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Type III interferon enhances the direct antitumor activity of type I interferon

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Summary

The antitumor activities of type III interferon (IFN) (interleukin (IL)-28 and IL-29) and the combination between type III IFN and type I IFN (IFN-α) were evaluated in *in vitro* and *in vivo* studies using human non-small cell lung cancer (NSCLC). The expression of IL-28 receptor (IL-28R) and IL-10Rß was detected in NSCLC lines. IL-29 significantly inhibited the *in vitro* growth of a wide range of NSCLC lines in a dose-dependent fashion. To a lesser degree, IL-28A also displayed the growth inhibitory activity. Cell cycle analysis revealed that IL-29 induced cell cycle arrest at the G1 phase and no significant apoptotic cell death. IL-29 up-regulated cyclin-dependent kinase inhibitor p21Waf1/Cip1 in IL-29-sensitive, but not in IL-29-insensitive cells, and knockdown of p21 with small interfering RNA largely attenuated the antiproliferative effect. Intratumoral and systemic administration of IL-29 inhibited NSCLC tumor growth in SCID mice in a dose-dependent manner. Immunohistochemical analyses demonstrated a marked up-regulated p21 expression in NSCLC tumors treated with IL-29. Furthermore, the cytokine combination between IL-29 and IFN-α displayed a more powerful antiproliferative effect *in vitro*, and a more increased p21 expression than each reagent alone. Additionally, the cytokine combination therapy suppressed the *in vivo* NSCLC growth more effectively than each reagent alone. These findings demonstrate that type III IFN can enhance type I IFN-mediated antitumor activities, and suggest the possibility that type III IFN may improve the efficacy and reduce the side effects of type I IFN therapy of cancer.

Introduction

IL-28 and IL-29, designated as type III IFN, are recently identified class II cytokine receptor ligands that are distantly related to members of the IL-10 family and the type I IFN family (1-3). The type III IFN expression is induced by virus infection or double-stranded RNA (1-3). Type III IFN signals through the same heterodimeric receptor complex that is composed of the IL-10Rß and a novel IL-28R (1-3). Type III IFN signaling induces many genes that are induced by signaling through IFN-α/ß receptors (1-3). These include genes such as myxovirus resistance-A (MxA), OAS1, a double-stranded RNA-dependent protein kinase R (PKR) and ADAR, which mediate at least some of the antiviral and antiproliferative activities of type I IFN (1-3).

IFNs are a large family of protein having a wide variety of biological properties (4). These include the inhibition of cell growth, activation of T cell and NK cell cytotoxicity, up-regulation of MHC class I molecules, promotion of T helper type I responses and inhibition of angiogenesis (5). Especially, IFN-α delivered either by recombinant protein or by viral or plasmid vectors has been displayed to have potent antitumor effects in mouse tumor models (5). To date, IFN-α has been approved for treatment of patients with renal cell carcinoma, metastatic melanoma, and chronic myelogeneous leukemia (6).

Type III IFN exerts bioactivities that overlap those of type I IFN (1-3). Similar to type I IFN, type III IFN has also been demonstrated to elicit antitumor activity (7-14). Nevertheless, to date, the studies to evaluate the antitumor action of type III IFN have been limited to a narrow range of pathological types of human tumors (7-10). In this study, using human NSCLC lines, we evaluated the direct antitumor activity of type III IFN alone and the combination between type III IFN and type I IFN in *in vitro* and *in vivo* studies.

Materials and Methods

Cells, mice and reagents. NSCLC lines were from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. SCID mice were from Charles River (Yokohama, Japan). IL-28A and IL-29 were from R&D Systems (Minneapolis, MN). IFN-α was provided by Sumitomo pharmaceuticals (Tokyo, Japan). Rabbit anti-asialo GM1 antiserum was from Wako Chemicals (Tokyo, Japan). Anti-p16 and anti-p27 antibodies were from Calbiochem (La Jolla, CA). Anti-p21 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

MTT assay. MTT assay was performed as described previously (15). The percentage of cell proliferation or reduction of cell proliferation was determined by the previously reported formula (16,17)

Apoptosis assay. Cells were stimulated with or without cytokine or cisplatin (18) at the indicated concentrations. To detect apoptotic cell death, a cell death detection ELISA assay kit (Roche Applied Science, Nutley, NJ) was used. Results for the negative control were set to 10, and all other measurements were normalized to the control (19). To identify cell death at an earlier stage, the Annexin V-PE apoptosis detection kit (BD Pharmingen, San Diego, CA) was used. Apoptotic cell death was analyzed on a FACScan (BD Biosciences, San Jose, CA).

Reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted using RNA Bee (TEL-TEST, Friendswood, TX), and used as template for one-step RT-PCR with the Superscript One-Step RT-PCR with a Platinum Taq kit (Invitrogen Corp., Carlsbad, CA, USA). The expression of OAS1, PKR, and ADAR was determined with quantitative RT-PCR using TaqMan Gene Expression Assays (Applied Biosystems, CA). Simultaneously, mRNA expression for GAPDH was determined using Pre-Developed TaqMan Assay

Reagents for GAPDH. Real-time RT-PCR application was carried out using a TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems). The expression levels of OAS1, PKR, and ADAR were normalized to GAPDH.

Cell cycle analysis. Cell cycle analysis was performed as described previously (20). In brief, cells were washed with PBS, suspended in a propidium iodide (PI) solution and then incubated for 30 min. Cell cycle distribution was determined by FACScan analysis.

Western blot analysis. Total cellular extracts were prepared using Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA). After electrophoresis, proteins were electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were incubated with respective antibodies, and then with horseradish peroxidase-conjugated secondary antibodies (Amersham, Arlington Heights, IL). The immunoreactive proteins were visualized with the ECL Plus chemiluminescence system (Amersham). The intensity of bands was analyzed using an image analysis software MultiGauge (Fujifilm, Tokyo, Japan).

RNA interference. Knockdown of p21 through the delivery of double stranded RNA molecules into the cells was performed using SignalSilence p21Waf1/Cip1 siRNA Kit (Cell Signaling Technology). The cells transfected with a p21-specific double stranded small inhibitory RNA (siRNA) oligonucleotide or with a negative control oligonucleotide were treated with or without IL-29 for the indicated time period.

Evaluation of *in vivo* tumor growth. To deplete NK cells in SCID mice, anti-asialo GM1 antiserum (Wako Chemicals) was administered 1 day prior to the tumor inoculation, and subsequently once every 5 days. Mice were inoculated subcutaneously with cells into the right flank. Tumor volume was calculated by using the formula $ab^2/2$ (a = largest diameter; b =

smallest diameter) (21). For intratumoral therapy, cytokine(s) or PBS was injected into tumors (120-170 mm^3) for consecutive 21 days. For systemic therapy, IL-29 or PBS was administered subcutaneously at the contralateral site to the tumor (80-100 mm^3) for consecutive 15 days.

Immunohistological examination. Immunostaining for Factor VIII related antigen was performed as described previously (21). For p21 immunostaining, sections were autoclaved and incubated with anti-p21 antibody. Immunohistochemistry was performed using a Histofine Simple Stain kit (Nichirei, Tokyo, Japan). Cells exhibiting distinct nuclear staining were considered positive for p21, and the percentages of positive-staining cells were determined.

Statistical analyses. Statistical analysis was performed using an unpaired two-tailed Student's *t*-test with a confirmation by parametric and *F*-tests. Differences were considered to be statistically significant when the *P* value was less than 0.05.

Results.

Type III IFN elicits direct antitumor activity. We first examined the expression of the receptor subunits for type III IFN in NSCLC lines by RT-PCR. The transcripts for IL-28R and IL-10Rß, which compose of the functional heterodimeric receptor complex for type III IFN, were expressed (Fig. 1). The expression of mRNA for IFN-αR1 and IFN-αR2 was also detected (Fig. 1). We next evaluated the growth inhibitory activity of type III IFN with MTT assay. IL-29 displayed the growth inhibitory activity against a wide range of NSCLC lines in a dose-response manner (Fig. 2, a-b and Table 1). To a lesser degree, IL-28A also showed the antiproliferative effect (Fig. 2, a-b).

Type III IFN does not induce the apoptotic cell death. To understand the underlying mechanisms of antitumor effect, we first evaluated the apoptotic cell death. No apoptotic DNA fragmentation in IL-29-treated cells was observed, whereas cisplatin induced a significant increase of DNA fragmentation in all cell types (Fig. 3a). Treatment with high concentration of IL-28A (Fig. 3a) or IL-29 for 48 hours (data not shown) did not induce the apoptotic DNA fragmentation. Moreover, we investigated the early apoptotic cell death using Annexin V assay by flow cytometry. Cells treated with cisplatin showed increased cell death, but IL-29 treatment did not increase the Annexin V-positive cells.

Type III IFN induces cell cycle arrest at the G1 phase. We next investigated the effect of IL-29 on cell cycle distribution. Cell cycle distribution analysis showed that IL-29 resulted in accumulation of cell numbers in G1 phase in a dose-dependent manner (Fig. 4a). OBA-LK1 cells treated with 50 ng/ml or 250 ng/ml of IL-29 increased the G1 population from 53% to 69% or 80%, and the identical treatment of 11-18 cells increased the G1 population from 30% to 61% or 79%, respectively. This increase of G1 population is accompanied by reduction of S and G2 populations (Fig. 4a).

Type III IFN up-regulates a cyclin-dependent kinase inhibitor p21Waf1/Cip1. To assess the mechanism of the cell cycle arrest induced by type III IFN, we first examined the OAS1, PKR, and ADAR expression levels (22-25). A quantitative RT-PCR analysis showed that IL-29 as well as IFN-α up-regulated the expression of OAS1 and ADAR (Fig 4b). These molecules were also up-regulated in A549 cells, which growth was little suppressed with type III IFN. Furthermore, the transcript of PKR was not influenced by any cytokines. These findings strongly suggested that some other molecules were mainly accountable for the antiproliferative effect of type III IFN.

We next focused on the cyclin-dependent kinase inhibitors including p16Ink4a,

p21Waf1/Cip1 and p27Kip1, which are involved in cell cycle arrest at the G1 phase. Western blot analyses showed that p21 and p27 were detected in NSCLC cells, and IL-29 led to a significant increase of p21 in OBA-LK1 and 11-18, but not in A549 (Fig 4c). The p21 expression in OBA-LK1 increased time-dependently in response to IL-29 (Fig 4d). On the other hand, the p27 expression was not influenced (Fig 4c). In addition, p16 was not up-regulated by IL-29 (data not shown).

Up-regulation of p21Waf1/Cip1 is required for the antiproliferative effect of type III IFN. To examine whether the observed p21 up-regulation is essential for the antiproliferative effect, we performed siRNA-mediated p21 knockdown. Western blot analysis revealed that introduction of the p21-siRNA (26) efficiently reduced the p21 expression for 48 hours after IL-29 treatment (Fig. 5a). The antiproliferative effect was largely alleviated in p21-siRNA transfected cells compared to non-transfected cells (Fig. 5b). Introduction of the control-siRNA did not affect the antiproliferative effect (Fig. 5), confirming that this alleviation of the antiproliferative effect depended on the p21-specifc siRNA and was not the result of any cell damage by transfection or by introduction of siRNA itself.

Type III IFN suppresses the *in vivo* tumor growth. To examine the antitumor activities of type III IFN *in vivo*, mice were implanted with NSCLC cells. Animals, which had developed palpable tumor nodules, were treated with daily intratumoral injections of various doses of IL-29. Mice treated with IL-29 displayed a significant reduction in tumor volume in a dose-dependent manner compared with mice treated with PBS (Fig. 6, a and b). Three weeks after treatment initiation, mice administered with 0.05, 0.4 or 1.5 μg of IL-29 showed a 30, 66 or 69% reduction in OBA-LK1 tumor volume (Fig. 6a), and a 22, 35 or 50% reduction in LK-1 tumor volume compared with controls (Fig. 6b). In addition, systemic treatment at a dose of 0.4, 1.5 or 7 μg per mouse per day resulted in a 20, 47 or 65% reduction in tumor

volume compared with controls (Fig. 6c). Moreover, in both strategies, type III IFN treatment did not appear to cause significant adverse reactions.

To assess the mechanism of the *in vivo* antitumor effect, we performed histological and immunohistochemical examination. There was little difference between IL-29-treated tumors and controls in terms of cellularity and presence of stroma (Fig. 7). Immunohistochemical staining for vascular endothelial cells to probe the possible contribution of the inhibition of angiogenesis to the observed growth suppression of tumors revealed that IL-29 did not affect the tumor vascularity (Fig. 7). On the contrary, p21 accumulated more in the tumors treated with IL-29 than the tumors treated with PBS (Fig. 7). The percentage of p21 positive-staining cells in the tumors treated with 7 μg of daily systemic therapy was significantly higher than that in the tumors treated with PBS (27.8 ± 3.0 % versus 18.3 ± 5.4 %, $p < 0.03$).

Cytokine combination elicits more potent antitumor effect than each reagent alone. Since type III IFN signals through the heterodimeric receptor complex, which is quite different from IFN-αR complex, we compared the growth inhibitory activity of increasing doses of type III IFN alone to the inhibitory action of increasing doses of type III IFN in combination with 10 ng/ml and/or 50 ng/ml of IFN-α. The cytokine combination showed a more powerful antiproliferative effect over the whole IFN-α concentration range tested (Fig. 8, a-d and Table 1).

Cytokine combination up-regulates p21 expression more efficiently and suppresses the *in vivo* tumor growth more effectively. Type III IFN enhanced the direct antitumor effect of IFN-α *in vitro*. Therefore, we evaluated the effect of cytokine combination on p21 expression, and found that treatment with the combination between IL-29 and TNF-α enhanced p21 expression more efficiently than each reagent alone (Fig. 9). This characteristic biological

function of type III IFN prompted us to further investigate the *in vivo* biological effect of this cytokine combination. OBA-LK1 tumors transplanted into SCID mice was treated with IL-29, IFN-α or a combination. All treatments resulted in significant retarded tumor growth kinetics, whereas the greatest antitumor activity was obtained with the cytokine combination therapy (Fig. 10).

Discussion

IFNs have direct antitumor activities including induction of cell death via apoptosis and antiproliferative effect on tumor cells. To date, type I IFN has been applied for the treatment of the patients with myelogenous leukemias, multiple myeloma, lymphomas, renal cell carcinoma, and metastatic melanoma. Newly identified antiviral cytokines IL-28 and IL-29 are considered to belong to the IFN family and designed as type III IFN. In addition to its primarily reported antiviral activity, type III IFN has been shown to have direct antitumor actions against a few human tumor lines including glioblastoma line (7), colon cancer line (8), neuroendocrine cancer line (9) and esophageal carcinoma lines (10). Nevertheless, to date, the efficacy and the precise molecular mechanisms of antitumor activities of type III IFN have not been fully elucidated. We therefore investigated the antitumor actions of type III IFN using NSCLC lines. Our findings indicated that type III IFN has a direct antitumor effect on a broad range of NSCLC lines, and that type III IFN has a unique biological feature to enhance the antitumor action of IFN-α. Our findings give rise to the possibility that type III IFN could improve the therapeutic efficacy of IFN-α although we now do not have evidences that type III IFN in combination with IFN-α can elicit an additive or a synergistic antitumor effect on other pathological types of tumors.

In our study, no significant cell death via apoptosis was induced by type III IFN. This finding is clear contrast with the result obtained from the studies using esophageal carcinoma lines, in which type III IFN mediated the apoptotic cell death (10). On the other hand,

treatment with a sufficient concentration of IL-29 resulted in the increment of cell population at the G1 phase. These results indicate that IL-29 mediated the growth inhibitory activity mainly through the cell cycle arrest at the G1 phase. To investigate the underlying mechanisms, we focused on the cyclin-dependent kinase inhibitors such as p16, p21 and p27, all of which have the possibility to be responsible for the cell cycle G1 arrest, and found that IL-29 significantly up-regulated p21 expression in IL-29-sensitive, but not in IL-29-insensitive cells. Moreover, the antiproliferative effect of IL-29 was largely alleviated by knockdown of p21 expression using siRNA specific for p21, indicating that the p21 up-regulation is crucial to the antiproliferative effect of IL-29 on NSCLC. These results may be in line with the previously reported findings that the increased p21 expression is responsible for cell cycle G1 arrest in many kinds of cells (27-29), and p21 overexpression in cells resulted in growth inhibition and G1 arrest (30-32).

For the *in vivo* studies, SCID mice were inoculated with NSCLC cells. Daily treatment with intratumoral injection of IL-29 resulted in a significant reduction of tumor growth in a dose-dependent manner. In LK-1 tumor model, IL-29 resulted in moderate antitumor effect compared to the result obtained from OBA-LK1 tumor model. Furthermore, systemic administration of IL-29 into mice with OBA-LK1 tumor resulted in significantly reduced tumor growth and enhanced p21 expression in xenogeneic tumors. In clear contrast to type I IFN (33), IL-29 did not inhibit the tumor angiogenesis. SCID mice treated with anti-asialo GM1 antiserum lack functional T cells, B cells and NK cells. Therefore, the growth inhibition in xenogeneic NSCLC tumor models is most likely due to a direct antitumor action of IL-29. These findings totally support the notion that the p21 up-regulation plays a major role in the *in vivo* antitumor action of IL-29. Actually, p21 expression in NSCLC tumors has been reported to be a predictor for favorable prognosis (34-36). It is important that the circulating IL-29 has a therapeutic application to human tumors because this type of treatment can be beneficial for patients with tumors that are difficult to access or with distant metastases.

Type III IFN displayed the biological property to augment the growth inhibitory effect of

IFN-α *in vitro*, and the combination between IL-29 and TNF-α up-regulated p21 expression more efficiently than each reagent alone. Therefore, we further evaluated the efficacy of cytokine combination therapy *in vivo*. The combination between IL-29 and IFN-α was more effective in inhibiting tumor growth than was either reagent alone. Although antitumor activity of type III IFN against various pathological types of tumors has not been fully evaluated, our findings raise the possibility that the cytokine combination therapy may not only surpass the therapeutic outcomes of IFN-α monotherapy but also reduce the side effects by decreasing the daily dose of IFN-α.

In conclusion, type III IFN can elicit direct antitumor effect on NSCLC *in vitro* and *in vivo* mainly through p21Waf1/Cip1 up-regulation. Type III IFN can also enhance the antitumor action of type I IFN. Considering that type III IFN has immunoregulatory activities, the application of type III IFN or cytokine combination therapy to immunocompetent cancer patients may possibly result in greater therapeutic effects.

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Figure Legends

Figure 1. Expression of mRNA encoding IL-28R, IL-10Rβ and IFN-α receptors.

The expression of mRNA encoding IL-28R, IL-10Rβ, IFN-α receptors and GAPDH was analyzed by RT-PCR. Lane 1: OBA-LK1, Lane 2: LK-1, Lane 3: 11-18, Lane 4: Sq-19, Lane 5: NCI-H23, Lane 6: A549.

Figure 2. Antitumor effect of type III IFN *in vitro*

a-b. The growth of cells cultured with the indicated concentrations of cytokine for 5 days was analyzed with MTT assay. Each value represents mean percent cell growth ± SD (n = 5). c-d. Growth inhibition by IL-28A, IL-29 or 10 ng/ml IFN-α was assessed for 5 consecutive days. Each value represents the mean absorbance ± SD (n = 5).

Figure 3. Effect of type III IFN on the apoptotic cell death.

a. DNA fragmentation in cells cultured with or without cytokine or cisplatin was analyzed using a Cell Death Detection ELISA. Each value is normalized to the negative control and represents mean relative DNA fragmentation ± SD (n = 5). b. OBA-LK1 cells cultured with or without cytokine or cisplatin for 12 hours were incubated with Annexin V-PE, and analyzed by flow cytometry.

Figure 4. Effect of IL-29 on cell cycle distribution and expression of cyclin-dependent kinase inhibitors

a. Cell cycle distribution of the cells cultured with or without IL-29 for 48 hours was analyzed on a FACScan. b. The expression of mRNA for OAS1, ADAR and PKR in cells cultured with or without the indicated cytokines were analyzed by quantitative RT-PCR. Each value is normalized to GAPDH and represents mean ± SD (n = 3; *, $p < 0.05$; **, $p < 0.001$ versus control). c-d. Induction of p21 and p27 in cells cultured with or without 50 ng/ml IL-29 for 24 hours was evaluated with western blot analysis. Time course analysis of p21 and p27

induction in OBA-LK1 cells treated with 50 ng/ml IL-29 was also performed. Relative p21 and p27 intensity normalized to β-actin are presented in the bar graph.

Figure 5. RNA interference inhibited IL-29-induced p21 up-regulation and counteracted the antiproliferative effect

a. Cells were transfected with or without a p21-siRNA oligonucleotide. The p21 expression was analyzed using western blot analysis. b. Cells were transfected with or without a p21-siRNA oligonucleotide or a non-targeted control oligonucleotide, and then cultured with incremental concentrations of IL-29 for 48 hours. Cell growth was analyzed with MTT assay. Each value represents mean percent cell growth ± SD (n = 5).

Figure 6. Antitumor effect of IL-29 *in vivo*. a-b. Intratumoral therapy of tumors with IL-29. Each value represents mean tumor volume (mm^3) ± SD for 5 mice per group (*, $p < 0.05$; **, $p < 0.001$; PBS versus IL-29, on day 21 or day 22 after therapy initiation). c. Daily systemic therapy of OBA-LK1 tumors with IL-29. Each value represents mean tumor volume (mm^3) ± SD for 4 mice per group (*, $p < 0.05$; **, $p < 0.0001$; PBS versus IL-29, on day 16 after therapy initiation).

Figure 7. Histological and immunohistochemical examination. Sections were stained with hematoxylin and eosin, or immunostained for Factor VIII related antigen or p21.

Figure 8. Antitumor effect of the cytokine combination *in vitro*. a-d, The effect of the cytokine combinations on the *in vitro* NSCLC growth was evaluated with MTT assay. Each value represents mean percent cell growth ± SD (n = 5).

Figure 9. Effect of cytokine combination on p21 expression. a. LK-1 cells were cultured with or without cytokine(s) for the indicated hours. Time course analysis of p21 induction was

performed using western blot analysis. 1. Control, 2. 10 ng/ml IFN-α, 3. 50 ng/ml IL-29, 4. 50 ng/ml IL-29 plus 10 ng/ml IFN-α

b. The relative p21 intensity normalized to β-actin was presented in the bar graph. (*, $p < 0.05$: IFN-α or IL-29 versus IL-29 plus INF-α)

Figure 10. Antitumor effect of the combination between IL-29 and IFN-α *in vivo*. Simultaneous administration of IL-29 and IFN-α inhibited the growth more effectively than each reagent alone (*, $p < 0.05$; PBS versus IL-29; **, $p < 0.03$ PBS versus IFN-α, ***, $p < 0.005$; IL-29 or IFN-α versus IL-29 plus IFN-α).

Table 1.

Antiproliferative activity of IL-28A, IL-29, IFN-α, IL-28A + TNF-α and IL-29 + INF-α on human NSCLC lines

Human NSCLC cell line	% reduction in cell growth				
	IL-28A	IL-29	IFN-α	IL-28A + TNF-α	IL-29 +TNF-α
Squamous cell carcinoma					
Sq-1	**17**	**33**	**42**	**53**	**57**
Sq-19	**< 15**	**18**	**27**	**40**	**50**
LK-2	**< 15**	**18**	**25**	**39**	**47**
LK-79	**< 15**	**20**	**26**	**36**	**52**
EBC-1	**< 15**	**< 15**	**71**	**79**	**82**
Adenocarcinoma					
11-18	**16**	**73**	**92**	**95**	**95**
LK-1	**15**	**42**	**36**	**52**	**55**
NCI-H23	**< 15**	**21**	**43**	**57**	**61**
Large cell carcinoma					
OBA-LK1	**48**	**71**	**87**	**92**	**94**
86-2	**22**	**56**	**77**	**83**	**85**
Lu99	**< 15**	**17**	**24**	**33**	**38**
Alveolar cell carcinoma					
A549	**< 15**	**< 15**	**< 15**	**23**	**25**

The antiproliferative effect of 10 ng/ml IL-28A, 10 ng/ml IL-29, 10 ng/ml IFN-α, 10 ng/ml IL-28A + 10 ng/ml IFN-α or 10 ng/ml IL-29 + 10 ng/ml IFN-α on human NSCLC lines was examined with MTT assay as described in Materials and Methods. The percentage of reduction in cell growth was determined by the following formula: % reduction in cell growth = (1 - A_{590} of cells cultured with cytokine(s) / A_{590} of cells cultured without cytokine(s)) x 100. IL-28A + IFN-α or IL-29 + IFN-α significantly inhibited the growth of NSCLC lines more efficiently than each reagent alone.

Figure 1.

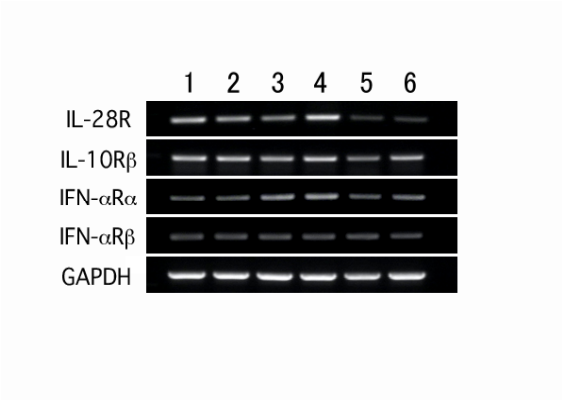
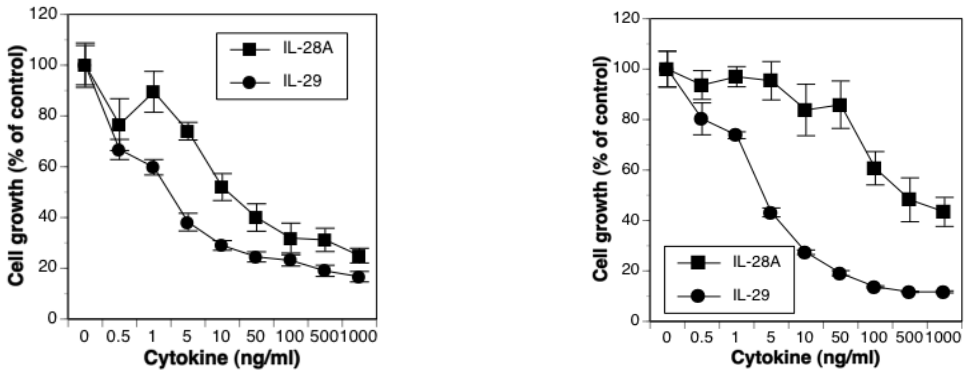


Figure 2.

(a) **OBA-LK1** **(b)** **11-18**



(c)

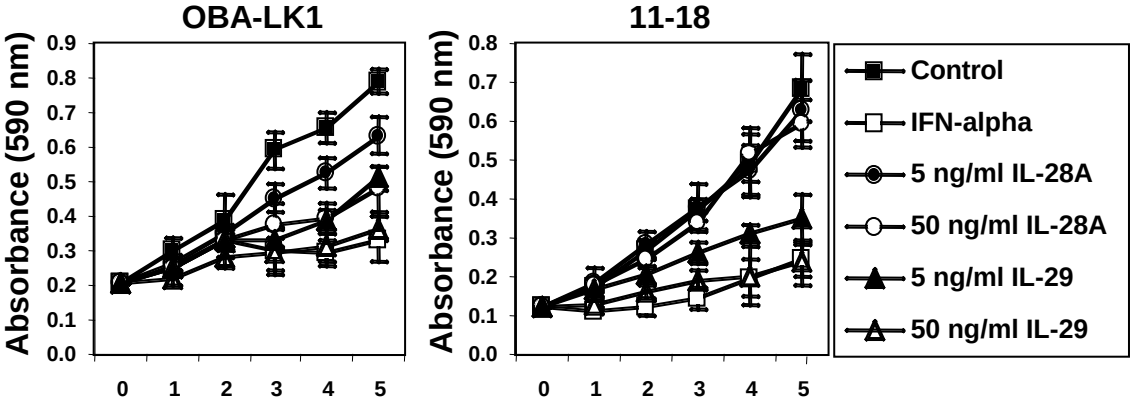
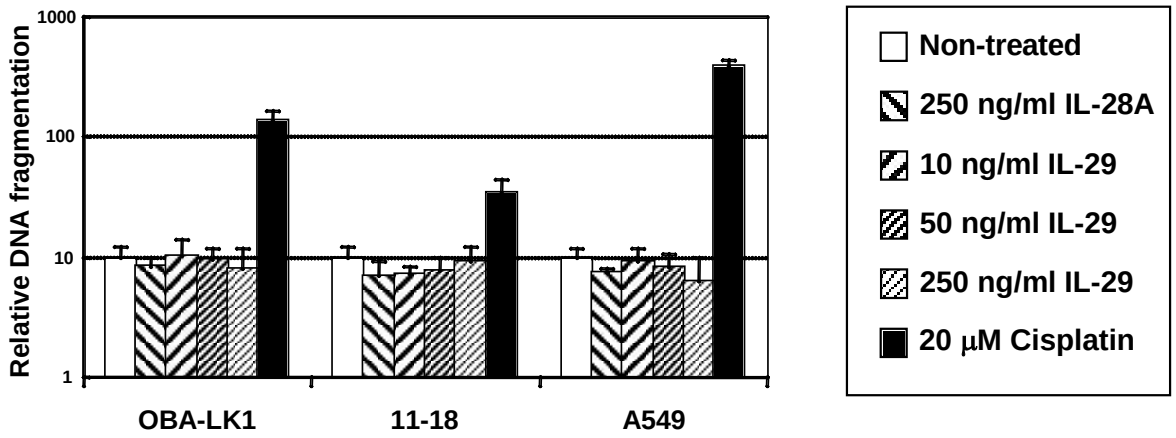


Figure 3.

(a)


Relative DNA fragmentation
1000
100
10
1
OBA-LK1
11-18
A549
Non-treated
250 ng/ml IL-28A
10 ng/ml IL-29
50 ng/ml IL-29
250 ng/ml IL-29
20 μM Cisplatin

(b)

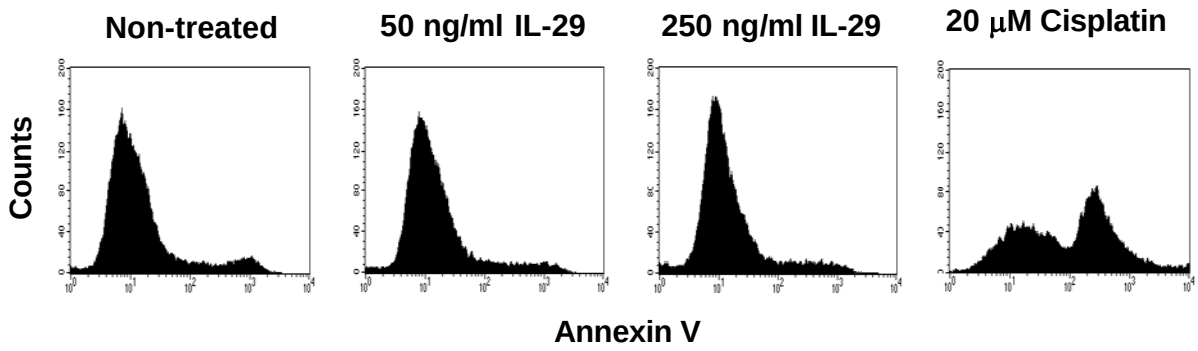
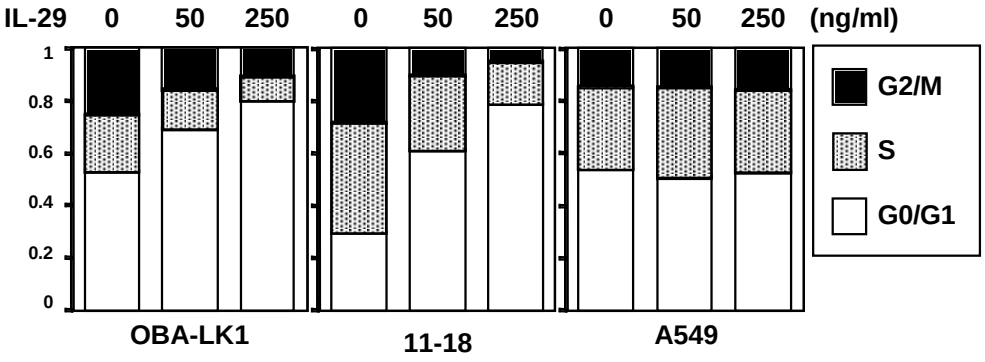
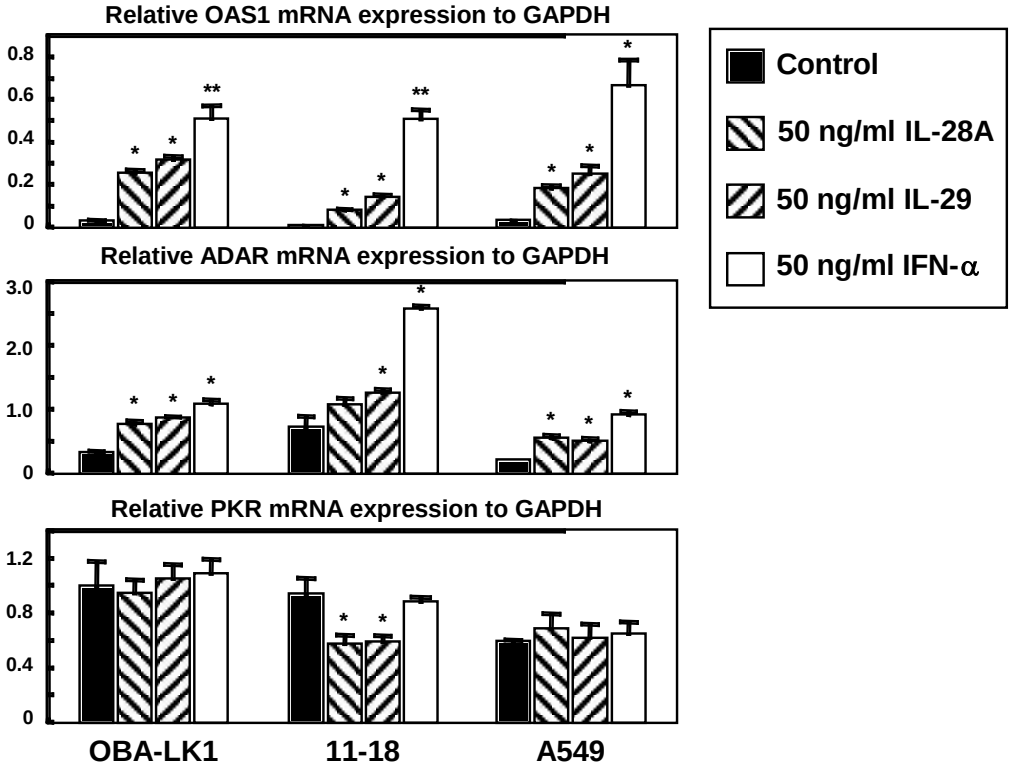

Non-treated
50 ng/ml IL-29
250 ng/ml IL-29
20 μM Cisplatin
Counts
Annexin V

Figure 4.

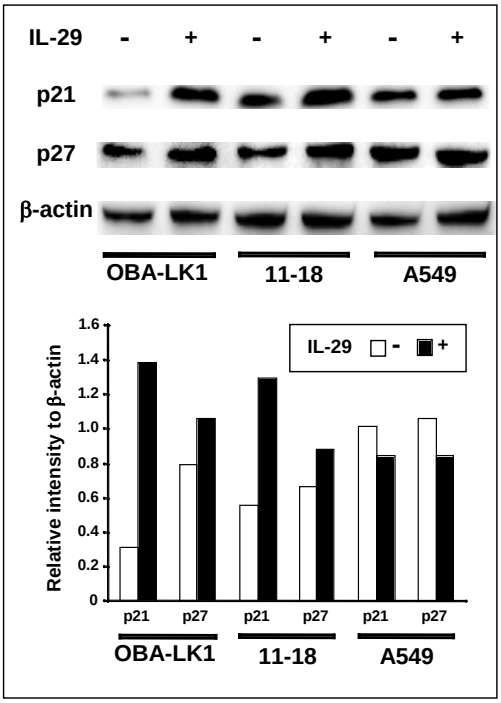
(a)


IL-29 0 50 250 0 50 250 0 50 250 (ng/ml)
1
0.8
0.6
0.4
0.2
0
G2/M
S
G0/G1
OBA-LK1
11-18
A549

(b)


Relative OAS1 mRNA expression to GAPDH
0.8
0.6
0.4
0.2
0
Relative ADAR mRNA expression to GAPDH
3.0
2.0
1.0
0
Relative PKR mRNA expression to GAPDH
1.2
0.8
0.4
0
OBA-LK1
11-18
A549
Control
50 ng/ml IL-28A
50 ng/ml IL-29
50 ng/ml IFN-α

(c)


IL-29 - + - + - +
p21
p27
β-actin
OBA-LK1
11-18
A549
Relative intensity to β-actin
IL-29 - +
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
p21 p27 p21 p27 p21 p27
OBA-LK1
11-18
A549

(d)

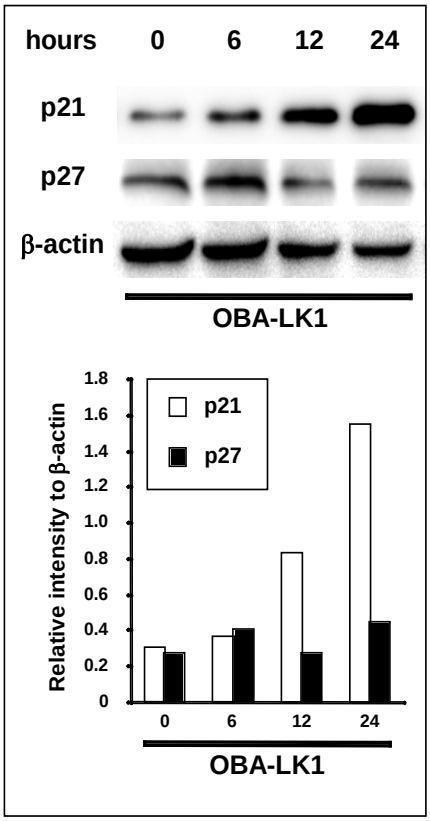
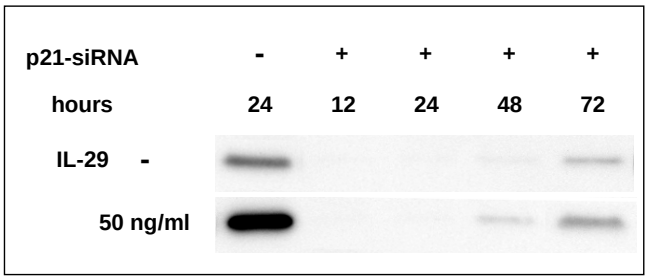

hours 0 6 12 24
p21
p27
β-actin
OBA-LK1
Relative intensity to β-actin
p21
p27
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0 6 12 24
OBA-LK1

Figure 5.

(a)


p21-siRNA - + + + +
hours 24 12 24 48 72
IL-29 -
50 ng/ml

(b)

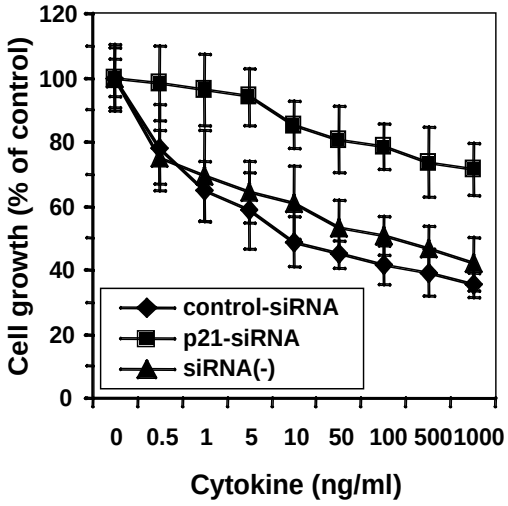
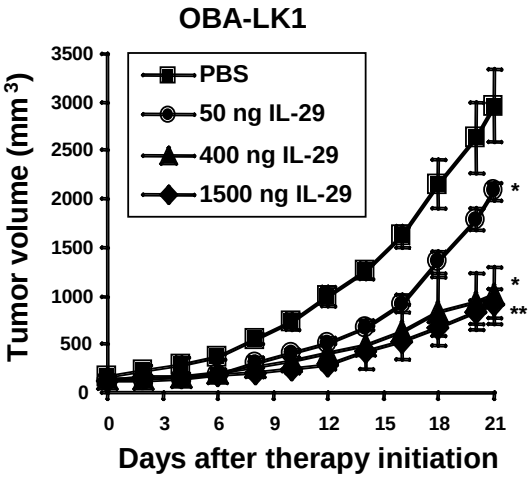
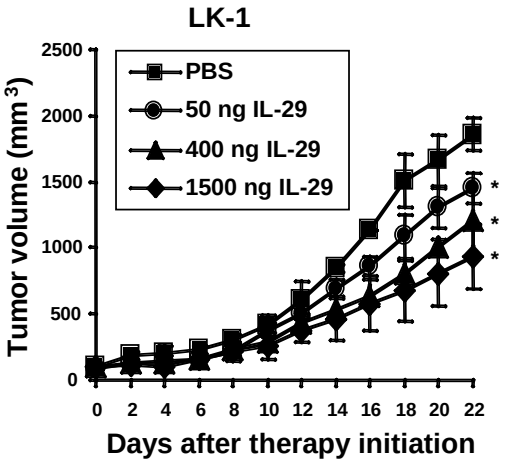

Cell growth (% of control)
120
100
80
60
40
20
0
control-siRNA
p21-siRNA
siRNA(-)
0 0.5 1 5 10 50 100 500 1000
Cytokine (ng/ml)

Figure 6.

(a)


OBA-LK1
Tumor volume (mm 3)
PBS
50 ng IL-29
400 ng IL-29
1500 ng IL-29
*
*
**
Days after therapy initiation

(b)


LK-1
Tumor volume (mm 3)
PBS
50 ng IL-29
400 ng IL-29
1500 ng IL-29
*
*
*
Days after therapy initiation

(c)

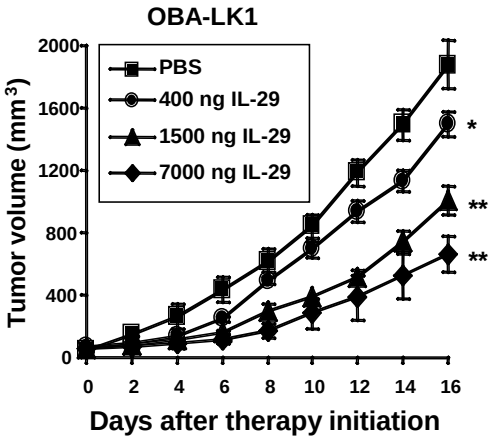

OBA-LK1
Tumor volume (mm 3)
PBS
400 ng IL-29
1500 ng IL-29
7000 ng IL-29
*
**
**
Days after therapy initiation

Figure 7.

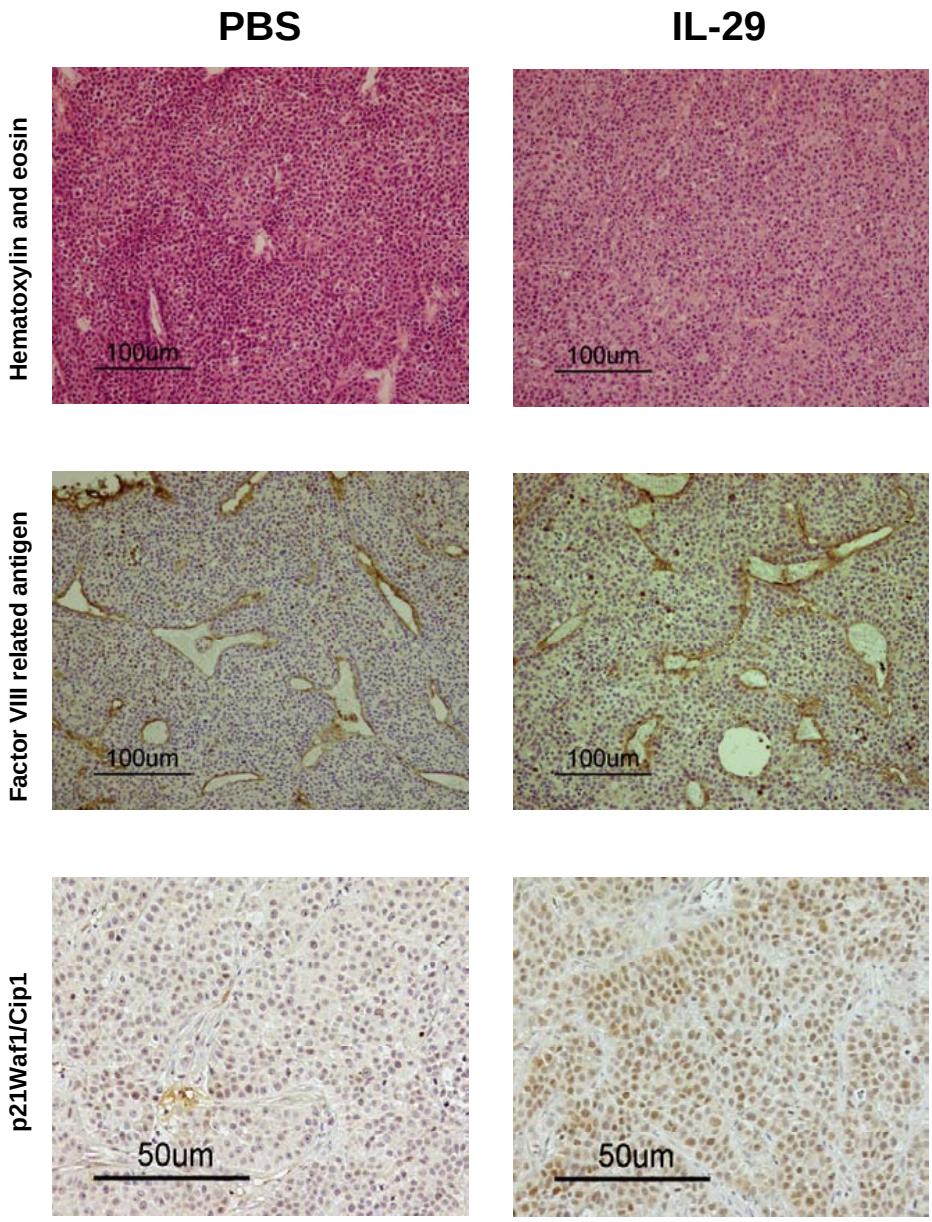
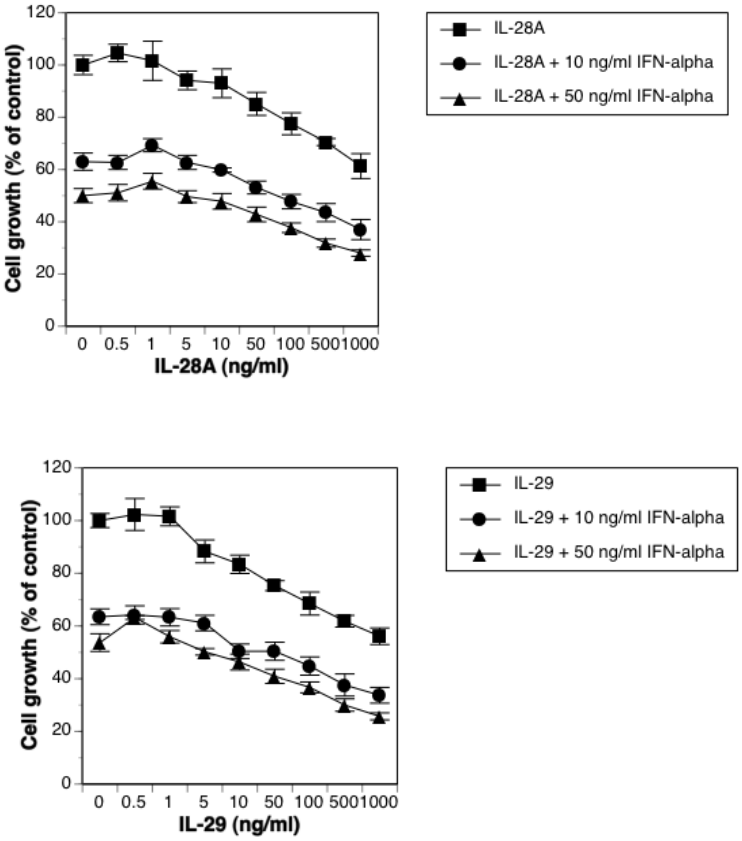
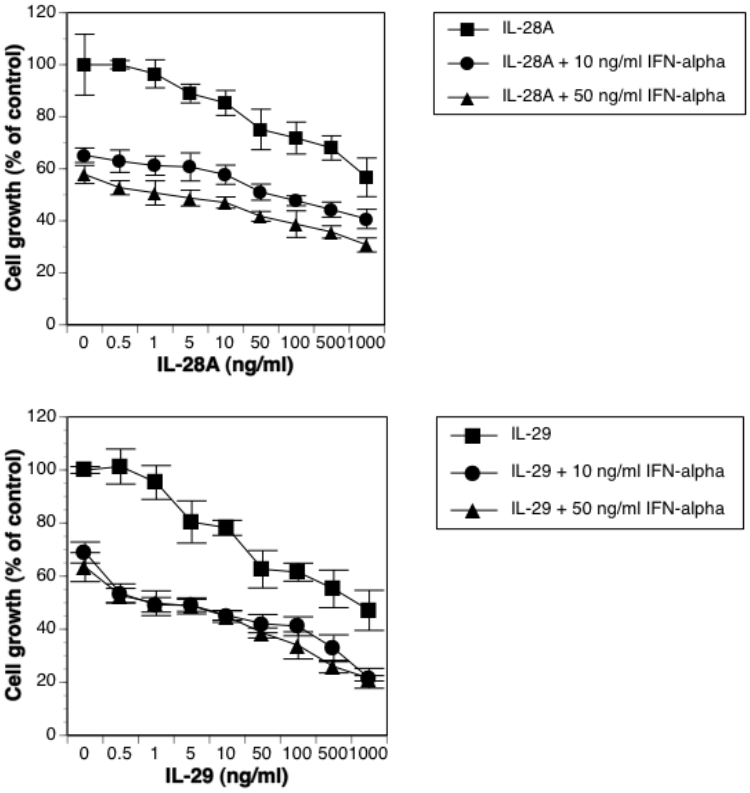

PBS
IL-29
Hematoxylin and eosin
Factor VIII related antigen
p21Waf1/Cip1
100um
100um
100um
100um
50um
50um

Figure 8.

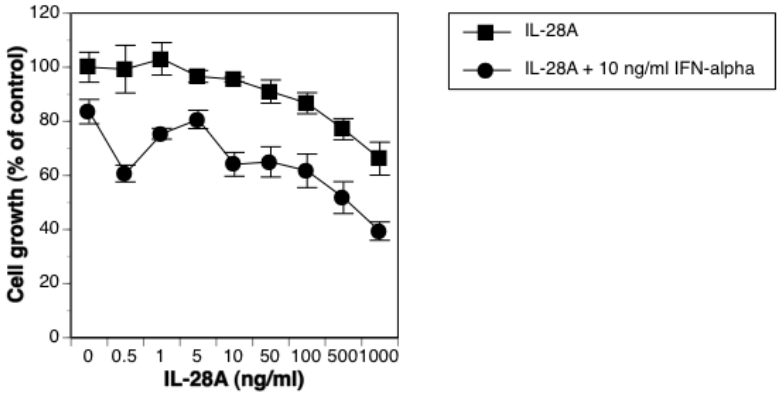
(a) Sq-19

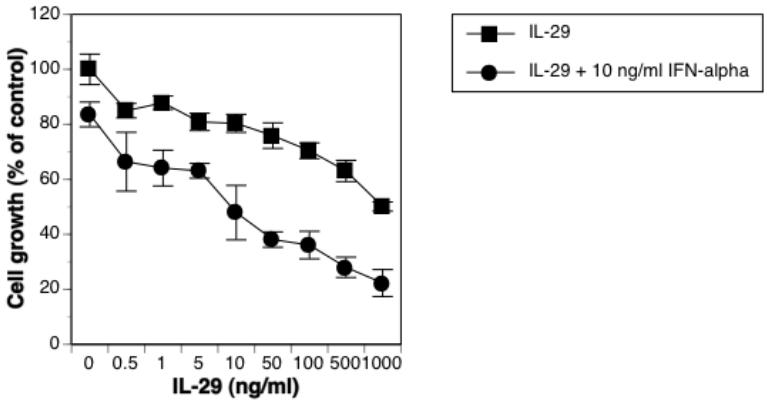


(b) LK-1

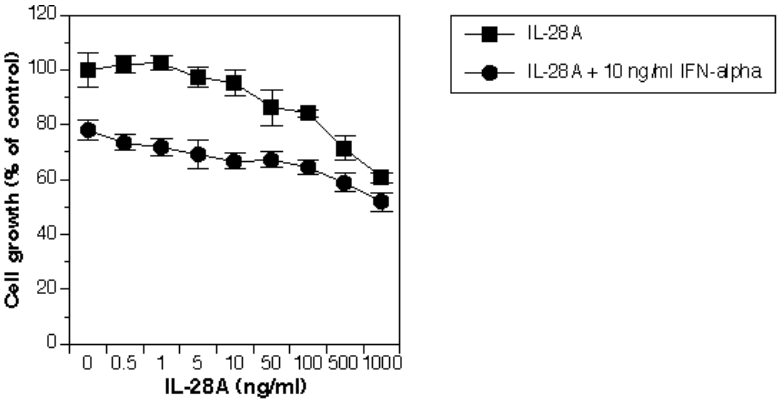


(c) LK-79


Cell growth (% of control)
120
100
80
60
40
20
0
0 0.5 1 5 10 50 100 5001000
IL-28A (ng/ml)
IL-28A
IL-28A + 10 ng/ml IFN-alpha


Cell growth (% of control)
120
100
80
60
40
20
0
0 0.5 1 5 10 50 100 5001000
IL-29 (ng/ml)
IL-29
IL-29 + 10 ng/ml IFN-alpha

(d) Lu99


Cell growth (% of control)
120
100
80
60
40
20
0
0 0.5 1 5 10 50 100 500 1000
IL-28A (ng/ml)
IL-28A
IL-28A + 10 ng/ml IFN-alpha

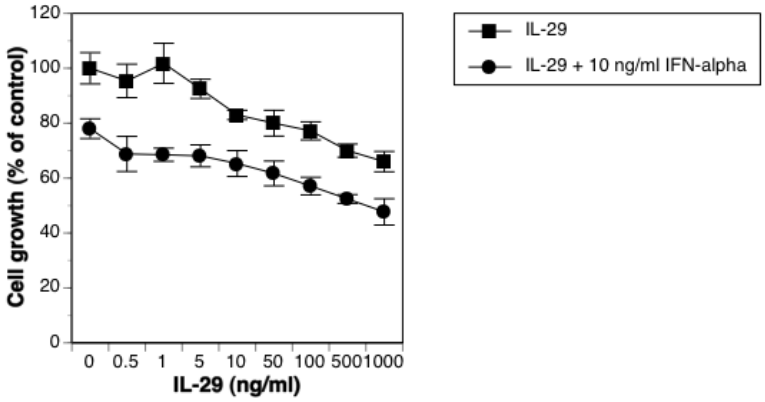
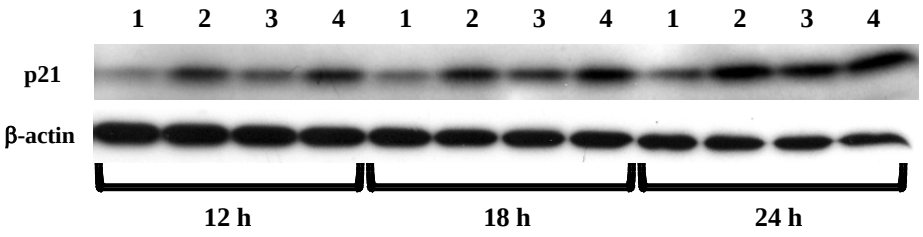

Cell growth (% of control)
120
100
80
60
40
20
0
0 0.5 1 5 10 50 100 5001000
IL-29 (ng/ml)
IL-29
IL-29 + 10 ng/ml IFN-alpha

Figure 9.

(a)


1 2 3 4 1 2 3 4 1 2 3 4
p21
β-actin
12 h
18 h
24 h

(b)

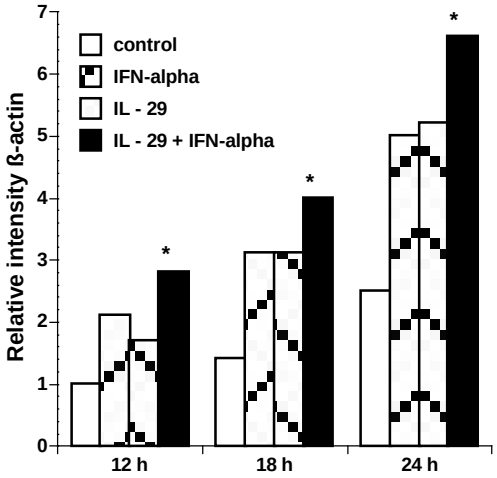
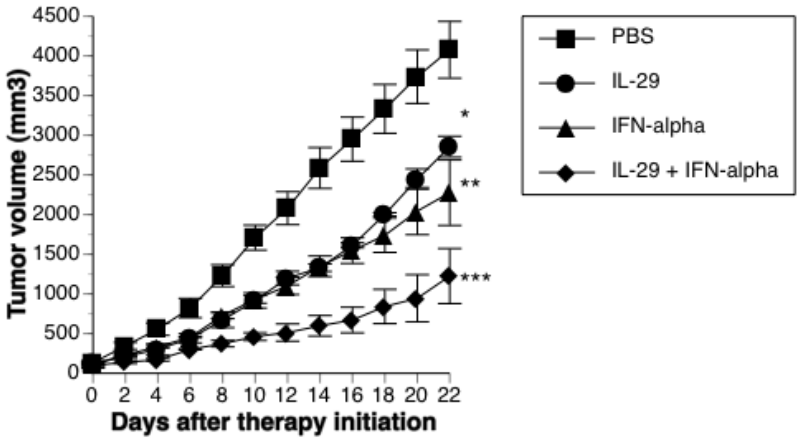

control
IFN-alpha
IL - 29
IL - 29 + IFN-alpha
Relative intensity β-actin
0
1
2
3
4
5
6
7
*
*
*
12 h
18 h
24 h

Figure 10.


Tumor volume (mm3)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0 2 4 6 8 10 12 14 16 18 20 22
Days after therapy initiation
*
**
***
PBS
IL-29
IFN-alpha
IL-29 + IFN-alpha