady W, Lieberman D, et al. Colorectal cancer. *Nat Rev Dis Primers*. 2015;1:15065.
16. Crusz SM, Balkwill FR. Inflammation and cancer: advances and new agents. *Nat Rev Clin Oncol*. 2015;12:584-596.
17. Janakiram NB, Rao CV. The role of inflammation in colon cancer. *Adv Exp Med Biol*. 2014;816:25-52.
18. Lasry A, Zinger A, Ben-Neriah Y. Inflammatory networks underlying colorectal cancer. *Nat Immunol*. 2016;17:230-240.
19. Tack J, Schumacher K, Tonini G, et al. The neurokinin-2 receptor antagonist ibodutant improves overall symptoms, abdominal pain and stool pattern in female patients in a phase II study of diarrhoea-predominant IBS. *Gut*. 2017;66:1403-1413.
20. McNab F, Mayer-Barber K, Sher A, Wack A, O'Garra A. Type I interferons in infectious disease. *Nat Rev Immunol*. 2015;15:87-103.
21. Trinchieri G. Type I interferon: friend or foe? *J Exp Med*. 2010;207:2053-2063.
22. Dunn GP, Bruce AT, Sheehan KCF, et al. A critical function for type I interferons in cancer immunoediting. *Nat Immunol*. 2005;6:722-729.
23. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol*. 2006;6:836-848.
24. Jelinek DF, Aagaard-Tillery KM, Arendt BK, Arora T, Tschumper RC, Westendorf JJ. Differential human multiple myeloma cell line responsiveness to interferon-alpha. Analysis of transcription factor activation and interleukin 6 receptor expression. *J Clin Invest*. 1997;99:447-456.
25. Grimley PM, Fang H, Rui H, et al. Prolonged STAT1 activation related to the growth arrest of malignant lymphoma cells by interferon-alpha. *Blood*. 1998;91:3017-3027.
26. Kitamura H, Kobayashi M, Wakita D, Nishimura T. Neuropeptide signaling activates dendritic cell-mediated type 1 immune responses through neurokinin-2 receptor. *J Immunol*. 2012;188:4200-4208.
27. Toyoshima Y, Kitamura H, Xiang H, et al. IL6 modulates the immune status of the tumor microenvironment to facilitate metastatic colonization of colorectal cancer cells. *Cancer Immunol Res*. 2019;7:1944-1957.
28. Ohno Y, Toyoshima Y, Yurino H, et al. Lack of interleukin-6 in the tumor microenvironment augments type-1 immunity and increases the efficacy of cancer immunotherapy. *Cancer Sci*. 2017;108:1959-1966.
29. Palma C. Tachykinins and their receptors in human malignancies. *Curr Drug Targets*. 2006;7:1043-1052.
30. Gharaee N, Pourali L, Jafarian AH, Hashemy SI. Evaluation of serum level of substance P and tissue distribution of NK-1 receptor in endometrial cancer. *Mol Biol Rep*. 2018;45:2257-2262.
31. Rosso M, Munoz M, Berger M. The role of neurokinin-1 receptor in the microenvironment of inflammation and cancer. *Scientific World J*. 2012;2012:381434.
32. Zhang L, Wang H, Yang T, et al. Formylpeptide receptor 1 mediates the tumorigenicity of human hepatocellular carcinoma cells. *Onco Targets Ther*. 2015;5:e1078055.
33. Gonzalez de Valdivia E, Broselid S, Kahn R, Olde B, Leeb-Lundberg LMF. G protein-coupled estrogen receptor 1 (GPER1)/GPR30 increases ERK1/2 activity through PDZ motif-dependent and -independent mechanisms. *J Biol Chem*. 2017;292:9932-9943.
34. Hoy JJ, Salinas Parra N, Park J, Kuhn S, Iglesias-Bartolome R. Protein kinase A inhibitor proteins (PKIs) divert GPCR-Gαs-cAMP signaling toward EPAC and ERK activation and are involved in tumor growth. *FASEB J*. 2020;34:13900-13917.
35. Smith JS, Pack TF, Inoue A, et al. Noncanonical scaffolding of Gαi and β-arrestin by G protein-coupled receptors. *Science*. 2021;371(6534):eaay1833.
36. Renzi D, Pellegrini B, Tonelli F, Surrenti C, Calabrò A. Substance P (neurokinin-1) and neurokinin A (neurokinin-2) receptor gene and protein expression in the healthy and inflamed human intestine. *Am J Pathol*. 2010;157:1511-1522.
37. Shi X, Gao NR, Guo QM, et al. Relationship between overexpression of NK-1R, NK-2R and intestinal mucosal damage in acute necrotizing pancreatitis. *World J Gastroenterol*. 2003;9:160-164.
38. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer*. 2015;5:263-274.
39. Jiang H, Dong L, Gong F, et al. Inflammatory genes are novel prognostic biomarkers for colorectal cancer. *Int J Mol Med*. 2018;42:368-380.
40. Ohtake J, Kaneumi S, Tanino M, et al. Neuropeptide signaling through neurokinin-1 and neurokinin-2 receptors augments antigen presentation by human dendritic cells. *J Allergy Clin Immunol*. 2015;136:1690-1694.
41. Rizza P, Moretti F, Capone I, Belardelli F. Role of type I interferon in inducing a protective immune response: perspectives for clinical applications. *Cytokine Growth Factor Rev*. 2015;26:195-201.
42. Zhang XW, Li L, Hu WQ, et al. Neurokinin-1 receptor promotes non-small cell lung cancer progression through transactivation of EGFR. *Cell Death Dis*. 2022;13:41.
43. Diebold AE, Boudreaux JP, Wang YZ, et al. Neurokinin A levels predict survival in patients with stage IV well differentiated small bowel neuroendocrine neoplasms. *Surgery*. 2012;152:1172-1176.
44. Turner GB, Johnston BT, McCance DR, et al. Circulating markers of prognosis and response to treatment in patients with midgut carcinoid tumours. *Gut*. 2006;55:1586-1591.
45. Fang W, Fu C, Chen X, et al. Neurokinin-2 receptor polymorphism predicts lymph node metastasis in colorectal cancer patients. *Oncol Lett*. 2015;9:2003-2006.

46. Uhlén M, Fagerberg L, Hallström BM, et al. Proteomics. Tissue-based map of the human proteome. *Science*. 2015;347:1260419.
47. Uhlen M, Oksvold P, Fagerberg L, et al. Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol*. 2010;28:1248-1250.
48. Joos GF, Vincken W, Louis R, et al. Dual tachykinin NK1/NK2 antagonist DNK333 inhibits neurokinin A-induced bronchoconstriction in asthma patients. *Eur Respir J*. 2004;23:76-81.
49. Nizam E, Erin N. Differential consequences of neurokinin receptor 1 and 2 antagonists in metastatic breast carcinoma cells; Effects independent of Substance P. *Biomed Pharmacother*. 2018;108:263-270.
50. Niu XL, Hou JF, Li JX. The NK1 receptor antagonist NKP608 inhibits proliferation of human colorectal cancer cells via Wnt signaling pathway. *Biol Res*. 2018;51:14.

SUPPORTING INFORMATION

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