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TUMOR GROWTH ARREST: INVOLVEMENT OF THE MUTATION IN THE CATARITIC REGION OF JAK1

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ABSTRACT

Interferon-γ is cytokine that has antiviral, antiproliferative and immunomodulatory effects. Since the original discovery of the classical JAK-STAT1 signaling pathway, it has become clear that the coordination and cooperation of multiple distinct signaling cascades are required for the generation of responses to IFN-γ. The receptor-associated JAK1 and JAK2 activities are important for interferon-γ response. In many cases, the lack of antigen presentation can be attributed to the down regulation of genes needed for antigen processing, such as the TAP1 and LMP2, which are greatly induced by IFN-γ signalling. The G871E mutation in ATP-binding region of JAK1 has already been identified in uterine leiomyosarcoma (Ut-LMS) cells, and possibly attribute to loss of IFN-γ inducible TAP1 and LMP2 expression. However, the effect of mutant JAK1 on sarcomagenesis and malignancy has not been fully understood. Here, the differential responsiveness to IFN-γ of the cells, which have exogenous mutant JAK1-G871E activity, was investigated. We now show the defective tyrosine kinase activity of mutant JAK1-G871E, suggesting the loss of IFN-γ inducible TAP1 and LMP2 expression. Importantly, it is likely that G871E mutation of JAK1 results in defective IFN-γ induced cell growth arrest due to loss of LMP2 expression. Understanding the mechanisms by which sarcoma cells circumvent cytokine signaling, thereby JAK1 mutation, rather than evading antitumor-specific immunity, markedly affects the cell proliferation.

Keywords: LMP2, JAK1, IFN-γ, Leiomyosarcoma, Growth arrest.

INTRODUCTION

Interferons (IFNs) are widely expressed cytokines that have potent antiviral and cell growth-inhibitory effects. These cytokines are the first line of defense against viral infections and have important roles in immunosurveillance for malignant cells. The IFN family includes two main classes of related cytokines: type I IFNs and type II IFN (Taylor 2004, Platanoas 2005). There are many type I IFNs, all of which have considerable structural homology. These include IFN-α and IFN-β. All type I IFNs bind a common cell-surface receptor, which is known as the type I IFN receptor (Taylor 2004, Platanoas 2005). By contrast, there is only one type II IFN, IFN-γ. IFN-γ binds a distinct cell-surface receptor, which is known as the IFN-γ receptor. IFN-γ receptor has multichain structures, which are composed of at least two distinct subunits: IFNGR1 and IFNGR2. Each of these receptor subunits interacts with a member of Janus kinase (JAK) family. In the case of the IFN-γ receptor, the IFNGR1 subunit associates with JAK1, whereas IFNGR2 is constitutively associated with JAK2 (Taylor 2004, Platanoas 2005). The initial step in IFN-γ-mediated signalling is the activation of these receptor-associated JAKs, which occurs in response to a ligand-dependent rearrangement and dimerization of the receptor subunits, followed by autophosphorylation and activation of the associated JAKs. As well as the activation of classical JAK-STAT (signal transducer and activator of transcription)-signalling pathways, the activation of IFN-receptor-associated

JAKs seems to regulate, either directly or indirectly, several other downstream cascades (Taylor 2004, Platanoas 2005). Such diversity of signaling is consistent with the pleiotropic biological effects of IFNs on target cells and tissues.

The transcription of IFN-γ-dependent genes is regulated by IFN-γ-activated site (GAS) elements, and STAT1 is the most important IFN-γ-activated transcription factor for the regulation of these transcriptional responses. After the engagement of the IFN-γ-receptor by IFN-γ, JAK1 and JAK2 are activated and regulate the downstream phosphorylation of STAT1 on the tyrosine residue at position 701 (Tyr701) (Darnell 1997, Stark 1998, Aaronson 2002). Such phosphorylation results in the formation of STAT1 homodimers, which translocate to the nucleus and bind GAS elements to initiate transcription (Darnell 1997, Stark 1998, Aaronson 2002). IFN-γ responsible gene products presumably function as mediators of host immune responses to infectious diseases or tumor progression (Fruh 1997, Ehrt 2001, Dunn 2005). The ability of tumor cells to present tumor-associated antigens on the cell surface via MHC class I molecules is necessary for the generation of an effective antitumor-specific antigen CTL response. Unfortunately, many tumor cells have lost this ability, thereby evading CTL-mediated immune surveillance and elimination (Algarra 2000, Marincola 2000, Dunn 2004, 2005). The lack of MHC class I surface expression in many cases can be attributed to the down-regulation of genes needed for antigen presentation, such as TAP1 and TAP2, and the proteasomal components LMP2 and LMP7, which are strongly induced by IFN-γ-signalling (Seliger 2000, Dunn 2004). The reduced expression and function of TAP1 and TAP2 and/or LMP2 and LMP7 have been found in several distinct tumor types, human cervical carcinoma, prostate cancer, melanoma, renal cell carcinoma (RCC), uterine leiomyosarcoma (Ut-LMS), and is associated with malignant transformation and disease progression (Seliger 1996, Delgado 2000, Dovhey 2000, Hayashi 2011). These data suggest that IFN-γ-induced restoration of antigen-processing machinery, such as TAP1 and LMP2, may improve antitumor-specific antigen CTL recognition in some patients; thus, approaches to activate this pathway may be of benefit to patients with TAP and/or LMP deficiencies. LMP2 deficient mice have been reported to exhibit the spontaneous development of Ut-LMS at a high level of incidence (Hayashi 2002). Because the importance and involvement of the IFN-γ-induced trans-activators, STAT1 and IRF-1, in the transcriptional regulation of the TAP1/LMP2 promoter have been established, serious mutation in the kinase activation domain of JAK1 molecule has been identified in a human Ut-LMS cell line (Hayashi 2011). In the studies described here, we demonstrate that the mutation in kinase activation domain of JAK1 is attributed to the loss of IFN-γ-inducibility TAP1 and LMP2 expression. In addition, the mutant JAK1 has been implicated in IFN-γ-induced cell growth-inhibitory effect because of the defective enhancement of LMP2. Evidence is presented that the higher cell proliferation characterized in malignant tumors is due to the loss of IFN-γ sensitivity associated with the defective JAK1 activity, demonstrating that IFN-γ may be a key determinant in tumor progression.

METHODOLOGY

Cell lines and Media - Mouse embryonic fibroblasts wild type ($JAK1^{+/+}$ MEFs), JAK1-deficient MEFs ($JAK1^{-/-}$ MEFs, a kind gift from Dr. RD. Schreiber, Washington University, St.Louis, MO), p65 deficient MEFs ($p65^{-/-}$ MEFs, a kind gift from Dr. A. Beg, Columbia University, NY, NY) and p50 deficient MEFs ($p50^{-/-}$ MEFs obtained from $p50^{-/-}$ mice by standard method) were maintained in Dalbeco's MEM supplemented with 0.6 % L-glutamine (Invitrogen Corporation, CA) and 10% fetal bovine serum (Sigma-Aldrich Co., MO).

Transfections - Diagrams of the JAK1-wt expression vector and their respective JAK1-GE mutant constructs are shown in Figure 1A. Point mutation in the JAK1 coding sequence (G871E) was constructed using Site-Directed mutagenesis System according to the manufacturer's protocol (Promega, WI). The DNA sequence of mutagenic oligonucleotides for the JAK-GE expression vector was as follows; gaggattcgtgacttggaagagggtcactttggg (g2612a). JAK1-deficient MEFs were transfected with the pRK5 control (2μg), JAK1wt expression vector (2μg) (a kind gift from Dr. J. Ihle, St. Jude Children's Research Hospital, Memphis, TN) or JAK-GE expression vector (2μg) with FuGENE6 Transfection Reagent (Roche, IN) according to the manufacturer's recommendations. IFN-γ was added at 24 h after transfection and the cells were incubated for an additional 48 h prior to harvesting. Co-transfections with pCMVβ-Gal were performed to normalize the transfection efficiency

Immunoprecipitation and Immunoblotting - Cytosolic extracts and nuclear extracts were prepared from 5 x 10^6 cells, treated and untreated with 250 units/ml mouse IFN-γ for the individual times indicated in each figure, essentially as previously described (Brucet 2004). The cells were harvested for 10 min at 1200 rpm, washed in 5 ml of ice-cold PBS and centrifuged for 5 min at 12000 rpm at 4˚C. The cells were pelleted and washed once in 0.4 ml of buffer A [10 mM Hepes, pH7.8; 10 mM KCl; 2 mM MgCl2; 1 mM DTT; 0.1 mM EDTA; complete protease inhibitor cocktail (Kirkegaard & Perr Lab. MD)] and incubated for 2 h at 4˚C. Then, 25μl of a 10% Nonidert P-40 solution was added, and the cells were vigorously mixed for 1 h at 4˚C and then centrifuged for 5 min at 12000 rpm. After the centrifugation, the supernatants were collected as cytosolic extracts and stored at -80˚C. Pelleted nuclei were resuspended in 40 μl of buffer C [50 mM Hepes, pH7.8; 50 mM KCl; 300 mM NaCl; 0.1 mM EDTA; 1 mM DTT; 10% (v/v) glycerol], mixed for 2 h at 4˚C, and centrifuged for 5 min at 12000 rpm at 4˚C. The supernatant containing the nuclear proteins was harvested, and then stored at -80˚C.

In order to detect STAT1, the phospho-STAT1(Try701), JAK1, JAK2, TAP1 and LMP2 expressions, the whole cell lysates or cytosolic extracts were resolved on a 10% SDS-polyacrylamide gel (SDS-PAGE), and immunoblotting were performed using the standard method with anti-STAT1 antibody, anti-phospho-STAT1(Try701) antibody (Santa-Cruz Biotechnol., CA), anti-JAK1 or JAK2 antibodies (Chemicon Int'l, CA), anti-TAP1 antibody (Stressgen, Canada) or anti-LMP2 antibody (Affinity Res. Products Ltd., Exeter). For the detection of IRF1 or IRF2 expression, the nuclear extracts were resolved on a 10% SDS-PAGE, and immunoblotting was performed using the standard method with an anti-IRF1 antibody (Transduction Lab., KY) or an anti-IRF2 antibody (Santa-Cruz Biotechnol., CA). The blots were visualized with the appropriate antibodies indicated in each figure, and subjected to alkaline phosphatase detection analysis according to the manufacturer's protocol (Promega Co., Madison, WI).

Whole cell extracts from 5 x 10^6 treated and untreated cells, with 250 units/ml IFN-γ for the individual times indicated in each figure were lysed in buffer containing 50 mM Tris-HCl, 0.1 mM EDTA, 200 mM NaCl, 10% glycerol, 0.5% NP-40, 1 mM DTT and complete protease inhibitor cocktail (Kirkegaard & Perr Lab. MD). Total cell lysate was pre-cleared with normal rabbit serum (Santa-Cruz Biotechnologies, CA) and 20 ml of protein-G sepharose (Amersham Biosciences, Sweden), then immuno-precipitated with 2 μg of either anti-JAK1 or JAK2 antibodies. The samples were resolved on a 10% SDS-PAGE and transferred onto Immobilon-P. The blots were visualized with a phosphotyrosine antibody 4G10 and alkaline phosphatase detection analysis following the manufacture's protocol

(Promega Co., WI). For the detection of IFN-γR1 chain expression, whole cell lysates were resolved as above. The blots were incubated with anti-IFN-γR1 chain antibody (PBL Biomedical Laboratories, NJ).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis - The expressions of TAP1, LMP2 and β-Actin transcripts were examined using RT-PCR. All tested cells were both untreated and treated with 250 units/ml human or murine IFN-γ (Pepro Tech, Inc., NJ) for 48 h before the RNA harvest. Total RNA was prepared from 5 x 10^6 cells using TRIzol reagent (Invitrogen Co., CA) according to the manufacturer's protocol. The RNA was reverse-transcribed with the Superscript II enzyme (Invitrogen Co., CA), the single-strand cDNA was used to amplify TAP1, LMP2 and β-Actin transcripts using PCR analysis, following a program of 35 cycles of 94ºC for 30s, 60ºC for 30s and 72ºC for 1.5 min with an additional 5 min for the extention of the transcripts. The PCR-amplified products were run on 2.0% agarose gel as described (Cabrera 2003, Miyagi 2003).

Reporter assay - The TAP1 (593-1), LMP2 (1-593) wild type and these gene constructions containing mutant IRF-E were generated by PCR amplification of the promoter region from LMP2-HGH/TAP1-CAT and IRF-E mutant bidirectional reporter constructs (Delgado 2000, Dovhey 2000) and cloned into pGL3-Basic (Promega Corp., Madison, WI) in both orientations. Diagrams of the TAP1 and LMP2 wt (TAP1 593-1/pGL3 and LMP2 1-593/pLG3) and their respective IRF-E mutant promoter construct are shown in figure 2. A total of 2 μg of these plasmid DNAs (a kind gift from Dr. K.L. Wlight, University of South Florida, Tampa, FL) were transfected into $JAK1^{+/+}$ MEFs or $JAK1^{-/-}$ MEFs by FuGENE 6 Transfection Reagent (Roche, IN) according to the manufacturer's recommendations. All transfections contained 200 ng of pCMVβ-Gal (Tropix, MA) as an internal transfection efficiency control. IFN-γ (to a final concentration of 250 units/ml) was added 24 h after transfection and the cells were incubated for an additional 24 h. The cells were harvested and assayed using Luciferase transcripts as a reporter gene, as the described previously.

Electrophoretic mobility shift assay (EMSA) - Nuclear extracts were prepared from the treated and untreated cells with IFN-γ for the individual times indicated in each figure, as above. EMSA was performed as previously described (Hayashi 1993), using DNA probes containing the STAT1 binding sequence or IRF-E. The DNA sequences of these synthetic oligonucleotides for the STAT1 binding site or IRF-E were as follows. Stat1, AAGCATTCCTGTAAGGACT; IRF-E, GGAAGCGAAAATGAAATTGACT.

DNA transfection and Isolation of stable transfectants - JAK1-deficient MEFs were co-transfected with pCEM9 plasmid plus the pRK5 control (2μg), JAK1wt expression vector (2μg) or JAK-GE expression vector (2μg) by FuGENE 6 Transfection Reagent (Roche, IN) according to the manufacturer's recommendation in serum-free D'MEM at 2.0 μg DNA/1 x 10^6 cells. Transfected cells were grown in D'MEM with 10% FCS for 48 h before the addition of a selection antibiotic, G418 sulfate (GIBCO, MD). Selected $JAK1^{-/-}$ MEFs were maintained in D'MEM with 10% FCS with G418 sulfate at 0.4 mg/ml for the pCEM9 plasmid. The transfected G418-resistant colonies were isolated as previously described (Kitayama 1989). IFN-α or IFN-γ was added when the plates were 60% confluent, for 48 h prior to harvesting. After IFN-α or IFN-γ treatement, the cells were scraped from the plates. Viable cells were counted by trypan blue exclusion (Beg 1996).

RESULTS

Non-induction of TAP1 and LMP2 expression by mutant JAK1 - IFN-γ stimulation significantly induces mRNA expression of multiple genes involved in the regulation of the cell growth. After the binding of IFN-γ to the type II IFN receptor, JAK1 and JAK2 are activated and phosphorylate STAT1 on the tyrosine residue at position 701 (Tyr701). The tyrosine-phosphorylated STAT1 forms homodimers that translocate to the nucleus and bind IFN-γ-activated site (GAS) elements, which are present in the promoters of IFN-γ-regulated genes. The IFN-γ-activated JAKs also regulate, through as-yet-unknown intermediates, the activation of the catalytic subunit (p110) of phosphatidylinositol 3-kinase (PI3K) (Ngyen 2001). The activation of PI3K ultimately results in the downstream activation of protein kinase C-δ (PKC-δ), which in turn regulates the phosphorylation of STAT1 on the serine residue at position 727 (Ser727) (Ngyen 2001). Tumor cells have been reported to have defective JAK1 kinase activity, specifically, the change of glycine to glutamic acid at amino acid position 871 (G871E) in the ATP-binding region of the JAK1 tyrosine kinase domain has been identified in human Ut-LMS cells (Fig.1A). It must therefore be demonstrated whether this mutation of the JAK1 results in an impaired TAP1 and LMP2 expression. The immunoblotting studies showed that TAP1 and LMP2 expression were not significantly induced by IFN-γ treatment in $JAK1^{-/-}$ MEFs, although its parental cells, $JAK1^{+/+}$ MEFs, underwent strong inductions of TAP1 and LMP2 following IFN-γ treatment (Fig.1B). JAK1 kinase activity is required for IFN-γ-inducibility of TAP1 and LMP2 expression. Furthermore, to evaluate whether the G781E mutation results in the loss of the transcriptional response of IFN-γ, the control vector, JAK1wt or JAK1-GE expression vector, was transiently transfected into $JAK1^{-/-}$ MEFs. Although exogenous JAK1wt markedly restored the IFN-γ inducibility of TAP1 and LMP2 expression, exogenous JAK1-GE did not (Fig.1B). All the $JAK1^{-/-}$ MEFs tested had exactly the same β-Actin expressions when compared with $JAK1^{+/+}$ MEFs as the control (Fig.1B). This amount of IFN-γ was sufficient to maximally induce the shared bidirectional promoter for both TAP1 and LMP2 genes in both MEFs (Fig.1B). The increasing amount of IFN-γ to 500 units/ml did not significantly induce the TAP1 and LMP2 expression in all tested $JAK1^{-/-}$ MEFs. Thus, the mutation of G871E in JAK1 resulted in the loss of their ability to up-regulate TAP1 and LMP2 expression with IFN-γ treatment.

To verify the non-induction of TAP1 and LMP2 expressions following IFN-γ treatment, RT-PCR analysis was performed using transfected-$JAK1^{-/-}$ and $JAK1^{+/+}$ MEFs (Fig.1C). As shown in Figure 1C, IFN-γ-treatment could not significantly induce the expressions of

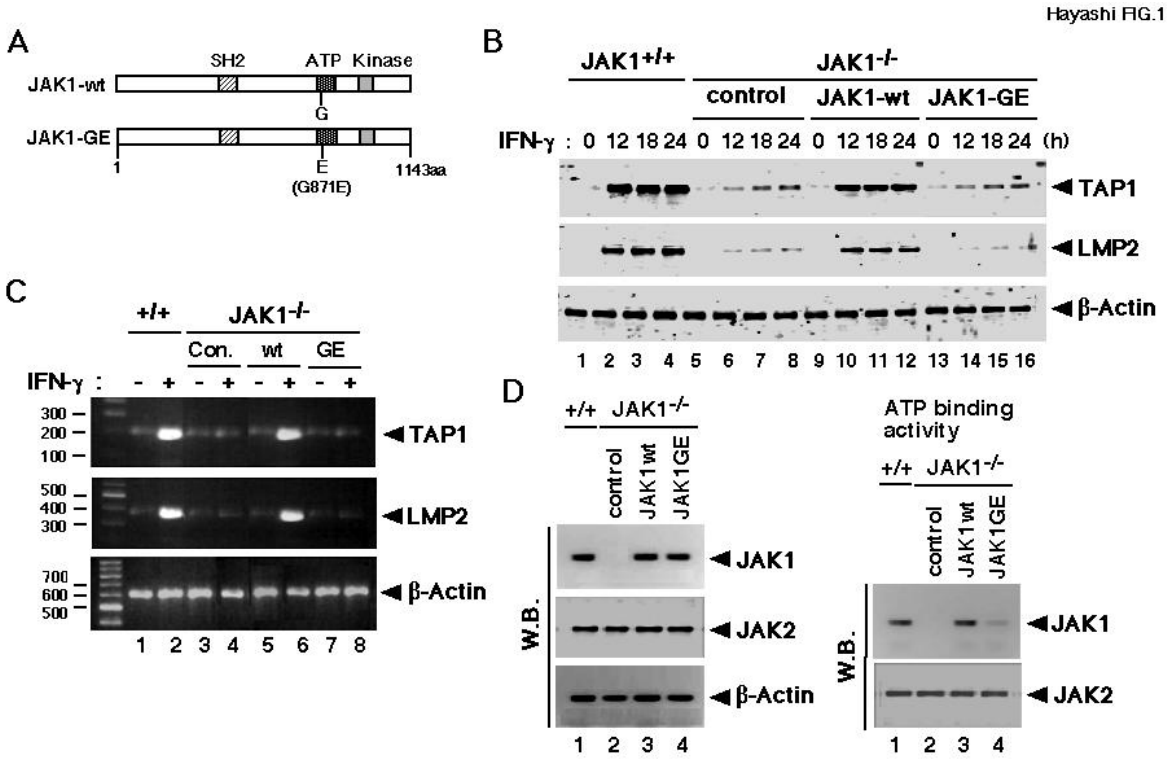

Figure 1: Exogenous JAK1-GE is not able to restore IFN-γ inducibility of TAP1 and LMP2 expression. (A) Schema of wild type JAK1 (JAK1wt) and mutant JAK1, which exhibits the G871E mutation in the ATP binding region. (B) JAK1$^{-/-}$ MEFs were transfected with control vector (2μg), JAK1wt expression vector (2μg) or JAK1-GE expression vector (2μg). IFN-γ was added at 24 h after transfection and the cells were incubated for an additional 24 h period prior to harvesting. Co-transfections with pCMVβ-Gal were performed to normalize the transfection efficiency. Western blotting revealed no recovery of the IFN-γ inducibility of TAP1 and LMP2 by exogenous JAK1-GE expression. (C) Examinations of mRNA expression for TAP1, LMP2 and β-actin in wild type MEFs, control vector-, JAK1-wt- or JAK1-GE-transfected JAK1$^{-/-}$ MEFs by RT-PCR. After culturing cells in the absence (-) or the presence of IFN-γ (250 units/ml, 48h), RT-PCR was performed with the appropriate primers indicated in the Materials and Methods section of this manuscript. The DNA products amplified by RT-PCR were loaded on agarose gel. The DNA size marker is indicated on the left side of the figure. (D) Loss of protein kinase ATP-binding activity of JAK1-GE. ATP binding activities of exogenous JAK1 derived from the wild type MEFs, control vector-, JAK1-wt- or JAK1-GE-transfected JAK1$^{-/-}$ MEFs. Western blotting demonstrated identical levels of expression of exogenous JAK1, endogenous JAK2 and β-actin, suggesting that the presence of the G871E mutation of JAK1 derived from JAK1-GE-transfected JAK1$^{-/-}$ MEFs results in a loss in protein kinase ATP-binding activity.

MRNA for both TAP1 and LMP2 in control vector or JAK1-GE transfected-JAK1$^{-/-}$ MEFs, although the expressions of mRNA for either TAP1 or LMP2 induced by IFN-γ treatment were clearly detected in JAK1$^{+/+}$ MEFs and JAK1-wt-transfected JAK1$^{-/-}$ MEFs. The expression of β-Actin mRNA as the internal control was detected in all of the cells tested at a similar basal expression level (Fig.1C). To confirm the functional effect of the mutation located in the ATP-binding region, the ATP-binding activities of JAK1 from all four MEFs were examined with agarose-conjugated ATP. The JAK1 of all MEFs demonstrated identical levels of expression of total JAK1, suggesting that the presence of the G871E mutation does not substantially alter the production or degradation (Fig.1D). The ATP-binding activities of JAK2 were detected in all tested MEFs (Fig.1D). In contrast, after the mixture with the cytosolic extract isolated from the JAK1$^{+/+}$ or JAK1-wt-transfected JAK1$^{-/-}$ MEFs, JAK1 was clearly detected in the protein/ATP complexes, whereas JAK1 was not detected in the protein/ATP complexes from the cytosolic extract isolated from the JAK1-GE- or control

vector-transfected JAK1$^{-/-}$ MEFs (Fig.1D). These findings suggested that the G781E mutation in the protein kinase ATP-binding region resulted in defective tyrosine kinase activity.

Loss of IFN-γ-induced shared bidirectional promoter activity for TAP1 and LMP2 genes by mutant JAK1 - The IRF-1 binds directly to a *cis*-acting element called the IRF enhancer (IRF-E) in the shared bidirectional promoter for both *TAP1* and *LMP2* genes (Wright 1995, White 1996, Brucet 2004). The IRF-E is located upstream of the nuclear factor κB (NF-κB) like binding site and GC1 box, as represented diagrammatically in Figure 2. The IRF-E was shown to be required for TAP1 and LMP2 up-regulation following IFN-γ treatment (Wright 1995, White 1996, Brucet 2004). To determine whether the mutation of G871E in JAK1 causes the defect in IFN-γ-induced activity to TAP1/LMP2 shared bidirectional promoter, JAK1$^{-/-}$ MEFs were transiently co-transfected with TAP1 and LMP2 wt, and IRF-E mut promoter-luciferase constructs plus JAK1-wt or JAK1-GE expression vector (Fig.2).

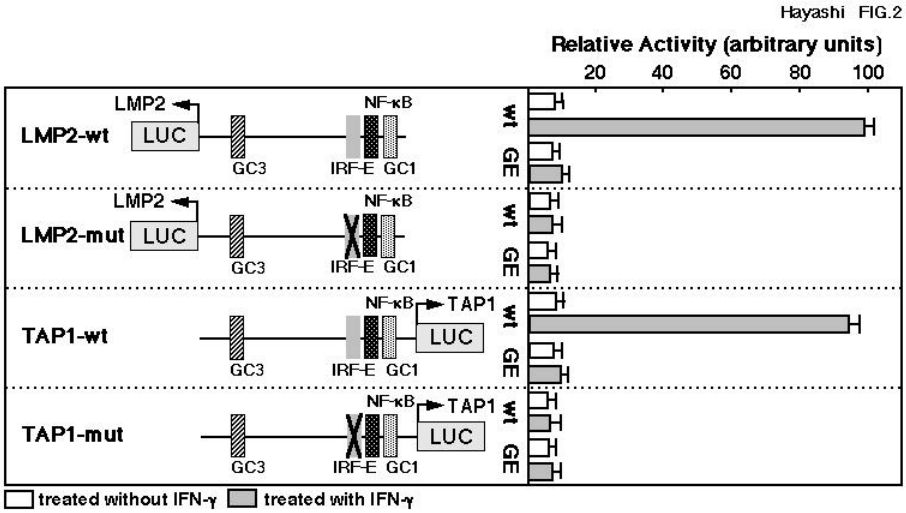

Figure 2: Differential activity of IFN-γ-induced TAP1 and LMP2 wt and IRF-E mt promoter in JAK1-wt- or JAK1-GE-transfected JAK1$^{-/-}$ MEFs. Diagrams of the TAP1 and LMP2 wt (TAP1 539-1/pGL3 and LMP2 1-593/pLG3) and their respective IRF-E mt promoter constructs are illustrated. JAK1-wt- or JAK1-GE-transfected JAK1$^{-/-}$ MEFs were transfected, IFN-γ was added 24 h later and the cells incubated for an additional 24 h prior to harvesting. Cotransfections with pSMV-βGAL were performed to normalize the transfection efficiency. Results are shown as relative TAP1 or LMP2 activity, normalized to constitutive TAP1 or LMP2 luciferase gene expression determined separately for JAK1-wt- or JAK1-GE-transfected JAK1$^{-/-}$ MEFs. Results shown are the average of three independent experiments, with the error bars representing the SE.

In the case of the JAK1-wt-transfected JAK1$^{-/-}$ MEFs, IFN-γ treatment markedly elicited around an eleven-fold induction of the LMP2 promoter activity and a ten-fold induction of the TAP1 promoter activity in an IRF-E dependent manner (Fig.2). However, no induction of the TAP1/LMP2 shared bidirectional promoter as a result of IFN-γ treatment was observed in JAK1-GE-transfected JAK1$^{-/-}$ MEFs (Fig.2). Mutation of the IRF-E abolished the ability of IFN-γ to induce TAP1/LMP2 gene expression (Fig.2). These research findings are consistent with the endogenous mRNA levels, showing the coordinate activation of *TAP1* and *LMP2* genes in the JAK1-wt-transfected JAK1$^{-/-}$ MEFs, but not in JAK1-wt-transfected JAK1$^{-/-}$ MEFs. The mutation of the IRF-E site slightly reduced the basal expression levels, indicating that this site also plays a role in constitutive expression. These research findings demonstrate that the mutation of G871E in JAK1 results in the defect in IFN-γ-inducibility of the TAP1/LMP2 shared promoter activity (Fig.2). IFN-γ-inducible transcriptional factor IRF-1 is activated by JAK1 kinase activity, and can then also strongly induce the transcriptional activation of both the *TAP1* and *LMP2* genes (Wright 1995, White 1996, Brucet 2004). Furthermore, research experiments with NF-κB subunit deficient cells, RelAp65$^{-/-}$ or p50$^{-/-}$

MEFs, demonstrated that NF-κB activation was not required for the IFN-γ-induced transcriptional activation of *TAP1*/*LMP2* genes, again, no effect was observed when the NF-κB binding site was mutated (data not shown).

Defective IFN-γ-induced JAK1 phosphorylation activity by mutant JAK1 - The IFN-γ-mediated tyrosine (Tyr701) phosphorylation activity of JAK1 and JAK2 significantly induces STAT1 activation. In order to examine the expressions and IFN-γ-mediated phosphorylation status of both JAK1 and JAK2, immunoblotting was performed with the appropriate antibodies. The results from immunoblotting showed that JAK2 was equally expressed in all tested MEFs; JAK1-GE-transfected MEFs had a significant exogenous JAK1 expression at a similar expression level in comparison with $JAK1^{+/+}$ and JAK1-wt-transfected MEFs (Fig.3A). To investigate the IFN-γ-induced JAK1 and JAK2 kinase activity, immuno-precipitation analyses with whole cell lysates prepared from all the MEFs stimulated with IFN-γ for the individual times were performed with either JAK1 and JAK2 specific antibodies and probed with an anti-phosphotyrosine antibody. The IFN-γ-induced tyrosine phosphorylation of JAK2 was dramatically detected in all tested MEFs (Fig.3B). On the other hand, the IFN-γ-induced tyrosine phosphorylation of JAK1 was not detectable in the JAK1-GE- or control vector-transfected $JAK1^{-/-}$ MEFs, whereas it was clearly found in the $JAK1^{+/+}$ or JAK1-wt-transfected $JAK1^{-/-}$ MEFs (Fig.3B). Thus, the defect in the IFN-γ-induced JAK1 activity resulted in an impaired IFN-γ-mediated signal pathway. To establish whether the lack of IFN-γR1 chain expression, which is the essential receptor for the IFN-γ signal pathway, may be responsible for the absence of the IFN-γ-mediated signal cascade. The anti-IFN-γR1 antibody detected an identical 90-kDa protein in each of the cell lines, consistent with their expected molecular weight (Fig.3A). IFN-γ stimulation has no effect on the IFN-γR1 expression levels compared with unstimulated MEFs. The results revealed that the IFN-γR1 was expressed in the JAK1-GE- or control vector-transfected $JAK1^{-/-}$ MEFs at similar levels in comparison with the $JAK1^{+/+}$ or JAK1-wt-transfected $JAK1^{-/-}$ MEFs (Fig.3A). Because the JAK1-GE- or control vector-transfected $JAK1^{-/-}$ MEFs had relatively comparable surface expressions of the IFN-γR1 chain to the $JAK1^{+/+}$ or JAK1-wt-transfected $JAK1^{-/-}$ MEFs, the loss of IFN-γ responsiveness in the JAK1-GE- or control vector-transfected $JAK1^{-/-}$ MEFs may not be attributable to the inadequate surface expression of this IFN-γ signal component.

Defective IFN-γ-induced Stat1 activation by mutant JAK1 - The loss of IFN-γ-mediated JAK-phosphorylation led us to examine where in the pathway the defect may reside. In an established IFN-γ-induced signal-cascade, STAT1 is markedly activated by IFN-γ treatment

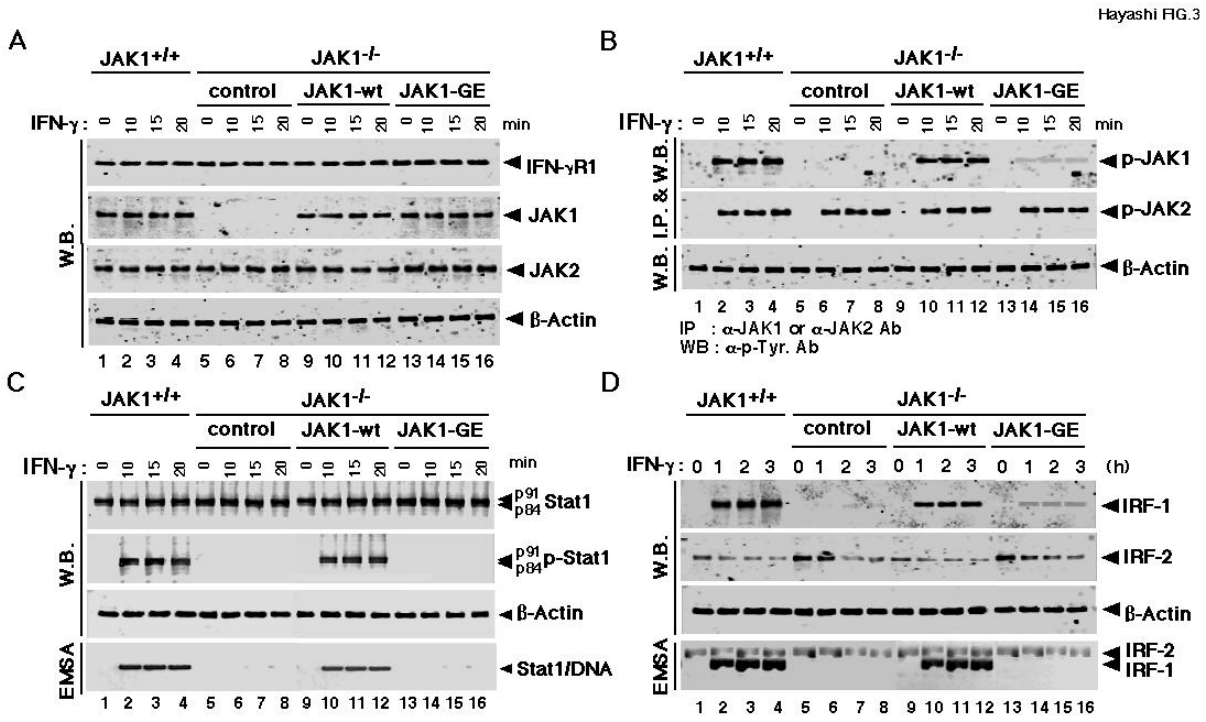

Figure 3: Defect in IFN-γ-induced STAT1 phosphorylation and IRF1 expression in JAK1-GE-transfected JAK1$^{-/-}$ MEFs. (A) Whole cell extracts were prepared from wild type MEFs, control vector-, JAK1-wt- or JAK1-GE-transfected JAK1$^{-/-}$ MEFs treated with IFN-γ (250 units/ml) for the individual times indicated in the figure, and 50 μg of whole cell extracts were resolved with 10% SDS-PAGE. The expression levels of IFN-γR1, JAK1, JAK2 and β-actin were examined by immunoblot analysis with appropriate antibodies. (B) whole cell extracts were prepared from wild type MEFs, control vector-, JAK1-wt- or JAK1-GE-transfected JAK1$^{-/-}$ MEFs treated with IFN-γ (250 units/ml) for the individual times indicated in the figure, and total hole cell extracts were immunoprecipitated with JAK1 and JAK2-specific antibodies, as already described. The immunoprecipitated-pellets were resolved with 10% SDS-PAGE, and then the immunoblot analyses were performed with an antiphospho-tyrosine-specific antibody. The expression levels of β-actin were examined using the cytosolic extracts prepared from all tested cells, the immunoblot analysis was performed using the standard procedure. (C) Whole cell extracts were prepared from wild type MEFs, control vector-, JAK1-wt- or JAK1-GE-transfected JAK1$^{-/-}$ MEFs treated with IFN-γ (250 units/ml) for the individual times indicated in the figure, and 50 μg of whole cell extracts were resolved by 10% SDS-PAGE. The expression levels of STAT1, phospho-STAT1(Tyr701) and β-actin were examined using immunoblot analysis with the appropriate antibodies indicated in the figure. The DNA binding activities of STAT1 were examined by EMSA with the appropriate DNA probes indicated in the Materials and Methods section of this manuscript. The expression levels of β-actin were examined using the cytosolic extracts prepared from all tested cells, and the immunoblot analysis was performed using the standard procedure. (D) The nuclear extracts were prepared from wild type MEFs, control vector-, JAK1-wt- or JAK1-GE-transfected JAK1$^{-/-}$ MEFs treated with IFN-γ (250 units/ml) for the individual times indicated in the figure, and 50 μg of the nuclear extracts were resolved by 10% SDS-PAGE. The expression levels of IRF1, IRF2 and β-Actin were examined by immunoblot analysis with appropriate antibodies. The DNA binding activities of IRF1 and IRF2 were examined by EMSA with the appropriate DNA probes indicated in the Materials and Methods section of this manuscript. Defect in IFN-γ-induced JAK1 kinase activity in JAK1-GE-transfected JAK1$^{-/-}$ MEFs.

through JAK kinase. In order to examine IFN-γ-induced STAT1 activation, immunoblotting was carried out. Whole cell lysates prepared from IFN-γ treated JAK1$^{-/-}$ and JAK1$^{+/+}$ MEFs were run on SDS-PAGE and blotted with appropriate antibodies. As expected, IFN-γ-treatment was able to dramatically induce the phosphorylation of STAT1 in JAK1$^{+/+}$ MEFs (Fig.3C). In contrast, the levels of IFN-γ inducible phospho-STAT1 in JAK1$^{-/-}$ MEFs remained unchanged compared with the uninduced phospho-STAT1 levels (Fig.3C). A similar level of phospho-STAT1 was detected in untreated JAK1$^{-/-}$ and JAK1$^{+/+}$ MEFs, a similar level of STAT1 was also observed in all tested MEFs (Fig.3C). Although IFN-γ-

treatment dramatically induced the phospho-STAT1 both in JAK1$^{+/+}$ MEFs and JAK1-wt-transfected JAK1$^{-/-}$ MEFs, the research result showed the complete absence of phospho-STAT1 in JAK1-GE- or control vector-transfected JAK1$^{-/-}$ MEFs (Fig.3C). To verify the IFN-γ-induced STAT1 DNA binding activity in all tested cells, EMSA was performed with nuclear extracts prepared from four types of MEFs treated with 250 units/ml of IFN-γ for the individual times. EMSA showed that the DNA-binding activity of phospho-STAT1 was dramatically detected in JAK1$^{+/+}$ MEFs and JAK1-wt-transfected JAK1$^{-/-}$ MEFs, whereas the JAK1-GE- or control vector-transfected JAK1$^{-/-}$ MEFs had undetectable STAT1/DNA complexes (Fig.3C). To specifically identify the protein complex with the DNA, the mutant DNA probes were used in EMSA. Incubation of the nuclear extract with the mutant DNA probes resulted in no significant DNA/protein complexes. Thus, these findings revealed that exogenous JAK1-GE lacked functional IFN-γ-induced STAT1 activation.

Defective IFN-γ-induced IFN-regulatory factor 1 activity by mutant JAK1 - IFN-γ-induced JAK kinase activity can directly phosphorylate STAT1, and the phospho-STAT1 plays a key role as a transcription factor for *IRF-1* gene expression. As shown in Figure 2, the mutation of G871E in JAK1 has no effect on IFN-γ-inducibility of the shared bidirectional promoter activation for the *TAP1/LMP2* gene; thus, it is likely to be due to a lack of IFN-γ-mediated IRF-1 activation. Immunoblotting with the appropriate antibodies was performed to assess the IFN-γ-induced IRF-1 activation in all trasfected JAK1$^{-/-}$ MEFs (Fig.3D). JAK1$^{+/+}$ MEFs show a low basal expression of IRF-1, yet a dramatic IFN-γ-inducibility of IRF-1 expression (Fig.3D). In comparison, JAK1-GE-transfected JAK1$^{-/-}$ MEFs demonstrated a similarly low basal expression of IRF-1, as well as JAK1$^{+/+}$ MEFs and JAK1-wt-transfected JAK1$^{-/-}$, but complete loss of IFN-γ-mediated induction of IRF-1 expression (Fig.3D). Nevertheless, IRF-2 is expressed in all tested MEFs (Fig.3D). To verify the defect in the IFN-γ-induced IRF-1 activation in JAK1-GE- or control vector-transfected JAK1$^{-/-}$ MEFs, EMSA was performed with nuclear extracts prepared from all tested MEFs stimulated or unstimulated with IFN-γ for the individual times indicated in Figure 3D. The migrating specific complexes (indicated as IRF-2) were observed with all the extracts tested (Fig.3D). Nuclear extracts from IFN-γ induced JAK1$^{+/+}$ MEFs and JAK1-wt-transfected JAK1$^{-/-}$ MEFs produced an additional prominent complex (indicated as IRF-1), but nuclear extracts prepared from JAK1-GE- or control vector-transfected JAK1$^{-/-}$ MEFs did not (Fig.3D). Antibody-blocking experiments confirmed the IRF-1 and IRF-2-binding activities in untreated and IFN-γ-treated extracts.

Defective IFN-γ-induced cell growth arrest due to mutation of JAK1 - Recent research findings have demonstrated that the JAK1-dependent signal pathway may partially limit the growth of wild-type cells. Thus, potential cytokine signal pathways are likely to be associated with the inhibition of cell growth. Furthermore, the research findings shown in this manuscript clearly demonstrate that the G781E mutation in the JAK1 molecule results in the impaired IFN-γ-inducible signalling pathway in JAK1$^{-/-}$ MEFs. Although partial inhibition of cell growth by the IFN-γ-inducible JAK1-dependent signal pathway was detected in JAK1-wt-transfected JAK1$^{-/-}$ MEFs, the JAK1-GE- or control vector-transfected JAK1$^{-/-}$ MEFs have no response to IFN-γ stimulation (Fig.4).

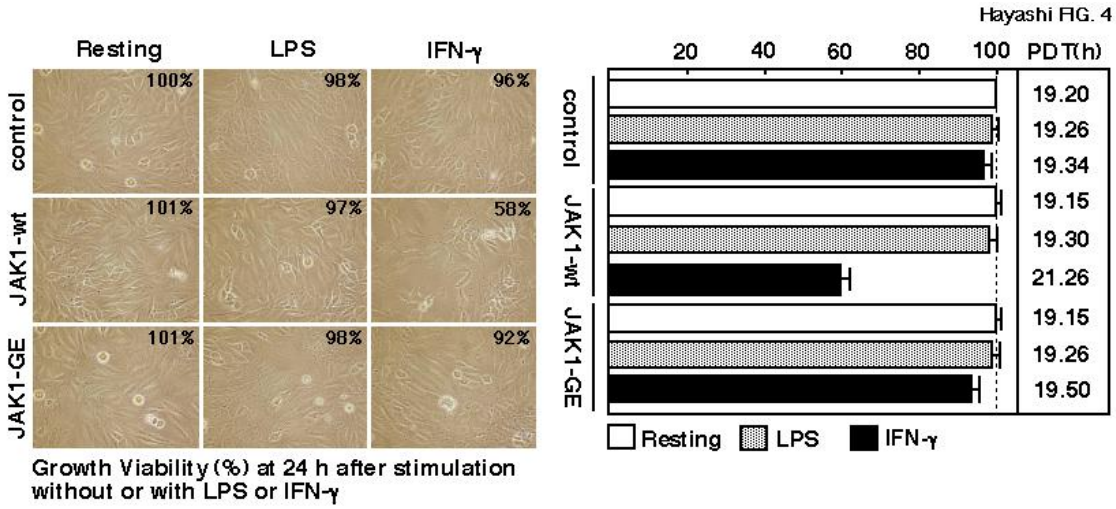

Figure 4: No effect of the exogenous mutant JAK1 in JAK1-GE-transfected JAK1$^{-/-}$ MEFs on the reaction to IFN-γ treatment with growth arrest. Growth viability (%) at 24 h after stimulation without or with LPS or IFN-γ. Population doubling times (PDT) of control vector-, JAK1-wt- or JAK1-GE-transfected JAK1$^{-/-}$ MEFs stimulated without or with LPS or IFN-γ. Each experiment was done in triplicate.

DISCUSSION

The proliferation, growth and apoptosis of normal and malignant cells are regulated by many different cytokines and growth factors. Such cytokines and growth factors exert their activities via interactions with specific cell-surface receptors. The binding of ligands to cytokine receptors triggers biological changes, such as phosphorylation and the activation of various signaling elements, ultimately resulting in the transcriptional regulation of specific genes that mediate cellular responses. Some cytokine receptors exhibit intrinsic tyrosine kinase activity, which allows them to directly initiate tyrosine phosphorylation cascades. However, the majority of cytokine-receptors lack intrinsic tyrosine kinase domain and instead, utilize cellular tyrosine kinases to regulate the phosphorylation of downstream proteins. The physiological relevance of the IFN-γ-dependent signal cascade through the JAK-STAT1 pathway, including PI3K, was established by generating and characterizing mice with a targeted disruption of the genes encoding STAT1 or JAK1 (Ihle 2001, O'Shea 2002, Ramana 2002, Sexl 2003). Significant advances have occurred over the last few years in the field of IFN-γ-induced JAK-STAT signaling cascade. The original identification of components of the JAK-STAT signalling cascade led to the development of important translational studies that have provided valuable information on the mechanisms by which different combinations of JAK kinase and their substrates participate in the regulation of malignant cell growth, survival and death (Verma 2003). Depending on the specific JAK kinase involved and the downstream effecter activated, different biological outcomes may occur. The accumulating evidence for the constitutive activation of various STATs in different cancers strongly suggests that JAK kinase, whose activities are generally required for STAT-activation, play critical roles in the pathogenesis of various human neoplastic diseases (Verma 2003). Recent research findings have demonstrated that JAK-STAT signalling cascade, under certain circumstances, negatively regulates neoplastic cell proliferation, such as in case of their activation by IFN-γ signaling (Verma 2003). In addition, loss of IFN-γ-inducible factor, LMP2 has been reported to lead to clearly enhance sarcomagenesis and malignancy (Hayashi 2002). The LMP2 deficient mouse exhibits the development of Ut-LMS (Hayashi 2002). Furthermore in our experiment, the loss of their ability to induce TAP1 and LMP2 expression has been demonstrated in human Ut-LMS SKN

cell line and other primary Ut-LMS cells treated with IFN-γ. This defect is localized to JAK1 activation, which acts upstream in the IFN-γ signal pathway since IFN-γ-treatment could not strongly induce JAK1 kinase activity (Hayashi 2011).

The sequencing analysis has clearly shown that the G-to-A base-pair change was predicted to change glycine to glutamic acid at amino acid position 871 (G871E) in the ATP-binding region of the JAK1 tyrosine kinase domain (Hayashi 2011). The JAK1 derived from SKN cells demonstrated identical expression levels of total JAK1, suggesting that the presence of the G871E mutation did not substantially alter the JAK1 production or degradation. The mutant JAK1 derived from SKN cells did not completely detect ATP-binding activity, suggesting that it is indispensable for the activation of JAK1 in STAT1 activation. It is likely that IFN-γ-inducible LMP2 and TAP1 expressions are impaired due to the G781E mutation in the ATP-binding region of JAK1. To evaluate whether the G781E mutation results in the loss of transcriptional response of IFN-γ, the JAK1-wt or JAK1-GE expression vector was transiently transfected into JAK1 deficient cells. Although exogenous JAK1-wt markedly restored the IFN-γ inducibility of TAP1 and LMP2 expression and phospho-STAT1, exogenous JAK1-GE did not (Fig.1). Thus, G781E mutation in the ATP-binding region of JAK1 results in the defect in IFN-γ□ inducible transcriptional activity.

Although IFN-γ treatment significantly induces cell growth arrest, the growth of JAK1-deficient cell lines is unaffected by IFN-γ treatment. Similarly, the cell cycle distribution pattern of freshly explanted tumor cells derived from JAK1-deficient tumors shows no response to IFN-γ treatment (Sexl 2003). In addition, the cells with defective JAK1 activation are also resistant to IFN-γ growth arrest (Sakamoto 1998, Yokota 2004, Ingley 2006). In our study, the growth of the control vector- or JAK1-GE-transfected $JAK1^{-/-}$ MEFs, which had the loss of JAK1 activity, was unaffected by IFN-γ treatment (Fig.4). In contrast, the growth of JAK1-wt-transfected $JAK1^{-/-}$ MEFs, which had strong exogenous JAK1 activity, was reduced by IFN-γ treatment (Fig.4). Interestingly, when LMP2-transfected $JAK1^{-/-}$ MEFs, which have marked LMP2 expression, were analyzed, the exogenous LMP2 expression resulted in moderate cell growth inhibition. Conversely, the growth of LMP2-transfected $JAK1^{-/-}$ MEFs was unaffected by IFN-γ treatment. Taken together, IFN-γ response to cell growth inhibition may be attributable to the IFN-γ inducibility of LMP2.

CONCLUSIONS

The down-regulation of MHC expression, including the *TAP1* and *LMP2* genes, is one of the biological mechanisms tumor cells use to evade host immune-surveillance (Singal 1996, Delgado 2000, Cabrera 2003). Recently, the incidence of IFN-γ unresponsiveness in human tumors was examined in several cancers, and it was revealed that approximately 33% of each group exhibited a reduction in IFN-γ sensitivity (Kaplan 1988). The G781E mutation in the ATP-binding region of JAK1, identified in human Ut-LMS, results in the defect in IFN-γ□ inducible transcriptional activity (Hayashi, 2011). Nevertheless, LMP2 expression, rather than providing an escape from immune surveillance, seems to play an important role in the negative regulation of cell growth. Defective LMP2 expression is likely to be one of the risk factors for tumor initiation, as it is in the LMP2 deficient mouse. Thus, gene therapy with LMP2 expression vectors may be a new treatment for tumors that exhibit a defect in LMP2 expression. Because there is no effective therapy for unrespectable tumors, including Ut-LMS, our results may bring us to specific molecular therapies to treat this disease.

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