NOTE TO USERS

Identification and characterization of the antitumor suppressor activity of interferon regulatory factor 3 (IRF-3) in B16 melanoma tumor model

by

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ABSTRACT

Delivery of transcription factor to cancer cells to reprogram gene expression may represent a novel strategy to augment the production of immune stimulatory cytokines and trigger a more potent antitumor response. In this study, we used a syngeneic mouse tumor model system involving the poorly immunogenic murine B16 tumor to evaluate whether delivery of the interferon regulatory factor 3 (IRF-3) can be used as an immunomodulator. The low immunogenicity of B16 melanoma cells may be due to their deficient cytokine expression, as well as their inefficient MHC-restricted epitope presentation. Gene-modified B16 melanoma cells were selected for their ability to express and to activate the IRF-3 protein. When injected into C57BL/6 mice, tumor growth was inhibited and tumors that developed from these mice had significant infiltration of inflammatory cells. Our observations demonstrated that gene transfer of IRF-3 into B16 melanoma could mediate important antitumor response by restoring both the deficient cytokine profile and the MHC class I protein expression.

RÉSUMÉ

Reprogrammer le code génétique des cellules cancéreuses par des facteurs de transcription peut devenir une nouvelle approche thérapeutique pour le traitement de plusieurs cancers. Ainsi, les cellules modifiées peuvent augmenter la production de leurs cytokines ou de molécules immunostimulatrices afin de monter une réponse immunitaire plus efficace. Dans cette étude, nous avons étudié les cellules tumorales B16. Cette lignée cellulaire est considérée faiblement immunogénique et nous a donc permis d'évaluer le role immunomodulateur du facteur de transcription IRF-3. Les B16 ont été transduites par un vecteur viral afin d'exprimer la protéine IRF-3 et ont par la suite été inoculées dans des souris C57BL/6. Un suivi sur la croissance des tumeurs démontre que les souris inoculées avec les cellules B16-IRF-3 développent des tumeurs 4 à 5 fois plus petites. En conséquence, nous avons démontré qu'IRF-3 est impliqué dans l'induction de cytokine pouvant être responsable dans le recrutement de cellules inflammatoires observées dans les tumeurs. De plus, IRF-3 semble être impliqué dans la régulation du complexe majeur d'histocompatibilité de classe I augmentant ainsi l'immunogénicité de ces cellules.

LIST OF ABBREVIATIONS

-/-	knockout
aa	amino acids
AD	activation domain
ATF	activating transcription factor
ATCC	American Type Culture Collection
CBP	CREB binding protein
CREB	c-AMP-responsive element binding factor
CTL	cytotoxic T lymphocyte
DBD	DNA binding domain
DMSO	dimethyl sulfoxide
DRAF	dsRNA-activated factor 1
dsRNA	double stranded RNA
ELISA	Enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
FasL	Fas ligand
FACS	Fluorescence-activated cell-sorting
GAF	IFNgamma activated factor
GAS	IFNgamma activated site
GFP	green fluorescent protein
HHV-8/ KSHSV	Human herpes virus-8/ Kaposi's Sarcoma
	Herpes simplex virus
HIV-1	Human immunodeficiency virus-1
HLA	human leucocyte antigen
HMG	high mobility group
IAD	IRF association domain
IB	immunoblotting
ICAM	intercellular adhesion molecule
ICS	interferon consensus site
ICSBP	interferon consensus binding protein/ IRF-8
IFN	interferon
IFNAR	interferon alpha/beta receptor (type I receptor)
IFNGR	interferon gamma receptor (type II receptor)
IκB	inhibitory factor kappa B
IKK	IκB kinase
IL	interleukin
IP	immunoprecipitation
IP-10	interferon-γ-inducible protein-10
IRF	interferon regulatory factor
IRF-E	interferon regulatory factor-element
ISG-15	interferon stimulated gene-15
ISGF	interferon stimulated gene factor
ISRE	interferon stimulated response element

JAK-STAT	Janus kinase-signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
LFA-1	lymphocyte function-associated 1
LMP	low molecular weight polypeptide proteasome subunit
LPS	lipopolysaccharide
LTR	long terminal repeat
MAL	MyD88 adapter-like
MAPK	mitogen activated protein kinase
MAPKK3	mitogen activated protein kinase kinase 3
MeV	Measle virus
MHC	major histocompatibility complex
MIP-1β	macrophage inflammatory protein 1-β
NDV	Newcastle's disease virus
NES	nuclear export signal
NF-κB	Nuclear factor-kappa B
NK	natural killer cells
NLS	nuclear localization sequence
NRE	negative regulatory domain
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
PKR	protein kinase R (dsRNA activated kinase)
PAMPs	pathogen-associated molecular patterns
Puro	puromycin
RANTES	regulated on activation normal T cell expressed
SDS	sodium dodecyl sulfate detergent
Ser	serine
Stat	signal transducer and activator of transcription
TAP	transporter for antigen presentation
TCR	T cell receptor
TGFβ	transforming growth factor β
Thr	threonine
TLRs	toll-like receptors
TNF	tumor necrosis factor
VAK	virus-activated kinase
VSV	vesicular stomatitis virus
WCE	whole cell extract

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CHAPTER I.

INTRODUCTION

I. 1. Gene Delivery Strategies

Gene therapy can be defined as the transfer of genetic material with therapeutic intent. Gene therapy consists essentially of gene cloning, target cell selection and gene transfer (128). Suitable genes can be inserted into various target cells, such as immune effector cells, tumor cells, and hematopoietic stem cells. The potential of gene therapy is expanding, as more information on cloned genes becomes available from various programs such as the Human Genome Project. Over the last decade, the impressive progress in the development of gene-transfer strategies has opened new perspectives for gene therapy of some human diseases, including cancer (Table.1) (151, 196).

Cytokine gene transfer into tumor cells has been regarded as a potentially useful approach for the treatment of some human malignancies (73). Most of the strategies of cytokine gene transfer have been based on the insertion of cytokine genes into tumor cells in order to promote their immunogenicity (129, 139, 140). In view of cancer vaccines, the major rationale for this strategy is: (i) to increase tumor cell immunogenicity; (ii) to enhance the anti-tumor immune reaction by the production of cytokines at the tumor site (151, 153). The genetic manipulation of tumor cells to express immunostimulatory molecules provides a current approach for the analysis of immune reactions against tumor cells *in vivo* (198). A key factor in the success of gene therapy is the development of gene delivery systems that are capable of efficient gene transfer in a broad variety of tissues, without causing any pathogenic effect.

Approach	Transgene	Target cell	Goal
Cancer vaccine	Immunostimulatory molecules or defined tumor antigens	Melanoma, renal cancer, other tumors	Stimulate antitumor immune response
Designer T cells	Chimeric T-cell receptor/ anti-CEA antibody	Adenocarcinomas	Target effector T cells to CEA-expressing tumors
Suicide gene	HSV-TK or cytokine deaminase	Mesothelioma, glioma, ovarian, colon, prostate cancer	Render tumor cells sensitive to ganciclovir or 5-fluorocytosine
Tumor-suppressor gene	Wild-type p53	Head and neck, lung, breast cancer	Inhibit proliferation, trigger apoptosis
Antisense	Antisense K-ras	Lung cancer	Inhibit oncogene expression
Myeloprotection	MDR-1, MGMT, DHFR	Hematopoietic stem and progenitor cells	Protect cells from cytotoxic therapy

Table 1. Selected gene transfer approaches for treatment of cancer. CEA, carcinoembryonic antigen; DHFR, dihydrofolate reductase; HSV-TK, herpes simplex virus thymidine kinase; MDR-1, multidrug resistance gene 1; MGMT, methylguanine methyltransferase. [a]Various immunostimulatory molecules (e.g., GM-CSF, IL-2, IL-4, IL-12, B7) and tumor antigens (e.g., MART-1, gp100, CEA, PSA) are under investigation. (Cornetta *et al*, 2000).

The term gene therapy vector refers to a system designed to transfer exogenous genetic material (transgene) into target cell. The critical determinants for choosing a particular vector system include: (a) host range and tissue specificity; (b) ability to

transfer genes to dividing versus nondividing cells; (c) capacity to integrate in the host genome versus episomal maintenance; (d) effects on target-cell viability and potential *in vivo* toxicity; (e) potential to generate replication-competent virus; (f) immunogenicity; (g) ease of manipulation; and (h) the amount of exogenous DNA that can be accommodated (198, 210). The main problem in gene therapy for various diseases still remains the effective and safe delivery of genes to the target tissue. There are two categories of gene delivery vectors: (1) non-viral and (2) virus-based gene delivery systems (Table 2) (210).

	Retrovirus RV	*Lentivirus LV*	*Adeno-virus AV*	*Adeno-Associated virus AAV*	*Cationic liposome CL*	*Plasmid DNA PD*
Carrying capacity (kb)	7-8	7-8	~30	3.5-4.0	NO LIMIT	NO LIMIT
Concentration (particules/ml)	$>10^8$	$>10^8$	$>10^{11}$	$>10^{12}$	NO LIMIT	NO LIMIT
Route of delivery	EX VIVO	EX/IN VIVO	EX/IN VIVO	EX/IN VIVO	EX/IN VIVO	EX/IN VIVO
Permanent integration	YES	YES	NO	YES/NO	NO	NO
Requirement of mitosis	YES	NO	NO	NO	NO	NO
Immuno-genecity	LOW	LOW MAY BE EXCEPT AIDS PATIENT	HIGH	NOT KNOWN	NONE	NONE
Duration of expression	LONG	LONG	SHORT	LONG	SHORT	SHORT
Manufacturing	PILOT SCALE (20-50L)	NOT KNOWN	EASY	DIFFICULT	EASY	EASY
Quality control	EXTREMELY DIFFICULT	EXTREMELY DIFFICULT	MODERATELY DIFFICULT	VERY DIFFICULT	SIMPLE	SIMPLE

Table 2. Gene delivery vectors. Different gene transfer vectors currently being developed for gene transfer into mammalian cells. These vectors can be divided into two categories; non-viral vectors which comprised cationic liposome (CL) and plasmid DNA (PD) and the viral engineered vectors: retrovirus (RV), lentivirus (LV), adenovirus (AV), and adeno-associated virus (AAV). (Jonas Araujo de Souza) (www.studentbmj.com/back_issues/1100/education/404.htlm)

Non-viral gene delivery systems depend on direct delivery of genetic information into target cell and include direct injection of naked DNA; particle bombardment, electroporation and encapsulation of DNA with cationic lipids or polymer (liposomes). These vectors are composed of naked DNA, usually in the form of plasmid DNA. Plasmids are designed to contain the gene of interest and regulatory elements that enhance gene expression. Although these delivery systems exhibit low toxicity, non-viral vectors are limited by low gene-transfer efficiency and are not well suited to systemic administration, as the DNA may be degraded before sufficient material is exposed to the target tissue. To address these limitations, several viruses have been engineered to transport genetic material (86).

Viral delivery systems are based on different viruses and can be defined as either integrating or nonintegrating vectors (86). Vectors based on adeno-associated virus and retrovirus (including lentivirus and foamy virus) have the ability to integrate their viral genome into the chromosomal DNA of the host cell, which will possibly achieve lifelong gene expression. Vectors based on adenovirus (Ad) and herpes simplex virus type 1 (HSV-1) represent the nonintegrating vectors. These vectors deliver their genomes into the nucleus of the target cell, where they remain episomal. Although transfection using these vectors is relatively high, several drawbacks such as low titers, possible immunogenicity and restricted gene insert size have to be taken into consideration (210).

	Murine retrovirus	Adeno-virus	AAV	Herpes Virus	Human Lentivirus
Genome	RNA	ds DNA	ss DNA	ds DNA	RNA
Transgene size	3-7	7-36	2.0-4.5	10-100	8-9
Titer	10^6-10^7	10^{11}-10^{12}	10^6-10^9	10^4-10^{10}	10^6-10^9
Host cell proliferation	Required	Not required	Improves efficiency	Not required	Improves efficiency
Stable integration	Yes	No	occasional	No	Yes
Immunogenicity	Low	High	Low	Variable	Not well studied

Table 3. Characteristics of viral vectors. Viral gene transfer vectors can be categorized on the basis of the fate of the introduced genetic material. Retroviral-, adeno-associated- and lentiviral-mediated gene transfer results in the integration of the vector into the host genome, whereas adenoviral and herpes mediated gene transfer results in episomal maintenance of the vector. Adapted from Cornetta *et al*, 2000.

Most of the current vectors used in gene therapy are viral, among which adeno- and retroviruses are commonly used. In this study, we choose the retroviral system to gene-modified mouse B16 melanoma cells to stably express the interferon regulatory factor 3 (IRF-3).

I. 1. 1. Retrovectors

Retroviruses are enveloped viruses, which contain 7-12kb RNA genomes. After virus entry to cells via specific cell-surface receptors, their genomes are reverse

transcribed into double-stranded DNA and subsequently integrated into the host in the form of provirus (Figure 1) (85). The provirus replicates as the host chromosome replicates and is transmitted to all of the progeny of the host cells.

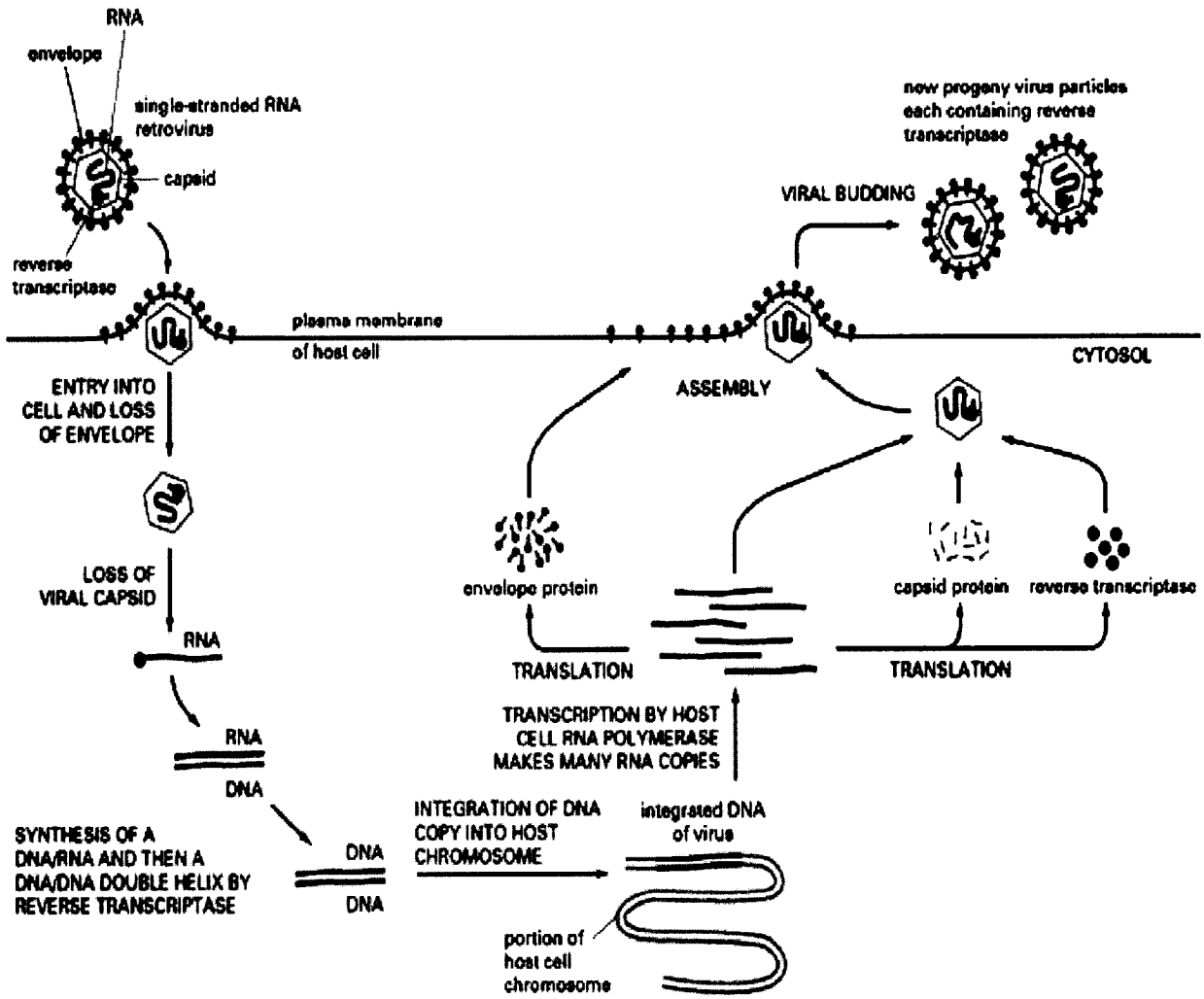


Figure 1. Life cycle of retroviruses. The virus life cycle is described by the following events, receptor binding, fusion with the cell membrane, uncoating of viral core, reverse transcription of the single-stranded RNA into double-stranded DNA, integration of proviral DNA into host genome, transcription and translation of viral proteins, assembly and budding of immature virions from the cellular membrane and finally maturation. (National Health Museum, www. accessexcellence.org/AB/GG/retro_life.html).

This ability of retroviruses to stably transfer genetic information is an attractive use of retroviruses as gene transfer vectors. The viral RNA contains three essential genes, *gag*,

pol, and *env*, and is flanked by long terminal repeats (LTR). The *gag* gene encodes for the core proteins capsid, matrix, and nucleocapsid, which are generated by proteolytic cleavage of the *gag* precursor protein (85). The *pol* gene encodes for the viral enzymes protease, reverse transcriptase, and integrase, and is usually derived from the *gag-pol* precursor. The viral tropism of retroviruses is determined by the envelope glycoprotein (*env*) (31). Because the retrovirus genome is relatively small and well characterized, it is possible to engineer vector-packaging systems, which produce only transgenes, and does not produce replication competent viruses (RCV) nor viral structural genes (105, 120). Absence of RCV is required for safety, while the lack of expression of any viral protein in recipient cells could be advantageous in many preclinical and clinical settings as viral proteins may elicit undesirable immune responses (28). Retroviral-mediated gene transfer requires a packaging cell line and a viral vector.

Retroviral vectors derived from Moloney murine leukemia retrovirus (MoMLV) have been widely used for efficient gene transfer to achieve long-term expression of a chosen therapeutic gene in mammalian cells (Figure 2). Replication-defective MLV vectors are generated by replacing all viral protein encoding sequences with the exogenous promoter-driven transgene of interest which is, in the case of this study, the interferon regulatory factor 3 (IRF-3) (49, 105). Besides the packaging signal, the viral LTRs and adjacent sequences, which are essential for reverse transcription and integration, remains in the vector (104, 118). In this system, vector RNA production is driven by U3 region of the LTR and results only in low titers of the vector due to the low transcriptional activity of the LTR.

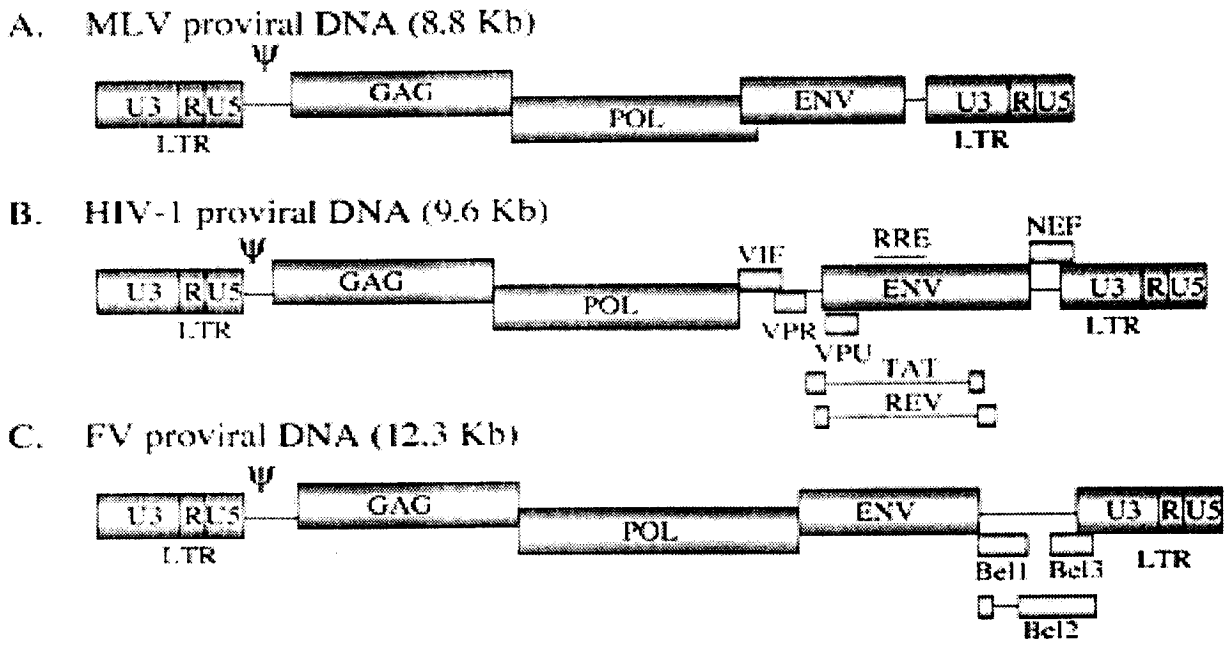


Figure 2. Schematic presentation of the proviral DNA organization of retroviruses. (*A*) The MLV provirus contains *gag*, *pol*, and *env* coding regions flanked by LTRs. The LTR is comprised of three regions, U3, R, and U5, which are essential for reverse transcription, proviral integration, and transcriptional activation. ψ indicates the packaging signal. (*B*) The HIV-1 proviral DNA encodes for six additional proteins *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef*, and contains the *cis*-acting element RRE. (C) The FV provirus contains three additional ORFs: *bel*1, *bel*2, and *bel*3. (Kootstra, 2003).

Therefore, the U3 of the 5' LTR is replaced by a CMV promoter resulting in a CMV/LTR hybrid with high transcriptional activity (41). The 3' U3 region of the LTR remained intact and is copied over to the 5' LTR during reverse transcription, allowing efficient integration and LTR-driven transgene expression in the transduced cell. The envelope G glycoprotein from the vesicular stomatitis virus (VSV-G) has been used to construct a pseudotyped MuLV with significant improvement in stability and transduction efficiency (117). This pseudotyped vector has a much broader host range than the vectors

with conventional amphotropic Env. It has been successfully used to transfer genes into human peripheral blood lymphocytes, leukocytes, hepatocytes, and vascular tissues. In addition, the green fluorescent protein (GFP) cDNA was introduced as a genetic marker, reporter, and selectable agent for engineered cells concurrently expressing the second, linked transgene (IRF-3). The bicistronic vector (pAP2-GFP) (49, 91) was designed by the insertion of the encephalomyocarditis virus internal ribosomal entry site (IRES) between the IRF-3 gene and the GFP reporter gene (53, 125). The expression cassette contains the two cDNAs and a single promoter that in combination with IRES allows the translation of the two open reading frames from one mRNA. GFP permits the non-invasive assessment of gene transfer efficiency since GFP expression could be determined by fluorescence microscopy and provides a way to select the gene-modified cells by FACS sorting (101).

For the packaging of retroviral vectors, the structural proteins are provided in *trans* in the packaging cells (Figure 3). The first packaging cell lines expressed *gag*, *pol*, and *env* from a complete proviral DNA lacking only the packaging signal (99, 105, 119). However, sequence homology between the vector and packaging constructs facilitated recombination, resulting in the generation of replication-competent virus. To prevent homologous recombination, packaging cells have been developed to express *gag/pol* and *env* from separate constructs (109, 110).

Furthermore, expression from the packaging constructs is no longer driven by the viral LTR, but by constitutive promoters, thus allowing a high level of virus production (31, 105).

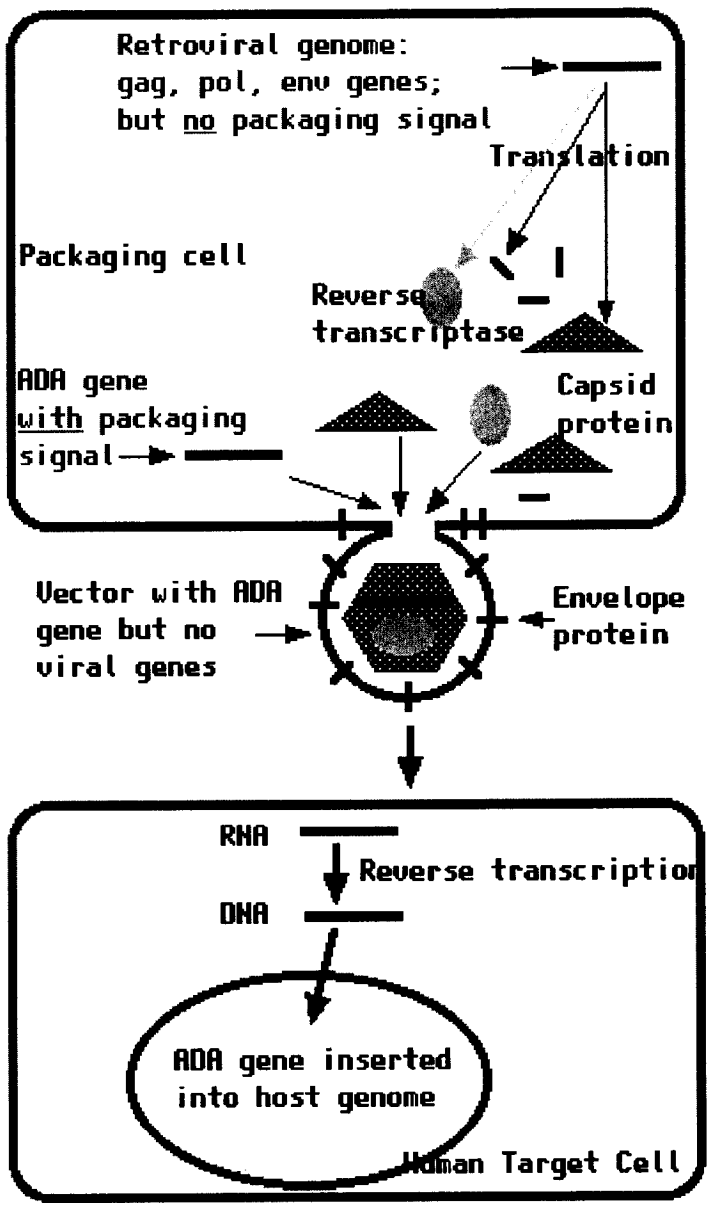


Figure 3. Packaging cell. Packaging cell lines are engineered so they express the missing sequences required at the RNA level for packaging of retroviral RNA's. These cells make all the retroviral gene products but do not package their own RNA into the cell culture medium since they lack the packaging signal, which is provided by the retroviral vector. The transfer of the retrovirus vector into these packaging cell lines is a complementation system, which allows for packaging of retrovirus vectors in the absence of replicating virus. (J.W. Kimball (1994) General Biology).

The disadvantages and risks associated with the use of retroviral vector encompass: 1) their capacity to infect only cells actively dividing, thus limiting the range of targeted cells (121), 2) their susceptibility to inactivation by serum complement, 3) the risk of replication competent virus arising in large-scale preparations of retroviral vectors, 4) their inability to infect certain cell type, 5) and considering that retroviral particules are generated in cell culture, that some cellular contaminants may thus coexist (86). In addition, the integration of the retrovirally transferred gene into the host cell genome is random and consequently there is theoretical risk of insertional mutagenesis (Table 4).

vector	advantages	disadvantages
retrovirus	• enters cells efficiently • viral genes absent • integrates stably	• hard to produce • limited insert size • random mutagenesis
adenovirus	• enters cells efficiently • produces high expression of therapeutic gene • does not integrate into host chromosome	• viral genes must be in vector • induces immune response
adeno-associated virus	• integrates into chromosome at specific site • does not produce immune response	• small insert size allowed • hard to produce
herpesvirus	• produced at high levels • targets nondividing nerve cells	• hard to produce • viral gene required

Table 4. Advantages and Disadvantages of viral vectors. Viruses vary in their usefulness as gene-therapy vectors. Some viruses are more adept at getting into cells, whereas others may ensure that corrective genes are expressed at higher levels or for longer periods. No one vector seems to combine all of the desirable properties. Adapted from Kootstra, 2003.

I. 2. Interferons

Interferons (IFNs) are a family of multi-functional secreted proteins that were first discovered as mediators of cellular resistance against viral infection. They were later

shown to play diverse roles in the immune response to pathogens, immunomodulation and hematopoietic development (134, 171). More recently, IFNs were divided into two major subgroups by their ability to bind to common receptor types (64, 116). Type I IFNs all bind to a type I IFN receptor, and include IFN-α, IFN-β, IFN-ω, and IFN-τ. IFN-γ is the sole type II IFN, and binds to a distinct type II receptor.

The genes and corresponding proteins of the type I IFN superfamily are structurally related, and the human genes are clustered within 400 kilobase (kb) on the short arm of chromosome 9. Fourteen genes comprise the human IFN-α family. Twelve IFN-α proteins of 165- or 166-amino acid residues are produced from these 14 genes (two of the genes are pseudogenes) (43). In primate and rodents, only a single IFN-β gene exists. Almost all cell types produce type I IFNs (33). The prototypical production sites for IFN-α and IFN-β are leukocytes and fibroblasts, respectively. Their induction usually follows exposure to viruses, double-stranded RNA, polypeptides, and cytokines. Type II IFN (IFNγ), a Th1 cytokine produced by activated T cells, natural killer cells and macrophages, is crucial in eliciting the proper immune response and pathogen clearance (Figure 4) (Table 5) (18, 39). IFNs elicit their effects through the transcriptional activation of target genes that possess specific consensus DNA-binding recognition sites within their promoters. These genes are regulated through the JAK-STAT signaling pathway and through the interferon regulatory factors (IRFs), a growing family of transcription factors with a broad range of activities (134). Recent reviews have detailed the discovery and characterization of both the JAK-STAT pathway and the IRF transcription factors (20, 103, 134, 171, 181).

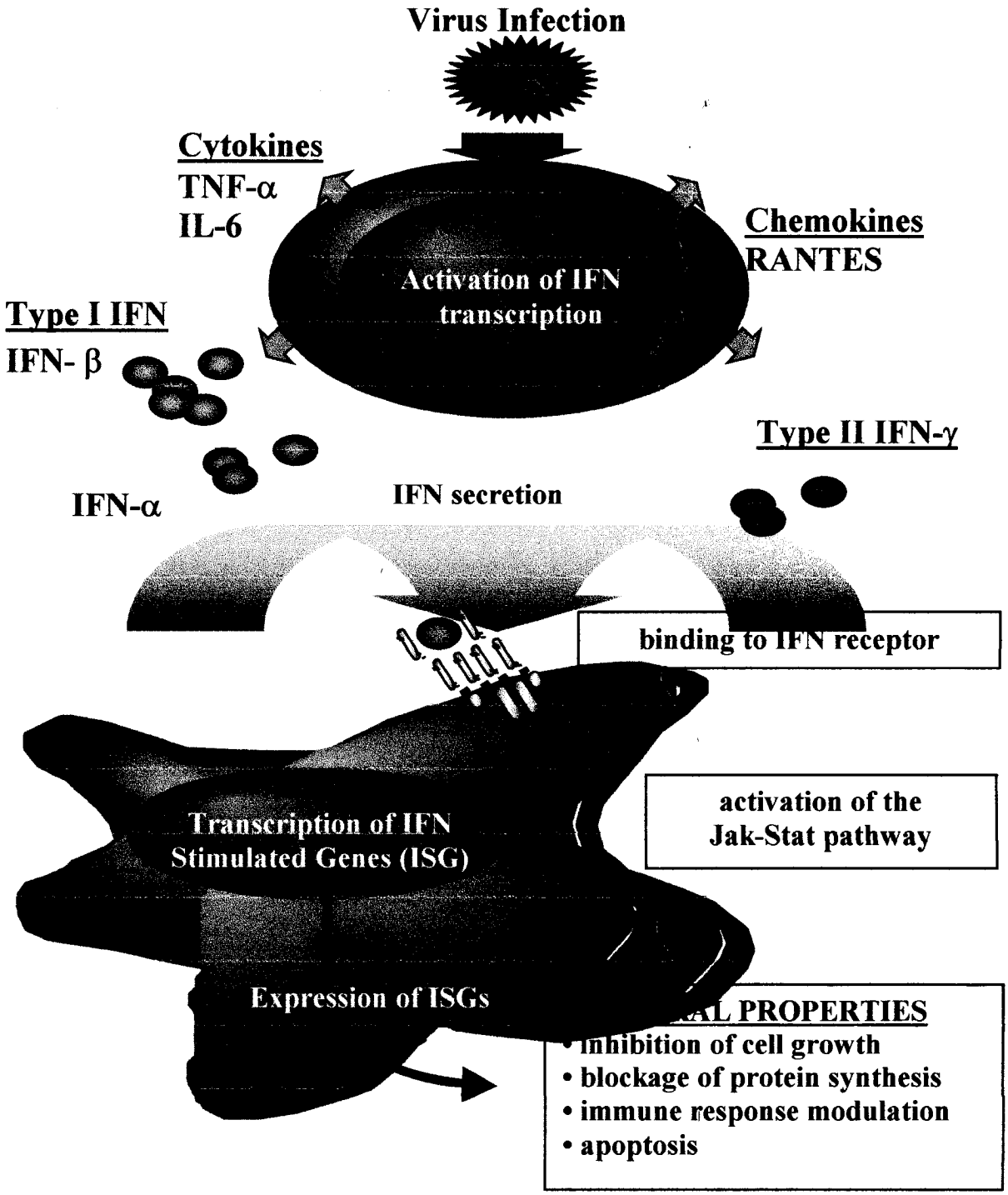


Figure 4. Interferon system and actions. Infection by pathogenic viruses leads to secretion of antiviral cytokines such as the IFNs, IL-6, TNF-α, IL-1 and other pro-inflammatory cytokines or chemokine. The signal triggered by IFN binding to specific membrane receptors leads to the activation of cytoplasmic factors that translocates to the nucleus and stimulates ISG expression. IFNs are also modulator of cell growth, block protein synthesis and induce apoptosis.

INTERFERONS	TYPE I INTERFERONS		TYPE II INTERFERONS
	IFN-α	IFN-β	IFN-γ
Principal Producing cells	Leukocytes Fibroblasts Macrophages Epithelial cells		T lymphocytes Macrophages
Inducing agents	Virus Double-stranded RNA		Mitogens Antigens Interleukine-2
Chromosomal locations	Human chr 9 Murine chr 4		Human chr 12 Murine chr 10
Gene Number	More than 15 human genes & 10 murine genes	1 & 1 murine gene	1 unique gene
Intron	0	0	3
Molecular weight (kDa)	16 to 27	28 to 35	20 to 25
aa (mature protein)	165	165	146

Table 5. Characteristics of Interferon proteins. IFNs are divided into Type I (IFN-α and IFN-β) and Type II (IFN-γ). IFN-α, previously called leukocyte IFN, is produced by peripheral blood leukocytes in response to viral infection or double-stranded RNA. This heterogeneous group of proteins, of molecular weights 16 to 27 kDa, shares high homology in their amino acid sequence (165 aa residues). IFN-β, also called fibroblast IFN, is a glycosylated protein of 28 to 35 kDa, produced by leukocytes or epithelial cells. Twenty-one non-allelic IFN-A genes and pseudo-genes encoding the different IFN-α proteins have been identified and are clustered together with IFN-B gene on the short arm of human chromosome 9. Similarly, 10 different murine IFN-A genes grouped in a proximal region of chromosome 4 centromer have been identified. Human and murine IFN-A genes are intronless and maintain a high degree of homology (80 to 95%) at the nucleotide sequence level, suggesting that the gene cluster was derived from a common ancestor gene by successive duplications. The gene encoding IFN-β is unique, intronless and is derived from the same ancestor gene as the IFN-A genes. The IFN-γ is encoded by a unique gene (IFN-G) containing 3 introns and located in the long arm of human chromosome 12. This gene has a weak homology with type I IFN genes. The mature protein is a mixture of two polypeptides of 20 to 25 kDa molecular weight, differing by differential use of N-glycosylation sites. Secretion of IFN-γ by lymphocytes is modulated by mitogenic stimuli, antigens or soluble mediators such as IL-1, IL-2 or IFN-γ itself. although IFN-γ possesses an antiviral activity, it is primarily an immune modulator than an antiviral agent. Adapted from Kalvakolanu and Borden, 1996.

I. 2. 1. Interferon signaling

Type I and type II IFNs possess their own cellular receptors on cell surfaces: IFNAR and IFNGR (Figure 5). IFNAR stimulation results in the activation of Janus family protein tyrosine kinases, Tyk2 and Jak1, which are associated with the IFNAR1 and IFNAR2 chains, respectively. This activation is followed by site-specific tyrosine phosphorylation of Stat1 and Stat2 transcription factors. These two phosphorylated Stats in combination with IRF-9/ ISGF3γ/ p48 form the heterotrimer transcription factor complex, ISGF3, which translocates to the nucleus and binds to ISRE to activate IFN-inducible genes (Figure 6) (64). In the case of IFNGR signaling, it involves the IFNGR1 and IFNGR2 chains. IFNγ binding to IFNGR results in the activation of Janus kinases, Jak1 and Jak2. This activation is followed by site-specific tyrosine phosphorylation of Stat1 and homodimerization.

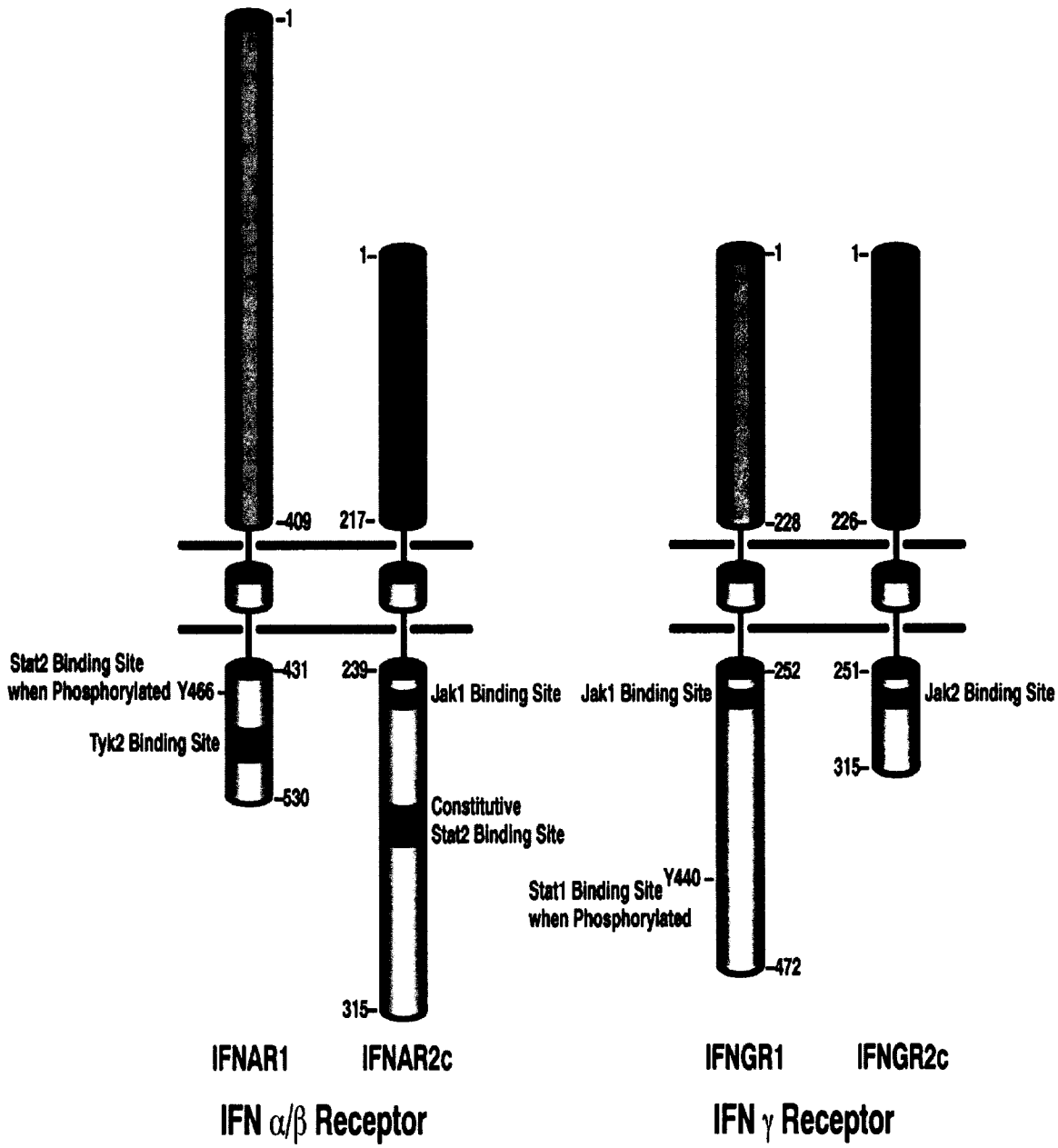


Figure 5. Interferon receptors. Type I receptor binds to IFN-α and IFN-β and type II receptor binds to IFN-γ. Signaling by IFNs is mediated by a pathway that includes the JAK kinases (Janus tyrosine kinases) and the STAT proteins (Signal Transducers and Activators of Transcription). (Wilks and Oates 1996, Larner and Finbloom 1995).

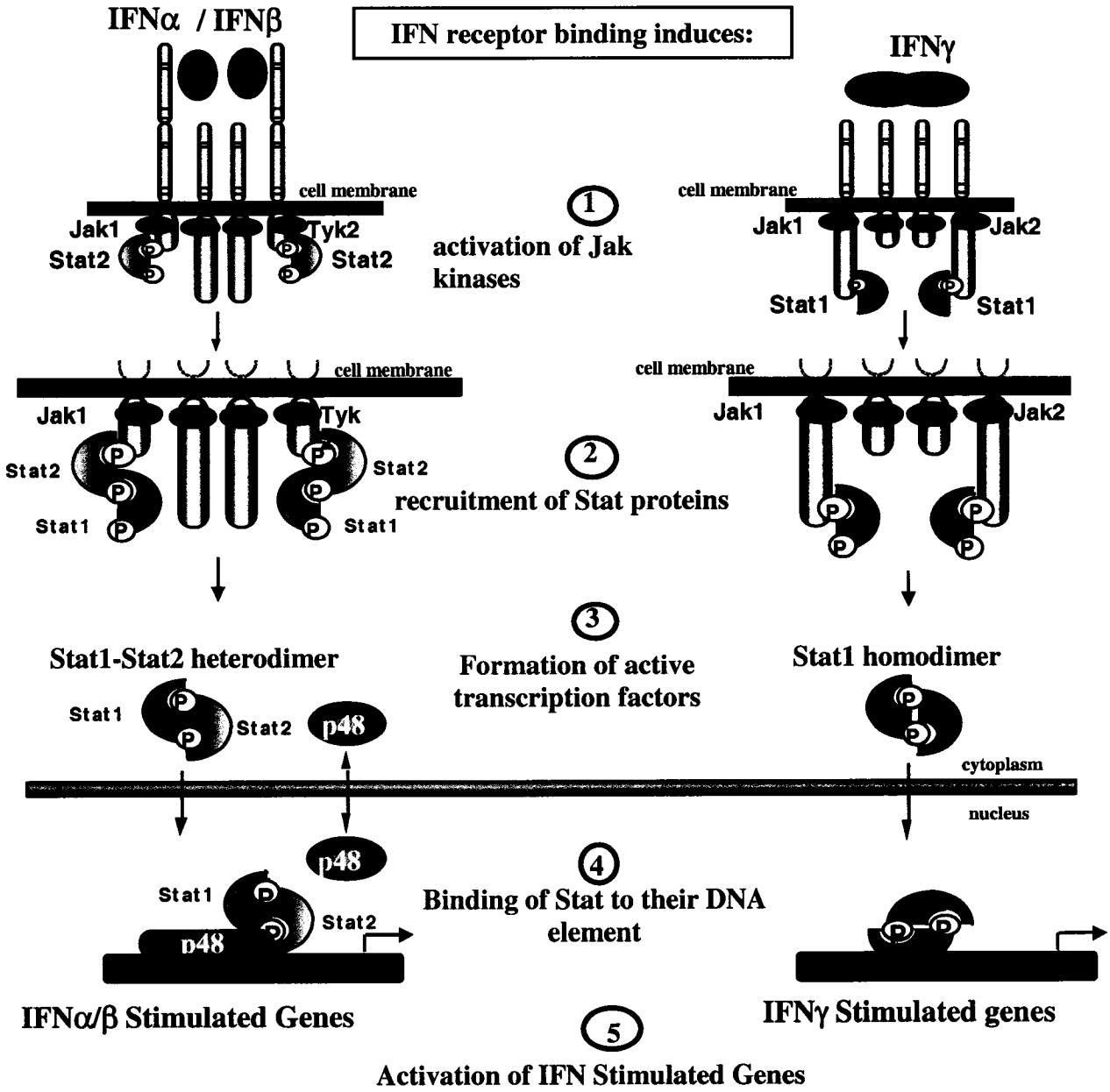


Figure 6. Interferons signal transduction. The binding of IFNs to the IFN receptors induces the Jak-STAT pathway. The binding of a cytokine to its receptor rapidly induces the tyrosine phosphorylation of the receptor by JAK kinases; these phosphorylated tyrosines provide a docking site for the STAT proteins. The STATs are phosphorylated by Jak kinases, released from the receptor and dimerize with one another. Dimeric STATs then translocate into the nucleus where they modulate expression of target genes by direct DNA binding. A remarkable feature of this system is that newly induced STAT-DNA binding activity can be detected in the nucleus within minutes of cytokine binding. This timing accurately reflects the rapidity of their activation and ability to exert biological actions. (Wilks and Oates, 1996; Larner and Finbloom, 1995; Bluyssen et al, 1996).

These two phosphorylated Stat1s form the transcription factor complex, GAF (IFN gamma-activated factor, which translocates to the nucleus and binds to GAS sites (IFN gamma-activated site; consensus sequence TTCNNNGAAA) to activate target genes (Figure 6) (Table 6) (64, 181).

There is in fact a novel form of cross talk which occurs between IFNα/β and IFNγ signaling, in which IFNγ is dependent on a weak IFNAR stimulation by spontaneously produced IFNα/β. Evidence has been provided for the physical association between IFNAR1 and IFNGR2 receptor chains. This docking site may be utilized by IFNγ–induced activation of the ISGF3 complex (181).

IFN α / β		IFNα / β and IFN-γ		IFNγ	
Gene	**Function**	**Gene**	**Function**	**Gene**	**Function**
ISG15	cytokine immuno-modulator	**CRG-2**	chemokine	**FcγRI**	IgG-Fcγ receptor
RNase L	mRNA degradation	**MHC class I**	Antigen presentation	**MHC class II**	Antigen presentation
2-5 (A) synthetase	Oligoadenylate synthesis	**Rbp-27**	Inhibition of Rev-dependent HIV activation	**iNOS**	Macrophage-specific effector
MxA	inhibition of virus	**GBP**	GTP binding	**Trp-tRNA synthetase**	Protein biosynthesis
PKR	Protein synthesis inhibitor	**IDO**	Tryptophan degradation	**Leucine AP**	exopeptidase
Lysyl oxidase	Reversion of ras-transformed phenotype	**IRF-1**	Transcription factor	**Mn-SOD**	superoxide scavenger
IRF-2	Transcription factor	**IFP-35**	Leucine zipper protein	**Phagocyte gp91-phox**	cytochrome b subunit of NADPH
6-16	unknown			**RING4**	Peptide transporter
				RING12	Component of the proteasome
				ICSBP	Transcription factor

Table 6. Interferon stimulated genes. A non-exclusive list of ISGs, classified according to the type of IFN inducer is presented in the table. The abbreviations correspond to: **AP**-amino peptide; **CRG**-cytokine responsive gene; **FCγRI**-IgG-Fc receptor; **GBP**-guanylate-binding protein; **ICSBP**-interferon consensus sequence binding protein; **IDO**-indoleamine-2,3-dioxygenase; **IFP**-interferon induced Protein; **iNOS**-inducible nitric oxide synthetase; **IRF**-interferon regulatory factor; **ISG**-Interferon stimulated gene; **MHC**-major histocompatibility complex; **Mx**-myxovirus inhibiting protein; **PKR**-RNA-dependent protein kinase; **Rbp**-RNA-binding protein; **RING**-really interesting protein; **SOD**-superoxide dismutase. Adapted from Kalvakolanu and Borden, 1996.

I. 3. Biological Properties of IFNs

I. 3. 1. Induction of Antiviral Functions by IFN

Interferons (IFNs) are a large family of multifunctional secreted proteins involved in antiviral defense, cell growth regulation and immune activation (17, 144). Viral infection induces transcription of multiple IFN genes (59); Newly synthesized IFN interacts with neighboring cells through cell surface receptors, resulting in the prompt and efficient synthesis of a group of over 30 new cellular proteins through the activation of the JAK-STAT family of cellular transcription factors (Figure 9) (59). These events represent the means by which IFNs induce the antiviral state that constitutes the primary host defense in innate immunity. The ability of IFNs to confer an antiviral state to uninfected cells is their defining activity (Figure 7). IFNs provide an early line of defense against viral infections—hours to days before cellular and humoral immune responses. This vital role has been demonstrated by numerous animal studies in which animals that cannot mount an IFN response succumb to infection by a non-lethal virus inoculation (171, 181).

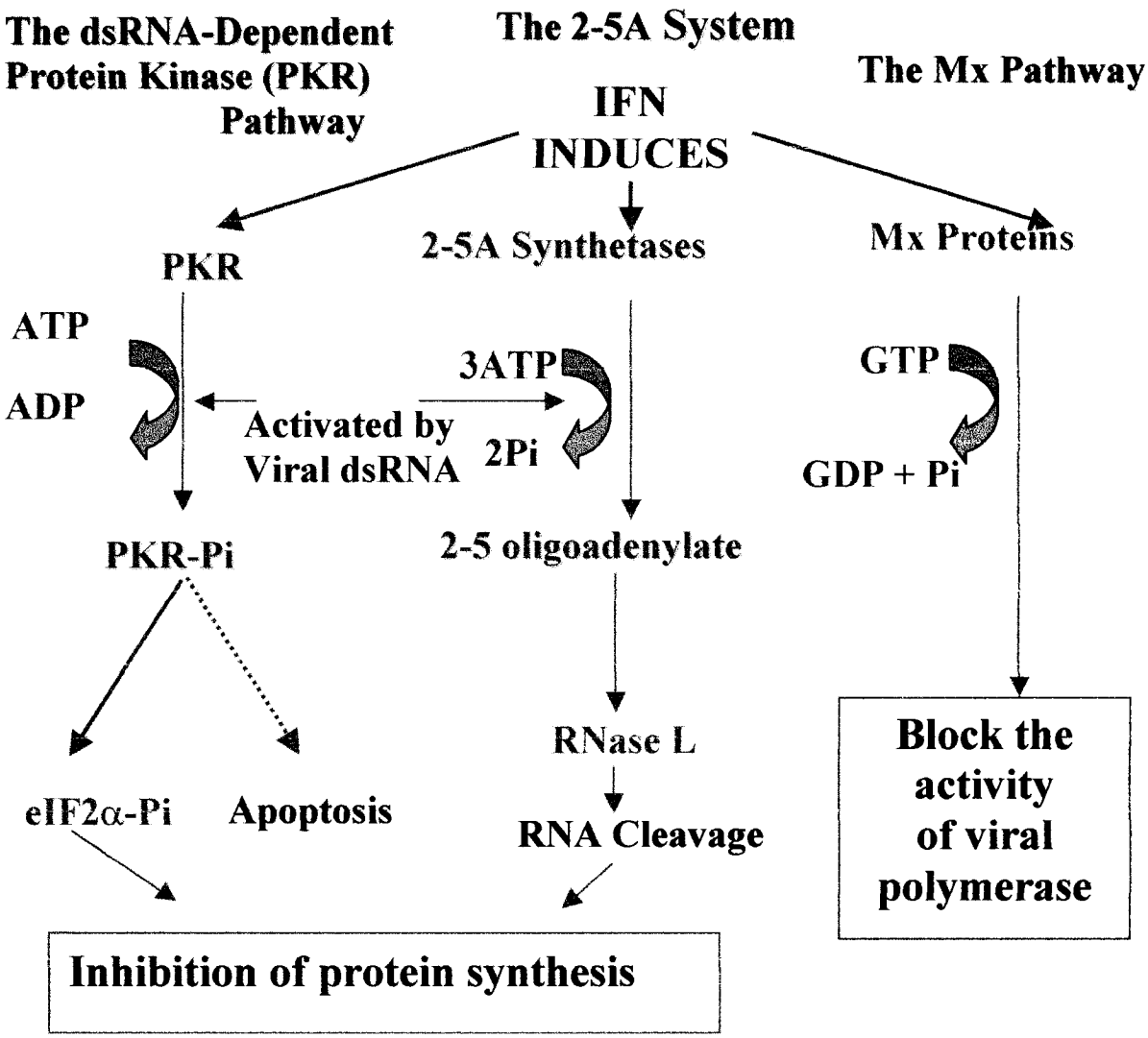


Figure 7. Antiviral mechanisms of interferons. The best-characterized IFN-induced antiviral pathways utilize the dsRNA-dependent protein kinase (PKR), the 2-5A system and the MX pathway. 2-5A oligoadenylate synthetase produces 2',5'-oligoadenylates (2-5A) which bind to inactive RNase L and induce its enzymatic activity of RNA degradation. PKR is normally inactive, but is activated by dsRNA and subsequently phosphorylates host substrates. The antiviral effect of PKR is due to its phosphorylation of eIF2, which is a component of the translation initiation complex. Phosphorylation results in rapid inhibition of translation. Mx proteins once induced have the ability to hydrolyze GTP. By this pathway, Mx proteins affects viral replication by interfering with the growth of influenza and other negative-stranded RNA viruses at the level of viral transcription (Williams, 2000).

I. 3. 2. Immunomodulatory Functions

a. IFN, antigen processing and presentation and development of CD8+

IFNs can have significant effects on innate and adaptive immune responses (35). Type I and type II IFNs enhance the expression of MHC class I proteins to promote the development of CD8+ T-cell responses (Table 7) (16, 135). In contrast, IFN-γ is uniquely able to induce the expression of MHC class II proteins that promotes enhanced CD4+ T-cell responses. Both type I and type II IFNs are able to upregulate the expression of the different protein components, which constitute the proteosome-protein processing pathway responsible for generating antigenic peptides (74, 208). Accordingly, IFNs can enhance immunogenicity by increasing the quantity and repertoire of peptides displayed in association with MHC class I proteins, which are dependent on an active proteosome-mediated pathway.

b. IFN and development of the CD4+ helper T-cell phenotype

Activated human and murine CD4+ T-cells can differentiate into two distinct T-cell subsets, which are defined by the cytokines they produce after stimulation. Th1 cells synthesize IFN-γ, IL-12 and lymphotoxin, and promote cell-mediated immunity. Th2 cells selectively produce IL-4, IL-5, IL-6, and IL-10 and thereby antibody production by B cells and the development of humoral immune responses (42). IFN has an important effect on Th1 cell development and plays a dual role in this process: 1) IFN facilitates Th1 production by enhancing the synthesis of IL-12 in antigen-presenting cells; and 2) IFN blocks the development of Th2 cells by inhibiting IL-4 production, which is required for Th2 formation (189).

IFN type	IFN categories	Receptor type	Prototypic cell of origin	Direct anti-proliferative effects	Stimulates MHC class I expression	Stimulates MHC class II expression	Stimulates NK cell activation
	Alpha (α)	I	Leukocyte	YES	YES	NO	YES
	Beta (β)	I	Fibroblast	YES	YES	SLIGHTLY	YES
	Omega (ω)	I	Leukocyte	YES	YES	NO	YES
	Tao (τ)	I	Ovine Trophoblast				
	Gamma (γ)	II	T-cells NK cells	YES	YES	YES	Less than type I IFNs, delayed

Table 7. Biological functions of Interferons. IFNs are divided into two major subgroups by virtue of their ability to bind to common receptor types. Type I IFNs all bind to a type I IFN receptor, and include IFN-α, IFN-β, IFN-ω, and IFN-τ. IFN-γ is the sole type II IFN and binds to a distinct receptor. Almost all cell types produce type I IFNs. The prototypical production sites for IFN-α and IFN-β are leukocytes and fibroblasts, respectively. Their induction usually follows exposure to viruses, double-stranded RNA, polypeptides and cytokines. The type II IFN-γ is produced in T cells and natural killer (NK) cells following a number of immunological stimuli including T-cell-specific antigens, staphylococcal enterotoxin A, and the combination of phytohemagglutinin and phorbol ester. Unlike IFN-α and IFN-β, it is not directly induced in cells following viral infection (Jonasch and Haluska, 2001).

I. 3. 3. Regulation of cell growth and Apoptosis

IFNs inhibit cell growth and induce apoptosis, activities which affect the suppression of cancer and infection (12, 112). Different cells exhibit varying degrees of sensitivity to the antiproliferative activity of IFNs (143). In some cases, growth arrest may be due to differentiation, particularly when IFNs are used in combination with other agents such as vitamin A derivatives (retinoids). Specific IFN-induced gene products have not been linked directly to antiproliferative activity; however IFNs targets specific

components of the cell-cycle control apparatus, including c-myc, pRB, cyclin D3, and cdc25A (203).

Today, IFNs (especially IFN-α) are mostly used as cytokines in patients. IFN-α is used worldwide in over 40 countries for the treatment of more than 14 types of cancer, including some hematological malignancies (hairy-cell leukemia, chronic myeloid leukemia, some B and T cell lymphomas) and certain solid tumors, such as melanoma, renal carcinoma and Kaposi's sarcoma (76). However, in spite of many years of intense work in animal tumor models, and considerable experience in the clinical use of IFN, the important mechanisms underlying the antitumor response are not fully understood. It is also unclear whether the current clinical use of IFN represents the most effective strategy for achieving optimal responses in patients with these cytokines or whether new delivery strategies can result in more pronounced and selective anti-tumor effects (144).

I. 4. Interferon Regulatory Factors

Gene activation in response to extracellular signals, environmental stresses, or infection by pathogens requires highly integrated signal transduction pathways that direct the transcriptional machinery to the appropriate sets of genes. This process is achieved in part by the coordinate activation of distinct sets of transcription activators and their assembly into multicomponent enhancer complexes (enhanceosomes). IFN regulatory factors (IRF), initially identified as regulators of IFN-α/β genes, constitute a family of transcription factors, the IRF family (Figure 8) (103, 134, 181). The members of this growing family commonly share significant homology in the amino-terminal 115 amino acids which comprise the DNA-binding domain (DBD); this region contains a characteristic repeat of five tryptophan residues spaced by 10-18 aa.

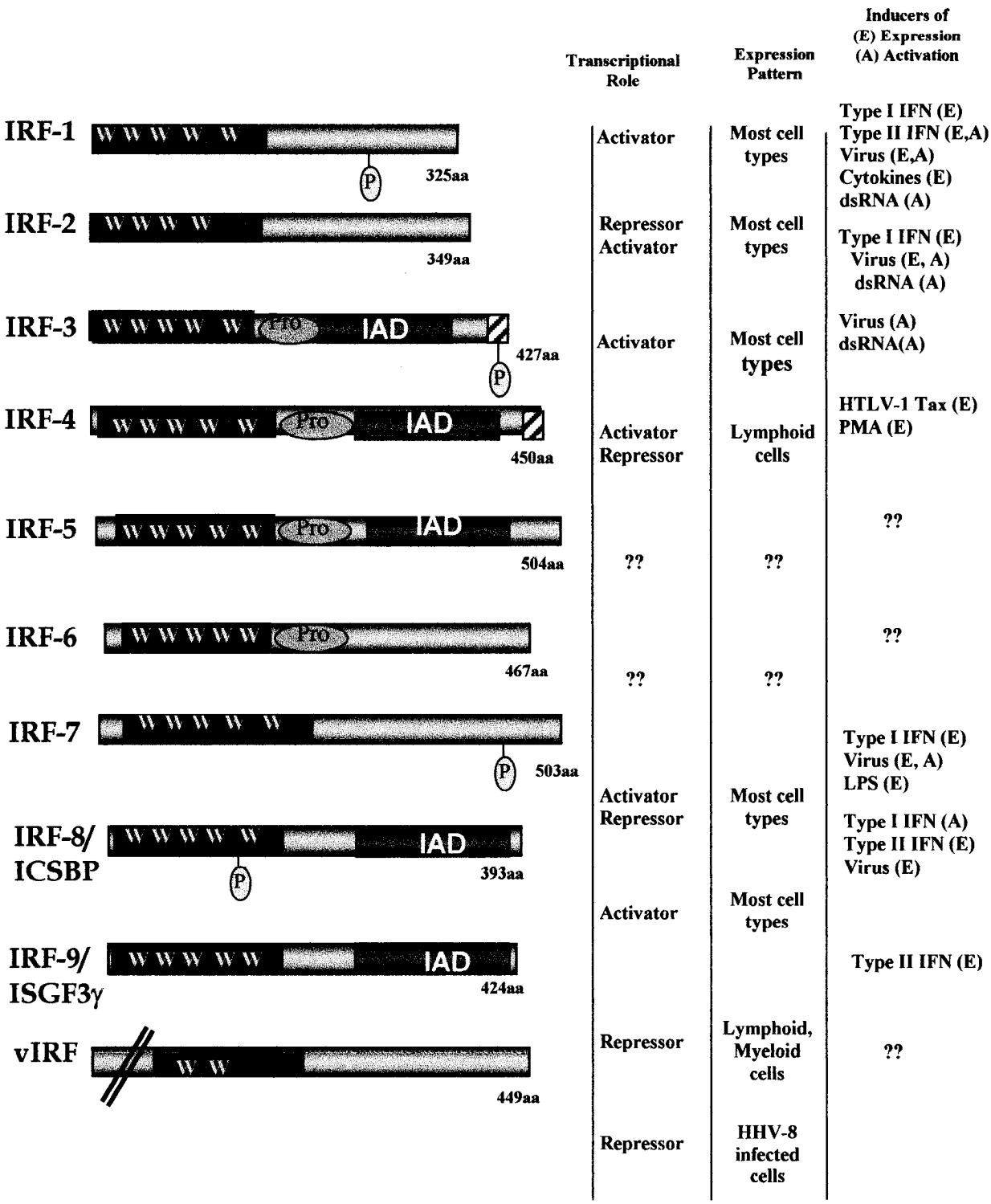


Figure 8. Interferon Regulatory Factor family members. The IRF family members; expression patterns and transcriptional roles. the conserved tryptophan repeats in the DNA binding domain (DBD) (black bar) are represented by W. Certain IRF family members possess a proline-rich domain by (Pro), an IRF Association domain (IAD), a C-terminal autoinhibitory domain (hatched bars) and phosphorylation sites designed by (P)

The DBD recognizes a similar DNA motifs termed Interferon Stimulated Response Element (ISRE; found in most IFN-inducible gene promoters, A/G NGAAANNGAAACT) (32), Interferon Consensus Sequence (ICS: the ICSBP recognition site found in the MHC class I promoter, G/A G/C TTTC) (34, 132, 197) or Interferon Regulatory Element (IRF-E or Positive Regulatory Domain (PRD) I and III in the IFN-β promoters, G(A)AAA G/C T/C GAAAG/C T/C) (179).

The best-characterized members of the IRF family, IRF-1 and IRF-2, were originally identified through transcriptional studies of the human IFN-β gene (46, 47, 65, 123). Their discovery preceded the recent expansion of this group of IFN-responsive proteins which now includes seven other members: IRF-3, IRF-4 (Pip/LSIRF/ICSAT), IRF-5, IRF-6, IRF-7, IRF-8/ICSBP, IRF-9/ISGF3γ/p48, (Figure 8) (103, 155, 181). All IRFs share a high degree of homology in the N-terminal DNA binding domain and generally bind the DNA sequence – GAAANNGAAANN; the C-terminal portion of the IRF proteins is unique to each member. Structurally, the IRF family also shares homology with the Myb oncoproteins that display the tryptophan repeat motif in their DNA binding domain. The best-characterized member, c-Myb regulates differentiation and proliferation in immature hematopoietic and lymphoid cells (56, 92), but the relationship of the c-Myb family to the interferon system remains undefined. Recently, virally encoded forms of IRF proteins in the genome of the Human Herpes Virus 8/ Kaposi Sarcoma Herpes Virus (HHV-8/KHSV) were identified; four open reading frames encoding proteins showing homology to cellular IRFs were found in the viral genome (124, 152).

I. 5. Interferon regulatory factor 3: IRF-3

IRF-3 was first identified through a search of an EST database for IRF-1 and IRF-2 homologs and was classified as a member of IRF family on the basis of (i) homology with other IRF family members and (ii) binding to the IFN-stimulated regulatory element (ISRE) of the *ISG15* promoter (6). This protein is distinct from cIRF-3, an avian protein which demonstrates homology to the IRF family members (60). At the amino acid level, IRF-3 has the highest homology to the IRF-8/ICSBP and IRF-9/ISGF3γ IRF members, with the homology extending into C-terminal domain.

Recently, both IRF-3 and IRF-3/IRF-9 knockout mice were established. The IRF-$3^{-/-}$ animals were more susceptible to viral infection, and the IFN levels in serum from encephalomyocarditis virus (EMCV)-infected mice were found to be significant lower in IRF-$3^{-/-}$ mice than in wild-type (WT) mice (155). Cells derived from IRF-3/IRF-9 knockout mice, in which both IRF-3 and IRF-7 is abrogated, showed an almost complete block in the induction of IFN-α and IFN-β genes, demonstrating cooperation between the two factors (Figure 9) (155). These results support the biochemical and molecular evidence that suggests that IRF-3 is critical for the host defense against viral infection.

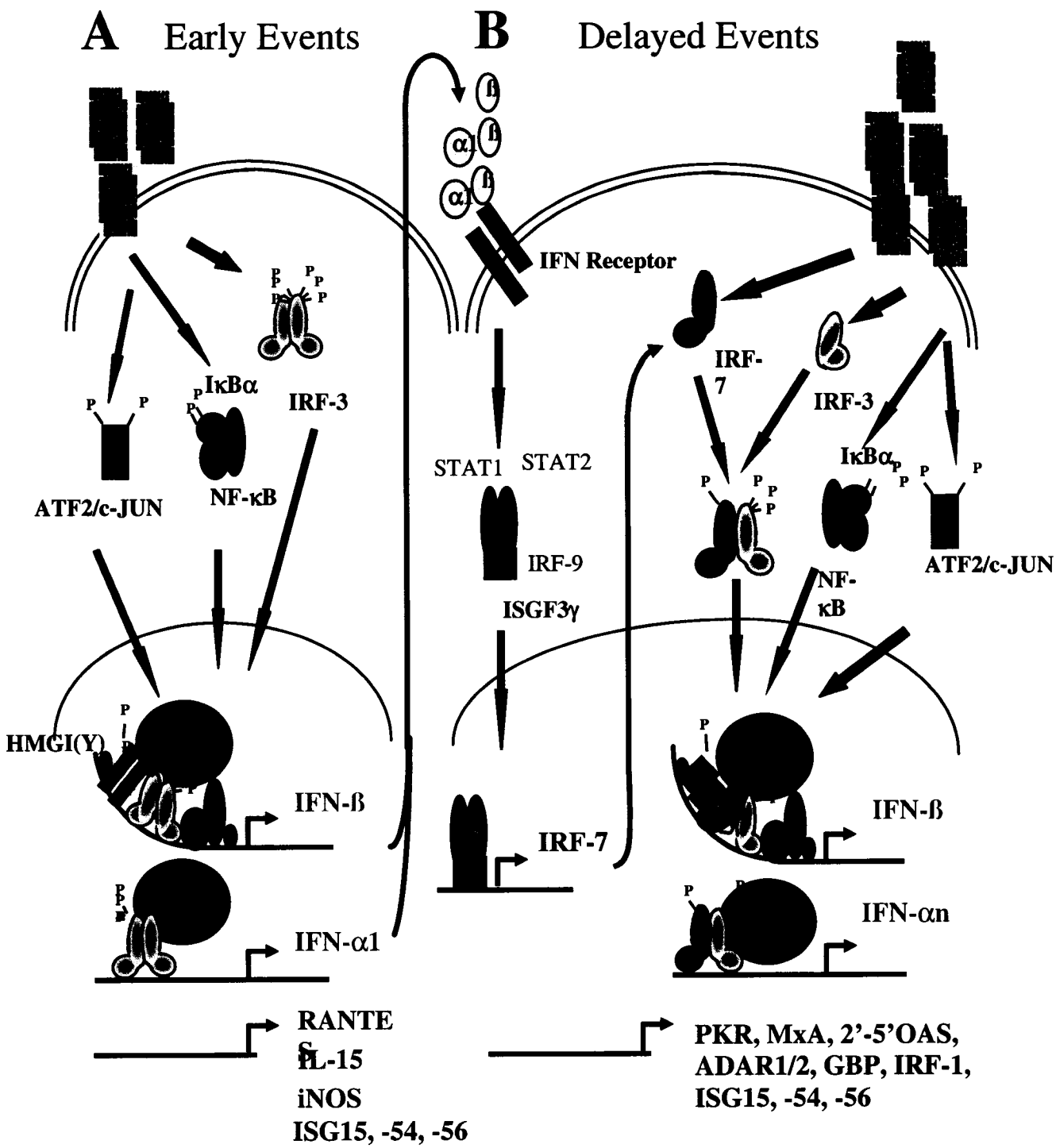

A Early Events
B Delayed Events
IFN Receptor
IκBα
IRF-3
ATF2/c-JUN
NF-κB
STAT1
STAT2
IRF-9
ISGF3γ
IRF-7
IRF-7
IRF-3
IκBα
ATF2/c-JUN
NF-κB
HMGI(Y)
IFN-ß
IFN-α1
IFN-ß
IFN-αn
RANTE
IL-15
iNOS
ISG15, -54, -56
PKR, MxA, 2'-5'OAS,
ADAR1/2, GBP, IRF-1,
ISG15, -54, -56

Figure 9. Early and delayed events of the IFN system. In response to virus infection, a number of signal transduction pathways are activated, ultimately leading to the activation of transcription factors that regulate immediate early genes, among which are the genes encoding type I IFN. Once secreted, IFN interacts with specific receptor at the surface of surrounding cells to induce the JAK/STAT signaling pathway, resulting in the activation of the ISGF-3 transcription factor and the production of IRF-7. Upon virus infection, IRF-3 and IRF-7 contribute to the expression and amplification of the IFN response by inducing delayed type I IFN genes and genes resulting in an antiviral state.

I. 5. 1. Functional domains of IRF-3

IRF-3 was characterized as a component of DRAF1 complex (89, 195). Among the IRF family, IRF-3 and IRF-7 have been identified as key regulators for the induction of IFNs (96, 97, 155, 181). The IRF-3 gene encodes a 427-amino acid protein of 55 kDa and is present as a single copy located to chromosome 19q13.3-13.4. (14). IRF-3 is constitutively expressed in every cell type tested to date, from immortalized/tumor cell lines to primary cells and freshly isolated tissues (96, 164, 195, 207). Unlike NF-κB, which is tightly associated with the regulatory subunit IκB in unstimulated cells, dormant IRF-3 presumably is free of associated molecules and is present in two forms (I and II) when resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (96, 164). Like all cellular IRF family proteins, IRF-3 possesses an amino-terminal DNA binding domain (DBD) that specifically binds to a conserved IFN-stimulated response element (ISRE motif) and a carboxyl-terminal IRF association domain (IAD) that mediates protein-protein interactions. IRF-3 also contains a transactivation domain (aa 134-394) and two autoinhibitory domains (ID) found within: the proline-rich sequence (aa 134-197) and the extreme C-terminal end (aa 407-414). Those two ID domains interact to generate a closed conformation that is likely to mask the C-terminal IAD, the DBD, and the nuclear localization sequence (NLS) of IRF3,

which prevents homodimerization and DNA binding in uninfected cells (Figure 10) (193). Nuclear export assay clearly demonstrated that the cytoplasmic localization of IRF-3 is a result of continuous export mediated by a nuclear export signal (NES) present in the middle part of the molecule (89, 207). Disruption of NES by mutagenesis resulted in nuclear IRF-3, but the mutant is incapable of gene activation, suggesting that initial cytoplasmic localization is crucial for the specific phosphorylation.

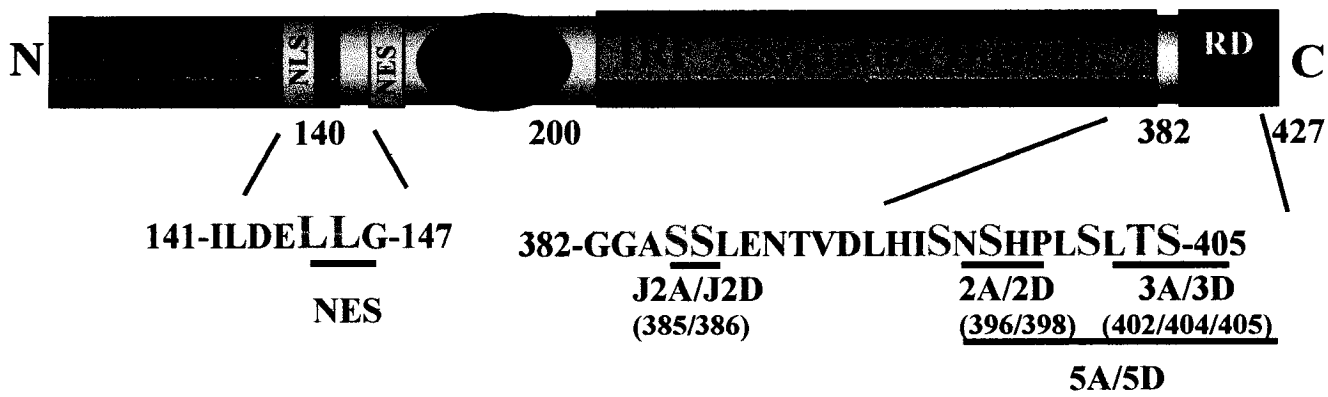


Figure 10. Schematic representation of IRF-3 transcription factor. Different domains are shown: the nuclear localization sequence (NLS), the nuclear export sequence (NES), the DNA-binding domain (DBD), the proline-rich sequence (Pro), the inhibitory domain (ID), the IRF association domain (IAD), and the signal response domain (RD). The sequence of aa 141-147 and the sequence of aa 382-405 are amplified below the schematic. The amino acids targeted for alanine or aspartic acid substitution are shown as larger letters, and point mutations are indicated below the sequence.

I. 5. 2. IRF-3 activation following virus infection

IRF-3 demonstrates a unique response to viral infection. Upon viral infection, IRF-3 is post-translationally modified and activated through phosphorylation within the C terminus of the protein on serines 385 and 386 (107) but also on serine residues 396, 398, 402, and 405 and threonine 404 (Figure 10) (96, 162, 164). A series of noncharacterized molecular events are involved in the activation of the virus-activated kinase (VAK), which seems to use the C-terminal end of IRF-3 as a substrate (Figure 11). Phosphorylation of the C-terminal Ser clusters and one Thr residue results in the appearance of slower migrating forms of IRF-3 (form III and IV) in SDS-PAGE (7, 96, 162, 164, 169, 195, 207). These modifications of IRF-3 induce conformational changes that relieve the intramolecular interaction between the two ID and thus reveal several important regions, including (1) the NLS, necessary for its import into the nucleus, (89) (2) the IAD, involved in homodimerization, (97) and (3) the DBD, which mediates stable association to promoter/enhancer regions containing cis-acting elements ISRE and positive regulatory domain I/II (PRDI/III), resulting in the induction of type I IFN and other cytokines, such as RANTES and interleukine 15 (Figure 11) (8, 51, 58, 78, 95, 96, 98, 157, 193, 195, 206).

Finally, virus-induced phosphorylation of IRF-3 is a signal for proteasome-mediated degradation of IRF-3, since mutations altering serine and threonine residues at S396, S398, S402, T404 and S405 to alanines inhibit virus-induced IRF-3 phosphorylation and degradation, indicating that serine or threonine phosphorylation subsequent to viral infection signals degradation of this IRF protein (96, 150).

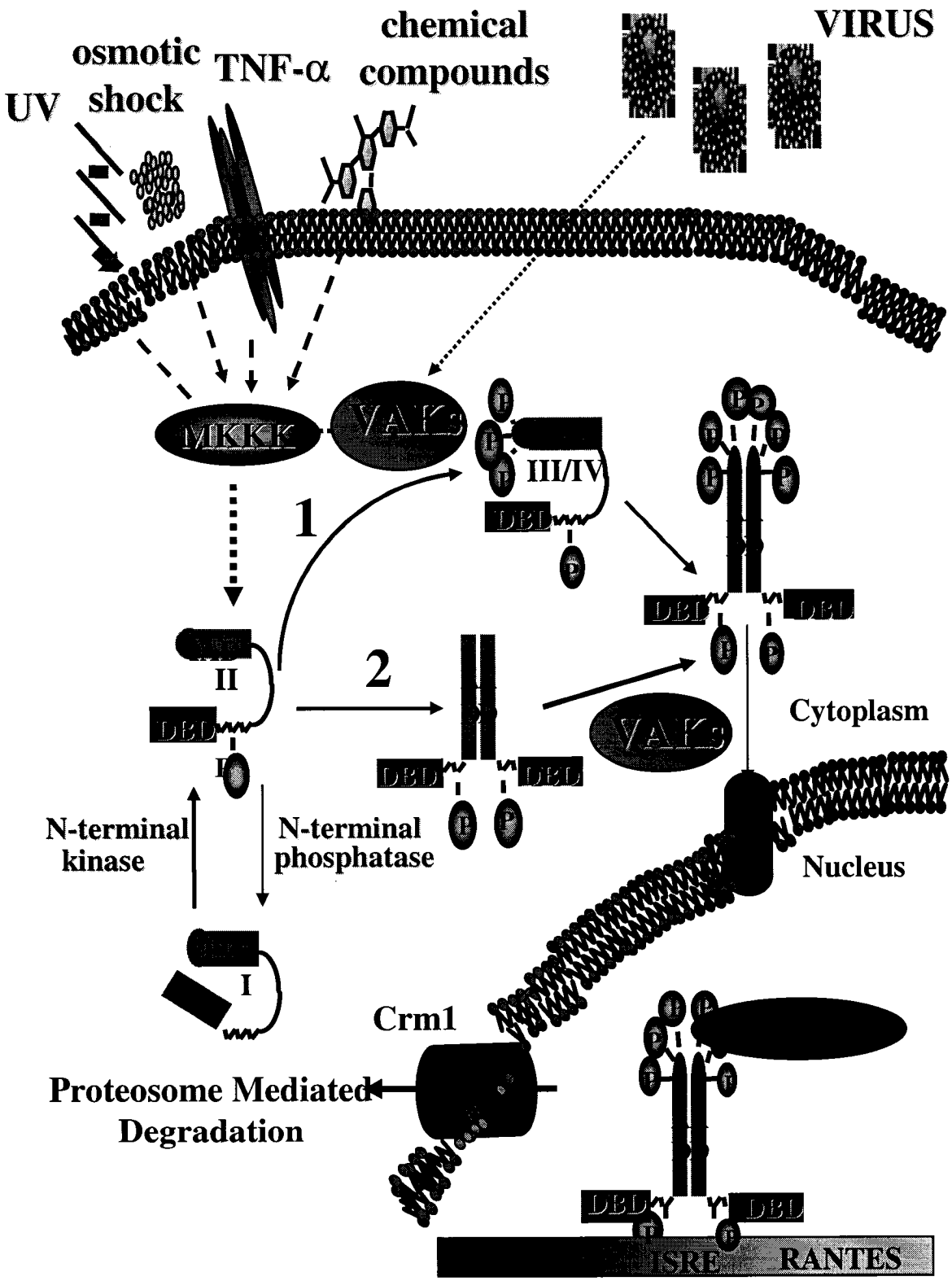

osmotic shock
UV
TNF-α
chemical compounds
VIRUS
MKKK
YAK3
III/IV
DBD
1
2
II
I
YAK3
Cytoplasm
N-terminal kinase
N-terminal phosphatase
Nucleus
Crm1
Proteosome Mediated Degradation
ISRE
RANTES

Figure 11. Schematic representation of IRF-3 activation following N-terminal and C-terminal phosphorylation. In uninfected cells, intramolecular association between the C-terminus and the DNA-binding domain (DBD) maintains IRF-3 in a latent state in the cytoplasm by masking the DBD, nuclear localization sequence (NLS) and IRF-association domain (IAD) regions of the protein (form I). Basal activities of both N-terminal kinase and phosphatase may affect the overall ratio between IRF-3 form I and form II. Treatment of cells with stress inducers, DNA-damaging agents, and growth factors activates an MAPKKK-related pathway involved in the positive regulation of the N-terminal kinase, resulting in an increase in the expression level of form II. N-terminal phosphorylation may induce a conformational change that reveals phosphoacceptor sites for virus-activated kinase (VAK) in the C-terminal end of IRF-3. C-terminal phosphorylation by VAK then relieves the intramolecular association between DBD and IAD, leading to homodimerization of IRF-3. Dimerized IRF-3 then accumulates in the nucleus and activates genes through DNA binding and CREB binding protein (CBP) association before proteasome pathway degrades IRF-3. Among the genes activated are IFN-β and IFN-α1. which act in both autocirne and paracrine fashion to induce the upregulation of a number of IFN-stimulated genes (ISG).

A variety of studies identifying viral activator of IRF-3 has shown that Sendai virus, measles virus (MeV), Newcastle disease virus, vesicular stomatitis virus, respiratory syncytial virus, sin nombre virus and Hantaan virus activate IRF-3 during the course of infection (23, 131, 164, 173). This list of viruses is restricted to closely related, negative-stranded, enveloped RNA viruses suggesting that the C-terminal phosphorylation of IRF-3 may be due to a specific component of the viral life cycle. Indeed, a recent study has shown that N nucleocapside (N) protein of MeV induced IRF-3 activation *via* the induction of the virus-activated kinase (VAK) responsible for IRF-3 phosphorylation (184). Interestingly, N protein physically interacts with IRF-3, implying that IRF-3 itself represents the molecule that detects the viral pathogen *via* nucleocapside recognition.

Mutagenesis of IRF-3 shows key residues for the activation of the C-terminal region ISNSHPLSLTSDQ of IRF-3 (Figure 10) (96). Substitutions by alanine of the serine and threonine residues at S396, S398, S402, T404 and S405 in the C-terminal

domain inhibits virus-induced IRF-3 phosphorylation, translocation and degradation, indicating that serine/threonine phosphorylation subsequent to viral infection signals activation of this IRF protein (96). Furthermore, recently, Ser396 has been shown to be phosphorylated *in vivo* following virus infection suggesting that the Ser396 residue is critical for IRF-3 activation (162). Mutation of the Ser-Thr sites to the phosphomimetic aspartic acid created a constitutively active form of IRF-3, termed IRF-3 (5D). IRF-3 (5D) behaves like virus-activated IRF-3, with the capacity to dimerize, translocates to the nucleus, associate with CBP/p300, bind to DNA, and activate the transcription of target genes in the absence of viral infection (8, 58, 95-98). IRF-3 5D has previously been shown to induce apoptosis when overexpressed in cell lines (70, 194).

Yoneyama & collaborators localized the carboxy-terminal phosphorylation sites to Ser385 and Ser386 (Figure 10) (207). Point mutations of either of these sites to alanine were generated and the mutants were no longer activated by virus infection (207). However, mutation of Ser385/Ser386 to phosphomimetic aspartic acid (IRF-3 (J2D)) does not generate a constitutively active form of IRF-3 (97, 162). As with Ser385/Ser386A mutation, the Ser385/Ser386D mutation blocks virus-induced dimerization, association with CBP/p300 coactivators, DNA binding, and transcriptional activity (97, 162). Therefore, phosphorylation of the Ser/Thr cluster between aa 395 and aa 407 of IRF-3 but not the Ser385 and Ser386 residues plays an important role in IRF-3 DNA binding and transactivation activity. The Ser385 and ser386 residues are also critical but may be involved in the interaction with the kinase(s) that ultimately phosphorylated IRF-3 at the downstream Ser/Thr sites or may be important for coactivator association (97, 162).

Double-stranded or highly structured RNA (dsRNA) plays a central role in the innate cellular antiviral response (11, 26, 29, 30, 142, 170). These RNA ligands are produced within cells infected with both DNA and RNA viruses. A synthetic compound called poly(I:C), which mimics the structure of dsRNA, also has the capacity to induce a subset of IFN-stimulated gene (ISG) and cytokines intended to impede viral replication and spread. Indeed, one of the ISRE-binding factors shown to be induced by dsRNA is DRAF1, which is composed of subunits including IRF-3, coactivators CBP/p300, and an uncharacterized tyrosine phosphorylated protein (194, 195). Recent studies have demonstrated that treatment with dsRNA was sufficient to activate IRF-3 (107, 108, 136, 191, 193, 195). However, no phosphorylation of IRF-3 in response to poly I:C treatment has been demonstrated. Recently, for the first time, a study demonstrated that Ser396 within the C-terminal Ser/Thr cluster of IRF-3 is targeted *in vivo* for phosphorylation following double-stranded RNA (dsRNA) (162).

I. 5. 3. Active form of IRF-3 associates with coactivators

IRF-3 activation results in the association with histone acetyltransferase (HAT) activity in a virus-dependent manner. CBP and p/300 were the first coactivators shown to be tightly associated with IRF-3 (89, 96, 97, 193, 195, 207). P300/CBP-associated factor (PCAF) and TBP-associated factor 250 (TAFII250), other proteins with HAT activity, were also shown to associate with IRF-3 after its activation (98). Chromatin remodeling through histone acetylation by the HAT activity of these proteins may be essential for constituents of the basal transcriptional machinery, such as transcription factor IID (TFIID), to gain access to transcriptionnally repressed chromatin containing IRF target promoters (191). Most importantly, the binding of IRF-3 to CBP seems to be a key step in

the nuclear accumulation of the transcription factor (89). In this context, CBP serves as a transcription coactivator but also as an anchor by preventing the export of IRF-3 back to the cytoplasm. The activated form of IRF-3, bound to CBP, induces transcription through distinct positive regulatory domains in the type I IFN promoters, and through select ISRE sites (96-98, 157, 193, 195, 207). Finally, IRF-3 is degraded by a proteasome-mediated mechanism since treatment with proteasome inhibitors also stabilizes IRF-3 protein levels (Figure 11) (96, 150).

The signaling pathway leading to IRF-3 phosphorylation and activation remain to be elucidated. IRF-3 is known to be activated in response to virus infection, but recent studies indicated that IRF-3 might also be a phosphorylation target following stimulation of cellular stress pathways or the engagement of TLR receptors.

I. 5. 4. IRF-3 phosphorylation following stimulation with stress inducers

Stress inducers and DNA damaging agents may also functionally activate IRF-3. Kim *et al*, (81, 82) demonstrated that treatment of Hela cells with stress inducers and genotoxic agents such as DNA damaging agents doxorubicin and UV radiation stimulated IRF-3 phosphorylation, nuclear translocation, CBP association, and transcriptional activation of an IRF-3 responsive promoter. These studies implicated MEKK1 in the activation of IRF-3 through the JNK pathway but p38 and IKK-independent. Phosphorylation of IRF-3 by DNA damaging agent doxorubicin, the osmotic shock inducer sorbitol, the stress inducer anisomycin, the phorbol ester PMA and by the overexpression of MEKK1 was observed at the N-terminal end between aa 186-198 (Figure 11) (164), which differs from the phosphorylation site(s) targeted by virus at the C-terminal. However, pharmacological and molecular studies suggest that

phosphorylation of IRF-3 occurring at the N-terminal following the treatment with the same stress-inducers and DNA damaging agent as Kim's group does not result in nuclear accumulation through CBP association nor does it transactivate the RANTES promoter (164). In this study, the N-terminal phosphorylation resulted in the accumulation of the form II in treated cells. Recently, N-terminal phosphorylation at residue Thr135 was shown to be the target of DNA-PK kinase after virus infection, which resulted in nuclear retention of IRF-3 (79). Those studies implicate different signaling pathways that lead to IRF-3 activation. The functions of N-terminal phosphorylation of IRF-3 remain to be elucidated.

I. 5. 5. IRF-3 activation following LPS treatment

In addition to virus or dsRNA, lipopolysaccharide (LPS) can induce production of IFN in certain cells, albeit usually at low levels (168). LPS or endotoxin is a predominant and structural component of the outer membrane of Gram-negative bacteria. Recently, the family of Toll-like receptors (TLRs) has been found to function as pattern-recognition receptors, which recognize Pathogen associated molecular patterns (PAMPs) (Figure 12) (66, 87, 138, 158). The TLR family is now comprised of at least 10 members in mammals (114, 147, 177). One of the members of TLRs, TLR4 has been shown to be essential for the recognition of LPS (Figure 13) (21, 22, 113, 175, 176). LPS/TLR4 interaction is transmitted to adapter molecules, MyD88, IRAK and TRAF6, and this event is followed by the triggering of an IκB kinase (IKK) and mitogen-activated protein (MAP) kinase cascade resulting in the activation of transcription factors NF-κB, AP-1 and ATF-2 (Figure 13) (1).

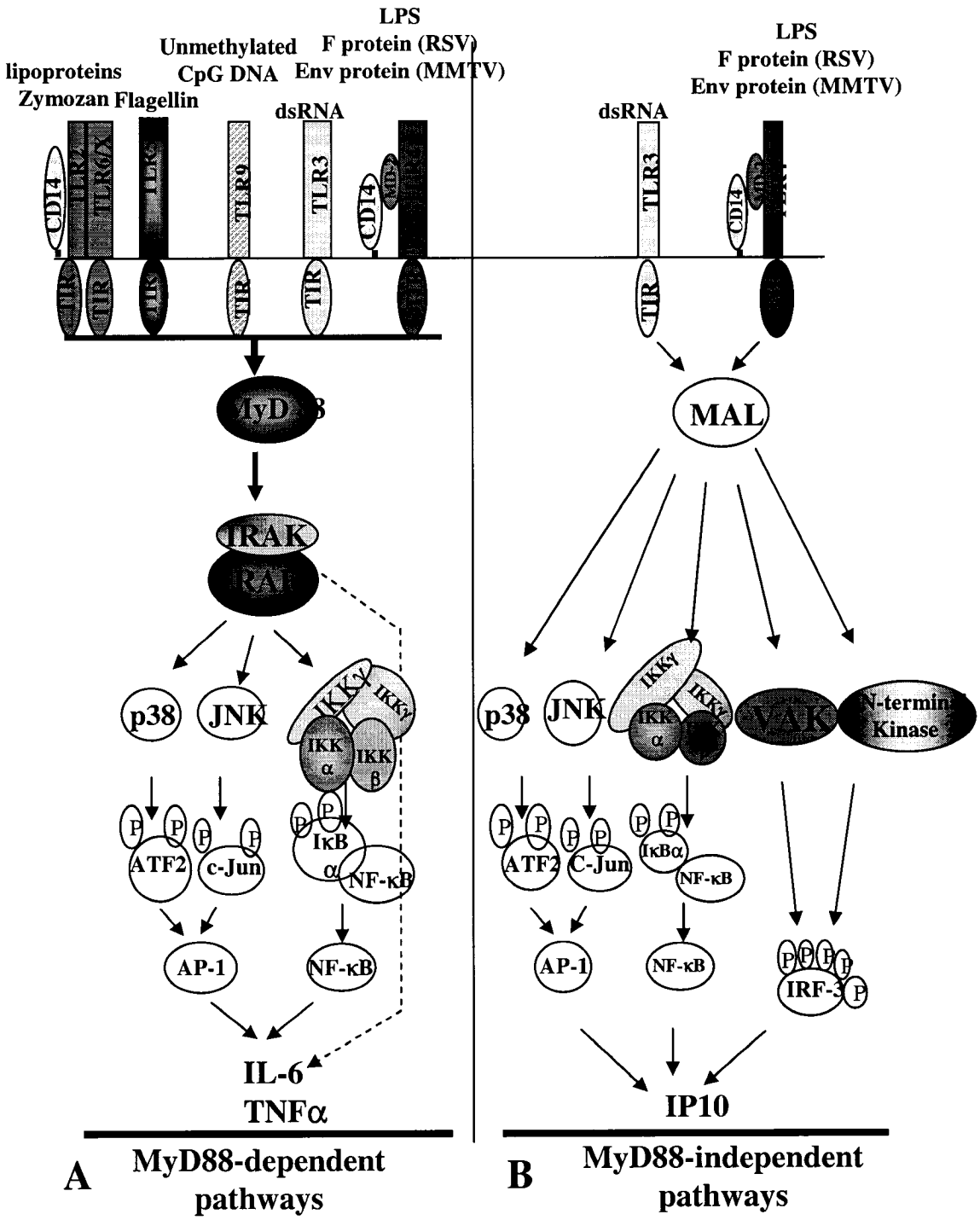

LPS
Unmethylated
F protein (RSV)
lipoproteins
CpG DNA
Env protein (MMTV)
Zymozan Flagellin
dsRNA
LPS
F protein (RSV)
Env protein (MMTV)
dsRNA
CD14
TLR2/
TLR6/X
TLR5
TLR9
TLR3
CD14
TLR4
TLR3
CD14
TLR4
TIR
TIR
TIR
TIR
TIR
TIR
TIR
MyD88
MAL
IRAK
RAI
p38
JNK
IKKγ
IKKγ
IKK
α
IKK
β
ATF2
c-Jun
IκB
α
NF-κB
AP-1
NF-κB
IL-6
TNFα
A
MyD88-dependent
pathways
p38
JNK
IKKγ
IKKγ
IKK
α
VAK
N-termin
Kinase
ATF2
C-Jun
IκBα
NF-κB
AP-1
NF-κB
IRF-3
IP10
B
MyD88-independent
pathways

Figure 12. Signaling through the Toll-like receptors. Molecular components involved in TLR signaling are shown (A and B).Activated TLRs associate with cytoplasmic adapter molecule, MyD88, through the homophilic interaction between their TIR domains. MyD88 interacts with the Ser/Thr kinase IRAK which subsequently activates the TRAF6 adapter. TRAF6 in turn activates the stress-related MAPK pathways JNK and p38, as well as the IKK complex leading to AP-1 and NF-κB activation respectively, and the induction of cytokines such as TNF-α and IL-6 (A). TLR-3 and TLR-4 alos activates MyD88-independent pathway (B). IKK, JNK and unidentified IRF-3 kinase can be induced in the absence of MyD88 and TRAF6 and lead to the induction of IFN-inducible genes, possibly trough the adapter molecule MAL. The fact that TNF-α and IL-6 production is totally abrogated in the MyD88-independent pathway under conditions where NF-κB and AP-1 are still activated suggests that an unidentified pathway in addition to p38/JNK and IKK is essential for cytokine production (dashed line in A).

Recent studies also demonstrated that TLR4 functions in the recognition of virus components. The innate immune response to RSV is mediated by the interaction of fusion protein of RSV with TLR4 and CD14 (90) and activation of B cells by mouse mammary tumor virus is accomplished by interaction between viral envelope protein and TLR4 (146).

Activation of IRF-3 by LPS was initially documented in the human astrocyte cell line U373 (130) where Navarro and David reported that LPS treatment of human U373 astrocytoma cells resulted in IRF-3 nuclear translocation and DNA binding activity *via* a TLR receptor and p38 dependent pathway. Furthermore, treatment of mouse macrophages with LPS results in the activation of IRF-3 in a MyD88-independent manner (80). Recently, Akira's group observed induction of the *IP-10* gene by lipid A, the functional moiety of LPS, in MyD88-deficient peritoneal macrophages in which production of TNF-α and IL-6 in response to LPS is completely impaired (80). In this model however, delayed activation of IKK and JNK and activation of IRF-3 were still observed. These results suggest that a MyD88-independent pathway(s) mediates NF-κB, JNK/p38, and IRF-3 activation in response to TLR4 signaling (Figure 12. 14). Furthermore, it implicates

that TLR4 is composed of at least two distinct pathways, a MyD88-dependent pathway that is critical to the induction of inflammatory cytokines and a MyD88/TNFR-associated factor 6-independent pathway that regulates induction of *IP-10* (Figure 12). Mal, the MyD88-adapter like, has been shown to be essential in TLR4 signaling (45). Mal is thus an attractive candidate as the adapter responsible for MyD88/TRAF6-independent pathway. The anti-Mal/TIRAP peptide used in the TIRAP study prevented induction of IFN-β (71, 72). This report is the first indication that the MyD88-independent pathway activated by TLR4, which leads to induction of IRF-3-dependent genes, requires Mal. It is possible that IRF-3 activation in response to LPS occurs *via* induction of IFN-β, with Mal having no role in IRF-3 activation, but participating in IFN-β induction *via* an unknown mechanism. However, Servant and collegues have shown that phosphorylation of Ser396 at the C-terminal cluster is not detected by the Ser396 phosphospecific antibody in LPS-treated cells, suggesting another pathway for IRF-3 activation following TLR-4 stimulation (162). Another target gene, called *UBP43* was also recently shown to be induced by LPS *via* IRF-3 in the murine macrophage-like cell line RAW 264.7 (102). In apparent contradiction to these observations, Pitha's group reported that LPS inhibits the virus-mediated activation of IRF-3 (77).

IRF-3 activation by LPS still remains unclear. Future research on TLR4 signaling pathway and on the phosphorylation states of IRF-3 after LPS treatment may clarify its precise mechanism of activation. Virus infection clearly induces C-terminal phosphorylation, whereas stress-inducers induce N-terminal IRF-3 phosphorylation. N-terminal phosphorylation might be induced by LPS treatment and therefore involve a

different kinase in IRF-3 activation. Thus, multiple signaling pathways might be involved in IRF-3 activation.

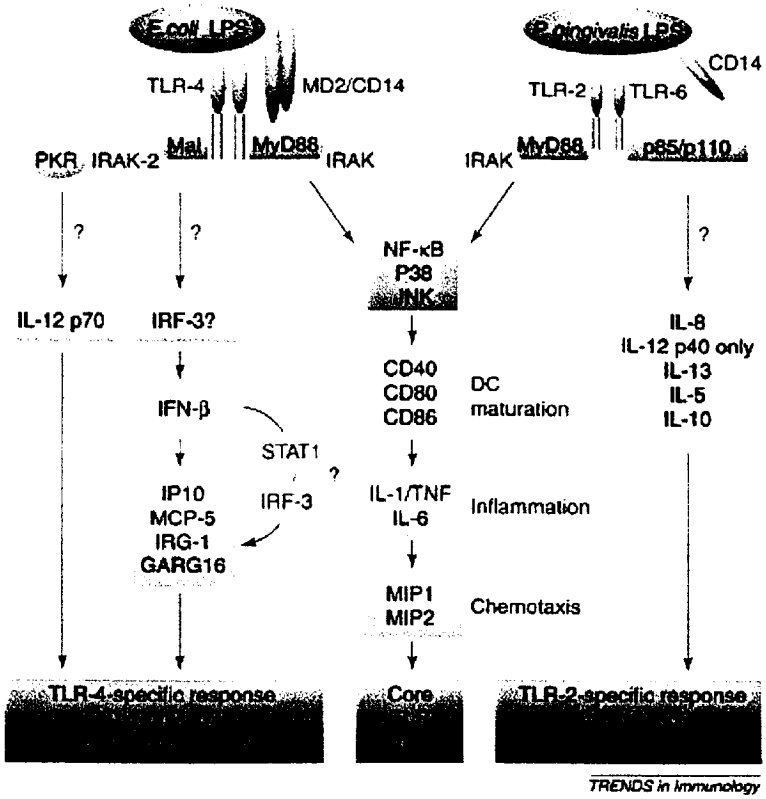


Figure 13. Three recent studies have demonstrated that Toll-like receptor (TLR-4) antagonists such as *E. coli* lipopolysaccharide (LPS) and TLR-2 agonists such as *P. gingivalis* LPS or peptidoglycan, whilst inducing common set of genes, also induce distinctive genes. The middle panel shows common signal activated by TLR-4 and TLR-2, which lead to induction of a range of genes. The receptor-proximal signals for this core TLR reponse are MyD88 and IRAK. On the left-hand side, the genes that are only induced by TLR-4 are shown. These may be elicited by MyD88-adapter-like (Mal), IRAK-2 and protein kinase R (PKR), and may involve activation of the transcrition factor IRF-3. The gene product induced specifically by TLR-4 induce Th1-like responses and may contribute to toxicity in sepsis. On the right-hand side, the genes that are only induced by TLR-2 agonists are shown. These may be elicited in part by the p85 subunit of PI3 kinase. The gene product will induce a Th2-like response and will induce lower toxicity. Mal is also able to regulate core signals such as NF-κB and p38, it can interact with MyD88 and can promote dendritic cell maturation. A signal exclusive to Mal that cannot be driven by MyD88 has yet to be described, although there is evidence pointing to IRF-3 and the genes on the left. Most recently, it has been shown that Mal but not PKR is involved in the induction of interferon-β, which may then drive genes such as IP-10 and MCP-5 in an autocrine loop. because IRF-3 is required for these genes, it is possible that Mal is not on the patway of IRF-3, but instead is required for induction of interferon-β via an as yet unknown machnism. Interferon-β would then activate IRF-3 (and STAT1) to induce the genes (O'Neill, 2002).

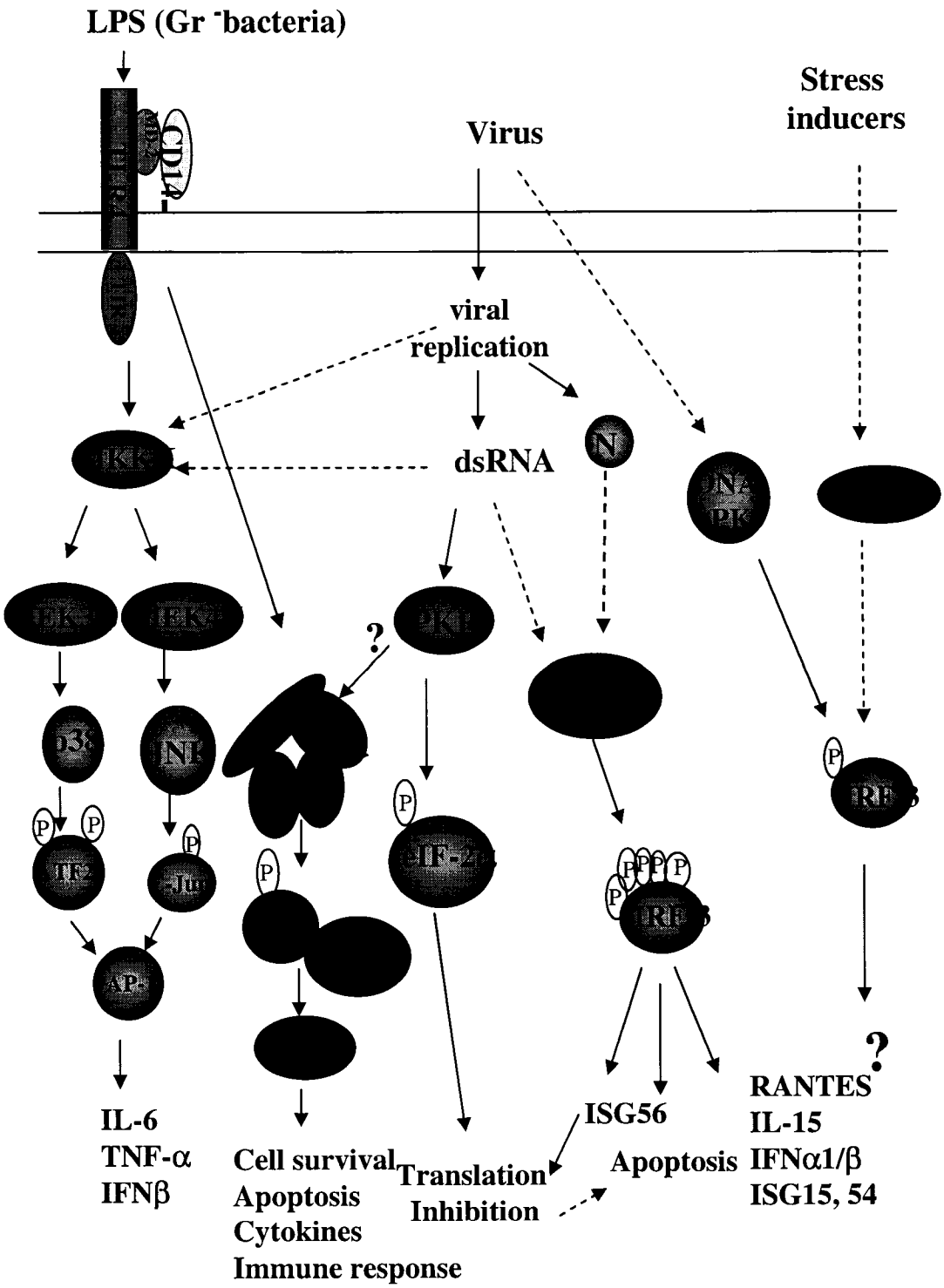

LPS (Gr -bacteria)
Virus
Stress inducers
viral replication
dsRNA
?
IL-6
TNF-α
IFNβ
Cell survival
Apoptosis
Cytokines
Immune response
Translation
Inhibition
ISG56
Apoptosis
RANTES
IL-15
IFNα1/β
ISG15, 54
?

Figure 14. A model of signaling cascades triggered by viruses and PAMPS. Virus infection results in the activation of multiple signaling cascades resulting in the phosphorylation of ATF-2, C-Jun, IRF-3 and NF-κB. Following virus replication ,the generation of dsRNA activates the stress-induced MAPK pathway p38/JNK resulting in the AP-1 activation. PKR is activated following binding to dsRNA, phosphorylates eIF-2α and inhibits protein translation. PKR may also associate with the IKKβ subunit. Viral nucleocapsid (N) and dsRNA activate VAK, a virus activated kinase, leading to C-terminal phosphorylation of IRF-3. IRF-3 activation stimulates target genes such as RANTES, IL-15 and IFNs. Some Interferon-stimulated genes (ISGs) are also induced, one of which ISG56 has been linked to inhibition of protein synthesis. Induction of DNA-PK and MKKK-related pathways following treatment with stress-inducers leads to N-terminal phosphorylation of IRF-3 to which no functional roles have been assigned yet. Components of bacterial cell wall, such as LPS, activate TLR-4 signaling, leading to cytokine production through the activation of JNK/p38 and IKK complex. Dashed lines represent uncharacterized signaling pathways.

I. 5. 6. Gene activation by IRF-3

Genes targeted by IRF-3 include classical IRF-responsive genes IFN-α1 (murine α4), and IFN-β promoter (78, 107, 156, 157), which are the immediate-early genes activated in response to viral infection by a protein synthesis-independent pathway. IRF-3 alone is not sufficient to induce expression of endogenous human IFN-α1 and IFN-β (70, 204, 205). Together with the transcription factors NF-κB and ATF-2/cJun, IRF-3 forms a transcriptionnally active enhanceosome complex at the IFN-β promoter (37, 38, 83, 115, 141, 185). In addition to its involvement in the transcriptional induction of immediate-early IFN genes, IRF-3 also directly controls the expression of the CC chemokine RANTES in response to paramyxovirus infection (51, 95) and the cytokine IL-15 (8). Furthermore, human cytomegalovirus (HCMV) and herpes simplex virus type 1 (HSV-1)-induced activation of the *ISG54* gene has been shown to be mediated by a transcriptional activator complex that contains IRF-3 (131, 145). Recently, another member of the IFN-stimulated gene family, *ISG56*, was shown to be a direct target of IRF-3 (58).

CHAPTER II.

HYPOTHESIS, OBJECTIVES, RATIONALE

The ability of IRF-3 to directly upregulates a member of the chemokine superfamily such as RANTES broadens the range of IRF-3 immunomodulatory targets and indicates that the role of IRF-3 is not solely restricted to the IFN system. Because of its critical role in the activation of the IFN cascade, the upregulation of chemokines and the induction of apoptosis, IRF-3 was selected as a prototype gene to modify B16 melanoma cells to determine whether ectopic IRF-3 expression could enhance cytokine production and triggers an antitumor response in syngeneic mice. Since B16 is a well characterized, poorly immunogenic subcutaneous tumor (160, 161), we focused our studies on this model to evaluate if IRF-3 could be used as an immunomodulatory transcription factor.

The first aim of the study was to characterize the gene-modified B16 cells to verify if expression of IRF-3 was stable and functional. Several experiments were performed to demonstrate IRF-3 activation in B16 melanoma cells. The second specific aim was to investigate the effects of IRF-3 expression into gene-modified tumor cells when injected into syngeneic mice.

Further analysis demonstrated that gene transfer of IRF-3 into B16 melanoma cells inhibited tumor development, where IRF-3 seems to play a role in the modulation of the cytokine profile of the tumors, the recruitment of inflammatory cells to the site of the tumor and the enhancement of the immunogenicity of the B16 melanoma cells by up-regulating MHC class I expression.

CHAPTER III.

MATERIAL AND METHODS

Animals

Specific pathogen-free female C57BL/6 mice and immunodeficient mice (SCID), 6-8 weeks old, were purchased from the Charles River (Montreal, Canada) and housed 6 per cage in a temperature-controlled and light-controlled environment. The animals were maintained on standard laboratory food and water *ad libitum* and housed according to the guidelines of the Canadian Council on Animal Care.

Cell culture, medium and reagents

B16.F0, a murine melanoma cell line of C57BL/6 origin, and the 293 GPG retroviral packaging cell line, were obtained as a generous gift from Dr. Jacques Galipeau. Cells were maintained in Dulbecco's modified Eagle Medium (Wisent) supplemented with 10% heat-inactivated fetal bovine serum (Wisent) and antibiotics at 37°C and 5% CO_2.

Generation of human IRF-3 B16 cell lines

Construction and characterization of the bicistronic AP2 green fluorescent protein retroviral vector has been described previously (49). cDNA of human IRF-3 was inserted into multiple cloning site of the retrovector AP2 (EcoR1blunt/XhoI). Pantropic retroviral supernatant was generated by transfection of the retrovirus vector p(AP2)-GFP or p(AP2) IRF-3-GFP into 293 GPG packaging cells using Lipofectamine (Life Technologies, Inc.) and sorted by fluorescence activated cell sorting (FACS) for GFP expression. For retroviral transduction, 10^6 B16 melanoma cells were seeded in 10cm plate and incubated with retroviral supernatants filtered from virus-producing cultures in the presence of lipofectamine (6μl/ml), which increases the transfection of the cells (49). The stable

transfectants, IRF-3-GFP B16 and AP2-GFP B16 (AP2-B16) cells were selected by FACS sorting and tested for IRF-3 expression by immunoblot analysis.

Immunoblot analysis

To prepare whole cell extracts, B16 cells (AP2 and IRF-3 B16 generated cell lines) and tumor samples (from mice injected with either IRF-3 B16 cells or AP2 B16 cells) were washed with phosphate-buffered saline (PBS) and lysed in 50mM Tris (pH 7.4), 150mM NaCl, 2mM EDTA, 30mM β-glycerophosphate, 10mM NaF, 10% glycerol, 1% Nonidet P-40, 0.1 mM Na3VO4 supplemented with a proteases inhibitors. Whole cell extracts from IRF-3 and AP2 B16 cell lines and from tumor samples were subjected to SDS-PAGE in a 7.5% polyacrylamide gel. Following electrophoresis, the proteins were transferred to a Bio-rad transfer membrane (BIO-Rad) in buffer containing 30mM Tris, 200mM glycine, and 20% methanol for 2h at 50V at 4°C. The membrane was blocked in 5% dried milk in Tris-buffered saline (PBS) for 1h at room temperature and was then probed with 1μg/ml of polyclonal IRF-3 antibody (Santa-Cruz) or from P. Pitha. The signal was detected with secondary antibodies conjugated to horseradish peroxidase at a dilution of 1:1000 and developed with chemiluminescence substrate (Amersham Inc.).

Immunocoprecipitation

For coprecipitation studies, 1μg of mouse CBP (mCBP)/p300 A-22 (Santa-Cruz) was covalently bound to Sepharose-protein A beads (Amersham) in 0.2 triethanolamine pH 9.0 with 5.2 mg of dimethyl pimelimidate (Sigma Inc.) for 2h at 4°C. The beads were combined with 400μg of whole cell extract from IRF-3-B16, AP2-B16 cells and from

tumor samples (IRF-3 and AP2) and incubated at 4°C for 4h. The beads were washed five times with Nonidet P-40 lysis buffer, resuspended in denaturing sample buffer, boiled and bound proteins were separated by SDS-PAGE and analyzed by immunobloting using IRF-3 antibody from Santa-Cruz. Immunocomplexes were detected by ECL, chemiluminescence-based system

Electrophoretic mobility shift assay (EMSA)

Equivalent amounts of hIRF-3 and AP2 B16 nuclear extracts from infected (Sendai virus 4hr, 40 HAU/10^6) and uninfected B16 cells were assayed for IRF-3 binding in a gel shift analysis using ^{32}P-labeled double-stranded oligonucleotide corresponding to the interferon-stimulated response element of the ISG15 gene (5'-GATCGGAAAGGGAAACCGAAACTGAAGCC3'). Complexes were formed by incubating the probe with 15μg of whole cell extract for 20 min at room temperature in 10 mM Tris-Cl (pH 7.5)-1 mM EDTA-50 mM NaCl-2 mM dithiothreitol-5% glycerol-0.5% Nonidet P-40-1μg/μl of poly(dIdC). Extract were run on a 5% polyacrylamide gel (60:1) cross-link) prepared in 0.28X Tris-borate-EDTA (TBE). After running at 160 V for 3h, the gel was dried and exposed to a Kodak film at –70°C overnight. To demonstrate the specificity of the detected signal, 1μg of anti-IRF-3 (Santa-Cruz FL-425) was incubated for 30 min on ice prior to the addition of the probe to observe a supershift in the complex formation. CBP complex formation was also analyzed by supershift assay using 1μg of anti CBP (Santa-Cruz A-22).

Assay of cell growth *in vitro*

Subconfluent cells were trypsinized, washed, and plated in triplicate in 6 well plates at 4 X 10^4 cells per well in 2 ml of the usual medium. Every day for each group of cells, 3 wells were trysinized and counted after trypan blue exclusion in a hemacytometer. Medium was changed every 3 days. Doubling time was determined by calculating the growth rate of exponentially growing cells.

Tumor Formation

Young (6-8 weeks) female C57BL/6 mice were shaved in the right flank area and injected s.c. with either $1x10^6$ mock-transfected AP2- B16 cells or hIRF-3 B16 cells in a total volume of 100μl of PBS. Tumor growth was followed by vernier caliper measurement every other day from day 7 after injection. All experiments included 7 mice per group. Tumor volume was calculated according to the formula $V = (a \times b^2)/2$ (a, largest superficial diameter; b, smallest superficial diameter).

Histology

Tumors were excised at day 19 (day of sacrifice) and used for subsequent RNA extraction and immunoblot analysis or fixed in formaldehyde, embedded in paraffin, and stained for histological evaluation (hematoxilin and eosin staining).

RNase Protection Analysis

Total RNA was isolated from various tumors (AP2-B16 and IRF-3-B16) at 19 days post injection (day of sacrifice) and from AP2-B16 and IRF-3 B16 melanoma cells after Sendai virus infection (40HAU) by using Trizol reagent (Life Technologies, Inc.) RNase

protection assays were performed using two Riboquant multiprobe template sets from Pharmingen. The mCK-3b and mCK-5 template was used for the T7 polymerase-directed synthesis of high specific activity [^{32}P]UTP-labeled antisense RNA probes. The probe set contained 12 probes for mCK-3b and 11 probes for mCK-5, including two housekeeping genes, *GAPDH* and *L32*. Probe ($3x10^5$ cpm) was hybridized with each RNA (10μg) sample overnight at 56°C. RNA samples were digested with RNase A and T1, purified, and resolved on 5% denaturing polyacrylamide gels. Internal housekeeping genes were analyzed to confirm equal RNA loading.

Flow cytometry analysis

IRF-3 and AP2 B16 cells were harvested by trypsinization and washed twice in FACS buffer (PBS + 0.5% BSA). 1 x 10^6 cells/well were aliquoted in round-bottomed 96-well plates and pelleted by centrifugation at 1500 rpm for 3min. The supernatants were removed and the cells were resuspended in FACS buffer + 20μg/ml Fc block (50μl/well) and incubated on ice for 15min. Antibodies were then added to the wells in 50μl of FACS buffer (20μg/ml for FITC-labeled antibodies and 8μg/ml for PE-labeled antibodies) and the plate was incubated on ice for 30 minutes in the dark. The cells were pelleted by centrifugation as before and washed twice with FACS buffer. After the final wash, the cells were resuspended in 200μl of FACS buffer and data was collected using a FACS Calibur and analyzed using the WinMDI software. All antibodies came from BD Pharmingen. The following antibodies used in this study were: FITC-B7.1, FITC-Fas, PE-B7.2, PE-FasL, PE-MHC class I and PE-MHC class II.

CHAPTER IV.

RESULTS

Characterization of the IRF-3 protein expression in B16.F0 melanoma cell line.

IRF-3- and AP2-GFP transduced B16.F0 cells were isolated by FACS analysis and expanded *in vitro*. To investigate whether the hIRF-3 protein is expressed in the retrovirally transduced B16 melanoma cells, whole cell extract were obtained from subconfluent monolayers and the cell lines were screened for hIRF-3 expression by immunoblot analysis using rabbit polyclonal human IRF-3 antibody (Figure 15, A). Empty vector control (AP2-GFP) B16 cells showed no expression of hIRF-3 (Fig. 15, lane 1) whereas the two described forms (I and II) of IRF-3 were identified in IRF-3 B16 cells (Fig 15, lane 2). The endogenous murine IRF-3 was poorly detected in the samples due to a lack of a good antibody, which could recognize the endogenous murine IRF-3 (data not shown).

Characterization of the IRF-3 expression in tumor samples.

IRF-3- and AP2- B16 cells were injected into C57BL/6 mice. Tumor were excised at day of sacrifice and lysed as described in *Material and Methods*. To test whether IRF-3 expression was maintained after injection of the IRF-3 B16 cells *in vivo*, whole cell extract obtained from tumor samples of AP2 (n=5) and IRF-3 (n=6) injected animals were analyzed for IRF-3 protein expression by immunoblot (Figure 15, B). As expected, no IRF-3 protein expression was detected in tumors samples from control animals, which received empty vector AP2-GFP B16 cells (Fig. 15 B, lanes 7-11). However, the two recognized bands (form I and II of IRF-3) were observed in tumor samples obtained from IRF-3 B16 injected animals (Fig.15, B, lanes 1-6). Surprisingly, the expression of IRF-3 is almost absent in 1 out of 6 tumor samples (lane 2) and decreased in other ones (lanes 3-5).

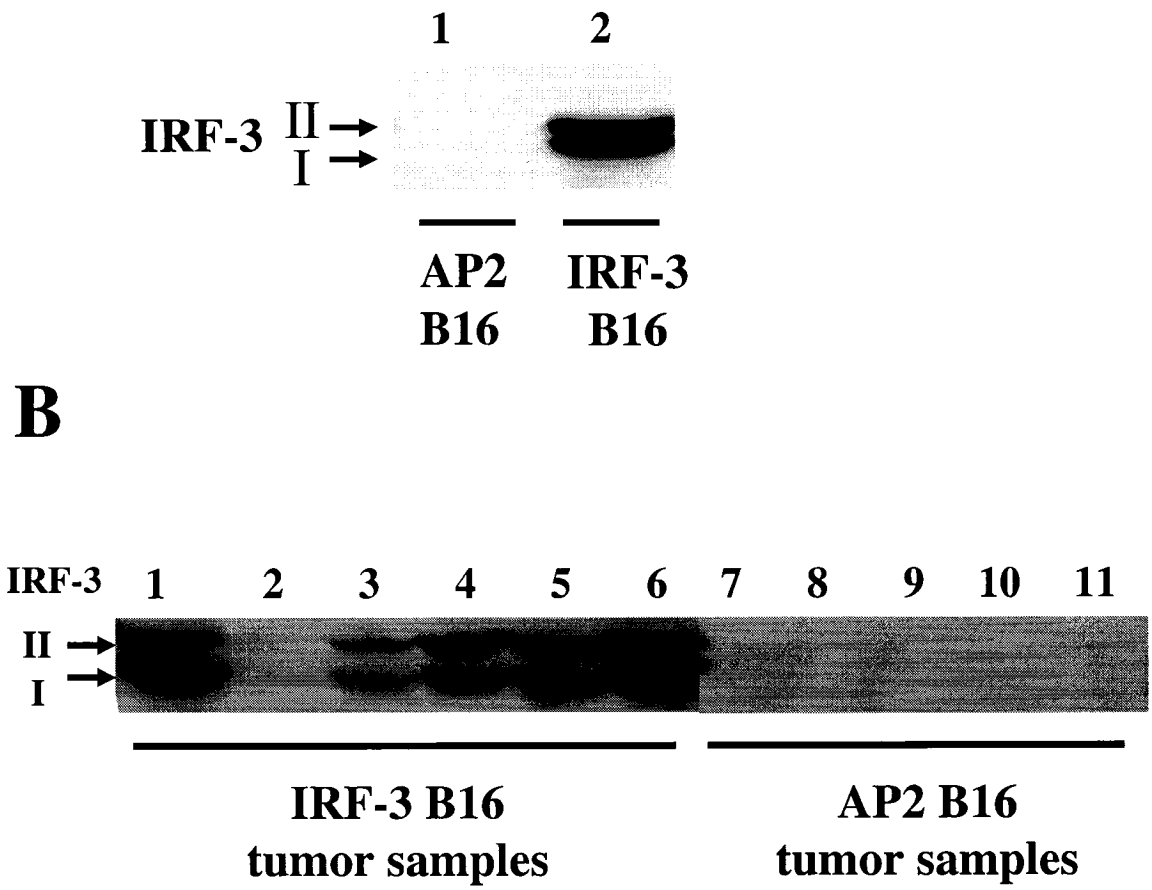


Figure 15. Expression of IRF-3 protein in retroviral transduced B16 melanoma cells and in tumors samples from mice injected with IRF-3, AP2-B16 cells. (**A**) IRF-3 and AP2-B16 cells, which were selected as described in Material and Methods, were analyzed for hIRF-3 expression by immunoblot analysis. Whole–cell extracts were fractionated by 7.5% SDS-PAGE and probed with anti-IRF-3 antibody. A 53 kDa protein (form I and II) corresponding to IRF-3 was detected in IRF-3 retrovirally transduced B16 cell line. (**B**) Whole-cell extract obtained from lysed tumor samples (IRF-3 B16 tumor samples n=6 and AP2 B16 tumor samples n=5) were subjected by immunoblot analysis. Extracts were fractionated by 7.5% SDS-PAGE and probed with rabbit polyclonal antibody anti-IRF-3 (P.Pitha).

Virus-induced phosphorylation of IRF-3 protein in IRF-3 B16 cells.

IRF-3 is expressed constitutively in various cells, and its expression is not enhanced by viral infection or by IFN treatment. In order to verify if hIRF-3 can be recognized and activated by the mouse cellular machinery, we investigated whether the hIRF-3 protein, expressed in mouse B16 melanoma cells, was capable of being phosphorylated by virus infection, a stimulus known to induce activation and phosphorylation of IRF-3 (96). IRF-3 B16 cells were infected with Sendai virus for 2, 4, 6, 8, 10, and 12 h or left uninfected (Figure 16). Whole cell extracts were then analyzed by immunoblot using polyclonal human IRF-3 antibody. Two forms of IRF-3 protein (designated I and II) were detected in uninfected cells and at 2hr post infection (Fig.16, lanes 1-2). Following virus infection, starting at 4h, a third slowly migrating form of IRF-3 was also detected (Fig. 16, lanes 3-6). Sendai virus infection resulted in two alterations in the expression of IRF-3 in B16 melanoma cells: an overall decrease in the amount of IRF-3 between 4 and 12h (Fig.16, lanes 3-7) reflecting the proteasome–mediated degradation of IRF-3 (96) and the generation of a more slowly migrating form of IRF-3 which was previously characterized (Fig.16, lanes 2-6) (96, 162, 164). The kinetics of Sendai virus infection observed in IRF-3 B16 cells clearly demonstrates that the hIRF-3 expression in B16 melanoma cells can functionally be phosphorylated by the mouse kinase homologue in the course of a viral infection.

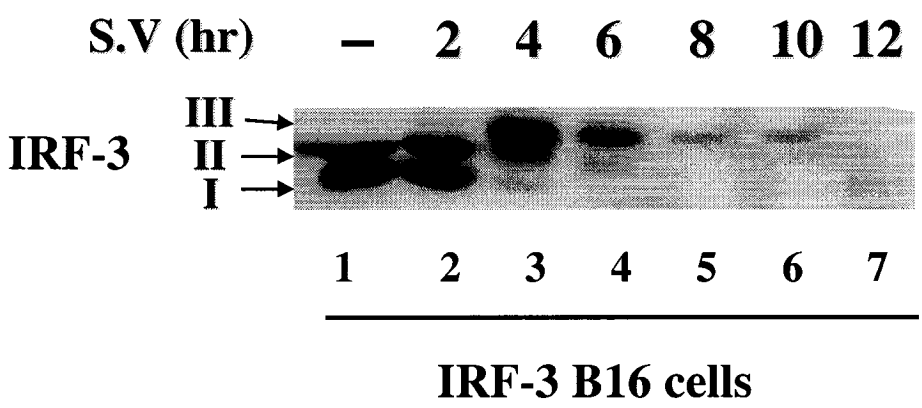


Figure 16. Sendai virus infection induces phosphorylation of IRF-3 protein in B16 melanoma cell line. IRF-3 B16 cells, which were selected as described in Material and Methods, were infected with Sendai virus (40 HAU/10^6 cells) for 2, 4, 6, 8, 10, or 12 hr (lane 2-7) or were left uninfected (lane 1). IRF-3 protein was detected in whole-cell extracts (60μg) by immunoblotting using rabbit polyclonal anti-IRF-3 antibody (Santa Cruz). Two forms of IRF-3, which were designated form I and form II, were detected (lane 1 and 2). Phosphorylated IRF-3 protein appears as distinct bands in immunoblots, migrating more slowly than IRF-3 forms I and II (lane 3 to 7).

Interaction between IRF-3 and CBP in IRF-3 B16 cells

The histone acetyltransferase coactivators CBP and p300 associate with C-terminally phosphorylated form of IRF-3 (96, 193, 195, 207). Since IRF-3 was shown to interact with co-activator CBP and p300, we next examined whether the expression of hIRF-3 in mouse B16 cells was able to associate with the co-activator CBP/p300. IRF-3 B16 cells were infected with Sendai virus for 4h or left uninfected. Co-immunoprecipitation of whole cell extract revealed that IRF-3 coimmunoprecipitated with CBP (Fig. 17, lane 2) from virus-infected IRF-3 B16 cells but not from uninfected cells (Fig.17, lane 1). This interaction IRF-3/CBP, was observed clearly in IRF-3 B16 infected cells *in vitro,* but was not seen when the immunoprecipitation was performed on the tumor samples obtained from AP2 and IRF-3 injected mice (Fig.17, lanes 3-6).

DNA-binding activity of hIRF-3 in transduced IRF-3 B16 melanoma cells.

To analyze the DNA-binding activity of IRF-3 in B16 cell lines, nuclear extract prepared from AP2- and IRF-3- B16 cells, infected with Sendai virus or uninfected, were subjected to electrophoretic mobility shift assay (EMSA) using ISRE probe from *ISG15* gene. In virus-induced IRF-3 B16 cells, a new protein-DNA complex was identified by EMSA (Fig.18, lane 4). This protein-DNA complex, which was previously characterized in detail (96, 195, 207), contained IRF-3 as confirmed by supershift analysis with IRF-3 antibody FL-325 (Fig 18, lane 8).

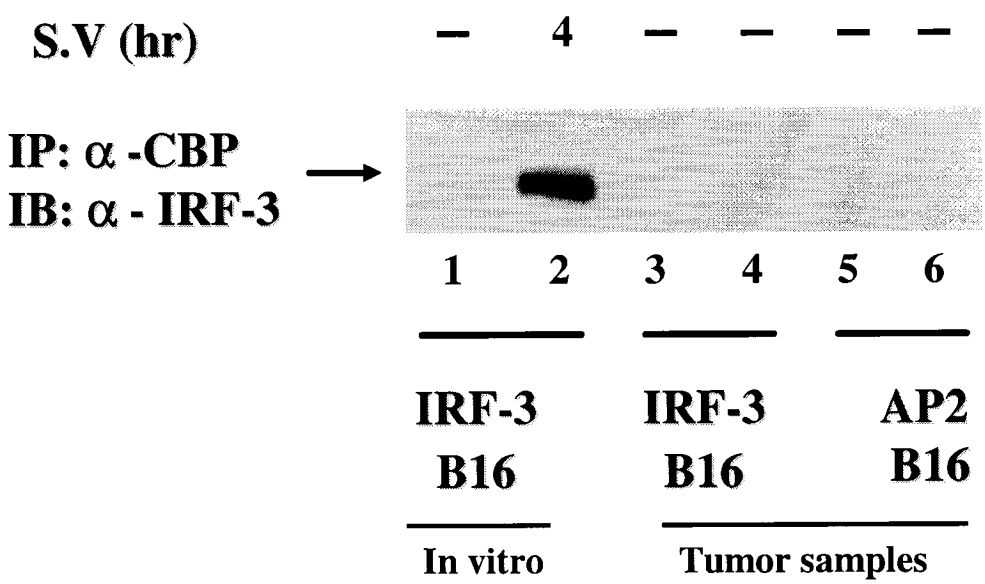


Figure 17. IRF-3 expression in transduced B16 cells is capable of binding co-activator CBP/p300 after Sendai virus infection. Whole-cell extract obtained from IRF-3 B16 cells, which were either uninfected or infected with Sendai virus (40 HAU/10^6 cells), and tumor samples (n=2 for IRF-3 and AP2- B16 tumor samples) were subjected to immunoprecipitation (IP) using the antibody CBP/p300 (Santa Cruz). Immunoprecipated proteins were separated by 7.5% SDS-PAGE gel and detected by immunobloting (IB) with rabbit anti-IRF-3 antibody (Santa Cruz)

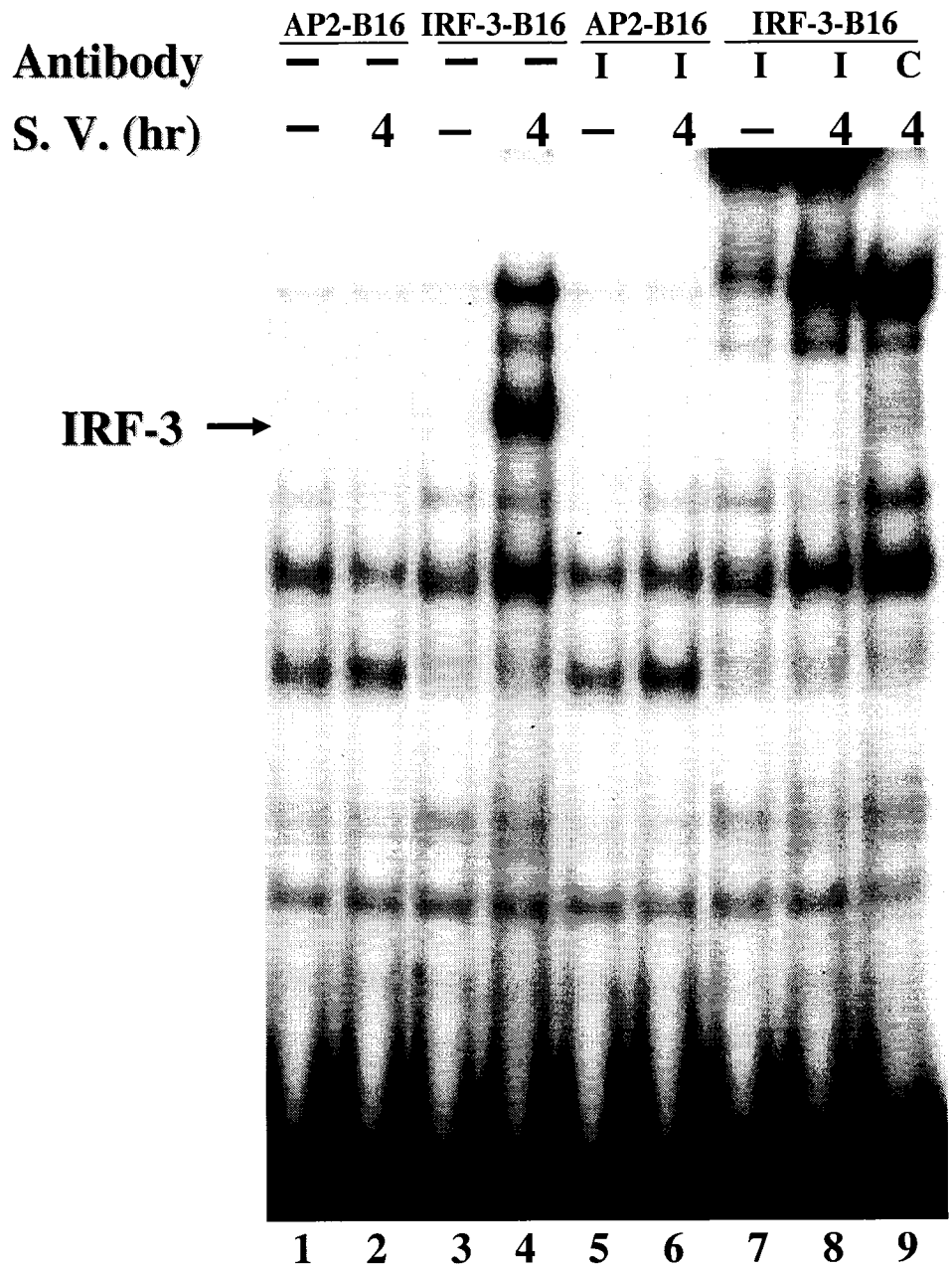


Figure 18. Expression of IRF-3 protein capable of binding IRF specific DNA sequence by gel mobility shift analysis. Nuclear extract was obtained from subconfluent cells and used to analyze IRF-3 DNA binding activity by electrophoretic mobility shift assay using ISRE of *ISG15* as the probe. Cells were left uninfected (lanes 1, 3, 5, 7, 9, 11, 13) or infected with Sendai virus (40 HAU/10^6 cells) (lanes 2, 4, 6, 8, 10, 12, 14, 15). Arrows indicate complex formation of IRF-3 as determined by supershift analysis (IRF-3 supershift (**I**), lanes 9-14) and complex formation of IRF-3 with CBP as determined by supershift analysis (CBP supershift (**C**), lane 15).

Addition of antibodies specific for IRF-3 to the DNA binding reaction inhibits the appearance of the complex, suggesting that hIRF-3 protein in mouse transduced IRF-3 B16 cells has a DNA binding activity upon viral infection. This complex was absent from uninfected IRF-3 B16 cells (Fig, 18, lane 3) and in both uninfected and virus-induced control AP2 B16 cells (Fig, 18, lanes 1-2). These observations demonstrate that expression of hIRF-3 in B16 cells, after virus infection, binds specifically to the ISRE site. However, in virus-induced control AP2 B16 cells, endogenous mouse IRF-3 does not exhibit DNA-binding activity for the ISRE site. Another hallmark of the activation of IRF-3 by virus/dsRNA is its association with co-activator CBP/p300, which is needed to expose the DNA binding activity of IRF-3 (96). Antibodies to CBP inhibited the formation of the IRF-3 complex observed in virus-infected IRF-3 B16 cells (Fig, 18 lane 9), indicating that hIRF-3 protein expression in the retrovirally transduced B16 cells was able to interact with CBP/p300 and bind DNA specific sequence.

In vitro growth properties of parental and retrovirally transduced B16 melanoma cells.

To test whether IRF-3 overexpression affects growth *in vitro* of the B16 melanoma cells, growth assay were performed. *In vitro* growth rate was assessed by cell counting and calculation of doubling time from exponentially growing cells. Table 8 is an average of 3 different assays. The retrovirally transduced B16 cells had virtually the same *in vitro* growth in terms of doubling time (15 +/- 0.7 hr) along with untransduced B16 cells.

Table 8. Doubling times of IRF-3 transduced B16.F0 cells. Cells were plated at 4 X 10^4 cells per well in 6 well plate, and 3 wells were counted daily by hemacytometer. This was repeated for each cell line. Doubling time was determined from growth rate of exponentially growing cells.

Cell line	Doubling time, hours (± SEM)
AP2- B16 cells	15.3 ± 0.66
IRF-3 B16 cells	16.9 ± 0.33

Injection of IRF-3 transduced B16 cells into syngeneic C57BL/6 mice.

Retroviral transduction of IRF-3 was performed *in vitro* with B16-F0 cells and GFP-positive cells were sorted by FACS to generate a stable IRF-3 transduced population. IRF-3 expressing B16 melanoma cells (IRF-3 B16) were inoculated subcutaneously into syngeneic C57BL/6 mice and tumor growth was monitored between the mock AP2 and IRF-3 transduced population. Expression of IRF-3 in the B16 melanoma cells resulted in slower tumor growth than injection of vector alone, mock-transduced cells (AP2-B16) (Figure 19). By day 19, at the time of sacrifice, the mean tumor volume in the AP2 B16 injected animals was approximately 1000 mm^3 whereas in animals inoculated with IRF-3 expressing B16 cells, the tumor volume on average was 200-300 mm^3. The slower tumor growth rate *in vivo* was of interest because the retrovirally transduced B16 cells had virtually the same *in vitro* growth in terms of doubling time (15 +/- 0.7 hr) (Table 8).

Tumor growth in immunodeficient mice (SCID)

To evaluate the role of host immunity in IRF-3-mediated antitumor responses; IRF-3 and AP2 transduced B16 tumor cells were injected s.c. into tumor bearing SCID beige C57BL/6 mice. IRF-3 expression in B16 cells did not alter tumor volumes in severe combined immunodeficient (SCID) mice (Figure 20).

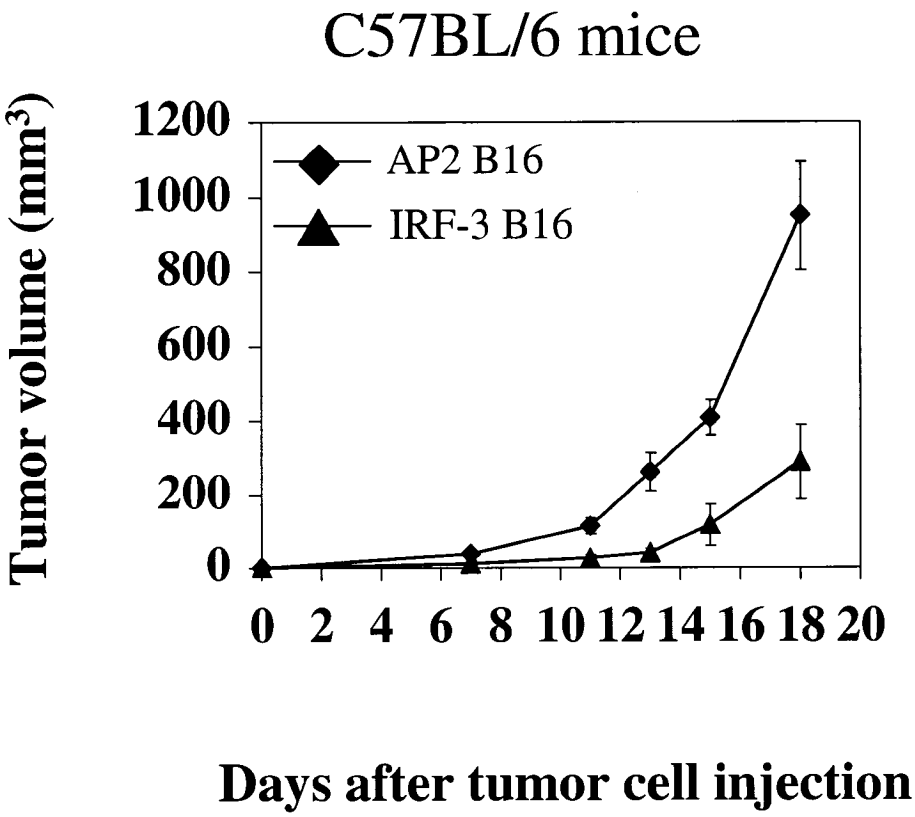


Figure 19. Expression of IRF-3 inhibits B16 tumor growth *in vivo*. IRF-3 B16 melanoma cells (▲) and mock-transduced AP2 B16 (◆) cells ($1x10^6$) were injected subcutaneously into female C57BL/6 syngeneic mice (7 mice per group). Tumor growth was followed by perpendicular caliper measurement every other day from day 7 after injection. Tumor samples from both groups of mice were collected at day of sacrifice (19 days).

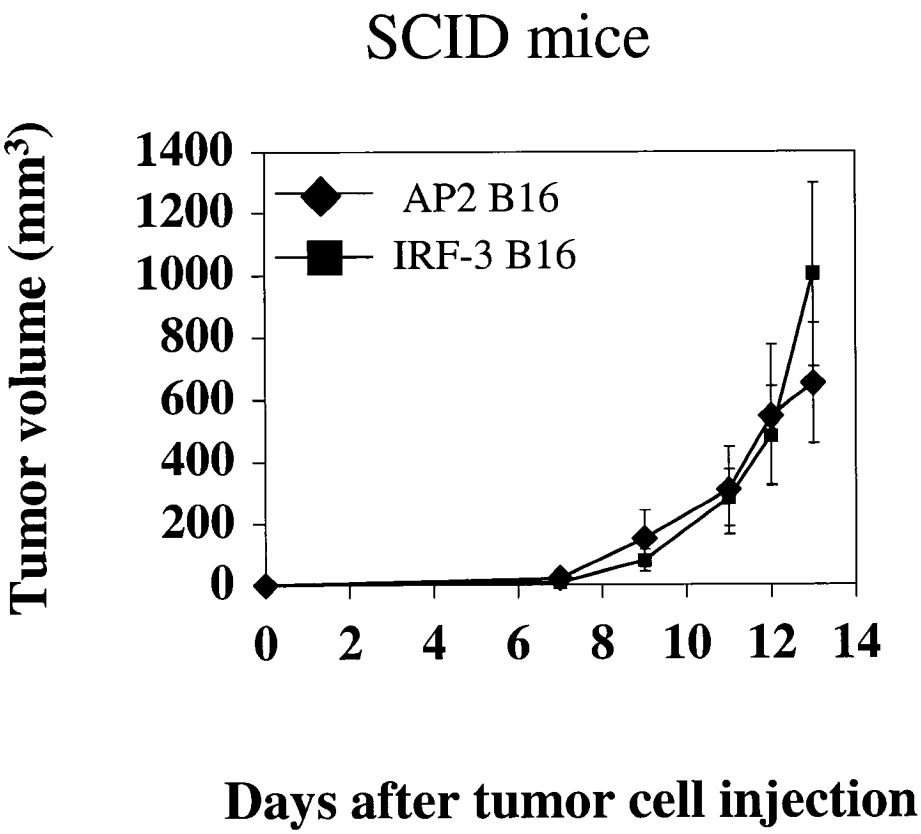


Figure 20. Tumor growth in SCID mice. Severe combined immunodeficient (SCID) mice were injected with IRF-3 transduced (■) and mock-transduced AP2 B16 melanoma cells (◆) ($1x10^6$). Tumor growth was followed by perpendicular caliper measurement every other day from day 7 after injection.

Expression of chemokines and cellular recruitment into tumor tissue.

Tumor progression has been previously shown to be modified by host cytokine expression, which in turn mediates leukocyte migration and activation (127, 140). Since IRF-3 plays an active role in the induction of IFNs and RANTES genes, we therefore analyzed the effect of IRF-3 expression on cytokine production. The profiles of several immunomodulatory chemokines were examined in tumor cells derived from mock-transduced AP2 (n=7) or IRF-3 transduced B16 tumors (n=10). Cytokine mRNA expression from solid tumor was determined by RNase protection assay. Interestingly, the IRF-3 B16 tumors displayed a 2-3-fold increase in basal mRNA levels of RANTES, IP-10, and MIP-1β compared with AP2 B16 tumors (Figure 21).

Infiltration of inflammatory cells to the site of the tumors

Since inflammatory chemokines such as RANTES, MIP-1β and IP-10 are specialized to recruit effector cells, including monocytes, granulocytes and effector T-cells, tumor infiltration was next evaluated by histology within the tumor cell mass, as well as at the margin of the tumor (Figure 22). Within the IRF-3 B16 tumor mass, moderate or dense lymphocyte infiltration was detected in 25% and 12% of the tumors, compared to mock-transduced AP2 B16 tumors (16% moderate infiltration) (Fig. 22, *A-B*). Interestingly, dense lymphocyte infiltration was clearly visible at the margins of the IRF-3 B16 tumors (75%) when compared with mock-transduced AP2 B16 tumors (12%) (Fig. 22, *C-D*). Although ectopic expression of IRF-3 had no direct inhibitory effect on B16 melanoma cells growth *in vitro*, a striking effect on local tumor infiltration *in vivo* was observed.

Histologically, AP2 B16 tumors demonstrated diffuse growth and a paucity of host inflammatory cells, which was not associated with areas of coagulation necrosis (Figure 22, *E-F*). In contrast, IRF-3 B16 tumors possessed a larger capsule, displayed dense infiltration of both neutrophils and lymphocytes and contained fewer blood vessels. Also apparent were areas of necrosis where tumor cells close to the inflammatory cells had degenerated. Tumor infiltration of both mononuclear cells and neutrophils may participate in killing of residual tumor cells, suggesting that one strategy to improve the efficacy of IRF-3-based therapy is to combine it with immunotherapy.

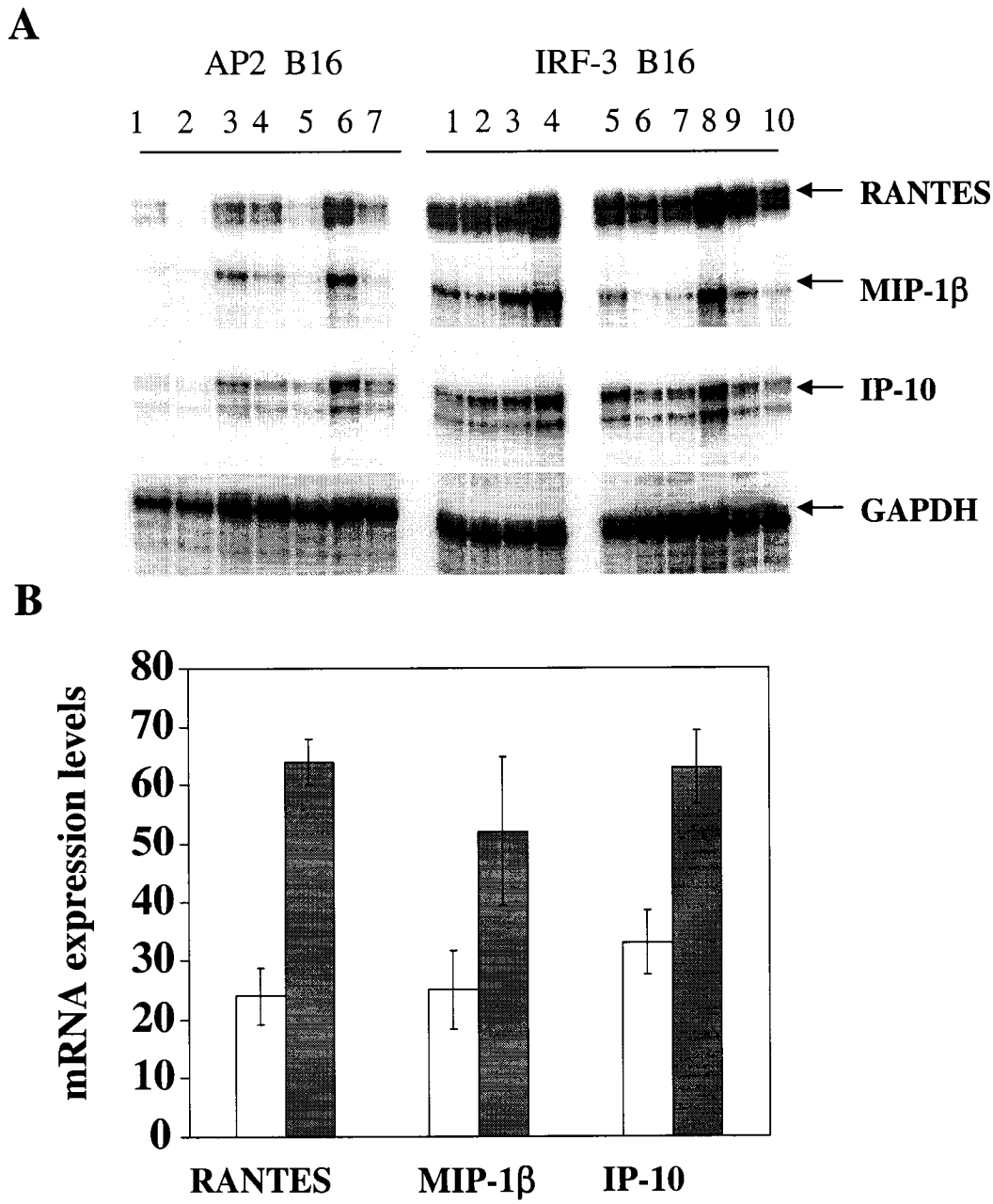


Figure 21. IRF-3 expression in B16 melanoma cells results in up-regulation of RANTES, IP-10, and MIP-1 β mRNA expression *in vivo*. *A*, Total RNA (10μg) was isolated from various transduced B16 tumors (mock-transduced AP2 B16 n=7, IRF-3 B16 n=10) and hybridized with the mCK-5 multiple probe before digestion with RNase. Separation of protected fragments was performed by 5% urea gel electrophoresis. Fragment assignment was determined by migration of protected fragments relative to internal standards. Induction of RANTES, IP-10, and MIP-1 β mRNA expression was confirmed by performing two additional RPA analyses. *B*, Cytokine mRNA expression levels were quantified relative to the internal control *GAPDH*.

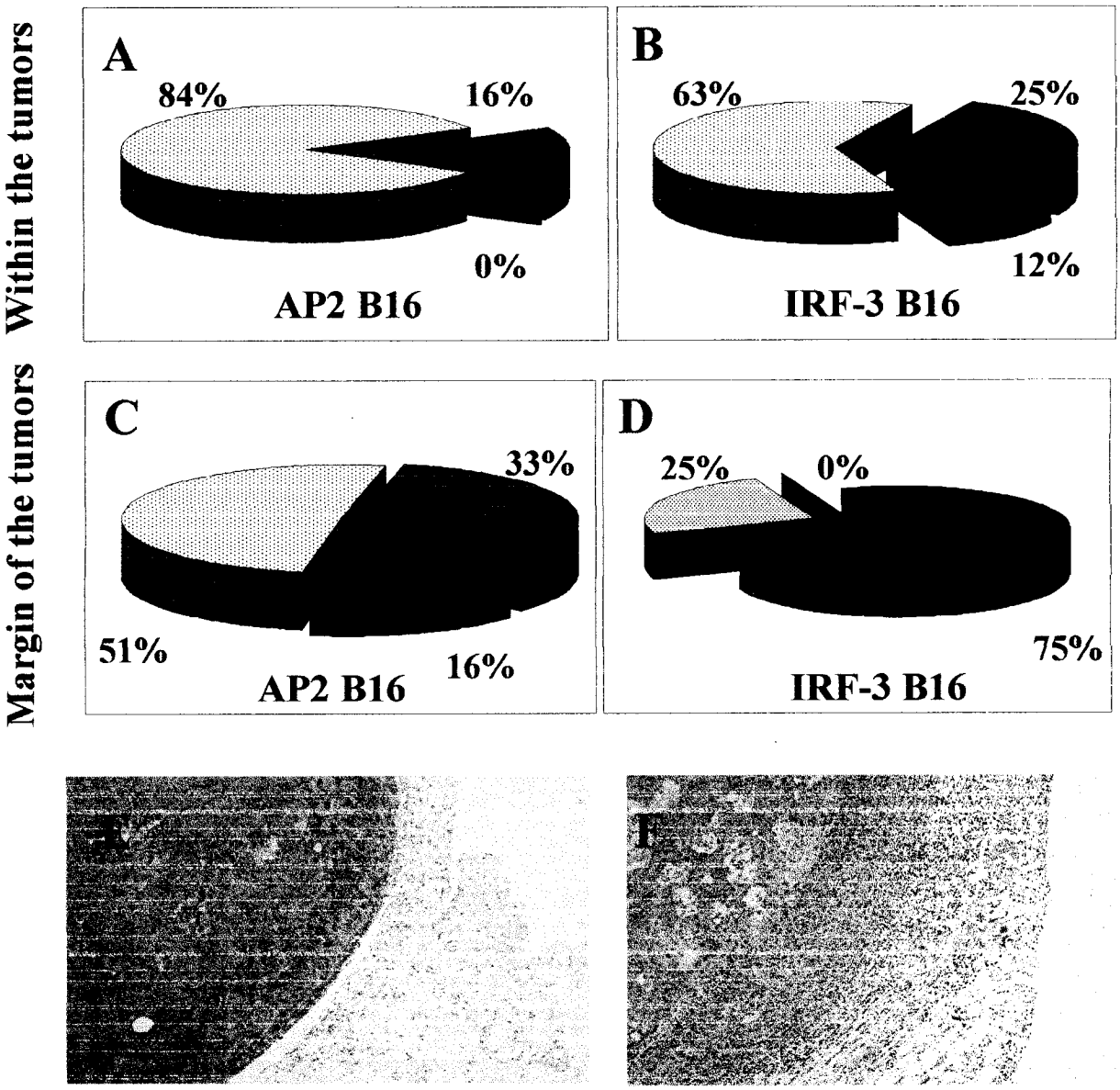
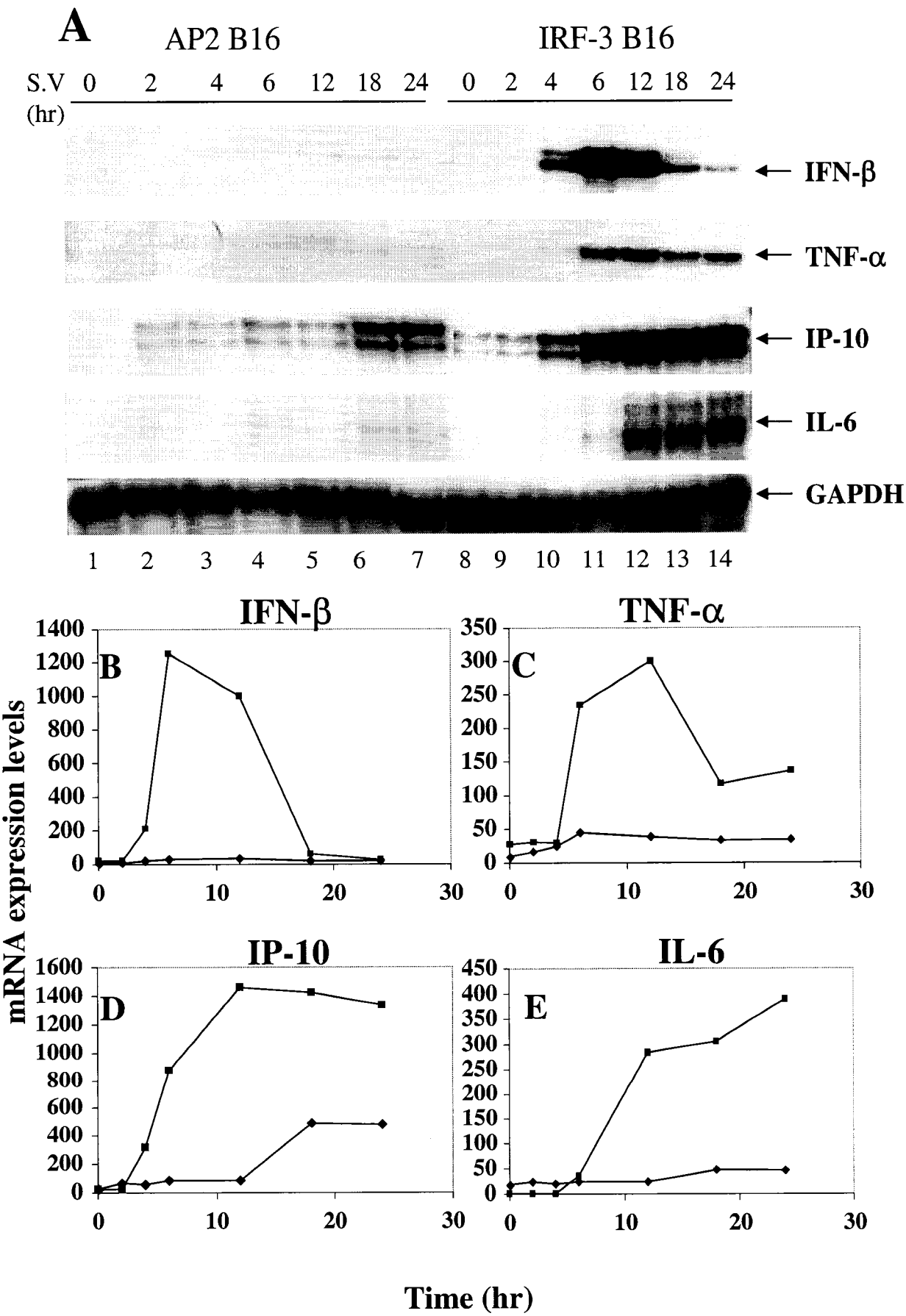


Figure 22. Recruitment of lymphocytes by IRF-3 transduced B16 melanoma cells. Histograms represent the percentage of infiltrating cells within (*A, B*) and at the margin (*C, D*) of the B16 melanoma tumors (AP2 and IRF-3): absence of lymphocyte is indicated by (■), moderate infiltration (▩) and dense infiltration (▩). *E* and *F*, histology of tumors growing s.c. from mock-transduced AP2 and IRF-3 B16 injected mice (*C57BL/6*) excised at day 19 (day of sacrifice). H&E stain; magnification X100.

Expression of cytokine mRNA in IRF-3 transduced B16 melanoma cells *in vitro*.

To test whether IRF-3 induced cytokine expression in B16 melanoma cells *in vitro*. At various times after infection with Sendai virus (a well-known inducer of cytokine gene expression and activator of IRF-3 function (96, 164)), samples were collected for RNase protection assay. Expression of TNF-α, IL-6, IP-10 and IFN-β mRNA was markedly enhanced in B16 cells expressing IRF-3. At their peak 4-12h after induction, IFN-β mRNA levels were enhanced more than a 1000 fold compared to the AP2-B16 population (Figure 23, lanes 10-12 and B), whereas in the AP-2 B16 cells, only a weak response to virus induction was observed (Fig. 23, lanes 2-7 and B), suggesting that IRF-3 expression reconstituted a defective IFN response. Although the magnitude of the response was decreased for TNFα and IL-6, the same enhanced production of mRNA was observed in the IRF-3-transduced B16 cells, and again IRF-3 expression appeared to restore an otherwise deficient TNF and IL-6 response (Fig. 23 A, C and E). The expression of Sendai-induced IP-10 was also enhanced in IRF-3 expressing B16 melanoma cells; IP-10 was induced at 18-24 hr after virus induction in control B16 cells, (Fig. 23A, lanes 6 and 7), whereas in IRF-3 expressing B16 cells, the kinetics and magnitude of IP-10 mRNA expression were dramatically enhanced, with mRNA induction beginning as early as 4h post-infection and reaching a peak at 12h p.i. (Fig. 23A, lanes 10-14 and D).

Figure 23. Kinetics of cytokine mRNA expression in IRF-3 transduced B16 melanoma cells *in vitro*. *A*, Mock transduced AP2 and IRF-3 B16 melanoma cells were infected with Sendai virus (40 HAU) and total RNA was prepared at different times after infection (0-24h) as indicated above the lanes. Total RNA (10μg) was subjected to RNase protection analysis using the mCK-3b and mCK-5 probe sets. The intensity of the bands was measured using NIH Image v1.6 and *GAPDH* protected probes were used for normalization. The fold induction of IFNb (*B*), TNFa (*C*) IP-10 (*D*) and IL-6 (*E*) mRNA was plotted for AP-2 B16 and IRF-3 B16 cells at each time point.


A
AP2 B16
IRF-3 B16
S.V (hr)
0 2 4 6 12 18 24
0 2 4 6 12 18 24
IFN-β
TNF-α
IP-10
IL-6
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IFN-β
TNF-α
IP-10
IL-6
B
C
D
E
mRNA expression levels
Time (hr)

Surface phenotype of retrovirally transduced B16 melanoma cells

As immune effector molecules, MHC class I molecules play important roles in the recognition and killing of tumor cells by effector cells. Abnormalities of MHC class I surface antigens are often associated with an immune escape of tumor cells. (50, 57, 161). B16 melanoma cell lines (derived from H-2^b C57BL/6 mice) cultured in serum-supplemented medium expressed only very low levels of the MHC-I molecules K^b and D^b and the MHC-II I-A^b on the cell surface. Since the level of expression of MHC class I molecules on tumor cells was an important parameter of immunogenicity, we analyzed the expression of the two types of MHC molecules (I and II) along with the co-stimulatory molecules (B7.1 and B7.2), Fas and the FasL by flow cytometry. As shown in Figure 24, the level of MHC class I molecule was strikingly up-regulated in cells transduced with IRF-3 (Fig. 24, panel B) compared to the control cells (AP2-B16) were only a small portion of cells expressed MHC class I molecule (Fig. 24, panel B). Several reports have demonstrated that tumor immunity can be enhanced by the provision of co-stimulatory signals other than the signal provided by contact between the Ag and its TCR (2, 48). Two co-stimulatory molecules, B7.1 and B7.2, enhance the activation of T cells by antigen presenting cells (APCs). We analyzed the surface expression of T-cell costimulating molecules in control cells AP2-B16 and in transduced IRF-3 B16 cells. Expression of those molecules in both cell lines was absent (Fig. 24, panel A and B). Next, we examine the expression of both the Fas receptor and its ligand on the AP2- and IRF-3- B16 cells. As depicted in Figure 24, the expression of the Fas receptor and FasL was barely detectable and unchanged in both AP2- and IRF-3- B16 cell lines.

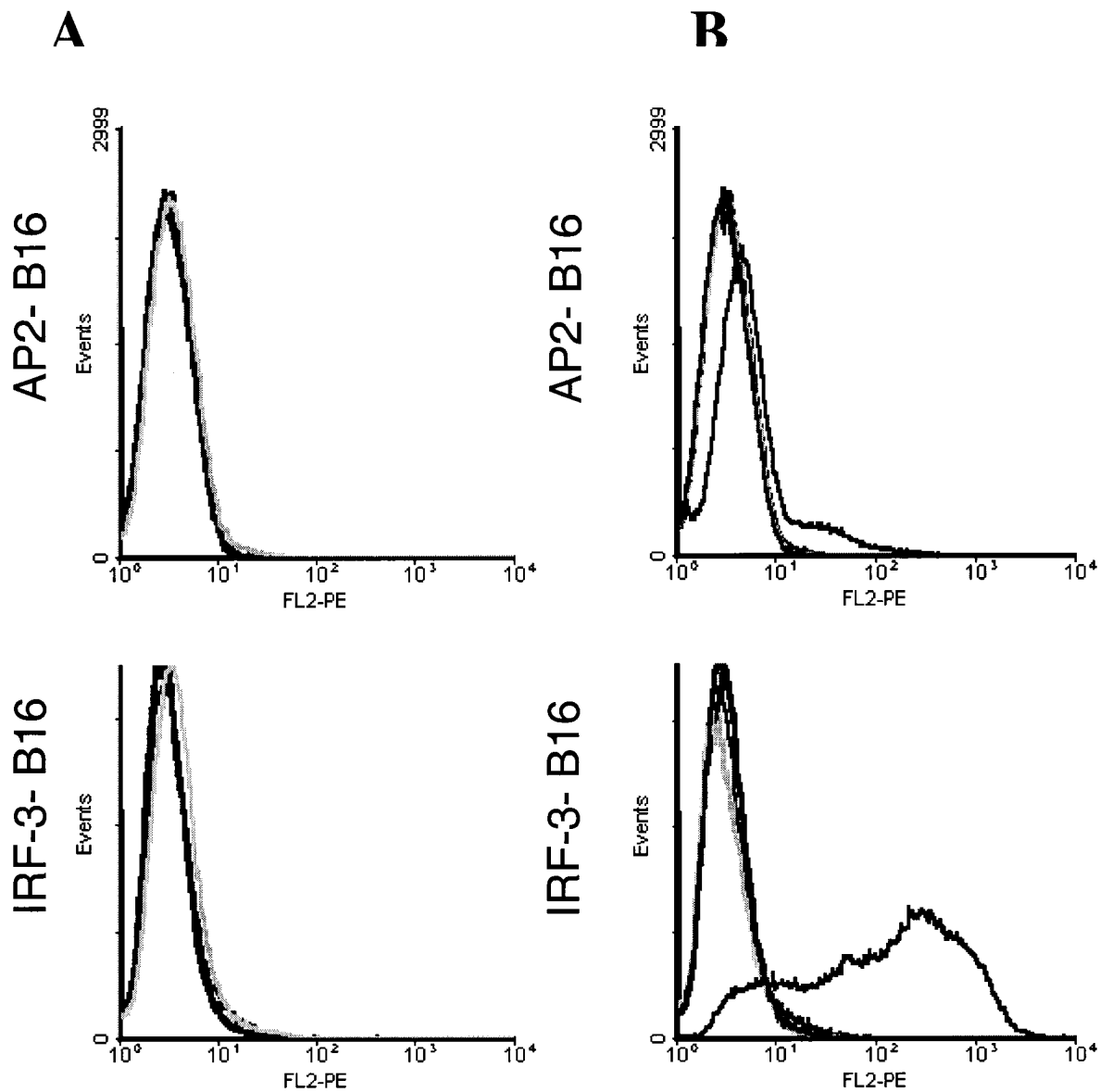


Figure 24. Expression of MHC class I, II, Fas, FasL, B7.1 and B7.2 in IRF-3 and AP2 transduced B16 melanoma cells. Panel A, cytofluorometric analysis of B7.1 (——) and Fas (——) expression for IRF-3- and AP2- transduced B16 cells. Panel B, cytofluorometric analysis of B7.2 (——), FasL (——), MHC class I (——) and class II (——) expression for AP2- and IRF-3- transduced B16 cells. Surface expression of those molecules was measured using mAb in the Material and Methods and compared to unstained cells (——).

CHAPTER V.

DISCUSSION

Transfection of transcription factor genes into tumor cells allows delivery of those genes at the site of tumor growth, where they might be expected to have a better therapeutic effect through an effective induction of antitumor immunity. This novel delivery strategy may render the tumor cells more sensitive to the microenvironment of the tumors, therefore more prone to be recognized and killed by the immune system. IRF-3 seems to mediate antitumor activity through at least two possibly related mechanisms: 1) attraction into malignant tissue of leukocytes by releasing chemotactic cytokines and 2) enhancing immunogenicity of the B16 melanoma tumor by upregulating components of the MHC class I antigen presentation pathway.

In this study, B16 melanoma cells were gene-modified to express the human IRF-3 protein. There is about 70% homology between the murine and the human protein. To verify the functionality of the human IRF-3 in B16 cells, several experiments were conducted. The ability of IRF-3 to be phosphorylated *in vitro*, to associate with the coactivator CBP/p300 and to bind to an ISRE DNA-binding site, reveals that IRF-3 can be functionally active in the B16 cells. During viral infection, IRF-3 is activated by phosphorylation but was not observed *in vivo* on tumor samples, suggesting that at this point of the study, it is not possible to know whether the antitumor activities requires phosphorylation of IRF-3 or overexpression of IRF-3 alone is sufficient. However, interestingly, IRF-3 can be detected in the nucleus in the absence of viral infection (204), and overexpression of IRF-3 in uninfected cells activates the expression of IFN-β and IFN-α (78).

Theoretically, integration of the retroviral vector as a provirus should lead to maintenance of the transgene in the infected cells and transfer to any progeny; however, expression of the transgene can be transient, probably as a result of down-regulation of transcription rather then gene loss (24). Therefore, it is possible to hypothesize that the diminish expression of IRF-3 in several tumor samples (Figure 15, B) recovered from IRF-3 B16 animals could lead to the impairment of the antitumor activity of IRF-3 in these mice, which could result, in part, for the tumor progression observed at day 15 (Figure 19). The IRF-3 B16 injected cells constitute a heterogeneous population of transduced cells. In order to address if the level of IRF-3 expression in the tumor correlates with the tumor growth, studies using expanded clones with different levels of IRF-3 should be evaluated. However, even though the level of IRF-3 differs from tumor samples, the reduction of tumor growth was almost the same for all the mice injected with the IRF-3 transduced B16 cells. It is possible that over time, the detection of IRF-3 is being lost. The effect of IRF-3 could therefore be assess earlier rather then the date of sacrifice.

Although gene modified IRF-3 and AP2- B16 cells have similar doubling times in *vitro* (Table 8), tumor growth was suppressed in animals injected with IRF-3 B16 cells. The *in vitro* doubling time does not completely rule out the possibility that the differences in tumor growth could be attributed to intrinsic discrepancies in the cell cycles of the different cell populations, as even a slight variation in doubling time could result in a 2-or 3 fold divergence after 20 days. However, if the lack of growth in AP2-B16 versus IRF-3 B16 melanoma in the syngeneic model was related to changes in the growth pattern of the tumor line, slower growth would have been expected in SCID mice. Since growth

suppression was observed only in immune competent animals, this observation provides evidence that growth inhibition is not due to growth pattern changes of the B16 IRF-3. Together, these observations suggest that the antitumor effects of IRF-3 do not involve differences in tumor growth properties, cell cycle or apoptosis in B16 melanoma cells, but rather indicate the requirement for a functional adaptive immune response as part of the antitumor response.

Usually solid tumors are infiltrated by mononuclear cells, which are mostly localized in the tumor stroma or in tissues immediately surrounding the tumor (13, 200). The intense infiltration of immuno-competent cells such as T lymphocytes, macrophages, or NK cells, within or around the tumor has been generally considered as evidence for a local antitumor immune response (192, 199). Thus, the dense tumor infiltration of both mononuclear cells and neutrophils observed in IRF-3 tumor samples (Figure 22) may participate in the killing of residual tumor cells. It has been demonstrated that the elimination of the tumor is closely related to the sensitivity of tumor cells to the cytotoxicity of immune effector cells (5, 36). Preliminary results using matrigel experiments where IRF-3 transduced B16 cells were injected along with a collagenase matrix revealed that the infiltration is composed mainly of CD8+ T-cells with fewer CD4+ T-cells when compared to AP2- B16 tumor samples (D.D., data not shown). This suggests that CD8+ cytotoxic T-cell could be an important factor in the killing of the B16 tumor cells. Further immunological analysis (e.g., immunostaining, knockout mice, intracellular cytokine analysis) will identify more specifically which type of cells is infiltrating the tumors and responsible for their elimination.

Melanomas are most frequently infiltrated by actively proliferating T-lymphocytes (186). Some of these T-cells are cytolytic and recognize peptide antigens derived from melanoma-specific antigens (149). However, the ongoing melanocytes-specific T-cell responses are most frequently incapable of controlling the growth of the tumor, resulting in the tumors escaping an immune T-cell response. One could argue that this phenomenon is responsible for the incomplete eradication of the IRF-3 B16 tumors. With the advent of this hypothesis, studies have shown that tumor-infiltrating mononuclear cells were inhibited in their proliferative activities (201). These observations led to formulating of a hypothesis that in the tumor microenvironment, immune cells lose their effectiveness, become partially or completely paralyzed, so that the balance between the immune system and tumor shifts in favor of the established tumor. Therefore, one strategy to improve the efficacy of IRF-3-based therapy is to combine it with immunotherapy.

To confirm the role of the immune system in the inhibition of tumor growth, we next evaluated the cytokines involved in the recruitment of tumor infiltrating cells. Infiltration of tumors with host cells is regulated by tumor-derived chemokines, a super-family of proinflammatory cytokines that is responsible for the selective recruitment and activation of mononuclear cells (148). Chemoattractants such as IP-10, RANTES and MIP-1β recruit lymphocytes into malignant tissue (19, 62, 75), which suggest that IRF-3 transduction into B16 melanoma enhanced cytokine release in the tumor microenvironment (Figure 21). Interestingly, RANTES and IP-10 are genes known to be regulated by IRF-3 (80, 95). RANTES is expressed relatively late after activation of peripheral blood T cells by antigen or mitogens but is rapidly induced in normal

fibroblasts and epithelial cells by TNF-α and IL-1β, suggesting that different control mechanisms may regulate RANTES transcriptional activation (133). The interferon-γ inducible protein-10 (IP-10), also called Cgr-2 (44, 137, 190) in mice, has been shown to attract only activated but not resting T lymphocytes and NK cells (100, 182, 183) and to impair tumoral angiogenesis (3, 4, 165). Thus, RANTES and IP-10 are chemotactic for memory T lymphocytes and monocytes (106, 182), MIP-1β may be chemotactic preferentially for CD8+ cells (180, 182). Whether IRF-3 overexpression is directly involved in cytokine secretion or additional inflammatory cells recruited to the microenvironment are required remains to be elucidated. Accordingly, it is possible that over time, the numbers of host-derived T and non-T immune cells significantly increase at the site of the tumor, which could correlate with an elevated production of RANTES, IP-10 and MIP-1β.

The potential of IRF-3-transduced B16 cells to secrete cytokines such as IFN-β, TNF-α, IP-10 and IL-6 upon *in vivo* stimulation could modulate the microenvironment of the tumor by attracting and activating inflammatory cell infiltration. These cytokines are known to be part of the repertoire of cytokines and growth factors that can be produce by melanomas (68, 88). The transduction of IRF-3 into the B16 cells seems to restore a somewhat deficient cytokine profile making the cells more prone to secrete those cytokines into the tumor microenvironment. TNF-α and IL-6 can inhibit melanocyte proliferation and melanogenesis and have been shown to up-regulate the expression of intercellular adhesion molecule-1 (ICAM-1) and major histocompatibility complex (MHC) class I (63, 111, 174). Transfection of interleukin-6 (IL-6) into B16 melanoma

cells causes growth retardation by arresting the cell cycle at G1/G0 boundary. Paracrine effects of IL-6 involve the influence of tumor angiogenesis and alteration of the activity of tumor infiltrating immune cells, upregulation of melanocyte and melanoma cell ICAM-1 expression (172). Despite the significant up-regulation of IFN-β and IP-10 which is barely detectable in the control AP2-B16 cells, the ability of IRF-3 to induce IL-6 and TNF-α in B16 melanoma cells (Figure 23) represent a novel role for IRF-3 in the NF-κB signalling pathway since IL-6 and TNF-α are known to be regulated by NF-κB (27, 94, 166, 167). IRF-3 could interact with NF-κB and other proteins to stimulate the transcription of IL-6 and TNF-α genes. The cooperation between IRF-3, NF-κB, AP2/c-JUN and HMG1(Y) has been demonstrated for the β-interferon (IFN) enhanceosome (185). Database analysis revealed several enhancer-binding sites, namely ISRE and NF-κB in the murine TNF-α promoter. Preliminary results have implicated IRF-3 in the transactivation of the murine TNF-α promoter (D.D., data not shown). However, further analyses are required to determine exactly the implication of IRF-3 in the regulation of TNF-α. Therefore, the precise role of IRF-3 in the regulation of IL-6 and TNF-α genes still remains to be demonstrated.

Recent studies have shown that in addition to viruses, multiple activators including lipopolysaccharide, cellular stress and DNA damage can activate IRF-3 function (163). Therefore it is conceivable that IRF-3 activation, albeit at a low level, may occur *in vivo* as a consequence of stress within the tumor microenvironment. Alternatively, simply increasing the intranuclear concentration of IRF-3 may be sufficient to increase the effect of IRF-3 on IFN and cytokine gene expression. IRF-3 along with IRF-7 have been detected in the nucleus in the absence of viral infections (204), and

overexpression of IRF-3 in uninfected cells activates the expression of IFN-β (78). Interestingly, Robinson's group demonstrated that IRF-3 induced IFN-γ-secreting CD8+ T cells when used as genetic adjuvant for influenza hemagglutinin (HA) (154). In this study, codelivery of plasmid encoding (HA) epitope and IRF-3 gene resulted in a significant increase of the CD8 T cell response associated with production of IFN-γ, suggesting that IRF-3 induces a Th1 response. Concurring with our study, whether the IRF-3 adjuvant effects required IRF-3 *in vivo* phosphorylation or resulted from overexpression of IRF-3 still remains a factor to determined.

It is generally accepted that cytotoxic lymphocytes, including natural killer (NK) cells and tumor-specific cytotoxic T lymphocytes (CTLs) eliminate tumor cells in a major histocompatibility complex (MHC)-dependent manner (15, 187)). Importantly, for tumor rejection to occur, antigens must be presented on HLA class I molecules of melanoma cells to be recognized by CD8+ CTL activation (52). The up-regulation of MHC class I surface expression in IRF-3 B16 cells could then be involved in the recruitment of effective CD8+ T cells observed in preliminary experiments (D.D., data not shown). Tumors expressing low or undetectable levels of HLA class I such as the B16 melanomas are believed to escape the immune system (178). Cell surface expression of HLA class I Ags is known to be increase by treatment with either type I or type II IFN (135). The immunogenicity of the wild-type tumor cells may be augmented by increases in the MHC class I level following H-2 gene transfection. The IRF-3 B16 cells showed a significant increase of the MHC class I surface expression compared to the parental tumor AP2-B16 cells (Figure 24), suggesting that the immunogenicity of IRF-3 gene-transduced B16

tumor cells was increased and that this provided a basis for the induction of an immune response. However, further analyses will be necessary to delineate the exact mechanisms by which IRF-3 is involved in this antigen-processing pathway. The expression of MHC class I molecules on tumor cells is regulated by many cytokines, including TNF-α, IL-1, IFN-γ, IL-2, IL-4 and IL-6 are all pleiotropic cytokines, not only regulating immune effector cells functions, but also directly affecting non-immune cells (9, 10, 126). Therefore, it is possible that the ability of IRF-3 to induce cytokines such as TNF-α, IL-6 and IFN-β in the B16 tumors cells can be implicated in inducing the somewhat deficient MHC class I surface expression.

Another explanation could also be attributable to the multiple components of the MHC class I pathway. The reduction or loss of MHC class I surface expression in human and murine tumors of distinct histologies could be attributable to structural alterations and/or dysregulation of various components of the MHC class I antigen-processing machinery (APM) (40, 50, 57, 159). B16 melanoma cells have been described as a poorly immunogenic tumor model due in part to the down-regulation of multiple APM components which able them to evade the immune surveillance (160, 161). Several of the proteins involved in class I assembly, i.e. β2m, tapasin, TAP1 and TAP2 and the class I heavy chain itself, are constitutively expressed at low levels and are upregulated by type II interferon (IFN)-γ reviewed in (18), and some are also regulated by type I interferons (IFN-α and IFN-β) owing to transcription factor binding elements in their promoter regions (55, 67, 84, 202). ISRE motifs have been described in the promoters region of earlier genes such as TAP1, LMP2 and tapasin (25, 54, 61, 122, 209). More interestingly,

Gobin *et al,* (54), has recently demonstrated that IRF-3 is a strong transactivator of the β2-microglobulin promoter, which contains along with an Ets/interferon-stimulated response element (ISRE), a kappaB site and an E box. The potential of IRF-3 to modulate the transcriptional control through the ISRE could be an important mechanism in the differential regulation of classical and non-classical MHC class I expression, which determines adequate Ag expression upon pathogenic challenge. Subsequent analyses on the profile of expression of the members of the antigen processing pathway could draw a phenotype of the B16 cells used in this study and help understand the mechanisms by which the IRF-3 B16 cells up-regulate the MHC class I surface expression.

Other possible reasons for the poor immunogenicity of tumors may be, in part, a consequence of failure to express costimulatory ligands necessary for activating CTLs. Several studies have demonstrated that tumor immunity can be enhanced by the provision of costimulatory signals, including B7.1, and ICAM-1 expressed on tumor cells (2, 48). Previous work has established that B7.1+ tumor cells not only induce protective immunity to subsequent challenge with the parental tumor but also result in elimination of preexisting tumor (93, 188). Low levels of B7.1 molecules have previously been reported to be present on the surface of cells from 3 to 10 human melanomas (69). However, in this study, IRF-3 and AP2- B16 cells do not expressed neither of the costimulatory signals B7.1, B7.2 and also failed to express Fas-L. These results suggest a different pathway that does not seems to involve IRF-3 in the regulation of those costimulatory signals.

CHAPTER VI.

CONCLUSION

In summary, we used a syngeneic murine B16 tumor model to evaluate the capacity of the transcription factor IRF-3 to re-program cytokine gene expression in order to modulate the tumorigenicity of a poorly immunogenic tumor model. In the present study, we were able to elicit potent antitumor response against the B16 melanoma tumors by gene modifying B16 melanoma cells with the IFN-regulatory factor 3.

The reconstitution of the cytokines profile in IRF-3 expressing B16 melanoma contributes to the enhanced recruitment of lymphocytes to the local tumor site compared to AP2-B16 in which cytokine induction remains defective. Recruitment of inflammatory effector cells to the tumor microenvironment as a consequence of cytokine release may inhibit tumor cell growth and metastasis by directed cell-mediated killing. Furthermore, our results demonstrate that IRF-3 expression in the murine melanoma context led to significant increase of the MHC class I surface expression, suggesting that IRF-3 is involved in the enhancement of the immunogenicity of the B16 melanoma cells which expose the tumors to the immune system.

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