Tanaka N, (小池)	Hepatitis C virus core protein induces spontaneous and persistent activation of peroxisome proliferator-activated receptor alpha in transgenic mice: Implications for HCV-associated hepatocarcinogenesis.	Int J Cancer	122	124-131	2008
Ishizaka N, (小池)	Impacts of changes in obesity parameters for the prediction of blood pressure change in Japanese individuals.	Kidney Blood Press Res	32	421-427	2009
Moriya K, (小池)	Tacrolimus ameliorates metabolic disturbance and oxidative stress caused by hepatitis C virus core protein: Analysis using mouse model and cultured cells.	Am J Pathol	175	1515-1524	2009
Tsutsumi T, (小池)	Proteomics analysis of mitochondrial proteins reveals overexpression of a mitochondrial protein chaperone, prohibitin, in cells expressing hepatitis C virus core protein.	Hepatology	50	378-386	2009
Yotsuyanagi H, (小池)	Chronic hepatitis C in patients coinfected with human immunodeficiency virus in Japan: a retrospective multicenter analysis.	Hepatol Res	39	657-663	2009
Ishizaka Y, (小池)	Association between changes in obesity parameters and incidence of chronic kidney disease in Japanese individuals.	Kidney Blood Press Res	32	141-149	2009
Murata M, (小池)	Hepatitis B virus X protein shifts hepatic Smad3-mediated signaling from tumor-suppression to oncogenesis in chronic hepatitis B.	Hepatology	49	1203-1217	2009
Yanagimoto S, (小池)	A single amino acid of toll-like receptor 4 that is pivotal for its signaltransduction and subcellular localization.	J Biol Chem	284	3513-3520	2009

Ishizaka Y, (小池)	Association between metabolic syndrome and carotid atherosclerosis in individuals without diabetes based on the oral glucose tolerance test.	Atherosclerosis	204	619-623	2009
Koike K (小池)	Steatosis, Liver injury and hepatocarcinogenesis in hepatitis C viral infection.	J Gastroenterol	44supl:	82-88	2009
Ichibangase T, (小池)	Limitation of immunoaffinity column for the removal of abundant proteins from plasma in quantitative plasma proteomics.	Biomed Chromatogr	23	480-487	2009
Hmwe SS, (小池)	Identification of hepatitis C virus genotype 2a replicon variants with reduced susceptibility to ribavirin.	Antiviral Res		2009 Dec 28. [Epub ahead of print]	2009
Ishizaka N, (小池)	Impact of changes pn waist circumference and BMI over one-year period on serum lipid data in Japanese individuals.	J Atheroscler Thromb		2009 Dec 23. [Epub ahead of print].	2009
Ishizaka N, (小池)	Changes in waist circumference and body mass index in relation to changes in serum uric acid in Japanese individuals.	J Rheumatol		2009 Dec 23. [Epub ahead of print].	2009
Watanabe S, (小池)	Prolonged treatment with PEG-IFN a-2b and ribavirin can improve SVR in chronic hepatitis C genotype 1 patients with late response in a clinical real-life setting in Japan.	Hepatol Res		2009 Sep 25. [Epub ahead of print].	2009
Moriya K, (小池)	Hepatitis C virus core protein compromises iron-induced activation of antioxidants in mice and HepG2 cells.	J Med Virol		In press	2010
Okuse C, (小池)	Effect of nucleoside analogue-interferon sequential therapy on patients with acute exacerbation of chronic hepatitis B.	Hepatol Res		In press	2010

Ishizaka N, (小池)	Association between gamma-glutamyl transferase levels and insulin resistance according to alcohol consumption and number of cigarettes smoked.	J Atheroscler Thromb		In press	2010
Koike K, (小池)	Lipid metabolism and pathogenesis of liver disease in hepatitis C viral infection.	Oncology		In press	2010
Koike K, (小池)	Animal models for hepatitis C and related liver disease.	Hepatol Res		In press	2010
Kanamori H, (小池)	RNA dependent RNA polymerase of hepatitis C virus binds to its coding region RNA stem-loop structure, 5BSL3.2, and its negative strand.	J Gen Virol		2010 Jan 6. [Epub ahead of print]	2010
Ishizaka N, (小池)	Effects of the AT(1) receptor blocker losartan and the calcium channel blocker benidipine on the accumulation of lipids in the kidney of a rat model of metabolic syndrome.	Hypertens Res		2010 Jan 8. [Epub ahead of print]	2010
上田啓次 (上田)	ヘルペスウイルス学のエッセンス	化学療法の領域	26	265-270	2010
Ohsaki E, (上田)	Accumulation of LANA at Nuclear Matrix fraction is important for Kaposi's Sarcoma-Associated Herpesvirus Replication in Latency.	Virus Research	139	74-84	2009
上田啓次 (上田)	Kaposi肉腫-最新の知見	皮膚科診療	30	1344-1348	2008
Uto H, (井戸)	Alanine aminotransferase flare-up in hepatitis C virus carriers with persistently normal alanine aminotransferase levels in a hyperendemic area of Japan	J Gastroenterol	42	673-80	2007
Abe H, (井戸)	Transgenic expression of osteoactivin in the liver attenuates hepatic fibrosis in rats	Biochem Biophys Res Commun	356	610-5	2007

Kanmura S, (井戸)	Early diagnosis potential for hepatocellular carcinoma using the SELDI ProteinChip system	Hepatology	45	948-56	2007
Takahama Y, (井戸)	Association of a genetic polymorphism in ectonucleotide pyrophosphatase/phophodiesterase 1 with hepatitis C virus infection and hepatitis C virus core antigen levels in subjects in a hyperendemic area of Japan	J Gastroenterology	43	942-50	2008
Kodama M, (井戸)	Endoscopic characterization of the small bowels in patients with portal hypertension evaluated by double balloon endoscopy	J Gastroenterol	43	589-96	2008
Kikkawa YS, (井戸)	Hepatocyte growth factor protects auditory hair cells from aminoglycosides	Laryngoscope	119	2027-31	2009
Uto H, (井戸)	Increased rate of death related to presence of viremia among hepatitis C virus antibody-positive subjects in a community-based cohort study	Hepatology	50	393-9	2009
Ido A, (井戸)	Translational research to identify clinical applications of hepatocyte growth factor	Hepatol Res	39	739-47	2009
Inaoka T, (井戸)	Local application of hepatocyte growth factor using gelatin hydrogels attenuates noise-induced hearing loss in guinea pigs	Acta Otolaryngol	50	393-9	2009
Kanmura S, (井戸)	Human neutrophil peptide 1-3 are useful biomarkers in patients with active ulcerative colitis	Inflamm Bowel Dis	15	909-17	2009
Nishida C, (井戸)	Clinical significance of alanine aminotransferase levels and the effect of ursodeoxycholic acid in hemodialysis patients with chronic hepatitis C	J Gastroenterol		(in press)	2009

Kanmura S, (井戸)	The complement component C3a fragment is a potential biomarker for hepatitis C virus-related hepatocellular carcinoma	J Gastroenterol		(in press)	2010
Nakamoto Y, (中本)	Combined Therapy of Transcatheter Hepatic Arterial Embolization with Intratumoral Dendritic Cell Infusion for Hepatocellular Carcinoma; Clinical Safety.	Clin. Exp. Immunol.	147(2)	296-305	2007
Tsuchiyama T, (中本)	Prolonged, NK cell-mediated antitumor effects of suicide gene therapy combined with monocyte chemoattractant protein-1 against hepatocellular carcinoma.	J. Immunol.	178(1)	574-83	2007
Oishi N, (中本)	Hepatitis B virus X protein overcomes oncogenic RAS-induced senescence in human immortalized cells.	Cancer Sci.	98(10)	1540-8	2007
Iida, N, (中本)	Tumor cell apoptosis induces tumor-specific immunity in a CC chemokine receptor 1- and 5-dependent manner in mice.	J. Leukoc. Biol.	84(4)	1001-1010	2008
Sakai, Y, (中本)	Common transcriptional signature of tumor-infiltrating mononuclear inflammatory cells and peripheral blood mononuclear cells in hepatocellular carcinoma patients.	Cancer Res.	68(24)	10267-10279	2008
Tsuchiyama, T, (中本)	Optimal amount of monocyte chemoattractant protein-1 enhances antitumor effects of suicide gene therapy against hepatocellular carcinoma by M1 macrophage activation.	Cancer Sci.	99(10)	2075-2082	2008
Mizukoshi E, (中本)	Enhancement of tumor-specific T-cell responses by transcatheter arterial embolization with dendritic cell infusion for hepatocellular carcinoma.	Int. J. Cancer	(in press)		2009

Baba T, (中本)	Crucial contribution of thymic Sirp alpha+ conventional dendritic cells to central tolerance against blood-borne antigens in a CCR2-dependent manner.	J. Immunol.	183 (5)	3053-3063	2009
Sakaki M, (広石)	Intrahepatic status of regulatory T cells in autoimmune liver diseases and chronic viral hepatitis	Hepatol Res	38 (4)	354-361	2008
Hiroishi K, (広石)	Immune Responses in HCV Infection and Mechanisms of HCV Persistence.	J Gastroenterol Hepatol	23 (10)	1473-1482	2008
Hiraide A, (広石)	Dendritic cells stimulated with CpG oligodeoxynucleotides and IFN-alpha -expressing tumor cells effectively reduce outgrowth of established tumors in vivo.	Cancer Sci	99 (8)	1663-1669	2008
Doi H, (広石)	Magnitude of CD8+ T-cell responses against hepatitis C virus and severity of hepatitis do not necessarily determine outcomes in acute hepatitis C virus infection	Hepatol Res	39	256-265	2009
Inokuchi M, (広石)	Infection of B cells with hepatitis C virus for the development of lymphoproliferative disorders in patients with chronic hepatitis C.	J Med Virol.	81 (4)	619-627	2009
Hiroishi K, (広石)	Strong CD8(+) T-cell responses against tumor-associated antigens prolong the recurrence-free interval after tumor treatment in patients with hepatocellular carcinoma.	J Gastroenterol		In press	2010
Takehara T, (竹原)	Natural killer cell-mediated ablation of metastatic liver tumors by hydrodynamic injection of IFN・gene to mice.	Int J Cancer	120	1252-1260	2007

Jinushi M, (竹原)	Natural killer cell and hepatic cell interaction via NKG2A leads to dendritic cell-mediated induction of CD4+ CD25+ T cells with PD-1-dependent regulatory activities.	Immunology	120	73-82	2007
Tatsumi T, (竹原)	Intrahepatic delivery of ・-galactosylceramide-pulsed dendritic cells suppresses liver tumor.	Hepatology	45	22-30	2007
Tatsumi T, (竹原)	Injection of IL-12 gene-transduced dendritic cells into mouse liver tumor lesions activates both innate and acquired immunity.	Gene Ther	14	863-871	2007
Yamaguchi S, (竹原)	Immunotherapy of murine colon cancer using receptor tyrosine kinase EphA2-derived peptide-pulsed dendritic cell vaccines.	Cancer	110	1469-1477	2007
Sakamori R, (竹原)	Signal transducer and activator of transcription 3 signaling within hepatocytes attenuates systemic inflammatory response and lethality in septic mice.	Hepatology	46	1564-1573	2007
Toyama T, (竹原)	A New Prognostic System for Hepatocellular Carcinoma Including Recurrent Cases: A Study of 861 Patients in a Single Institution.	J Clin Gastroenterol	42	317-322	2008
Yamaguchi S, (竹原)	Dendritic cell-based vaccines suppress metastatic liver tumor via activation of local innate and acquired immunity.	Cancer Immunol Immunother	57	1861-1869	2008
Kohga K, (竹原)	Serum levels of soluble MHC class I-related chain A in patients with chronic liver diseases and the changes during transcatheter arterial embolization for hepatocellular carcinoma.	Cancer Sci	99	1643-1649	2008
Tatsumi T, (竹原)	Decreased expressions of CD1d molecule on liver dendritic cells in subcutaneous tumor bearing mice.	J Hepatol	49	779-786	2008

Sasakawa A, (竹原)	Activated liver dendritic cells generates strong acquired immunity in ・-galactosylceramide treatment.	J Hepatol	50	1155-1162	2009
Hikita H, (竹原)	Mcl-1 and Bcl-xL cooperatively maintain integrity of hepatocytes in developing and adult murine liver.	Hepatology	50	1217-1226	2009
Kohga K, (竹原)	Anti-cancer therapy inhibits MICA ectodomain shedding by downregulating ADAM10 expression in hepatocellular carcinoma.	Cancer Res	69	8050-8057	2009
Hikita H, (竹原)	BH3-only protein Bid participates in the Bcl-2 network in healthy liver cells.	Hepatology	50	1972-1980	2009
Uemura A, (竹原)	Natural killer cell is a major producer of interferon ・ that is critical for the IL-12-induced anti-tumor effect in mice.	Cancer Immunol Immunother	59	453-463	2010
Kohga K, (竹原)	Sorafenib inhibited the shedding of MICA on hepatocellular carcinoma cells by downregulating ADAM9.	Hepatology			in press
Itose I, (考藤)	Involvement of dendritic cell frequency and function in virological relapse in pegylated interferon-a2b and ribavirin therapy for chronic hepatitis C patients.	J Med Virol	79	511-521	2007
Abe T, (考藤)	Hepatitis C virus nonstructural protein 5A modulates the toll-like receptor-MyD88-dependent signaling pathway in macrophage cell lines.	J Virol	81	8953-8966	2007
Kanto T, (考藤)	Virus-associated innate immunity in liver.	Front Bioscience	13	6183-6192	2008
Miyazaki M, (考藤)	Impaired cytokine response in myeloid dendritic cells in chronic hepatitis C virus infection regardless of enhanced expression of Toll-like receptors and retinoic acid inducible gene-I.	J Med Virol	80	980-988	2008

Kohga K, (考藤)	Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A in patients with chronic liver diseases and changes during transcatheter arterial embolization for hepatocellular carcinoma	Cancer Sci	99	1643-1649	2008
Toyama T, (考藤)	A new prognostic system for hepatocellular carcinoma including recurrent cases: a study of 861 patients in a single institution.	J Clin Gastroenterol	42	317-322	2008
Kurashige N, (考藤)	Initial viral response is the most powerful predictor of the emergence of the YMDD mutant virusin chronic hepatitis B patients treated with lamivudine.	Hepatology Research	38	450-456	2008
Kanada A, (考藤)	Early emergence of entecavir-resistant hepatitis B virus in a patient with hepatitis B virus/human immunodeficiency virus coinfection	Hepatology Research	38	622-688	2008
Shinzaki S, (考藤)	IgG oligosaccharide alterations are a novel diagnostic marker for disease activity and the clinical course of inflammatory bowel disease.	Am J Gastroenterol	103	1173-1181	2008
Egawa S, (考藤)	Upregulation of GRAIL is associated with remission of ulcerative colitis.	Am J Physiol Gastrointest Liver Physiol	295	G163-G169	2008.
Itose I, (考藤)	Enhanced ability of regulatory T cells in chronic hepatitis C patients with persistently normal alanine aminotransferase levels than those with active hepatitis.	J Viral Hepat	16	846-852	2009

Oze T, (考藤)	Pegylated interferon alpha-2b (Peg-IFN alpha-2b) affects early virologic response dose-dependently in patients with chronic hepatitis C genotype 1 during treatment with Peg-IFN alpha-2b plus ribaviri n.	J Viral Hepat	16	578-585	2009
Inoue Y, (考藤)	Factors affecting efficacy in patients with genotype 2 chronic hepatitis C treated by pegylated interferon alpha-2b and ribavirin: reducing drug doses has no impact on rapid and sustained virological responses.	J Viral Hepat			2009
Hiramatsu N, (考藤)	Ribavirin dose reduction raises relapse rate dose-dependently in genotype 1 patients with hepatitis C responding to pegylated interferon alpha-2b plus ribaviri n.	J Viral Hepat	16	586-594	2009
Kurokawa M, (考藤)	Effect of interferon alpha-2b plus ribavirin therapy on incidence of hepatocellular carcinoma in patients with chronic hepatitis.	Hepatol Res	39	432-438	2009

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
上田啓次	ウイルスによるがん	谷口直之、杉山治夫、松浦成昭、三善英知	がん医学入門	中山書店	東京	2008	pp80-95
上田啓次	カポジ肉腫関連ヘルペスウイルス	山口恵三、戸塚恭一	Key Word 感染症　第2版	先端医学社	東京	2008	pp192-193
上田啓次	HHV8	成田和男	病原細菌・ウイルス図鑑	北海道大学出版会	札幌		印刷中

Ⅳ. 研究成果の刊行物・別刷

Int. J Cancer · **120**, 1252–1260 (2007)

Natural killer cell-mediated ablation of metastatic liver tumors by hydrodynamic injection of IFNα gene to mice

Tetsuo Takehara†, Akio Uemura†, Tomohide Tatsumi, Takahiro Suzuki, Ritsuko Kimura, Ai Shiotani, Kazuyoshi Ohkawa, Tatsuya Kanto, Naoki Hiramatsu and Norio Hayashi*

Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka, Japan

Interferon (IFN) α is a pleiotropic cytokine acting as an antiviral substance, cell growth inhibitor and immunomodulator. To evaluate the therapeutic efficacy and mechanisms of IFNα on hepatic metastasis of tumor cells, we hydrodynamically injected naked plasmid DNA encoding IFNα1 (pCMV-IFNa1) into Balb/cA mice having 2 days hepatic metastasis of CT-26 cells. Single injection of pCMV-IFNa1 efficiently enhanced the natural killer (NK) activity of hepatic mononuclear cells, induced production of IFNγ in serum and led to complete rejection of tumors in the liver. Mice protected from hepatic metastasis by IFNα therapy displayed a tumor-specific cytotoxic T cell response and were resistant to subcutaneous challenge of CT-26 cells. NK cells were critically required for IFNα-mediated rejection of hepatic metastasis, because their depletion by injecting anti-asialo GM1 antibody completely abolished the antimetastatic effect. To find whether NK cells are directly activated by IFNα and are sufficient for the antimetastatic effect, the responses to IFNα were examined in SCID mice lacking T cells, B cells and NKT cells. IFNα completely rejected hepatic metastasis in SCID mice and efficiently activated SCID mononuclear cells, as evidenced by activation of STAT1 and a variety of genes, such as MHC class I, granzyme B, tumor necrosis factor-related apoptosis-inducing ligand and IFNγ, and also enhanced Yac1 lytic ability. Study of IFNγ knockout mice revealed that IFNγ was not necessary for IFNα-mediated NK cell activation and metastasis protection. In conclusion, IFNα efficiently activates both innate and adaptive immune responses, but NK cells are critically required and sufficient for IFNα-mediated initial rejection of hepatic metastasis of microdisseminated tumors.

Key words: DNA; innate; adaptive; immunity NK

The liver is the most common site of metastatic malignancy and the status of this organ is an important determinant of survival in patients with advanced disease. The risk of hepatic metastasis remains high in many patients after potentially curative surgery at primary sites.[1] This suggests that the spread of tumor cells can occur in the liver even when they cannot be detected by current diagnostic modalities. To suppress the incidence of liver metastasis, whole liver therapy against microdisseminated tumors should be considered.[2] Since the liver contains an abundance of immune cells, the cytokine-meditated activation of these cells may be a promising approach toward this end.[3,4]

Interferon (IFN) α is a pleiotropic cytokine acting as an antiviral substance, cell growth inhibitor and immunomodulator. IFNα as well as IFNγ are primarily induced in response to viral infection of cells and ligate a cognate receptor for the Type 1 IFN expressed on target cells.[5] On the other hand, Type 2 IFN, IFNγ, is produced predominantly by T lymphocytes, natural killer (NK) cells and NKT cells and uses a distinct receptor. IFNα-mediated antiviral activity includes induction of 2′-5′ oligoadenylate synthetases, double-stranded RNA-activated protein kinase (PKR) and Mx proteins. IFNα can exert direct effects on tumor cells by inhibiting proliferation, inducing apoptosis and inhibiting the release of proangiogenic factors such as vascular endothelial growth factor.[6] IFNα-mediated immunomodulation includes dendritic cell maturation, NK cell activation, MHC Class I induction and cytokine production.[7] Most, if not all, of these actions are mediated by the Jak-STAT signaling pathway downstream of the Type 1 IFN receptor.[8–10] Type 1 IFN receptor upon ligand ligation phosphorylates Jack1 and then phosphorylates STAT1, which activates a variety of IFN-regulated genes. IFNα and IFNβ have been shown to elicit antitumor effects in various murine models of cancer.[11–14] IFNβ was also shown to be effective for retarding metastatic tumor growth in murine liver, but the underlying mechanisms have not been elucidated.[15]

In the present study, we investigated the efficacy of hydrodynamics-based expression of IFNα in the liver against a murine model of hepatic metastasis of CT-26 colon cancer cells and the mechanisms of an IFNα-mediated therapeutic effect of hepatic metastasis. Mice treated with IFNα completely rejected hepatic metastasis and became resistant to rechallenge by CT-26 cells. Although IFNα induced a variety of host responses including increased NK activity, increased IFNγ production and tumor-specific T cell responses, the initial rejection of hepatic metastasis was solely dependent on NK cells. Our study has shed light on NK cell activation as an important mechanism by which IFNα ablates microdisseminated tumors in the liver.

Material and methods

Mice

Specific pathogen-free female Balb/cA mice, SCID mice and their wild-type control mice were purchased from Clea Japan, Inc. (Tokyo, Japan). Rag2 knockout (Rag2 KO) mice were purchased from Taconic (Germantown, NY). IFNγ knockout (GKO) mice with a Balb/cA background was kindly provided by Dr. Yoichiro Iwakura (Institute of Medical Science, University of Tokyo).[16] All mice were used at the age of 5 to 8 weeks. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care, and the study protocol complied with the institution's guidelines.

Tumor models

Intrasplenic injection of tumor cells was used to establish microdisseminated liver tumors in mice.[17] CT-26 colon cancer cells originating from Balb/cA mice were maintained in DMEM supplemented with 10% FCS. Syngeneic mice were anesthetized with pentobarbital and given a cut on the left side flank. CT-26 cells (1×10^5) were suspended in 150 μl of PBS and injected into the spleen. For subcutaneous tumor models, CT-26 cells (5×10^5) were injected into the back of the mice under light anesthesia.

NK cell depletion

For depletion of NK cells *in vivo*, anti-asialo GM1 antibody (Wako, Osaka, Japan) was intraperitoneally administered.[17] We

Grant sponsor: The Ministry of Education, Culture, Sports, Science and Technology, Japan; Grant sponsor: The Ministry of Health, Labor and Welfare of Japan.

†Both authors contributed equally to this work.

***Correspondence to:** Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Fax: 81-6-6879-3449.
E-mail: hayashin@gh.med.osaka-u.ac.jp

Received 5 April 2006; Accepted after revision 22 May 2006
DOI 10.1002/ijc.22152
Published online 12 December 2006 in Wiley InterScience (www.interscience.wiley.com).

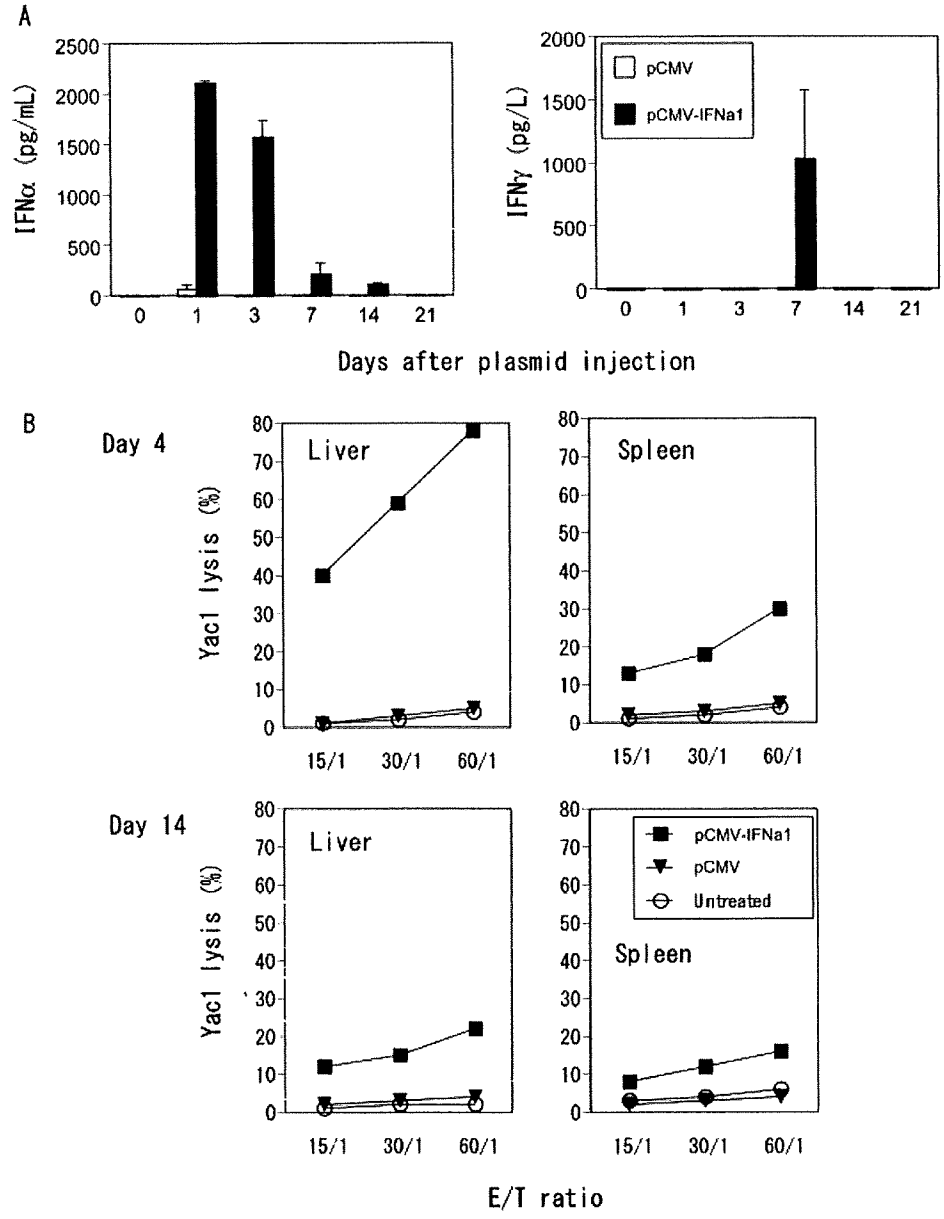

FIGURE 1 – Effects of hydrodynamic injection of IFNα-expressing plasmid. (*a*) Serum IFNα and IFNγ concentration. Balb/cA mice were hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) and bled at indicated time points to measure the levels of serum IFNα and IFNγ. The results are indicated as mean and SD (n = 3/group). Shown are representative data for 2 independent experiments. (*b*) Yac1 lytic ability. Hepatic or splenic mononuclear cells were isolated from naive Balb/cA mice (open circles) and those injected with either pCMV-IFNa1 (closed squares) or pCMV (closed triangles). Yac1 lytic ability was measured by a standard chromium-release assay at indicated effector and target ratios (E/T ratio). All experiments were performed at least 3 times and representative data are shown.

determined the appropriate dosing to be 500 μg/mouse (50 μl when dissolved according to the manufacturer's instructions) based on FACS analysis of hepatic mononuclear cells. Injection of this dose of anti-asialo GM1 antibody depleted more than 95% of DX-5 positive, TCRβ-negative cells (NK cells) in the liver. NKT cells were less affected than NK cells, because 40% of Cd1d-tetramer positive cells, which are invariant NKT cells, still remained in the liver after the treatment. Anti-asialo GM1 antibody was injected 1 day after tumor inoculation and then every 5 days. For the control, the same amount of normal rabbit immunoglobulin (DAKO, Copenhagen, Denmark) was intraperitoneally administered.

Injection of naked plasmid DNA

A plasmid coding the murine IFNa1 gene, pCMV-IFNa1, was generously provided by Dr. Daniel J. J. Carr (University of Oklahoma, Health Science Center).[18] Plasmid DNA was prepared using an EndoFree plasmid system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Hydrodynamic injection of plasmid DNA was performed as previously described.[19] In brief, 25 μg of plasmid DNA was diluted with 2.0 ml of lactated Ringer's solution and injected into the tail vein, using a syringe with a 30-gauge needle. DNA injection was completed within 8 to 15 sec.

ELISA

Blood samples were serially obtained from the venous plexus in the retro-orbita under light anesthesia. The levels of serum IFNα and IFNγ were measured using commercially available ELISA kits (Biomedical Laboratories for murine IFNα; Endogen for murine IFNγ).

Mononuclear cells

Mononuclear cells were isolated from the liver or spleen as previously described.[20] The NK activity of mononuclear cells was assessed with standard 4-hr ^{51}Cr-releasing assay using Yac1 cells as targets. To examine CT-26-specific responses, splenocytes were stimulated with CT-26 cells for 5 days in the presence of 30 U/ml

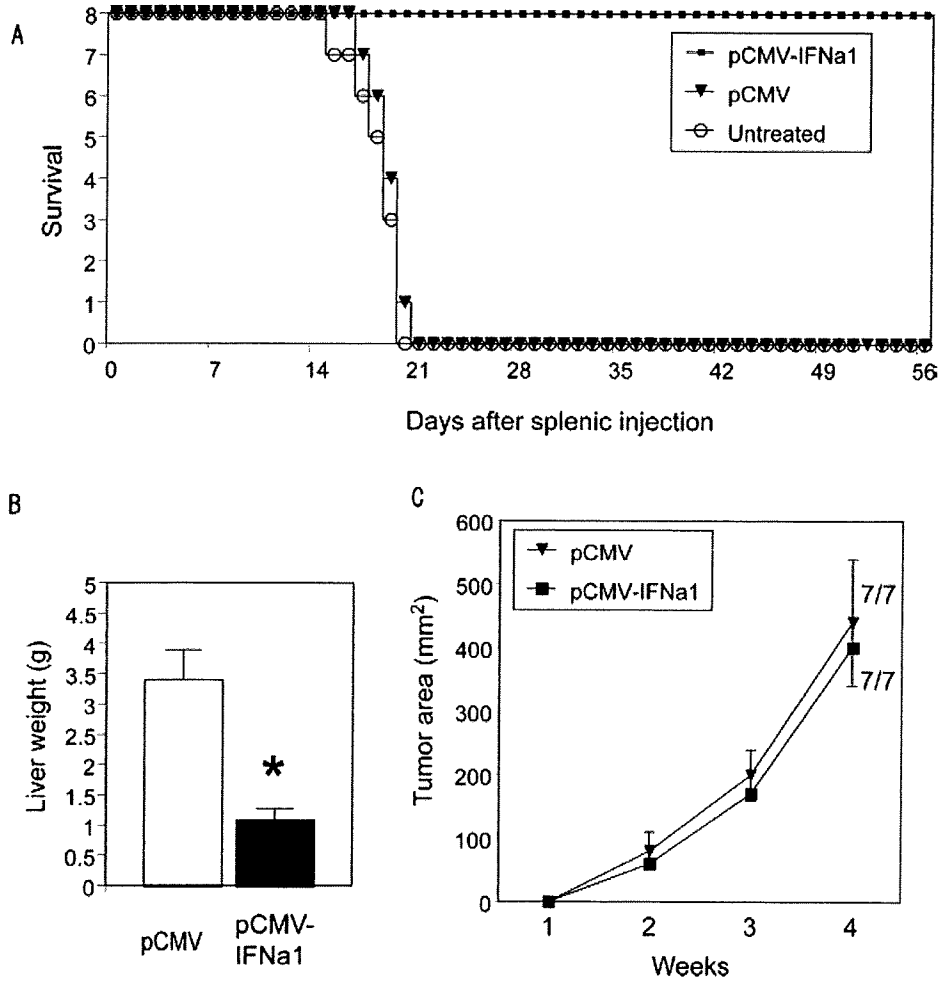

FIGURE 2 – Anti-tumor effects of IFNα therapy. (*a*) Survival. Balb/cA mice were intrasplenically injected with CT-26 cells. Two days later, the mice were randomly assigned to 3 groups and received hydrodynamic injection of either pCMV-IFNa1 (closed squares) or pCMV (closed triangles) or untreated (open circles). The number of survivors in each group was monitored. (*b*) Anti-metastatic effects. Balb/cA mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) 2 days later. At 14 days after the splenic injection, the mice were sacrificed to examine liver tumor development by measuring liver weight. All experiments were performed at least 3 times and representative data are shown. *, $p < 0.05$ *vs.* pCMV injection group. (*c*) Anti-tumor effects on subcutaneous tumors. Balb/cA mice were subcutaneously injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed squares) or pCMV (closed triangles) 2 days later. Tumor growth was examined every week. Tumor size was expressed as the mean tumor size of only those mice bearing tumors. Each data point represents the mean tumor size and SD. The ratio of the number of mice bearing tumor/the number challenged for each treatment group at 4 weeks is shown in the figure.

of murine IL-2 and subjected to analysis for lytic activity against CT-26 cells or BNL A.7 murine hepatoma cells by 4-hr ^{51}Cr-releasing assay. In some experiments, mononuclear cells were separated into CD90-positive cells (T cells) and CD90-negative cells (non-T cells) using the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Western blotting

Mouse recombinant IFNα was generously provided by Fujisaki Institute, Hayashibara Biochemical Laboratories (Okayama, Japan). Mononuclear cells were treated with or without IFNα. Whole cell lysate was prepared from mononuclear cells from mice, and 20 μg of protein was separated by SDS-PAGE and transferred to PVDF membrane. The membrane was stained with anti-STAT1 antibody (Upstate Biotechnology, Lake Placid, NY) or antiphospho-sepcific STAT1 (Y701) antibody (Upstate Biotechnology) and visualized by chemiluminescence. The specificities of STAT1 and phosphorylated STAT1 signals were confirmed by siRNA experiment using BNL A.7 cells in the presence or absence of IFNα treatment (data not shown). Anti-STAT antibody recognizes STAT1α, whereas antiphospho-STAT1 antibody recognizes phosphorylated form of both STAT1α and STAT1β.

Microarray analysis

Total RNA was isolated from cultured SCID splenocytes in the presence or absence of IFNα by ISOGEN. RNA was analyzed using the GeneChip Mouse Genome Array 430 2.0 (Affymetrix, Santa Clara, CA). Analysis of difference expression was performed by GeneChip Operating Software Ver. 1.1. Genes were considered to be significantly upregulated according to the following criteria: (*i*) the mean fold increase was more than 4-fold; (*ii*) the expression of a gene was significant in NK cells after IFNα treatment; (*iii*) a significant increase was registered based on the algorithm of the software.

Statistics

Data are represented as mean ± SD. Comparisons between groups were analyzed by unpaired *t*-test with Welch's correction or ANOVA for experiments with more than two subgroups. *Post hoc* tests were done using the Bonferroni's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Single intravenous injection of IFNa1 gene enhances NK activity and completely rejects hepatic metastasis of CT-26 cells

Hydrodynamics-based gene delivery establishes efficient foreign gene expression predominantly in the liver, especially in hepatocytes.[21,22] Serial measurement of serum IFNα demonstrated that pCMV-IFNa1 injection led to substantial IFNα production on Day 1. The levels of serum IFNα then declined but were still detectable at Day 14 (Fig. 1*a*). To examine biological effects of the produced IFNα, we evaluated the NK activity of mononuclear cells from the liver and spleen. pCMV-IFNa1 injection, but not

control pCMV injection, increased Yac1 lytic activity of hepatic mononuclear cells and, to a lesser extent, splenic mononuclear cells at 4 days. The levels of Yac1 lytic activity declined but were still higher at 14 days after the injection (Fig. 1*b*). We also measured IFNγ production in serum, since IFNα is known to activate IFNγ production.[23,24] pCMV-IFNa1 injection, but not pCMV injection, increased serum IFNγ at 7 days (Fig. 1*a*). Since serum IFNγ increased relatively at a later time point, it may be an indirect effect rather than a direct effect of IFNα. These data indicated that hydrodynamic injection of pCMV-IFNa1 efficiently produced biologically active IFNα for a while in mice.

To evaluate the therapeutic effects of IFNα, Balb/cA mice were intrasplenically injected with CT-26 cells. Two days later, the mice were randomized into 3 groups and intravenously injected with either pCMV-IFNa1 or pCMV or were not treated. All pCMV-injected mice or untreated mice died within 3 weeks (Fig. 2*a*). They exhibited massive liver tumor in the liver. In contrast, all mice receiving pCMV-IFNa1 survived more than 2 months. To evaluate tumor metastasis, we sacrificed another cohort of mice at 2 weeks after tumor inoculation. There were no macroscopic or microscopic liver tumors in the pCMV-IFNa1-injected mice. In contrast, livers from pCMV-injected mice had massive tumors and were significantly heavier than those from pCMV-injected mice (Fig. 2*b*).

These results clearly indicated the striking therapeutic effects of IFNα on hepatic metastasis of CT-26 cells. To examine this therapeutic effect at a site other than the liver, Balb/cA mice were subcutaneously injected on the back with CT-26 cells and hydrodynamically injected 2 days later with pCMV-IFNa1 or pCMV. No difference in tumor growth was noted between pCMV-IFNa1-injected mice and pCMV-injected mice (Fig. 2*c*).

Mice protected from hepatic metastasis by IFNα gene therapy were resistant to subcutaneous challenge of CT-26 cells and exhibited a tumor-specific T cell response

We next investigated the possibility of IFNα-mediated rejection of hepatic metastasis being followed by induction of an adaptive immune response to the original tumor. To this end, we subcutaneously injected CT-26 cells into the mice that had been protected from CT-26 hepatic metastasis by IFNα therapy. The mice were rechallenged with CT-26 cells 1 month after the initial splenic injection. The controls were naïve Balb/cA mice as well as those receiving pCMV-IFNa1 but not CT-26 splenic inoculation. The incidence of tumor formation was lower in mice that had rejected hepatic metastasis by IFNα therapy than in the control mice. Even if they developed subcutaneous tumors, tumor size was significantly smaller than in the control mice (Fig. 3*a*).

To examine the tumor-specific response, splenocytes were isolated 3 weeks after tumor inoculation and restimulated *in vitro* with CT-26 cells. Splenocytes isolated from CT-26 bearing mice treated with IFNα showed significant levels of killing ability against CT-26 cells, but not against BNL A.7 cells (Fig. 3*b*). When mice were intrasplenically injected with UV-irradiated CT-26 cells, the splenocytes did not show significant killing activity regardless of the subsequent IFNα therapy (Fig. 3*c*). Separation of effector cells into T cells and non-T cells based on CD90 expression revealed that this killing ability was mediated by T cells, but not by non-T cells (data not shown). Thus, a tumor-specific cytotoxic T cell response was established in mice that had rejected hepatic metastasis of CT-26 cells by IFNα therapy.

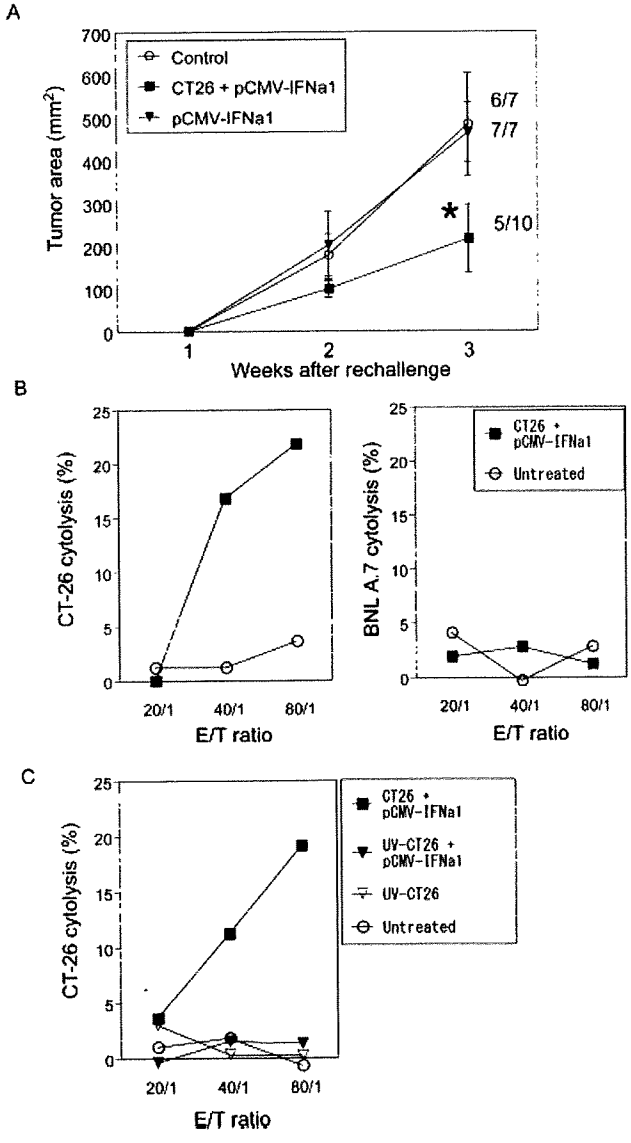

FIGURE 3 – Systemic immunity and tumor-specific T cell response. (*a*) Anti-tumor effects on rechallenged tumors. Balb/cA mice that had rejected hepatic metastasis of CT-26 cell by IFNα (closed squares), those treated with IFNα alone (closed triangles), and naïve mice (open circles) were challenged with subcutaneous injection of CT-26 cells 1 month after the previous treatment. Subcutaneous tumor growth was examined every week by measuring tumor area. Tumor size was expressed as the mean tumor size of only those mice bearing tumors. Each data point represents the mean tumor size and SD. The ratio of the number of mice bearing tumor/the number challenged for each treatment group at 3 weeks is shown in the figure. *, $p < 0.05$ *vs.* control or pCMV-IFNa1 injection only group. (*b*) *In vitro* tumor-specific killing ability. Balb/cA mice were intrasplenically injected with CT-26 cells and then treated with pCMV-IFNa1 2 days later. Splenocytes were isolated from CT-26 plus pCMV-IFNa1-injected mice at 3 weeks (closed squares) or naïve mice (open circles), restimulated with CT-26 cells for 5 days and then subjected to analysis for the lytic ability against CT-26 cells (left) or BNL A.7 cells (right). Shown are representative data for 3 independent experiments. (*c*) Requirement of CT-26 cells and IFNα on induction of tumor-specific killing ability. Balb/cA mice were intrasplenically injected with live CT-26 cells (squares) or UV-irradiated CT-26 cells (triangles) and then treated with (closed symbols) or without (open symbols) pCMV-IFNa1 2 days later. Splenocytes were isolated from mice at 3 weeks, restimulated with CT-26 cells for 5 days and then subjected to the analysis for the lytic ability against CT-26 cells. Mice injected with live CT-26 cells without following injection of pCMV-IFNa1 did not survive for 3 weeks naïve mice were included as controls (open circles). Shown are representative data for 3 independent experiments.

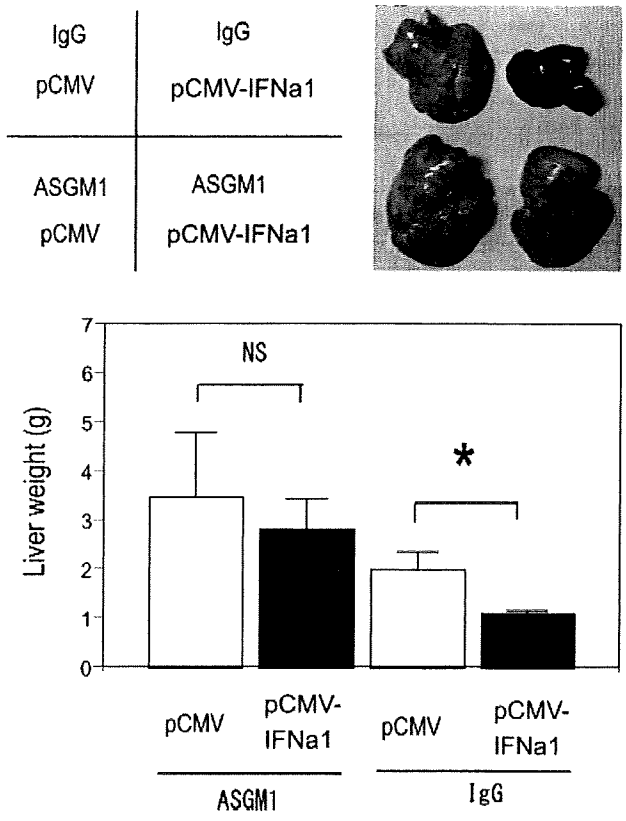

FIGURE 4 – Requirement of NK cells on IFNα-mediated anti-metastatic effects. Balb/cA mice were intrasplenically injected with CT-26 cells, intraperitoneally injected with either anti-ASGM1 or control IgG at 1 day, and hydrodynamically injected with either pCMV-IFNa1 (closed bars, n = 8/group) or pCMV (open bars, n = 7/group). Mice were sacrificed at 14 days to examine tumor growth in the liver. Top, a representative picture of the liver in each group. Bottom, comparison of liver weight among treatment groups. Experiments were performed at least 3 times and representative data are shown. *, $p < 0.05$. NS, not significant.

NK cells are required for IFNa-mediated initial rejection of hepatic metastasis

To examine whether the observed increase in NK activity of hepatic mononuclear cells is involved in the complete rejection of hepatic metastasis, we induced depletion of NK cells by injecting anti-asialo GM1 antibody. pCMV-IFNa1 injection completely abrogated hepatic tumor formation in control immunoglobulin-injected mice. In sharp contrast, pCMV-IFNa1 injection did not offer antimetastatic effects in anti-asialo GM1 antibody-injected mice, suggesting the critical contribution of NK cells to the antimetastatic effects of IFNα (Fig. 4). We examined the possibility that hepatic mononuclear cells can serve as direct effectors cells for CT-26 eradication. Although CT-26 cells were more resistant to hepatic mononuclear cells than Yac1 cells, pCMV-IFNa1 injection clearly enhanced the killing ability of hepatic mononuclear cells against CT-26 cells (data not shown). This result indicated that CT-26 is potentially susceptible to hepatic mononuclear cells upon IFNα therapy.

IFNα directly activates NK cells

IFNα is known to be able to activate a variety of immune cells. To examine whether NK cells can be directly activated by IFNα, we analyzed SCID mice that lack T cells, B cell and NKT cells due to spontaneous DNA-dependent protein kinase point mutation.[25] SCID or wild-type splenocytes were cultured with IFNα and examined for STAT1 phosphorylation, which peaked at 30 min and declined at 6 hr after IFNα stimulation in both mice (Fig. 5*a*). However, the signals of STAT1 phosphorylation were weaker in SCID splenocytes than in wild-type cells. Of interest is the finding that STAT1 expression was reduced in SCID cells compared to wild-type cells. Similar data were also obtained from experiments on Rag2 KO mice, another model of deficiency for T cells, B cells and NKT cells. To examine the reasons for SCID or Rag2 KO cells expressing low levels of STAT1, we separated wild-type splenocytes into T cells and non-T cells based on CD90 expression. The levels of STAT1 expression were weaker in non-T cells than in T cells (Fig. 5*b*). Taken together, the difference in the levels of STAT1 expression among lymphocyte subsets could explain the reduced phosphorylation signals after IFNα treatment in SCID or Rag2 KO cells.

To examine the gene profiles activated by IFNα in NK cells, we used Affymetrix DNA array analysis on SCID hepatic mononuclear cells. Six hours treatment of IFNα (1,000 U/ml) upregulated 243 of 45,101 genes in SCID cells by more than 4-fold. They included well known IFNα-regulated genes such as H2, 2′-5′ oligoadenylate synthetases, Mx1, IRF and suppressor of cytokine signaling (SOCS). Among the effector molecules for cytotoxicity, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and granzyme B were activated. Various cytokines such as IL-15 and IFNγ were also upregulated. These data revealed that NK cells upon IFNα stimulation produced well-characterized IFN-inducible genes and others that are relatively specific to killer cells or immune cells.

pCMV-IFNa1 injection completely suppressed tumor formation in the liver in SCID mice

We examined the *in vivo* effects of IFNα in SCID mice. In agreement with SCID cell activation *in vitro*, pCMV-IFNa1 injection enhanced the Yac1 lytic ability of hepatic mononuclear cells in SCID mice (Fig. 5*c*). To examine whether NK cells are sufficient for IFNα-mediated rejection of hepatic metastasis, we injected pCMV-IFNa1 or pCMV into mice that had been intrasplenically injected with CT-26 cells 2 days earlier. pCMV-IFNa1 completely suppressed tumor formation in the liver (Fig. 5*d*). As described in the *Material and methods* section, anti-asialo GM1 injection reduces the number of NKT cells. However, this SCID experiment clearly showed that NKT cells are not required for NK cell activation by IFNα and its antimetastatic effects.

pCMV-IFNa1 injection completely suppressed tumor formation in the liver in GKO mice

IFNγ has been established as an endogenous inhibitor of tumor development and progression.[26] Exogenous administration of IFNγ suppresses tumor formation in a variety of models.[15,27] To examine the possibility of IFNγ being involved in antimetastatic effects on IFNα, we injected pCMV-IFNa1 or pCMV plasmid into GKO mice exposed to 2 days of metastasis of CT-26 cells. IFNα treatment led to complete rejection of CT-26 cells in GKO mice (Fig. 6*a*). pCMV-IFNa1 injection, but not pCMV injection, augmented the Yac1 lytic ability of mononuclear cells (Fig. 6*b*).

Discussion

Here we report that a single injection of pCMV-IFNa1 could lead to complete rejection of preexisting hepatic metastasis of colon cancer cells. This partly agrees with a previous report by Kobayashi et al.,[15] who hydrodynamically injected IFNβ- or IFNγ-expressing plasmid into CT-26 bearing mice and reported the antimetastatic effects of IFNβ or IFNγ. In contrast to our study, all mice died within 45 days due to metastasized tumor growth even if plasmid injection was began one day after tumor inoculation and repeated every other day. The complete protection against hepatic metastasis observed in the present study allowed

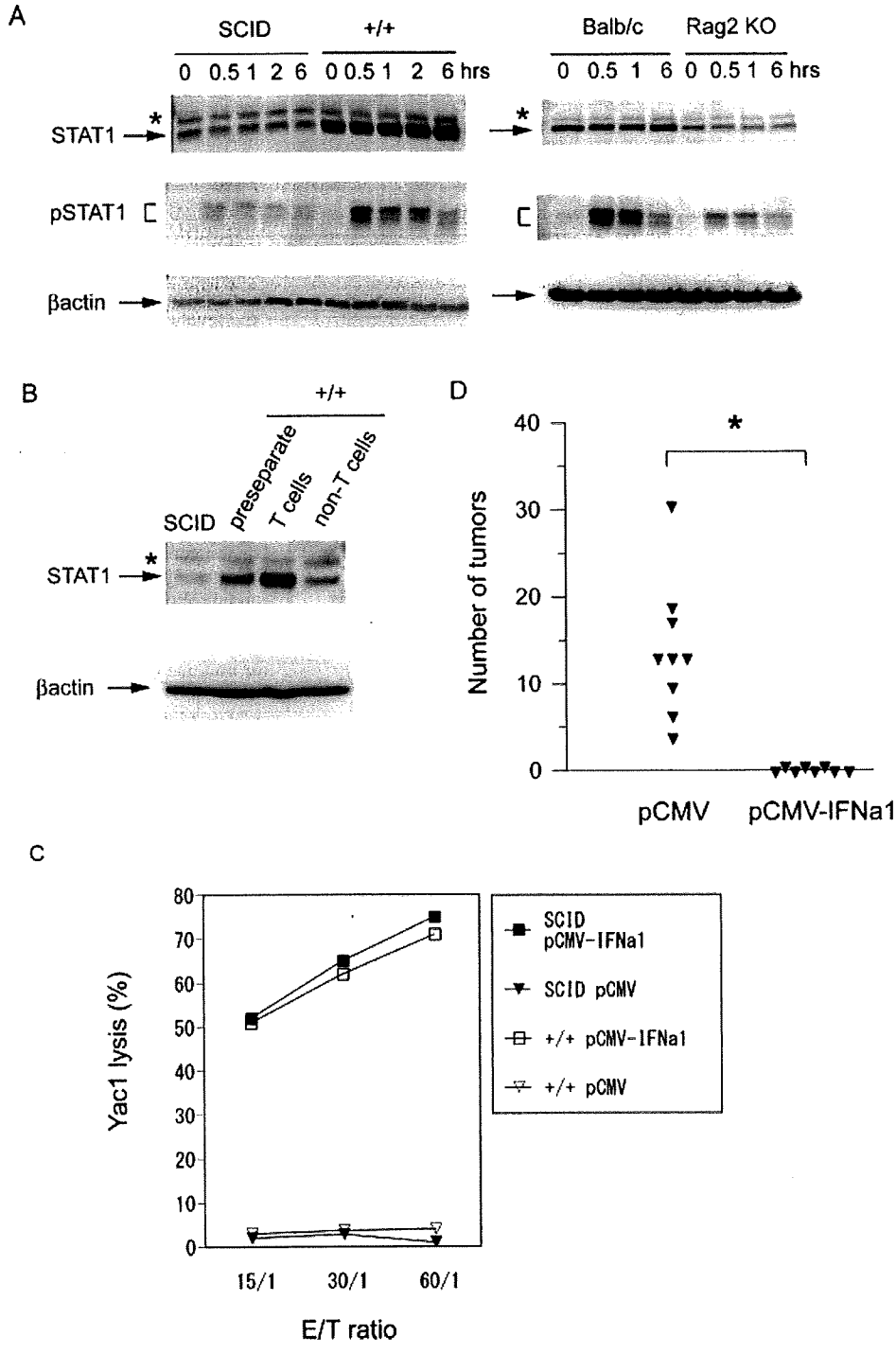

FIGURE 5 – IFNα-mediated NK cell activation and anti-metastatic effects in SCID mice. (*a*) STAT1 and phospho-STAT1 expression of splenocytes from SCID or Rag2 KO mice. Splenocytes were treated with 1,000 U/ml of IFNα and lysed at indicated time points (0 to 6 hr). Expression of STAT1 and phospho-STAT1 (pSTAT1) was analyzed by Western blot. +/+ and Balb/c indicate wild-type controls for SCID and Rag2 KO mice, respectively. *, non-specific band (see the *Material and methods* section). (*b*) STAT1 expression in T cells and non-T cells. Splenocytes from wild-type mice (+/+) were separated into T cells and non-T cells based on expression of CD90. Expression of STAT1 was analyzed by Western blot. SCID and preseparated wild-type splenocytes were included as controls. *, non-specific band (see the Material and methods section). (*c*) Yac1 lytic ability. SCID mice (closed symbols) or wild-type mice (open symbols) were hydrodynamically injected with either pCMV-IFNa1 (squares) or pCMV (triangles). Four days later, splenocytes isolated form the mice were examined for the lytic ability for Yac1 cells. Experiments were done at least 3 times and representative data are shown. (*d*) Anti-metastaic effects. SCID mice or wild-type mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 or pCMV 2 days later. After 14 days, mice were sacrificed to examine tumor development in the liver. The numbers of hepatic tumors were compared among the groups. Experiments were performed 3 times and representative data are shown. *, $p < 0.05$.

us to investigate the adaptive response after antimetastatic effects. Mice that had rejected CT-26 cells by IFNα showed a tumor-specific T cell response and suppressed tumor growth of rechallenged skin tumor. Therefore, pCMV-IFNa1 injection not only caused initial rejection of metastasized tumors but also induced durable and systemic adaptive immunity. Interestingly, splenic injection of UV-irradiated CT-26 cells, even if followed by pCMV-IFNa1 injection, did not elicit significant tumor-specific T cell responses. Therefore, the efficient induction of adaptive T cell responses requires IFNα-mediated rejection of live tumor cells and cannot be recapitulated by simple injection of dead tumor cells and IFNα.

NK cells are present in a high percentage in the liver.[28] In the present study, we focused on NK cells which were rapidly activated by IFNα to examine the cellular mechanisms of protection against hepatic metastasis. Critical requirement of NK cells was demonstrated by anti-asialo GM1 antibody-injected mice which did not show protection against CT-26 metastasis. In contrast, T cells, B cells or NKT cells were dispensable for IFNα-mediated antimetastatic effects since IFNα therapy did show antimetastatic

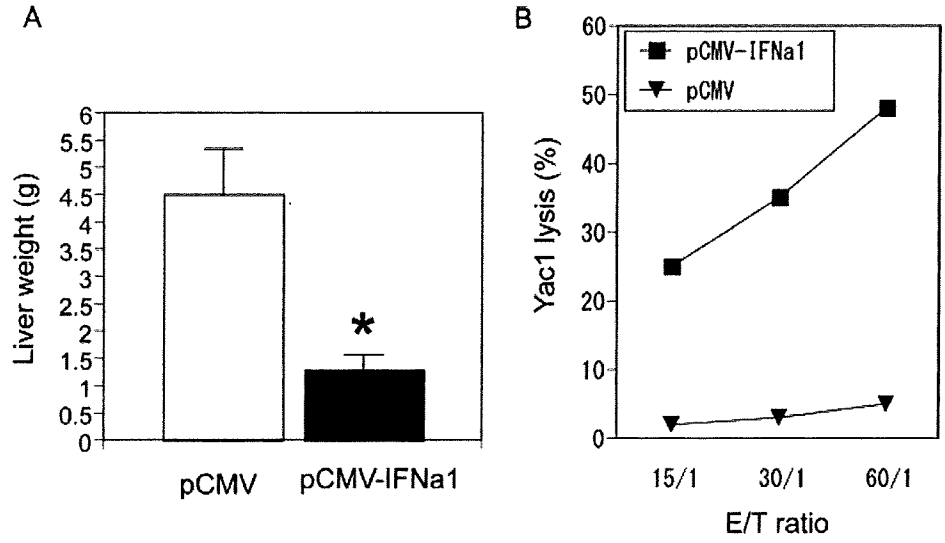

FIGURE 6 – NK cell activation and anti-metastatic effects in GKO mice. (*a*) Anti-metastatic effects. GKO mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) 2 days later. After 14 days, the mice were sacrificed to examine tumor development in the liver. The liver weight was compared between the groups ($n = 8$/*group*). Experiments were performed 3 times and representative data are shown. *, $p < 0.05$ *vs.* pCMV injection group. (*b*) Yac1 lytic ability. GKO mice were hydrodynamically injected with either pCMV-IFNa1 (closed squares) or pCMV (closed triangles). Four days later, splenocytes isolated from the mice were examined for the lytic ability for Yac1 cells. All experiments were performed at least 3 times and representative data are shown.

effects in SCID mice. Research using a variety of murine models has revealed the direct effects of tumor cells[11] and the CD8 T cell response[12–14] involved in the antitumor effects of IFNα. A recent study[29] using STAT1-deficient animals and STAT1-deficient tumor cells revealed that IFNα activation of host cells, but not tumor cells, is required for antitumor effects in a peritoneal model of melanoma. They also showed the involvement of NK cells in their model. Our data demonstrated that NK cells are critically required and sufficient for IFNα-mediated protection from liver metastasis. However, NK cells are not effective for controlling tumor growth at extrahepatic sites, because IFNα activated splenic (systemic) NK activity but did not elicit antitumor effects against subcutaneously injected CT-26 cells. Subcutaneous tumor growth appeared to be controlled by adaptive immunity rather than innate pathway.

The reason that IFNα-mediated activation of NK cells leads to such a strong antitumor effect in the liver but not under the skin is not known. In the present study, we applied hydrodynamic injection of the IFNα gene to obtain efficient and stable expression of IFNα. Since the hydrodynamic procedure leads to predominant expression of foreign genes in the liver, the concentration of IFNα may be greater in the liver than in circulation. This may be related to the observed strong antitumor effects in the liver. Another possibility is that NK cells are more numerically abundant and functionally potent in the liver than in other organs.[30] In any case, the hydrodynamic injection of the IFNα gene led to higher activation of the NK lytic ability of hepatic mononuclear cells than that of systemic mononuclear cells. This may be related to the stronger antitumor activity in the liver.

An earlier study on STAT1 knockout mice revealed that STAT1 is a critical signaling molecule for IFNα in macrophage and T cells.[9] STAT1-deficient mice showed impairment of NK activity.[31] STAT1-deficient splenocytes did not show increase in NK lytic activity upon IFNα stimulation.[29] Therefore, STAT1 should also play an important role in IFNα-mediated NK cell activation. However, the significance of STAT1 in NK cells on IFNα action had not been fully proven, because splenocytes consist of a variety of lymphocyte subsets. In the present study, we found that NK cells express lower levels of STAT1 than T cells, which is associated with lower levels of STAT1 expression in SCID splenocytes than those in wild-type splenocytes. Importantly, IFNα phosphorylated STAT1 in SCID splenocytes with similar kinetics to that in wild-type splenocytes even if the signal intensities in the former were lower than those in the latter. In agreement with this, IFNα was capable of activating a variety of genes in SCID mononuclear cells. Thus, IFNα does not require other lymphocyte subsets to activate NK cells and to induce NK cell expression of IFN-regulated genes.

IFNγ was shown to be produced in lymphocytes upon IFNα administration, which is dependent on STAT4 signaling.[32] In the present study, IFNγ was produced in serum after pCMV-IFNa1 injection. Furthermore, the IFNγ gene was activated in SCID NK cells upon IFNα stimulation. However, IFNγ is not necessary for NK cell activation in terms of killing ability as well as an IFNα-mediated antimetastatic effect. NK cells, upon IFNα stimulation, expressed well-established IFN-regulated genes[33] as well as killer cell-specific molecules granzyme B or TRAIL. Although our data showed that hepatic mononuclear cells from mice receiving IFNα can kill CT-26 cells *in vitro*, it remains unclear whether NK cells serve as direct effector cells for ablating CT-26 cells *in vivo*. Further study is needed to find whether killer cell-specific molecules are actually involved in the antimetastatic effects of IFNα.

IFNα has achieved a long record of clinical use in the treatment of hematological malignancy and solid tumors such as melanoma, renal cell carcinoma and Kaposi's sarcoma.[34,35] In therapy for colon carcinoma, special attention has been paid to the use of IFNα in the combination with 5-FU, since IFNα has been shown to modulate 5-FU metabolism and to enhance its cytotoxic activity.[36] Although several clinical trials have evaluated the 5-FU plus IFNα combination for adjuvant therapy of colon carcinomas with encouraging results,[37,38] recent randomized trials revealed that addition of IFNα to 5-FU + levamisole marginally increased the recurrence-free survival time compared to 5-FU + levamisole alone, but did not alter the over-all survival.[39] Therefore, use of IFNα as a modulator of 5-FU activity may have some limitations in future clinical use. In the present study, we demonstrated that

IFNα activates both innate and adaptive immunity and ablates microdisseminated colon carcinoma cells in the liver. There may be a variety of reasons which can explain the difference between the present study and the clinical use in the therapy of metastasizing colon cancer. We found CT-26 far less sensitive to NK cells than Yac1 cells but human colon carcinoma cells might be more resistant to NK cells activated by IFNα in a clinical setting. Systemic administration of recombinant IFNα may be less effective than enforced expression of IFNα gene in the liver. In any way, we used CT-26 cells just as a murine model of hepatic metastasis and observed similar therapeutic effect of the IFNα gene when using another cell line such as BL6 melanoma cells in a C57/BL6 background (our unpublished data). Our study raised the possibility that IFNα therapy may be a promising approach for developing future adjuvant therapy for metastatic liver tumors arising from various organs. Immunological aspect of IFNα is important when considering antimetastatic effect of this cytokine.

In conclusion, IFNα-mediated protection of CT-26 hepatic metastasis critically requires NK cells. NK cells, upon IFNα stimulation, do not require other immune cells such as T cells, B cells and NKT cells for their activation and protection against hepatic metastasis. NK cell production of IFNγ is not involved in the increase in NK activity and antitumor effect. Our study has shown NK cells to be important mediators in ablating microdisseminating tumors in the liver in IFNα therapy. Eradication of microdisseminated tumor cells by IFNα led to long-lasting adaptive immune responses which may be important for suppressing tumor growth in extrahepatic sites and overall antitumor effects.

Acknowledgements

We thank Dr. Daniel J.J. Carr (University of Oklahoma, Health Science Center) for providing the pCMV-IFNa1 plasmid, Dr. Yoichiro Iwakura (University of Tokyo, Institute of Medical Science) for providing GKO mice, Dr. R.S. Blumberg (Brigham and Women's Hospital) and Dr. M Kronenberg (La Jolla Institute for Allergy and Immunology) for providing the CD1d tetramer and Fujisaki Institute, Hayashibara Biochemical Laboratories for providing mouse recombinant IFNα. We also thank K. Kobayashi for excellent technical assistance.

References

1. Sasson AR, Sigurdson ER. Surgical treatment of liver metastases. Semin Oncol 2002;29:107–18.
2. Ruan DT, Warren RS. Liver-directed therapies in colorectal cancer. Semin Oncol 2005;32:85–94.
3. Seki S, Habu Y, Kawamura T, Takeda K, Dobashi H, Ohkawa T, Hiraide H. The liver as a crucial organ in the first line of defense: the roles of Kupffer cells, natural killer (NK) cells and NK1.1 Ag T+ cells in T helper 1 immune responses. Immun Rev 2000;174:35–46.
4. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer 2004;4:11–14.
5. Taniguchi T, Takaoka A. The interferon-α/β system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. Curr Opin Immunol 2002;14:111–6.
6. Marschall Z, Scholz A, Cramer T, Schafer G, Schirner M. Effects of interferon α on vascular endothelial growth factor gene transcription and tumor angiogenesis. J Natl Cancer Inst 2003;95:437–48.
7. Biron CA. Interferon α and β as immune regulators: a new look. Immunity 2001;14:661–4.
8. Durbin JE, Hackenmiller R, Simon MC, Levy DE. Targeted disruption of the mouse *Stat1* gene results in compromised innate immunity to viral disease. Cell 1996;84:443–50.
9. Meraz MA, White JM, Sheehan KCF, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greenlund AC, Campbell D, Carver-Moore K, DuBois RN et al. Targeted disruption of the *Stat1* gene in mice reveals unexpected physiologic specificity in the JAK-STA signaling pathway. Cell 1996;84:431–42.
10. Gil MP, Bohn E, O'Guin AK, Ramana CV, Levine B, Stark GR, Virgin HW, Schreiber RD. Biological consequences of STAT1-independent IFN signaling. Proc Natl Acad Sci USA 2001;98:6680–5.
11. Qin XQ, Tao N, Dergay A, Moy P, Fawell S, Davis A, Wilson JM, Barsoum J. Interferon-β gene therapy inhibits tumor formation and causes regression of established tumors in immune-deficient mice. Proc Natl Acad Sci USA 1998;95:14411–6.
12. Coleman M, Muller S, Quezada A, Mendiratta SK, Wang J, Thull NM, Bishop J, Matar M, Mester J, Pericle F. Nonviral interferon α gene therapy inhibits growth of established tumors by eliciting a systemic immune response. Hum Gene Ther 1998;9:2223–30.
13. Horton HM, Anderson D, Hernandez P, Barnhart KM, Norman JA, Parker SE. A gene therapy for cancer using intramuscular injection of plasmid DNA encoding interferon α. Proc Natl Acad Sci USA 1999; 96:1553–8.
14. Li S, Zhang X, Xia X, Zhou L, Breau R, Suen J, Hanna E. Intramuscular electroporation delivery of IFN-α gene therapy for inhibition of tumor growth located at a distant site. Gene Ther 2001;8:400–7.
15. Kobayashi N, Kuramoto T, Chen S, Watanabe Y, Takakura Y. Therapeutic effect of intravenous interferon gene delivery with naked plasmid DNA in murine metastasis models. Mol Ther 2002;6:737–44.
16. Yoshimoto T, Okamura H, Tagawa Y, Iwakura Y, Nakanishi K. Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-γ production from activated B cells. Proc Natl Acad Sci USA 1997;94:3948–53.
17 Miyagi T, Takehara T, Tatsumi T, Kanto T, Suzuki T, Jinushi M, Sugimoto Y, Sasaki Y, Hori M, Hayashi N. CD1d-mediated stimulation of natural killer T cells selectively activates hepatic natural killer cells to eliminate experimentally disseminated hepatoma cells in murine liver. Int J Cancer 2003;106:81–9.
18. Harle P, Noisakran S, Carr DJ. The application of a plasmid DNA encoding IFN-α 1 postinfection enhances cumulative survival of herpes simplex virus type 2 vaginally infected mice. J Immunol 2001;166: 1803–12.
19. Takehara T, Suzuki T, Ohkawa K, Hosui A, Jinushi M, Miyagi T, Tatsumi T, Kanazawa Y, Hayashi N. Viral covalently closed circular DNA in a non-transgenic mouse model for chronic hepatitis B virus replication. J Hepatol 2006;44:267–74.
20. Miyagi T, Takehara T, Tatsumi T, Suzuki T, Jinushi M, Kanazawa Y, Hiramatsu N, Kanto T, Tsuji S, Hori M, Hayashi N. Concanavarin A injection activates intrahepatic innate immune cells to provoke an anti-tumor effect in murine liver. Hepatology 2004;40:1190–6.
21. Liu F, Song YK, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther 1999;6: 1258–66.
22. Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injection of naked plasmid DNA. Hum Gene Ther 1999;10:1735–7
23. Sareneva T, Matikainen S, Kurimoto M, Julkunen I. Influenza A virus-induced IFN-α/β and IL-18 synergistically enhance *IFN-γ* gene expression in human T cells. J Immunol 1998;160: 6032–8.
24. Cousens LP, Peterson R, Hsu S, Dorner A, Altman JD, Ahmed R, Biron CA. Two roads diverged: interferon α/β- and interleukin 12-mediated pathways in promoting T cell interferon γ responses during viral infection. J Exp Med 1999;189:1315–28.
25. Danska JS, Holland DP, Mariathasan S, Williams KM, Guidos CJ. Biochemical and genetic defects in the DNA-dependent protein kinase in murine acid lymphocytes. Mol Cell Biol 1996;16:5507–17.
26. Ikeda H, Old LJ, Schreiber RD. The roles of IFNγ in protection against tumor development and cancer immunoediting. Cytokine Growth Factors Rev 2002;13:95–109.
27. Sun WH, Burkholder JK, Sun J, Culp J, Turner J, Lu XG, Pugh TD, Ershler WB, Yang NS. In vivo cytokine gene transfer by gene gun reduces tumor growth in mice. Proc Natl Acad Sci USA 1995;92: 2889–93.
28. Doherty DG, O'Farrelly C. Innate and adaptive lymphoid cells in the human liver. Immunol Rev 2000;174:5–20.
29. Lesinski GB, Anghelina M, Zimmerer J, Bakalakos T, Badgwell B, Parihar R, Hu Y, Becknell B, Abood G, Chaudhury AR, Magro C, Durbin J et al. The anti-tumor effects of IFN-α are abrogated in a STAT1-deficient mouse. J Clin Invest 2003;112:170–80.
30. Vermijlen D, Luo D, Froelich CJ, Medema JP, Kummer JA, Willems E, Braet F, Wisse E. Hepatic natural killer cells exclusively kill splenic/blood natural killer-resistant tumor cells by the perforin/granzyme pathway. J Leukoc Biol 2002;72:668–76.
31. Lee C-K, Rao DT, Gertner R, Gimeno R, Frey AB, Levy DE. Distinct requirements for IFNs and STAT1 in NK cell function. J Immunol 2000;165:3571–4.
32. Nguyen KB, Watford WT, Salomon R, Hofmann SR, Pien GC, Morinobu A, Gadina M, O'Shea JJ, Biron CA. Critical role for STAT4 activation by type 1 interferons in the interferon-γ response to viral infection. Science 2002;297:2063–6.
33. Der SD, Zhou A, Williams BRG, Silverman RH. Identification of genes differentially regulated by interferon α, β, or γ using oligonucleotide arrays. Proc Natl Acad Sci USA 1998;95:15623–8.

34. Belardelli F, Ferrantini M, Proietti E, Kirkwood JM. Interferon-α in tumor immunity and immunotherapy. Cytokine Growth Factor Rev 2002;13:119–34.
35. Kirkwood J. Cancer immunotherapy: the interferon-α experience. Semin Oncol 2002;29:18–26.
36. Grem JL, van Groeningen CJ, Ismail AA, Johnston PG, Alexander HR, Allegra CJ. The role of interferon-α as a modulator of fluorouracil and leucovorin. Eur J Cancer 1995;31A:1316–20.
37. Wadler S, Lembersky B, Atkins M, Kirkwood J, Petrelli N. Phase II trial of fluorouracil and recombinant interferon α-2a in patients with advanced colorectal carcinoma: an Eastern Cooperative Oncology Group study. J Clin Oncol 1991;9: 1806–10.
38. Dufour P, Hussenı F, Dreyfus B, Cure H, Martin C, Prevost G, Olivier JP, Dumas F, Duclos B, Olivares R, Leszler A, Bergerat JP et al. 5-fluorouracil versus 5-fluorouracil plus α-interferon as treatment of metastatic colorectal carcinoma. a randomized study. Ann Oncol 1996;7:575–9.
39. Link KH, Kornmann M, Staib L, Redenbacher M, Kron M, Beger HG. Increase of survival benefit in advanced resectable colon cancer by extent of adjuvant treatment: results of a randomized trial comparing modulation of 5-FU + levamisole with folinic acid or with interferon-α. Ann Surg 2005;242:178–87.