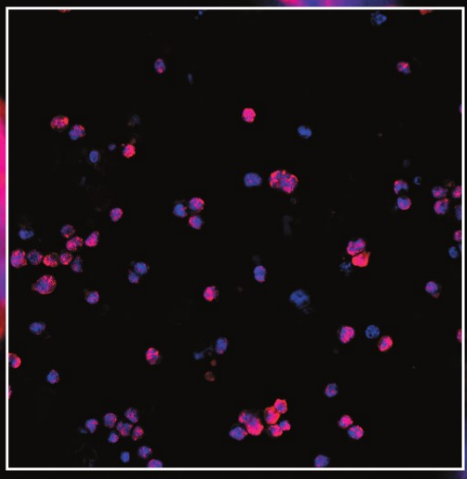
CURRENT TOPICS IN MICROBIOLOGY AND IMMUNOLOGY

Paula M. Pitha
Editor

Interferon: The 50th Anniversary



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Current Topics in Microbiology and Immunology

Paula M. Pitha (Ed.)

Interferon: The 50th Anniversary

With 35 Figures and 8 Tables

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Cover Illustration: The picture shows the expression of KSHV encoded VIRF-3 in B cells.

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Preface

This monograph has been written against the background of the 50-year anniversary of the discovery of interferon (IFN), with the aim of acknowledging some of the initial work as well as sharing the recent discoveries and placing emphasis on the important insights in the rapidly developing area of the innate antiviral immune response. The monograph is not an attempt to be comprehensive, but rather selective in presenting the innovative and challenging results in this area. We hope that this volume will have an impact on stimulating the new generation of scientists to enter the ever-evolving IFN field.

Over the last half century, IFN, originally discovered as an antiviral protein, has developed from an inhibitor of viral replication to a major force in the antiviral response. Initially studied only by a few virologists, IFN was generally considered a poorly defined protein of limited importance. The development of molecular techniques led to the identification of a family of IFN genes and has shown an unexpected complexity of type I IFN genes and their expression. Presently, some aspects of the pathogen-mediated induction of IFN gene expression are understood at the molecular level, while others are still at the stage of description. Both Toll-like receptors and cytoplasmic RNA helicases were shown to recognize viral nucleic acids, and the basis of a distinct recognition of viral nucleic acids from cellular nuclear acids is emerging. Important insights into the virus recognition entity, leading to the expression of IFN and IFN-induced genes, have been gained recently, which are challenging the accepted concept of the critical role of dsRNA in IFN induction. The availability of genetically modified mice that are lacking either essential components of the IFN-inducing pathway or the IFN signaling pathway fully confirmed the original idea, made soon after its discovery, that IFN is the first cellular defense against viral infection.

One reason that made the study of the IFN system very attractive was its potential application to the treatment of viral diseases. However, the initial search for the IFN-induced antiviral protein, able to inhibit replication of all groups of viruses, failed, as well as the hope of using IFN for treatment of a broad spectrum of viral infections. Instead, IFN was shown to induce a

number of distinct IFN-stimulated proteins (ISG), able to specifically inhibit various groups of viruses at different stages of the viral replication cycle. Rather unexpected was the observation that both type I IFN (IFNα/β) and type II IFN (IFNγ) induce massive de-repression of the cellular genome and stimulate expression of a large number of cellular genes of many different functional categories. It is therefore not surprising that IFNs are not exclusively antiviral proteins, but that they are also involved in many different cellular functions. Why a small polypeptide like IFN is responsible for the stimulation of expression of such a vast amount of cellular genes is not yet clear.

The host response to pathogens is a combination of the innate immune response and cellular and humoral immunity. Although originally discovered as a component of the innate antiviral response, the interaction of type I IFN with the acquired immune system is clearly emerging and thus the distinction between the selective roles of type I and type IIIFN in the innate and the acquired immunity is starting to disappear. The importance of the strict regulation of IFN induction and its timely synthesis is clearly demonstrated by the findings that deregulated IFN synthesis is associated with autoimmune diseases. Thus there seems to be a fine balance between the positive and negative effects of IFN. The future challenge will be to understand the role of IFN in a broader context, not only in a cellular defense against pathogens, but also the basis of its detrimental role in autoimmune disease. We hope that this monograph, which includes chapters dealing with the current highlights of IFN research, will illustrate how the simple questions in science can gain substantial complexity with time, and how critical is the basic research for advancement in translational research and clinical applications.

I wish to thank Mike Oldstone for initial work on developing this monograph and Anne Clauss for advice and support in producing this volume. I would also like to thank all the authors, who took time from their busy schedule to contribute to this volume, which has made this monograph unique by giving both historical and future perspectives. Finally I would like to dedicate this book to the memory of Jacqueline De Maeyer-Guignard and Edward De Maeyer, who made major contributions to the development and progression of the IFN field.

Paula Pitha

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(Addresses stated at the beginning of respective chapters)

Part I: Reflections

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How to Chase a Red Herring and Come up with a Smallmouth Bass

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Abstract The collaboration between Alick Isaacs and myself started in the summer of 1956. Our initial project was to show, by electron microscopy, that interference between inactivated influenza virus and live virus involved the transfer of material from the interfering virus to the host cell. This approach failed for technical reasons. However, in the course of this work it appeared that more interfering activity remained in the system than we were entitled to expect. One possible explanation was that a substance, not identical with the initial interfering virus, was being generated. Subsequent experiments, aimed at checking this hypothesis, led to the description of interferon.

Suppose we were living in a world where every scientific project is a success and yields exactly the results expected. This would be terribly boring, and for my part, I would have chosen a different career. Fortunately, most projects fail, which of course is not very satisfactory either. But occasionally a project, pursued more or less energetically, opens a vista onto a side issue, irresistibly seductive and dangerous. Dangerous, because being seduced is always dangerous, as attested by many novels and operas.

The project, when the collaboration between Alick Isaacs (1921–1967) and me started in midsummer 1956, was the following: we knew that influenza virus particles, inactivated by heat and irreversibly attached to red cells, were capable of inducing interference against challenge with live influenza virus in chick embryos. I had finished this work before I reached Mill Hill in July 1956, but it was still unpublished (Mooser and Lindenmann 1957). Alick thought that we could use this technique to ask the following question: how do the influenza virus particles, with one of their sides firmly attached to the carrier red cells, leaving only their opposite side free to interact with the host cells, induce interference? One possibility was that something was being transferred from the virus to the host. What could that be? From bacteriophage work, it was known that the phages inject their nucleic acid into the host bacterium, the rest of the phage remaining outside. If something similar went on in the induction of interference, one might have a chance to observe this. Before inducing interference the virus particles would be inflated by their nucleoprotein content,

but after having induced interference they ought to be empty, to have collapsed. Their fixation on red cells might offer a way to observe this.

This idea, by the way, is an illustration of an interpretation I have offered elsewhere (Lindenmann 1999): scientists do not do experiments in order to answer the most important questions, but they only ask those questions that suggest possible experiments.

Because Alick Isaacs had already worked with R.C. Valentine (1928–1968), an excellent electron microscopist, we were hoping to be able to document this sequence of events by electron microscopy. We would have a first look at virus particles immediately after their fixation to red cells, before any interaction with host cells had taken place. Then the virus-coated red cells would be allowed to interact with host cells, thereby inducing interference. After that, a second look with the electron microscope would reveal, hopefully, collapsed, empty virus particles still hanging on the red cell carriers.

So this was the project, but to realize it we had to modify the technique I had used, which had been to stick the inactivated virus to intact red cells and do the interference experiment in entire embryonated eggs. Electron microscopy required that we use red cell ghosts, transparent to the electron beam, instead of erythrocytes full of hemoglobin. Furthermore, a very simple technique, based on the use of chorioallantoic membrane fragments from embryonated eggs rather than the entire eggs, was envisaged. This allowed measuring six to eight experimental points (virus hemagglutinin titers) per egg, instead of just one point, thus realizing an economy of material and money and allowing greater freedom of manipulations.

Our first concern was to see whether, with the modifications mentioned, measurable interference indeed occurred. This proved to be the case: red cell ghosts coated with heat-inactivated influenza virus and brought into contact with chorioallantoic membrane fragments induced a state of interference, as measured by the degree of inhibition suffered by a subsequently applied challenge virus, similar to the effect of the free virus that we included as a control.

Had we been obliged to apply for a grant in order to continue our project, what I have just explained would probably have been the basis of our grant proposal. Such a proposal might have been reviewed by an expert in electron microscopy and one in virology. The virologist would have seen nothing very exciting in the proposal, and the electron microscopist would have foreseen difficulties in interpreting the pictures. Both might have suggested that radioactive labeling of the virus contents would offer better chances of success. This is advice we could not easily have ignored. So we should have embarked on a rather demanding additional technique, and before we could have mastered it my fellowship would have expired—even in the unlikely event of our grant application being immediately answered. But we had the good fortune of not having to apply for a grant. The financing of our project was secure: Isaacs's

salary was paid by the Influenza Centre, mine by a fellowship from the Swiss Academy of Medicine, and the (modest) running expenses were covered by the institute's budget.

The experiments I had done in Zurich in 1955 had one major flaw, which was in fact the reason why my boss, Prof. Hermann Mooser (1891–1971), was still brooding over my results: I had assumed that the inactivated virus remained firmly bound to the red cells during the whole experiment. This was not an unreasonable assumption, but it had not yet been proved. How could we show that the virus indeed remained where we wanted it to stay? With Valentine the following exceedingly simple experiment was done: red cell ghosts were prepared and one-half of them were brought in contact with heat-inactivated virus, then washed; these were the virus-loaded ghosts. Three samples were incubated overnight in roller drums at 37°C (the same conditions used to induce interference, except that the membrane fragments were left out): (a) untreated red cell ghosts; (b) red cell ghosts loaded with virus; (c) a mixture of equal parts of a and b. From these three samples electron microscopic grids were prepared. In due time, the pictures were ready to be analyzed and revealed the following: as expected, the untreated ghosts (a) showed very clean and neat surfaces free of any particles resembling influenza virus. The ghosts loaded with virus (b) showed, again as expected, numerous typical virus particles distributed over their surface. However, the pictures from c might have led us to abandon the whole project. They showed, side by side, ghosts containing many virus particles (as in b), empty ghosts (as in a), but, in addition, some ghosts which had a few unmistakable virus particles attached. This probably meant that, in collisions between virus-loaded and empty ghosts, some virus particles had changed their place. Obviously, the attachment of the virus to the ghosts was not as irreversible as we had hoped.

Fortunately, the whole electron microscopy took some time before interpretable pictures were laid on Valentine's desk, and in the meantime we had not been idle. We wondered if the interference-inducing capacity of virus-loaded ghosts could become exhausted. So after a first round of interference induction, the membrane fragments were removed and replaced with fresh membranes to see whether they, in turn, would show interference. We had three reasons to expect this second round of interference to be substantially weaker than the first round: Alick knew from previous experiments that heat-inactivated influenza virus held at 37°C lost its interfering capacity after a relatively short time. Further, we reasoned that if interference had been caused by the virus injecting its nucleic acid into the host cells the remaining empty virus particles would lack activity. Finally, Alick knew that the membranes released an inhibitor into the fluid that impeded the interfering activity of heated virus (Isaacs and Edney 1950). However, contrary to our expectation, this second round of interference was very nearly as strong as the first round.

Now here intuition, or perhaps less mysteriously simply recollection enters into the picture. Our common chief, C.H. (later Sir Christopher) Andrewes (1896–1988) had written in 1942 in a paper on interference between live viruses in tissue cultures:

> The most obvious explanation of the phenomenon is probably the correct one—that the virus first upon the scene uses up some essential foodstuff in the cells. An alternative to the hypothesis of an exhaustion of food-supply would be, of course, the generation within the cell of some poorly diffusible inhibitory substance.
>
> C.H. Andrewes 1942

I don't recall that Alick and I specifically discussed this paper, but we probably had it at the back of our minds. What we certainly did discuss was the wish to see if there was some "generation" of an inhibitory substance ("of course," as Andrewes had written), which might be an explanation for the unexpected persistence of interfering activity. In the seminal paper (Isaacs and Lindenmann 1957), we wrote: "In an effort to explain the results of the last experiment the possibility was considered that fresh interfering activity was produced by the membrane" (p 263). In order to discuss this hypothesis we had to give the unknown substance a name. I suggested "interferon," and Alick thought that this was because I was jealous of my colleagues in experimental physics who were playing with things like electrons, myons, neutrons, baryons, mesons etc.

Until then, our project had involved three elements: (1) the red cell ghost, (2) inactivated virus, and (3) membrane fragments. It now dawned upon us that, under our new tentative hypothesis, the red cell ghosts, always meant to passively carry the virus, could be dispensed with—forget the ghosts. So an experiment was started in which the heat-inactivated virus was brought in contact with the chick membrane fragments for 2 h (bath no. 1). The membranes were then washed free of virus and incubated with fresh fluid (bath no. 2). After having spent a number of hours in this fresh fluid, the membranes were removed and placed in a third bath of fresh fluid (bath no. 3) and challenged with live influenza virus to see if they showed the phenomenon of interference—they did. So far there is nothing new in this experiment. However, now comes the justification for Alick's labeling of this experiment "in search of an interferon" (November 6th, 1956; from Alick's lab journal now kept at the National Library of Medicine in Bethesda, MD): into bath no. 2 a fresh, naive set of membranes was placed, left therein for several hours and then challenged with live influenza virus. To our delight (because by that time we had already become partial, which is a dangerous moment in any investigation), these membranes, which had, as far as we could tell, never been in direct contact with virus, showed clear-cut interference.

The next, rather hectic, few weeks were mainly concerned with the elimination of possible artifacts or of trivial explanations. To give an example of one possible trivial explanation, bath no. 2 could have been depleted of, to use again Andrewes's words lurking at the back of our minds, "some essential foodstuff," although our use of inactivated influenza virus made this proposition less likely than in Andrewes's case, who had been using live virus. Or bath no. 2 might have been teeming with virus particles released from the membranes after temporary capture.

What of the electron microscopy? This proved disappointing, although by that time we were, in this respect, beyond disappointment: the electron microscopic pictures of the ghosts taken after they had been in contact with the membrane fragments could not be interpreted, because they were obscured by cellular debris, so that a distinction between "full" and "empty" virus particles was impossible—the red herring.

I presented some of our results, those involving the red cells, at a meeting of the Swiss Society for Microbiology in Interlaken on June 22, 1957 (Lindenmann and Isaacs 1957). By that time, our two papers in the Proceedings of the Royal Society were still in print, so that this Interlaken meeting was the first official emergence of interferon.

The first metaphor I have used in the title of this paper, the chasing of a red herring (which can be defined as "to follow a distracting clue"), means that we embarked upon an experiment which seemed doable but met with unexpected difficulties. In my second metaphor, the smallmouth bass is described as a game fish which gives the angler a good fight and jumps spectacularly up and down—and I don't have to tell those in the interferon business how many ups and downs they have been through over the past 50 years.

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Interferon Research BC (Before Cloning)

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Abstract As we approach the 50th anniversary of the publications describing the discovery of interferon, it is appropriate to look back at some of the trials and tribulations marking the early days of interferon research. This brief chapter, drawn largely from the author's own experiences, relates how progress was achieved in some key areas of interferon research in the 1960s and 1970s despite the lack of analytical tools that had become available only after the successful cloning of interferon genes. One of the topics discussed concerns the evolution of the idea that interferon synthesis is controlled both at transcriptional and posttranscriptional levels. I also recount some of the early work that led to the identification of IFN-α and IFN-β as the two major type I interferon species.

1 The Beginnings

When the editor of this volume, Dr. Paula Pitha, asked me to contribute a chapter, she said, "Why don't you summarize the beginnings of the interferon field from your perspective?" Easier said than done. Fortunately, I do meet one important condition: I am old enough to remember the early days of the interferon field. By coincidence, I graduated from medical school in the town of Bratislava in former Czechoslovakia (now Slovakia) in 1957, the same

year that Isaacs and Lindenmann, working at the Medical Research Council Laboratories in Mill Hill, outside London, published their first report of the discovery of interferon (Isaacs and Lindenmann 1957). I did not hear about interferon when I was a medical student, but I do clearly remember how I first learned about the molecule that would become a major focus of my professional interest for several decades. As fate would have it, Alick Isaacs visited Bratislava in 1958 and gave a lecture on his and his colleagues' work concerning viral interference and interferon. By then I was a research fellow at the Institute of Virology in Bratislava, eager to master the science of virology. I recall vividly Alick's description of interferon as a cell-derived protein responsible for the interference between heat-inactivated and live influenza virus in cultured chick embryo chorioallantoic membranes. At the time, Alick Isaacs, though only in his mid-thirties, was already well known for his studies on influenza virus and virus interference. He was a young-looking man, bubbling with energy, wit, and personal charm. I was fortunate to become personally acquainted with Alick during his visit to Bratislava and to be able to maintain friendly contacts with him afterwards. (Sadly, Isaacs died in 1967 at the age of 45.)

I did not make a decision to work on interferon right after hearing Alick Isaacs's lecture. My project at the time was aimed at developing a tissue culture assay for tick-borne encephalitis virus (TBE), a flavivirus of some medical importance in Central and Eastern Europe. Although the virus multiplied readily in many types of cultured cells, it did not cause a cytopathic effect, so that virus-infected cells were morphologically indistinguishable from uninfected cells and the presence of the virus was difficult to determine. To remedy the situation, I followed the advice of another prominent visitor to Bratislava, Albert Sabin, who is best known for the development of the live oral polio vaccine. During a visit in 1959, Albert suggested that I inoculate cultures with TBE virus, wait 2–3 days and then challenge the same cells with another virus that normally kills cells in the process of its replication. Multiplication of TBE virus may induce a state of interference, Sabin said, so that the challenge virus might fail to produce a cytopathic effect. Thus, the absence of cell death upon inoculation of the challenge virus would provide an indication of the presence of TBE. I followed Albert Sabin's advice and, eureka, the experiment turned out to be successful. As Albert had predicted, the presence of TBE virus suppressed the multiplication and the appearance of cytopathic effect of the challenge virus (I used Western equine encephalomyelitis virus), thus providing an indirect method for the assay of TBE virus (Vilcek 1960a). But what was the mechanism of this interference? Could it be due to the production of a substance similar to interferon, shown to be responsible for interference between inactivated and live flu virus in chick chorioallantoic membranes (Isaacs and Lindenmann

1957)? (I should add that at the time, in mid-1959, the role interferon in virus interference was not yet widely recognized and, very likely, I would not have thought of this possibility had I not heard Isaacs's lecture about a year earlier.) I completed a few experiments and soon it became obvious that a substance produced by TBE virus-infected cells, unrelated to the virus itself, was indeed mediating the interference phenomenon. To be on the cautious side, I termed the mediator "an interferon-like substance" (Vilcek 1960b). Around the same time, other investigators in Europe and the US were describing the production and action of what appeared to be interferon in other virus-cell systems (Chany 1960; Dinter 1960; Henle et al. 1959; Ho and Enders 1959; Kaplan et al. 1960). Thus the new field of interferon research was born and I had the privilege of becoming one of its first members!

2
Posttranscriptional Control of Interferon Production

2.1
Preformed Interferon?

Fast-forward to 1965. By then I was a newly appointed assistant professor in the Department of Microbiology at New York University School of Medicine and many more investigators have joined the interferon bandwagon. In the 1960s, it was already accepted that interferon proteins are encoded by cellular (and not viral) genes and that, like other cellular proteins, interferon production requires synthesis of cellular mRNA followed by its translation into the interferon protein. This conclusion was based mainly on the demonstration that inhibitors of DNA-dependent RNA synthesis (usually actinomycin D) and various inhibitors of protein synthesis blocked interferon production in virus-stimulated cells. Subsequent experiments showed that actinomycin D also inhibited interferon production stimulated in cultured cells by the double-stranded RNA, poly(I).poly(C), suggesting that interferon production induced by nonviral interferon inducers also required de novo synthesis of cellular mRNA and protein (reviewed in Burke 1966; Vilcek 1969).

The validity of these conclusions was called into question by the observations made in animals stimulated to produce interferon by injection with bacterial endotoxin (Ho and Kono 1965; Youngner and Stinebring 1966). Surprisingly, unlike virus-induced interferon, production of endotoxin-induced interferon was not suppressed by inhibitors of RNA synthesis (actinomycin D) or protein synthesis (cycloheximide or puromycin). Later, Youngner and colleagues found that cycloheximide not only did not suppress, but actually increased poly(I). poly(C)-induced circulating interferon in mice (Youngner and Hallum 1968).

On the basis of these findings, the authors proposed that endotoxin-induced and poly(I).poly(C)-induced interferon in animals is not newly synthesized, but represents preformed interferon that is merely released by the action of the inducer.

Similar paradoxical effects of metabolic inhibitors on interferon production were also seen in cell cultures. My first graduate student at NYU, Toby Rossman, observed that the addition of actinomycin D to chick embryo cells 10–14 h after inoculation with Chikungunya virus (an RNA-containing alphavirus) slightly increased subsequent interferon release when compared to cells not treated with the inhibitor of RNA synthesis (T. Rossman, unpublished observations). We then examined the effects of metabolic inhibitors on poly(I).poly(C)-induced interferon production in cultures of rabbit kidney cells (Vilcek et al. 1969). Actinomycin D addition before poly(I).poly(C) blocked interferon production, but when added 3.5 h after poly(I).poly(C), the inhibitor markedly enhanced the interferon yield. Rather than simply invoking the existence of preformed interferon, we proposed that the increased release of interferon from cells treated with actinomycin several hours after exposure double-stranded RNA may be explained by the blocking of an endogenous inhibitor. We pointed out that these findings were similar to observations of the paradoxical effect of actinomycin D on the synthesis of the glucocorticoid-inducible enzyme tyrosine amino-transferase and that in the latter case the enhancing effect was ascribed to the blocking of endogenous cellular inhibitors acting at the level of protein translation.

More difficult, at least initially, was the interpretation of the effects of inhibitors of protein synthesis on poly(I).poly(C)-induced interferon production, which we also studied in rabbit kidney cell cultures. Puromycin either failed to inhibit or inhibited interferon production only when added together with actinomycin at 3.5 h after poly(I).poly(C) (Vilcek et al. 1969). Another inhibitor of protein synthesis, cycloheximide, caused a slight initial suppression of interferon production, but at later time points caused a marked increase in the amount of interferon produced (Fig. 1). It seemed difficult to reconcile these observations with the idea that interferon in these cultures was newly synthesized. How could significantly more interferon be synthesized in the presence of cycloheximide that decreased the rate of protein synthesis by 90% or more? Eventually, our experiments led to the conclusion that the paradoxical enhancement of interferon production was due to the greatly increased availability of interferon mRNA, apparently an indirect consequence of the suppressed synthesis of an endogenous inhibitory protein that either degraded interferon mRNA or prevented its translation (Vilcek and Ng 1971). Thus, if accumulation of interferon mRNA were to be enhanced 100-fold in the presence of cycloheximide, a 90% inhibition of

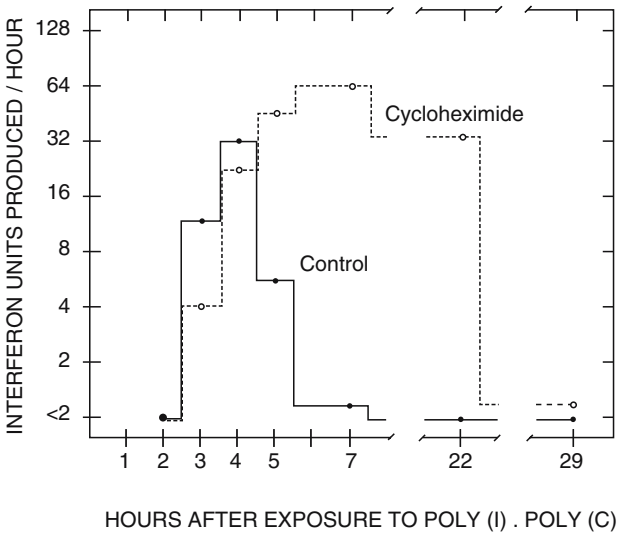

Fig. 1 Interferon production in the presence and absence of cycloheximide in rabbit kidney cell cultures stimulated with poly(I).poly(C). The dose of cycloheximide used (20 μg/ml) inhibited total protein synthesis by about 90%, and yet total interferon production was enhanced, mainly because cycloheximide prevented the termination (shut-off) of interferon production, which in control cultures occurred by about 5 h. It is believed that the presence of cycloheximide prevents the synthesis of a posttranscriptional inhibitor that causes inactivation of interferon mRNA. (Reproduced from Ng and Vilcek 1972, with permission)

protein synthesis would still result in a tenfold net increase in the *synthesis* of interferon protein.

In later years, the action of metabolic inhibitors was studied mainly in the model of normal human diploid fibroblasts stimulated to produce IFN-β by poly(I).poly(C). These studies showed more directly that stimulation of cells with double-stranded RNA in the presence of inhibitors of protein synthesis resulted in a marked stabilization of interferon mRNA (Cavalieri et al. 1977; Dinter and Hauser 1987; Raj and Pitha 1981 1983; Tan et al. 1970; Vilcek and Havell 1973; Vilcek and Ng 1971). By the early 1980s, it became accepted that a labile cellular protein produced during the first few hours of stimulation with poly(I). poly(C), causing a selective degradation of IFN-β mRNA, is the target of the inhibitory action of metabolic inhibitors.

2.2 Superinduction

One practical consequence of these experiments was the design of a protocol for the production of high yields of IFN-β in cultures of human diploid fibroblasts (Billiau et al. 1973; Havell and Vilcek 1972; Tan et al. 1970). To produce high yields of interferon, cells were first treated with poly(I). poly(C) in the presence of an inhibitor of protein synthesis (usually cycloheximide). Several hours later, cells were pulsed with actinomycin D. Then, upon removal of the inhibitors, cells released a large burst of interferon. The amount of interferon produced after these treatments was up to 100-fold greater than after stimulation with poly(I).poly(C) alone (Fig. 2). For many

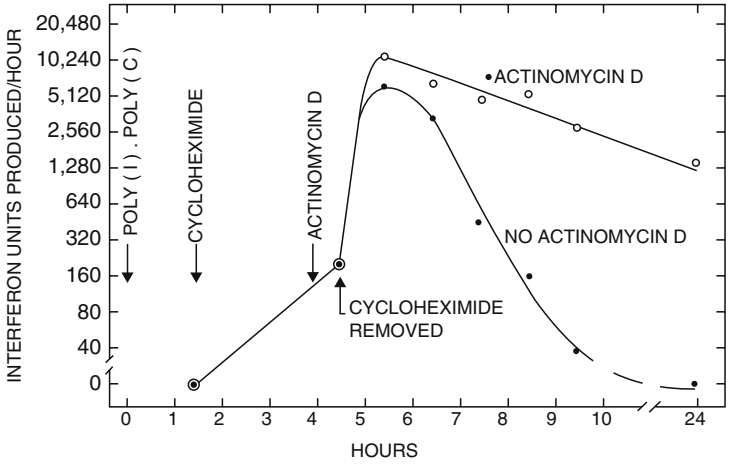

Fig. 2 Evidence for posttranscriptional control of interferon production. Rabbit kidney cell cultures were stimulated with poly(I).poly(C) and then treated with cycloheximide (10 μg/ml). In addition, another group of cultures was also treated with actinomycin D (5 μg/ml) as indicated. Subsequently, the inducer and the inhibitors were removed and fresh medium was added. It is believed that the presence of cycloheximide suppresses the synthesis of a posttranscriptional inhibitor that causes inactivation of interferon mRNA. Therefore, large amounts of interferon mRNA can accumulate in the presence of cycloheximide. The accumulated interferon mRNA is efficiently translated following removal of cycloheximide. Addition of actinomycin D, an irreversible inhibitor of RNA synthesis, at 4 h would prevent subsequent synthesis of the posttranscriptional inhibitor and therefore result in a greater and more sustained interferon synthesis from interferon mRNA that had accumulated in the presence of cycloheximide. (Reproduced from Vilcek and Ng 1971, with permission)

years, this superinduction protocol was used, mainly in Europe and Japan, for the production of natural human IFN-β for clinical applications. This superinduction protocol was also used for the isolation of IFN-β mRNA that served as a template for the cloning of human IFN-β cDNA and elucidation of its complete sequence (Derynck et al. 1980; Taniguchi et al. 1980) Eventually, recombinant DNA techniques replaced the use of human diploid fibroblasts and the superinduction protocol for the routine production of human IFN-β.

A better understanding of the molecular mechanisms of posttranscriptional control of IFN-β expression was achieved only in the 1980s and 1990s. Since this article focuses on interferon research in the BC (before cloning) era, I will allude only briefly to these more recent developments. About two decades ago, AU-rich elements (AREs) present in the 3′ untranslated region (UTR) of some inherently unstable cytokine and oncoprotein mRNAs were shown to be responsible for rapid mRNA degradation (Shaw and Kamen 1986). It is now known that a multi-subunit particle called an exosome is required for rapid degradation of ARE-containing RNAs (Chen et al. 2001). ARE recognition requires certain ARE binding proteins that can also bind to the exosome and recruit it to the RNAs, thereby promoting their degradation. The components of the machinery responsible for the recognition and degradation of some mRNAs through this pathway are only beginning to be understood.

Human IFN-β mRNA contains an ARE in the 3′ UTR and another AU-rich destabilizing sequence, termed coding region instability determinant (CRID), within the coding region (Paste et al. 2003; Raj and Pitha 1993; Whittemore and Maniatis 1990). Independent replacement of either ARE or CRID resulted in a moderate stabilization of IFN-β mRNA, while removal of both elements caused a major increase in message stability. A 65-kDa cytoplasmic protein was shown to bind to both elements, suggesting that it plays a role in the degradation of IFN-β mRNA (Raj and Pitha 1993). A more complete characterization of the proteins interacting with the IFN-β mRNA ARE and CRID has apparently not been attempted.

While enormous progress has been made in our understanding of the molecular mechanisms controlling IFN-β (and, to a lesser degree, IFN-α) gene transcription (Honda et al. 2006), very few studies analyzing the molecular mechanism responsible for the posttranscriptional regulation interferon synthesis—the main target of superinduction—have been published in recent years. Given the apparent importance of posttranscriptional regulation in the control of interferon (especially IFN-β) synthesis, the neglect of this subject is surprising.

3
How Many Interferons?

Touted as a potentially useful antiviral therapeutic, the study of interferon from the beginning attracted wide attention. Already in 1960, the then popular Flash Gordon magazine featured a comic strip in which medics inside a space ship used interferon to save a dying patient afflicted with a mysterious viral infection. In real life, things turned out to be somewhat more complicated. The main obstacle to the launching of meaningful clinical trials with interferon was the lack of technologies for the production of sufficient quantities of human interferon.

One promising system for the large-scale production of human interferon was pioneered by Kari Cantell and his colleagues at the National Blood Transfusion Center in Helsinki, Finland. There, in the early 1960s, they established a laboratory for the production of human interferon that employed large-scale cultures of normal human leukocytes challenged with Sendai virus (Strander and Cantell 1966). The cells used to produce interferon were obtained from fresh buffy coats, isolated from units of blood collected from healthy donors. Until replaced in the 1980s by interferons produced by recombinant DNA techniques, human leukocyte interferon generated in Finland was the main source of material used in clinical trials in Europe and the United States.

Another technology, which became available for large-scale production of interferon in the early 1970s, utilized cultured lines of normal human diploid fibroblasts, usually derived from neonatal foreskins. To obtain high yields of interferon, cultures were stimulated with poly(I).poly(C) and sequentially treated with cycloheximide and actinomycin D under the superinduction protocol, as outlined in the preceding section (Fig. 2).

Interferon preparations produced in buffy coat-derived leukocytes and in diploid fibroblasts became known as leukocyte and fibroblast interferons, respectively. Initially, it was not known whether leukocyte and fibroblast interferons were or were not qualitatively different. Of course, it was known since 1959 that interferons showed species specificity, i.e., that interferons produced in cells of one species may or may not show antiviral activity in cells of a heterologous animal species. These findings indicated that interferons produced in cells of different animal species were not identical. However, for many years it was believed that interferons were not tissue specific, i.e., the consensus was that interferons produced in different cells of the same species were probably identical (reviewed in Vilcek 1969). (An exception was lymphocyte-derived type II, or immune, interferon, which was known to be pH2-labile and, therefore, suspected to be different from conventional interferon (Wheelock 1965)).

This view started to change when it was found that human leukocyte and fibroblast interferons can show significant differences in their heterospecific antiviral activities. For example, human leukocyte interferon, but not fibroblast interferon, was found to have a high degree of antiviral activity in bovine and porcine cells (Gresser et al. 1974).

A more direct indication of the existence of different molecular species of human interferon was obtained through the analysis of antigenic properties of interferons produced in leukocyte and fibroblast cultures. In the late1960s and early1970s, it took a heroic effort to produce enough partially purified interferon for the successful immunization of animals and production of neutralizing antibodies. A pioneer in the effort to generate antibodies to interferon was Kurt Paucker with his colleagues at the Medical College of Pennsylvania in Philadelphia. (Sadly, Kurt Paucker, a close friend and colleague, died prematurely in 1980.) They observed that antibodies generated by immunization of rabbits with leukocyte interferon, which neutralized the activity of homologous interferon, usually (though not always) also neutralized human fibroblast interferon. In contrast, antibodies produced in rabbits against human fibroblast interferon completely failed to neutralize leukocyte interferon (Berg et al. 1975). Edward Havell in my laboratory, using our own antisera to human leukocyte and fibroblast interferons, confirmed these findings. At first these results were puzzling. Why would antigenic cross-reactivity be only unidirectional, i.e., why did antisera to fibroblast interferon completely fail to neutralize leukocyte interferon?

In order to solve this puzzle Edward Havell and I joined forces with Kurt Paucker and his colleagues (Havell et al. 1975a). Based on some preliminary observations made in both laboratories, we came up with a hypothesis that would explain these findings: we postulated that human fibroblast and leukocyte interferons are in fact antigenically distinct and the antibodies they elicit do not cross-react. We further postulated that the reason for the apparent unidirectional antibody cross-reactivity is that leukocyte interferon preparations contain a mixture of two distinct species of interferon molecules: (1) a major component that is characteristic for leukocyte interferon and (2) a minor component that is identical to fibroblast interferon. Thus, when used for immunization, leukocyte interferon preparations would usually elicit the generation of antibodies to both leukocyte and fibroblast interferons, whereas immunization with fibroblast interferon preparations (which contain little or no leukocyte interferon) would produce only antibodies to fibroblast interferon.

To confirm our hypothesis, we took serum from a rabbit immunized with leukocyte interferon that showed neutralizing activity to both leukocyte and fibroblast interferons, and we passed it through an affinity column of human fibroblast interferon covalently bound to Sepharose (Table 1). We predicted that if the

Table 1 Neutralization of fibroblast and leukocyte interferons by antiserum generated by the immunization of a rabbit with leukocyte interferon: evidence that crude antiserum can be separated into two distinct antibody populations that are specific for fibroblast and leukocyte interferons, respectively (adapted from Havell et al. 1975a)

Fraction of serum tested for neutralizing activity	Neutralization of leukocyte IFN	Neutralization of fibroblast IFN
Original crude serum	+	+
Serum fraction passed through column of fibroblast IFN bound to sepharose	+	–
Serum fraction retained and eluted from column of fibroblast IFN bound to sepharose	–	+

antiserum contained distinct antibodies to leukocyte and fibroblast interferons, only the latter antibodies should stick to the column. Thus after passage through the column, the antibody should no longer neutralize fibroblast interferon, but still react with leukocyte interferon. In contrast, if neutralization of fibroblast interferon is due to antigenic cross-reactivity, the effluent antibody should have decreased reactivity with both fibroblast and leukocyte interferon. The results clearly supported the existence of two separate antibody populations, specific for leukocyte and fibroblast interferons (Havell et al. 1975a). We proposed to designate the major component of human leukocyte interferon preparations "Le interferon." We also proposed that the species which forms the minor component of leukocyte interferon and the major or exclusive component of fibroblast interferon be designated "F interferon." Shortly thereafter, the Le and F interferon species were actually isolated from interferon preparations generated in cultures of buffy coat-derived human leukocytes, corroborating our conclusions (Havell et al. 1975b; Paucker et al. 1975).

The scientific community accepted the proposed nomenclature. Some years later, a special Interferon Nomenclature Committee decided to introduce designations IFN-α and IFN-β instead of Le and F interferons, respectively (Stewart 1980). The same committee also introduced the designations of type I and type II interferons. These designations are still in use today.

A great deal has happened since these developments. We now know that there are 12 distinct human IFN-α proteins, produced from 14 genes (reviewed in Pestka et al. 2004). In addition to the IFN-β gene and protein (a single gene in humans and most other mammalian species), there are numerous other type I interferons, comprising IFN-δ, IFN-ε, IFN-κ, IFN-τ, IFN-ω and limitin. (IFN-δ, IFN-τ, and limitin have not been found in humans.) There is also a single IFN-γ or type II IFN gene and protein. The most recent addition to the interferon family

are the type III interferons (also called IFN-λ or IL28/29 family), comprised of three related genes and proteins (Kotenko et al. 2003; Sheppard et al. 2003). Members of the type I, II, and III interferon families interact with three different heterodimeric receptors, respectively, to activate somewhat distinct but overlapping JAK-Stat signal transduction pathways (Pestka et al. 2004).

4 The Next Fifty Years

What is in store for interferon research in the next 50 years? Judging from the continuing boom in interferon-related publications, the interest in interferon is not going to wane any time soon. However, the pace and nature of the discovery process are likely to be different. It took over 20 years from the time of their discovery for interferon proteins to be purified to homogeneity. Then, in the latter part of the twentieth century, the pace of discovery in all of biomedical science, and especially in the interferon field, has accelerated substantially—to a large extent due to the introduction of molecular cloning and other methods of molecular biology.

In the early days of interferon research, scientists were seeking to answer three broad fundamental questions: (1) what is the structure of interferon molecules? (2) What are the mechanisms that regulate interferon induction and synthesis? and (3) What are the mechanisms of interferon action? In addition, there has always been the desire to understand the role of interferon in the intact organism. At the more applied level, a great deal of effort has been devoted to the development of interferons into useful therapeutic drugs.

Work is likely to continue at all of these levels, but with different intensities. Of the three questions listed in the preceding paragraph, much recent effort has been directed toward the elucidation of molecular mechanisms of interferon induction, partly because of significant progress in the elucidation of the roles of toll-like receptors (TLR), interferon regulatory factor (IRF) proteins, and other transcriptional mediators in this process (reviewed in Honda et al. 2006; Stetson and Medzhitov 2006). Despite recent progress, much remains to be learned about molecular mechanisms of type I interferon production as it is becoming clear that TLRs are not the only molecular sensors triggering interferon production (Yoneyama et al. 2004). Molecular pathways utilized by certain inducers of type I interferon production, e.g., some viral glycoproteins and other microbial components, have not yet been discovered. Much is also still to be learned about the molecular mechanisms regulating IFN-γ induction.

Efforts toward a more complete realization of the therapeutic potential of interferons will also continue, with most attention likely to be devoted to improvements in the pharmacokinetic properties of interferons and to decreasing their side effects. However, the most fertile area of future research could become the exploration of physiological and, more importantly, pathophysiological roles of interferons. Although the idea that interferons can contribute to the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus, was introduced in the 1970s and 1980s (Hooks et al. 1982; Preble et al. 1984; Skurkovich and Eremkina 1975), the molecular mechanisms have only recently started to be elucidated (Banchereau and Pascual 2006). The latter studies could usher in a new branch of interferon research: the development of therapeutically useful antagonists of interferon.

So what will articles commemorating the 100 th anniversary of the discovery of interferon be talking about? Let's wait and see.

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Purification and Cloning of Interferon Alpha

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Abstract Interferon alpha (IFN-α) was approved by the US Food and Drug Administration on June 5, 1986 and paved the way for development of many other cytokines and growth factors. Nevertheless, we have barely touched the surface of understanding the multitude of human IFNs. This paper reviews the history of the purification of human leukocyte IFN, the cloning of the IFN-αs, and the current state of knowledge of human interferon alpha genes and proteins.

1 The Interferons

The interferons are proteins with antiviral activity (Isaacs et al. 1957; Isaacs and Lindenmann 1957; Nagano and Kojima 1958; Pestka et al. 1987, 2004a; Sen and Lengyel 1992; Stark et al. 1998). There are two types of human interferons, type I and type II, and interferon-like cytokines (Krause and Pestka

Table 1 The human type I interferons and interferon–like proteins[a]

Class	Names
Type I IFNs	IFN–α
	IFN–β
	IFN–ε
	IFN–κ
	IFN–ω
	IFN–ν
Interferon–like cytokines	IL–28A, IL–28B, IL–29

[a]This table summarizes interferon and interferon-like proteins. Human type I IFN members are clustered together, as are the IL-28A, IL-28B, and IL-29 cytokines

2005; Pestka et al. 2004a, 2004b). Type I human interferons consist of six classes: IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, and IFN-ν (Table 1). However, a large number of type I interferons are found in other animal species, which will not be discussed in this report, but they have been described in another publication (Krause and Pestka 2005). Type II interferon consists only of IFN-γ. In addition, three related human interferon-like cytokines have been reported: IL-28A, IL-28B, and IL-29 (Table 1) (Kotenko et al. 2003; Sheppard et al. 2003). There is only one IFN-β, IFN-ε, IFN-κ, IFN-ω, and IFN-ν, but the IFN-α proteins consist of twelve individual proteins (Table 2).

Although partial purification of the interferons as bands on sodium dodecyl sulfate-polyacrylamide gels was reported by a number of groups, it was not until 1978 and thereafter that any interferon had been purified to homogeneity in solution in sufficient amounts for its chemical and physical characterization (Friesen et al. 1981; Rubinstein et al. 1978a, 1979c, 1981; Stein et al. 1980). The introduction of reverse-phase and normal-phase high-performance liquid chromatography to the purification of proteins (Friesen et al. 1981; Rubinstein et al. 1979c; Stein et al. 1980) led to the first successful purification of IFN-α and IFN-β so that sufficient amounts were available in solution without detergent for their chemical, biological, and immunological studies.

2 Purification of Human Leukocyte Interferon

Since the discovery of interferons, many attempts were made to purify the interferons with little success until 1978. In fact, interferon used in experiments as well as in initial human clinical trials was essentially a crude protein fraction less

Table 2 Human interferon alpha genes (14) and proteins (12/13)[a]

Genes	Proteins
IFNA1	IFN–αD, IFN–α1
IFNA2	IFN–αA (IFN–α2a), IFN–α2 (IFN–α2b), IFN–α2c
IFNA4	IFN–α4a (IFN–α76), IFN–α4b
IFNA5	IFN–αG, IFN–α5, IFN–α61
IFNA6	IFN–αK, IFN–α6, IFN–α54
IFNA7	IFN–αJ, IFN–αJ1, IFN–α7
IFNA8	IFN–αB2, IFN–αB, IFN–α8
IFNA10	IFN–αC, ΨIFN–α10, ΨIFN–αL, IFN–α6L
IFNA13	IFN–α13 (sequence identical to IFN–α1)
IFNA14	IFN–αH, IFN–αH1, IFN–α14
IFNA16	IFN–αWA, IFN–α16, IFN–αO
IFNA17	IFN–αI, IFN–α17, IFN–α88
IFNA21	IFN–αF, IFN–α21
IFNAP22	ΨIFN–αE

[a]The genes for human IFN–α are given in the left column and the proteins in the right column. Allelic forms of the IFN–α proteins are shown in the right column (Pestka 2000). Designations in parentheses represent alternate names for identical proteins

than 1% of which by weight consisted of interferon. Because of the use of such crude interferon-containing material, it was not clear what activities of these preparations were indeed due inherently to the interferon present and what activities were due to the numerous other contaminating proteins. By definition, the antiviral activity was due to the interferon. However, these crude preparations exhibited antiprotozoal and antibacterial activities, inhibited cellular growth (antiproliferative activity), blocked antibody synthesis, and were ascribed to have many other activities. However, without high-purity preparations of interferon, it was not possible to demonstrate definitively whether or not a particular activity was due to the interferon protein molecule itself. Thus, it was essential to obtain purified interferon to determine what activities were intrinsic to the interferon molecule. Since very little was known about the size and structure of the interferons, the isolation of purified interferons was necessary to establish their chemical composition and structure as well as their biological activities.

2.1 Production

We began purification of interferon from human leukocytes in 1977. This human leukocyte interferon (Hu-IFN-α) was produced by incubating human white blood cells with Newcastle disease virus or Sendai virus for 6–24 h (Familletti et al. 1981a; Familletti and Pestka 1981; Hershberg et al. 1981; Waldman et al. 1981). The procedure was a combination of methods that had previously been reported (Cantell and Tovell 1971; Wheelock 1966). The antiviral activity was found in the cell culture medium after overnight incubation of the leukocytes. We substituted milk casein for human or bovine serum in the culture medium, as had been described (Cantell and Tovell 1971). The use of casein, a single protein, instead of serum, which contains many different and uncharacterized proteins, simplified the initial concentration and purification steps. We used leukocytes from normal donors as well as from patients with chronic myelogenous leukemia. These leukemic cells made substantial amounts of human leukocyte interferon when induced with Newcastle disease virus or Sendai virus (Familletti et al. 1981a; Hadhazy et al. 1967; Lee et al. 1969; Rubinstein et al. 1979b); however, less than 0.1% of the starting medium consisted of leukocyte interferon (Rubinstein et al. 1981). Although leukocyte interferon consisted predominantly of IFN-α, small amounts of IFN-β and IFN-ω were also present (Adolf et al. 1990; Cavalieri et al. 1977b).

The cytopathic-effect inhibition assay for interferon as originally described took 3 days. Other assays for interferon were even longer. A more rapid assay was necessary to proceed with the purification expeditiously. A cytopathic effect inhibition assay that could be done in 12–16 h was developed and accelerated the purification immensely (Familletti et al. 1981b).

2.2 High-Performance Liquid Chromatography for Protein Purification

Prior to purification by high-performance liquid chromatography (HPLC), interferon in media after production was concentrated, then passed through a Sephadex G-100 fine gel filtration column to isolate protein in the 20,000-mw range as described (Rubinstein et al. 1978a, 1979c, 1981). Because standard methods for protein purification were not significantly successful in purification of the human interferons from blood cells, we applied HPLC to the purification of proteins. Udenfriend and co-workers (Bohlen et al. 1975; Stein et al. 1973; Udenfriend et al. 1972) had developed sensitive fluorescent techniques for detection of amino acids and peptides and had achieved the separation of peptides by reverse-phase HPLC (Lewis et al. 1978; Rubinstein et al. 1978b; Stein

et al. 1978), but separation of proteins had not yet been accomplished. After gel filtration, the major interferon fraction was applied directly to a Lichrosorb RP-8 column. The column was washed in 1 M sodium acetate buffer, then the interferon was eluted with *n*-propanol gradients, as reported and described in detail (Rubinstein et al. 1978a, 1979c, 1981). By changing the pH of the elution buffer, a completely different separation could be achieved during elution of the same reverse-phase column with *n*-propanol. As subsequently demonstrated with fibroblast interferon (Friesen et al. 1981), a large number of different columns and solvent systems could be used to effect resolution of proteins. By applying normal-phase chromatography with a diol silica column between the two reverse phase columns, it was possible to use just three sequential HPLC steps to purify human leukocyte interferon (IFN-α) to homogeneity. Sufficient amounts were purified in high yield for initial chemical characterization of the protein and for determination of amino acid composition. The amino acid composition of the human leukocyte interferon species γ2 (our nomenclature at the time for what is now designated as one of the IFN-α species) was the first reported for any purified interferon (Rubinstein et al. 1979c). Originally, the natural interferons that were isolated from the mixture present in leukocyte interferon by high performance liquid chromatography (Pestka 1983a; Rubinstein et al. 1978a, 1979c, 1981) were then designated α1, α2, β1, β2, β3, γ1, γ2, γ3, γ4, γ5, and δ. Unfortunately, the same Greek letters were later used to designate leukocyte, fibroblast, and immune interferons, respectively, as IFN-α, IFN-β, and IFN-γ, causing a great deal of misunderstanding for many years.

The initial steps for purification of IFN included selective precipitations and gel filtration (Fig. 1) followed by HPLC. The HPLC steps were reverse-phase chromatography (Fig. 2A) at pH 7.5 on LiChrosorb RP-8, normal partition chromatography on LiChrosorb Diol (Fig. 2B), and reverse-phase chromatography at pH 4.0 on LiChrosorb RP-8 (Fig. 2C, D). Gradients of *n*-propanol were used for elution of interferon from these columns (Fig. 2A, C, D). The overall purification was approximately 80,000-fold and the specific activity of purified interferon was $2–4\times10^8$ units/mg (Rubinstein et al. 1979c). Interferon prepared by this procedure yielded a single band of MW 17,500 on polyacrylamide gel electrophoresis. The antiviral activity was associated with the single protein band (Rubinstein et al. 1978a). The specific activity of this peak was 4×10^8 units/mg.

Several reports had previously described high-performance liquid chromatography of proteins, mainly on ion exchange and size exclusion columns (Chang et al. 1976; Regnier and Noel 1976). However, those systems were either not commercially available or had a low capacity. With proper choice of eluent and pore size, octyl and octadecyl silica could be used for high-resolution reverse-phase HPLC of both peptides and proteins. Accordingly, with *n*-propanol

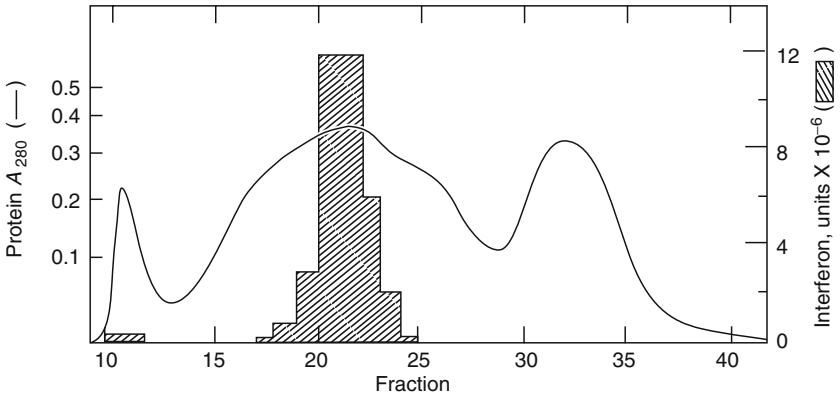

Fig. 1 Initial steps in purification of leukocyte interferon. Cells and debris were removed by low-speed centrifugation from the medium containing interferon. Casein was used as a serum substitute. By acidification of the medium to pH 4 with hydrochloric acid, the bulk of the casein was precipitated. The supernatant containing the interferon was concentrated as described (Rubinstein et al. 1979c). The concentrated solution containing relatively crude interferon was applied to a column of Sephadex G-100 fine, then the column eluted with 4 M urea/0.1 sodium acetate, pH 7.5. The detailed procedures were described previously (Rubinstein et al. 1979c). The fractions with interferon activity were pooled and purified by HPLC (Fig. 2). Similar procedures were carried out as we purified leukocyte interferon species over several years (Hershberg et al. 1981; Pestka 1983a; Rubinstein et al. 1979c, 1981; Waldman et al. 1981). (From Rubinstein et al. 1979c)

as eluent, the use of LiChrosorb RP-8 (octyl silica) columns for protein fractionation was a major factor in the success of the purification (Fig. 2A, C, D). In addition, LiChrosorb Diol, which is chemically similar to glycophase resins, which have been used for exclusion chromatography of proteins, was introduced as a support for normal partition chromatography of proteins (Fig. 2B). High recoveries of interferon activity were obtained in each chromatographic step, a requirement when small amounts of initial starting material are present. Although the initial experiments were conducted with leukocytes from normal donors (Rubinstein et al. 1978a, 1979c), it was found that leukocytes from patients with chronic myelogenous leukemia (CML), who were undergoing leukapheresis to lower their peripheral white blood cell counts, were a rich source of interferon that appeared to be essentially identical to the human leukocyte interferon purified from leukocytes from normal donors (Rubinstein et al. 1979a). As with HPLC of interferon from normal leukocytes on the Diol column (Fig. 2B), three major peaks of activity, labeled α, β, γ, were observed with

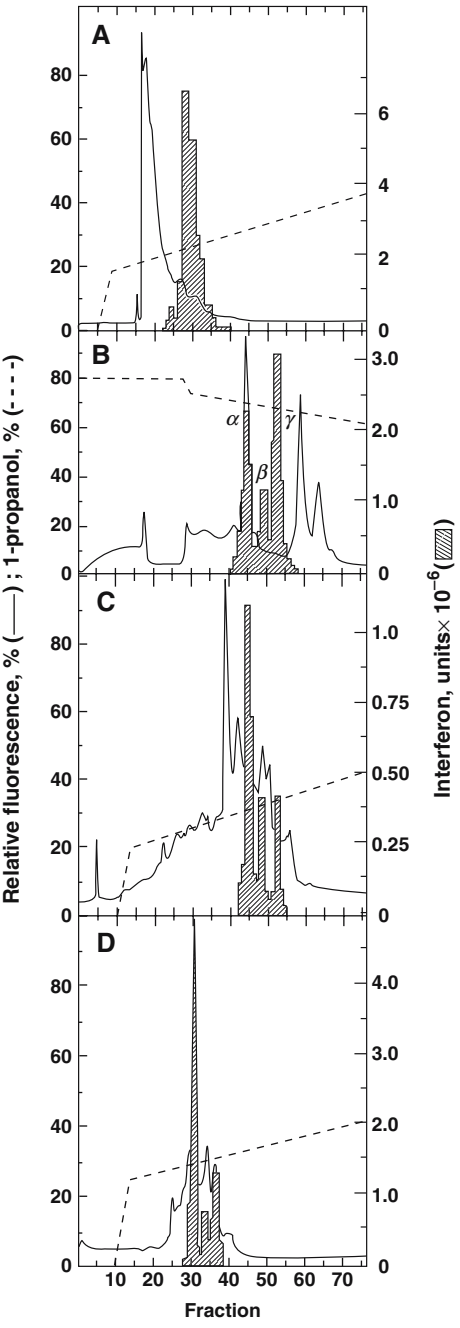

Fig. 2A–D High-performance liquid chromatography of leukocyte interferon. **A** Chromatography on Lichrosorb RP-8 at pH 7.5. **B** Chromatography on Lichrosorb diol at pH 7.5. **C** Chromatography on Lichrosorb RP-8 at pH 4.0. **D** Rechromatography on Lichrosorb RP-8. The conditions were similar to those of step **C**. Several preparations carried through step **C** were pooled (13×10^6 units) and applied to the last column. The gradations on the abscissa correspond to the end of the fractions. Further details were reported previously (Rubinstein et al. 1979c). (From Rubinstein et al. 1979c)

interferon prepared from CML cells. Although the protein profiles were almost identical, the activity profiles showed that the amount of activity under peak α was lower in preparations from leukemic cells compared to normal leukocytes (Rubinstein et al. 1979a, 1979c). However, even from normal leukocytes, the ratio of peaks α, β and γ varied from one preparation to another.

2.3 Multiple Species of Leukocyte Interferon

During the purification of leukocyte interferon, it became evident that multiple species existed. Peptide mapping and sequencing of these species supported this concept. As additional leukocyte interferon species were isolated from cultured myeloblasts (Hobbs et al. 1981; Hobbs and Pestka 1982) and other sources (Allen and Fantes 1980; Berg and Heron 1981; Zoon 1981), the concept that IFN-αs are a family of interferons was established.

2.4 Carbohydrate Content

Five purified species of leukocyte interferon that were initially isolated and interferon produced by Namalwa cells were found to contain no detectable carbohydrate (Allen and Fantes 1980; Rubinstein et al. 1981). A more extensive analysis of the carbohydrate content of the species of human IFN-α subtypes derived from patients with chronic myelogenous leukemia (CML) and from normal donors was determined (Adolf et al. 1991; Labdon et al. 1984). Amino sugar content was measured by HPLC and fluorescamine detection of acid hydrolysates of each sample (Labdon et al. 1984). *O*-linked glycosylation was also detected by a combination of HPLC, enzymatic analysis, and SDS-polyacrylamide gel electrophoresis (Adolf et al. 1991). Two species showed significant amounts of glucosamine (Labdon et al. 1984). Most of the purified species of leukocyte interferon from a myeloblast cell line were also tested and two species were found to contain sugar residues. These forms also differed from the CML interferons in that they revealed the presence of greater amounts of galactosamine. The apparent lack of carbohydrate in some of the higher-molecular-weight species of interferon implicated factors other than glycosylation for the molecular-weight differences. The results indicated that some species of IFN-α are glycosylated to various degrees. It was later shown that a natural form of Hu-IFN-α2 was *O*-glycosylated (Adolf et al. 1991) and that Hu-IFN-ω is glycosylated (Adolf et al. 1990). Considering that the recombinant human IFN-α species produced in *Escherichia coli* do not contain carbohydrate, it was useful to discover that most of the human IFN-α species were devoid of carbohydrate.

3
Identification and Cloning of the Recombinant Human IFN-α Species

Because recombinant DNA technology offered an opportunity to produce large amounts of Hu-IFNs economically, many scientific teams set out to clone them in bacteria. Several groups isolated recombinants for several Hu-IFN-α species (Maeda et al. 1980; Nagata et al. 1980) and for IFN-β (Derynck et al. 1980; Goeddel et al. 1980a; Houghton et al. 1980; Maeda et al. 1980; Taniguchi et al. 1980), obtaining the clones by somewhat different but analogous approaches. The cloning and expression of Hu-IFN-αA (Hu-IFN-α2a) as an illustration of these procedures is described.

Isolating Hu-IFN DNA sequences was a formidable task since it meant preparing DNA recombinants from cellular mRNA that was present at a low level. This task had never been accomplished previously from a protein whose structure was unknown. In addition, in order to reconstruct DNA recombinants which would express natural IFN, it is useful to know the partial amino acid sequence of the proteins, particularly at the NH_2- and COOH-terminal ends. Without this information, synthesis of natural Hu-IFN in bacterial cells would not have been definitive. Thus, purification of the Hu-IFNs and determination of their structure (Allen and Fantes 1980; Hobbs et al. 1981; Hobbs and Pestka 1982; Knight et al. 1980; Levy et al. 1981; Rubinstein et al. 1978a, 1979c, 1981; Shively et al. 1982; Zoon et al. 1979) assisted us in these efforts.

To isolate recombinants containing the human DNA corresponding to IFN-α, we developed a number of procedures. First, it was necessary to isolate and measure the IFN mRNA. This was accomplished several years earlier when IFN mRNA was translated in cell-free extracts (Pestka et al. 1975; Thang et al. 1975) and in frog oocytes (Cavalieri et al. 1977a; Cavalieri et al. 1977b; Cavalieri and Pestka 1977; Reynolds et al. 1975). The next step was to prepare sufficient mRNA from cells synthesizing IFN, and this was accomplished with both fibroblasts and leukocytes (Familletti et al. 1981a; McCandliss et al. 1981a). A library of complementary DNA (cDNA) was prepared from a template of partially purified mRNA isolated from human leukocytes synthesizing IFN. Next was to find in this vast library of recombinant plasmids those which contained DNA encoding IFN. We devised an indirect two-stage procedure to identify clones containing interferon sequences. In the first stage, we screened all the bacterial colonies to find those with cDNA made from the RNA of induced cells; among these there might have been some carrying IFN cDNA. We therefore screened all the recombinants for their ability to bind to mRNA from cells synthesizing IFN (induced cells), but not to mRNA from uninduced cells (those not producing IFN). To do this, individual transformed colonies were screened by colony

hybridization for the presence of induced specific sequences with ^{32}P-labeled IFN mRNA (mRNA from induced cells) as probe. In the presence of excess mRNA from uninduced cells, recombinants that were representative of mRNA sequences existing only in induced cells should be evident on hybridization. This screening procedure allowed us to discard about 90% of the colonies: since their plasmids carried no induced cDNA, these could not encode IFN (Maeda et al. 1980, 1981).

In the second stage, we identified those recombinants containing the IFN DNA sequences among the remaining 10%. To accomplish this, we pooled the recombinant plasmids in groups of ten and examined these for the presence of IFN-specific sequences by an assay that depends upon hybridization of IFN mRNA to plasmid DNA (Maeda et al. 1980; McCandliss et al. 1981b). Plasmid DNA from ten recombinants was isolated and covalently bound to diazobenzloxymethyl (DBM) paper. The mRNA from induced cells was hybridized to each filter. Unhybridized mRNA was removed by washing. After the specifically hybridized mRNA was eluted, both fractions were translated in *Xenopus laevis* oocytes. Once a positive group had been found (one in which the specifically hybridized mRNA yielded IFN after microinjection into frog oocytes), it was necessary to identify the specific clone or clones containing IFN cDNA. The individual colonies were grown, the plasmid DNAs were prepared, and each individual DNA was examined by mRNA hybridization as above. By these procedures, a recombinant, plasmid 104 (p104), containing most of the coding sequence for a Hu-IFN-α, was identified (Maeda et al. 1980). The DNA sequence was determined and found to correspond to what was then known of the amino acid sequence of purified Hu-IFN-α (Levy et al. 1980; Levy et al. 1981). The cDNA insert in plasmid p104 contained the sequence corresponding to more than 80% of the amino acids in IFN-αA, but not for those at its amino-terminal end. It was, therefore, used as a probe for finding a full-length copy of the IFN cDNA sequence that could be used for expression of Hu-IFN-αA in *E. coli* (Goeddel et al. 1980b). In addition, p104 DNA was used to isolate DNA sequences corresponding to other IFN-α species directly from a human gene bank.

Examination of the coding regions of the IFN-α genes that have been isolated in our laboratory and others have shown that these correspond to a family of homologous proteins (Pestka 1983a; Rubinstein et al. 1979c) that are closely related to each (Table 2). Thus, the previously discovered heterogeneity in Hu-IFN-α was at least in part the result of distinct genes representing various expressed Hu-IFN-α sequences. The cloned Hu-IFN-αA (Hu-IFN-α2a), the first one we isolated, corresponds to one of the natural Hu-IFN-αs that we purified by HPLC. By procedures similar to those described for plasmid p104, plasmid p101 was shown to contain the sequence for Hu-IFN-β. Thus, the nucleotide sequences coding for Hu-IFN-α and Hu-IFN-β were identified.

4
The Recombinant Human IFN-α Genes and Proteins

A summary of the IFN-α genes and proteins is listed in Table 2. There are in essence 14 human genes that comprise the IFN-α family. Minor variants consisting of one or two amino acid differences account for the multiple alleles (Diaz et al. 1994; Krause and Pestka 2005; Pestka 1983a, 1983b, 1986). Excluding the pseudogene IFNAP22, there are 13 genes. One of them, IFNA10 is also a pseudogene in one allelic form. There are 13 proteins expressed from these genes. The protein produced from gene IFNA13 is identical to that produced from IFNA1. Thus, there are 12 separate IFN-α proteins (and allelic forms) produced from these 14 genes (Table 2).

5
Concluding Summary

This review concentrated on the purification and cloning of IFN-α. Several extensive reviews (Pestka 1983a, 1983b, 2000) provide further details with tables and figures that would be useful to the reader. The recombinant proteins produced in *E. coli* have properties substantially equivalent to the proteins produced by human cells. Predominantly, only one recombinant IFN-α protein is used therapeutically (IFN-α2a, IFN-α2b, and IFN-αc, allelic variants) so that the remaining IFN-α species remain an untapped reservoir of opportunity. Why the body produces so many of these interferons remains unanswered. As our understanding of the mechanism of their receptor interactions develops, some of these answers should be forthcoming.

Although purification of the interferons to homogeneity remained elusive for about two decades after their discovery, they are now available in purified form. The availability of these proteins for laboratory and clinical studies has already catalyzed extensive new developments with these agents and it is likely that we will gain new insights into their actions and develop new applications for their use in the near future.

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Part II: Interferons and Their Signaling Pathways

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Type I Interferon: The Ever Unfolding Story

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Abstract Since the discovery of interferon 50 years ago, the understanding of the mechanism of the virus-mediated induction of type I IFN and its function has been under intensive investigation. Remarkable progress has been made in recent years both in the identification of cellular receptors detecting the viral infection and in the understanding the signaling pathways resulting in the induction of interferon and interferon-induced genes. In this review of type I interferon, we aim to summarize not only the historical site of the interferon induction and its antiviral function, but also the complexity of the signals that lead to activation of expression of interferon genes and the expanding repertoire of this multifunctional protein.

1
Introduction and Historical Perspective

The innate antiviral response developed as a rapid and regulated defense mechanism of the host against the viral intruder. Antiviral cytokines and chemokines, activated as an early response to viral infection, play a critical role in both the outcome of the infection and its pathogenicity. It has been 50 years since the antiviral protein interferon (IFN) was first described (see the chapter by Lindenmann, this volume). When it was found that the activity of IFN is species-specific, but not virus-specific, it was assumed that IFN will have broad antiviral therapeutic application. However, the IFN system was more complex than originally anticipated and it was later shown that IFN preparations had not only antiviral (Paucker and Cantell 1962), but also many anticellular effects (Gresser et al. 1969). It was also shown that there were at least two antigenic distinct subtypes of type I interferon induced by viral infection: IFNα, produced in leukocytes and IFNβ produced in fibroblasts (Havell et al. 1975). Another antiviral protein was found to be induced in mitogen-activated T lymphocytes and was named IFNγ, or type II IFN (Whellock and Sibley 1965) (see the chapter by Young and Bream, this volume).

Surprisingly, it was found that in fibroblasts, IFNβ could be induced not only by infection with different types of viruses but also by treatment with synthetic double-stranded (ds) RNA, poly rI.rC (Field et al. 1967). Interestingly enough, only ds polyribonucleotides and not single-stranded (ss) polyribonucleotides or polydeoxynucleotides were effective IFN inducers (Colby and Chamberlin 1969; Pitha and Carter 1971; Carter et al. 1972). The structural modification of polyrI.rC aimed at generating a super inducer generally failed (Pitha and Pitha 1971, 1972), leading to the assumption that the IFN-inducing entity in infected cells was the dsRNA intermediate of viral replication (Long and Burke 1971; Sekellick and Marcus 1982). It took another 30 years before the molecular mechanism of the cellular response to dsRNA was uncovered and some of the original observations about the structure of nucleic acid recognized by the cells were challenged.

In precloning times, the biological assay of IFN was the only available endpoint measurement, and therefore it was not clear whether virus- or poly rI.rC-mediated stimulation occurred at the transcriptional or post-transcriptional level (see the chapter by Vilcek, this volume). The first evidence that stimulation of IFN synthesis in infected cells occurs at the transcriptional level was suggested by studies with actinomycin D (Wagner 1964), and it was shown later that IFN mRNA is present only in poly rI.rC-induced and not in uninfected cells. Since this was done before cloning techniques were developed, IFN

mRNA was detected by transfection and translation of cellular RNA in chick cells (de Maeyer-Guignard et al. 1972; Reynolds and Pitha 1974) and later in *Xenopus* oocytes (Reynolds et al. 1975).

The oocyte translation assay was very efficient and facilitated the cloning of human IFNα cDNAs and IFNβ cDNA (Nagata et al. 1980; Derynck et al. 1980a). The cloning of the *IFN* genes opened a Pandora's box. Not only could IFN now be produced in sufficient amounts for clinical studies, but unexpectedly, instead of a single IFN*A* gene, both in humans and mice, the type I IFN family consisted of multiple IFN*A* genes and pseudogenes, and only one IFN*B* gene (Derynck et al. 1980b; Gray et al. 1982; Kelly and Pitha 1985; Zwarthoff et al. 1985) (see the chapter by Pestka, this volume). Both IFN*A* and IFN*B* lack introns (Kelly and Pitha 1985; Weissman and Weber 1986) and are clustered on the short arm of human chromosome 9, or mouse chromosome 4 (Diaz et al. 1994; Kelly et al. 1985). The human IFN*A* family is composed of 15 active genes and 11 pseudogenes, which share about 96% homology. Expansion of the IFN*A* cluster is likely to have occurred by unequal crossing over of the duplicated *IFN* genes (Henco et al. 1985). Although all IFNα are antiviral, functional diversity between individual variants is starting to emerge (Ortaldo et al. 1984; Hilkens et al. 2003). In addition, the human IFN*Ω* family consists of about five pseudogenes and one full gene expressed in leukocytes (Hauptmann and Swetly 1985) and one IFN*K* gene expressed in keratinocytes (LaFleur et al. 2001). These *IFN* genes are also part of the interferon cluster on chromosome 9. All type I IFNs share a common receptor (see the chapter by Uzé et al., this volume). The type II, IFN*G*, gene which contains three introns and maps to the long arm of chromosome 12, signals through its own distinct receptor (see the chapter by van Boxel-Dezaire and Stark, this volume). Lastly, a new group of IL10-related antiviral proteins, type III IFN, were identified in infected cells and named IFNλ (λ 1–3) (Kotenko et al. 2003; Sheppard et al. 2003). While these proteins share some antiviral and immunomodulatory functions with type I IFN, they signal via a distinct receptor (Kotenko et al. 2003).

There has been remarkable progress made in recent years in the identification of the cellular receptors detecting viral infections and in the understanding of the signaling pathways leading to the stimulation of type I *IFN* gene expression. The importance of type I IFN to the activation of effector cell populations and adaptive immunity is also emerging. In this review, we will focus on type I IFN, with an emphasis on IFNα, and we will attempt to summarize our present knowledge about the regulation of IFN*A* transcription, its cell type-specific expression and its general role in the innate immune response against viral infection. We will also discuss the possible harmful effects IFNα and the adverse interplay between the expression of IFNα and autoimmune disorders.

2
Regulation of Type I IFN Gene Expression

2.1
Cellular Recognition of Infection

Type I IFN is rapidly produced in response to viral infection in almost all nucleated cells. Two classes of cellular receptors recognize intracellular viral nucleic acids. Toll-like receptors (TLR), present in the endosomal compartments of the immune cells, detect viral RNA or DNA (Akira et al. 2006; O'Neill 2006). TLR3 detects dsRNA, a common replication intermediate of both DNA and RNA viruses, as well as viral dsRNA released from the apoptotic cells (Alexopoulou et al. 2001; Schultz and Williams 2005). Single-stranded viral genomic RNA, which is enriched in uridine or guanosine residues, is detected by TLR7 and TLR8 (Diebold et al. 2004) and DNA viruses are recognized by TLR9, which detects the unmethylated CpG regions in viral genomic DNA (Lund et al. 2003). The endosomal localization of TLR is essential for the discrimination between viral and cellular nucleic acids (Barton et al. 2004). The second classes of receptors that detect dsRNA produced in infected cells are RNA helicases containing the caspase recruitment domain (CARD), RIG-I and MDA5 (Yoneyama et al. 2004) (Fig. 1). They are expressed ubiquitously in all cell types (see the chapter by Onomoto et al., this volume). Binding of the nucleic ligand to its respective receptor activates signaling pathways leading to the activation of latent transcription factors of the NFkB and interferon regulatory factor (IRF) families that are involved in the activation of the early inflammatory genes (see the chapter by Severa and Fitzgerald, this volume)

2.2
The IRF Family

The molecular mechanism of virus-mediated type I IFN gene induction served as a model for the study of inducible transcription. The 5′ regions of both human and mouse IFN*A* and IFN*B* contain a domain called the interferon regulatory element or virus responsive element (VRE), which has multiple GAAANN repeats (Weidle and Weissman 1983; Goodbourn et al. 1986; Fujita et al. 1987). In addition, IFN*B* VRE contains an NFkB site that binds NFkB/Rel transcription factors (Thanos and Maniatis 1995). Stimulation of IFN*B* transcription is mediated by a ternary complex enhanceosome consisting of NFkB, activated interferon responsive factors (IRFs), and activated protein 1 (AP-1), which are recruited to the VRE of the *IFN* promoter (Du et al. 1993; Merika et al. 1998; Thanos and Maniatis 1995). Since not all IFN*A* subtypes are expressed at the same levels, it was not initially clear whether their levels were regulated

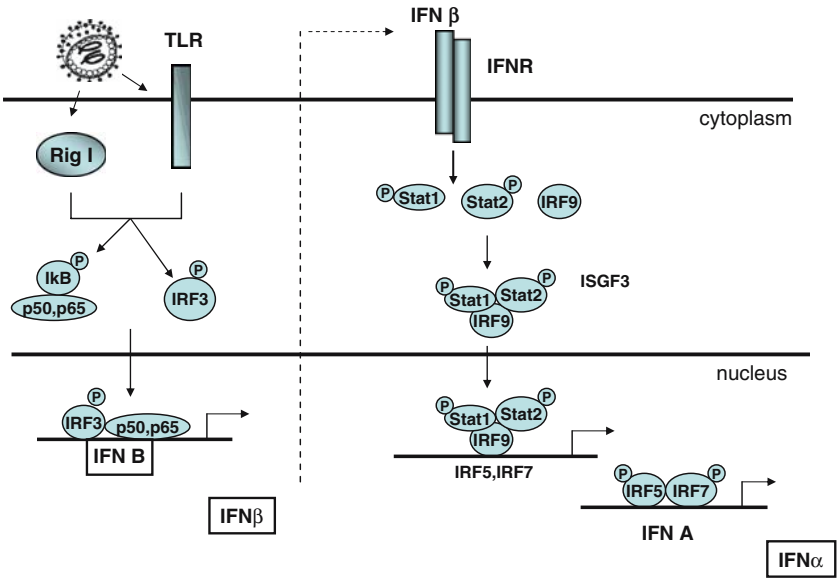

Fig. 1 The IRF family plays a critical role in the antiviral response. In most cells, the antiviral response occurs in two phases. In the first phase, the viral or bacterial infection stimulates phosphorylation of constitutively expressed IRF-3, which together with NFkB binds to VRE in the interferon promoter, resulting in the expression of the IFN*B* gene. In the second phase, the IFNβ binds to the type I IFN receptor, which results in the phosphorylation of STAT1 and STAT2 and their interaction with IRF-9, forming the ISGF3 complex that activates expression of large number of interferon stimulated genes including IRF-7 and IRF-5. Activated IRF-7 binds either alone or together with IRF-3 to the VRE of the IFNA promoters and induces expression of IFN α, which leads to the amplification of the antiviral effect

at the transcriptional or posttranscriptional levels (Kelly and Pitha 1985; Yeow et al. 2000; Zwarthoff et al. 1985). It was eventually shown that each IFN subtype contains a distinct promoter that regulates expression of individual IFN*A* in infected cells (Bisat et al. 1988). In contrast to IFN*B*, the VRE of the IFN*A* promoters does not contain an NFkB site, but shows the presence of multiple repeats of the AANNGAA sequence that can bind activated IRFs. Thus while the inducible activation of IFN*B* transcription is dependent on both NFkB and IRF, activation of IFN*A* seems to depend mainly on IRF.

The IRF family consists of nine cellular *IRF* genes (*IRF-1*to *IRF7*, *IRF-8/ICSBP* and *-IRF-9/p48/ISGF3*) (Honda and Taniguchi 2006). In addition, Kaposi's sarcoma herpes virus (KSHV) encodes viral analogs of IRF (Chang et al.

1994). Cellular IRFs share a significant homology in the N-terminal region. This region comprises a helix-turn-helix DNA-binding domain containing five tryptophane repeats, which bind the GAAA and AANNGAA domains in the VRE of type I IFN promoters (Escalante et al. 1998). The C-terminal of most IRFs contains an IRF association domain (IAD) that is important for homo- and heterodimeric interactions and a gene activation domain (Moustakas and Heldin 2003). The first member of the IRF family, IRF-1, was discovered through its ability to bind the VRE of the IFN*B* promoter (Miyamoto et al. 1988). While ectopic overexpression of IRF-1 in undifferentiated EC cells stimulated the expression of type I *IFN* genes, it failed to bind the VRE of IFN*A* (MacDonald et al. 1990; Au et al. 1992). Furthermore, homozygous deletion of IRF-1 did not impair the virus-mediated induction of IFN*A* (Ruffner et al. 1993). An IRF binding site (IRF-E) in the VRE of IFN*A* promoters plays an important role, as a single nucleotide mutation in the IRF-E abolishes the inducibility of the murine IFN *A4* promoter. Also, priming with IFN restores IFN*A* induction in IRF-1-null cells, indicating that another IFN-induced protein is the critical factor, later shown to be IRF-7 (Au et al. 1998).

The search for another IRF-E binding protein has led to the identification of IRF-3, IRF-7, and IRF-5 (Au et al. 1995, 1998; Barnes et al. 2001; Marie et al. 1998). The identification of IRF-3 and IRF-7 and their role in the activation of type I IFN genes had a major impact on the understanding of the inducible expression of type I IFN (Juang et al. 1998; Sato et al. 2000; Sobel and Ahvazi 1998; Weaver et al. 1998; Yeow et al. 2000; Yoneyama et al. 1998) (Fig. 1). Ubiquitously expressed IRF-3 is activated in the TLR-3, TLR-4, or RIG-I/MDA5 signaling pathways by two noncanonic IkB kinases: TBK-1 and IKKε (Fitzgerald et al. 2003; McWhirter et al. 2004; Sharma et al. 2003). IRF-3 homodimerizes or heterodimerizes with IRF-7 and translocates to the nucleus, where it associates with CREB binding proteins CBP/p300. Activated IRF-3 stimulates transcription of IFN*B*, as well as of some interferon-stimulated genes (ISG) (Grandvaux et al. 2002; Juang et al. 1998; Lin et al. 1999). While expression of IRF-3 alone is sufficient to activate the promoter of IFN*B*, the IFNB enhanceosome contains not only IRF-3, but also IRF-7 (Wathelet et al. 1998). Mice with a homozygous deletion of IRF-3 show impairment in the NDV-mediated induction of type I IFN and an increased susceptibility to EMCV infection (Sato et al. 2000).

IRF-7, which is constructively expressed only in some lymphoid cells and pDC (Izaguirre et al. 2003), is critical for the induction of IFN*A*. Reconstitution of IRF-7 expression in infected human fibroblasts that expressed only IFNβ conferred expression of several IFN*A* genes (Yeow et al. 2000). Mice with a homozygous deletion of IRF-7 were unable to express type I IFN genes upon viral infection or activation of TLR9 by CpG-rich DNA, indicating that IRF-7 is a master regulator of type I IFN expression (Honda et al. 2005). Virus-induced

expression of distinct IFN*A* subtypes is determined by the organization of IRF-3 and IRF-7 recognizing domains in the VRE of the IFN*A* promoters. Distortion in the GAAA core sequence of these binding domains affects the cooperativity of IRF-3 and IRF-7 binding and their synergistic activation. The differential expression of the individual IFN*A* subtypes has been shown to be due to a distinct nucleotide substitution in these domains (Au et al. 1993, 2001; Civas et al. 2006; Morin et al. 2002) and by the presence of negative regulatory sequences (DNRE) located in the upstream regulatory region of some IFN*A* subtypes (Lopez et al. 2000). IRF-3 and IRF-7, together with histone transacetylases, have been shown to be part of the transcriptionally active human IFNA1 enhanceosome (Au et al. 2001), whereas the murine IFN *A11* promoter, which that is not activated by IRF-3, binds only IRF-7 homodimers (Civas et al. 2006). These data indicate that the level of IRF-3 and IRF-7 in cells determines the relative levels of expression of individual IFN*A* subtypes.

2.3 The Role of IRF-5

An unexpected finding was that type I IFN, together with number of cytokines, could be induced in PBMCs not only by dsRNA or viral infection, but also by a compound of the imidazoquinoline family, imiquimod-(R848). The profile of R848-induced cytokines was very similar to that induced by Sendai virus infection (Megyeri et al. 1995). Although it was shown that this compound activates NFkB, the mechanism of induction was unclear and it was not until the TLR7-null mice became available that R848 stimulation of the TLR7-mediated pathway was shown (Hemmi et al. 2002). In contrast to TLR3 and TLR4, TLR7 activates IRF-5 and IRF-7, but not IRF-3 (Schoenemeyer et al. 2005) (see the chapter by Severa and Fitzgerald, this volume). The question therefore arises as to what role is played by IRF-5 in the antiviral response. Many splice variants of human IRF-5 have been identified, yet not all of these were transcriptionally active (Mancl et al. 2005). Like IRF-7, ectopic expression of IRF-5 (AY 504946) rescued induction of several IFN*A* subtypes; however, the subtypes of IFNα induced by IRF-5 and IRF-7 were distinct. While IFNα1 was the major subtype induced by NDV in IRF-7-expressing cells, IRF-5-expressing cells expressed IFNα 8 as the major subtype. Like IRF-1 and IRF-3, IRF-5 induced apoptosis and expression of several pro-apoptotic genes (Barnes et al. 2002). Interestingly, IRF-5 overexpression also upregulated a number of early inflammatory proteins including RANTES, MIP-1β, I-309, MCP-1, and IL-8. This suggested an important role for IRF-5 in the regulation of the expression of the early inflammatory cytokines and chemokines (Barnes et al. 2004). IRF-5 expression could be induced not only by viral infection and type I IFN, but also by the

tumor suppressors p53, thus connecting IRF-5 and p53 induced pro-apoptotic pathways (Mori et al. 2002). Like p53, IRF5 has tumor suppressing activity as well. Not only does IRF5 stimulate the cyclin-dependent kinase inhibitor p21, while repressing cyclin B1, but it also stimulates the expression of the pro-apoptotic genes Bak1, Bax, caspase 8, and DAP kinase 2, thus indicating an ability to promote cell cycle arrest and apoptosis independently of p53. As a consequence, it was suggested that IRF5 might provide an additional line of therapeutic intervention, in particular in the case of tumors resistant to apoptosis due to a loss of p53 function (Hu and Barnes 2006).

However, unlike IRF-7-null mice that show a major defect in type I IFN induction, pDC from IRF-5-null mice did not show any defect in the MyD88-mediated induction of type I IFN, instead demonstrating downregulation of IL-6, TNFα, and IL-12p40 expression (Takaoka et al. 2005). This supports our earlier observation that in cells expressing ecotopic IRF-5, viral infection induced the expression of pro-inflammatory cytokine genes (Barnes et al. 2004). Whether IRF5 is required only for the synthesis of pro-inflammatory cytokines and not for type I IFN is not clear yet. There are, however, a few differences between the human IRF-5 (variant 4) and mouse IRF-5. Unlike the human IRF-5, which is expressed in multiple spliced variants (Mancl et al. 2005), C57BL/6 J mice express only a single , dominant transcript and very low levels of one IRF-5 splice variant in bone marrow. This variant was not express in spleen or established mouse cell lines and shows a transactivation capability similar to full-length IRF-5. Also, the mouse IRF-5, while activated by MyD88 and TBK-1, does not seem to be activated efficiently by NDV infection (P.M. Pitha, unpublished results). The differences in the IRF-5-mediated activation of human IFN*A* genes in vitro and in MyD88-stimulated induction in mice, as well as the discordant effect of IRF-5 on the activation of *IFN* genes in cells expressing ectopic IRF-5 and mouse cells lacking IRF-5 expression, are unexpected. These results indicate that in the presence of high levels of activated IRF-7, the contribution of IRF-5 to the induction of IFN genes may be negligible, and its role may be limited to the induction of inflammatory chemokines and cytokines that are not stimulated by IRF-7. The MyD88-mediated activation of both IRF-5 and IRF-7 involves the formation of a tertiary complex consisting of MyD88, TRAF6, and IRF-5 or IRF-7 (Takaoka et al. 2005), and it is likely that this complex preferentially assembles with IRF-7 than with IRF-5. It was also shown that IRF-4 completes the binding of IRF-5 to MyD88 (Negishi et al. 2005) and therefore in cells expressing IRF-4 such as pDC or B cells, IRF-5 may be not efficiently activated. Thus the role of IRF-5 in the stimulation of type I IFN genes may be limited to the cells that do not express IRF-4 or activated IRF-7 and may also depend on a distinct, concentration-dependent activation of IRF-5 and IRF-7. While NDV activates IRF-7 by the

RIG-I pathway, this pathway does not seem to lead to the activation of IRF-5 (P.M. Pitha, unpublished results). Thus additional studies of differences in the MyD88-dependent and -independent signaling pathways leading to the activation of IRF-5 are clearly warranted. Lastly, a connection between IRF-5 and IFNα induction has been suggested by the observation that one of the genetic risk factors of systemic lupus erythematosus (SLE), which is associated with a constitutive IFNα production, was identified as a mutated IRF-5 haplotype, which drives an increased expression of IRF-5 (Graham et al. 2006) (see the chapter by Crow, this volume).

2.4 Interferon-Stimulated Genes: Mediators of Antiviral Effects

The binding of type I IFN to its cellular receptor initiates receptor-mediated signaling pathways, resulting in the induction of IFN-stimulated genes (*ISG*) (Darnell et al. 2004). The major signaling pathway involves the activation of two JAK kinases (JAK1 and Tyk2), which are associated with the type I IFN receptor, and the consequent tyrosine phosphorylation of the preexisting signal transducers and activators of transcription (STAT). Upon phosphorylation, STAT1 and STAT2 assemble together with interferon responsive factor 9 (IRF-9) into a multimeric complex (ISGF3), which interacts with interferon-responsive elements (ISRE) present in the 5′ flanking region of *ISG* (Darnell et al. 2004; Improta et al. 1994) (see the chapter by Uzé et al., this volume). Type I IFN also stimulates the formation of STAT1 homodimers, which bind to a slightly different DNA domain, the IFN-γ-activated site (GAS), present in the promoters of *ISG* that can be induced both by type I IFN and IFNγ. In addition, the STAT2-IRF-9 heterodimer is also an activator of transcription (Kraus et al. 2003) and in LCMV infection, induction of type I IFN has been shown to depend only on STAT2 (Ousman et al. 2005). Signaling by type I IFN can also activate both the MAPK and PI3K pathways (Platanias 2005); however, the contribution of these two pathways to the antiviral response in vivo is not clear. Interestingly, while IFNλ binds to a different receptor than IFNα/β, it nonetheless signals through the JAK/STAT pathway to ISRE domains (Kotenko et al. 2003; Sheppard et al. 2003). Analysis of the transcription signature of type I IFN-induced genes shows that IFN stimulates a major upregulation of cellular genes expression, which encode proteins with diverse functions including antiviral properties, pro-apoptotic functions and modulators of ubiquitination pathways (de Veer et al. 2001).

Although the antiviral function of the majority of ISG has yet to be determined, several of the interferon-induced antiviral proteins have been identified (Samuel 2001). The earliest characterized ISG were 2′, 5′-oligoadenylate

synthetase (2′, 5′-OAS), RNA-dependent protein kinase (PKR), Mx-GTPase, and the RNA-specific adenosine deaminase (ADAR). The 2′,5′-OAS pathway leads to RNA degradation. It consists of 2′,5′-OAS, which, when activated by dsRNA, polymerizes ATP into pppA(2′p5′A)n, (2′,5′A oligoadenylates); in turn it activates a cellular endonuclease (Kerr and Brown 1978). RNase L degrades both cellular and viral RNAs at UU or AU nucleotides. Expression of 2′, 5′-OAS in cells leads to the establishment of an antiviral state, which results in the selective inhibition of the replication of picornaviruses such as encephalomyocarditis virus (EMCV) (Chebath et al. 1987). Another endonuclease induced by IFN is ISG20, which has specificity for ssRNA. When overexpressed, ISG20 inhibits replication of VSV, influenza virus, EMCV, and HIV-1 (Espert et al. 2003). The interferon-induced antiviral gene *PKR* has been given a lot of attention. *PKR* is activated by dsRNA-mediated autophosphorylation. Activated PKR catalyzes phosphorylation of several substrates, including the α subunit of the initiation factor eIF-2 (eIF-2α) (Samuel 1979), which is implicated in the inhibition of viral protein synthesis, as well as the transcription factor inhibitor IκB (Kumar et al. 1994). PKR-deficient mice exhibit an increased susceptibility to VSV infection (Stojdl et al. 2000), whereas their antiviral response to influenza virus and Vaccinia virus (VV) is not impaired, again demonstrating viral specificity among the ISG (see the chapter by Sadler and Williams, this volume).

The Mx proteins are GTPases induced by IFNα/β but not by IFNγ. Overexpression of Mx confers a high degree of antiviral activity and resistance to infection by a large group of viruses, including influenza A and C, Hantana virus and measles virus, but not picorna viruses. The inhibition of influenza virus replication by Mx proteins is due to the inhibition of primary transcription mediated by a virion-associated polymerase.

Two nucleic acid-editing enzymes, adenosine deaminase acting on RNA (ADAR) and APOBEC3G, have also been shown to be ISG. ADAR-mediated transition of nucleotides from A to I disrupts base pairing and the AU base pair is replaced by the less stable IU pair, which destabilizes dsRNA. This A-I editing has been found in multiple viral RNA sites of negative-strand RNA viruses, and it has been associated with persistent infection (Murphy et al. 1991). APOBEC3G is a cytosine deaminase that converts cytidine to uridine in single-stranded proviral DNA, which results in hypermutation of the HIV-1 genome. Expression of APOBEC3G has been shown to be upregulated by IFNα (Chen et al. 2006; Yu et al. 2003). Thus both APOBEC3G and ADAR are IFN-induced antiviral proteins that can induce hypermutation of the viral genome and decrease viral fitness.

During the early days of IFN research, it was assumed that the interferon-mediated inhibition of viral replication was caused by a common mechanism affecting a large number of viruses. Instead, it has become clear that the antiviral

effect is due to the combinatory effects of many proteins and that any given antiviral protein may show specificity for a distinct group of viruses.

3 Innate Response to Pathogens

3.1 Cell Defense Against Viral Offense

The use of genetically modified mice deficient for the type I IFNAR or components of the IFN signaling pathway, such as STAT1, clearly establish the importance of type I IFN in the resistance to viral infection in vivo. Both IFN-α/β and STAT1 knockout mice are highly susceptible to viral infection and unable to establish an antiviral state. Similarly, the availability of genetically manipulated mice lacking either individual TLR receptors or cytoplasmic receptors has advanced the understanding of the cellular recognition of invading pathogens. However, it is still not completely clear what determines the specificity of the recognition and whether the receptor recognizes both the viral genome and replication intermediate. As shown with HSV-1, which is an effective IFN inducer, recognition may be complex. The unmethylated HSV-1 DNA genome is a very effective inducer of IFNα in human pDC, and this induction is dependent on TLR9 (Lund et al. 2003), while in human PBMCs, HSV-1 glycoprotein D alone can induce synthesis of IFNα. Another virus of the herpes group, mouse cytomegalovirus (MCMV), induces type I IFN through recognition of both TLR9 and TLR3 (Krug et al. 2004), but the recognition by CMV is through TLR2 (Compton et al. 2003).

dsRNA has been long considered the recognition entity for viral infection, and in the majority of the cells, RIG-I and MDA5 are important for the recognition of most RNA virus infections. However, not all viruses generate a significant amount of dsRNA intermediates and still are recognized by RIG-I (Pichlmair et al. 2006). The RIG-I pathway is induced by most of the viruses tested, whereas MDA5 is required for the response against picornaviruses (Kato et al. 2006). Recent observations indicate that this distinction is based on the specific recognition of the 5′-triphosphate viral RNA structure (Hornung et al. 2006). The uncapped 5′-triphosphate end of ssRNA and positive-strand RNA viruses or their transcripts is recognized by Rig I (Kato et al. 2006; Hornung et al. 2006). In contrast, the 5′ end of picornavirus transcripts remains associated with the protein used as a primer and therefore uncapped 5′ RNA is absent during picornavirus replication (Lee et al. 1977). RIG-I also plays a major role in the induction of the antiviral response to the hepatitis C virus (HCV) RNA

replicative intermediate in cultured hepatocytes (Sumpter et al. 2005) (see the chapter by Loo and Gale, this volume).

There are several indications that in the central nervous system (CNS), where TLR3 is expressed at high levels, the inflammatory response is initiated by TLR3. For instance, the inflammatory response initiated by TMEV and West Nile virus is dependent on TLR3 (Wang et al. 2004). The antiviral response to the ssRNA genomes of VSV and influenza virus in pDC was shown to depend on TLR7 (Lund et al. 2003) and there are some indications that the viral envelope can also be recognized by TLR4, which is a primary receptor for lipopolysaccharides (LPS). A TLR4-mediated antiviral response was induced by the fusion protein of RSV (Kurt-Jones et al. 2000) and by the envelope protein of MMTV (Burzyn et al. 2004). Altogether, these data indicate that both viral nucleic acids and glycoproteins are capable of generating the antiviral response and that the multiple patterns of recognition may enhance the antiviral response and its duration. However, in order to be able to replicate and establish infection, viruses develop various strategies for evading the innate immune response of the host, as is discussed by Haller in this volume.

Type I IFNs are not only essential for antiviral defense, but they also exert a number of immunoregulatory effects. They modulate the expression of major histocompatibility complex (MHC) antigens, and it is via this mechanism that IFN-α/β increases susceptibility of vaccinia virus (VV), or lymphocytic choriomeningitis virus (LCMV) -infected fibroblasts to lysis by cytotoxic T lymphocytes (CTL) (Bukowski and Welsh 1985). IFNα/β can also downregulate expression of IL-12 in human dendritic cells (DCs) and monocytes (Karp et al. 2000), stimulate expression of IFN-γ in response to influenza virus infection (Sareneva et al. 1998), and induce expression of IL-15 (Durbin et al. 2000). IFNα has also multiple effects on the function of immune cells (Garcia-Sastre and Biron 2006) including enhancement of NK cell activity (Biron et al. 1999), activation of $CD8^+$ T cells during the early steps of infection (Zhang et al. 1998), and protection of $CD8^+$ T cells from antigen-induced cell death (Marrack et al. 1999). Human IFNα promotes the differentiation of dendritic cells (Santini et al. 2000), the upregulation of IFN-γ expression, and stimulation of B cell differentiation in both a DC-dependent and -independent manner (Biron 2001; Santini et al. 2000). Recent data demonstrate that type I IFN can directly stimulate the B cell response during the early stages of influenza virus infection (Coro et al. 2006). IFNα has been also shown to induce the differentiation of human monocyte-derived DCs, which are able to induce Th1 polarization both in vitro and in vivo (Cella et al. 2000; Santini et al. 2000) and stimulate B cell proliferation and Ig class switching (Le Bon and Tough 2002). Thus, while in the past the IFN system was considered as only a part of the host innate immunity,

recent data indicate that type I IFN has an important role in bridging innate and acquired immunity (Biron 2001).

3.2 Positive and Negative Role of Type I IFN in Bacterial Infection

Although synthesis of type I IFN was originally associated with viral infection, the production of type I IFN is also induced as an immediate innate response to bacterial infection, where interferon has been shown to modulate an innate antibacterial response (Bogdan et al. 2004). Like viruses, bacteria can be recognized by membrane-bound receptors and cytoplasmic receptors. The binding of a specific ligand to a given TLR recruits specific adaptors and initiates cellular signaling pathways, leading to the activation of the IRFs and the NFκB family of transcription factors. LPS present on Gram-negative bacteria is recognized by TLR4 and initiates the association of TLR4, either with TRIF and TRAM adaptor proteins, leading to the activation of IRF-3 and IRF-7 or with MyD88 adaptor, which leads to NFκB activation. Several Gram-negative bacteria, such as *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia* spp., stimulate type I IFN synthesis after the invasion of the cell (Bogdan et al. 2004). The unmethylated bacterial DNA is recognized by endosomally expressed TLR9. Binding of dsDNA to TLR9 occurs in the endosomal compartment and results in the activation of IRF-5 and IRF-7. However, there is also cytoplasmic recognition of B-form DNA, which occurs in the cytosol and results in the activation of IRF-3. Thus an intracellular Gram-positive bacterium that has a cytoplasmic life cycle phase, such as *Listeria monocytogenes*, probably activates the IFN response via the cytosolic DNA recognition pathway (O'Connell et al. 2005; Stetson and Medzhitov 2006). Bacterial flagellin is a TLR5 ligand, which mediates signaling through MyD88, resulting in the activation of NFκB factors and the induction of inflammatory cytokines (Hayashi et al. 2001). Whether TLR5 also activates IRFs and type I IFN has not been yet determined.

Type I IFN can also modulate the outcome of bacterial infection. It is important to realize, however, that interferon can be both protective and detrimental to the host. Type I IFN inhibits intracellular replication of *Legionella pneumophila* (Schiavoni et al. 2004) and contributes to the clearance of pathogens in *Leishmania* infection (Diefenbach et al. 1998). Type I IFN also increases resistance against Gram-positive bacteria such as *Streptococcus pneumoniae* and *Bacillus anthracis* (Gold et al. 2004; Weigent et al. 1986). In contrast, during *Listeria* infection, type I IFN synthesis increases the susceptibility of lymphocytes to infection (Carrero et al. 2006). IFN treatment also reduces host resistance to *L. monocytogenes* infection and has a negative impact on the survival of infected mice (Auerbuch et al. 2004).

Taken together, these data indicate that the effect of type I IFN on bacterial infection is complex. On one hand, it contributes to the clearance of pathogens; on the other, it can have harmful effects on the host.

3.3 Super IFN Producers: pDC

Although type I IFN can be produced essentially by any infected cell, most infected cells produce low levels of IFN that can act in an autocrine manner, or protect only cells localized in close proximity to the focus of infection. In human PBMCs, there is a rare type of cells, designated as natural interferon-producing cells, that produce very high levels of IFNα in response to viral infection and therefore can generate a systemic response (Fitzgeral-Bocarsly et al. 1988). Further characterization of these cells revealed that these cells are a CD123 and $CD4^+CD11c^+Lin^-$ subset of DCs referred to as plasmacytoid DCs (Siegal et al. 1999). Later, a pDC subset was also identified in mice; however, murine pDCs do not express CD123, but can be defined as CD11b-CD11c low $B220^+$ cells that also express Ly6C (Colonna et al. 2004). PDCs differ from the monocyte derived DCs (mDCs), not only by their phenotype but also by their migration pattern (Penna et al. 2002). PDCs are recruited to the site of inflammation, where they are activated, while immature mDCs in peripheral tissues migrate after maturation to lymphatic tissues (Jahnsen et al. 2002). mDCs and pDCs express a distinct set of TLRs and therefore recognize different pathogens. TLRs expressed in pDCs are those associated with recognition of viral or bacterial DNA and viral RNA, namely TLR7/8 and TLR9. The induction of the antiviral response is dependent on the co-adaptor MyD88 (Fig. 2). mDCs express relatively high levels of TLR3 and low levels of TLR4, the induction of the IFN response is through the adaptor TRIF, and it is MyD88-independent (see the chapter by Severa and Fitzgerald, this volume).

The IFNα subtypes and their relative level of expression induced in pDCs appear to be virus specific. While HSV-1 induced approximately 10- to 100-fold higher levels of IFNα in pDCs than in mDCs, the difference in the relative levels of IFNα induced by Sendai virus in pDCs and mDCs was much smaller. Since HSV-1 is recognized by TLR9 in pDCs, but by RIG-I in other cell types (Melchjorsen et al. 2005), the above observation indicates that the antiviral response induced by TLR9 is much stronger. Furthermore, the subtypes of IFNα induced in Sendai virus and HSV-1-infected cells were distinct (Izaguirre et al. 2003). The difference in the profile of IFNα subtypes expressed in pDC upon stimulation of TLR9 and TLR7 has not been yet determined. Both human and mouse pDCs also express high levels of IRF-8. While IRF-8 plays a critical role in pDC development its role in the activation of type I IFN is not yet clear (Tamura et al. 2005).

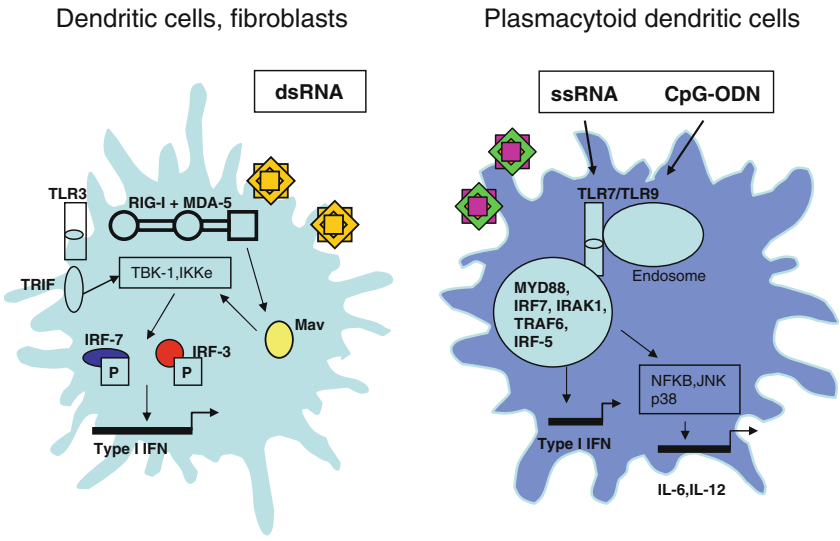

Fig. 2 The distinct difference in the induction of the antiviral response in pDCs and other cell types. In fibroblast and conventional DCs, ds viral RNA and viral transcripts are recognized by cytoplasmic RNA helicase RIG I (or MDA5) or TLR3. The TLR3 and RIG I pathways are mediated by cofactor TRIF or MAV, respectively. Both of these pathways activate TBK1 and IKKε and consequently IRF-3 and IRF-7. In pDCs, the antiviral pathway is mediated either by TLR7, which recognizes ss viral RNA, or TLR9 recognizing the unmethylated viral DNA. The activation of the respective TLRs leads to an assembly of multicomponent complex containing MyD88, IRF-7, IRAK 1, and TRAF-6 and activation of constitutively expressed IRF-7 and IRF-5

Several factors may contribute to the high production of IFNα in pDCs. In cells that constitutively express only IRF-3, an autocrine IFNβ feedback is required for an efficient production of IFNα (Marie et al. 1998; Prakash et al. 2005). However, in pDCs, which express relatively high levels of IRF-7, this autocrine feedback is not required (Dai et al. 2004; Izaguirre et al. 2003). The degradation of IRF-7 also seems to be attenuated in pDCs (Prakash et al. 2005). However, it has recently been suggested that the main reason for the high IFN production in pDCs is the distinct intracellular localization of TLR and TLR/MyD88 complexes in pDCs and other cell types. While in cells other than pDCs the TLR9-MyD88-TLR7 complex is rapidly translocated to lysosomes and degraded, in pDCs it is retained in the endosomal compartment for a longer period of time (Honda et al. 2005).

The specific impact of pDCs on innate and acquired immunity in vivo is virus-dependent. In MCMV or VSV infection, pDCs are the major producer of type I IFN (Dalod et al. 2003). In contrast, pDCs do not contribute to type I IFN synthesis in mice infected with LCMV or West Nile virus (Colonna et al. 2004; Dalod et al. 2003), and the cells producing type I IFN in either one of these viral infections have not yet been identified. Compared to other type I IFN-producing cells, pDCs have two unique functions: they can rapidly produce high levels of type I IFN and the induction of the antiviral response does not require direct viral infection. Since pDCs can respond to noninfectious viral particles or viral nucleic acid, the induction of the antiviral response in these cells is not subjected to viral mimicry (Hengel et al. 2005). The downside of this property is that the ability of pDCs to respond to exogenous nucleic acids or nucleic acid–protein complexes can result in the unregulated production of IFNα and inflammatory cytokines, such as that associated with autoimmune and inflammatory diseases (see the chapter by Crow, this volume).

4
The Good, the Bad, and the Promising

4.1
IFN in the Treatment of Viral Infections

IFNα/β has a long history of clinical use for the treatment of viral infections. Our understanding of the molecular mechanisms by which IFN exerts its remarkably pleiotropic effects is constantly being refined, from the intricate cascade of phosphorylations, which characterize its signaling pathways, to the identification of an ever-growing array of antiviral ISGs. The greater understanding of their virus-specific antiviral functions may provide a new approach to antiviral therapy.

In hepatocytes, hepatitis C virus (HCV) triggers the induction of IRF-3 and NF-κB, via the signaling cascade initiated by HCV genomic RNA. It has been shown that TLR7 confers immunity against HCV via IFN-dependent and -independent pathways. Thus TLR7 agonists might present an alternative to IFN in the treatment of chronic HCV infection (Lee et al. 2006). Clinically, polyethylene glycol-modified IFNα2a in addition to ribavirin is currently the treatment of choice for chronic HCV infection, which leads to cirrhosis and hepatocellular carcinoma. IFNα has been shown to suppress HCV replication. In most patients, a sustained inhibition of HCV genotype 2 and 3 replication is achieved after 24 weeks of treatment (Dalgard and Mangia 2006).

In spite of the efficacy with which IFN inhibits HCV, chronic infection can be established in the liver, mainly because HCV has been remarkably successful

in evolving mechanisms to evade these defenses. The HCV-encoded NS3/4A protease is an effective antagonist of both the RIG-I and TLR3 signaling pathways that are induced by dsRNA regions of secondary structure in the ssRNA HCV genome. Not only does NS3/4A inhibit direct signaling for IFN secretion, but it also prevents IFN amplification via the autocrine and paracrine loops (Foy et al. 2003). HCV core protein induces in vitro expression of suppressor of cytokine signaling (SOC) proteins, which downregulate the JAK-STAT pathway (Bode et al. 2003). Lastly, because the HCV polymerase lacks a proofreading function, a number of viral variants can be generated during the course of a persistent infection, thus affording a great deal of viral complexity and variable sensitivity to IFN (Gale and Foy 2005). The understanding of the molecular strategies employed by the virus to evade immune surveillance will provide novel targets for therapeutic control of HCV (see the chapter by Loo and Gale, this volume).

IFNα was shown to inhibit angiogenesis in Kaposi sarcoma and reactivation of KSHV in primary effusion lymphoma cells (Albini et al. 2000; Marchisone et al. 1999) and, in combination with antiviral therapy, it was used in patients with AIDS-associated Kaposi sarcoma (KS) (Krown et al. 2006).

Another viral infection where IFN has been used therapeutically is respiratory papillomatosis associated with human papilloma virus (HPV) infection (Gerein et al. 2005). Interferon inducer imiquimod has also been used topically (Aldara cream) for treatment of genital warts caused by HPV (Slade et al. 1998).

However, the use of the recombinant IFN at therapeutically effective doses is generally associated with side effects and toxicity and thus a novel method of delivery or use of interferon analogs with higher specific activity that would allow a lower well-tolerated dose are being developed.

4.3 Role of IFN in Autoimmune Diseases

Constitutive production of IFN has been associated with the pathogenesis of some autoimmune diseases, whereas while in others, IFN treatment seems to be beneficiary. There is a preponderance of evidence for an association of IFN with the pathogenesis of systemic lupus erythematosus (SLE) and that of insulin-dependent diabetes mellitus (IDDM). In both diseases, serum levels of IFN are increased, but no initial inducer, be it endogenous or exogenous, has been identified so far. In addition, either disease may appear as an unintended consequence of IFN treatment for an unrelated condition (Devendra and Eisenbarth 2004).

The role of IFN on IDDM appears to be dependent on the stage of the disease. Initially, IFN might be responsible for an aberrant autoimmune response to a viral inducer with pancreatic tropism. As mentioned earlier, no such inducer has been identified to date, but pro-inflammatory products of damaged cells and secretion of other cytokines may induce local IFN secretion and

IFN-mediated pancreatic tissue damage. In later stages of IDDM, proliferation and survival of reactive T cells appears to be suppressed by IFN. Type 1 diabetes has been reported in association with IFN treatment of unrelated disorders, such as cancer and chronic hepatitis (Fabris et al. 2003) and in association with elevated IFN levels during coxsackievirus B infection (Chehadeh et al. 2000). In mice, transgenic expression of type I IFN in beta cells of the pancreas resulted in the destruction of the beta cells (Stewart et al. 1993). However, in NOD mice IFN had a beneficial effect (Sobel and Ahvazi 1998).

In spite of the conspicuous absence of a known inducer, a model has been proposed whereby SLE is the result of sustained activation of myeloid dendritic cells at the instigation of IFN secreted by pDCs in a predisposed background. It remains unclear whether a predisposing background is due to hypersensitivity to stimuli, a greater number of IFN-producing cells, or the existence of a particularly effective inducer (Theofilopoulos et al. 2005). Recently, global gene expression profiling of PBMCs from SLE patients has shown induction of ISG as a hallmark of SLE (Bennett et al. 2003). Moreover, two novel autoantigens have been identified in CD1 lupus mice and found to be IFNα-inducible (Hueber et al. 2004) (see the chapter by Crow, this volume).

The use of IFNβ in the treatment of multiple sclerosis (MS) is well established, although its mechanism of its action is mostly unknown. The beneficial effects of IFNβ in preventing relapsing episodes may be due to a combination of anti-inflammatory, antiproliferative, and pro-apoptotic responses (Hafler 2004). Both experimental rheumatoid arthritis and myasthenia gravis also appear to benefit from treatment with IFN (Deng et al. 1996).

Given its pleiotropic effect in both innate and adaptive immunity, it is not surprising that IFN would play a pivotal role in the pathogenesis of autoimmunity as well. By the same token, IFN provides both a privileged and vulnerable target for therapeutic intervention.

Type I IFN has been shown to have both positive and negative modulatory effects on autoimmune diseases, yet what needs to be established is the nature of the inducer in distinct autoimmune disease and which of the IFNα variants subtypes are induced.

5
Reflections and Considerations

Recent studies clearly established that type I IFN has not only a critical role in the innate antiviral response, but it also provides a stimulus for the adaptive immune response. It has also become clear that type I IFN can have a critical

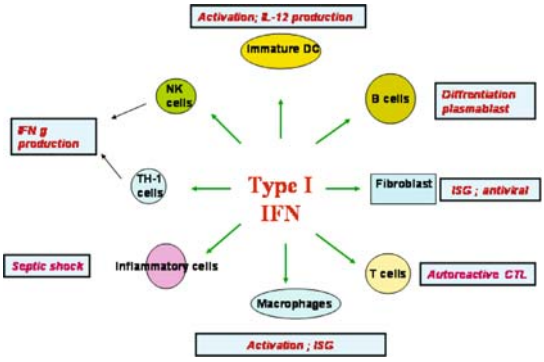

Fig. 3 Multiple functions of type I IFN

role in the pathogenicity of viral infection and in autoimmune disease (Fig. 3). However, a number of questions remain.

Thus the specificity and redundancy of the different type I IFN subtypes needs to be further clarified. In particular, the mechanisms involved in specific induction of distinct type I IFN in vitro have yet to be determined.

While it is easy to see the advantage of the redundancy of antiviral proteins in the defense against a wide variety of pathogens, it is not readily evident why IFN evolved to control so many cellular functions in addition to its role in immune responses. The ability to control the cellular life cycle of a pathogen and to initiate cell death is undoubtedly a powerful way to limit and ultimately eliminate infection, but it is puzzling why so much control over so many cellular mechanisms belongs to one small family of cytokines. One has to wonder then, whether the vast multiplicity of IFN-regulated genes mirrors the multiplicity of means by which pathogens are using the cellular machinery for their survival and propagation.

The felicitous exercise of IFN control over a vast array of cellular genes implies a need for tight regulation of the IFN signaling pathway to prevent unintended consequences. Autoimmune disorders are a glaring example of the deleterious consequences wrought on the organism, when IFN deregulation occurs. The identification of negative regulators of the type I IFN signaling pathway should facilitate the pharmacological manipulation of IFN function.

While the increased understanding of the interferon pathway and its cross-talk with the other inflammatory cytokine has revealed an ever-increasing degree of complexity in the mechanism of type I IFN action, the identification of IFN-stimulated proteins involved in the regulation of many cellular mechanisms and functions makes them very tempting agents for therapeutic

intervention. Future studies will no doubt expand the repertoire and function of these multiple proteins induced by the IFN signaling pathway even more.

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The Receptor of the Type I Interferon Family

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Abstract All type I IFNs act through a single cell surface receptor composed of the IFNAR1 and IFNAR2 subunits and two associated cytoplasmic tyrosine kinases of the Janus family, Tyk2 and Jak1. A central issue in type I IFN biology is to understand how a multitude of subtypes can generate similar signaling outputs but also govern specific cellular responses. This review summarizes results from the last decade that contributed to our current state of knowledge of IFN-receptor complex structure and assembly.

1 Introduction

Interferon turns 50! (Isaacs and Lindemann 1957). This year is the half-century birthday of a family of virally induced cytokines now designated as type I IFNs to distinguish them from the 8 years younger type II IFN. This latter, better known as IFNγ, was first described by Wheelock as an IFN-like substance released by leukocytes stimulated with PHA (Wheelock 1965). Today the type I IFN family also needs to be distinguished from the recent type III IFNs (Kotenko et al. 2003). The common feature of all IFNs is, by definition, their antiviral activities, which also suggests the existence of common signaling elements. The simplest criterion to classify IFNs is based on their receptor usage. Type I IFNs (several α, β, ω, κ, ε, τ subtypes) use a heterodimeric receptor composed of the IFNAR1 and IFNAR2 chains, whereas type II IFN (γ) and type III IFNs (several λ) use a receptor formed by IFNGR1/IFNGR2 and IFNLR1/IL10R2, respectively. This review will deal solely with the type I IFN system.

The concept of a cellular IFN receptor was established in the 1970s, a time when peptide hormones were shown to induce their biological effects through specific binding to cell surface receptors. Among the first groups demonstrating the existence of a cellular IFN receptor (reviewed by Aguet 1980; Stewart 1979), Gresser et al. (1974) showed that IFN can be recovered from the surface of IFN-sensitive mouse leukemia L1210 cells exposed to IFN, but not from similarly treated IFN resistant L1210 cells. A posteriori this was the first demonstration of the existence of a cellular IFN receptor, since later it was shown that L1210R cells do not bind iodinated IFN (Aguet and Mogensen 1983) and carry a deletion in the IFNAR1 gene (Lutfalla and Uze 1994). A variety of somatic cell genetic studies establishing many aspects of the genetic control of IFN sensitivity (reviewed by Slate et al. 1981), as well as the cloning, production, and purification of several subtypes of IFNα (reviewed by Weissmann and Weber 1986) and detailed studies of the IFN binding-cellular response relationship (reviewed by Branca 1988; Mogensen et al. 1989), all ultimately led to the cloning of IFNAR1 (Uze et al. 1990). However, it rapidly became evident that an additional component ought to exist (Uze et al. 1992). The soluble form of IFNAR2 was purified in 1994 (Novick et al. 1994). In the following year, the functional IFNAR2 isoform was described (Domanski et al. 1995; Lutfalla et al. 1995).

Studies in the 1990s were of considerable importance as they established two concepts that formed the basis of our current understanding of how an IFN-receptor complex works: first, the definition of a helical cytokine and the molecular description of the helical cytokine receptor families to which the IFN receptor system belongs (reviewed by Walter 2004); and second, the discovery

of the signal transduction elements of the so-called Jak/STAT pathways (Darnell 1997; Kisseleva et al. 2002; Stark et al. 1998). Altogether, these findings led to the schematic representation shown in Fig. 1.

The binding of IFN to both IFNAR1 and IFNAR2 brings IFNAR2-associated Jak1 in close proximity of IFNAR1-associated Tyk2. Tyk2 and Jak1 are activated by reciprocal trans-phosphorylation and phosphorylate-specific tyrosine

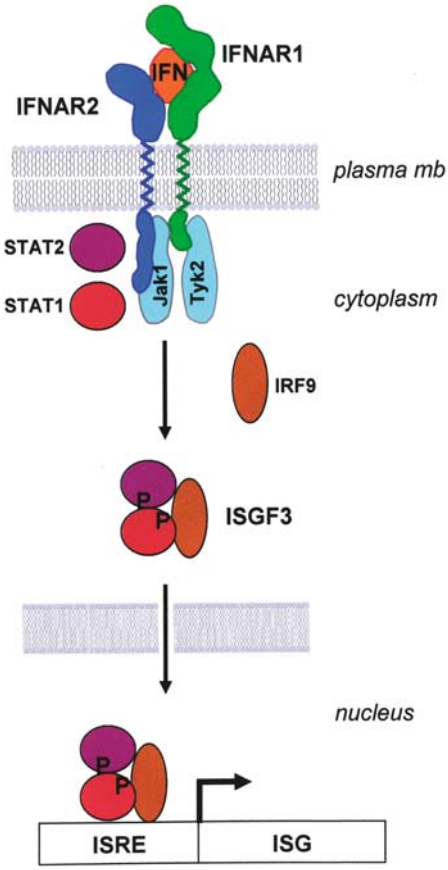

Fig. 1 The classical Jak/STAT signaling pathway activated by type I IFNs. IFNAR1-associated Tyk2 and IFNAR2-associated Jak1 phosphorylate STAT2 and STAT1, which form, together with IRF9, the ISGF3 transcription factor. The latter migrates into the nucleus and activates the transcription of a large number of IFN-stimulated genes carrying an ISRE sequence in their promoter

residues in IFNAR1 and IFNAR2. STAT1 and STAT2 are then recruited to the receptor complex, are tyrosine phosphorylated and heterodimerize to form with IRF9 the prominent transcription complex that will activate the expression of a large number of IFN-stimulated genes (ISGs) carrying an IFN-stimulated response element (ISRE) in their promoter. Other IFN-activated STATs have been described which bind to the IFNγ-activated or GAS element present in the ISG promoters. In addition, accessory signaling cascades are engaged that may operate in specific cellular contexts, in concert with or independently from STATs (Brierley and Fish 2002, 2005; Darnell 1997; Platanias 2005). The current view is that these parallel pathways may be required to sustain complex biological responses.

Type I IFN is unique within the helical cytokine family for its high level of complexity in all eutherian mammals. All mammalian orders possess at least one IFNα and one IFNβ gene (Krause and Pestka 2005). In humans there are 13 α, one β, one κ, one ω, and one ε subtypes, all interacting with the same receptor and activating the common Jak/STAT pathway described above. The selective pressure for the maintenance of this multigene family during evolution is still unknown. Such a multitude of genes may provide the necessary flexibility to control biological activities as diverse as antiviral effects, antiproliferative and antiangiogenic activities, as well as complex functions in cellular differentiation, inflammation, innate resistance, adaptive immunity and regulation of bone homeostasis (Dunn et al. 2005; Takayanagi et al. 2005; Tough 2004). This hypothesis is supported by the differential activities among type I IFN subtypes that can be measured in selected in vitro systems (see Sect. 5).

An important issue yet to be solved is how a single receptor complex can generate differential signaling. In this review, we will address this issue in light of recent data concerning the structure of the type I IFN receptor complex, the dynamics of its formation, and several mechanisms regulating receptor expression and cellular responsiveness.

2
Structure of the IFN-Receptor Complex

2.1
The Receptor Binding Sites on IFNs

IFNαs are among the first proteins that were heterologously expressed in *Escherichia coli* and purified. This enabled the study of mutant forms of IFN and the partial determination of the location of the receptor-binding sites. These data were put in context with the publications of the structures of α and β IFNs. The first structure of a type I IFN (mu-IFNβ) was reported in 1992 (Karpusas

et al. 1997; Senda et al. 1992), followed by the x-ray and NMR structures of hu-IFNα2b (Klaus et al. 1997; Radhakrishnan et al. 1996). Type I IFNs consist of a five helix bundle (see Fig. 4), with an additional functionally important long loop that connects helices *A* and *B*. The C-ter tail (beyond residue 158) is unstructured in all IFNα2 structures, but not in IFNβ.

The structural information obtained, together with mutagenesis data, gave a clear framework of the location of the IFNAR2 binding site on IFNα2, and, to a lesser extent, of the IFNAR1 binding site (Piehler et al. 2000). Figure 2 shows how the two receptors bind on opposite sides of the IFNα2 molecule (Roisman et al. 2001, 2005). As determined from mutagenesis studies, the energetic picture of the IFNAR2 binding site has a classical arrangement, with the hotspot residues in the center, surrounded by residues of lesser importance (Fish 1992; Mitsui et al. 1993; Piehler et al. 2000; Piehler and Schreiber 1999; Uze et al. 1994). The IFNα2 *E*-helix is located at the center of the IFNAR2 binding site, flanked by residues on the *A*-helix and the *AB*-loop. The contributions of residues of the *D*-helix seem to be marginal. Six binding hotspot residues were found on IFNα2, L30, and R33 on the *AB* loop and R144, A145, M148, and R149 on the *E*-Helix. Systematic mutational analysis of hu-IFNβ has identified parts of the

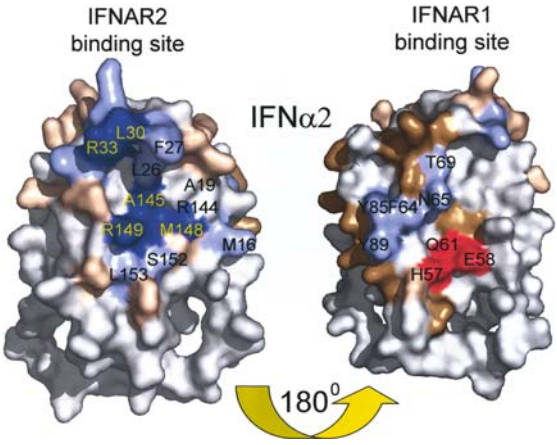

Fig. 2 The functional epitope for binding IFNAR2 and IFNAR1 on IFNα2 (solution structure of IFNα2, residues 1–161). The IFNAR1 binding surface is a 180° rotation from the IFNAR2 binding site. Residues that, upon mutation, increase or decrease the binding affinity by more than twofold are colored *red* or *blue*, respectively. *Dark blue* is for hotspots, where binding is decreased more than tenfold upon mutation. The figure was drawn using PyMol

A helix, the *AB* loop, and the *E* helix as the binding epitope to IFNAR2, and parts of the *B*, *C*, and *D* helices and the *DE* loop as being responsible for binding IFNAR1. The interaction between IFNα2 and IFNAR2 has an affinity in the nM range, the interaction of IFNβ being about tenfold tighter (Cutrone and Langer 1997; Piehler and Schreiber 1999). The rate of association of these complexes is 1 and $5\times10^7 M^{-1}s^{-1}$, and their rate of dissociation is 0.006 and 0.002 s^{-1}, respectively (Lamken et al. 2004; Peleg-Shulman 2004). The binding data for this interaction are similar to those found for other cytokine-receptor interactions such as growth hormone (Cunningham and Wells 1993), IFNγ (Walter et al. 1995), and IL10 (Walter 2004). Interestingly, the IFNAR2 binding site overlaps the largest continuous hydrophobic patch on IFNα2 (Piehler et al. 2000), suggesting that hydrophobic interactions play a significant role in stabilizing this interaction. The unstructured C-ter tail of different IFNs has a large variation in its net charge, from neutral to +4 (Slutzki et al. 2006), variation that contributes to an up to 20-fold difference in binding affinities to IFNAR2. Double mutant cycle analysis as well as constrained docking placed the tail near a negatively charged loop on IFNAR2, comprising of residues E132–134, suggesting that the IFN tail gains structure upon receptor binding (Slutzki et al. 2006).

The interaction of hu-IFNα2 with IFNAR1 is in the μM range, i.e., approximately 1000-fold weaker than the interaction with IFNAR2. The only hu-IFN that binds reasonably tight to IFNAR1 is IFNβ (K_D=100 nM) (Cutrone and Langer 1997; Lamken et al. 2004; Roisman et al. 2005). Surprisingly, both human and bovine IFNα bind bovine IFNAR1 at a 10 nM affinity (Langer et al. 1998). The interaction of IFNAR1 on IFNα2 was mapped on the *B* and *C* helices, opposite the binding site for IFNAR2 (Fig. 2). No binding hotspots were found in the interface, with individual mutations having up to a fivefold effect on binding (Roisman et al. 2005). The residues on IFN identified to reduce binding and biological activity are F64, N65, T69, L80, Y85, and Y89 (IFNα2 sequence numbering) (Hu et al. 2001; Roisman et al. 2005; Runkel et al. 2000). Conversely, H57, E58, and Q61, located on the *B*-helix, conferred tighter binding to IFNAR1 when mutated to Ala. Interestingly, these three residues, are conserved in all IFNα proteins (Fig. 3), suggesting that weak binding is important for IFNα action, apparently to sustain a differential level of biological activities compared to IFNβ. An engineered triple Ala IFNα2 mutant (termed HEQ) confers a 30-fold higher binding affinity toward IFNAR1, comparable to that measured for IFNβ (Jaitin et al. 2006). Indeed, this IFNα2 mutant is almost indistinguishable from IFNβ in its biological activity. One may view the interaction of IFNAR1 with IFNα2 as an example of an architecture of a weak protein–protein binding site, which lacks hotspots for binding and is not optimized for high affinity. The extensive mutagenesis work done on IFNs suggests that the functional epitopes on IFNβ are similar, but not identical, to those on IFNα2 (Runkel et al. 1998, 2000).

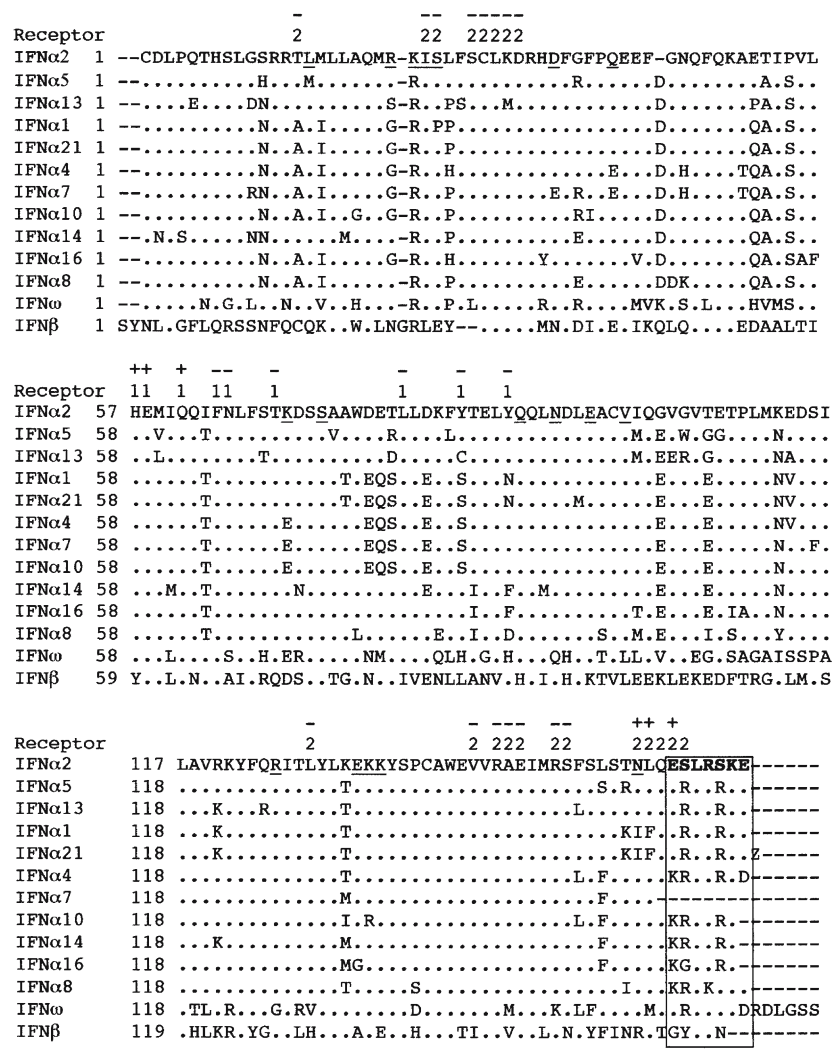

Fig. 3 Alignment of human interferons. Residues that, upon mutation to Ala, reduced or increased the binding affinity are marked with a *number and plus or minus sign above the letter*. The *number* represents the relevant receptor subunit, *1* for IFNAR1 and *2* for IFNAR2. *Underlined letters* represent mutations that did not confer a change in binding affinity. The *box* marks the IFN tail

2.2 The Ligand Binding Site on IFNAR2

The structure of the extracellular domain (EC) of IFNAR2 was solved using NMR. The residue numbering scheme used here is the one used in the NMR structure (PDB id 1N6U), which is different from the numbering in some earlier publications (Lewerenz et al. 1998; Piehler and Schreiber 1999; Roisman et al. 2001). IFNAR2-EC is comprised of two fibronectin type III (FNIII) modules (residues 13–99 and 111–203) connected by a linker segment (residues 100–110, Fig. 4). The fibronectin domains are characterized by seven β-strands arranged in a β-sandwich. The terminal residues 1–11 and 206–212 are unstructured. A striking feature of IFNAR2-EC is the mutually perpendicular orientation of its two FNIII domains, which is approximately 90°. This angle is different from that found in IFNγR (Walter et al. 1995) and tissue factor (Harlos et al. 1994), but similar to that in IL-10R1 (Walter 2004).

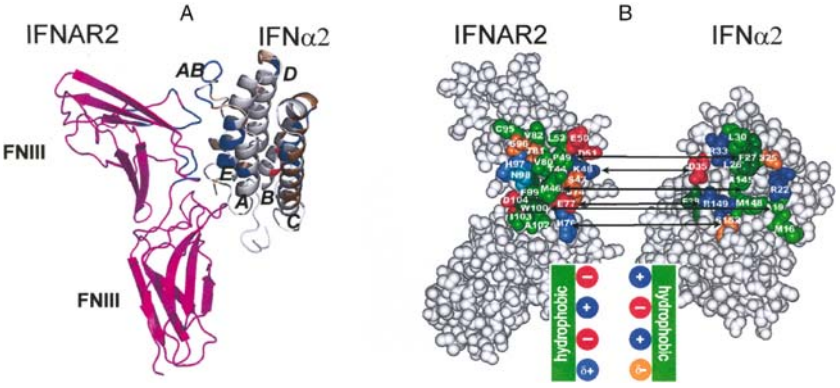

Fig. 4 Modeling the IFNAR2/IFNα2 complex. **A** Ribbon representation of the complex. Areas of interaction (as determined by mutagenesis) are colored *blue* (for mutations that reduce affinity) and *red* (for mutations to Ala that increase affinity). Helices *A*–*E* and the *AB* loop are labeled. The IFNAR1 binding site was mapped opposite the IFNAR2 binding site, onto helixes *B* and *C*. **B** Open book representation of the IFNAR2/IFNα2 binding site portrayed in spacefill mode. The six interacting pairs of residues (determined by double-mutant cycles) are connected by the *arrows*. Coloring is according to the chemical nature of the side chain, showing the high degree of complementarity of the interface

On IFNAR2, the IFN binding site is located on loops 43–53 and 76–80, and the interdomain residues 100–110 (Fig. 4) (Chill et al. 2003; Chunthaprapai et al. 1999; Roisman et al. 2001). Deletion of the C-terminal domain of IFNAR2 had only a small effect on IFNα2 binding. The largest hydrophobic patch on the protein surface is within the IFN binding site, surrounded by polar and charged residues (Piehler et al. 2000). Although IFNα2 and IFNβ bind competitively to the same functional epitope, mutational analysis revealed distinct binding centers for these IFNs on IFNAR2. Particularly, M46 is a specific hotspot for IFNα2, while W100 is a specific hotspot for IFNβ binding. Two other differentially binding residues are H76 and N98 on IFNAR2. Mutating these two residues to Ala increased the binding affinity of IFNβ to IFNAR2 by 40-fold, while the affinity to IFNα2 was unchanged (Peleg-Shulman et al. 2004). At low protein concentrations, the H76A, N98A IFNAR2 mutant is a perfect candidate for a specific carrier protein for IFNβ (but not IFNα2), substantially prolonging its half-life in circulation. Conversely, at high protein concentration the mutant IFNAR2 may serve as a specific antagonist for IFNβ (Peleg-Shulman et al. 2004).

2.3 The Ligand Binding Site on IFNAR1

IFNAR1 is a member of the cytokine receptor superfamily, sharing conserved structural FNIII building blocks that form the extracellular ligand-binding domain. In IFNGR1, IFNGR2, growth hormone receptor, tissue factor, and IFNAR2, there are two FNIII domains, each containing 100 amino acids with seven β-strands and connecting loops. The extracellular domain of IFNAR1 is atypical, consisting of a tandem array of four FNIII domains, here denoted subdomains 1 through 4 (SD1–4; beginning from the N terminus) (Kotenko and Langer 2004; Mogensen et al. 1999; Pestka 1997). The four-domain structure of IFNAR1 appears to represent a tandem duplication of the more common two-domain structure.

The low intrinsic affinity of huIFNAR1 for IFNs has previously hampered studies seeking to identify residues involved in ligand binding and specificity. The bovine IFNAR1 (bo-IFNAR1) homolog was found as an attractive target for mutagenesis and analysis of the IFN binding site, as human IFNs display uniformly high binding and biological activity on bovine cells. This reflects the ability of bo-IFNAR1 to bind human type I IFNs with moderately high affinity (Cutrone and Langer 1997). In vitro studies have shown that the three N-terminal FNIII domains of the ectodomain of IFNAR1 (IFNAR1-EC) are required for ligand recognition, which is very atypical for cytokine receptors (Lamken et al. 2005). Recent studies by Piehler et al. have indicated substantial

conformational changes of IFNAR1-EC upon ligand binding, which are propagated to the membrane-proximal FNIII domain. Strikingly, the membrane-proximal domain of IFNAR1 is not involved in ligand binding, but is absolutely critical for formation of a functional signaling complex (Lamken et al. 2005). Modeling the mutagenesis results from bo-IFNAR1 on the human homolog suggested that four aromatic residues located on SD2 and SD3 (W129, F136, Y157, and W253) constitute an important part of the IFN binding epitope (Cutrone and Langer 2001). On the other hand, residues 62–70, which are recognized by the 64G12 monoclonal Ab may participate in binding, but are not crucial (Cutrone and Langer 2001). However, a report published in 2004 claimed that these residues are essential for IFN binding; therefore this issue will await further investigations (Cajean-Feroldi et al. 2004). The importance of both SD2 and 3 was corroborated by fragment studies of IFNAR1, showing that subunits SD1–2 and SD3–4 did not bind IFN (Lamken et al. 2005). Fragment SD1–2-3 retained almost normal binding affinity, while SD2–3-4 had no binding activity. Binding competition experiments have shown that IFNα and β bind the same epitope on IFNAR1. The comparison of the energetics of the mutual binding epitopes on IFNα2 and IFNAR1 is somewhat perplexing. As mentioned, no hotspots for IFNAR1 binding were found on IFNα2, contrary to a number of hotspot mutations found on IFNAR1 (particularly W129, F136, Y157, and W253). Additional structural knowledge is needed to explain this discrepancy.

2.4 Modeling the IFNα2/IFNAR2 Complex

Double-mutant cycle analysis measures the coupling energy between two residues. Coupled mutations are most often spatially close, making it feasible to apply this information as distance constraints between the two residues for docking. In some sense, this is similar to the use of NOEs in calculating an NMR structure. Extensive double-mutant cycle mapping between residues on IFNα2 and on IFNAR2-EC yielded a number of interacting residue pairs (Fig. 4). Docking of IFNα2 and IFNAR2 using these distance constraints resulted in a structural model of the complex, which accounted well for the single-mutation data (Chill et al. 2003; Roisman et al. 2001). The striated motif observed for the IFNAR2-EC binding surface interacts with a highly complementary array of hydrophobic and hydrophilic patches on the binding surface of IFNα2. Receptor residues ^{R}E50, ^{R}K48, ^{R}H76, and ^{R}E77 of the hydrophilic strip interact with a matching array of alternating charges upon the ligand formed by residues $^{\alpha}$R33, $^{\alpha}$D35, $^{\alpha}$S152, and $^{\alpha}$R149. The resulting overall pattern of four intermolecular electrostatic interactions of alternating polarity on the surface of both receptor and ligand is particularly striking (Fig. 4), and provides a good

explanation for the fast rate of association observed for this complex. At the heart of the binding interface are the two complementary hydrophobic strips, with receptor residues ^{R}M46, ^{R}P49, ^{R}V80, ^{R}V82, ^{R}W100, and ^{R}I103 interacting with ligand residues $^{\alpha}$M16 and $^{\alpha}$A19 of the *A* helix, $^{\alpha}$L26, and $^{\alpha}$L30 of the *AB* loop, and $^{\alpha}$A145 and $^{\alpha}$M148 of the *E*-helix.

As mentioned above, the split of binding energies of mutations on IFNAR2 was significantly different for binding to IFNα2 versus to IFNβ. Superimposing the unbound structure of IFNβ onto the structure of IFNα2 in the model of the complex placed W22 of IFNβ at the same location occupied by A19 in IFNα2 (Fig. 4) and suggests a direct interaction between $^{\beta}$W22 and ^{R2}W100. As mentioned above, the mutation ^{R2}W100A had a much larger effect on IFNβ binding than on IFNα2 binding. A clear validation of the similarity of the IFNα2 and IFNβ binding sites on IFNAR2 was obtained by mutating Ala 19 on IFNα2 to Trp and showing a clear interaction between the A19 W mutation on IFNα2 and W100 on IFNAR2. (Slutzki et al. 2006). This suggests that differential activation between IFNα2 and IFNβ is apparently not a result of differences in the structure of this complex.

A number of attempts have been made to model the IFN-IFNAR1 interaction also (Cajean-Feroldi et al. 2004; Mogensen et al. 1999). However, because of lack of cohesive structural data on the IFNAR1 receptor, and the partial mapping of the IFN binding site on IFNAR1, these models are still quite speculative and their validation will have to await further experimental studies.

2.5 Ternary IFN-Receptor Complex Assembly

As detailed in Sect. 2, extensive structure – function analysis of IFNs and the receptor subunits IFNAR1 and IFNAR2 by mutagenesis and binding studies clearly shows that essentially the same binding sites are recruited by all type I IFNs. These data are consistent with a 1:1:1 complex, with a very similar architecture for the different members of the family. Thus, differences in the structure of the ternary complex probably do not account for the differential signal activation by IFNs. The key difference among type I IFNs in terms of recognition by their receptor are the affinities and the rate constants of their interaction with the receptor subunits. While all IFNs bind IFNAR2 faster and with higher affinity than IFNAR1, a range of equilibrium constants between 100 nM (IFNα1) and approximately 100 pM (IFNβ) have been observed. Differences in binding affinities stem from large differences in both association and dissociation rate constants. Substantial differences have also been observed in the binding affinity toward IFNAR1. Here, similar binding affinities in the micromolar

range were observed for the IFNα subtypes. In contrast, IFNβ binds IFNAR1 with an equilibrium dissociation constant of approximately 50 nM. The key role that these differences in affinity to the receptor subunits play in differential signaling has been clearly confirmed by protein engineering (see Sect. 5).

Understanding the consequences of differential affinities and complex stabilities on signaling requires a detailed mechanistic analysis of the assembly of the ternary ligand–receptor complex on the plasma membrane. In vitro studies with the ectodomains of IFNAR1 and IFNAR2 tethered onto solid supported membranes corroborated the ligand-induced receptor assembly mechanism (Lamken et al. 2004), which was proposed earlier by Rubinstein and co-workers based on cross-linking experiments (Cohen et al. 1995). As no interaction between the receptor subunits could be observed (Lamken et al. 2004), a preassembled complex on the plasma membrane—as proposed for several other cytokine receptors—can be excluded. Furthermore detailed in vitro studies clearly established that the ligand interacts independently with the two receptor subunits (Lamken et al. 2004). Based on detailed binding studies with the extracellular domains of the receptor subunits tethered onto solid-supported membranes, a two-step binding mechanism was experimentally confirmed, where the ligand binds first to one of the receptor subunits and then recruits the second subunit only on the membrane surface (Fig. 5) (Gavutis et al. 2005). Two

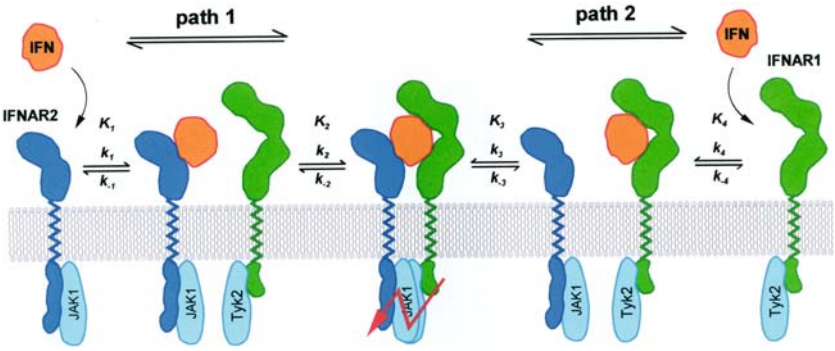

Fig. 5 Two-step assembling of the ternary complex. After ligand binding to one of the receptor subunits, the second receptor subunit is recruited by lateral interaction on the membrane. Two assembling pathways are possible. Since the first step of ligand binding to one of the receptor subunits is rate-limiting, the population of the two pathways depends only on the association rate constants *k1* and *k4*, and the relative concentrations of IFNAR1 and IFNAR2. The ternary complex is in a dynamic equilibrium with the binary complexes, which is determined by the affinity constants *K2* and *K3*. In particular for the IFNα subtypes, the low affinity toward IFNAR1 (*K2*) limits its recruitment into the ternary complex

possible assembly pathways are depicted in Fig. 5. While intuitively binding to the high-affinity subunit IFNAR2 seems to be much more likely (pathway 1), a systematic biophysical analysis identified the relevance of pathway 2 (Gavutis et al. 2006), because ligand binding to one of the receptor subunits is the rate limiting step in the two-step assembly mechanism. Since all interactions are transient, a dynamic complex is formed on the plasma membrane, with binary and ternary complexes being in a dynamic equilibrium. In light of the different affinities of different IFNs for the receptor subunits, the dynamic nature of the signaling complex has important consequences. The equilibrium concentrations of binary and ternary complexes are differently populated for different IFNs due to their different K_2 and K_3 (see Fig. 5). The efficiency of recruitment of the receptor subunits depends on the absolute and relative concentration of the receptor subunits. The concentration of IFNAR1 and IFNAR2 in human cells (500–5,000 binding sites per cell) suggests that in particular the recruitment of IFNAR1 into the ternary complex is limited by the low affinity of the IFNα subtypes (K_2 in Fig. 5). In contrast, the affinity of IFNβ to both receptor subunits is so high that ternary complex assembling is quantitative, even at relatively low concentrations of the receptor subunits. This mechanism has the consequence that the responsiveness of cells to different IFNs is modulated by the surface concentrations of the receptor subunits: if the concentration of IFNAR1 is too low, the affinity of IFNα is not sufficient to recruit it. Strikingly, this affinity cannot be compensated by an increased dose, since the limiting event is not ligand binding to the receptor, but the concentration of IFNAR1. This hypothesis is supported by the observation that some cell lines with very low levels of IFNAR1 are responsive to IFNβ, but not to IFNα (Lewerenz et al. 1998; Vitale et al. 2006). As further detailed in Sect. 5, this effect may play a key role in differential signaling.

Besides these equilibrium effects, the different interaction rate constants of IFN with the receptor subunits have implications for the dynamic state of the ternary complex. The mechanism shown in Fig. 5 implies that the ternary complex is transiently formed in a "kiss and run" fashion. It is therefore possible that a ligand molecule bound to the cell surface interacts consecutively with several different copies of IFNAR1 or IFNAR2, and the lifetime of individual ternary complex vastly differs among different IFNs. Thus, the lifetime of a ternary complex is much higher for IFNβ (~100 s) than for IFNα (1–5 s), as also observed in cells (Platanias et al. 1996). While the minimum lifetime of a ternary complex to signal has not been established yet, it is conceivable that the lifetime affects the efficiency of recruitment and activation of cytoplasmic effectors. It is, therefore, quite possible that different signaling pathways require different stabilities of the ternary complex, which may be a reason for differential signal activation (see Sect. 5). Interestingly, the stability of the ternary

complex also affects receptor downregulation: upon stimulation with IFNα, downregulation of only IFNAR1 is observed, whereas upon stimulation with IFNβ, both IFNAR1 and IFNAR2 are downregulated (Jaitin et al. 2006).

Our current understanding of ternary complex assembly mostly stems from in vitro studies with the extracellular domains of the receptor subunits. While these measurements provide conceptual mechanistic insight, they cannot fully mimic the processes on the plasma membrane. For example, lateral organization of the plasma membrane into microdomains may play an important role. FRAP experiments have demonstrated the very low mobility of both receptor subunits, which appear highly confined in membrane microdomains, as identified by single molecule tracking (J. Piehler et al., unpublished results). Therefore, detailed studies on the receptor assembly, dynamics, and signal activation in live cells will be of key importance in order to understand the biophysical basis of differential signal activation.

3 The Intimate Relationship Between IFNARs and JAKs

Like all receptors for helical-bundled cytokines, the components of the type I IFN receptor do not possess a catalytic domain but rely on the Janus tyrosine kinases (Jaks) to activate STAT and other signaling pathways (Kotenko and Pestka 2000; Pestka et al. 2004). The accepted model of Jak/STAT activation by cytokines was originally drawn from genetic and biochemical studies of type I IFN-initiated events (Fig. 1), which were facilitated by obtaining IFN-resistant HT-1080-derived mutants deficient in key signaling components (Darnell et al. 1994; Pellegrini et al. 1989). The steps can be summarized as follows: ligand-mediated dimerization of the receptor subunits brings the associated Jaks into a proper orientation (or high local concentration), allowing their cross-phosphorylation and conversion to a catalytically active "on" state. While homodimeric-type receptors were found to engage two Jak2 molecules, heteromeric-type receptors, such as IFN receptors, activate two different Jaks, which in turn proceed to phosphorylate receptor tyrosine residues. STATs and other effector molecules are then recruited via their SH2 domain to the phosphotyrosine-based receptor motifs in order to be eventually phosphorylated.

Jak proteins possess a large amino-terminal region, a centrally located kinase-like (KL or JH2) domain and a carboxy-terminal tyrosine kinase (TK or JH1) domain. Concordant work from several laboratories determined that the amino-terminal region of Jak, via FERM and SH2-like domains, is involved in recognition of the membrane-proximal portion of the receptor. The KL domain plays a pivotal role in the control of both the resting (off) and the activated (on)

receptor/kinase complex; the TK domain, bearing a genuine activation loop, exerts catalytic activity. Molecular modeling studies of Jak2 suggested how the JH2 domain might prevent activation of catalytic JH1 domain (Lindauer et al. 2001). The prediction attributed a critical role to a short loop within JH2. Interestingly, a Val residue is located within this loop and its substitution to Phe (V617F) has been found in a large number of patients with myeloproliferative diseases (Goldman 2005). The crystal structures of the TK domain of Jak3 complexed with a staurosporine analog (Boggon et al. 2005) and of the TK domain of Jak2, complexed with a Jak-specific inhibitor (Lucet et al. 2006) have been solved. However, more structural data are awaited to better understand the mechanism by which Jak kinases are regulated.

The IFNAR1 and IFNAR2 subunits of the IFN receptor interact with Tyk2 and Jak1, respectively. Upon IFN binding, both enzymes undergo tyrosine phosphorylation and catalytic activation in an interdependent manner. To identify residues involved in the switch to the "on" state and to understand the basis of the interdependence of Tyk2 and Jak1, mutated versions of each kinase were studied in the corresponding Jak-null cells. For instance, it was shown that substitution of the $Y_{1054–1055}$ in the activation loop of Tyk2 precludes ligand-dependent activation of the enzyme, without abolishing its basal catalytic potential. Mutation of K_{930} in the ATP binding site generates a catalytically inactive protein, which, however, can be phosphorylated at $Y_{1054–1055}$ upon IFNα treatment (Gauzzi et al. 1996). These results demonstrated that Tyk2 switches to an "on" state when phosphorylated in the activation loop by the neighboring Jak1.

Using the human Tyk2-null cell line as a model system, a noncatalytic role of Tyk2 toward receptor functioning was uncovered. Indeed, Tyk2 null cells have a reduced level of IFNAR1 protein and, as a consequence, binding of IFNα is severely affected. Weak binding and signaling activity of IFNβ is, however, detectable, suggesting that Tyk2 contributes structurally to the binding capacity of the receptor more toward α than β IFN. Studies of deleted forms of Tyk2 revealed that an intact amino-terminal region (FERM and SH2-like domains) rescues the level of IFNAR1, but not the full binding activity of the receptor, for which the KL domain is required (Gauzzi et al. 1997). While the KL and the TK domains of Tyk2 and Jak1 are interchangeable, the subdomains of the amino-terminal region cannot be swapped without a loss of function (Richter et al. 1998). Interestingly, in the absence of Tyk2, IFNAR1 accumulates into a perinuclear endosomal compartment, where it is degraded. Conversely, when complexed to Tyk2, IFNAR1 is stabilized at the plasma membrane. Tyk2 acts by reducing the basal internalization rate of IFNAR1, most likely by masking basal internalization motif(s) (Ragimbeau et al. 2003). The chaperone-like role of Tyk2 toward IFNAR1 was confirmed using RNA interference in human cell lines other than fibrosarcoma cells.

The comprehensive study on Tyk2/IFNAR1 has thus revealed new aspects of the function and regulation of Jak enzymes and in particular the intimate relation and high level of dependence that can exist between a receptor subunit and the associated kinase. Studies of other cytokine receptor complexes have shown that Jaks, apart from being critical signaling components, may promote receptor maturation and delivery to the cell surface (Huang et al. 2001; Radtke et al. 2002). As of today, it appears that the chaperone-like function of a Jak may be more or less critical depending on the properties of the receptor itself and the cellular context. In the case of IFNAR1, the role of Tyk2 may be more critical toward cellular responsiveness to specific IFN subtypes or in the presence of low concentrations of ligand.

In spite of many studies, the surfaces of interaction and the molecular determinants of specificity in a receptor:Jak (R:Jak) complex remain ill defined. The biochemical demonstration of the association of the two partners in cells is not straightforward and a possible contribution of membrane components toward the stability of a R:Jak complex is plausible.

Recent data showed that, within a few minutes of IFN binding, IFNAR1 is phosphorylated on serine residues (S535 and S539) located within the cytoplasmic tail (Kumar et al. 2003), a region previously shown to contain negative regulatory sequences (Basu etal. 1998; Gibbs et al. 1996). This phosphorylated motif is recognized by the F-box-containing β-TrCP2/HOS protein. With Skp1 and Cullin1, β-TrCP2 is part of the SCF ubiquitin ligase complex and is known to interact with a large number of phosphorylated substrates, including the IκB, β-catenin, and Cdc25a proteins. Once ubiquitinated, these proteins are targeted to proteasome-dependent degradation (Fuchs et al. 1999). In the case of IFNAR1, β-TrCP2 is involved in IFN-induced ubiquitination and proteolysis of IFNAR1 via the lysosomal pathway (Kumar et al. 2003, 2004). While it is clear that ligand-induced ubiquitination precedes receptor degradation, the precise role of ubiquitin conjugation is unclear. Ubiquitination may generate an internalization signal, which is recognized by the endocytosis machinery, and it may also direct the postinternalization fate of IFNAR1 and ensure efficient endosomal sorting. It appears that IFNAR1 ubiquitination and degradation can occur via a ligand-dependent and a ligand-independent pathway. S535 phosphorylation, ubiquitination, and degradation of endogenous IFNAR1 are robustly stimulated by IFN and Tyk2 catalytic activity, which is essential for this stimulation (Marijanovic et al. 2006). Yet, when highly expressed, IFNAR1 is S535 phosphorylated and ubiquitinated in the absence of ligand, in a manner that does not require Tyk2 activity. Thus, a ligand/Tyk2-independent pathway may be at work to regulate the level of expressed IFNAR1 in a cell-type specific fashion.

Little is known about the fate of ligand-engaged IFNAR2. Proteolytic cleavage of membrane-associated IFNAR2 and consequent liberation in the cytosol of a portion of the receptor intracellular region has been reported to occur upon phorbol ester treatment (Saleh et al. 2004). An interesting novel player involved in downregulating the IFN response and acting on the receptor is Ubp43, an IFN-inducible ISG15 deconjugating enzyme. IFN hypersensitivity was described in ubp43$^{-/-}$ mice, suggesting a function of Ubp43 in downregulation of IFN responses. Interestingly, Ubp43 functions in type I IFN signaling independently of its isopeptidase activity, by downregulating the Jak-STAT pathway at the level of the IFN receptor. Ubp43 appears to bind to the membrane-proximal region of IFNAR2, previously shown to be essential for the interaction with Jak1 (Domanski et al. 1997; Usacheva et al. 2002). A likely possibility is that Ubp43 competes with Jak1 for receptor binding (Malakhova et al. 2006).

4
Differential Activities Between Type I IFN Subtypes

Since the original report by Ortaldo et al. showing that an IFNα subtype (α7) was not able to stimulate NK cells (Ortaldo et al. 1984), several studies reported differential activities of type I IFNs, but there is little evidence for an absolute specialization in the function of IFN subtypes. A differential effect between type I IFN subtypes is therefore defined by a lack of correlation between two specific activities. For example, IFNα2 and IFNβ exhibit comparable specific activities for the antiviral effect against VSV replication on WISH cells; however, the IFNβ is much more potent than IFNα2 to inhibit the proliferation of these cells (Jaitin et al. 2006). IFNα2 and IFNβ thus have differential activities for this specific function in this specific cellular context. In a similar manner, differences in antiproliferative activities of IFNα2/α21 hybrids do not correlate with their ability to activate the STAT1/2 transduction pathway (Hu et al. 1999).

In human primary cells, IFNα 8 was shown to be unable to affect T cell motility, whereas IFNα2 does (Foster et al. 2004). The chemokine CXCL10 was found to be differentially induced in dendritic cells by IFNα1, α2, and α21 (Hilkens et al. 2003). In monocytes undergoing RANKL-induced osteoclastic differentiation, IFNβ is 100-fold more potent that IFNα2 at inhibiting differentiation, whereas the two subtypes are equally potent in activating early transcriptional responses (Coelho et al. 2005). This differential effect results from a stronger IFNβ transcriptional induction of CXCL11, a chemokine known to be upregulated preferentially by IFNβ also in other cell types (Rani

et al. 1996) and found to inhibit osteoclastogenesis. The IFN-induced expression of both CXCL10 and CXCL11 was shown to be dependent on activation of p38 and NFκB, respectively, in addition to the STAT1/2 signaling pathway (Hilkens et al. 2003; Rani et al. 2002). IFNβ also exhibits a greater potency than IFNα2 to induce apoptosis of human tumoral cells (Leaman et al. 2003; Vitale et al. 2006). Whereas most clinical successes have been obtained using IFNα2 or IFNβ, in light of these observations, more research is necessary in order to choose the best IFN subtype to be used for a given pathology.

The molecular basis of type I IFN differential responses has been extensively studied by testing the possibility of different structures of the IFN-receptor complexes, different orientations of the complexes, or different complex stoichiometries. However, these approaches failed to highlight differences between the IFNα2 and β-receptor complexes. Functional differences among type I IFNs are instead related to different affinities and kinetics of the interaction with receptor subunits (see Sects. 2 and 3). In line with this hypothesis, the HEQ mutant of IFNα2, resulting in an affinity to IFNAR1 similar to that of IFNβ, recapitulates the IFNβ's unique activities on WISH cells (Jaitin et al. 2006). Thus, the emerging picture is that different biological properties of type I IFN subtypes are most likely related to the affinities to the receptor subunits—in particular to IFNAR1—and not to other structural features. It is to be noted that the biological activities for which the IFN subtypes act differentially (antiproliferative, cellular differentiation, etc.) require several days of IFN stimulation. Conversely, with the exception of STAT3 and STAT5, which were found differentially activated by IFNα subtypes in a murine erythroblast cell line (Cull et al. 2003), no significant differential STAT phosphorylation was observed in response to IFNs that in fine act differentially (Hilkens et al. 2003).

Interestingly, in vitro studies on artificial membranes indicated that, given the low affinity of IFNα2 toward IFNAR1, the recruitment of IFNAR1 into the ternary complex may be limited by the IFNAR1 surface concentration (see Sect. 3). This is not the case for IFNβ, which binds IFNAR1 more tightly and therefore efficiently recruits IFNAR1 on the membrane, even at very low concentrations (Jaitin et al. 2006). It is therefore tempting to speculate that the cell surface expression level of IFNAR1 could be a determining factor for a specific cellular response to an IFN subtype. Cell surface IFNAR1 is stabilized by its interaction with Tyk2 (see Sect. 4). Ectopic expression in HT1080 cells of IL12Rβ1, another Tyk2-interacting receptor, dampens the concentration of Tyk2 available to interact with IFNAR1 (Dondi et al. 2001). As a consequence, the surface level of IFNAR1 is decreased and cells are selectively impaired in their response to IFNα2 but not to IFNβ (Dondi et al. 2001). Recently, Severa et al. (2006) showed that, while immature dendritic cells are equally sensitive to IFNα2 and β, LPS-matured dendritic cells fully respond to IFNβ but not to

IFNα2. This differential desensitization correlates with a poor IFNAR1 expression level in LPS-matured dendritic cells. The cell surface level of IFNAR1 could thus be an important cellular parameter for a fine tuning regulation of the responsiveness to type I IFN subtypes.

5 Conclusion

In the end, we can tentatively propose that type I IFN receptor system is perhaps simpler than was initially suspected. All experimental data indicate that one IFN molecule is sandwiched between one IFNAR1 and one IFNAR2 chain and that only minute differences in the architecture of the complex engaged with different IFN subtypes are expected. However, we still have to fully understand how IFNα and β act differentially. The affinity toward the receptor subunits may represent the key factor (Jaitin et al. 2006). How differential affinities may translate into differential signaling remains speculative. Some possible explanations have been discussed in Sects. 3 and 5. However, the interplay between receptor assembly, signal activation, and downmodulation events further complicates the picture. Since, upon its formation at the cell surface, the ternary IFN-receptor complex is rapidly internalized (Branca 1988), it would be interesting to investigate whether signal specificity and/or diversity can be generated at the level of endosomes, as described in other ligand-receptor systems (Miacynska et al. 2004). Once internalized, the IFNα2 and the IFNβ ternary complexes may follow different endosomal routes, thus generating subtype-specific responses. During its endocytic itinerary, the more stable IFNβ-receptor complex may connect to local interactors and/or persist to convey longer-lasting signals. The notion that signaling regulates traffic and vice versa that the spatial-temporal distribution of receptors can influence signaling, qualitatively and/or quantitatively, has become widely recognized (Polo and Di Fiore 2006; Sorkin and Von Zastrow 2002). Cell type-intrinsic properties undoubtedly represent important parameters that shape the IFN responses. Hence, we still have work to do to draw a detailed spatiotemporal picture of IFN-induced signaling in live cells.

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IFN-γ: Recent Advances in Understanding Regulation of Expression, Biological Functions, and Clinical Applications

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Abstract Interferon-gamma (IFN-γ) is a key immunoregulatory protein that plays a major role in the host innate and adaptive immune response. Also known as type II interferon, IFN-γ is a single-copy gene whose expression is regulated at multiple levels by the host. Transcription control is regulated through epigenetic mechanisms as well

as the accessibility of chromatin and the binding of activating and inhibitory proteins to promoter and enhancer elements. Post-transcriptional control is mediated through mRNA localization and mRNA stability while post-translational control occurs through the activation of protein kinase R by the 5′ portion of the mRNA, protein folding within the endoplasmic reticulum and the possible interaction of the mRNA with microRNAs. The biological effects of IFN-γ are widespread, as almost every cell type is altered upon interaction with this protein. Thus it has become very apparent that IFN-γ is a multipotent cytokine whose regulation and effects are complex and essential to host survival.

1 Introduction

Regulation of IFN-γ expression is under strict control in order to provide the host protection from a variety of pathogens, while limiting the damage caused by unrestrained inflammatory responses and effector T cell functions. A number of complex, interwoven molecular mechanisms have evolved to assure appropriate tissue-specific expression of IFN-γ, which include various epigenetic events, inducible transcription, as well as post-transcriptional and post-translational modifications. The topic of this review is to summarize the recent literature pertaining to control mechanisms of IFN-γ expression, as well as to describe some new insights into the biological role if IFN-γ in the development and progression of cancer. In addition, we briefly summarize some current clinical applications of this important immunoregulatory molecule.

2 Epigenetic Control

In an effort to maintain appropriate cellular and kinetic expression of IFN-γ, the *ifng* gene and its flanking genomic sequence lay in a dormant, transcriptionally inactive state in most resting cell types in which the *ifng* gene is DNA-methylated and the associated chromatin is hypoacetylated. Early reports indicated that the chromatin structure around the *ifng* gene correlated with tissue-selective expression of IFN-γ. For example, Hardy and colleagues identified the presence of certain DNase I hypersensitivity sites (HS) in the *ifng* gene only in cell types competent to express IFN-γ (Hardy et al. 1985). Additional analysis indicated that other HS sites in the *ifng* gene were inducible in T cells, which suggested a link between genomic structure and gene induction may be part of a molecular program to impart tissue-specific regulation of IFN-γ expression (Hardy et al. 1987).

2.1 DNase I HS and Genomic Requirements (Transgenics)

Concentrated work in recent years has offered a more complete picture of the genomic requirements for cell- and signal-specific regulation of IFN-γ (Fig. 1). Transgenic mouse models of IFN-γ regulation suggest that regions distal to the *ifng* gene are required to confer normal IFN-γ expression profiles (Soutto et al. 2002; Young et al. 1989). An 8.6-kb fragment of human genomic DNA containing the full-length *ifng* gene and 2.7 kb of upstream sequence fails to provide tissue-specific IFN-γ expression. Meanwhile, a bacterial artificial chromosome (BAC) transgene containing a 191-kb fragment of genomic DNA surrounding the *ifng* gene recapitulates cell-specific and optimal signal-specific IFN-γ expression patterns, thereby indicating a requirement for distal regulatory sites (Soutto et al. 2002).

In this context, several putative regulatory regions have been identified in and around the *ifng* gene based on DNase I HS analysis. Three HS sites are located in introns (HS I, II, and III), with two of these regions preferentially appearing in T helper 1 (Th1) cells (Agarwal and Rao 1998; Hardy et al. 1987). These sites fall within the previously mentioned 8.6-kb genomic fragment that

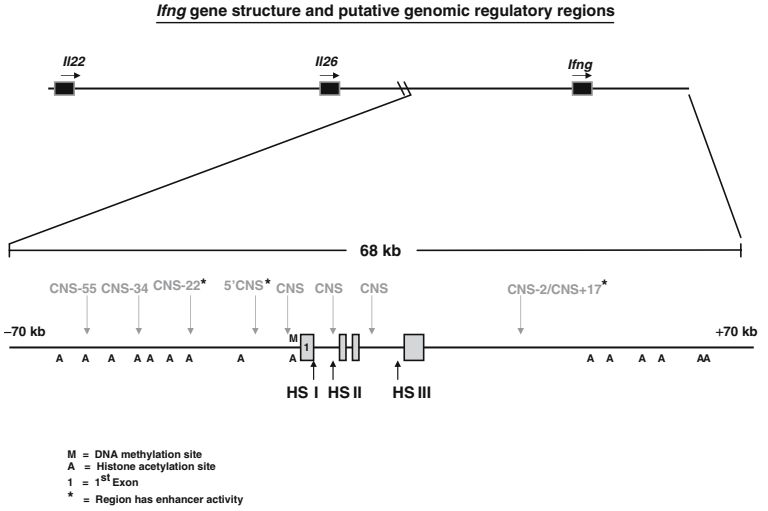

Fig. 1 Epigenetic structure of the human *ifng* locus on chromosome 12. Exons are represented by *gray boxes* with exon 1 indicated. *HS*, DNaseI hypersensitivity site; *CNS*, conserved noncoding sequence. Known sites of DNA methylation and histone acetylation are indicated. See text for associated references

is not completely sufficient to support appropriate IFN-γ expression. Recent studies have characterized additional regulatory sites that are located outside the 8.6-kb fragment, which have begun to provide some insight into the cooperative effects of distal and proximal regulatory elements in controlling IFN-γ gene expression profiles.

Early efforts to identify regulatory regions in the *ifng* gene were hampered by insufficient sequence information beyond the proximal portions of the *ifng* gene. However, with the completion of the human, mouse, rat, and other species' genomes, comparative sequence analysis is now possible on a larger scale and has already been proven as a powerful analytical tool to identify genomic regions of homology that are likely to harbor regulatory sequences (Gu and Su 2005; Nardone et al. 2004). For instance, utilizing DNA sequence comparisons between species as a tool to identify conserved sequence motifs in the *ifng* gene (generally greater than 100 bp in length with approximately 70% sequence identity; Dermitzakis et al. 2005), Lee and colleagues have found another HS site that is approximately 5 kb upstream of the *ifng* gene (5′CNS) (Dermitzakis et al. 2005; Lee et al. 2004). Interestingly, these analyses also revealed that previously identified HS sites in the *ifng* intronic regions are situated within or in close proximity to conserved noncoding sequences (CNS). Taking a similar approach, Shnyreva et al. have confirmed the 5′CNS and identified a conserved site 18 kb downstream of the mouse *ifng* gene (CNS2) that has Th1-specific HS activity (Shnyreva et al. 2004). Our group has identified another HS 5′ to the *ifng* gene, located–approximately 3.5 kb upstream, that is responsive to cytokine stimulation in human NK cells (Bream et al. 2004). Although this region does not map to a CNS site, we did identify a conserved Stat5 binding motif within the HS region that is near the same genomic position upstream of both the mouse and human *ifng* genes. In a more recent publication, Hatton et al. reported on additional CNS sites at –22 kb, –34 kb and –55 kb. They found that the CNS at –22 kb also contained a number of transcription factor binding sites, was found to bind T-bet, and enhanced promoter activity. When this CNS site was deleted using a transgenic approach, it eliminated $CD4^+/CD8^+$ T cell and NK cell IFN-γ expression in response to T cell receptor signaling or interleukin signaling, respectively (IL-12 + IL-18) (Hatton et al. 2006).

2.2 Acetylation

Post-translational modifications to histone proteins associated with genomic DNA is another indicator of chromatin structure and DNA accessibility (Fischle et al. 2003). The acetylation state of residues contained within histone tails is a well-accepted marker of epigenetic status at any given loci and is

functionally linked to the capacity of a gene to be transcribed (Avni et al. 2002; Gribnau et al. 2000; Li et al. 2006). The technique of chromatin immunoprecipitation (ChIP) permits the analysis of protein/DNA interactions and has become a useful tool in characterizing the chromatin structure of the *ifng* gene based on acetylation levels at sites across the locus.

The first studies to assess histone acetylation levels in the *ifng* gene focused on the proximal promoter region in T cells going through the process of activation and Th1/Th2 differentiation (Avni et al. 2002; Fields et al. 2002). In naïve T cells, there is very low to nondetectible histone H3 and histone H4 acetylation at both the IFN-γ and IL-4 promoters. However, during the process of T helper differentiation, there is a progressive, selective increase in IFN-γ promoter acetylation in Th1 as compared to Th2 cells (Morinobu et al. 2002). The enhanced acetylation of the IFN-γ promoter is abrogated in Stat4-deficient T cells, indicating a role of IL-12 in initiating and/or maintaining a permissive chromatin structure in differentiating Th1 cells (Fields et al. 2002). Interestingly, Stat4 has also been found to directly bind to the IFN-γ promoter (Nguyen et al. 2002) and recently, the repositioning of nucleosomes in the proximal IFN-γ promoter was shown to be Stat4-dependent in Th1 cells (Zhang and Boothby 2006).

Several reports have extended these findings to distal regions in the *ifng* locus, supporting a role for region-specific histone acetylation in IFN-γ-competent cells. In Th cells, there is a progressive loss of histone methylation in the 5′CNS region located approximately 5 kb upstream of the *ifng* gene, which curiously does not segregate between Th1 and Th2 cells (Lee et al. 2004). This same region was found have selective histone acetylation in $CD4^+$ Th1 cells and $CD8^+$ T cells (Shnyreva et al. 2004). Furthermore, the Th1 and $CD8^+$ selective hyperacetylation profiles were more pronounced in regions from the proximal promoter, third intron, and at another CNS site (CNS2) located 18 kb downstream. We have also characterized a region approximately 3.6 kb upstream of the *ifng* gene that is a site of preferential histone acetylation in Th1 cells and NK cells (Bream et al. 2004). Chang and Aune have broadened the search for sites of localized histone acetylation distal to the *ifng* gene by targeting up to 70 kb of genomic DNA flanking the gene and have identified a profile of Th1-specific acetylation over the *ifng* locus that extends well beyond the *ifng* gene (Chang and Aune 2005).

Thus, it is clear that tissue-specific IFN-γ transcription is regulated, at least in part, by a series of carefully timed, receptor-dependent epigenetic events that occur both proximal and distal to the *ifng* gene. In fact, during Th1 differentiation, the chromatin structure around the *ifng* gene is permissive to the point that proximal and distal regions of the *ifng* gene come into close, physical proximity with one another (Eivazova and Aune 2004). This would permit, for example, the association of distal enhancer/repressor elements with

the transcriptional machinery to fine-tune gene transcription (Smale 2001). Building on this concept, Spilianakis and colleagues have remarkably demonstrated that despite being positioned on different chromosomes, the *ifng* locus and the co-regulated *il4* locus are in close contact in naïve T cells (Spilianakis et al. 2005). This suggests that control of the chromatin structure within the *ifng* (and *il4*) locus may not be autonomously regulated but rather coordinated with other genomic loci and mediated by intra- as well as interchromosomal interactions.

3
Transcription Factors

Alterations of the chromatin structure at various sites across the *ifng* locus have also been linked with the recruitment of DNA-binding proteins, often to regions of chromatin remodeling, thus serving as genomic substrates in establishing competency for acute IFN-γ transcription. For many years, it was thought that a single transcription factor (TF) or TF family would ultimately account for IFN-γ transcription profiles and function as a "master regulator" of IFN-γ expression. In fact, reports have accumulated over time, implicating a variety of TF in controlling IFN-γ transcription, focusing primarily in the proximal IFN-γ promoter and intronic regions. Notably, the NFAT, AP-1, and NF-κB families are known to have prominent roles in regulating IFN-γ expression (Aune et al. 1997; Kiani et al. 2001; Penix et al. 1993, 1996; Rao and Avni 2000; Sica et al. 1997; Sweetser et al. 1998; Tato et al. 2003; Thierfelder et al. 1996; Zhou et al. 2004). This paper, however, will not focus on these TFs, as their role(s) in IFN-γ regulation will be the subject of a forthcoming review (Young et al., unpublished data). Nonetheless, a few key TF will be discussed, which have emerged as important downstream effector molecules in controlling cell-specific and to a degree, signal-specific IFN-γ expression.

3.1
Stat4

As indicated above, this review is intended to put more recent findings into a genomic context to enhance our understanding of IFN-γ gene regulation through the interface between TF recruitment and chromatin structure. In this regard, the IL-12/Stat4 pathway has emerged as a critical regulator of IFN-γ based in part on the fact that Stat4-deficient mice have severe deficits in IFN-γ expression and Th1 development (Kaplan et al. 1996; Thierfelder et al. 1996). Stat4 is known to control IFN-γ in several ways, which include binding

directly to the IFN-γ promoter (Nguyen et al. 2002). Stat4 is also known to recruit other transcription factors such as AP-1 to the promoter, thereby influencing IFN-γ expression (Barbulescu et al. 1998; Nakahira et al. 2002). The recruitment of AP-1 and cooperative binding with Stat4 to the IFN-γ promoter has been reported only in relation to IL-12/IL-18-induced synergy on IFN-γ expression. AP-1, in addition to IL-18, is also a downstream target of T cell receptor (TCR) signaling, and mimicking TCR triggering has been shown to induce binding of AP-1 to the same proximal promoter sites (Penix et al. 1993, 1996). It would be interesting to determine if this Stat4-dependent mechanism of IFN-γ regulation is conserved between the IL-18 receptor and TCR.

As mentioned, Stat4-dependent mechanisms are also in play with respect to chromatin remodeling events around the *ifng* gene (Chang and Aune 2005; Fields et al. 2002). Recently, Zhang and Boothby reported that the Switch (Swi)-sucrose nonfermenter (SNF) component Brahma-related gene 1 (*Brg1*) is recruited to the *ifng* gene in developing Th1 cells in a Stat4-dependent manner (Zhang and Boothby 2006). This provides a direct link between Stat4 and epigenetic alterations at the IFN-γ promoter by the recruiting of a member of the Swi-SNF complex that is recognized as an important group of chromatin-modifying proteins (Narlikar et al. 2002).

3.2 T-bet

The T-box containing the protein T-bet is another critical transcription factor implicated in the regulation of IFN-γ expression (Mullen et al. 2002; Szabo et al. 2000, 2002). Like Stat4, T-bet has been shown potentially to have multiple mechanisms of action in governing tissue-specific IFN-γ expression profiles. Like Stat4, T-bet deficiency results in defects in Th1 development and IFN-γ expression, resulting in host susceptibility to numerous pathogens (Matsui et al. 2005; Ravindran et al. 2005; Sullivan et al. 2005). In addition, forced overexpression of T-bet in Th2 cell populations simultaneously repressed IL-4/IL-5 expression and induced IFN-γ production, suggesting an indispensable role for T-bet in $CD4^+$ T cell differentiation as well as imparting Th1-like IFN-γ expression patterns (Szabo et al. 2000, 2002). The mechanism(s) by which T-bet exerts its biological effects, however, has been the topic of debate.

Functional T-bet binding sites have been identified in multiple regions of the IFN-γ promoter, which also correspond to known sites of chromatin remodeling in Th1 cells. In two separate papers, T-bet half-sites were shown to be flanked by NFAT and/or ATF/AP-1/CEBP binding elements that appear to function cooperatively in binding to and trans-activating the IFN-γ promoter (Lee et al. 2004; Tong et al. 2005). These data suggest that T-bet's mechanism

of action is by binding to sequences in the IFN-γ promoter and interacting with other DNA-binding proteins to block the recruitment of transcriptional repressors (such as mSin3a) and/or to bind directly to induce IFN-γ transcription. The recent finding by Hatton and co-workers (Hatton et al. 2006) that T-bet binding to a CNS site 22 kb upstream of the *ifng* gene, which results in enhanced IFN-γ promoter activity, and that the T-bet binding site is near a number of other conserved transcription factor binding sites further supports the model of cooperation between T-bet and other transcription factors in regulating IFN-γ expression.

Analogous observations that forced expression of the Th2-specific transcription factor GATA3 blocked the development of IFN-γ-expressing Th1 cells created some confusion since seemingly both GATA3 and T-bet possessed the capacity to simultaneously and preferentially activate one T cell differentiation program while repressing the other (Ferber et al. 1999; Nawijn et al. 2001; Ouyang et al. 1998). The mechanisms by which T-bet and GATA3 could regulate both Th1 and Th2 developmental pathways remained unclear until very recently. While there are potential binding sites in the IFN-γ promoter, it has been reported that GATA-3 does not in fact directly interact with the *ifng* genomic DNA but may function by antagonizing Stat4 levels (Kaminuma et al. 2004; Usui et al. 2003). Furthermore it has been reported that T-bet does not directly control IFN-γ transcription but rather acts by downregulating GATA3 function (Hwang et al. 2005) and/or levels (Usui et al. 2003, 2006). These latter reports seem to be in conflict with studies describing T-bet directly upregulating the IFN-γ promoter in transient transfection studies. More studies are needed to clarify the multiple reported mechanisms of T-bet action in controlling IFN-γ expression. The recent observation of T-bet and GATA-3 binding sites belong in close proximity in the −22 kb CNS, that is in an open chromatin conformation (Hatton et al. 2006), suggests that there may be a dynamic balance between T-bet and GATA-3 interactions within this regulatory locus. Regardless, it is clear that T-bet is required for optimal IFN-γ expression in $CD4^+$ Th1 cells and NK cells (Bream et al. 2003; Lee et al. 2006; Townsend et al. 2004).

3.3 Eomesodermin

It should be noted that the $CD4^+$ Th1/Th2 system is the predominant model in which IFN-γ regulation has been studied, even though $CD8^+$ T cells, NK, and NKT cells are important cellular sources of IFN-γ. Nevertheless, recent findings have suggested cell-subset-specific regulation of IFN-γ in $CD8^+$ T cells. Given that T-bet is a critical component of IFN-γ regulation and Th1 differentiation, it was curious that $CD8^+$ cells in the T-$bet^{-/-}$ mice had seemingly normal

IFN-γ expression. Eomesodermin (Eomes), a paralog of T-bet, was identified as a determinant of IFN-γ expression in $CD8^+$ T cells (Nawijn et al. 2001). Eomes is also implicated in other $CD8^+$ effector T cell functions including the development of the cytotoxic machinery although the mechanism(s) through which Eomes regulates IFN-γ expression have yet to be determined. Nevertheless, this work has been extended to indicate that T-bet and Eomes may have a cooperative role in determining some NK cell and $CD8^+$ T cell-specific functions by regulating CD122 expression, which is part of the IL-2/IL-15 receptor complex (Intelkofer et al. 2005). The hypothesis of cooperation between T-bet and Eomes received support from the work of Hatton et al. (Hatton et al. 2006), who demonstrate by using a transgenic bacterial artificial chromosome (BAC) model to delete a CNS site that the deletion of the −22 kb CNS resulted in a loss of IFN-γ expression in both $CD4^+$ and $CD8^+$ T cells in response to both T cell receptor and cytokine (IL-12 + IL-18) signaling. These developments provide another potential link between cytokine regulation of cell lineage decisions and gene expression profiles. In addition, the technology for manipulating BACs by bacterial recombineering, creating BAC transgenic mice, and using Cre-lox models has become more standardized and available, thus making it feasible to evaluate the discrete contributions of distal elements for appropriate gene expression profiles using in vivo models. This is preferable to transfection systems that cannot replicate the complex intrachromasomal interactions that are likely critical to distal enhancer function. Thus, the use of these in vivo tools is proving to be a robust approach for evaluating the genomic requirements for gene expression.

4 Inhibition of IFN-γ Expression

4.1 SMAD Proteins

The role of transcription factors involved in inhibition of IFN-γ expression has not been widely studied. Earlier work demonstrated a role for YY1 in inhibiting IFN-γ gene expression (Ye et al. 1996), and a recent study reported that the transcription factor DREAM can directly bind to the promoter and inhibit IFN-γ expression (Savignac et al. 2005). In a more detailed study, Yu and co-workers defined the mechanisms by which transforming growth factor-β (TGF-β) inhibits IFN-γ expression (Yu et al. 2006). These investigators showed that TGF-β downregulates T-bet mRNA through the direct interaction of SMAD proteins with the TGF-β promoter. SMAD proteins also interact with a region from −204 to −138 of the IFN-γ promoter to directly inhibit activation of the IFN-γ promoter by

T-bet, although it is not completely clear if this effect is direct or indirect. Supporting this model, these investigators also found that NK cells from SMAD3$^{-/-}$ mice produced more IFN-γ than similar cells obtained from WT mice. Thus, inhibition of IFN-γ expression may also result from protein/DNA interactions.

4.2 DNA Methylation

The direct methylation of CpG islands of genomic DNA is another well-defined epigenetic mechanism to regulate gene expression. In general, hypermethylation is associated with gene repression, while hypomethylation is correlated with gene expression (Refaeli et al. 2002). Early studies on the methylation status of a particular CpG site at position −53 in the proximal IFN-γ promoter, revealed it to be predictive of Th1 and Th2 cells' ability to transcribe the *ifng* gene (Young et al. 1994). This has been corroborated by other studies indicating the IFN-γ promoter CpG site exists in a hypermethylated state in Th2 cells but is hypomethylated in IFN-γ-expressing cell populations such as Th1 cells, memory CD8 cells, and NK cells (Fitzpatrick et al. 1998, 1999; Jones and Chen 2006; Tato et al. 2004; Winders et al. 2004; Yano et al. 2003). In addition, methylation of CpA residues in the IFN-γ promoter also correlate with IFN-γ expression patterns (White et al. 2002). Interestingly, the coding region of the *ifng* gene is also a target of DNA methylation, as hypermethylation patterns have been observed in naïve CD8$^+$ T cells, naïve CD4$^+$ T cells, and thymocyte precursors (Jones and Chen 2006). Upon T cell activation, however, the *ifng* gene undergoes a rapid loss of DNA methylation, coinciding with the ability of *ifng* to be transcribed (Kersh et al. 2006; Northrop et al. 2006). The process of transferring methyl groups is controlled by a family of DNA methyltransferases and deletion of one such member, DNA methyltransferase 1 (Dnmt1), results in augmented levels of IFN-γ in Th1 cells (Lee et al. 2001). Overall, it is clear that active methylation/demethylation of the IFN-γ promoter and gene is a critical step in the competency of IFN-γ to be expressed within the lymphoid compartment.

5 Post-transcriptional Control

5.1 mRNA Stability

Post-transcriptional control is another mechanism of IFN-γ regulation that has been reported by a number of labs, although the precise mechanism by which this occurs remains to be defined. While mRNA stability in response to

cytokine and other activation signals is p38 MAPK- and/or Stat4-dependent (Rao and Avni 2000; Ravindran et al. 2005; Rafaeli et al. 2002; Robertson 2005), a recent report provides evidence that mRNA stability observed upon IL-12 + IL-18 treatment of cells involves the 3′ untranslated region of the *ifng* gene (Mavropoulos et al. 2005). In studies that offer a mechanistic insight into the mRNA stabilization process, it was demonstrated that the increase in IFN-γ mRNA observed upon treatment of Jurkat cells with anti-LFA1 and anti-CD3 was abrogated by treating the cells with siRNA to HuR, a protein that binds to the 3′ untranslated portion of a number of cytokine mRNAs (Wang et al. 2006). Whether or not there is direct interaction of HuR with the IFN-γ mRNA remains to be determined.

In preliminary studies, we (HAY) have found that deletion of the 100-bp region in the 3′ UTR, which contains the AUUA repeats, results in significantly higher levels of expression of IFN-γ in vivo when heterozygous mice are treated with interleukins 12 or 18 (H.A. Young, unpublished observations). While this result is consistent for a role for these elements in mRNA stabilization, proof of this model awaits further validation.

Thus, while IFN-γ mRNA stabilization is a consequence of treatment of cells with numerous activation signals, exactly how the p38MAPK pathway is responsible for this effect and the role of known/unknown RNA-binding proteins in mediating mRNA stabilization remains a target of future study.

5.2 microRNAs

As yet, there have been no reports demonstrating a direct role for micro RNAs in controlling IFN-γ expression. However, a recent study utilizing mice where Dicer, a gene essential for microRNA processing, was selectively eliminated from the T cell compartment, demonstrated that these mice contained an increased number of IFN-γ^+ $CD4^+$ T cells (Muljo et al. 2005). This effect was apparent even if cells were differentiated into the Th2 cells. Furthermore, upon treatment with PMA/ionomycin, the T cells produced roughly twice as much IFN-γ as compared to cells from normal mice.

In our laboratory, bioinformatic analysis has suggested that the *ifng* gene may be a strong target for microRNA interaction. These preliminary observations, along with the work reported by Muljo and colleagues (Muljo et al. 2005), certainly offer the possibility that microRNAs may be involved in regulating IFN-γ gene expression. Proof of this hypothesis awaits further experimentation.

5.3
Post-translational Control

Post-transcriptional control of IFN-γ transcripts has only recently been proposed as a mechanism by which IFN-γ expression can be regulated. Fourteen nucleotides in the 5′ untranslated portion of the human IFN-γ mRNA have been found to form a pseudoknot. This pseudoknot has been shown to activate PKR, an interferon inducible kinase that is normally activated by double-stranded RNA (Ben Asouli et al. 2002; Kaempfer 2006). Upon activation, PKR phosphorylates EIL2α, a consequence of which is the inhibition of IFN-γ mRNA translation. It was also demonstrated that mutations that destabilize this pseudoknot increase IFN-γ protein expression. Interestingly, while the sequence of the 5′ region of the mRNA does not appear to be highly conserved through evolution (e.g., in mice), the pseudoknot itself does appear to be maintained, thus suggesting that evolutionary pressure has maintained post-translational mechanisms of control for IFN-γ.

A second mechanism by which IFN-γ expression may be controlled at the post-translational level has recently been reported (Vandenbroeck et al. 2006). In this report, it was demonstrated that protein folding in the endoplasmic reticulum was ATP/N-glycosylation-dependent and upon heat shock, the protein folding was maintained by calreticulin. IFN-γ protein release then took place upon cell recovery at 37°C. This report offers a model where IFN-γ protein may be sequestered by the cell during stress but then rapidly released upon restoration of normal conditions. The observation that the proprotein convertase, furin, is also required for efficient IFN-γ protein expression in Th1 T cells (Pesu et al. 2006) also indicates that regulation of protein folding may be another novel mechanism controlling IFN-γ. protein expression.

In summary, post-translational mechanisms involved in regulating IFN-γ.expression offer additional new mechanisms to further control host exposure to this important immunoregulatory molecule.

6
Biological Effects of IFN-γ

As a search of PubMed with the key word “interferon-gamma” revealed over 46,000 hits, a thorough review of the biological functions of IFN-γ is beyond the scope of this article. The effects of IFN-γ on the expression of numerous genes, both positive and negative, have been extensively reported. The signaling pathway triggered by IFN-γ is well defined, as the receptor has two chains and receptor–ligand interaction triggers activation of the Janus kinases 1 and 2 with

subsequent phosphorylation of Stat1. Upon phosphorylation of Stat1, dimers are formed, translocate to the nucleus, and activate gene transcription primarily through the interaction with GAS elements (gamma-activated sequences) or in some cases, interferon stimulated response elements (ISREs). Other transcription factors, including NF-κB and c-Jun, have also been identified as playing a role in IFN-γ signaling and Stat1-independent pathways have been identified and characterized (for a review see Ramana et al. 2002). Furthermore, it has been reported that the IFN-γ protein is actually transported to the nucleus and this nuclear localization plays a role in the specificity of IFN-γ effects on gene expression (for reviews see Ahmed et al. 2003; Ramana et al. 2002).

IFN-γ production is characterized as the hallmark of the Th1 phenotype and IFN-γ has been shown to downregulate the generation of IL-4- and IL-10-producing Th2 T cells (reviewed in Szabo et al. 2003). Interestingly, IFN-γ has been shown to enhance Th2 polarization and the survival of IL-4 producing cells if present during the initial T cell priming (Bocek et al. 2004). Most recently IFN-γ has been shown to inhibit the development of a new subset of T cells (Harrington et al. 2005), characterized by their ability to produce IL-17 (Bettelli et al. 2006; Harrington et al. 2005). These cells play an important role in the development of a number of autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE) (Bocek et al. 2004). The inhibition of their development by IFN-γ begins to shed new light on the role of IFN-γ in the development and progression of these diseases.

There is also evidence that IFN-γ can control the generation and activation of $CD4^+/CD25^+$ regulatory T cells (Tregs). Tregs suppress a wide variety of immune responses and induce immune tolerance (see review Maloy and Powrie 2001). Furthermore, a recent report demonstrated that pretreatment of mice with IFN-γ prevented the development of Tregs reactive to immunized self antigens (Nishikawa et al. 2005). Surprisingly, Treg formation appears to be normal in *ifng*$^{-/-}$ and *ifngR*$^{-/-}$ mice, indicating that it is not required for Treg development (Kelchtermans et al. 2005; Sawitzki et al. 2005). Furthermore, Tregs can themselves produce IFN-γ and this may trigger apoptosis in naïve and/or Th2 effector T cells (Dalton et al. 2000; Rafaeli et al. 2002), thus indicating that IFN-γ may have a more generalized role in regulating host immunosuppression. These new findings, taken together with the classical roles of IFN-γ in the pro-inflammatory response, demonstrate the widespread role of IFN-γ in regulating the host immune response.

The role of IFN-γ in the host immune response to cancer has recently been reevaluated by Robert Schreiber's laboratory (Dunn et al. 2005). This laboratory has found that the tumor response to IFN-γ is critical for an effective host response, as they demonstrated that in mice deficient in the IFN-γ response (e.g., *Stat1*$^{-/-}$ or *ifngR1*$^{-/-}$ mice), there was a higher incidence of chemically

induced and spontaneous tumors. Insensitivity to IFN-γ at the level of the tumor was a major factor contributing to the increased tumor incidence, as IFN-γ is required to increase tumor recognition by inducing MHC class 1 antigen processing and presentation pathway. A more thorough description of the role of IFN-γ in the host response to tumor challenge and development can be found elsewhere (Dunn et al. 2005).

7 Clinical Applications

Despite the multitude of papers that have investigated the properties of IFN-γ, the clinical use of IFN-γ is still somewhat limited. In the USA, IFN-γ has been approved for only two specific uses: treatment of chronic granulomatous disorder (CGD), as these patients are more susceptible to fungal and bacterial infections, and severe osteoporosis. Current clinical trials are limited, with a major effort being made in the use of IFN-γ for the treatment of idiopathic pulmonary fibrosis. In this condition, IFN-γ administration resulted in a survival benefit in certain subgroups. Other trials involving IFN-γ include analysis of the effects on lung immune function in *Mycobacterium tuberculosis*-infected patients; tolerance and toxicity of IFN-γ alone or in combination with tumor necrosis factor in AIDS-related complex patients; the effects of IFN-γ in hepatitis C patients that do not respond to IFN-α; the use of adenovirus vectors expressing IFN-γ in cancer patients; and evaluation of antifibrotic activity in hepatitis C patients with severe liver fibrosis or compensated cirrhosis. In addition, the efficacy of IFN-γ-treated tumor cells as a vaccine in combination with cyclophosphamide and intralymphatic immunotherapy is being analyzed. For a more complete listing of trials involving IFN-γ, readers are referred to http://www.controlled-trials.com/mrct/search.asp or www.clinicaltrials.gov with "interferon-gamma" as the search criterion. Interestingly, there are a number of studies where antibodies that neutralize IFN-γ have been evaluated for the treatment of chronic inflammatory diseases, including a number of autoimmune diseases (for a recent review, readers are referred to Skurkovich and Skurkovich 2006). Thus, inhibiting IFN-γ activity may have as much clinical efficacy as direct administration of the cytokine itself.

In summary, while IFN-γ currently has limited use in the clinic, it may well be anticipated that more localized administration, use in combination with other treatments, or inhibition of its activity will allow clinicians to target this molecule in the appropriate clinical setting.

8 Conclusions

Given the essential role of IFN-γ in both innate and adaptive immunity, it may not be considered surprising that there are multiple mechanisms by which the expression of this gene is regulated. Regulation at the nucleic acid level can occur at chromatin accessibility, transcription factor activation and availability, mRNA stability, and possible microRNA interactions. Post-translational mechanisms involved in regulating the levels of IFN-γ protein released from cells further demonstrate the breadth of host control that has evolved. As more is understood at the basic science level regarding the effects of IFN-γ during the host response to infection, cancer, and autoimmune diseases, a clearer understanding of immune system development, maturation, and function will result. It is anticipated that such an understanding will lead to a more targeted clinical approach focused on harnessing the effects of this critical immunoregulatory molecule.

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Cell Type-Specific Signaling in Response to Interferon-γ

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Abstract Type II interferon-γ (IFN-γ) is a pleiotropic cytokine that regulates many different cellular functions. The major signaling pathway activated by IFN-γ involves sequential phosphorylation of the tyrosine residues of the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) proteins, providing the primary mechanism through which gene expression is induced. However, recent work has revealed that the responses are complex, as shown by the activation of kinases in addition to JAKs, differential patterns of activation of STAT1, STAT3, and STAT5 in different cells, and activation of transcription factors other than STATs. This complexity is used to regulate biological functions differentially in a cell type-specific manner, by activating different specific signals and patterns of gene expression.

1 Introduction and Historical Perspective

Interferons (IFNs) are pleiotropic cytokines that were originally discovered because of their antiviral activity. Three major types have been described. The type I IFNs include α, β, ω, τ, δ, κ, and ε. The predominant forms are IFN-α, of which there are 12 separate proteins in humans, and a single IFN-β (Pestka et al. 2004). The recently identified type III IFNs, alternatively named IFN-λ, consist of interleukin-(IL)-28 and IL-29 (reviewed by Ank et al. 2006). Type II IFN, represented only by IFN-γ, is produced by NK cells, $CD4^+$ and $CD8^+$ T cells, in contrast to IFN-β, which is produced by most types of cells. IFN-γ has important immunomodulatory properties in addition to helping to protect cells from viral, bacterial, and parasitic infections (Dupuis et al. 2001; Lu et al. 1998). It is one of the major cytokines responsible for upregulating MHC class I on the surfaces of many cells and for inducing MHC class II on endothelial cells and a variety of leukocytes (Fruh et al. 1999; van den Elsen et al. 1998). In addition, IFN-γ is crucial for activating mononuclear phagocytes in the battle against infectious agents and for stimulating antigen-presenting cells to produce IL-12 and tumor necrosis factor (TNF)-α (Schroder et al. 2004). Furthermore, IFN-γ regulates the humoral immune response by effecting IgG heavy chain switching (Finkelman et al. 1988). Of note, IFN-γ exerts profound antiproliferative effects on a variety of normal and tumor cells (Schroder et al. 2004). It also plays a major role in tumor immunosurveillance, part of a more general process called cancer immunoediting, which is responsible both for eliminating tumors entirely and for sculpting the immunogenic phenotypes of the tumors that eventually do form in immunocompetent hosts (reviewed by Dunn et al. 2004a, 2004b). New, exciting results suggest that IFNs signal in a cell type-specific manner. A recent review summarizes the evidence for type I IFNs (van Boxel-Dezaire et al. 2006), and the current review focuses on IFN-γ-dependent signaling.

Investigation of the signaling pathways activated by type I and II IFNs by genetic and biochemical means led to the discovery of the JAKs and STATs, tyrosine kinases and latent transcription factors that drive the major responses to the IFNs (Darnell et al. 1994; Stark et al. 1998). Further research revealed that JAK1 and JAK2 bind tightly to the two subunits of the IFN-γ receptor, IFNGR1 and IFNGR2, respectively (Sakatsume et al. 1995; Behrmann et al. 2004). Ligand binding induces the assembly and activation of the IFNGR complex (Bach et al. 1996), leading to the cross-phosphorylation and activation of JAK1 and JAK2, and phosphorylation of the cytoplasmic domain of IFNGR1 (Igarashi et al. 1994), providing docking sites for the SH2 domains of STATs. After STAT1 has been phosphorylated on tyrosine 701, it dissociates from the receptor, forming

a homodimer through reciprocal phosphotyrosine-SH2 interactions. STAT1 homodimers translocate to the nucleus and bind to gamma-activated sequence (GAS) elements in the promoters of most IFN-responsive genes (ISGs). Similarly to IFN-γ, type I IFNs can also activate the formation of STAT1 homodimers, but they primarily activate the formation of the trimeric IFN-stimulated gene factor 3 (ISGF3), a complex of activated STAT1, STAT2, and IRF9. The IFN-stimulated regulatory element (ISRE) present in promoters of certain ISGs (Friedman and Stark 1985) binds to ISGF3 in response to type I IFNs (reviewed in Stark et al. 1998). However, some ISREs can also bind to a complex of STAT1 homodimers plus IRF-9 upon IFN-γ stimulation (Bluyssen et al. 1995; Majumber et al. 1998). Interestingly, type I IFNs can activate all seven mammalian STATs in a cell type-specific manner, all of which bind to GAS elements in ISGs (van Boxel-Dezaire et al. 2006). Notably, not only can IFN-γ activate STAT1 homodimers, but STAT1-independent pathways must also exist (Ramana et al. 2002). This aspect is discussed extensively below.

2 IFN-γ Receptor Functions

The IFNGR was initially characterized in the early 1980s by the binding of radiolabeled ligands in a variety of different cell types (reviewed by Farrar et al. 1993). These experiments indicated that most primary cells and cultured cell lines express high-affinity binding sites for IFN-γ. In addition, it was found that human and murine IFN-γ bound to their respective receptors in a strictly species-specific manner and therefore induced biological responses in species-matched cells only (reviewed in Bach et al. 1997). Pestka and colleagues (Jung et al. 1987) demonstrated, using stable murine–human somatic cell hybrids containing all the murine chromosomes but only a few human chromosomes, that all the hybrids containing human chromosome 6 bound to IFN-γ with high affinity. However, only hybrids containing both human chromosomes 6 and 21 demonstrated a biological response to IFN-γ. The human and murine genes for the ligand-binding component of the IFNGR were cloned and are localized on chromosomes 6 and 10, respectively (Aguet et al. 1988; Gray et al. 1989). This chain is IFNGR1, or IFN-γ receptor α chain. The second subunit, designated IFNGR2 or IFN-γ receptor β chain, was subsequently cloned, and the human and murine genes are localized on chromosomes 21 and 16, respectively (Soh et al. 1994; Hemmi et al. 1994).

The activation of STAT3 in response to IFN-γ is more clearly seen in murine than human cells (Costa-Pereira et al. 2005). In rat astrocytes, the GTPase RAC1 associates with IFNGR1 and is activated by JAK1 after IFN-γ stimulation.

Notably, RAC1 deficiency abolishes STAT3 activation and diminishes STAT1 activation in IFN-γ-stimulated rat astrocytes, suggesting that RAC1 may serve as an auxiliary mediator of IFN-γ signaling (Park et al. 2004). If, for instance, RAC1 were to associate only with murine IFNGR1, it could explain perhaps why STAT3 is activated more abundantly by murine IFN-γ. However, to our knowledge, no data are available concerning the possibility that RAC1 might be involved in IFN-γ-dependent signal transduction in human cells. Guanine nucleotide exchange factors such as VAV activate RAC1 by exchanging GDP for GTP. Interestingly, in murine RAW 264.7 macrophages, IFN-γ triggers the prompt, dose-dependent tyrosine phosphorylation of VAV, which is essential for activating lymphocytes. In addition, VAV binds to the SRC-related kinase HCK in murine macrophages, and antisense oligonucleotides specific for murine HCK block IFN-γ-mediated VAV phosphorylation (English et al. 1997). Whether the phosphorylation of VAV is involved in any way in the activation of STATs or other signaling pathways induced by IFN-γ is not known. It is possible that differential involvement of adaptor proteins or expression of cell-surface proteins that modify receptor functions might explain species- or cell type-specific differences in IFN-γ-dependent signaling, as discussed below.

Our recent work has revealed that the IFN-γ-induced activation of both STAT1 and STAT3 depends totally on the phosphorylation of tyrosine 419 in the cytoplasmic domain of murine IFNGR1 (Qing and Stark 2004). In contrast, when the corresponding tyrosine 440 in the cytoplasmic domain of human IFNGR1 is mutated, STAT1 is no longer phosphorylated on serine 727, but low levels of STAT1 phosphorylation of tyrosine 701 and STAT3 activation are still detectable (Costa-Pereira et al. 2005). Notably, these IFNGR1 mutant human cells are still able to induce IRF-1, MHC class I, and CIITA after IFN-γ stimulation, although at reduced levels. Because Costa-Pereira et al. (2005) used human IFNGR1$^{-/-}$ diploid fibroblasts to express human IFNGR1 mutants, whereas IFNGR1$^{-/-}$ mouse embryonic fibroblasts (MEFs) were used to express murine IFNGR1 mutants (Qing and Stark 2004), the different outcomes might be explained by differences between species, cell type-specific differences, or both. However, when mutant human Y440F IFNGR1 was expressed in murine fibroblasts, the upregulation of MHC class I, induction of IFN regulatory factor 1 (IRF1) mRNA and stimulation of nitric oxide production were totally abolished (Farrar et al. 1992), suggesting that some of the differences noted above might indeed be species-specific.

Sustained exposure to IFN-γ is harmful (Starr et al. 1998; Naka et al. 1998; Marine et al. 1999) and, therefore, it is of the utmost importance to have a variety of mechanisms in place that tightly regulate the activation of STAT1 by IFN-γ (reviewed by Wormald and Hilton 2004). Many studies show that suppressor of cytokine signaling (SOCS)-1 is the most potent inhibitor of STAT1-mediated

signaling in response to IFN-γ (reviewed by Alexander and Hilton 2004). SOCS-1 inhibits the kinase activity of JAK2 by binding directly to the active site loop domain in vitro (Yasukawa et al. 1999). In addition, SOCS-1 can polyubiquinate VAV and JAK2, which then become targets of proteasome-mediated degradation (Kile et al. 2002). By employing mutants of IFNGR1, we investigated the roles of the other tyrosines in the cytoplasmic domain of mouse IFNGR1, namely Y285, Y370, and Y441. Stronger activation of STAT1 and enhanced antiviral activity was observed only with IFNGR1 variants mutated in Y441 (Qing et al. 2005). In addition, constitutive overexpression of SOCS-1 inhibited IFN-γ-dependent signaling only in cells expressing mutants that retained Y441 and Y419. Interestingly, mutation of Y441 blocked the ability of SOCS-1 to bind to IFNGR1 in response to IFN-γ. Based on these results, we proposed that the phosphorylation of Y441 creates a docking site for SOCS-1, which then binds to JAK2 to partially inhibit JAK2 phosphorylation. Furthermore, the binding of SOCS-1 to Y441 also blocks the access of STAT1 to Y419, and this effect may be the principal mechanism of inhibition of IFN-γ-dependent signaling (Qing et al. 2005). In addition to SOCS-1, other negative regulators of IFN-γ-dependent signaling are important. SHP-2, an SH2 domain-containing protein tyrosine phosphatase that is constitutively associated with IFNGR1, inhibits STAT1 activation, probably by affecting JAK1, but without affecting the phosphorylation of IFNGR1 (You et al. 1999). Protein inhibitor of activated STAT1 (PIAS-1) acts by blocking the binding of phosphorylated STAT1 dimers to DNA and thus inhibits STAT1-mediated gene activation in response to IFN-γ (Liu et al. 1998). Finally, the nuclear phosphatase TC45 dephosphorylates STAT1 in the nucleus (ten Hoeve et al. 2002).

Other important sites in the cytoplasmic domain of IFNGR1 are leucine 270 and isoleucine 271, which play a critical role in directing receptor trafficking after ligand binding (Greenlund et al. 1994), and proline 267, which plays a dominant role in constitutive JAK1 association and thus is crucial for IFN-γ-dependent signal transduction (Kaplan et al. 1996). The cytoplasmic domain of IFNGR2 is much shorter than that of IFNGR1 (66 amino acids versus mouse or human IFNGR1, 200 and 221 amino acids, respectively). Within this domain, two closely spaced sequences, at 262–267 and 270–274, block IFNGR2 function when mutated to alanine residues (Bach et al. 1996). Co-precipitation studies showed that these sequences are necessary for the specific and constitutive association of the IFNGR2 chain with JAK2. The IFNGR1 promoter contains a GC-rich region with no TATA box, like promoters for uninducible housekeeping genes (Bach et al. 1997), suggesting that IFNGR1 expression is not regulated by external stimuli, which has been largely confirmed experimentally. However, the critical region −128 to −109 of the IFNGR1 promoter appears to possess a phorbol ester-responsive element. Binding of the transcrip-

tion factor SP1 to this element causes the upregulation of IFNGR1 expression, explaining why phorbol ester causes upregulation of IFNGR1 in differentiating monocytes (Sakamoto and Tanaguchi 2001). In contrast, the transcription of IFNGR2 seems to be tightly regulated.

Differential expression of IFNGR2 is another cause of cell type-specific responses. Potential binding sites in the mouse IFNGR2 promoter were found for SP1, AP-2, NF1, EGR, and NF-kB (Ebensperger et al. 1996). Whereas IFNGR1 is highly expressed on membranes of T, B, and myeloid cells, IFNGR2 seems to be expressed highly only on myeloid cells, moderately on B cells, and poorly on T cells (Bernabei et al. 2001). In contrast to Th2 cells, Th1 cells, which are characterized by high IFN-γ production, are unresponsive to IFN-γ because they do not express IFNGR2 (Pernis et al. 1995). Unresponsiveness is due to IFN-γ-dependent downregulation of IFNGR2 and is not directly linked to T cell differentiation: human peripheral blood T cells and murine Th2 cells also downregulate IFNGR2 upon exposure to IFN-γ (Bach et al. 1995). Notably, ligand-dependent IFNGR2 downregulation did not occur in certain fibroblast cell lines, suggesting that IFN-γ regulates the expression of IFNGR2 and concomitant sensitivity only in certain cell types. Other causes of selective IFNGR2 downregulation on T cells are stimulation with insulin-like growth factor (IGF)-1 (Bernabei et al. 2003) and uptake of iron by the transferrin receptor (Regis et al. 2005). Interestingly, primary T cells downregulate IFNGR2 but still express IFNGR1 after ligation of the T cell receptor (TCR) and IL-2 stimulation and are unresponsive to IFN-γ (Sakatsume and Finbloom 1996). Expression of IFNGR2 and thus sensitivity to IFN-γ can be restored by secondary TCR ligation or by treatment with phorbol ester, showing that the expression of IFNGR2 can also be positively regulated. Therefore, as T cells progress from primary TCR activation through IL-2-dependent proliferation, followed by secondary TCR stimulation, their responsiveness to IFN-γ varies, and this may affect their ability to participate in an ongoing immune response (Sakatsume and Finbloom 1996). Other factors that upregulate IFNGR2 on T cells are serum deprivation (Bernabei et al. 2001), exposure to nitric oxide (Allione et al. 1999), or low extracellular pH (Bosticardo et al. 2001). It is not clear why T cells are particularly sensitive to regulation of IFNGR2 expression in contrast to other cell types.

3
Activation of Kinases Other than JAKs

JAK-STAT signaling alone is not sufficient to explain all the biological effects of IFN-γ and several other kinase pathways have emerged as critical additional components of IFN-γ-induced signal transduction. First of all, the phos-

phorylation of STAT1 on Y701 is not enough to induce the full expression of IFN-γ-induced genes: additional phosphorylation of serine 727 is necessary (Wen et al. 1995; Kovarik et al. 2001; Varinou et al. 2003). Serine phosphorylation of STAT1 facilitates the association of chromatin-bound STAT1 with the co-activator CBP and the subsequent recruitment of histone acetylases, important for chromatin remodeling (Varinou et al. 2003). Inhibition of p38 mitogen-activated protein kinase (P38MAPK) led to defective serine phosphorylation of STAT1 in fetal brain astrocytes after stimulation with IFN-γ (Lee et al. 2003). In addition, the function of serine-phosphorylated STAT1 was dependent on P38MAPK activation after stimulation of human epithelial cells or mouse fibroblasts with type I or type II IFN (Goh et al. 1999). MKK6 turned out to be the upstream activator of P38MAPK in these cells (Goh et al. 1999). Of note, dsRNA-activated protein kinase (PKR)-deficient MEFs show defective phosphorylation of S727 upon IFN-γ stimulation, suggesting that PKR might function upstream of P38MAPK in these cells (Ramana et al. 2000b). In several different cell types activation of phosphatidylinositol 3-kinase (PI3K) and subsequently AKT by IFN-γ are needed to phosphorylate S727 (Nguyen et al. 2001). A member of the protein kinase C (PKC) family, PKC-δ, is rapidly activated in human promyelocytic cells downstream of PI3K and associates with STAT1, which then is phosphorylated on S727 (Deb et al. 2003). Notably, the activation of PKC-δ and serine phosphorylation of STAT1 are crucial for induction of pro-apoptotic genes and mitochondria-dependent apoptosis (DeVries et al. 2004). Other PKC family members might also be involved in cell type-specific responses to IFN-γ. An IFN-γ-induced PI3K/PKC-ε/MAPK signaling pathway is involved in S727 phosphorylation in mesangial cells (Choudhury 2004). In contrast, in human embryonic kidney cells, PCK-ε seems to be involved in the tyrosine phosphorylation of STAT1 (Ivaska et al. 2003), but this might occur through the activation of SRC-family kinases instead of MAPKs, as discussed below. In T cells, IFN-γ activates a PI3K/mTOR/PKC-θ/MKK4 signaling pathway, which does not affect the tyrosine phosphorylation of STAT1. However, since the transcription of GAS-containing genes is enhanced by this pathway, it is likely that this enhancement is also a result of increased phosphorylation of S727 (Srivastava et al. 2004). Although which MAPK is activated downstream of PI3K and PKC activation was not investigated in any of the above-mentioned studies, it is possible that a serine-threonine kinase such as P38 (Goh et al. 1999) or perhaps c-Jun kinase (JNK; Zhao et al. 2005) is directly responsible for serine phosphorylation of STAT1. The IFN-γ-stimulated signaling pathway that is emerging from all of these data is: PI3K→AKT→PKC(-δ, -ε, or -θ)→MKK(4 or 6)→P38MAPK→ serine phosphorylated STAT1. The cell type-specific variation of this proposed pathway seems to be the activation of different PKC and MKK family members.

The adaptor protein that couples the activated IFNGR to PI3K activation is presently unknown, but the CRK/CBL adaptor protein complex has been proposed to play this role (Platanias 2005).

Different cells are likely to employ alternative strategies to phosphorylate STAT1 on S727. IFN-γ has been shown to elicit a calcium ion flux in thyroid cells, microglia, neutrophils, T cells, monocytes, and fibroblast-like cells, suggesting that an increase of free calcium ions is involved in IFN-γ-dependent signaling in several cell types (Aas et al. 1999; Koide et al. 1988; Kung et al. 1995; Buntinx et al. 2002; Franciosi et al. 2002; Nair et al. 2002). In response to IFN-γ, human fibrosarcoma cells and MEFs activate calcium/ calmodulin-dependent kinase (CAMK) II, which can interact directly with STAT1 and induce the phosphorylation of S727 in vitro (Nair et al. 2002). In keratinocytes, an increase in free calcium ions leads to activation of the annexin II/PYK2/ MEKK4/ MKK6/P38 MAPK/ATF2 signaling pathway upon IFN-γ stimulation (Halfter et al. 2005). It is likely that P38 MAPK activation through this pathway also increases the serine phosphorylation of STAT1 and the consequent enhancement of ISG-transcription, although the authors did not investigate this point. Because the serine-threonine kinase CAMKII and the calcium-regulated tyrosine kinase PYK2 are both sensitive to an increase in calcium ions, it is possible that these two pathways are intertwined, particularly since it has also been described that transcription factors such as CREB, ATF, and C-EBP-β are substrates of CAMKII (White et al. 1998; Cruzalegui et al. 2000). Indeed, IFN-γ also activates CREB and C-EBP-β in addition to ATF2 (see Sects. 6.1 and 6.2). However, more research is needed to comprehend the individual, and possibly overlapping signaling pathways that lead to the activation of these transcription factors and subsequent cell type-specific transcription. It is possible that the activation of PYK2 in certain cell types leads to downstream enhancement of the serine phosphorylation of STAT1 in addition to the activation of additional transcription factors. Interestingly, PYK2 activation by IFN-γ leads to the activation of another MAPK, ERK2, eventually leading to the serine phosphorylation of STAT1 and maximal transcriptional activation in MEFs (Takaoka et al. 1999). The adaptor protein GRB2 complexed with SOS might couple the activation of PYK2 to ERK activation in response to IFN-γ (Blaukat et al. 1999). However, the coupling of PYK2 with another adaptor protein such as CRK leads to activation of JNK (Blaukat et al. 1999), suggesting that IFN-γ-activated PYK2 might be involved in the activation of multiple downstream signaling pathways by coupling to different adaptor proteins.

Although, as described above, IFN-γ-induced PKC activation leads to MAPK activation, PKC also seems to be involved in activating SRC-family tyrosine kinases. In human alveolar epithelial cells, IFN-γ activates PLC-γ2 via an upstream tyrosine kinase to induce the activation of PKC-α and c-SRC or LYN,

resulting in the activation of STAT1 and expression of ICAM-1, and thus the initiation of monocyte adhesion (Chang et al. 2002). SRC family kinases are required for IFN-γ to activate STAT3 (but not STAT1) by tyrosine phosphorylation, whereas JAK1 and JAK2 are required to activate both STAT1 and STAT3 in MEFs (Qing and Stark 2004). FYN could be involved in STAT3 activation, because this SRC-family member associates through its SH2 domain with activated JAK2 upon IFN-γ stimulation (Uddin et al. 1997). Interestingly, the tyrosine kinase PYK2 amplifies c-SRC-dependent STAT3 activation in response to epidermal growth factor (Shi et al. 2004), and it is possible that it does the same in response to IFN-γ, because PYK2 becomes phosphorylated upon stimulation of MEFs by IFN-γ (Takaoka et al. 1999).

In addition to affecting the activation of STATs and other transcription factors, the activation of kinases other than JAKs seems to be involved in activating other signaling pathways. For instance, the activation of mTOR downstream of PI3K leads to selective regulation of the translation of IFN-γ-induced mRNAs, but not transcription, by activating p70S6K and phosphorylating the S6 ribosomal proteins, and by phosphorylating the repressor of mRNA translation EIF4E-binding protein 1 (4EBP1), which deactivates 4EBP1, leading to its dissociation from EIF4E and the subsequent initiation of translation (Platanias 2005). In addition, treatment with IFN-γ leads to the tyrosine phosphorylation of CBL, followed by the sequential activation of C3G and RAP1, resulting in subsequent growth inhibitory effects in promyelocytic cells (Alsayed 2000). Furthermore, studies performed with MEFs that lack both the α and β subunits of IKK revealed that a subset of IFN-γ-induced genes is dependent on IKK activation (Sizemore et al. 2004). The IKK complex is best known as a regulator of NF-κB-dependent signaling and its effect on IFN-γ-dependent signaling is currently being studied in our laboratory. Finally, IFN-γ induces a MEKK1/MEK1/ERK/ C/EBP-β signaling pathway to induce the transcription of GATE-dependent ISGs (see also Sect. 6.2 and Roy et al. 2002). In summary, it is well accepted that, in addition to the JAKs, several different kinases are activated in response to IFN-γ, and one can safely predict that the cell type-specific expression of these kinases and their substrates will help to determine cell type-specific responses.

4
Differential Activation of STATs in Different Cell Types

Type I IFNs are unique for their ability to activate all seven known mammalian STATs. Cell-type specific activation of various STATs by type I IFNs has been reviewed recently (van Boxel-Dezaire et al. 2006). IFN-γ mediates its important

antiproliferative effects through the activation of STAT1 and the downstream induction of IRF-1, FAS/CD95, and proteolytic cleavage of caspases 2, 3, and 7 (Bromberg et al. 1996; Porta et al. 2005; Sironi et al. 2004). Although it was first thought that STAT1 was the sole mediator of responses to IFN-γ, more recent work has shown that biologically significant STAT1-independent pathways must be active in addition (Ramana et al. 2000a, 2002). For instance, STAT1-deficient mice are more resistant to murine cytomegalovirus (MCMV) and Sindbis virus than are IFNGR, IFNAR double knockout mice (Gil et al. 2001). In addition, IFN-γ suppresses the cell growth induced by growth factors and cytokines, but enhances the proliferation of STAT1-deficient bone marrow macrophages and MEFs (Gil et al. 2001; Ramana et al. 2000b). Stimulation of cell growth could be explained partly by the fact that only in STAT1-deficient cells are the immediate-early genes *c-myc* and *c-jun* induced (Ramana et al. 2000b). Of note, our more recent data indicate that the activation of STAT3 is increased and prolonged in STAT1-deficient MEFs, and this effect is probably responsible for activating most or all of the IFN-γ-induced ISGs in these cells (Qing and Stark 2004; Ramana et al. 2005). Y419 of murine IFNGR1 is needed to activate both STAT1 and STAT3 in wild-type MEFs upon IFN-γ stimulation, revealing that STAT1 and STAT3 compete for the same binding site (Qing and Stark 2004). A previous study showed that the SH domain of STAT1 has a much higher affinity for tyrosine 419 of IFNGR1 than does the SH domain of STAT3 (Greenlund et al. 1995), explaining why STAT1 is preferentially activated in response to IFN-γ. Of note, when STAT1 levels are low or when STAT1 is absent, STAT3 becomes more highly activated in MEFs (Qing and Stark 2004). In contrast, bone marrow macrophages from STAT1-deficient mice activate even less STAT3 than do the cells from wild-type mice (Gil et al. 2001), suggesting that different cell types have different intrinsic capacities to activate STAT3 in response to IFN-γ.

Interestingly, in addition to STAT1, STAT3 becomes abundantly activated in IFN-γ-activated rat astrocytes. In these cells, STAT3 activation is totally dependent on RAC1 expression (Park et al. 2004), which suggests that species and or cell type-specific activation of STAT3 by IFN-γ might depend on differential adaptor protein expression. Murine adipocytes activate both STAT1 and STAT3 in response to IFN-γ (Stephens et al. 1998). Notably, eosinophils and (mature) monocytes isolated from peripheral human blood activate only STAT1, whereas human neutrophils activate both STAT1 and STAT3 in response to IFN-γ (Caldenhoven et al. 1999). Furthermore, IFN-γ delays the apoptosis of human neutrophils by activating STAT3 and subsequently inducing cellular inhibitor of apoptosis 2 (cIAP2) mRNA and protein expression

(Sakamoto et al. 2005). Notably, the activation of STAT3 and ERK1/2 by IFN-γ is crucial for antagonizing signaling by TGF-β in human renal tubular epithelial cells (Giannopoulou et al. 2006). Interestingly, retinas from mouse embryos and neonates activate STAT3 but not STAT1 after stimulation by IFN-γ (Zhang et al. 2005). Immature mouse macrophages show reduced binding of STAT1 to GAS elements, despite normal activation of STAT1 by IFN-γ, and high constitutive expression of PIAS-1 in immature macrophages could explain these findings (Coccia et al. 2002). In contrast, human neonatal monocytes and monocyte-derived macrophages do not activate STAT1 in response to IFN-γ, despite normal expression levels of IFNGR and STAT1 (Marodi et al. 2001). The authors suggest deficient IFNGR signaling as an explanation, but activation of other STATs was not investigated. Notably, another study showed that neonatal macrophages respond selectively to IFN-γ, in that CIITA mRNA was not induced but other mRNAs were, such as IRF-1 (Lee et al. 2001). Since the induction of CIITA mRNA by IFN-γ depends on activated STAT1, USF-1, and IRF-1 (Muhlethaler-Mottet et al. 1998; O'Keefe et al. 2001), whereas IRF-1 mRNA can be induced by activated STAT1, STAT3, or STAT5 (Heim et al. 1999), these data collectively suggest that neonatal monocytes activate STAT3 or STAT5 instead of STAT1 in response to IFN-γ.

Interestingly, IFN-γ activates STAT5b in human promyelocytic cells, but not in human epithelial cells, whereas IFN-α activates STAT5a in both cell types (Meinke et al. 1996). IFN-γ, which promotes differentiation but not proliferation of human promyelocytic cells, activates STAT5b only in immature monocytes; monocytic differentiation leads to a strong decrease in IFN-γ-mediated activation of STAT5b but not of STAT1 (Meinke et al. 1996). Of note, STAT5 activation by IFN-γ requires Y419 or Y440 of mouse or human IFNGR1, respectively, and the binding of SOCS-3 to this tyrosine residue inhibits STAT5 activation by IFNGR (Woldman 2001). The biological significance of STAT5 activation by IFN-γ is also demonstrated by the fact that activation of STAT5b is crucial to induce IGF-I production in primary human dermal fibroblasts (Hwa et al. 2004). Interestingly, IFN-γ preferentially activates STAT5b in these cells, but in the absence of STAT5b IFN-γ will activate STAT5a, which does not lead to IGF-1 induction. In summary, it is becoming clear that STAT3 and STAT5 are also activated in response to IFN-γ, often in a cell type-specific or maturation stage-specific manner. However, much more research is needed to understand how and why different cell subtypes activate different STATs and also to comprehend fully how the activation of each specific STAT in each specific cell type leads to the induction of specific genes, thus contributing to specific biological responses.

5 Priming

5.1 How Prior Exposure to Other Cytokines Affects Responses to IFN-γ

Exciting new data have emerged indicating that, especially during inflammation, pre-exposure to certain cytokines profoundly changes how cells respond subsequently to IFN-γ. One obvious mechanism through which priming can effect IFN-γ-dependent signaling is by enhancing the expression or activity of specific transcription factors. Notably, priming of human macrophages with low doses of IFN-γ for 2 days leads to increased STAT1 activation when the macrophages are subsequently restimulated with a slightly higher dose of IFN-γ for 10 min (Hu et al. 2002). This phenomenon could be explained by increased STAT1 levels due to IFN-γ priming (Hu et al. 2002), in line with previous results showing that activated STAT1 and IRF-1 drive the expression of STAT1 mRNA and STAT1 protein (Pine et al. 1994; Harada et al. 1994; Wong et al. 2002). These finding are particularly significant, because ligand-induced feedback inhibition and desensitization occur normally, as demonstrated by the priming effects of type I IFN on subsequent stimulation by type I IFN (Sakamoto et al. 2004). Of note, IFN-γ-primed macrophages also show a changed pattern of IFN-γ-induced gene activation. Expression of the STAT1-dependent genes *CCR2*, *IP10*, and *IRF1* are increased, but expression of the STAT3-dependent genes *EGR2*, *BCL2A1*, *IL1B*, *IL6*, *MMP1*, *RANTES*, *VCAM1*, and *FAS*, implicated in tissue destruction during inflammation, is attenuated (Hu et al. 2002, 2005). In this manner, priming by IFN-γ may deliver a homeostatic signal by attenuating the IFN-γ-dependent induction of certain tissue-destructive genes (Hu et al. 2005). One way that activated STAT3-dependent gene expression is attenuated is by downregulation of STAT3 function by increased STAT1 levels, as predicted by our finding that STAT1 and STAT3 compete for binding to Y419 of IFNGR1 (Qing and Stark 2004). For that matter, since the activation of STAT5 by IFN-γ also depends on Y419 (Woldman 2001), priming by IFN-γ could also lead to diminished STAT5 activation upon subsequent stimulation by IFN-γ due to increased competition by STAT1.

In contrast to IFN-γ, stimulation by IL-6 leads to increased STAT3 levels (Narimatsu et al. 2001; Yang et al. 2005). Based on our findings, we predict that priming by IL-6 would result in enhanced STAT3 activation by IFN-γ. In accordance, priming of human neuroblastoma cells for 5 h with cytokines from the GP130 family (such as CNTF or IL-6) leads to the activation of STAT3 in addition to STAT1 after stimulation by IFN-γ, whereas unprimed nerve cells activate only STAT1 in response to IFN-γ (Kaur et al. 2003). However, priming

by CNTF or IL-6 for 5 h left STAT3 levels unchanged. Expression of mutant GP130 revealed that a functional STAT binding site and a functional SHP-2 binding domain are both needed for subsequent IFN-γ-induced STAT3 activation. In addition, treatment with kinase or protein synthesis inhibitors during the priming phase revealed that MAPK and PKC activation, in addition to new protein synthesis, were crucial for STAT3 activation by IFN-γ in nerve cells (Kaur et al. 2003). The authors proposed that cross-talk between GP130 cytokines and IFN-γ involves the induction of an as yet unknown gene that requires STAT, PKC, and MAPK activation, and that the product of this gene is necessary to promote the interaction of STAT3 with the IFNGR. More studies are needed to determine the mechanism of these phenomena in nerve cells, and also to investigate whether increased STAT3 levels in response to IL-6 lead to increased STAT3 activation by IFN-γ in other cell types.

Similarly to IFN-γ, stimulation with IFN-β leads to a subsequent increase in STAT1 levels through activation of STAT1 homodimers (Pine et al. 1994; Harada et al. 1994). However, priming by IFN-β decreases the transcriptional activation of genes normally induced by IFN-γ in human fetal astrocytes and mouse macrophages (Gao et al. 2000; Hua et al. 2002), indicating that increased levels of STAT1 do not automatically lead to increased STAT1-dependent transcriptional activation by IFN-γ. The transcriptional defect caused by IFN-β priming could be related to decreased STAT1 activation or decreased binding of activated STAT1 to GAS-containing ISGs after IFN-γ stimulation (Revel et al. 1995; Gao et al. 2000; Hua et al. 2002). Furthermore, the decrease in STAT1 phosphorylation concerns only the transactivating form of STAT1 (STAT1α), whereas phosphorylation of STAT1β, which lacks the transactivation domain and thus is potentially a negative regulator, is even slightly increased in macrophages (Gao et al. 2000). Notably, priming macrophages with IFN-β results in an increase in the kinetics of STAT1α dephosphorylation, suggesting that priming causes these changes by increasing protein tyrosine phosphatase activity (Gao et al. 2000). These results are in line with data showing that the desensitizing effect of priming by IFN-β on subsequent IFN-β stimulation of fibroblasts is due to increased activity of the tyrosine phosphatase TC-PTP (Sakamoto et al. 2004). However, decreased activity of a tyrosine kinase due to IFN-β priming cannot be excluded. Indeed, prolonged incubation with LPS also leads, via the intermediate production of IFN-β, to diminished IFN-γ-induced STAT1 activation (Crespo et al. 2002; Dalpke et al. 2003). In these studies, the IFN-β-stimulated induction of CIS, SOCS-1, and SOCS-3 could be related to decreased STAT1 activation by inhibited tyrosine kinase activity of the JAKs. In summary, priming can positively or negatively change the quantity and quality of signal transduction by IFN-γ, and several possible mechanisms have been described. Therefore, the nature of the IFN-γ response can be altered

or reprogrammed. This flexibility is certain to play an important role during infection and inflammation, since in both situations cells are exposed repeatedly to bursts of cytokines. It remains to be established whether IFN-γ-induced signaling can be altered similarly in various blood cell subsets after priming, especially with IFN-β, IL-6, or IFN-γ. More work is also needed to determine exactly how long the effects of priming last.

5.2 Receptor Cross-talk

In mouse cells, IFN-γ and IL-6 cannot signal properly in the absence of type I IFN receptors, indicating constitutive cross-talk between these cytokine receptors. The low levels of IFN-β always present in tissues cause IFNAR1 to interact with IFNGR2 and GP130 in caveolar domains of plasma membranes (Taniguchi and Takaoka 2001). The associated IFNAR1 may provide an additional docking site that promotes the dimerization of STATs and concomitant increases in DNA binding and transcription in response to IFN-γ or IL-6, or transphosphorylation of the receptors may lead to enhanced STAT activation (reviewed by Taniguchi and Takaoka 2001; Ivashkiv et al. 2003). Of note, all of the data cited above were generated with mouse cells, and it is not known whether the same receptor cross-talk also takes place in human cells, or whether it is restricted to certain cell types. Tanaguchi and Takaoka (2001) suggest in their "revving-up model" that low-dose priming with type I IFN is necessary and results in normal IL-6 and IFN-γ signaling. In contrast, as discussed above, higher doses of type I IFNs abolishes STAT1 activation, and perhaps also STAT3 and STAT5 activation by IFN-γ. It is also not known whether priming by IL-6 via GP130 influences IFN-γ-dependent signaling via the proposed connection to IFNAR1. Alternatively, the lipid raft-STAT signaling hypothesis suggests that membrane complexes that contain STATs may be sites where the combinatorial effects of different cytokines and different activation pathways are integrated (Sehgal et al. 2002).

We discuss above that stimulation by LPS through TLR4 negatively influences IFN-γ-dependent signaling via the intermediate production of IFN-β and via priming by IFN-β. Nevertheless, IFN-γ signaling can be enhanced by the simultaneous stimulation of defined TLR agonists or whole-bacterial lysates and IFN-γ (Dalpke et al. 2003). Short-term co-stimulation results in the amplification of IFN-γ-dependent signaling attributable to P38MAPK-dependent serine phosphorylation of STAT1 in macrophages (Dalpke et al. 2003). The data suggest that cross-talk between IFNGR and TLRs can also positively influence IFN-γ-dependent signaling. Similarly, IFN-γ and LPS synergistically induce

the expression of pro-inflammatory factors such as IL-1, IL-6, IL-12, NO, and TNF-α (Zhao et al. 2006). Comparable synergism is observed between IFN-γ and peptidoglycan (a TLR ligand) or poly(I:C) (a TLR3 ligand) in inducing the IL-12 promoter. Notably, IRF-8-deficient macrophages do not show enhancement of LPS-induced ERK and JNK phosphorylation by IFN-γ, and the synergistic production of pro-inflammatory factors by LPS and IFN-γ is also lost. Zhao et al. (2006) suggest that the observed interaction between IRF-8 and TRAF6 modulates TLR signaling and may contribute to the cross-talk between the IFN-γ and TLR signaling pathways.

In addition to the known synergism between simultaneous TNF-α and IFN-γ signaling, leading to macrophage activation, there seems to be cross-talk between TNF-α- and IFN-γ-induced signaling. Normally, activated TNF-α receptor 1 (TNFR1) recruits TRADD, which in turn triggers two opposing pathways, leading to either caspase activation and induction of apoptosis or to NF-κB activation and induction of anti-apoptosis genes. In human epithelial cells, the association of STAT1 with TRADD and FADD increases after TNF-α binds to TNFR1 (Wang et al. 2000). STAT1-deficient cells show an apparent increase in the formation of TNF-α-induced TRADD–Rip and TRADD–TRAF2 complexes, leading to enhanced NF-κB activation (Wang et al. 2000). These data indicate that STAT1 acts as a negative regulator of signaling through TNFR1. Interestingly, the binding of IFN-γ to IFNGR causes nuclear translocation of STAT1, which might inhibit STAT1 from being recruited to the TNFR1, providing an explanation for why TNF-α-dependent activation of NF-κB is stronger in the presence of IFN-γ signaling (Wesemann and Benveniste 2003). Notably, stimulation by IFN-γ causes the formation of a TRADD-STAT1α complex in the nucleus. Because IFN-γ-mediated STAT1 activation is prolonged in macrophages in which the expression of TRADD is knocked down, TRADD is identified as a negative regulator of STAT1 activation by IFN-γ (Wesemann et al. 2004).

Finally, there is evidence of cross-talk between the IFNGR and the B cell antigen receptor (BCR) or T cell antigen receptor (TCR). Cross-linking of the BCR or TCR enhances the serine phosphorylation of STAT1 and consequent increased induction of STAT1 target genes (Zu et al. 2005). Enhancement of STAT1 serine phosphorylation by cross-linking of BCR involves the activation of P38MAPK and CaMKII, indicating that signaling pathways other than those stimulated by cytokines can also modulate signaling by IFN-γ (Xu et al. 2005). Interestingly, T cell activation 4 h in advance of IFN-γ stimulation leads to the loss of STAT1 phosphorylation, most likely due to the induction of SOCS1 (van de Wiele et al. 2004). However, loss of sensitivity to IFN-γ is still apparent after 3 days, and a mechanism other than inhibition of STAT1 activation by SOCS1 seems to be the reason.

6
New Signaling Pathways

6.1
Activation of Transcription Factors Other than STATs

In addition to differential STAT activation in various cell types (discussed in Sect. 4), differential activation of transcription factors other than STATs is likely to be another strategy used by various cell types to generate specific responses to IFN-γ. Notably, Oncostatin M activates STAT1, but does not activate the STAT1-dependent genes TAP1 and IRF-1, as does IFN-γ, suggesting that signals in addition to STAT1 activation must emanate from the IFNGR (Mahboubi et al. 2002). Interestingly, the constitutive transcription factor SP1 collaborates with STAT1 in inducing certain IFN-γ-stimulated genes. Examples are the *ICAM-1* and *MCP-1* genes in human epithelial and astrocytoma cells (Look et al. 1995; Zhou et al. 1998). Activated STAT1 and SP1 bind, respectively, to GAS elements and GC-rich sequences (GC boxes) to induce transcription (Zhou et al. 1998). Interestingly, the transcription factor AP-1 is critical for the constitutive and IFN-γ-stimulated expression of IFI16 (Clarke et al. 2003). It is likely that AP-1 collaborates with STAT1 in the IFN-γ-dependent induction of IFI16, because two GAS-like elements have been identified in the promoter of this gene (Trapani et al. 1994). Constitutively expressed transcription factors can also negatively regulate IFN-γ-dependent transcription. The promoter of MCP-1 contains an IFN-responsive-inhibitory sequence (IRIS) that consists of a 13-bp CT-rich sequence adjacent to the GAS site (Valente et al. 1998). In gel-shift assays, STAT1 and an as-yet-unknown constitutive transcription factor present in nuclear extracts from IFN-γ-stimulated osteoblastic cells bind to a probe containing the GAS/IRIS.

Transcription factors of the IRF family also collaborate with STAT1 for the induction of certain IFN-γ-dependent genes. IRF-1 is expressed weakly in most cells, but after IFN-γ stimulation IRF-1 is induced quickly by the binding of activated STAT1α to the GAS element in the promoter (Pine et al. 1994). IRF-1 is subsequently involved in the induction of many IFN-γ-stimulated genes through binding to ISREs (Dror et al. 2007). Of note, the following genes are expressed because of the cooperative action of IRF-1 and activated STAT1: *STAT1* (Pine et al. 1994; Wong et al. 2002), *CIITA* (Muhlethaler et al. 1998), *GP91PHOX* (Kumatori et al. 2002), indoleamine 2,3-dioxygenase (*IDO*) (Chon et al. 1996), *vcam1*, *icam1*, *mig*, *ena78*, *itac*, and *ip10* (Jaruga et al. 2004). IFN-γ activates the transcriptional activity of IRF-1 by activating PKC-α (at least in mouse macrophages), which results in post-translational modifications of IRF-1 such as tyrosine phosphorylation, but perhaps also acetylation (Sharf

et al. 1997; Giroux et al. 2003). Additional transcription factors can assist activated STAT1 and IRF-1 in inducing some of these genes in response to IFN-γ. For instance, the induction of the transactivator CIITA also requires the cooperation of USF-1, a constitutively and ubiquitously expressed transcription factor (Muhlethaler et al. 1998). CIITA is subsequently involved in inducing MHC Class II by IFN-γ (reviewed by van den Elsen 2004). As mentioned above, the expression of ICAM-1 needs the cooperative action of the constitutive transcription factor SP1. Furthermore, the induction of IDO requires the activation of PKR and NF-κB and MIG/CXCL9 induction requires NF-κB activation in addition to activated STAT1 and IRF-1 after stimulation by IFN-γ (Du et al. 2000; Hiroi et al. 2003).

All the transcription factors mentioned above are not expressed in a cell type-specific manner, and thus are unlikely to be responsible for cell type-specific gene induction by IFN-γ. GP91(PHOX), an essential component of NADPH oxidase, which is crucial for generating superoxide anions that kill ingested microorganisms, is an example of a gene that is expressed only in differentiated cells of the myeloid linage (Royer-Pokora et al. 1986). Study of *GP91PHOX* transcription might provide clues about the way IFN-γ induces cell type-specific genes. Interestingly, in addition to the activation of STAT1 and IRF-1 (Kumatori et al. 2002), serine/threonine phosphorylation of PU.1 by PKC-α- or PKC-βI is needed for *GP91PHOX* expression in monocytes (Mazzi et al. 2004). Notably, PU.1, a member of the ETS family, is selectively expressed in neutrophils, monocytes, and B cells (Suzuki et al. 1998), probably explaining why *GP91PHOX* is stimulated by IFN-γ only in myeloid cells. In addition, in myeloid cell lines, PU.1 interacts with IRF-1, IRF-8, and CBP to form the complex hemopoietic-associated factor-1 (HAF-1), which plays an important role in activating the *GP91PHOX* promoter in response to IFN-γ in myelomonocytic cells (Eklund et al. 1995). IRF-8 is also expressed selectively in myeloid cells and functions primarily in macrophages and some types of dendritic cells (reviewed by Kanno et al. 2005). Therefore, the myeloid-specific activation of PU.1 and IRF-8 can explain why GP91(PHOX) is selectively induced in monocytes/macrophages by IFN-γ. Interestingly, IRF-8 expression also directs the differentiation of myeloid progenitor cells into mature macrophages (Tamura et al. 2000) and drives the development of plasmacytoid dendritic cells (Tsujimura et al. 2003). Based on all of these results, we propose that combinatorial interactions between cell type-specific and developmentally restricted transcription factors and ubiquitous factors account for cell type-specific gene expression in response to IFN-γ.

PU.1 also cooperates with STAT1 in inducing FcγRI upon stimulation by IFN-γ of myeloid cells. Interestingly, PU.1 is required for both basal and IFN-γ-induced promoter activity, whereas activated STAT1 is needed only for

IFN-γ-induced activation of *FCGRI* (Aittomaki et al. 2002). Furthermore, the STAT1-mediated activation of the *FCGRI* promoter critically requires CBP/p300 (Aittomaki et al. 2002), and this requirement is likely to be important for the expression of many IFN-γ-dependent genes (Zhang et al. 1996). In human monocytic cells, additional requirements for FcγRI induction by IFN-γ are PKR-dependent serine phosphorylation of STAT1 and activation of the NF-κB pathway (Karehed et al. 2007). Another transcription factor that collaborates with STAT1 and CBP/p300 is GATA-1, which is involved in the IFN-γ-mediated induction of HLA-E. Notably, a cell type-restricted enhancer has been identified in the promoter of HLA-E, which binds to GATA-1 upon stimulation with IFN-γ (Barrett et al. 2004). Activation of the annexin II/PYK2/MEKK4/MKK6/P38 MAPK pathway by IFN-γ in keratinocytes is needed to activate the transcription factor ATF2 (Halfter et al. 2005). The authors propose that ATF2 activation by IFN-γ might induce the expression of COX2, but this has not been proven yet. ATF2 will certainly not induce this expression by itself, because others have shown that COX2 induction in response to IFN-γ depends on binding of IRF-1 to ISRE elements in the *cox2* promoter (Blanco et al. 2000). Finally, the transcription factor CREB becomes activated in murine macrophages through the activation of cAMP and PKA following stimulation by IFN-γ (Liu et al. 2004). It is not yet clear which IFN-γ-stimulated genes depend on transcriptional activation by CREB or ATF2, or whether these transcription factors operate totally independently of STAT1. We speculate, based on the papers discussed above, that transcription factors in addition to STATs, such as PU.1, IRF-8 and perhaps CREB, and additional yet-to-be-defined factors are necessary for cell type-specific gene induction in macrophages in response to IFN-γ. These unique macrophage-related signaling pathways must also play a role in the IFN-γ-dependent induction of genes involved in the novel cell type-specific antiviral activation of these cells, which does not involve the induction of IFN-α/β, TNF-α, PKR, RNASEL, or MX1 (Presti et al. 2001).

6.2 STAT-Independent Signals

In Sect. 6.1, we described many examples of collaborative action between STAT1 and other transcription factors needed for the expression of certain genes in response to IFN-γ. Similarly, it is likely that IFN-γ-activated STAT3 or STAT5 cooperates with other transcription factors. However, it is possible that the activation of additional transcription factors leads to the induction of genes without the help of any STAT. It remains to be discovered whether the IFN-γ-dependent, STAT1-independent pathways such as PI3K-dependent monocyte adhesion and induction of concentrative nucleoside transporters

(CNT) in macrophages are truly independent of any STAT (Navarro et al. 2003; Soler et al. 2003). Importantly, a novel type of GAS element with an IRF/ETS binding site has been described that functions independently of STAT1 (Contursi et al. 2000). In a murine macrophage-like cell line, PU.1 and IRF-8 bind to the GAS element present in the promoter of *irf8* (Contursi et al. 2000; Kanno et al. 2005). DNA sequence motif comparison revealed that GAS elements can be divided into two subtypes. One is the classical AAA/TTT palindrome, which contains no IRF/ETS binding motif; examples from this group are PML-GAS and GBP-GAS. The other includes a novel IRF/ETS composite element (Kanno et al. 2005), and examples are GAS elements in the promoters of *irf8*, *irf1*, *CCL2*, *TLR3*, and *cathepsin E preprotein* (Kanno et al. 2005). Notably, all of these genes are important for immune cell functions, particularly in macrophages and dendritic cells.

Other IRF family members can also mediate IFN-γ-stimulated gene induction in a STAT-independent manner. IFN-γ stimulates the expression of the polymeric immunoglobulin receptor (PIGR), which is expressed constitutively on the basolateral surfaces of secretory epithelial cells, where it directs polymeric IgA and pentameric IgM to exocrine secretions (Piskurich et al. 1997). There are three ISRE elements in the promoter of *PIGR*; the two upstream elements are bound to constitutive transcription factors and the third binds to IRF-1 upon IFN-γ stimulation of a human epithelial cell line (Piskurich et al. 1997). Furthermore, constitutive expression of the exonuclease ISG20 depends on the constitutive transcription factors SP1 and USF-1. In contrast, IFN-γ-stimulated expression of ISG20 depends on the binding of IRF-1 to the ISRE element in the promoter, which contains no functional GAS elements (Gongora et al. 2000). These two IFN-γ-activated promoters are driven by the binding of activated IRF-1 to ISRE elements independently of any STAT.

It is becoming clear also that not all ISRE elements are equivalent. Sequence alignment of ISREs has revealed three subtypes thus far (Meraro et al. 2002). The classical ISRE can recruit only IRF dimers, in addition to ISGF3. In contrast, some ISRE subtypes harbor an ETS/IRF binding site named EIRE (ETS/IRF response element; Meraro et al. 2002), different in composition from the ETS/IRF binding element, called EICE, described earlier by Brass et al. (1996). Of note, IRF-8 expression is high in myeloid and B cells, whereas IRF-4 is highly expressed in T and B cells. EICE binds to the immune cell-restricted factor PU.1, which forms a complex with either IRF-4 or IRF-8, and is therefore present in genes whose expression is restricted to immune cells (Kanno et al. 2005). IRF-4 and IRF-8 do not bind effectively to the ISRE element alone, but can do so only when in a complex with PU.1. In contrast, other members of the IRF family (IRF-1, 2, 3, and 7) bind to ISRE elements directly (Honda et al. 2006). Both EICE and EIRE have only one ETS binding site, but EIRE possesses

two IRF binding sites, in contrast to EICE which has only one (Meraro et al. 2002). Because of this difference, promoters that contain EIRE can also become activated after binding to IRF dimers, whereas both types of promoters can become activated by IRF4 or IRF-8/PU.1 heterocomplexes (Meraro et al. 2002). In contrast, IRF heterocomplexes consisting of IRF-8 with either IRF-1 or IRF-2 can bind only to classical ISREs and EIREs and not to EICEs and have been suggested to function as repressors (Bovolenta et al. 1994; Sharf et al. 1995). Interestingly, classical ISRE-containing genes in macrophages, and possibly in other immune cells, can be repressed by IFN-γ through protein–protein interaction between IRF-8 and another ETS family member, TEL, resulting in recruitment of the histone deacetylase HDAC3 (Kuwata et al. 2002). Examples of human genes with EIREs are *ISG15*, *6–16*, *9–27*, *IP10*, *ISG54*, and *CCYBB* (encoding GP91(PHOX)). However, other ways to activate EIRE-containing genes by IFN-γ have also been described. For instance, the human *IP10* and *ISG54* genes possess only an EIRE and no GAS in their promoters and both can bind to STAT1 and IRF-9, probably as a STAT1 homodimer/IRF-9 complex (Majumber et al. 1998; Bluyssen et al. 1995). In addition, the mouse *ip10* promoter can bind to STAT1 and IRF-1 in hepatocytes, but it is not clear what kind of complex is formed (Jaruga et al. 2004). Importantly, the ability of EIRE motifs to recruit not only IRFs but also PU.1/IRF heterocomplexes predicts that some of the genes harboring such elements might be regulated differentially by IFN-γ in immune cells, which constitutively express IRF-4, IRF-8, and PU.1, as has been shown for the *ISG15* gene in macrophages (Meraro et al. 2002) and the *CYBB* gene in myelomonocytic cells, the latter gene showing cooperation with PU.1/IRF-1/IRF-8/CBP (Eklund et al. 1995).

A strong increase in IRF-8 in response to IFN-γ was observed originally in macrophages (Politis et al. 1994), probably because activated PU.1 and IRF-8 binding to the IRF/ETS composite GAS element present in the *irf8* promoter (Contursi et al. 2000; Kanno et al. 2005). Because of the increase in IRF-8 expression, a model was suggested in which IRF-8 and PU.1 play a role in amplifying the expression of genes containing the IRF/ETS composite GAS element by generating a second wave of transcription (Kanno et al. 2005). In addition, it is likely also that the expression of EICE- or EIRE-containing genes is amplified in a macrophage-specific manner through a second wave of transcription. Although it was believed that IRF-8 is only expressed in certain immune cells, it has been shown recently that IFN-γ-stimulated primary colon carcinoma cells also express IRF-8, probably because activated STAT1 binds to the composite GAS element in the *IRF8* promoter (Liu et al. 2003). Similarly, an increase in IRF-1 levels after stimulation by IFN-γ in nonimmune cells is a result of the binding of STAT1 to the IRF/ETS composite GAS element in the *IRF1* promoter. Therefore, since nonimmune cells do

not express PU.1, an increase in IRF-8 may have a quite different effect on classical ISRE or EIRE-containing genes, because complexes formed between IRF-8 and IRF-1 or IRF-2 might function as repressors (Bovolenta et al. 1994; Sharf et al. 1995). However, it cannot be excluded that a concomitant increase in IRF-8 and IRF-1 in immune cells might also lead to the repression of certain genes. However, when IRF-8 levels are not too highly induced in non-immune cells, an increase in IRF-1 homodimers will certainly lead to increased expression of genes containing an ISRE or EIRE element. Moreover, activated STATs may collaborate with IRF-1 to induce the expression of genes that harbor both a classical GAS and an ISRE element. Summarizing these results, we propose that the availability of STATs, IRF, or ETS family members, constitutively present or induced, will determine how a certain cell type responds to IFN-γ, and that the balance among these transcription factors will determine which subtype of ISRE- or GAS-containing genes will be turned on in the first and second phases of transcriptional activation.

CIITA and IRF-9 are examples of transcription factors whose expression is induced by IFN-γ and which therefore play an important role in the second wave of the IFN-γ response. A novel IFN-responsive cis-acting enhancer element, γ-IFN-activated transcriptional element (GATE), distinct from GAS and ISRE, but partly homologous to ISRE, has been identified in the promoter of the *irf9* gene (Weihua et al. 1997). Two transcription factors bind to GATE, GBF1, and GBF2, after they are both synthesized de novo in response to IFN-γ. GBF2 was identified subsequently as C-EBP-β. Its induction by IFN-γ is JAK1- and STAT1-dependent and is probably mediated by the binding of STAT1 to a putative GAS element in the promoter of the *cebpb* gene (Roy et al. 2000). IFN-γ activates C-EBP-β expression by activating the MEKK1/MEK1 or MEK2/ERK1 or ERK2 cascade, resulting in the phosphorylation of C-EBP-β at threonine 294 in the consensus ERK phosphorylation site of the regulatory domain RD2 (Hu et al. 2001). Furthermore, MLK activation by IFN-γ leads to the dephosphorylation of C-EBP-β at serine residue 64 in the transcription activation domain, permitting recruitment of transcriptional co-activators such as p300 (Roy et al. 2005). To date, it is not known whether the MLK-driven dephosphorylation is caused by enhanced phosphatase activity or by inactivation of the kinase that normally phosphorylates serine 64 constitutively (Kalvakolanu et al. 2005). GBF-1 is a novel transcription factor and the gene is located at human chromosome 9q34.13 (Hu et al. 2002). It is not clear which signals from the activated IFNGR induce GBF-1 expression. GBF-1 possesses glutaredoxin-like, PTP-like, RNA Pol II-like, and ribonucleoside diphosphate reductase-like domains, but its precise mechanism of action is not yet known (Hu et al. 2002). The fact that GBF-1 does not bind to monomeric GATE, but does bind to multimeric GATE in a DNA screen, shows that it has very weak DNA binding activity by itself.

However, similarly to IRF-8, which does not bind to DNA alone, GBF-1 possesses strong transactivating activity (Hu et al. 2002). Recent data indicate that C-EBP-β interacts with GBF1 after phosphorylation of C-EBP-β at threonine 294 (Meng et al. 2005). Just as IFN-β induces IRF-9, the induction of IRF-9 by IFN-γ seems to be independent of activated STAT1, but dependent on unphosphorylated STAT1 through an as-yet-unidentified mechanism (Rani et al. 2005). Unphosphorylated STAT1 and STAT3, upregulated in response to IFN-γ or GP130-linked cytokines, respectively, induce sets of genes distinct from those that respond to phosphorylated STAT1 and STAT3 (Chatterjee-Kishore et al. 2000; Yang et al. 2005).

In summary, we describe several IFN-γ-stimulated signaling pathways that involve the activation of different IRF and ETS family members and that function either independently of any STAT or collaborate with STAT1 for the induction of certain genes. These signaling pathways play a very important role in the response of immune cells to IFN-γ. It is not yet known which transcription factors collaborate with activated STAT3 or STAT5 in specific cell types in response to IFN-γ. We expect that many more IFN-γ-activated transcription factors will be discovered that either collaborate with STATs or induce gene expression totally independently of any STAT.

7 Conclusions and Perspective

We propose that different patterns of STAT activation in different cell types contribute to the activation of "STAT only genes" (Fig. 1). In addition to the activation of JAKs, additional kinases such as PI3K, P38, ERK, PYK2, PKC, SRC family members, CaMKII, PKR, MEKK4, and MKK6 become activated. Depending on the cell type, some of these kinases also contribute to the serine phosphorylation of STATs. In addition, they (and other kinases yet to be identified) lead to the activation of transcription factors other than STATs (GATA-1, USF-1, CREB, NF-κB, AP-1, IRF-1, IRF-8, PU.1, and ATF-2). These activated transcription factors either collaborate with STATs on the promoters of "STAT + transcription factor (TF) genes" or function totally independently of any STAT on the promoters of "TF-only genes" (Fig. 1). Furthermore, the differential activation of TFs other than STATs by kinases other than JAKs is an important aspect of cell-type specificity (Fig. 1). Therefore, depending on which STATs and TFs become activated in each cell type, IFN-γ, in addition to its well known antiviral activity, can promote either apoptosis and growth inhibition or survival and proliferation, induce IgG switching in B cells, activate macrophages, and regulate cancer immunoediting. Importantly, priming

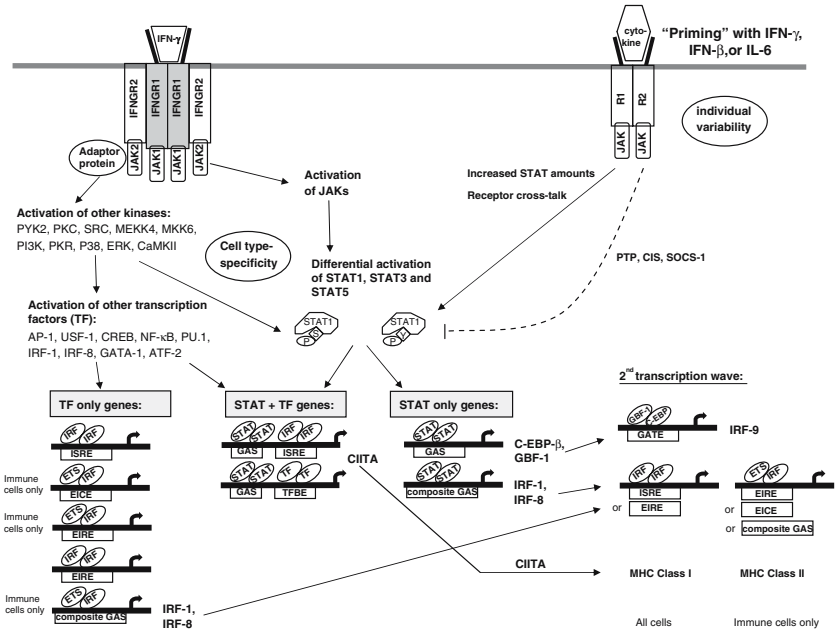

Fig. 1 The complexity of IFN-γ-dependent signaling helps to explain cell type specificity and individual variability in responses. See Sect. 7, "Conclusions and Perspective," for a description of the details

with a cytokine (for example, IFN-γ, IL-6, IFN-β) can either positively (by increasing STAT levels or through receptor cross-talk) or negatively (through PTPs, SOCS-1, and CIS) influence the tyrosine phosphorylation and activation of STATs (Fig. 1). More importantly, priming leads to increased or decreased activation of specific STATs, and thus is likely to have a major impact eventually on which STAT-only genes or STAT + TF genes will be induced (Fig. 1). We propose that priming may well be an important cause of altered IFN-γ-dependent signaling in different individuals (Fig. 1). Notably, the transcription factors IRF-1, IRF-8, CIITA, GBF-1, and C/EPB-β are induced in response to IFN-γ, and play an important role in the secondary transcriptional wave. We propose that the availability of STATs, IRF, or ETS family members, constitutively present or induced, will determine how a certain cell type responds to IFN-γ, and that the balance among these transcription factors will determine which subtype of ISRE- or GAS-containing genes will be turned on in the first and second phases of transcriptional activation.

We hope that the model of Fig. 1 provides a framework for understanding the complex modulation of IFN-γ-dependent signaling, but much is still to be learned. Individual cell types need to be studied in order to understand how and why certain STATs are activated preferentially, and whether different cell types are equally susceptible to priming. Also, the activation of kinases other than JAKs in each individual cell type needs to be linked to the activation of TFs other than STATs, and eventually to the induction of specific genes. More research is also needed to identify cis-acting elements in the promoters of STAT + TF genes and TF-only genes. All of this information is needed to understand fully how different individuals and different cell types react to IFN-γ during infection and inflammation.

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Interferon Research: Impact on Understanding Transcriptional Control

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Abstract Celebrations are certainly in order for the 50th anniversary of the Isaacs/Lindenmann discovery of interferon as a secreted substance of probable cellular origin that conferred resistance to at least four viruses of distinctly different character—influenza, Sendai, Newcastle's disease, and vaccinia. Personal pride, excusable, I hope, leads me to recall also that following the trail of how interferon works its magic led 15 years ago this year to the discovery of the STATs and their activation by the Jak kinases. These later findings were stimulated by and in turn further stimulated an ever deepening interest in how transcription figured in changing cell behavior. Here are presented some further reflections on these topics.

1 Introduction

The history of interferon (IFN) research, beginning with the Lindenmann and Isaacs discovery in 1957 (Isaacs and Lindenmann 1957; Isaacs et al. 1957), justifiably shows a central concern with the great importance of interferon in innate immunity, evolution's first and perhaps most important invention for the survival of animals. A second fundamental role of IFN, the discovery of which around 1970 portended a wider importance of these proteins in cell biology, is the inhibition of growth, first documented by Ion Gresser and colleagues (1970). And study of these biologically crucial aspects of interferon research continue apace today as featured in most chapters in this book.

But research using interferon has also had a great impact on a fundamental area of cellular and molecular biochemistry—the molecular basis of signal transduction and the mechanisms of immediate and specific gene activation. Only in 1977 was it shown that the transcription of integrated MMTV (mouse mammary tumor virus, a retrovirus whose DNA was incorporated into

a host cell) was increased in cells treated with glucocorticoids (Fingold et al. 1977; Young et al. 1977). This was the best evidence at the time of a change in the transcription rate of specific integrated chromosomal DNA in eukaryotes brought about by an extracellular substance. But, of course, steroids enter through the cell membrane and MMTV was not a "normal" gene. Thus, prior to 1984 it was not known how or if extracellular signaling proteins affected gene expression. Only as a matter of convenience did our research (and to some extent that of George Stark and Ian Kerr who were actually interested in the anti-viral state) use interferon to approach this question. With cDNA cloning, it had become possible to score the transcription rate of individual mammalian genes, and purified type 1 IFN was provided to us by Ernest Knight and his colleagues from E.I. DuPont, who had shown with two-dimensional gel electrophoresis the actinomycin-inhibited appearance of new proteins in IFN-treated cells (Knight and Korant 1979). Therefore, we undertook to find out if IFN effected a quick and direct change in transcription of a set of selected genes. Copies of individual mRNAs (cDNAs), whose presence was greatly increased in IFN-treated human cells, were identified (Friedman et al. 1984; Larner et al. 1984, 1986). The transcription rate (assayed by run-on labeling of nuclear RNA followed by hybridization to cloned cDNA) of the genes encoding these mRNAs was increased 10- to 100-fold within 15–30 min of treatment. Initially, we neither knew nor cared what role, if any, the proteins encoded by these inducible mRNAs played in the physiologic effects of interferon. However, these results established a responsive, cultured cell system in which the elements could be discovered that controlled an extracellular polypeptide-induced transcriptional response (Levy and Darnell 1990). The biochemistry/molecular genetics that followed identified interferon-sensitive promoters, followed by identification of interferon-induced site-specific binding factors, purification of these factors and cloning of the genes encoding the proteins in these factors. Thus STAT1 and STAT2, the latent cytoplasmic IFN-activated transcription factors, were uncovered (reviewed in Darnell 1997; Darnell et al. 1994; Stark et al. 1998). (Less germane to this book, the availability of STAT1 and STAT2 accelerated the discovery of the remaining five STAT proteins and established this family of proteins as major respondents to cytokine signaling in general as well as in other tyrosine kinase pathways; Levy and Darnell 2002.)

The certainty that STAT1 and STAT2 were necessarily the DNA-binding nuclear proteins that effected interferon-induced transcriptional response came through a collaboration with the Kerr and Stark labs. Our cDNAs complemented their interferon-unresponsive mutant cell lines that were shown to lack STAT1 or STAT2 (Leung et al. 1995; Muller et al. 1993). It was soon established that activation of STATs required tyrosine phosphorylation (Schindler et al. 1992) that occurred through IFN-activation of JAK kinases bound non- covalently to IFN

receptors (Ihle 1996; Stark et al. 1998). These findings gave rise to the idea of a set of receptor-triggered JAK-STAT pathways that are the basis of signaling by many different cytokines (Schindler and Darnell 1995).

The voluminous research in this field now reported not only in the general literature but in a dedicated journal, *The Journal of Interferon and Cytokine Research,* has spilled over into virtually all areas of modern cell biology and genetics. Specifically featured have been biochemical studies on protein interactions at the receptor, and in the cytoplasm to effect STAT activation and nuclear accumulation (Levy and Darnell 2002; Stark et al. 1998), and STAT action in the nucleus to stimulate transcription (Hartman et al. 2005; Horvath 2004a, 2004b). Further, structural analysis of the STATs, with and without phosphorylation, has been invaluable for insight into how STATs function (Becker et al. 1998; Chen et al. 1998; Mao et al. 2005; Neculai et al. 2005). Structural analysis both helps to explain how already observed mutations cause their effects and points the way to the generation of further mutations to test functional ideas. A recent striking example is the solution of nonphosphorylated STAT1 (Mao et al. 2005) and later STAT5 (Neculai et al. 2005), which together with mutagenesis experiments (Mertens et al. 2006; Zhong et al. 2005) argue for a dramatic approximately 180° rotation of STAT1 monomers during dephosphorylation. Figure 1 and Table 1 provide a summary of phenotypes associated with known STAT1 mutations in the various structural domains of that protein.

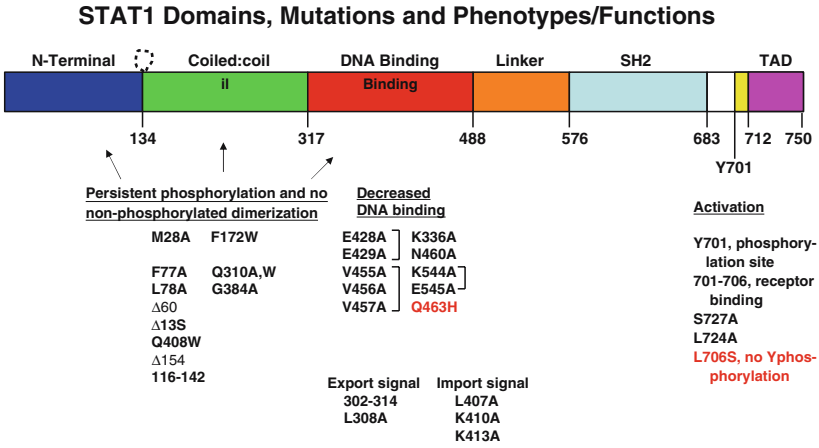

Fig. 1 Mutations and phenotypes of STAT1 mutants were created by site-specific mutagenesis except Q463H and L7065, which were discovered in humans (references as given in Table 1)

Table 1 References to STAT1 mutations and phenotypes

N–terminal domain	Reference
M28A	Chen et al. 2003
F77A	Zhong et al. 2005
F78A	Chen et al. 2003
W37A	Vinkemeier et al. 1998
Δ60	Shuai et al. 1996
Δ135	Mertens et al. 2006
Δ154	Haspel & Darnell. 1999
116–142 variants	Mertens et al. 2006
F172W	Zhong et al. 2005
302–314	Begitt et al. 2000
L308A	Begitt et al. 2000
K336A	Yang et al. 2002
Q340A,W	Mertens et al. 2006
G384A	Mertens et al. 2006
L407A	McBride et al. 2002
Q408W	Mertens et al. 2006
K410A, K413A, K416A	Fagerlund et al. 2002
E428, E429A	Horvath et al. 1995
V455A, V456, V457AA	Horvath et al. 1995
N460A	Yang et al. 2002
Q463H	Chapgier et al. 2006
K544A, K545A	Yang et al. 2002
R60A	Shuai et al. 1994
Y701F	Schindler et al. 1992
	Shuai et al. 1992
L706S	Dupuis et al. 2001
S727	Wen et al. 1995; Zhang et al. 1998
L724A	Nair et al. 2002
K724I	Nair et al. 2002

The phenotypes of the mutations listed here are given in Fig. 1

A most important aspect of transcriptional signaling from cell-surface receptor ligand interactions is that in normal circumstances it is transient. That several classes of proteins (SOCs, PIAS, tyrosine and serine phosphatases) regulate the level and duration of STAT activity represent major discoveries

(Alexander and Hilton 2004; Shuai and Liu 2005). Precisely how each of these negative regulators manage their task is an active area of investigation.

In the genetic/physiologic area, making mice that lack components of the receptor-JAK-STAT pathways, both global and cell-specific deletions, has led the way in establishing the essentiality of each component in the interferon response in animal physiology (Levy and Darnell 2002; O'Shea et al. 2002; Stark et al. 1998). Other crucial studies on the broad cellular effects of IFNs have featured the determination of the target genes of IFN activation (de Veer et al. 2001; Der et al. 1998; Hartman et al. 2005). How some of the encoded proteins of IFN-induced genes effect cell changes has been firmly established by deletion studies in mice. The crucial role of the receptor-JAK-STAT pathway in interferon response was clearly settled by knockouts that rendered mice exquisitely sensitive to either bacterial or viral infection (O'Shea et al. 2002). In recent years, important work on IFN activation of serine kinase pathways that do not require STATs (Platanias 2005; Pokrovskaja et al. 2005; Rani and Ransohoff 2005) has broadened the fabric of interferon responses. All of these studies have resulted in explaining the interferon response as deeply or more deeply than any other signaling pathway. Reports on updated aspects of these extensive studies make up the text of this book.

Our own continuing interest in this field stimulates me to list some unsolved important problems, some of which are general to much of regulatory biology.

A central issue in modern cellular biochemistry is how a balance is achieved between interacting proteins that lead to what we observe as a dichotomous decision, e.g., an IFN-treated cell is either resistant to a virus or it is not; after appropriate stimulus a cell either goes around the cell cycle or it does not; or it undergoes apoptosis or it does not. The STATs play some role in all of these decisions. How many activated STAT1 molecules acting through what length of time assures the antiviral state or inhibits growth? The competing forces of activation and inactivation or direct inhibition of all activated transcription factors are probably finely balanced and the STATs are no exception. Since STAT1 is proapoptotic, does IFN treatment that is sufficient to induce the antiviral state sometimes or always result in cell death? Since STAT3 is anti-apoptotic (and can in some cells at least be activated by IFN-α), do STAT1 and STAT3 duel it out in determining the outcome? When both STAT1 and STAT3 are activated, a phosphodimer of STAT1:STAT3 is prominently formed. Is this heterodimer active on some, none, or all of the same gene targets? These questions beg for a deeper understanding of the precise transcriptional output regulated by the STATs.

We have too little comprehensive knowledge about the state of chromatin in genes that are poised for immediate activation by cytokines. Are the promoters of such poised genes in a special ready-to-go chromatin state? A number of years ago, we found evidence for a prebound protein prior to IFN activation of

the GBP gene, which apparently departed upon activation (Mirkovitch et al. 1992). Is this a general property of all quickly inducible genes?

The recent progress in chromatin (histone) modification that accompanies gene transcription and gene silencing needs to be more thoroughly studied in IFN-inducible genes. The quick induction and return to a preinduction transcriptional level of some IFN-inducible genes and the induction followed by secondary sustained transcription of other genes offers among the very best experimental material to approach the problem of reversibly inducible genes. This latter set of problems is particularly intriguing since not only histone acetylation but also de-acetylation are apparently required for full IFN responses (Nusinzon and Horvath 2005).

Most of the above discussion has dealt with transcriptional activation by the canonical STAT activation through tyrosine phosphorylation by JAK kinases. Recent evidence shows conclusively that the full IFN response also entails activation of other signaling pathways. In fact, an entire issue (December 2005) of *The Journal of Interferon and Cytokine Research* was devoted to this topic.

Finally, it is amply demonstrated that STATs can play a role in transcription without being phosphorylated (Chatterjee-Kishore et al. 2000; Ramana et al. 2000; Yang et al. 2005). How does this occur? Since it is unlikely that the nonphosphorylated STAT can on its own specifically bind DNA, the answer undoubtedly lies in the labor-intensive task of discovering all of the proteins associated with the promoters of IFN responsive genes and their interaction with STATs during IFN-dependent induction and de-induction. This short list of open questions attests to the continuing opportunities offered in this field. So too do the series of interesting papers that follow to report new results.

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Part III: Recognition of Pathogens, Molecular Mechanism of Interferon Beta Induction

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TLR-Mediated Activation of Type I IFN During Antiviral Immune Responses: Fighting the Battle to Win the War

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Abstract Toll-like receptors (TLRs) are crucially important in the sensing of viral infections and viral nucleic acids. TLR triggering leads to the induction of specific intracellular signaling cascades that result in the activation of two major families of transcription factors; the IFN-regulatory factors (IRFs) and nuclear factor-kappa B (NF-κB). IRFs and NF-κB work together to trigger the production of type I interferons (IFNα/β) or inflammatory cytokines leading to the maturation of dendritic cells and the establishment of antiviral immunity. This review will focus on the most recent findings relating to the regulation of IRF activity by TLRs, highlighting the increasing complexity of TLR-mediated signaling pathways.

1 Introduction

The mission of a virus inside the host is to multiply. This task is counteracted by strong and precise host immune responses. The first warriors to combat virus infections were discovered 50 years ago by Isaacs and Lindenmann as soluble proteins released by almost all cell types capable of interfering with virus replication, and referred to as the interferons (IFNs) (Isaacs and Lindenmann 1957). Type I IFNs belong to a multiprotein family that consists of about 30 members sharing a variable degree of structural homology (Hardy et al. 2004; Pestka et al. 2004; van Pesch et al. 2004). Type I IFNs include multiple *Ifn-αs*, *Ifnb*, *Ifnω*, *Ifnκ*, and *Ifne* genes; during viral and bacterial infections, the main type I IFNs that are synthesized are IFN-αs and IFN-β (Bogdan et al. 2004; Coccia et al. 2004). In the past few years, the regulation and function of these IFNs have been extensively characterized.

The discovery of the Toll-like receptors (TLRs) represents a key milestone in understanding how virus-infected cells recognize and react to invading pathogens (Janeway and Medzhitov 2002). At present, 13 TLRs have been identified: TLR1–9 are common to mouse and human, while TLR10 is unique to humans and TLR11–13 are unique to the mouse (Tabeta et al. 2004; Takeda et al. 2003; Zhang et al. 2004). TLRs play a key role in detecting microbial products derived from a broad range of pathogens, often referred to as pathogen-associated molecular patterns (PAMPs). Several lines of evidence indicate that the TLRs involved in the recognition of molecular structures unique to bacteria and fungi (TLR1, TLR2, TLR4, TLR5, TLR6) are localized to the plasma membrane and can be recruited to the phagosome, whereas the TLRs that detect viral and bacterial nucleic acids (TLR3, 7, 8, and 9) are localized in the endosomal compartment. Bacterial and viral double-stranded (ds) DNA is detected by TLR9. TLR7 and TLR8 are closely related and are involved in recognizing virus-derived single-stranded (ss) RNAs. Furthermore, dsRNA, which is generated in infected cells as an intermediate of virus replication, triggers TLR3.

TLRs are transmembrane proteins: their extracellular domains contain a repetitive structure rich in leucine residues, the leucine-rich repeats (LRRs), that are involved in ligand recognition. The intracellular region includes a common structure to all TLRs and IL-1 receptor family members, and is referred to as the Toll/IL-1 resistance (TIR) domain, which is essential for signal transduction. Every TLR triggers a specific cellular activation program via the recruitment of different combinations of specific adaptor molecules to its TIR domain. These adapters include myeloid differentiation factor 88 (MyD88) (Muzio et al. 1997), MyD88 adapter-like (Mal) (Fitzgerald et al. 2001) (also

called TIRAP; Horng et al. 2001), TIR-domain-containing adapter inducing interferon-β (TRIF) (Yamamoto et al. 2002; Hoebe et al. 2003) (also called TICAM1; Oshiumi et al. 2003a) and TRIF-related adapter molecule (TRAM) (Fitzgerald et al. 2003b) (also called TICAM2; Oshiumi et al. 2003b). Only recently, another TIR-domain-containing adapter has been described, SARM (SAM- and ARM-containing protein), which contains sterile alpha (SAM) and HEAT/Armadillo (ARM) motifs, as well as a TIR domain (Liberati et al. 2004). SARM has recently been shown to act as a negative regulator of TLR signaling (Carty et al. 2006). The recruitment of these TIR-domain-containing adapters to the TIR domain of activated TLRs leads to the activation of several transcription factors, including NF-κB and the IFN-regulatory factors (IRFs), with the subsequent induction of type I IFNs and IFN-dependent responses.

In this review, we have focused on the role of TLRs and associated signaling molecules in innate immunity to viruses in order to give a complete overview of how TLRs are involved in sensing and initiating immune responses to viruses.

2
ER-Localized TLRs: The Specialists in Virus Recognition

2.1
TLR3

The innate immune system is the first line of defense against virus infection and involves the release of proinflammatory cytokines, type I IFNs, and activation of adaptive immune responses. A number of viral products are sensed by cells of the innate immune system; among them, dsRNA is a common signature of viral replication and is generated in infected cells by most (if not all) viruses. In 2001, it was described for the first time that TLR3 mediates responses to poly (I:C), a synthetic analog of dsRNA. Indeed TLR3 knockout mice were resistant to poly (I:C)-induced shock compared to wild-type mice (Alexopoulou et al. 2001). Since the inhibition of endosomal acidification abrogates poly (I:C) signaling, it has been assumed that TLR3 is localized to the endosomal compartment. In fact, TLR3 has been shown to reside in multivesicular bodies, a subcellular compartment situated in the endocytic trafficking pathway in dendritic cells (DC) and could not be detected on the cell surface (Matsumoto et al. 2003). This intracellular localization of TLR3 is thought to be important for encountering dsRNA.

TLR3 has been implicated in the immune response to several viruses. TLR3 controls inflammatory cytokine and chemokine production in respiratory syncytial virus (RSV)-infected cells (Rudd et al. 2005). RSV-induced CXCL10 and

CCL5 production, but not CXCL8 production or viral replication, were shown to be impaired in the absence of TLR3. Hoebe et al. reported that mice homozygous for the *Lps2* mutation, a distal frameshift error in TRIF, are hypersusceptible to mouse cytomegalovirus (MCMV) (Hoebe et al. 2003), and a role for TLR3 in the response to MCMV was confirmed using TLR3 knockout mice (Tabeta et al. 2004). A major function for TLR3 in antiviral responses involves its role in promoting the cross-priming of cytotoxic T lymphocytes (CTLs). This occurs in cells that are themselves not directly infected. Murine CD8α^+ DCs can be activated in this manner by dsRNA present in virally infected cells taken up by phagocytosis (Schulz et al. 2005). These observations may explain the subcellular localization pattern of TLR3 in the endosomal compartment.

In some circumstances, the TLR3-mediated response can be detrimental to the host. During infection with West Nile Virus (WNV), a mosquito-borne ssRNA flavivirus, TLR3-deficient mice were found to be more resistant to lethal WNV infection. TLR3-deficient mice had increased viral load in the periphery (Wang et al. 2004). TLR3-dependent inflammatory response modulates the ability of WNV to invade the central nervous system after replicating in the periphery by inducing a reversible breakdown of the blood–brain barrier. TLR3 knockout mice also have an unexpected advantage upon influenza A virus challenge: a reduction in TLR3-mediated inflammatory response reduces the clinical manifestation of the influenza A-induced pneumonia (Le Goffic et al. 2006). In both of these cases, the virus appears to benefit from its interaction with TLR3.

In addition to viral RNA, heterologous RNA released from or associated with necrotic cells, likely through secondary structure, also stimulates TLR3 and induces immune activation (Kariko et al. 2004). Thus, RNA escaping from damaged tissues or contained within endocytosed cells could serve endogenous danger signals and be sensed by TLR3.

2.2 TLR7 and TLR8

TLR7 and TLR8 have been shown to recognize viral nucleic acids. Firstly, TLR7 and 8 were shown to trigger IFN production in response to the imidazoquinolines, imiquimod, and resiquimod (or R-848). These are low–molecular–weight immune response modifiers with potent antiviral and antitumor properties that are used clinically in the treatment of external genital warts caused by human papilloma virus infection (Hemmi et al. 2002). Using MyD88 and TLR7 knockout mice, Hemmi et al. showed that the imidazoquinolines activate murine immune

cells in a TLR7- and MyD88-dependent manner. Moreover, R-848 can be recognized either by human and murine TLR7 or human TLR8 but not murine TLR8, suggesting that TLR8 is not functional in mice, in accordance with the observation that TLR7-deficient mice do not respond to R-848, even though TLR8 is present (Jurk et al. 2002). Since this initial discovery, the immunostimulatory action of several additional guanine nucleoside analogs has been shown to be controlled exclusively via TLR7 (Lee et al. 2003) and this activity in human cells appeared to require endosomal acidification.

The first evidence of TLR7 and 8 triggering by physiological ligands was reported by Heil et al. (2004). Indeed they described the ability of guanosine- and uridine-rich ssRNA oligonucleotides derived form immunodeficiency virus-1 (HIV-1) to stimulate DCs and macrophages to secrete IFN-α and proinflammatory cytokines via murine TLR7 and human TLR8. In the same issue of *Science*, another group also reported the capacity of TLR7 to sense synthetic ssRNA (polyU) or ssRNA derived from wild-type Influenza virus (Diebold et al. 2004). Viral genomic ssRNA could substitute for intact Influenza in triggering IFN-α and cytokine production by murine plasmacytoid DCs (pDCs) and only background levels of IFN-α were measured in pDCs derived from TLR7$^{-/-}$ and MyD88$^{-/-}$ mice, further supporting the hypothesis that ssRNA is a TLR7 ligand. The recognition of another ssRNA virus, vesicular stomatitis virus (VSV), was also shown to be TLR7/MyD88-dependent (Lund et al. 2004).

Influenza virus, like VSV, is internalized into an endocytic compartment where viral fusion and release into the cytosol occurs; this suggests that the recognition by TLR7 might occur in the endosomal compartment. In fact, both Diebold and Heil's reports showed that virus-induced IFN-α production in pDCs required intact endocytic pathways (Diebold et al. 2004; Heil et al. 2004). This is consistent with the idea that viral nucleic acids would be sensed from an intracellular compartment.

Because GU-rich sequences are found in viral as well as endogenous RNA, TLR7 and 8, as has been described for TLR3, may also detect self-RNA acting in this way as sensors of endogenous danger signals (Heil et al. 2004). Accordingly, small nuclear ribonucleoproteins (snRNPs), which are a major component of the immune complexes associated with the pathogenesis of the autoimmune disease systemic lupus erythematosus (SLE) activate human pDCs to produce IFN-α, proinflammatory cytokines and to upregulate costimulatory molecules when the U1snRNA is intact (Savarese et al. 2006). The recognition of U1snRNA is dependent on TLR7. Therefore in certain circumstances, detection of self-RNA by these TLRs can contribute to autoimmune disease.

2.3 TLR9

Unmethylated CpG motifs are a feature of bacterial but not vertebrate genomic DNA and TLR9 was originally shown to be activated by these molecules (Hemmi et al. 2000). Oligodeoxynucleotides (ODNs) containing CpG motifs activate host defense mechanisms leading to innate and acquired immune responses. The concept of immunostimulatory DNA was borne as a result of studies on attenuated mycobacteria bacillus Calmette Guerin (BCG)-mediated tumor resistance. The component of BCG for activating natural killer (NK) cells and inducing tumor regression in mice was subsequently found to be the DNA (Tokunaga et al. 1984). Purified BCG DNA induced NK cell activity and the production of type I and II IFNs in vitro (Yamamoto et al. 1988). Cloning and synthesizing mycobacterial genes helped to elucidate that certain self-complementary palindromes in these ODNs were responsible for the immune stimulatory effects (Yamamoto et al. 1992). The active palindromes contained at least one CpG dinucleotide. CpG dinucleotides are more common in the bacterial genome (Kuramoto et al. 1992) and are not methylated in bacterial DNA but are routinely methylated at the 5′ position of the cytosines in vertebrate DNA (for extensive reviews see Krieg 2002 and Tokunaga et al. 1999). Several groups reported that the immunostimulatory CpG-ODNs directly activate macrophages (Sparwasser et al. 1997; Stacey et al. 1996) and murine DCs (Sparwasser et al. 1998) to upregulate co-stimulatory molecules and produce proinflammatory cytokines. Interestingly, expression patterns for TLRs differ between different subpopulations of dendritic cells. Plasmacytoid DCs (pDCs) predominantly express TLR7 and TLR9, whereas myeloid DCs express TLR1–6 and TLR8, but not TLR7 and TLR9 (Hornung et al. 2002; Jarrossay et al. 2001; Kadowaki et al. 2001). Accordingly, only human pDCs (as well as human B cells) respond to CpG-DNA.

The CpG motifs are also found in abundance in some viral genomes, such as the dsDNA virus, Herpes simplex virus (HSV). The pDCs respond to HSV-1 by secreting high levels of type I IFNs, releasing IL-12 and upregulating co-stimulatory molecules (Dalod et al. 2002) and the pDC responsiveness to HSV-1 in vitro is indeed mediated by the TLR9/MyD88 pathway (Krug et al. 2004). Similar results have been reported in HSV-2-infected pDCs (Lund et al. 2003); in this case, however, they also demonstrated that purified HSV-2 DNA was able to trigger IFN-α production in pDCs. In $TLR9^{-/-}$ mice infected with HSV-2, no IFN-α was detected (Lund et al. 2003). Moreover, the recognition of HSV-2 by pDCs is dependent on an intact endocytic pathway, since inhibitors of endosomal acidification such as chloroquine or bafilomycin inhibit these responses. This is consistent with the fact that TLR9 is located and signals from an intracellular

endosomal compartment (Ahmad-Nejad et al. 2002; Latz et al. 2004). Ahmad-Nejad et al. and Latz et al. reported that CpG-ODNs move into early endosomes and are then transported to a tubular lysosomal compartment. In accordance with this, TLR9 redistributes from the ER to these structures where the CpG-ODNs are located and where MyD88 can also accumulate.

It is highly likely that other large DNA viruses whose genomes are rich in CpG motifs are also recognized by TLR9. Only very recently, Basner-Tschakarjan et al. reported that the dsDNA virus, adenovirus efficiently activates pDCs in a TLR9-dependent manner, resulting in maturation and IFN-α production (Basner-Tschakarjan et al. 2006).

Another intriguing aspect of TLR9 function is that its activation can also be triggered by self-DNA. DNA-containing immune complexes (ICs) isolated from sera of SLE patients have been shown to trigger TLR9 (Boule et al. 2004; Leadbetter et al. 2002; Means et al. 2005), and this stimulation is inhibited either by agents that block TLR9 signaling or by directly inhibiting TLR9 itself (Leadbetter et al. 2002). Thus, a mechanism must exist to ensure that TLRs involved in nucleic acid recognition (TLR3, 7, 8, 9) can discriminate between foreign and self nucleic acids. Recently, Barton et al. very elegantly described that a chimeric TLR9 receptor, which localizes to the cell surface, responded normally to synthetic CpG-DNA but not to nucleic acids contained in viral particles. However the relocated chimeric TLR9 gained the ability to recognize self-DNA, which does not stimulate wild-type TLR9 (Barton et al. 2006). So, it appears that the intracellular localization of TLR9 is not required for ligand recognition as was initially proposed but instead controls access of the receptor to different sources of DNA. Viral DNA can be methylated as is the case for self-DNA; therefore the immune system has adopted a strategy for viral recognition: the recognition of viral nucleic acids within endosomal compartments. This can be a critical mechanism to properly discriminate between self or foreign nucleic acids and to maintain homeostasis within the immune system.

In addition to the recognition of viral nucleic acids, it has also been reported that several viral proteins are detected by TLRs located on the surface of host cells. The hemagglutinin (HA) protein of measles virus activates human cells in a TLR2-dependent manner (Bieback et al. 2002). Human cytomegalovirus (HCMV) has also been shown to trigger TLR2 signaling (Compton et al. 2003). A role for TLR4 in virus recognition was first described in the case of the fusion (F) protein of RSV (Kurt-Jones et al. 2000). More recently, the envelope proteins (env) from both mouse mammary tumor virus (MMTV) and Moloney murine leukemia virus (MMLV) (Burzyn et al. 2004) activate murine monocytes and bone-marrow-derived macrophages, respectively, in a TLR4-dependent manner.

In conclusion, there is ample evidence that TLRs participate in viral recognition. TLR2 and TLR4 recognize viral glycoproteins on virions while the intracellular TLR3, 7, 8 and 9 detect naked viral nucleic acids.

3
IFN Gene Induction During Viral Infections: Pathways Activated by TLRs

3.1
MyD88 or TRIF? This Is the Question!

Among the five different adapter molecules containing the TIR domain, MyD88 was the first identified and shown to be critical for TLR and IL1R family signaling (Kawai et al. 1999). MyD88 can associate with all TLRs (Medzhitov et al. 1998) with the exception of TLR3 (Oshiumi et al. 2003a; Yamamoto et al. 2003). MyD88 has an amino terminal death domain (DD) and a carboxy-terminal TIR domain. The TIR domain is involved in the interaction with TLRs and other adapters (see below) while the death domain associates with members of the IL-1R-associated kinase (IRAK) family (Martin and Wesche 2002). IRAK-1 is recruited to MyD88 via DD–DD interactions within a complex with another protein termed Toll-interacting protein (Tollip) (Burns et al. 2000). This IRAK1-MyD88 association triggers hyperphosphorylation of IRAK1 by itself as well as phosphorylation by the related kinase, IRAK-4 (Cao et al. 1996; Li et al. 2002). These events lead to the dissociation of IRAK1 from MyD88 and Tollip and its interaction with the downstream adaptor tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF-6) (Burns et al. 2000). TRAF-6, a RING domain ubiquitin ligase activates the TAK1 kinase through K63-linked polyubiquitination (reviewed in Chen 2005). TAK1 in turn activates the IKK complex, which phosphorylates IκBs and targets these NF-κB inhibitors for ubiquitination and degradation by the proteosome. NF-κB is then released and translocates to the nucleus where it can induce several hundred target genes (Medzhitov et al. 1997; O'Neill 2002).

The diversity of TLR signaling pathways was revealed following the analysis of the response of MyD88-deficient macrophages to Gram-negative bacteria-derived lipopolysaccharide (LPS) (Kawai et al. 1999). LPS, which signals via TLR4 and MD2, can still trigger the activation of NF-κB and MAPK in cells from MyD88 knockout mice, albeit with delayed kinetics compared with wild type cells, whereas most other TLR ligands are completely ineffective at triggering these events in the absence of MyD88. Although MyD88-deficient mice lose their ability to induce proinflammatory cytokines in response to LPS, they are still able to upregulate co-stimulatory molecules and induce type I

IFNs and IFN-inducible genes (ISGs) (Kaisho et al. 2001; Kawai et al. 2001). Subsequent studies from several groups identified another adapter TRIF that regulates these MyD88-independent pathways (Fitzgerald et al. 2003b; Hoebe et al. 2003; Yamamoto et al. 2003). TRIF knockout mice are compromised in the induction of type I IFNs and the expression of ISGs in response to LPS and the dsRNA mimetic poly(I:C), a TLR3 ligand. Both TLR4 (Navarro and David 1999) and TLR3 (Fitzgerald et al. 2003b; Oshiumi et al. 2003a; Yamamoto et al. 2002) signaling cascades activate the nuclear translocation and DNA binding of the transcriptional regulator, IRF3, a key regulator of IFN-β and ISGs, a process mediated solely by TRIF in the case of TLR3 signaling (Fitzgerald et al. 2003b; Hoebe et al. 2003; Yamamoto et al. 2003). In the case of TLR4 signaling, an additional adapter, TRAM is also required to recruit TRIF to TLR4 (Bin et al. 2003; Fitzgerald et al. 2003b; Oshiumi et al. 2003b). TRAM is modified by N-terminal myristoylation, which is important in tethering TRAM to the plasma membrane, where it co-localizes with TLR4 (Rowe et al. 2006). This function of TRAM appears to be important in recruiting TRIF to membrane-localized TLR4. A fourth adapter molecule Mal (also called TIRAP) also participates in TLR4 signaling. In contrast to TRIF and TRAM, however, Mal appears to be important in the recruitment of MyD88 to TLR4 to regulate inflammatory cytokine genes (Fitzgerald et al. 2001; Horng et al. 2001; Kagan and Medzhitov 2006).

TLR3-mediated NF-κB activation is also triggered by a TRIF-dependent mechanism. The C-terminus of TRIF associates with the serine threonine kinase receptor interacting protein-1 (RIP1) through a RIP homotypic interaction motif (Meylan et al. 2004). RIP-1-deficient cells fail to activate NF-κB in response to poly (I:C) (Meylan et al. 2004), whereas IRF3 activation remains intact (Cusson-Hermance et al. 2005). The TRIF N-terminal region has also been shown to associate with TRAF6 in overexpression systems (Sato et al. 2003). Studies using macrophages from TRAF6-deficient mice, however, suggest that the exact requirement for TRAF6 in the TLR3 response to NF-κB is still a little unclear, probably due to functional redundancy with other TRAF proteins in certain cell types (Gohda et al. 2004). TAK-1 is also involved in TLR3-mediated NF-κB and MAPK activation (Sato et al. 2005). Recent studies have also shown that TRIF and MyD88 can bind to a second TRAF family member TRAF3, which activates IRFs to induce type IFNs. TRAF3 does not appear to be required for the induction of proinflammatory cytokines, however (Hacker et al. 2006; Oganesyan et al. 2006).

Transcriptional regulation of the IFN-β gene requires the activation of IRF3, ATF-2/c-Jun, and NF-κB. These transcription factors form a multiprotein complex, the enhanceosome on the IFN-β enhancer (Maniatis 1986). In the resting state, IRF3 is localized to the cytoplasm. In response to a viral challenge, IRF3 is phosphorylated on multiple serine/threonine residues, which control

its dimerization. In this active form, IRF3 then translocates to the nucleus and associates with the coactivators CREB-binding protein (CBP)/p300 on the IFN-β enhancer. The IκB-related kinases, inhibitory protein κB kinase (IKK)ε (also called IKK*i* ;Shimada et al. 1999) and TANK-binding kinase (TBK1) (also called NAK [Tojima et al. 2000] or T2K [Bonnard et al. 2000]), phosphorylate IRF3 (Fitzgerald et al. 2003a; Sharma et al. 2003). IKKε and TBK1 are structurally related to IKKα and IKKβ. but, unlike IKKα or IKKβ, do not appear to be involved in NF-κB activation (McWhirter et al. 2004; Sharma et al. 2003). Sharma et al. and Fitzgerald et. al. showed that blocking IKKε and TBK1 activity using RNA interference prevented Sendai virus-induced IRF3 phosphorylation and subsequent activation of the IFN promoter (Fitzgerald et al. 2003a; Sharma et al. 2003). Fitzgerald et al. also described a requirement for IKKε and TBK1 in poly (I:C)-induced IRF3 activation via TLR3 and TLR4 (Fitzgerald et al. 2003a; McWhirter et al. 2004). TBK1$^{-/-}$ embryonic fibroblasts fail to activate IRF3 and induce IFN-β, IFN-α, or ISGs in response to virus, LPS or poly (I:C) (McWhirter et al. 2004). TBK1 is ubiquitously expressed, while IKKε expression is restricted to lymphoid cells, even if it can be inducible in several other cell types. Moreover, IKKε may be functionally redundant with TBK1 in cells where both are expressed (Hemmi et al. 2004; Perry et al. 2004). Perry et al. showed that the Sendai virus-induced IFN response in TBK1$^{-/-}$ embryonic fibroblasts could be partially restored by reconstitution with wild-type IKKε but not with a mutant lacking the kinase activity (Perry et al. 2004).

A schematic representation of the signaling pathways downstream of TLR3 and TLR4 and the role of the adapters TRIF and TRAM in regulating these events are shown in Fig. 1.

3.2 MyD88-Dependent Pathways in pDCs

The first report that described cells with plasma cell morphology in the T cell areas of human reactive lymph nodes was published in 1958 (Lennert and Remmele 1958). These cells were named T-associated plasma cells. Only in 1999, after much debate and several controversial manuscripts, Siegal et al. (1999) reported that the plasmacytoid DCs indeed represented the previously characterized IFN-producing cells (Fitzgerald-Bocarsly 1993; Svensson et al. 1996). In the intervening years, the morphology and functions of pDCs have been fully characterized, together with their intracellular signaling cascades (Barchet et al. 2005; Liu 2005). Following viral infections, human and mouse pDCs are capable of producing up to 10 pg/cell of type I IFNs, making them 10- to 100-fold more efficient than other cell types, including mDCs (Fitzgerald-Bocarsly et al. 1988; Siegal et al. 2001). Moreover, unlike mDCs, pDCs do not express

TLR2, TLR3, TLR4, or TLR5, and therefore they do not respond to the ligands of these TLRs. Remarkably, the TLRs expressed by pDCs are restricted to those that enable recognition of DNA and RNA viruses. In fact, human and murine

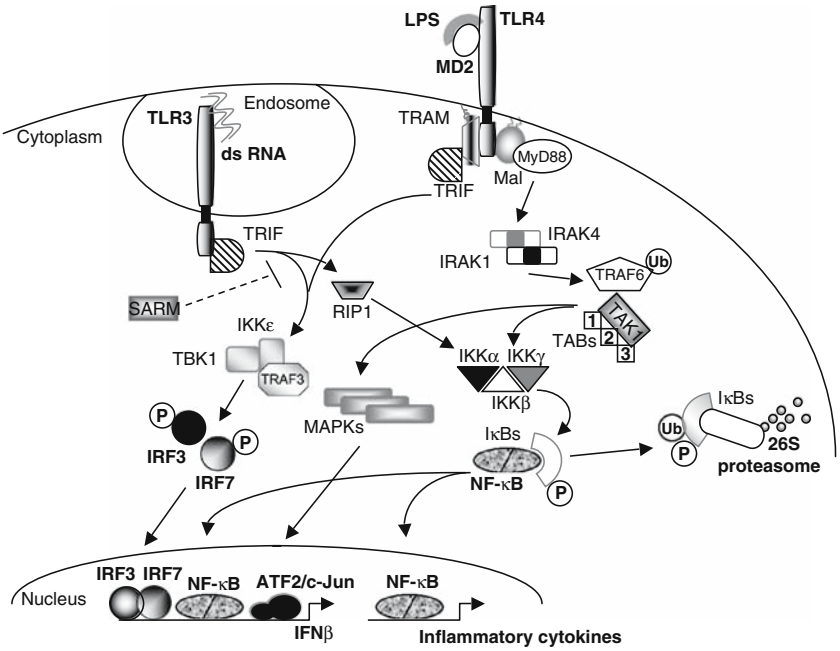

Fig. 1 TRIF-dependent pathways regulating TLR3- and TLR4-mediated activation of IRF3/7 and NF-κB. The adapter molecule Mal/TIRAP contains a phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain, which is important in mediating the recruitment of MyD88 to TLR4. MyD88 associates with the downstream serine/threonine kinases IRAK-1 and -4. A dimeric E2 (or ubiquitin conjugating enzyme) consisting of Ubc13 and Uev1A polyubiquitinates target proteins, including TRAF6. K63-polyubiquintated TRAF6 mediates activation of TAK1-associated proteins TAB2 and TAB3, which interact with K63-ubiquitin chains. The IKK complex is then activated, leading to NF-κB activation. TLR3 signaling to this pathway bypasses MyD88 and IRAKs and possibly TRAF6. Instead TLR3 uses RIP1, which may also be ubiquitinated by TRAF6. Both TLR3 and TLR4-mediated activation of IRF3/7 and the induction of IFN-β take place in a MyD88-independent manner and require TRIF and the IKK-related kinases, IKKε and TBK1. The adapter TRAM (TRIF-related adaptor molecule) is tethered to the plasma membrane via N-terminal myristoylation, which is required to recruit TRIF to the TLR4 cytoplasmic domain. IRF7 is also activated by the IKKε/TBK1 pathway, although it is unclear if transcriptional regulation via IFN-β is required or if this is direct. The TRIF-dependent pathways are negatively regulated by SARM

pDCs express only TLR7 and TLR9 (Bauer et al. 2001; Boonstra et al. 2003; Iwasaki and Medzhitov 2004; Jarrossay et al. 2001; Kadowaki et al. 2001; Krug et al. 2001) and can promptly produce large amounts of type I IFNs in response to either imidazoquinoline compounds (Ito et al. 2002), ssRNA-ODNs, ssRNA viruses (Heil et al. 2004), or CpG-ODNs and DNA viruses (Kadowaki et al. 2001; Krug et al. 2001).

TLR7 is closely related to TLR9 phylogenetically and as such these two receptors have several features in common (Wagner 2004). The signaling pathways activated by these TLRs are completely dependent on MyD88, and there is no evidence that other TIR-domain-containing adapters are involved (Hemmi et al. 2003). In contrast to what was observed in TLR3- and TLR4-activated signaling to IFN genes, TRIF is completely dispensable for type I IFN gene induction in the TLR7 and TLR9 pathways (Hemmi et al. 2000, 2002, 2000). Because the induction of type I IFNs is crucially dependent on the activation of IRFs, this raised the intriguing question of how these TLRs could activate IRFs without the help of TRIF. Compared to mDCs, pDCs express constitutively very high levels of IRF7 (Coccia et al. 2004; Izaguirre et al. 2003). Most cell types, including mDC, require upregulation of IRF7 in response to type I IFN feedback signaling, in order to secrete IFN-α subtypes. In contrast, pDCs are capable of rapidly secreting IFN-α even in the absence of the IFN autocrine loop due to this high basal expression of IRF7 (Barchet et al. 2002). Come clarity to this issue was provided by the observation that the engagement of TLR7 and TLR9 did not lead to the activation of IRF3, but instead activated the related factors IRF7 (Honda et al. 2004; Kawai et al. 2004) and IRF5 (Schoenemeyer et al. 2005). In a key paper from Honda et al., IRF7 has been named the master regulator of type I IFN-dependent immune response (Honda et al. 2005). Using splenic pDCs purified from IRF7 knockout mice, the authors demonstrated that the induction of IFN-α and IFN-β upon HSV-1 and VSV infection, which activate TLR9 (Krug et al. 2004) and TLR7 (Lund et al. 2004), respectively, is completely dependent on IRF7, whereas no difference was observed in IRF3-deficient pDCs. Type I IFN induction was also completely IRF7-dependent when the cells were stimulated with the TLR9 ligand, CpG-ODNs (Honda et al. 2005). Thus in the pDCs, IRF7 and not IRF3 is the key mediator of IFN-α and IFN-β gene expression.

Major advances in understanding how type I IFN production is triggered in the TLR7 and TLR9-activated pathways have been made with the discovery that IRF7 interacts directly with MyD88 to form a complex in the cytoplasm (Honda et al. 2004; Kawai et al. 2004). Moreover, this complex involves the IRAK1/4 kinases and TRAF6 (Honda et al. 2004; Kawai et al. 2004). Data from Kawai et al. has suggested that in addition to being phosphorylated, IRF7 is also ubiquitinated and that the ubiquitin ligase activity of TRAF6 is important

for this event (Kawai et al. 2004). Although IRF7 activation can occur via phosphorylation through the action of the IKKε and/or TBK1 kinases as part of the secondary feedback loop (Caillaud et al. 2005; Sharma et al. 2003), it is unclear at present if either of these kinases participate in TLR7/9 signaling to IRF7 in pDCs. What is clear is that the IRAK kinases participate in the phosphorylation of IRF7 in pDCs (Uematsu et al. 2005). IRAK1 interacts with and phosphorylates IRF7 in vitro and the kinase activity of IRAK1 is necessary for the activation of IRF7. TLR7 and TLR9 ligands are severely impaired in their ability to activate IRF7 and induce IFN-α in IRAK1- and IRAK4-deficient pDCs. A very recent study has also identified a role for IKKα in IRF7 activation in TLR7/9 signaling (Hoshino et al. 2006). Hoshino et al. demonstrated that TLR7/9-induced IFN-α production was severely impaired in IKKα-deficient pDCs and a kinase-deficient IKKα blocked the ability of MyD88 to activate the IFN-α promoter in synergy with IRF7 in overexpression experiments. All of these findings highlight the importance of IRF7 in TLR7 and TLR9 signaling and are summarized in Fig. 2.

3.3 IRF5: The Outsider

Many members of the IRF family are important in innate and/or acquired immunity. Although they share a similar DNA-binding domain at their N-terminus, the different IRFs possess unique characteristics that result in unique protein–protein and protein–DNA interactions leading to unique functions. In most viral infections, dsRNA and LPS signaling can activate IRF3 and IRF7 (Doyle et al. 2002; Fitzgerald et al. 2003b; Kawai et al. 2001). In contrast, the activation of IRF5 is much more restricted. It occurs upon infection with Newcastle disease virus (NDV), VSV, and HSV (Barnes et al. 2002, 2003), while no effect has been detected following Sendai virus infection or dsRNA treatment (Schoenemeyer et al. 2005). Recently, an important role for IRF5 in TLR signaling has been emphasized (Schoenemeyer et al. 2005; Takaoka et al. 2005). IRF5 seems to be highly involved in the induction of proinflammatory cytokines, such as TNF-α, IL-12, and IL-6; in fact, their expression is severely impaired upon TLR4, 5, 7, and 9 triggering in various cells from IRF5 knockout mice (Takaoka et al. 2005). Putative IFN-stimulated response elements in the promoters of these inflammatory cytokines are suggested to bind IRF5. TLR7 and 8 triggering by the imidazoquinoline R-848 induced nuclear translocation of IRF5 in murine macrophages (Schoenemeyer et al. 2005), whereas IRF5 could not be activated by either the TLR3/TRIF pathway or upon SV infection. Data from several groups have shown that SV is detected by the recently identified RNA helicase RIG-I (Rothenfusser et al. 2005; Yoneyama et al. 2004).

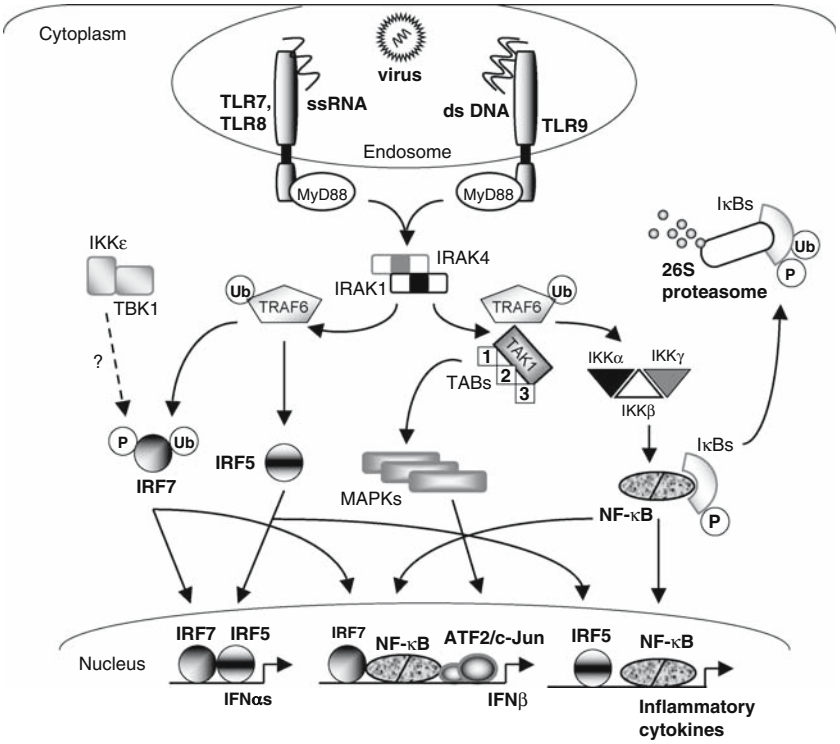

Fig. 2 MyD88-dependent pathways in pDCs. Recognition of viral ssRNA and dsDNA via TLR7/8 and TLR9, respectively, triggers the recruitment of MyD88, which in turn interacts with IRAKs and TRAF6. TRAF6-mediated ubiquitination leads to the activation of TAK1 and ultimately to NF-κB and MAPK activation. IRF5 and IRF7 are also activated, via MyD88. IRAK1 is required to phosphorylate IRF7. IRF7 is also ubiquitinated via K63-polyubiquintation. The activated form of IRF7 can translocate to the nucleus and activate the transcription of IFN-β and IFN-α genes. TRAF6 and IRAK1 are also involved in the activation of IRF5, which is essential for inflammatory cytokine gene induction. IRF5 is activated by all TLRs which signal via MyD88

Several earlier studies had shown that IRF5 and IRF7 could regulate the expression of overlapping as well as distinct IFN-α subtypes (Barnes et al. 2002). In human cells, Schoenemeyer et al. demonstrated that ectopic expression of IRF5 enabled type I IFN production following TLR7 triggering and that knockdown of IRF5 by siRNA in human monocytes reduced this response. In contrast, Takaoka et al. showed that the induction of IFN-α in response to the TLR9

ligand, CpG-ODNs was normal in pDCs derived from IRF5-deficient mice. Observations from Mancl et al. identified nine distinct alternatively spliced IRF5 mRNAs (V1-V9) that have cell type-specific expression, localization, inducibility, and function in virus-mediated type I IFN gene induction (Mancl et al. 2005). Further investigations are needed to better understand the exact role of IRF5 in IFN induction in different pathways and in different cell types.

Consistent with a role for IRF5 in the regulation of inflammatory cytokine production, Schoenemeyer et al. showed that IRF5 is part of a complex with MyD88 and TRAF6 (Schoenemeyer et al. 2005), similarly to IRF7 (Kawai et al. 2004). This resemblance between MyD88-mediated activation of IRF7 and IRF5 is further enforced by the observation that IRAK-1 kinase is important in IRF5 activation (Schoenemeyer et al. 2005). IRF5 can also be phosphorylated and activated upon ectopic expression of TBK1 and IKKε (Cheng et al. 2006). The physiological relevance of these observations remains to be clarified, however, since inflammatory cytokine production (which is controlled by IRF5) is induced normally in TBK1 or IKKε knockout cells (Hemmi et al. 2004; N. Goutagny and K.A. Fitzgerald, unpublished data). MyD88 also interacts with IRF4, which appears to negatively regulate the IRF5 signaling pathway (Negishi et al. 2005). IRF4 deficiency does not affect the ability of TLR7/9-stimulated pDCs to secrete IFN-α but caused overproduction of inflammatory cytokines. This was accompanied by enhanced activation of NF-κB and MAPKs. This hyperreactivity is observed not only in TLR7/9 but also in TLR2/4 signaling. IRF4, but not IRF7, can compete with IRF5 for association with MyD88, which can account for this phenotype of the IRF4 knockout mice. Our current understanding of the role of IRF5 in the antiviral immune responses is shown in Fig. 2.

3.4 Negative Regulators of MyD88 and TRIF Signaling

Several endogenous negative regulators of TLR signaling have been described for the MyD88-dependent pathway. MyD88s is the short form of MyD88 and its overexpression inhibits IL-1- and LPS- but not TNF-induced NF-κB activation (Janssens et al. 2003). Another inhibitor of the MyD88-mediated pathway is IRAK-M, a member of the IRAK kinase family (Wesche et al. 1999), which has been shown to block the formation of IRAK1–TRAF6 complexes (Kobayashi et al. 2002). A different level of regulation occurs through SOCS1, one of eight members of the SOCS family important in suppressing cytokine signaling (Alexander 2002). SOCS1 represses LPS-induced NF-κB activation in a TLR4- and MD2-dependent manner (Kinjyo et al. 2002), and Mansell et al. demonstrated recently that SOCS1 is required for the ubiquitin-proteasome-mediated degradation of Mal (Mansell et al. 2006). The inhibitory effect of

SOCS1 on TLR signaling can also be indirect by blocking type I IFN signaling itself (Baetz et al. 2004; Gingras et al. 2004). Several additional negative regulators of the MyD88 pathway have been described, including PI3K (Fukao et al. 2002), Tollip (Zhang and Ghosh 2002), A20 (Boone et al. 2004), ST2 (Brint et al. 2002), SIGIRR (Wald et al. 2003), and RIP105 (Divanovic et al. 2005), all acting at different levels of the intracellular cascade.

Much less is known about negative regulation of the TRIF-IRF3 response. Carty et al. recently demonstrated that the fifth TIR-domain containing adapter SARM acts as a negative regulator of TRIF signaling (Carty et al. 2006). SARM interacts directly with TRIF leading to a block in gene induction downstream of TRIF. SARM does not target the MyD88 pathway. Knockdown of SARM by siRNA leads to enhanced TRIF-dependent cytokine and chemokine induction.

As discussed above, the IKK-related kinases TBK1 and IKKε are involved in IRF3 activation downstream of TRIF. SIKE (for suppressor of IKKε) is a protein that interacts with both TBK1 and IKKε and dissociates from them upon viral infection or TLR3 stimulation (Huang et al. 2005). Overexpression of SIKE blocks the interaction of TBK1 and IKKε with TRIF and IRF3, but does not influence the interaction of TRIF with TRAF6 or Rip1, essential for NF-κB activation. siRNA targeting of SIKE potentiated virus- and TLR3-induced IRF3 responses. Very recently, Saitoh and colleagues demonstrated that the peptidyl prolyl isomerase Pin1 also negatively regulates the IRF3 pathway. Pin1 associates with activated IRF3 and promotes the ubiquitin-mediated degradation by the proteosome. Phosphorylation of IRF3 on Ser339/Pro440 upon stimulation with poly (I:C), LPS or Newcastle virus is associated with this destabilization of IRF3 (Saitoh et al. 2006). IRF3 and Pin1 interact only when IRF3 is phosphorylated on Ser339. Ectopic expression of Pin1 blocks IRF3 activation and IFN-β production downstream of TLR3 and 4 and the RIG-I pathway. As expected, Pin1-deficient mice produce much more IFN-β in response to dsRNA compared to wild type mice in vivo.

4 Concluding Remarks and Some Speculations

In the last few years we have witnessed an enormous improvement in our understanding of the delineation of TLR signaling, particularly in relation to the pathways that regulate IRF activation. The TLR pathway is particularly important in the pDCs for the detection of viral RNA and DNA associated with endocytosed viral particles. However, it is now becoming increasingly clearer that, in most other cell types, TLR-independent sensors are more critical for antiviral defenses. These TLR-independent sensors include the recently

discovered cytoplasmic RNA helicases, RIG-I (Yoneyama et al. 2004) and Mda-5 (Kang et al. 2004) and a putative cytosolic DNA sensor, which remains to be defined. Signaling through these cytoplasmic receptors converges on many of the same signaling intermediates as those employed by the TLRs. Elucidation of the cross-talk between these different sensors and pathways in the response to a given virus remains a key challenge in our quest to understand innate immunity to viruses.

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Regulation of Antiviral Innate Immune Responses by RIG-I Family of RNA Helicases

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Abstract The recognition of viral nucleic acids with pattern recognition receptors (PRRs) is the first step in inducing the innate immune system. Type I interferons (IFNs), central mediators in antiviral innate immunity, along with other cytokines and chemokines, disrupt virus replication. Recent studies indicated at least two distinct pathways for the induction of type I IFN by viral infection. Toll-like receptors (TLRs) are extracellular or endosomal PRRs for microbial pathogens, whereas retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are novel intracellular PRRs for the viral dsRNA. In this review, we describe the distinct mechanisms inducing type I IFNs through TLRs and RIG-I/MDA5 pathways.

1 Introduction

Higher organisms including humans are equipped to counteract infecting viruses using two kinds of immune responses: innate and adaptive immunity. Unlike adaptive immunity, which is characterized by its specificity and memory, innate immunity is provoked early in infection and is critical for an initial

antiviral response. The type I interferon (IFN) system plays a major role in antiviral innate immunity (Samuel 2001; Stetson and Medzhitov 2006). Upon viral infection, type I IFN is secreted in body fluid and expands IFN response signals, resulting in the activation of various enzymes that prevent viral replication. In addition to antiviral activity, type I IFN has been known to exert various biological effects such as cell cycle regulation, differentiation, and immune modulation. Furthermore, innate immune responses lead to the activation of specific cells with antigen-presenting functions to facilitate the initiation of adaptive immunity.

The triggering of the IFN system is the activation of IFN genes. Since the initial discovery of type I IFN, the activation mechanism of the type I IFN genes has been a major focus of many researchers. Although several double-stranded (ds) RNA-binding proteins such as protein kinase-activated by RNA (PKR) have been attributed to the detection of replicating viral RNA, gene knockout studies do not support its role (Yang et al. 1995). Recent functional analyses revealed that TLRs function as pathogen receptors including those of viral origin (Takeda and Akira 2005). TLR3 has been identified as a receptor for exogenous dsRNA (Alexopoulou et al. 2001); however, TLR3-deficient cells can still activate type I IFN genes (Diebold et al. 2003; Yoneyama et al. 2004), suggesting the existence of other receptor(s). Screening of an expression cDNA library identified RIG-I as an essential receptor for virus-derived dsRNA (Yoneyama et al. 2004). In this article, we describe the recently identified function of the RIG-I family of RNA helicases in innate immune reactions to infecting viruses.

2
The Role of TLR and RIG-I Family Helicases in Viral Infection

2.1
TLR Detects Extracellular Pathogen-Associated Molecular Patterns

Toll was first identified as a transmembrane receptor regulating insect morphogenesis (Hashimoto et al. 1988). Toll mutation also results in increased sensitivity to fungi in *Drosophila* (Lemaitre et al. 1996), leading to the identification of mammalian Toll-like receptors (TLRs) as sensing receptors of various pathogen-associated molecular patterns (PAMPs) (Medzhitov et al. 1997). Ten members of human TLRs are expressed in a tissue-specific manner and many are expressed in dendritic cells (DCs) and macrophages (Takeda and Akira 2005). Although each TLR detects a distinct set of PAMPs, a common extracellular leucine-rich repeat (LRR) motif is responsible for sensing. When LRR

detects a pathogen, a signal is generated in the cytoplasm, which is mediated by the cytoplasmic domain of the receptor. TLR activation results in the production of various cytokines, leading to the activation of innate immune responses (as described in this volume by Severa and Fitzgerald). Upon TLR activation, macrophages and DCs differentiate into antigen-presenting cells initiating antigen-specific acquired immunity. Viral infection is sensed by three TLRs: TLR3 (Alexopoulou et al. 2001), TLR7/8 (Diebold et al. 2004; Heil et al. 2004; Lund et al. 2004), and TLR9 (Hemmi et al. 2000; Krug et al. 2004; Lund et al. 2003), which are mostly expressed on the endosomal membrane (Fig. 1). Double-stranded RNA (dsRNA), single-stranded RNA, and unmethylated CpG DNA are detected by TLR3, TLR7/8, and TLR9, respectively (Fig. 2). This subset of TLRs activates transcription factors including NF-κB, IRF-3, and

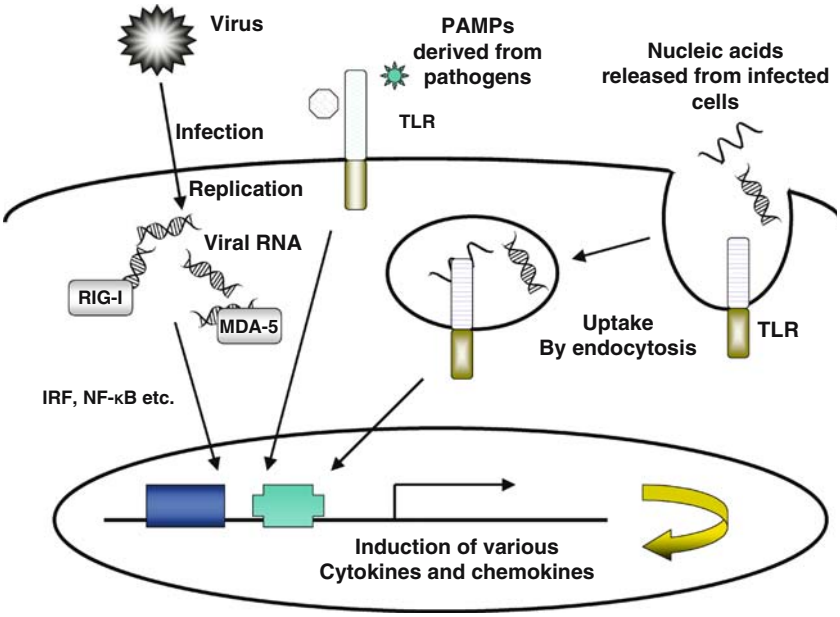

Fig. 1 Recognition of PAMPs by TLR and RIG-I family helicases. Transmembrane receptor TLR is expressed on the plasma or endosomal membranes and senses extracellular PAMPs. RIG-I family helicases detect viral RNA in the cytoplasm. Activation of these receptors transduces signals resulting in overlapping, but in a different set of target genes, including cytokines and chemokines

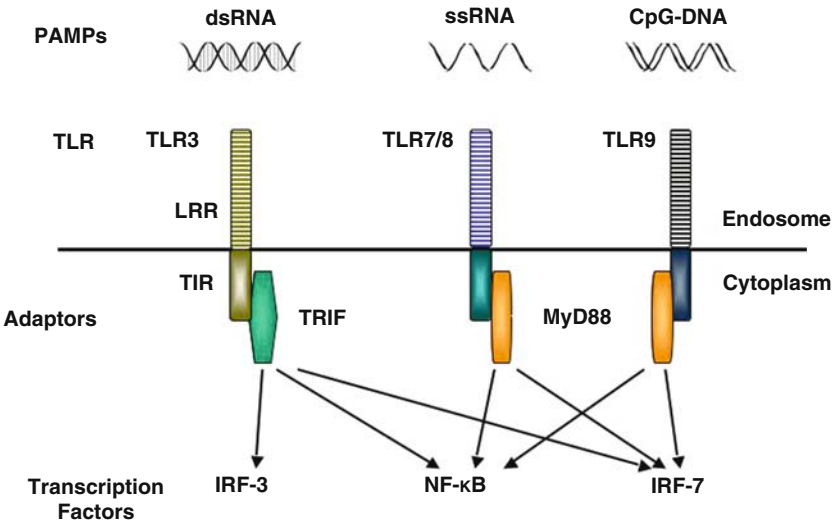

Fig. 2 Recognition of viral nucleic acids by different TLRs. TLR3, TLR7/8, and TLR9 detect dsRNA, ssRNA, and unmethylated CpG DNA. MyD88 adaptor is indispensable for signaling by TLR7/8 and 9, whereas another adaptor TRIF is essential for TLR-3 signaling

IRF-7 through common and distinct cytoplasmic adaptor molecules (Takeda and Akira 2005).

2.2 Cytoplasmic Receptor, RIG-I Helicase Family

Since dsRNA such as polyI:polyC is known to induce IFN synthesis, it is generally accepted that dsRNA is the major viral product responsible for the activation of innate immune responses. TLR3 was first shown to confer responsiveness to exogenously added polyI:polyC in HEK293T cells (Alexopoulou et al. 2001), and is thus hypothesized to function as a physiological sensor of replicating viruses. However, TLR-3-deficient cells are still responsive to viral infection or poly I:poly C transfection (Yoneyama et al. 2004), suggesting an alternative cytoplasmic sensor.

Functional screening identified human RIG-I as putative positive regulator of IFN genes (Yoneyama et al. 2004). RIG-I is a putative RNA helicase containing two repeats of caspase recruitment domain (CARD) at the N-terminal region and a DExH/D box helicase homology region at its C-terminal region (Fig. 3). RIG-I exhibits specific binding activity to dsRNA. Overexpression of

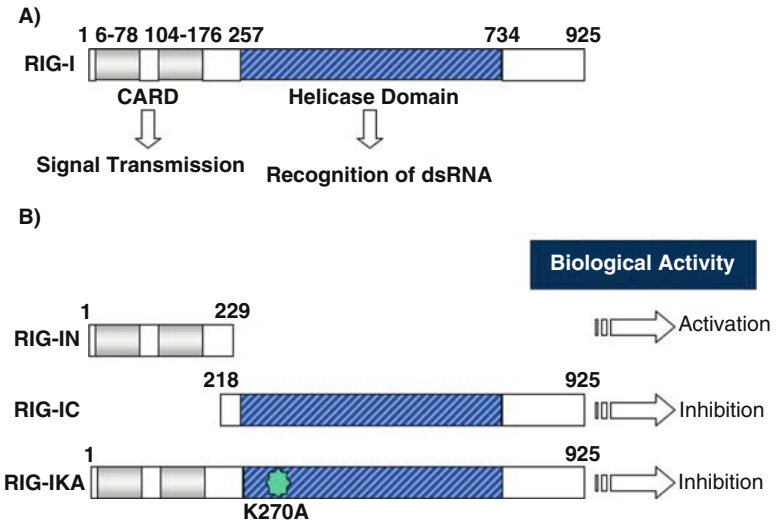

Fig. 3 A, B Structure–function relationship of RIG-I. **A** Structure of RIG-I. **B** Biological activity of RIG -I mutants

RIG-I in cultured cells did not significantly activate the IFN promoter; however, overexpression of the N-terminal region containing two CARD repeats alone constitutively activated the IFN promoter. This suggests that CARD is essential and sufficient for signaling, and is under negative regulation by the C-terminal region. Full-length RIG-I is present as an inactive form; however, it can be activated by viral infection or transfection of dsRNA. This supports the speculation that inhibition of CARD by the C-terminal region is reversed by dsRNA. Interestingly, RIG-I lacking CARD acts as a dominant-negative inhibitor of virus-induced activation of IFN-β promoter. Furthermore, K270A mutant, which has disrupted ATP binding motif within the conserved helicase domain, also functions as a dominant inhibitor. These observations suggest that, in addition to dsRNA binding, ATP hydrolysis is necessary for the induced unmasking of CARD.

In the human genome database, there are two other genes encoding RIG-I-related helicases, MDA5 and LGP2 (Yoneyama et al. 2005). MDA5 exhibits a similar domain structure as RIG-I, characteristic of two repeat CARDs and the helicase domain (Fig. 4). The third helicase LGP2 lacks CARD. Functional analyses of these helicases, using cell culture, revealed that MDA5 functions as a positive signaling regulator, similar to RIG-I. Recent studies using gene disruption of RIG-I and MDA5 revealed that these helicases detect different viruses (Gitlin et al. 2006; Kato et al. 2005, 2006). MDA5 was essential for detection of picorna virus infection,

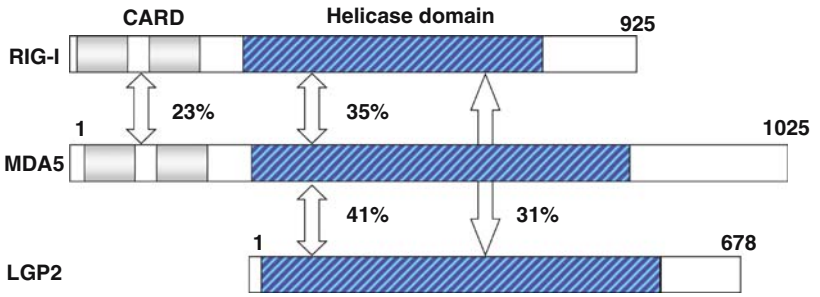

Fig. 4 Structure of RIG-I family helicases. Human and mouse RIG-I family consists of RIG-I, MDA5, and LGP2. Percentage indicates amino acid identity between corresponding domains

while RIG-I was critical for detection of other viruses types tested. Interestingly, this virus specificity likely reflects different RNA species generated by respective viruses. At present, the chemical basis of this difference is not known. Functional analyses of LGP2 in cell culture revealed that LGP2 dominantly inhibits the virus-induced activation of IFN genes (Rothenfusser et al. 2005; Yoneyama et al. 2005). Since LGP2 is transcriptionally induced by autocrine IFN, its function as a feedback negative regulator has been suggested.

3 Signaling Cascades of Antiviral Innate Responses

A comparison of signaling cascades initiated by the detection of dsRNA by TLR3 and RIG-I/MDA5 is illustrated in Fig. 5. TLR3 activation by dsRNA occurs in the endosome and the signal is transmitted through TRIF (Hoebe et al. 2003; Oshiumi et al. 2003; Yamamoto et al. 2002, 2003), TBK-1 (NAK, T2K)/IKKi (IKK-ε) kinases (Fitzgerald et al. 2003; Hemmi et al. 2004; McWhirter et al. 2004; Perry et al. 2004; Sharma et al. 2003). The latter kinases are responsible for a specific phosphorylation and activation of IRF-3. It was shown that TBK-1/IKKi kinases are under positive and negative regulation by NAP1 and SIKE, respectively (Huang et al. 2005; Sasai et al. 2005). RIG-I/MDA5 activates a novel adaptor IPS-1 (MAVS, Cardif, VISA) containing a single copy of CARD (Kawai et al. 2005; Kumar et al. 2006; Meylan et al. 2005; Seth et al. 2005; Sun et al. 2006; Xu et al. 2005). Interestingly, IPS-1 is anchored on the outer membrane of mitochondria via its C-terminal transmembrane domain (Seth et al. 2005). Although a mitochondrial association is critical for the signaling, its mechanism is elusive. IPS-1 apparently activates the

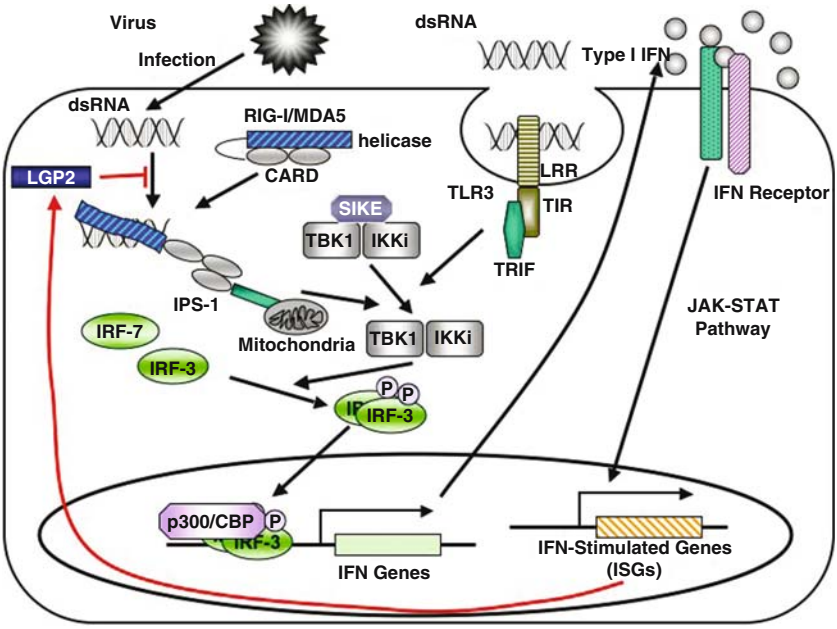

Fig. 5 Signaling cascade induced by dsRNA. TLR3 recognizes extracellular dsRNA in endosomes. Upon dsRNA binding, the cytoplasmic domain of TLR3 transmits a signal to an adaptor, TRIF. Cytoplasmic dsRNA is recognized by RIG-I and MDA5. CARD of these helicases interacts with an adaptor, IPS-1, which localizes on the outer membrane of mitochondria. Signals mediated by TRIF and IPS-1 activate common protein kinases TBK-1 and IKK-I, resulting in phosphorylation-mediated activation of transcription factor IRF-3. IRF-3, as a complex with co-activator CBP or p300, activates target genes including type I IFN genes. Secreted IFN activates secondary signals through IFN receptor and JAK-STAT pathway to activate ISGs

IRF-3 kinases TBK-1/IKKi. Thus, RIG-I/MDA5 activates a distinct signaling cascade from TLR3 and the signal is converged at TBK-1/IKKi (Fig. 5).

4 Cell-Type-Specific Function of TLRs and the RIG-I Family

As mentioned earlier, TLR7/8 and TLR9 detect distinct viral PAMPs and activate signaling cascades, MyD88, IRAK1, and IRF-7 (Takeda and Akira 2005). So far, this signaling has been showed to be specific for plasmacytoid DCs (pDCs), which are responsible for the production of high levels of serum IFN-α (Fig. 6).

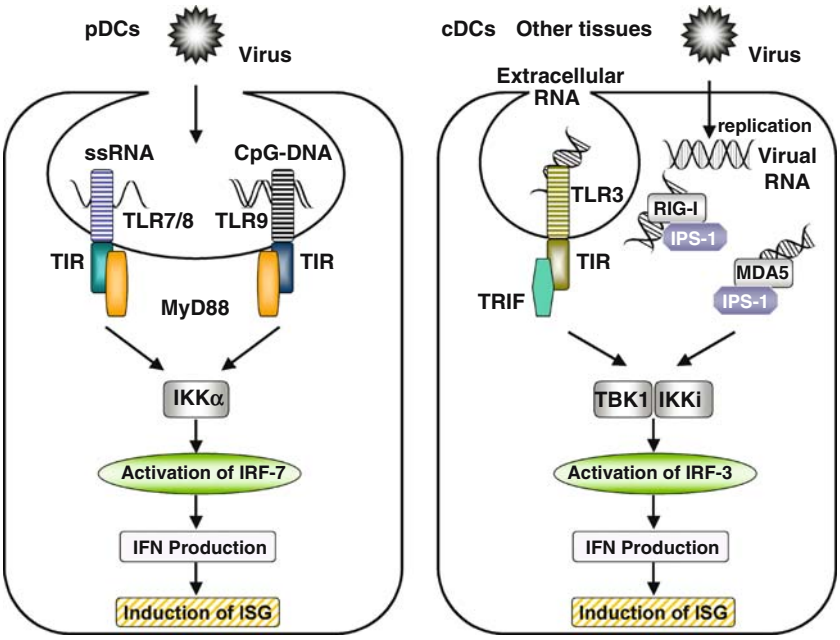

Fig. 6 Tissue-specific signaling cascade for IFN gene activation. Virus infection triggers a distinct signaling cascade in pDCs and other cell types including cDCs. TLR7/8 and TLR9 are specifically expressed in pDCs, whereas TLR3 is expressed in cDCs. IFN induction in pDC is dependent on MyD88, IKKα, and IRF-7, whereas these adaptors are dispensable in other cell types

pDCs and other cell types, including cDCs, use distinct pathways in a mutually exclusive manner to sense viral infections. As revealed by analysis using knockout mice, MyD88 but not RIG-I is essential in pDCs, and RIG-I but not MyD88 is critical in cDCs (Kato et al. 2005, 2006) (Fig. 6). The biological significance of TLR3 function in viral infection is not well established.

5 Viral Evasion Strategies for Antiviral Responses

With the elucidation of host antiviral response mechanisms, it has become evident that replication-competent viruses are equipped to counteract the antiviral mechanisms. It is well known that acutely infecting viruses, which undergo a lytic infection, selectively inhibit host macromolecular syntheses collectively

known as shut off. Apart from this nonspecific blockade, viruses encode inhibitory proteins, which target specific processes of the antiviral signaling.

V proteins of paramyxoviruses bind to MDA5 and inhibit its signaling (Andrejeva et al. 2004; Yoneyama et al. 2005). V protein of Sendai virus specifically binds to MDA5 but neither interaction nor blockade was observed with RIG-I; however, in light of the fact that paramyxoviruses are specifically detected by RIG-I, as evidenced by RIG-I knockout mice, its physiological relevance is controversial.

Hepatitis C virus (HCV) is known to be poorly adaptable to tissue culture for replication. One reason is its high sensitivity to IFN-mediated reaction: HCV replication requires host cell mutations that inactivate RIG-I signaling (Sumpter et al. 2005). HCV encodes a protein complex, NS3/4A, which acts as RNA helicase and protease. NS3/4A protease cleaves IPS-1 at its cytoplasmic domain, thus releasing it from mitochondria (Lin et al. 2006; Loo et al. 2006; Meylan et al. 2005). As IPS-1 is an essential adaptor for both RIG-I and MDA5 signaling and its association with mitochondria is obligatory, this cleavage completely blocks RIG-I/MDA5 signaling. Indeed, IPS-1 mutation at the cleavage motif or NS3/4A protease inhibitor restores the activation cascade stimulating the IFN genes.

NS1 protein of influenza A virus has been implicated in the inhibition of IFN gene activation. Using influenza A virus with NS1 mutation and RIG-I knockout mice, it was shown that NS1 blocks the signaling cascade triggered by RIG-I (Kato et al. 2006). NS1 is a dsRNA binding protein, thus sequestration of RIG-I from its ligand is one mechanism; however, the dsRNA-binding-deficient mutant of NS1 remains inhibitory (Donelan et al. 2003), suggesting multiple actions of this protein.

Ebola virus VP35 protein is another dsRNA binding protein inhibiting RIG-I-mediated signaling (Cardenas et al. 2006). Like NS1 of influenza A virus, VP35 may have dual inhibitory functions: in addition to dsRNA sequestration, it may be inhibiting steps downstream of IPS-1and IRF-3 kinases.

Since RIG-I and MDA5 are IFN-inducible and positive feedback is an important trait of the system, inhibition of IFN action, including IFN-R, by the JAK-STAT pathway remotely inhibits RIG-I and MDA5. In this regard, viral proteins that target IFN action are also inhibitory for IFN production.

6 Ligands for RIG-I and MDA5

In vitro binding studies revealed that RIG-I exhibits a specific binding activity to dsRNA, such as poly I:C, poly A:U, 5′ or 3′ non-coding genomic RNA of HCV synthesized in vitro, but not to poly A, tRNA, single-stranded region of HCV genomic RNA and dsDNA (Sumpter et al. 2005; Yoneyama et al. 2004).

MDA5 exhibits a much weaker binding activity to poly I:C. Inconsistent with the in vitro binding, functional analysis using knockout mice and cells deficient in either RIG-I or MDA5 revealed that dsRNA produced by in vitro transcription and poly I:C are specifically detected by RIG-I and MDA5, respectively (Kato et al. 2006). Furthermore, the RNA viruses tested were classified into two groups; picorna viruses (including EMCV) are specifically sensed by MDA5 and other viruses (including VSV, influenza virus and Sendai virus) by RIG-I. The specificity arises from different classes of RNA structure, as suggested by the results that RNA extracted from VSV and EMCV viral particles activated RIG-I and MDA5, respectively. This result includes noteworthy facts: VSV genomic RNA is unlikely to be highly double-stranded; under certain circumstances, viral replication may not be necessary to activate RIG-I. For dsRNA recognition, one report suggests the importance of end structure for selective activation of IFN genes or RNA interference (Marques et al. 2006). The search and elucidation for true ligands present in virus-infected cells for RIG-I and MDA5 is absolutely necessary to further our understanding of how self and non-self is recognized at the RNA level. At present there is no reasonable explanation to satisfy all these observations.

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Beyond Double-Stranded RNA-Type I IFN Induction by 3pRNA and Other Viral Nucleic Acids

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Abstract Production of type I IFN is the key response to viral infection. Since the discovery of type I IFNs in 1957, long double-stranded RNA formed during replication of many viruses was thought to be responsible for type I IFN induction, and for decades double-stranded RNA-activated protein kinase (PKR) was thought to be the receptor. Recently, this picture has dramatically changed. It now became evident that not PKR but two members of the Toll-like receptor (TLR) family, TLR7 and TLR9, and two cytosolic helicases, RIG-I and MDA-5, are responsible for the majority of type I IFNs induced upon recognition of viral nucleic acids. In this review, we focus on the

molecular mechanisms by which those innate immune receptors detect viral infection. Based on the recent progress in the field, we now know that TLR7, TLR9, and RIG-I do not require long double-stranded RNA for type I IFN induction.

1 History of Type I IFN Induction

Type I IFNs (IFN-α isoforms and IFN-β) are regarded as the dominant mediators of antiviral defense in vertebrates. Since their initial discovery half a century ago as acid-stable, soluble factors "interfering" with viral proliferation in cultured cells (Isaacs and Lindenmann 1957; Nagano and Kojima 1958), intense research has focused on type I IFN receptor signaling and the plethora of type I IFN-mediated effects (Theofilopoulos et al. 2005). For the host, an intact type I IFN response is critical for the survival of many viral infections (Gresser et al. 1976; Muller et al. 1994). Sensing of viral replication has been proposed to be responsible for triggering the production of type I IFNs by infected host cells. However, the specific host immune receptors and their respective molecular ligands remained elusive until very recently. Moreover, to mount an appropriate antiviral response, the innate immune system must distinguish viruses from bacteria, fungi, and multicellular parasites. Charles Janeway was the first to propose that the detection of highly conserved pathogen-associated molecular patterns (PAMPs) may be mastered by a limited number of germline-encoded pattern recognition receptors (PRRs) (Janeway 1989). A few years later, the first experimental evidence of such a receptor came from the fruit fly (Lemaitre et al. 1996). Shortly afterwards, a member of the family of toll-like receptors (TLR), the mammalian homolog of *Drosophila* toll, was demonstrated to be responsible for detecting lipopolysaccharides (LPS), a characteristic component of the cell walls of Gram-negative bacteria (Medzhitov et al. 1997; Poltorak et al. 1998). This observation was confirmed by the subsequent generation of TLR4-deficient mice (Hoshino et al. 1999).

Parasites, bacteria, and fungi rely on a multitude of molecules that are distant in evolutionary terms from the mammalian organism, and are thus readily discernible as non-self by members of the Toll-like receptor (TLR) and NOD-like receptor (NLR) families (reviewed in Meylan et al. 2005). In sharp contrast, all components of viruses are produced within the infected host cell, and therefore lack distinguishable non-self molecular patterns. Nevertheless, viruses are promptly recognized by the innate immune system and elicit pronounced antiviral type I interferon and cytokine responses. Shortly after the discovery of type I interferons, it was proposed that viral nucleic acids could be stimulating the type I IFN response (Isaacs et al. 1963). Many viruses synthesize

double-stranded RNA (dsRNA) during their replication cycle (Baltimore et al. 1964; Montagnier and Sanders 1963), whereas dsRNA was thought to be absent in uninfected cells. Therefore dsRNA formed during viral infection was postulated to be the molecular signature of viral infection. In support of this hypothesis, the enzymatically generated double-stranded RNA polynucleotide polyinosinic:polycytidylic acid (poly I:C) was found to be a potent inducer of type I IFN (Field et al. 1967). Although the authors carefully emphasized that all other double-stranded polynucleotides were inactive, the notion that long viral double-stranded RNA elicits type I IFN became commonplace, and poly I:C has been used as an interferon-inducing mimic of viral dsRNA ever since.

2
Long Double-Stranded RNA and the Activation of Antiviral Effector Molecules

In early attempts to uncover the inducers of interferon and of other mediators of antiviral activity, IFN-α and poly I:C-treated or reticulocyte extracts were analyzed. Chromatographic separation of lysates revealed proteins that were increased by preincubation with IFN-α, and whose enzymatic activity depended on the presence of dsRNA (usually poly I:C) (Farrell et al. 1978; Hovanessian et al. 1977; Zilberstein et al. 1978). Two proteins, interferon inducible double-stranded RNA-activated protein kinase (PKR) and the 2′,5′ oligoadenylate synthetase (OAS) could be affinity purified using poly I:C-cellulose (Farrell et al. 1978; Hovanessian et al. 1977). Both activated PKR and OAS were found to block translation of viral RNA by distinct mechanisms. In the presence of poly I:C, OAS catalyzes the synthesis of 2′,5′ oligomers of adenosine (2-5As) (Hovanessian et al. 1977; Zilberstein et al. 1978), which activate RNase L (Farrell et al. 1978). RNase L in turn degrades single-stranded viral and cellular RNAs (Farrell et al. 1978) in a sequence-independent manner (Minks et al. 1979). Consequently RNase L-deficient mice displayed a reduced antiviral activity of IFN-α, as well as impaired apoptosis (Zhou et al. 1997). In contrast, the serine threonine kinase PKR was found to more specifically block the translation of viral RNA (Farrell et al. 1978) by phosphorylation of the eukaryotic translation initiation factor eIF2a.

Besides its function in limiting translation of viral protein, PKR was also reported to activate NF-κB (Kumar et al. 1994). PKR was therefore proposed as a key receptor mediating virus- and dsRNA-induced production of type I interferons (Kumar et al. 1994). However, these findings remained controversial, as other studies that examined PKR-deficient mice and cells (Chu et al. 1999;

Iordanov et al. 2001; Maggi et al. 2000; Smith et al. 2001; Yang et al. 1995) found no defects in the induction of interferon in response to poly I:C or viral infection that could not be overcome with type I IFN pretreatment.

Further analysis revealed that PKR is not only activated by poly I:C but is able to interact with dsRNA as short as 11 bp. However, at least 30 bp are required to activate PKR kinase activity (Manche et al. 1992). In another study (Zheng and Bevilacqua 2004), recombinant PKR could also be activated by RNA oligonucleotides containing a 16-bp dsRNA stem loop in combination with a more than 11-bp-long single-stranded RNA part at the 5′ or 3′ end. All these studies question the often quoted requirement of a dsRNA molecule longer than 30 bp; furthermore, it became evident that the translational shut-down by PKR is not linked to the induction of type I IFN synthesis and secretion. The finding that $PKR^{-/-}$ cells still produce type I IFNs spurred further research on receptors capable of recognizing long double-stranded RNA. Such investigations led to a member of the Toll-like receptor (TLR) family, TLR3, which was proposed to bind to long dsRNA and to induce IFN-β (Alexopoulou et al. 2001). TLRs are transmembrane receptors that were shown to recognize a variety of conserved pathogen-associated molecular patterns (PAMPs) of bacterial, fungal, and parasitic origin. The study of Alexopoulou et al. was the first to demonstrate a role for TLRs in the recognition of viruses. TLR9 was found to be the receptor for unmethylated CpG motifs in DNA (Hemmi et al 2000); however, CpG-DNA at first was thought to be characteristic for bacterial DNA, and the role of TLR9 in detecting DNA viruses was only proposed later (Krug et al. 2004a, 2004b; Tabeta et al. 2004). Upon engagement with their specific ligands, TLRs trigger signaling pathways that lead to the activation of NF-κB and IRFs (signaling of TLRs reviewed in Moynagh 2005). TLR3 was found to induce type I IFNs upon poly I:C stimulation by activation of the kinase TBK1, which phosphorylates the transcription factor IRF3, resulting in the induction of IFN-β (Doyle et al. 2002; Fitzgerald et al. 2003; Sharma et al. 2003). Another group reported that TLR3 is activated by ssRNA (Kariko et al. 2004b); however, TLR3-deficient mice and mice deficient in the signaling adapter TRIF (Gitlin et al. 2006; Kato et al. 2006) still responded to poly I:C. Moreover, dendritic cells derived from TLR3-deficient mice were still stimulated by dsRNA transfected into the cytosol (Diebold et al. 2003).

3 Type I IFN Induction by Nucleic Acids in Immune Cells

Unlike tumor cell lines, which were examined in early studies on type I IFN and dsRNA, primary immune cells such as peripheral blood mononuclear cells (PBMCs) express a wide spectrum of functional TLRs. Different immune cell

subsets express distinct patterns of TLRs (Hornung et al. 2002). Plasmacytoid dendritic cells (PDCs) (reviewed in Colonna et al. 2004) are the major producers of early type I IFN production upon viral infection. PDCs express TLR7 and TLR9 but not TLR3. Both TLR7 and TLR9 are located in the endosomal compartment and signal via the adaptor molecules MyD88, IRAK1, and TRAF6, leading to activation of IRF7 and the induction of type I interferons as reviewed by Moynagh (2005). In addition, recent studies show that TRAF3 plays a crucial role in the MyD88-dependent signaling cascade (Hacker et al. 2005; Oganesyan et al. 2005). Single-stranded DNA and the small antiviral compound R848 had been shown to induce IFN in PDCs dependent on TLR9 and TLR7, respectively (Hemmi et al. 2000, 2002; Jurk et al. 2002; Krug et al. 2001b; Rothenfusser et al. 2002). TLR9 detects unmethylated so-called CpG motifs in single-stranded DNA (Hemmi et al. 2000). Different classes of synthetic CpG oligodeoxynucleotides (ODN) were developed based on the distinct effects on the two TLR9-expressing immune cell types: PDCs and B cells (Hartmann et al. 2003; Hartmann and Krieg 2000; Krug et al. 2001a).

In contrast to TLR3, both TLR7 and 9 depend on the signaling adapter MyD88. Accordingly, PDCs derived from TLR9- or MyD88-deficient mice are unable to produce type I IFN in response to DNA viruses such as herpes simplex viruses (HSV) and murine cytomegalovirus (MCMV) (Krug et al. 2004a, 2004b; Lund et al. 2003; Tabeta et al. 2004). While TLR9 was responsible for detecting viral DNA, TLR7 was shown to recognize RNA: TLR7 detects synthetic short (20–27 bases) single-stranded RNA (Diebold et al. 2004; Heil et al. 2004) and short interfering double-stranded RNA (siRNA) (Hornung et al. 2005; Judge et al. 2005; Sioud 2005; reviewed in Schlee et al. 2006). The amount of type I interferon induction was dependent on the RNA sequence. Ironically, Hornung and colleagues came across a very potent type I interferon inducing small RNA sequence core motif (5′-GUCCUUCAA-3′) in the attempt to knock down the interferon inducer TLR9 in PDCs using the siRNA technology (Hornung et al. 2005). It was demonstrated that these siRNAs induce systemic immune activation in mice, and that the immunological activity required TLR7. Of note, the same siRNA did not induce type I interferon in immortalized human embryonic kidney cells (HEK293), which produced type I interferon in response to poly I:C. In subsequent studies, similar findings were reported by Judge et al. (2005) (identifying a core motif 5′-UGUGU-3′) and Sioud (2005). In all three studies, transfection with cationic lipids (e.g., DOTAP, lipofectamine) or cationic polymers (e.g., PEI, polyethylenimine) was essential for the immunological activity of siRNA. The same applies for the immunological activity of single-stranded RNA (Diebold et al. 2004; Heil et al. 2004; Scheel et al. 2005).

4
RNA Modifications Contribute to the Distinction of Self- Versus Non-self RNA in Immune Cells

While for short RNA oligonucleotides the immunological activity is clearly sequence dependent (Hornung et al. 2005; Judge et al. 2005), for long RNA molecules such as mRNA, sequence specificity of immunological activity is less prominent (Scheel et al. 2005). This raises the question of how the immune system is able to distinguish between self and non-self (for example viral) RNA. This question was addressed recently by Kariko and colleagues (2005) who showed that human mitochondrial RNA, when transfected into monocyte-derived dendritic cells, provoked secretion of TNF-α at similar quantities compared to total RNA isolated from *Escherichia coli*. In contrast, RNA of other cellular compartments showed no immunological activity. The authors proposed that mammalian RNA is masked by naturally occurring nucleoside modifications that are expected to be similar in closely related species. According to this concept, mitochondrial RNA is stimulatory since it resembles bacterial rather than mammalian RNA. In healthy cells, mitochondrial RNA will not be released. In contrast to other self-RNA, mitochondrial RNA never enters the cytosolic compartment. As a consequence, mitochondrial RNA under healthy conditions is not detected by cytosolic mechanisms of detection. Only if the cell is lysed can mitochondrial RNA enter the endosomal compartment of immune cells via phagocytosis. Indeed, the stimulatory effect of in vitro RNA transcripts composed of unmodified nucleotides in their study could be abrogated by incorporation of modified nucleosides such as pseudouridine, 5-methylcytidine, N6-methyladenosine, inosine, and N7-methylguanosine. In order to examine modification sensitivity of different TLRs, HEK293 cells expressing TLR3, TLR7, TLR8, or TLR9 were transfected with RNA containing modified nucleosides. Transfection of unmodified RNAs stimulated IL-8 production (sensitive readout for immunoactivation of HEK293 cells) in HEK293 cells overexpressing TLR3, 7, and 8. Interestingly, RNA recognition by TLR3, TLR7, and TLR8 is suppressed by the presence of different types of modified nucleotides within the RNA ligand. TLR3 was the least sensitive receptor with regard to suppression by nucleoside modifications. Furthermore, the authors showed that in monocyte-derived dendritic cells, 5%–10% of modified nucleosides were sufficient to inhibit TNF-α secretion by 75%–90%. Together, these results show that RNA modification contributes to the distinction of self versus non-self RNA by the immune system.

5
Type I IFN Induction by RNA Mediating RNA Interference

Further insight into the properties that render RNA molecules stimulatory to the immune system is driven by siRNA technology. Based on studies by Tuschl and colleagues (Elbashir et al. 2001), siRNA is now used worldwide as a robust tool for target-specific gene silencing in cell lines and human primary cells. However, depending on the mode of synthesis and the sequences used to generate siRNA, also nonspecific, so-called nonspecific off-target effects of siRNAs were observed.

To overcome limitations with the transfection of synthetic siRNA, vector-based (e.g., lentiviral) expression systems for the introduction of short hairpin siRNAs (shRNA) mimicking siRNAs were developed (Brummelkamp et al. 2002; Harborth et al. 2003; Paddison et al. 2002). The most commonly used shRNA expression system consists of a RNA-polymerase III dependent promoter driving the expression of two complementary 19- to 29-bp RNA sequences linked by a short loop of 4–10 nt. The resulting transcript is exported to the cytoplasm and processed by dicer. Lentiviral vectors haboring the Pol III-shRNA expression cassette (Li et al. 2003; Rubinson et al. 2003; Tiscornia et al. 2003) allow RNAi-mediated gene silencing via siRNA in cells that are otherwise difficult to transfect.

Sequence specificity of gene silencing by such shRNA was questioned by Bridge et al. (Bridge et al. 2003), who demonstrated that infection of human lung fibroblasts with Pol III-shRNA containing lentivirus directed against the gene MORF4L1 not only silenced MORF4L1 but also stimulated interferon-inducible genes such as 2′,5′-OAS, an indicator of type I interferon. The IFN-inducing effect was dependent on the sequence and the dose of the vector; seven of 23 shRNAs targeting different genes exhibited IFN induction. In contrast, transfection of synthetic siRNA with the same putative IFN-inducing sequences led to sequence-specific silencing without triggering an IFN response. Northern blot analysis of shRNA showed that the majority of shRNA transcripts were correctly processed to 20 nt transcripts. The authors speculated that remaining unprocessed transcripts could be detected by cytosolic RNA sensing receptors. In the follow-up paper, the group of Iggo (Pebernard and Iggo 2004), further correlated the U6 promoter sequence with OAS induction. This study revealed that the region between –2 (the end of the promoter) and +2 (the start of RNA transcript) is crucial for the immune stimulatory effect, which was lost when they used the endogenous human sequence (CCGA). Further mutations leading to a partial mismatch in the shRNA (predicted to create a 14-bp duplex) suggested that stimulation required more than a 14-bp duplex.

William´s group (Sledz et al. 2003) described the induction of IFN target genes by transfection of synthetic siRNAs into a human glioblastoma cell line (T98G) or a renal carcinoma cell line (RCC). When comparing the two studies from Bridge and colleagues and from Sledz and colleagues, it is important to note that different cell lines (Bridge, human lung fibroblasts; Sledz, RCC and T98G) and different ways of siRNA generation (Bridge, synthetic siRNAs and shRNAs; Sledz, synthetic siRNA and T7-phage-polymerase siRNA) were used.

Using mouse embryonic fibroblasts (MEFs) with different gene deficiencies related to the IFN response system, Sledz et al. proposed that PKR was the interferon-inducing receptor for siRNAs. Later, the same group postulated a different siRNA receptor (RIG-I, see below) in T98G cells (Marques et al. 2006). Of note, in the two studies published by Bridge et al. (2003) and Sledz et al. (2003), type I interferon was not analyzed at the protein level.

Kariko et al. (2004a) suggested that TLR3 was responsible for the induction of type I IFN by siRNA. These data are based on keratinocyte (HaCaT) and HEK 293 cells, which responded to synthetic siRNA but not to the single-stranded components (ssRNA) by secretion of low amounts of IFN-β that was comparable to stimulation with poly I:C. Overexpression of TLR3 in HEK 293 cells resulted in fourfold higher induction of type I IFN secretion in response to transfected siRNA. However, overexpression of NF-κB-inducing receptors such as TLR3 may also contribute indirectly to the enhanced type I IFN response induced by siRNA, for example by upregulating IFN-inducible cytosolic RNA receptors. For example, TLR3 overexpressing HEK 293 cells secrete more IL-8 than empty vector or TLR9 overexpressing HEK 293 cells (Kariko et al. 2005); consequently, such studies do not necessarily provide evidence for a direct interaction between siRNA and TLR3.

Kim et al. (2004) showed that the induction of type I IFN by siRNA depended on the use of T7-RNA polymerase (T7 RNAP) for siRNA generation. In contrast to Bridge et al. (2003) and Sledz et al. (2003), in the study by Kim and colleagues, type I IFN was measured at the protein level, which is less sensitive than measuring IFN-dependent responses on the transcriptional level and thus underscores the magnitude of the IFN response they reported. In their study, Kim and colleagues examined siRNAs targeting the early ICP4 gene of HSV-1. Only T7 RNAP-derived transcripts but not synthetic siRNA elicited a potent antiviral activity when transfected into HEK 293 cells. The same antiviral activity was observed by transfection of T7 transcripts with unrelated sequences. Analysis of supernatants revealed the presence of substantial amounts of IFN-α and IFN-β protein. These results were reproduced in HeLa cells, as well as K562, CEM, and Jurkat cells. It is well known that unlike capped mammalian mRNA, the 5′ ends of T7 transcripts harbor a triphosphate GTP-nucleotide. Treatment

of T7 transcripts with RNase T1 (with the 5′ end p-GGG removed, which was single-stranded in their case) and alkaline phosphatase was sufficient to completely abrogate interferon-inducing activity. Additional experiments using T3 and Sp6 phage RNA polymerases demonstrated similar induction of type I IFN. The examination of multiple cell lines by Kim et al. (2004) pointed to a powerful ubiquitously expressed sensor for short triphosphate RNA.

6
Detection of RNA in the Cytosol: 3pRNA Is the Ligand for RIG-I

Yoneyama and colleagues identified the interferon-inducing cytoplasmic DexD/H box RNA helicase RIG-I, containing a caspase recruitment domain (CARD) (Yoneyama et al. 2004). Expression of the CARD domain sensitized cells to activate the transcription factor IRF3, leading to the induction of the IFN-β promoter. Later on it was shown that this pathway involves the IRF3 kinase TBK1, which is activated by the newly characterized adaptor protein IPS-1, also known as Cardif, MAVS, or VISA (Kawai et al. 2005; Meylan et al. 2005; Seth et al. 2005; Xu et al. 2005; reviewed in Sen and Sarkar 2005). In overexpression experiments, RIG-I was shown to bind poly I:C. However, overexpression of a dominant negative mutant of RIG-I impaired IRF3 activation by Newcastle disease virus (NDV), a negative-strand RNA virus, while IRF3 activation by poly I:C was not inhibited. Subsequent studies with RIG-I$^{-/-}$ mice and MEFs (Kato et al. 2006) showed no defect in the response to poly I:C.

Hornung and colleagues (2006) demonstrated that RIG-I detects in vitro transcribed RNA. RNA with a triphosphate at the 5′ end (now termed 3pRNA), which is generated during in vitro transcription, was identified to be the ligand for RIG-I. The minimal length of 3pRNA was 19 nucleotides. The activity of 3pRNA was independent of double-strand formation. Both exogenous 3pRNA transfected into the cell and endogenously formed 3pRNA (expression of T7 RNA polymerase) activated RIG-I. Genomic RNA prepared from a negative-strand RNA virus and RNA prepared from virus-infected cells, but not RNA from non-infected cells, triggered a potent IFN-α response in a 5′-triphosphate-dependent manner. Binding studies of RIG-I and 3pRNA revealed a direct molecular interaction. The 5′ capping or incorporation of modified nucleotides such as pseudouridine, 2-thiouridine, and 2′-O-methylated uridine in place of uridine in short 3pRNA strongly diminished IFN-α induction. In a parallel study, Pichlmair et al. (2006) attributed the inhibitory effect of the influenza virus protein NS1 to its binding and inhibition of the RIG-I triphosphate RNA complex.

7 Virus Specificity of 3pRNA Recognition

These results provide evidence that uncapped unmodified 3pRNA is detected by RIG-I in the cytosol of eukaryotic cells. Of note, all primer-independent RNA transcripts in a normal uninfected cell initially contain a 5′-triphosphate end. However, most if not all self-RNA species entering the cytosol lack a free 5′-triphosphate end. Before self-RNA leaves the nucleus, RNA is further processed, which applies to RNA transcripts of all three DNA-dependent RNA polymerases (pol) in eukaryotes. Pol I transcribes a large polycistronic precursor of ribosomal RNA (rRNA) that contains the sequences for the mature rRNAs (18, 5.8S, 25–28S rRNA), two external transcribed spacers, and two internal transcribed spacers. This primary transcript is subjected to endo- and exonucleolytic processing steps to produce the mature rRNAs. The net result of this maturation process is a monophosphate group at the 5′ end of all pol I transcribed rRNAs (Fromont-Racine et al. 2003). Messenger RNAs (mRNAs) and small nuclear RNAs (snRNAs), which are transcribed by pol II, receive a 7-methyl guanosine group that is attached to the 5′-triphosphate of the nascent RNA by a process called capping (Shatkin and Manley 2000). Thus, upon export into the cytoplasm, no free triphosphate groups are found in pol II transcripts. All mature tRNAs (pol III) have a 5′-monophosphate (Xiao et al. 2002), as it is likely to apply to 5S rRNA. U6 RNA receives a γ-monomethylphosphate cap structure following transcription. However, 7SL RNA (pol III) has a triphosphate at the 5′ end, and is present at high copy numbers in the cytosol. Therefore, the presence or absence of a 5′ triphosphate might not be the only structural feature of RNA responsible for the distinction of self and viral RNA.

It is well known that eukaryotic RNA undergoes significant modifications to its nucleosides and its ribose backbone. Among all nucleoside modifications, pseudouridinylation is one of the most common post-transcriptional modifications of RNA that appears to be universal among rRNAs and small stable RNAs such as splicing small nuclear RNAs (snRNAs), tRNAs, and small nucleolar RNAs (snoRNAs). However, the frequency and location of pseudouridinylated nucleotides vary phylogenetically. Intriguingly, eukaryotes contain far more nucleoside modifications within their RNA species. Human ribosomal RNA, for example, the major constituent of cellular RNA, contains ten times more pseudouridine and 25 times more 2-O-methylated nucleosides than *E. coli* rRNA (Rozenski et al. 1999). The same applies to eukaryotic tRNAs, the most heavily modified subgroup of RNA with up to 25% of modified nucleosides. The host machinery that guides nucleoside modifications and 2′-O-methylation of the ribose backbone is located in the nucleolus, and consists of RNA–protein

complexes containing snoRNAs and several associated proteins (snoRNPs) (Decatur and Fournier 2003). Information on nucleolus-specific nucleoside modifications or ribose 2′-O-methylation of viral RNA genomes is limited. Since most RNA viruses do not replicate in the nucleus and modification is tightly confined to the sequence and structure of their target, extensive modification of viral RNA seems unlikely. Altogether, post-transcriptional modifications of eukaryotic RNA such as 5′ processing or capping, as well as nucleoside modifications or ribose backbone methylation, provide the molecular basis for the distinction of self-RNA generated in the nucleus from viral RNA of cytosolic origin containing 5′-triphosphate (3pRNA).

The mRNAs of viruses infecting eukaryotic cells also commonly contain 7-methyl guanosine cap-structures at their 5′ ends and poly(A) tails at their 3′ends (Furuichi and Shatkin 2000). Some viruses make use of the host transcription machinery to acquire caps and poly(A) tails. RNA viruses that do not rely on the host transcriptional machinery produce their own capping enzymes or utilize other mechanisms such as snatching the 5′-terminal regions of host mRNAs. Despite these adaptations of viruses to the host transcriptional system, viral RNA synthesis leads to transient cytosolic RNA intermediates with an uncapped 5′-triphosphate end. With notable exceptions such as the Picornavirus family (see below), viral RNA-dependent RNA polymerases (RdRp) initiate polymerase activity de novo, without a specific primer (Kao et al. 2001). As a consequence, these RdRp-dependent transcripts start with an uncapped 5′-triphosphate. This has been studied in great detail for the replication of positive-strand RNA viruses of the family of Flaviviridae (including the genera *Flavivirus*, *Pestivirus*, and *Hepacivirus*); members of all of these virus genera were reported as being recognized via RIG-I (Honda et al. 1998; Kato et al. 2006; Sumpter et al. 2005). Segmented NSV rely on a cap-snatched primer for mRNA transcription, yet initiate genomic and the complementary antigenomic RNA replication by a primer-independent de novo mechanism resulting in a 5′-triphosphate-initiated transcript (Honda et al. 1998; Neumann et al. 2004). NSV with a nonsegmented genome (order Mononegavirales), including the Paramyxoviruses and Rhabdoviruses, initiate both replication and transcription de novo leading to 5′-triphosphate RNA in the cytosol. Both the full-length replication products, vRNA and cRNA, and a short leader RNA, which is abundantly synthesized during initiation of transcription, maintain their 5′-triphosphate (Colonno and Banerjee 1978; Whelan et al. 2004), while the virus-encoded mRNA transcripts are further modified at their 5′ ends by capping and cap methylation. Consequently, genomic RNA from NSVs per se is expected to trigger an IFN-response without the need for replication and presumed dsRNA formation.

Consistent with this notion, not only live virus but also RNA purified from NSV virions (VSV) has been shown to trigger strong type I interferon responses depending on RIG-I (Kato et al. 2006). Hornung and colleagues confirmed and extended these observations by demonstrating that dephosphorylation of the viral RNA isolates completely abolished the IFN-response, thereby indicating that the 5′-triphosphate moiety is strictly required for recognition (Hornung et al. 2006).

A notable exception are the viruses in the Picornavirus-like supergroup (picornavirus, potyvirus, comovirus, calicivirus, and other viruses), which exclusively employs a protein known as viral genome-linked protein (VPg) as a primer for both positive- and negative-strand RNA production. This protein primer is part of the precursor RdRp and is cleaved off as elongation of the initial complex occurs, usually to become a 5′-genome-linked protein (Lee et al. 1977). Thus during the life-cycle of Picornaviruses uncapped, triphosphorylated 5′ ends are absent. Consequently, based on our studies, RIG-I is expected to be involved in the detection of Flaviviridae and NSV but not Picornaviruses. This is confirmed in a recent study (Kato et al. 2006).

A number of studies suggested that the helicases MDA-5 and RIG-I recognize dsRNA (Andrejeva et al. 2004; Rothenfusser et al. 2005; Yoneyama et al. 2004). The results in the work of Hornung and colleagues (2006) demonstrated that double-strand formation of RNA is not required for RIG-I-RNA interaction, and that dsRNA is not sufficient for RIG-I activation. These results further demonstrate that MDA-5 is not involved in 5′-triphosphate RNA recognition. Although there is convincing evidence that MDA-5 is activated by the long dsRNA mimic poly I:C, activation of MDA-5 by natural long dsRNA is still controversial (Kato et al. 2006). Taken together, TLR3 is so far the only receptor that induces type I IFN upon binding of the natural molecule long dsRNA, but the contribution of TLR3 to type I IFN induction and viral clearance in vivo seems to be weak (Rudd et al. 2006).

There is good evidence that short dsRNA such as siRNA generated by Dicer-mediated cleavage of long dsRNA does not elicit a type I IFN response in non-immune cells (Elbashir et al. 2001; Hornung et al. 2005; Kim et al. 2004). A recent study suggests that the two-nucleotide overhang at the 3′ end of dicer cleavage products are essential for the lack of immunorecognition of short dsRNA (Marques et al. 2006). The same study proposed that synthetic blunt-end short dsRNA is recognized via RIG-I. The conclusion that RIG-I is the receptor for blunt end short dsRNA is based on experiments using RIG-I overexpression and using anti-RIG-I siRNA (short dsRNA with two-nucleotide 3′overhangs) on top of stimulation with blunt end short dsRNA stimulation. RIG-I-deficient cells have not been examined in this study. This experimental design does not

provide clear-cut evidence for the primary involvement of RIG-I in type I IFN induction by blunt-end short dsRNA. Furthermore, in the study by Hornung and colleagues, 5′ triphosphate blunt-end RNA and 5′ triphosphate 2-nt overhang RNA showed identical RIG-I ligand activity, suggesting that the molecular feature 2-nt overhang does not inhibit RIG-I-mediated recognition (Hornung et al. 2006).

8 MDA-5 Recognizes Poly IC

MDA-5 is structurally related to RIG-I, as it also contains two CARD domains and a helicase domain. MDA-5 was originally identified as a type I IFN-inducible molecule mediating cell cycle arrest and apoptosis in melanoma cells (hence the name melanoma differentiation antigen 5) (Kang et al. 2002, 2004; Kovacsovics et al. 2002). A first indication of a role for MDA-5 in virus recognition came from the observation that a paramyxoviral protein that mediated immune evasion bound to MDA-5 (Andrejeva et al. 2004). In overexpression experiments, MDA-5 was shown to bind poly I:C, and enhanced the interferon response to poly I:C as well as several viruses. Conversely, siRNA mediated knock-down blocked type I IFN induction in response to these stimuli (Yoneyama et al. 2005). MDA-5 was then shown to play an essential role in the detection of Picornaviruses such as encephalomyocarditis virus (EMCV) or Theiler's virus (Gitlin et al. 2006; Kato et al. 2006). In addition, mice deficient in MDA-5 were found to be highly susceptible to EMCV. Although the nature of the natural RNA ligand that engages MDA-5 has so far remained obscure, a surprising observation was that cells derived from MDA-5-deficient mice, as well as MDA-$5^{-/-}$ mice stimulated in vivo were found unable to mount a type I IFN response to poly I:C, establishing MDA-5, rather than the several other receptors that bind, or have been shown to be activated by poly I:C, as the dominant receptor mediating the interferon response to poly I:C (Gitlin et al. 2006; Kato et al. 2006). However, the natural viral ligand for MDA-5 has not yet been identified.

In addition to RIG-I and MDA-5, another cytosolic receptor may exist for detecting DNA. Until recently TLR9 was the only innate sensor for detecting microbial DNA. Recent studies indicate that DNA is detected in the cytosol independently of TLR9 (Okabe et al. 2005; Stetson and Medzhitov 2006), but the receptor has not been identified yet. The cytosolic receptor mediating recognition of B-form DNA, unlike RIG-I and MDA-5, signals independently of IPS-1.

9
Cell-Type-Dependent Sensing of Viral RNA

As discussed in the previous sections, immune and nonimmune cell types express characteristic patterns of nucleic acid receptors (Table 1). For example, Melchjorsen and colleagues reported that activation of innate defense against a paramyxovirus is mediated by RIG-I, TLR7, and TLR8 in a cell-type-specific manner (Melchjorsen et al. 2005). They found that nonimmune cells relied entirely on RNA recognition through RIG-I for activation of an antiviral response. In contrast, immune cells such as myeloid cells utilized TLR7 and TLR8. Unlike

Table 1 Nucleic acid receptors

Receptor	Nucleic acid	Ligands	Role in IFN–α/β response	Inhibitory modification
Endosomal Toll–like receptors				
TLR3	dsRNA	Poly I:C	(+)[a]	
TLR7	ssRNA/siRNA	Poly U, G, U–rich, motifs	+++ (PDC only)	2′–O–methylation[c]
TLR8	ssRNA/siRNA	G, U–rich	-	2′–O–methylation
TLR9	(ss)/dsDNA	CpG motifs[b]	+++ (PDC only)	^{m}CG Methylation
Cytosolic receptors				
PKR	dsRNA	Poly I:C, >30 bp, stem loop	(+)[a]	
RIG–I	ss/dsRNA	5′ triphosphate ends	+++	5′ m^{7}guanosine cap 2′–O–methylation Pseudouridinylation
MDA–5	dsRNA	Poly I:C	+++	
(dsDNA–R)	dsDNA	B–form DNA, poly dAdT: dTdA	+++	

[a]Both TLR3 and PKR have been reported to induce type I IFN production in response to poly I:C. However, compared to MDA-5, their contribution to the type I IFN response in vivo is rather weak

[b]CpG-containing oligonucleotides (ODN) that induce a strong type I IFN response include CpG-A and CpG-C ODN

[c]Modifications that have been shown to prevent detection by the receptors indicated and that are frequently found in mammalian nucleic acids

in nonimmune cells, RNA sensing in paramyxovirus-infected myeloid cells was independent of RIG-I, TLR3, and PKR. Kato and colleagues also found cell-type specific involvement of RIG-I in antiviral immune response. In their study type I IFN induction in both fibroblasts and myeloid dendritic cells was RIG-I-dependent, while type I IFN induction in PDC was RIG-I-independent (Kato et al. 2005). It is important to note that the mechanisms used for RNA sensing may not only be cell-type-dependent but may also depend on the type of virus and its strategy to enter the target cell and to evade immune recognition. In contrast, recognition of synthetic RNA or of RNA transcribed from vector systems is more predictable because there is no immune evasion and because the mode of delivery is known. Of note, the use of cationic lipids and polycationes leads to both endosomal and cytosolic delivery (Almofti et al. 2003; Boussif et al. 1995) and thus both TLR- and RIG-I-mediated RNA sensing is triggered, provided these receptors are expressed in the cell type examined, and the appropriate RNA ligand is delivered. Of note, subcellular localization of TLR3 is cell-type-specific (Matsumoto et al. 2003): in fibroblasts, TLR3 is located on the cell surface, and the TLR3-mediated activity can be blocked by anti-TLR3 antibodies. In myeloid dendritic cells, TLR3 is found in the cytosolic compartment. A more detailed analysis in TLR3-transfected B cells revealed that TLR3 is detectable in multivesicular bodies, a subcellular compartment situated in the endocytic trafficking pathway (Matsumoto et al. 2003).

10 Conclusion and Future Directions

Bacteria, fungi, or cellular parasites are recognized via conserved molecules typical for the respective type of pathogen. In contrast, all virus components are formed within the infected host cell; consequently, a virus-specific detection system is more difficult to achieve. It is now evident that host cells are equipped to detect viral nucleic acids. For viral infection in vivo, the following picture is evolving: large parts of the early type I IFN response upon viral infection are due to TLR7 and TLR9 expressed in PDCs; in fact, PDCs are the only considerable source of TLR7- and TLR9-induced type I IFN production upon viral infection. The major advantages of this PDC response are that the presence of viral particles is sufficient for recognition, that viral infection of cells is not required for detection, and that viruses are recognized before viral proteins have a chance to mediate immune evasion. This first wave of type I IFN production plays an important role in limiting viral spread by PDC-derived direct antiviral mechanisms early on, and by sensitizing yet uninfected cells for cytosolic recognition of viral nucleic acid via strong upregulation of the two

cytosolic helicases, RIG-I and MDA-5. These two cytosolic receptors are then responsible for the second and prolonged wave of type I IFN production and for the induction of apoptosis of virally infected cells. For all four receptors, distinction of self from viral nucleic acid is based on a combination of localization and molecular structure. In this sophisticated system of virus detection, the following situations signal viral danger:

1. Appearance of unmodified RNA in the endosomal compartment of PDCs
2. Appearance of DNA containing unmethylated CpG motifs in the endosomal compartment of PDCs
3. Unmodified RNA with a triphosphate group at the 5′ end (3pRNA) in the cytosol of any cell type
4. DNA in the cytosol of any cell type

It is still unclear whether long dsRNA in the cytosol is sufficient to elicit an antiviral response via one of the receptors known to date. Although poly I:C is a ligand for MDA-5, long double-stranded RNA seems insufficient as a ligand, and the natural ligand still needs to be identified. In addition to RNA-detecting receptors, the cytosolic receptor for DNA may add new perspectives in therapeutic viral mimicry. With regard to viruses that perform inside the nucleus such as HBV and HIV, uncovering molecular mechanisms of sensing viral nucleic acids in the nucleus appears on the radar of scientific challenges.

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Part IV: Interferon-Induced Genes

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The Interferon-Stimulated Genes: Targets of Direct Signaling by Interferons, Double-Stranded RNA, and Viruses

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Abstract The interferon system plays a profound role in determining the outcome of viral infection in mammals. Viruses induce the synthesis of interferon, which, in turn, blocks virus replication by inducing the expression of antiviral proteins encoded by interferon-stimulated genes. It is not widely appreciated that without the participation of interferon, many of the same genes can also be induced by a variety of virus-related agents, such as double-stranded RNA and viral proteins. In this chapter, we discuss different signaling pathways, activated by these agents, that lead to the induction of partially overlapping sets of genes, including the interferon-stimulated genes. We also review the biochemical and cellular properties of the protein products of a selected number of these genes including ISG56, ISG54, and ISG15.

1 Introduction

Interferons (IFN) are defined by their ability to block virus replication. In addition to this property, these potent cytokines have many other effects on cellular physiology, especially in cells of the immune system (Biron and Sen 2001; Samuel 2001). Most, if not all, of these effects are mediated by the products of cellular genes, whose expression is highly induced by interferon treatment of cells. These IFN-stimulated genes (ISGs) number in the hundreds, but the biochemical and cellular functions of only a handful of them have been delineated as yet (Sarkar and Sen 2004). However, much is known about how IFNs induce transcription of these genes, the identities of the proteins that mediate and regulate the signaling pathways and how dysregulation of these pathways in cancer cells or in virus-infected cells contribute to pathogenesis. In this chapter, we review the observations demonstrating that many ISGs can be induced by a number of alternate signaling pathways activated by not only IFNs, but also viral proteins, RNAs and DNAs, bacterial lipopolysaccharides, and most notably double-stranded RNA. In addition, the biochemical and cellular functions of the proteins encoded by a few most highly induced ISGs are discussed.

2 Historical Perspective

Viruses and the interferon system, especially type I IFNs, are connected at many levels. It was clear from the time of its discovery that synthesis and secretion of IFN can be induced by virus infection of cells (Nagano and Kojima 1954; Isaacs and Lindenmann 1957; Watanabe 2004). Since then, much information has been gathered regarding the biochemical pathways, activated by viruses, which lead to IFN gene induction (Garcia-Sastre and Biron 2006). A critical discovery was the finding that synthetic double-stranded RNA could mimic virus infection and induce IFNs. Viral dsRNA is often produced in infected cells as byproducts of viral genome replication (Jacobs and Langland 1996); hence, for a long time it was thought that synthetic dsRNA serves as a surrogate of viral dsRNA. As will be elaborated below, although partially overlapping, the pathways activated by viruses and dsRNA are not identical, nor do they lead to the induction of an identical set of genes. IFNs, of course, inhibit virus replication, creating the powerful loop of antiviral innate immunity: induction of IFNs by virus infection and inhibition of virus replication by IFNs. Since without going through the above IFN loop, dsRNA and virus infection can directly

induce many of the same antiviral genes that IFN can induce, virus replication in the primary infected cell can be directly attenuated by the proteins encoded by these genes (Elco et al. 2005; Sen and Sarkar 2005b). Finally, many viruses, if not all, encode RNA or proteins that can interfere with various components of the IFN system: IFN synthesis, IFN signaling and functions of IFN-induced proteins. Often, the same virus can block more than one such processes, thus ensuring efficacy (Haller et al. 2006). The plethora of interplays outlined above gives rise to the host–virus equilibrium observed in a virus-infected cell. In vivo, additional factors, such as other virus-induced cytokines and activated cells of the immune system, contribute to homeostasis as well.

After the discovery of the ability of dsRNA to induce IFN synthesis, the second major discovery connecting the two was the finding that dsRNA could activate two enzymes, PKR and 2-5 OAS, which are induced by IFNs (Lengyel 1987). Thus, for the action of these enzymes in virus-infected cells, concerted actions of both IFN and dsRNA are needed, one inducing the synthesis of the proteins and the other functioning as their co-factor for imparting enzyme activity (reviewed in Sarkar and Sen 2004). Cloning of many IFN-stimulated genes (ISGs) and subsequent identification of many more ISGs using microarray analyses provided important tools for studying their modes of induction (Der et al. 1998). Such studies surprisingly revealed that dsRNA or virus infection could induce many ISGs directly, without the participation of IFNs (Geiss et al. 2001). In some cell lines, dsRNA and IFNs can synergize their induction as well. The definitive proof of IFN-independent induction of ISGs came from experiments that used cell lines genetically incapable of synthesizing IFNs or responding to IFNs. For example, in human gliomas, the type I IFN locus is often deleted during the development of the disease. Thus, cell lines derived from these tumors cannot produce IFNs, but many ISGs can be induced in them, upon dsRNA treatment or virus infection (Geiss et al. 2001). These genes, although originally classified as ISGs, can be legitimately called dsRNA-stimulated genes (DSGs) or viral-stress-inducible genes (VSIGs) as well.

The signaling pathways used by various agents to induce these genes are distinct, with some overlaps. The single common feature is the cis-element in the promoters of these genes that receives the signaling. It was originally identified as the IFN-stimulated response element (ISRE), to which the IFN-activated trimeric transcription factor, ISGF3, binds and induces transcription. ISGF3 is composed of three proteins, STAT1, STAT2, and IRF9, and it is the latter protein that specifically recognizes ISRE (Stark et al. 1998). IRF9 is a member of the large IFN regulatory factor (IRF) family of proteins, all of which can bind to ISRE or ISRE-like elements. Soon it was discovered that dsRNA or virus infection could activate other IRF members, such as IRF-3, IRF-7, and IRF-5 (Barnes et al. 2002). Upon activation, these proteins translocate

from the cytoplasm to the nucleus, bind to the ISRE sites in the promoters of the target genes, and induce their transcription. Thus, the ISRE–IRF axis is the common link of gene induction by IFNs, viruses, and dsRNA. The latter two agents can efficiently activate other transcription factors, such as NFκB and AP-1, as well (Sen and Sarkar 2005b). Consequently, genes regulated by those transcription factors are induced by dsRNA or virus infection, but not by IFNs. Certain genes, such as the human IFN-β gene, have complex promoters, and they require for induction the co-ordinate actions of IRF-3–IRF-7, NFκB, and AP-1 (Maniatis et al. 1998). Consequently, viruses and dsRNA can induce their transcription, but IFNs cannot. However, induced transcription of genes with simple promoters, such ISG56 and ISG15, is driven by ISRE only, and as a result, all agents that can activate transcription factors containing IRF proteins can efficiently induce these genes. Because of the ease of analysis, we have used the latter genes extensively for delineating the signaling pathways activated by IFNs, dsRNA, and viruses (Peters et al. 2002; Sarkar et al. 2004; Elco et al. 2005). These studies have revealed additional cross-talks among the different inducers of ISGs. Many genes, encoding proteins that are components of the signaling pathways, are ISGs themselves. For example, synthesis of TLR3, a receptor for dsRNA, is strongly induced by IFN (Heinz et al. 2003). Similarly, STAT1, an essential component of the IFN-signaling pathways, is an ISG. Conversely, some ISGs encode proteins, such as SOCS, that block the signaling pathways and limit the duration of the gene induction process (Alexander and Hilton 2004). Thus, ISGs not only affect the antiviral state and other properties of the cell but also regulate, both positively and negatively, their own expression.

3
IFN-Independent Induction of ISGs

Signaling pathways triggered by many viral components, or their mimics, lead to the induction of ISGs. These pathways initiate at different points but converge at various nodes and all cause activation of IRF-3 or IRF-7, the transcription factors that are the common denominators of these pathways (Fig. 1). The Toll-like receptors have emerged as major sensors of viral components (Akira et al. 2006; Stetson and Medzhitov 2006). Viral nucleic acids are recognized by TLRs present in the endosomal membranes (Kawai and Akira 2006; Meylan and Tschopp 2006). TLR3 initiates signaling by binding dsRNA (Alexopoulou et al. 2001), whereas TLR7 and TLR8 recognize viral single-stranded RNA (Diebold et al. 2004); viral DNA is recognized by TLR9 (Tabeta et al. 2004). TLR2 and TLR4, present on the cell surface, are recognized by some viral glycoproteins (Boehme and Compton 2004). In addition to TLR3, two cytoplasmic

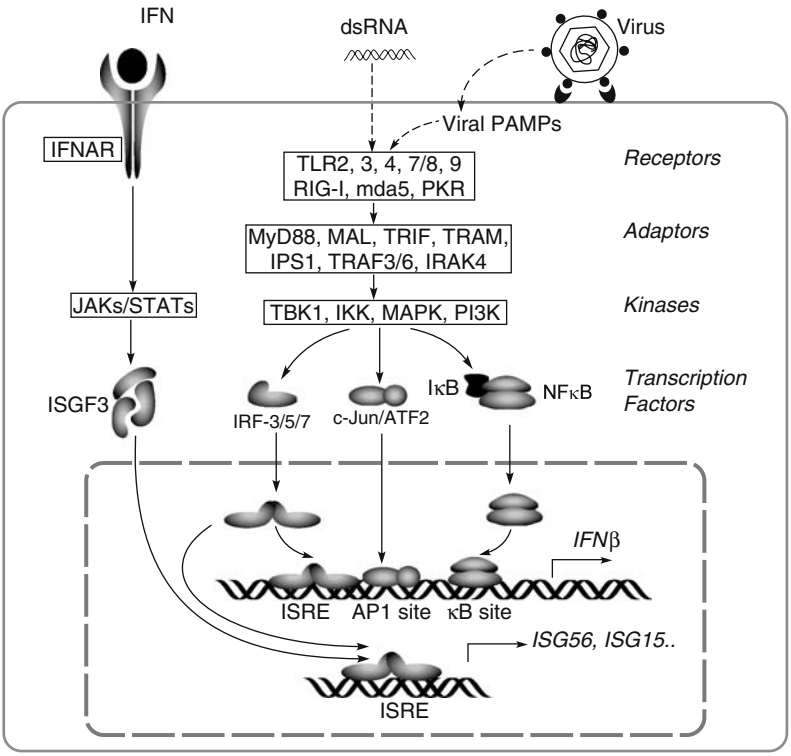

Fig. 1 Signal integration for viral-stress-inducible gene (VSIG) expression. Schematic diagram shows major signaling pathways stimulated by virus, IFN, and dsRNA that induce transcription of a common set of genes. Virus, dsRNA, and viral pathogen-associated molecular patters (PAMP) are sensed by Toll-like receptors or RNA helicases. The signaling pathway finally leads to the induction of interferons as well as several IFN inducible genes (ISGs). IFNs are then secreted and signals through IFN receptor (IFNAR) and JAK/STAT pathway to induce ISGs to generate antiviral state

RNA helicases, RIG-I and Mda-5, can recognize dsRNA and signal (Meylan and Tschopp 2006). It is anticipated that additional cytoplasmic receptors may recognize other viral components and start similar signaling cascades. IRF-3 activation by these pathways requires the action of the protein kinase TBK1, which is recruited to different receptors by different adaptor proteins (Fitzgerald et al. 2003). For TLR3, the critical adaptor protein is TRIF (Yamamoto et al. 2002; Oshiumi et al. 2003), whereas RIG-I and Mda-5 use the mitochondrial

protein IPS-1 (Sen and Sarkar 2005a). TLR7, TLR8, and TLR9 all require the major adaptor of TLR pathways, MyD88. TLR4 uses both MyD88 and TRAM, the latter recruiting TRIF to bring in TBK1 to the signaling complex (Kawai and Akira 2006).

4
Induction of ISGs by TLR3 Signaling

TLR3 is present mostly on the endosomal membrane, although in some cell types its presence on the plasma membrane has been noted (Matsumoto et al. 2003). Its ectodomain specifically recognizes endosomal dsRNA through ionic interactions between the negatively charged ligand and positively charged amino acid residues present on both sides of a canyon in which the dsRNA perfectly fits (Bell et al. 2005; Choe et al. 2005; Bell et al. 2006). Extracellular dsRNA has to be endocytosed to reach TLR3, as revealed by the chloroquine sensitivity of the process (de Bouteiller et al. 2005). Because many viruses enter the cell through endocytosis, their genomic RNAs may encounter TLR3 in the endosome. The first step in TLR3 signaling is its dimerization, which presumably leads to a conformational change of its cytoplasmic domain to initiate the signaling process (Fig. 2).

The most novel feature of TLR3 signaling is the need for receptor Tyr-phosphorylation (Sarkar et al. 2003, 2004). Although phosphorylation of specific Tyr residues located in the cytoplasmic domains of receptors for growth factors and cytokines is quite common, this feature is unique for TLR3 among the Toll-like receptors. There are five Tyr residues in the cytoplasmic domain of human TLR3 and several of these residues, if not all, are phosphorylated at the beginning of the signaling process. The functional roles of these residues have been assessed by mutating them, individually or in combinations. At least two of the five are essential for signaling; one of them has to be Tyr759, the other one can be Tyr858 or Tyr733. Tyr-phosphorylation of TLR3 is a ligand-dependent process, but the responsible protein kinase has not yet been identified. Tyr759 or 858, after phosphorylation, can recruit the signaling complex. The main adaptor protein is TRIF, but TRAF3 is needed as well (Hacker et al. 2006; Oganesyan et al. 2006). The different branches of signaling bifurcate from TRIF (Jiang et al. 2004). A complex containing TRAF6, TAB1, TAB2, and TAK1 activates the protein kinases JNK, P38, and IKK (Jiang et al. 2003). Another adaptor, RIP-1, is also recruited by TRIF and it is required for NFκB activation (Meylan et al. 2004). JNK, P38, and IKK activate the transcription factors c-Jun, ATF2, and NFκB, respectively. A separate branch of signaling originating from TRIF is triggered by the recruitment of the protein kinases TBK1 or IKKε,

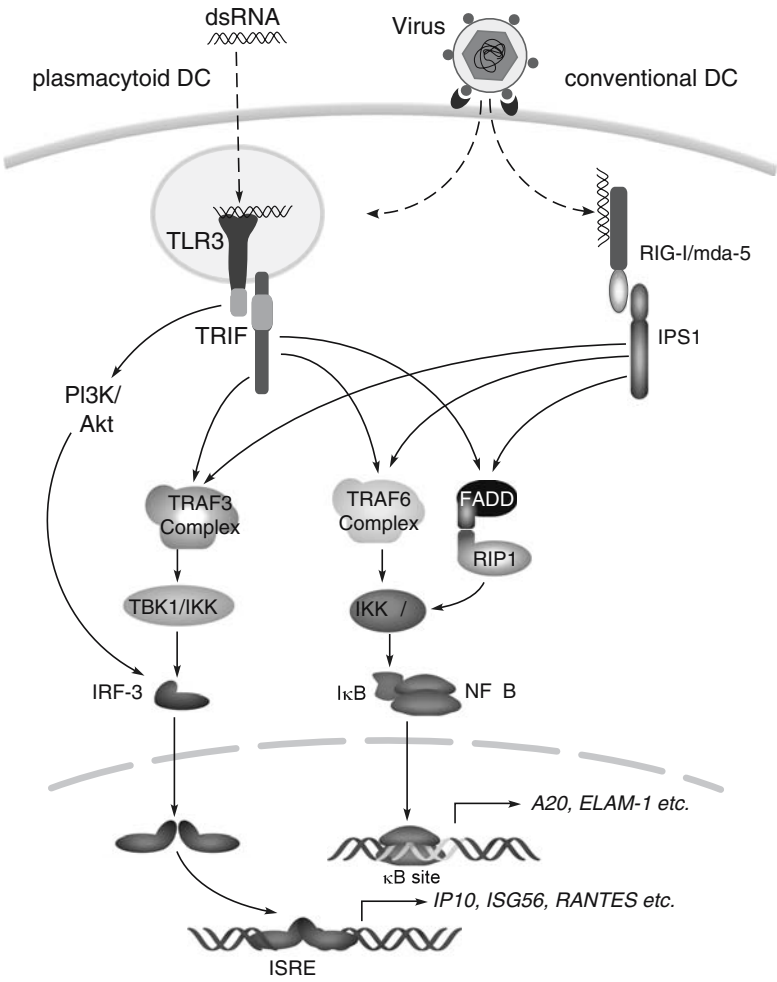

Fig. 2 Signaling pathways activated by viruses and dsRNA. Depending on cell type, viral dsRNA can signal either through Toll-like receptor 3 or RNA helicases: RIG-I/mda-5. Through different sets of adaptors, the signal causes activation of two major transcription factors, IRF-3 and NF-κB, followed by induction of specific sets of genes

which directly phosphorylate IRF-3 (Fitzgerald et al. 2003; Sharma et al. 2003). IRF-3 phosphorylation leads to its dimerization and translocation to the nucleus where it binds to the ISRE sites in the promoters of the target genes

and induces their transcription. The histone deacetylase, HDAC6, is required for IRF-3 to function as a transcription factor (Nusinzon and Horvath 2006).

As mentioned above, Tyr759 of TLR3 is absolutely needed for complete signaling by this receptor. When this residue is mutated to Phe, NFκB- and IRF-3-driven genes are not induced by dsRNA. Surprisingly, in dsRNA-treated cells expressing the mutant receptor, NFκB is released from IκB and translocated to the nucleus but it does not drive gene transcription (our unpublished observation); similarly, IRF-3 is dimerized and translocated to the nucleus but it is transcriptionally inactive (Sarkar et al. 2004). Investigation of the underlying molecular mechanisms has revealed that the activation of both transcription factors, IRF-3 and NFκB, is a two-step process and the second step is defective in cells expressing the Y759F mutant of TLR3. Phosphorylated Tyr759 recruits PI3 kinase, probably indirectly, to the TLR3 complex, PI3 kinase is activated, it phosphorylates Akt, which leads to additional phosphorylation of the TBK1-activated IRF-3. The tyrosine kinase, Src, which is known to be activated by Akt, may be a participant in this pathway, because Src is activated by TLR3 signaling and its presence is needed for gene induction by TLR3 (our unpublished observation). As expected from the above description, inhibitors of PI3 kinase, Akt and Src, both block IRF-3-mediated gene induction by TLR3. They have the same effect as the Tyr759 mutation of TLR3, namely incomplete phosphorylation of IRF-3, as revealed by two-dimensional gel analysis of nuclear IRF-3 isolated from dsRNA-treated cells expressing Y759F TLR3. Chromatin immunoprecipitation assays demonstrate that unlike fully phosphorylated IRF-3, underphosphorylated IRF-3 cannot bind tightly to the promoter and interact with co-activators, such as CBP (Sarkar et al. 2004).

In the NFκB pathways, the first step of activation is mediated by the phosphorylation of IκB by the IKK complex and the consequent release of NFκB and its translocation to the nucleus. TLR3 Tyr759 is not required for the above process or for the phosphorylation of NFκB P65 protein in Ser276 and Ser536 resides. However, it is required for additional phosphorylation of P65 as revealed by two-dimensional gel analysis of nuclear P65. Underphosphorylated P65 cannot bind to the promoters of the target genes tightly and drive their transcription. Surprisingly, the PI3 kinase pathway is not required for the second step of P65 phosphorylation (our unpublished observation). The above studies highlight the two-step nature of the activation of both IRF-3 and NFκB, although the details are different. The first step is initiated by the phosphorylation of Tyr858 of TLR3, leading to the release of NFκB from IκB and the dimerization of IRF-3 as a result of its partial phosphorylation. The second step is initiated by the phosphotyrosine 759 of TLR3. It leads to further phosphorylation of IRF3 and its full activation and complete phosphorylation of NFκB P65 and its full activation.

5
Partially Overlapping Repertoire of Genes Induced by Viruses, IFN, and dsRNA

In order to identify the primary target genes induced by viruses, type I IFNs and dsRNA, microarray analyses have been performed by many investigators (reviewed in Sarkar and Sen 2004). However, it is important to realize that because the different inducing agents can induce one another, the results of the above analyses, unless carefully designed, are often difficult to interpret. For example, virus replication can produce both dsRNA and IFNs, and dsRNA treatment can produce IFN, thus complicating the pictures. Mutant cell lines have helped to circumvent this problem considerably. Human cell lines that cannot synthesize IFNs, because of the deletion of the type I IFN locus in their genomes, have been particularly useful to identify dsRNA-regulated genes. Such an analysis using cDNA microarray identified 175 dsRNA-stimulated genes in a human glioma cell line. On the other hand, expression of 95 other genes was repressed by dsRNA treatment of these cells (Geiss et al. 2001). A subset of dsRNA-induced genes was also induced by inflammatory cytokine, IFNs, and viruses, indicating that these genes have broad functions (Elco et al. 2005). Induction of some of these genes required ongoing protein synthesis, while others did not, and the dsRNA-induced genes functionally covered all aspects of cellular metabolism. Mutant cell lines that are incapable of responding to IFNs or dsRNA have also been effectively used to untangle the overlapping signaling pathways. When cells from mice carrying targeted gene disruptions are used for this purpose, the results are unequivocal. In contrast, the results from mutagenized human cell lines, selected for IFN-unresponsiveness or dsRNA-unresponsiveness, need cautious interpretations. For example, the HT1080-derived U series of cells have been widely used for examining the roles of specific components of the IFN-signaling pathways in the cellular responses to other inducers. Using these lines, we initially concluded that none of the components of the type I IFN signaling pathways, other than STAT1, is needed for gene induction by dsRNA (Bandyopadhyay et al. 1995). The need of STAT1 was based upon the observed failure of U3A cells to respond to dsRNA. But recent in-depth analysis revealed that the original conclusion was erroneous; STAT1 is not needed for dsRNA signaling; U3A cells cannot respond to dsRNA because, unlike the parental line, they do not express TLR3. STAT1 restoration in U3A cells did not restore basal TLR3 expression and dsRNA-responsiveness, indicating that a different mutation is responsible for this phenotype. Ectopic expression of TLR3 or induction of the resident TLR3 gene by IFN treatment imparted dsRNA responsiveness to U3A cells (C.P. Elco and G.C. Sen, unpublished observation). The same was true for HeLaM cells, providing an explanation

for our old observation that IFN pretreatment makes these cells responsive to dsRNA (Tiwari et al. 1987). The above example should remind investigators that the mutagenized cell lines carry many mutations in addition to the ones for which they have been selected and a given phenotype and the known mutation cannot be causally connected, without rigorous testing.

A series of mutant cell lines was used to investigate the gene induction pathways activated by Sendai virus infection. Microarray analysis revealed that the same genes were induced by SeV in the presence or the absence of TLR3 (Elco et al. 2005). In TLR3-expressing cells, dsRNA and SeV did not induce completely overlapping sets of genes, although many genes were induced by both indicating that the two inducers have distinct properties. Induction of some of the virally induced genes required IFN signaling, suggesting that they are probably induced secondarily by IFN produced upon virus infection. Induction of some genes required NFκB, others required IRF-3, whereas a third group required both transcription factors. An unexpected observation was that increasing levels of IRF-3 inhibited the induction of some, but not all, NFκB-dependent genes (Elco et al. 2005). The molecular basis of this cross-talk between the two signaling pathways remains unknown.

6
The ISG56 Family

Members of the ISG56 (IFIT1) gene family are very strongly induced in response to IFN, dsRNA, or virus infection. These genes show up at or near the top of all microarray databases inquiring into the nature of cellular genes induced upon infection with a variety of viruses (Sarkar and Sen 2004). Our early investigation demonstrated that type I IFN, dsRNA, or infection with encephalomyocarditis virus or vesicular stomatitis virus could independently induce ISG56 mRNA and the encoded protein, P56, in human glioma cells (Kusari et al. 1987; Tiwari et al. 1987). Because the human ISG56 gene promoter contains two ISREs and no other identifiable transcription factor binding sites, it has been very useful for analyzing, in isolation, the IRF-mediated signaling pathways activated by different inducers. There are four members of the human ISG56 gene family IFIT-1 or ISG56, IFIT-2 or ISG54, IFIT-4 or ISG60, and IFIT-5 or ISG58. In mouse, there are three genes, ISG56 (IFIT1), ISG54 (IFIT2), and ISG49 (IFIT-3). Phylogenetically, human/mouse ISG56, human/mouse ISG54, and human ISG60/mouse ISG49 are the cognate genes in the two species.

Induction patterns of human and mouse ISG56 and ISG54 have been studied in vitro and in vivo (Terenzi et al. 2005, 2006; F. Terenzi et al., unpublished

observations). Although they are usually induced with similar characteristics, interesting and unexpected differences have been noted. In human HT1080 cells, IFN-β treatment strongly induced both mRNAs, but the level of ISG54 mRNA declined quickly, whereas the level of ISG56 mRNA remained constant over a 24-h period. In response to dsRNA, the ISG56 mRNA was induced much more efficiently, but the kinetics of induction of the two mRNAs was very similar. Sendai virus infection induced both mRNAs strongly, but the levels of both mRNAs declined rapidly. In contrast, in another cell line (HEK293), SeV caused strong and sustained induction of both mRNAs for 24 h. These results demonstrated inducer-specific and cell-type-specific differential regulations of ISG56 and ISG54 induction, some of which could be functioning at a post-transcriptional level.

The mouse ISG56 and ISG54 genes are induced by IFN β and dsRNA equally well in bone-marrow-derived macrophages, but in mouse embryo fibroblasts dsRNA cannot induce them, probably because these cells do not express TLR3. Interesting differences were noted when IFNα, IFNβ, dsRNA, or VSV were injected to mouse tail veins. In most tissues, both genes were induced in response to all inducers, but in spleen B cells, only ISG54 was induced. In liver, both genes were induced by dsRNA and IFNβ, whereas only ISG56 was induced by IFNα and VSV. These results indicate that the regulation of induction of the two closely related genes, ISG56 and ISG54, is complex and governed by both tissue-specific and inducer-specific processes (F. Terenzi et al., unpublished observation).

All members of the ISG56 family of genes encode proteins with multiple tetratricopeptide (TPR) motifs. These are degenerate protein–protein interaction motifs and they often function in combinations. Each TPR adopts a helix-turn-helix structure and adjacent TPR motifs pack in parallel, forming a spiral of repeating anti-parallel helices (Lamb et al. 1995; Blatch and Lassle 1999). Most TPR-containing proteins bind to cellular multi-protein complexes and regulate their functions. Although human and mouse ISG56 proteins have only 50% sequence identity, they both have six identifiable TPR motifs that are located along the linear protein sequences at similar positions. In contrast, the ISG54 proteins of both species have four TPR motifs located in the N-terminal halves of the protein (Sarkar and Sen 2004).

One multi-protein complex, to which the ISG56 related proteins bind, is the translation initiation factor eIF-3. Binding of the P56 proteins to eIF3 causes inhibition of the protein synthesis initiation (Guo et al. 2000). A 12-subunit protein complex, eIF3 catalyzes many steps of initiation of protein synthesis. One of these steps is the stabilization of the ternary complex, eIF2.GTP.tRNA-Met_i; this function of eIF3 is inhibited by both HuP56 and HuP54, but not by

the mouse homologs. Another function of eIF3 is to facilitate the formation of the 48S complex composed of the 40S ribosomal subunit and the 20S complex containing eIF3, ternary complex, eIF4F, and mRNA. The latter function of eIF3 is blocked by both mouse P56 and mouse P54; it is also blocked by HuP54. None of these proteins blocks the formation of the 20S complex or many other functions of eIF3. The high selectivity of the affected eIF3 functions probably reflects the fact that different regions of this large protein complex mediate different functions and the P56 proteins bind only to specific regions of eIF3 and affect the functions carried out by those regions. Support for the above concept comes from the observation that different members of the P56 family bind to specific subunits of eIF3 (Hui et al. 2003, 2005). Human P56 binds to the eIF3e protein (also known as Int-6 or P48). This protein contains both a nuclear localization signal and a nuclear export signal and its presence in both the cytoplasm and the nucleus has been noted (Guo and Sen 2000). Although its cytoplasmic function is easily attributable to its property as a subunit of eIF3, its nuclear function remains an enigma. In this context, it is worth noting that this protein was discovered as the product of the mouse Int-6 locus, whose disruption by the integration of a mouse mammary tumor virus genome causes mammary carcinoma in mice; however, the biochemical basis of that pathogenesis is unknown (Marchetti et al. 1995). HuP56 interacts with the shared C-terminal domain of both the cytoplasmic and the nuclear isoforms of eIF3e. This domain contains a PCI motif that is responsible for the P56 interaction. The PCI motif, a long α-helix, is present in different subunits of three large protein complexes: the regulatory subunit of proteasome (P), the COP9/signalosome (C) complex, and the translation initiation (I) factor 3 (Hofmann and Bucher 1998). Similar to eIF3e, eIF3c contains a PCI motif, and both mouse P56 and mouse P54 interact with eIF3c, but not with eIF3e. Human P56 does not interact with eIF3c, human P54 interacts with both subunits, and human P58 and P60 interact with neither (Terenzi et al. 2006). The interaction between eIF3c and HuP54 is mediated by at least two domains of eIF3c: the PCI domain at the C-terminal and another domain present at its N-terminal. In contrast, using its own N-terminal region, mouse P54 interacts only with the PCI domain of eIF3c and mouse P56 interacts only with the N-terminal region of eIF3c. Thus, different P56-family proteins can interact with more than one region of eIF3c (Hui et al. 2005; Terenzi et al. 2005, 2006). P56 can inhibit translation of not only capped cellular mRNAs but also viral mRNAs, such as hepatitis C mRNAs, translation of which is initiated at internal ribosomal entry sites (IRESs). There is strong evidence for the regulation of Hepatitis C protein synthesis by P56 in IFN-treated or untreated virus-infected cells (Wang et al. 2003).

7
Functions of ISG15

ISG15 is another human gene that is strongly induced by IFNs, dsRNA, and viruses (Martensen and Justesen 2004). It encodes a 15-kDa ubiquitin-like protein, P15 or ISG15. Like ubiquitin, ISG15 protein is linked to target proteins by isopeptide linkages between lysine side chains of the targets and its own carboxyl terminus. Many ISG15-conjugated proteins are present in IFN-treated cells; some are constitutively expressed and some are IFN-induced, such as ISG56. They functionally cover many aspects of cell metabolism (Zhao et al. 2005). Like ubiquitination, ISG15ylation of proteins requires the participation of three families of enzymes, E1, E2, and E3. These have been identified as UbeIL, UbcH8, and HERC5, respectively (Yuan and Krug 2001; Zhao et al. 2005; Dastur et al. 2006); However, additional enzymes with the same properties may exist. The genes encoding these enzymes are IFN-inducible as well, and the proteins themselves are targets of ISG15ylation. It is not yet known how the target proteins of ISG15ylation are selected because they apparently do not contain any common signature motifs. An enzyme, UBP43, which can remove ISG15 from these target proteins has also been identified, although its specificity is not as stringent as originally claimed (Malakhova et al. 2006).

The primary protein product of ISG15 is a 17-kDa precursor, which is cleaved to produce P15, a 15-kDa protein, upon the removal of eight carboxyl-terminal residues (Potter et al. 1999). The functional consequences of ISG15ylation of proteins remain elusive. Some information is available in this regard in the context of cells infected with different viruses. For example, the NS1B protein of influenza B virus specifically blocks ISGylation of proteins, suggesting that this process allows the virus to evade an antiviral effect of ISG15 (Yuan and Krug 2001). In another study, ISG15 was implicated to be the protein that mediates the action of IFN against HIV-1 morphogenesis (Su et al. 1995; Okumura et al. 2006). IFN inhibits the release of HIV-1 virions without affecting viral protein synthesis and this effect can be mimicked by ectopic expression of ISG15 and its activating enzymes. HIV-1 morphogenesis requires ubiquitination of its Gag protein and its interaction with the cellular protein TsgI01. ISG15 inhibits the interaction between the two proteins as well as their ubiquitination. A potential role of ISG15 in blocking the replication of Sindbis virus in mice was suggested in a study utilizing a chimeric Sindbis virus to express ISG15. ISG15 expression protected mice against Sindbis virus-induced lethality and virus replication (Lenschow et al. 2005). In contrast, $ISG15^{-/-}$ mice were perfectly capable of mounting an IFN-induced antiviral response against vesicular stomatitis virus and lymphocytic choriomeningitis virus (Osiak et al. 2005). In the future, further

evaluation of these genetically modified mice, for their ability to mount antiviral effects against other viruses, may be illuminating.

8 Future Perspectives

With the realization of the diversity of viral agents that can induce the same genes by triggering different converging pathways, the time is right to evaluate the relative contributions of these pathways in virus-infected organisms. Already there are indications that, even for a single virus, different pathways may be dominant in different cell types. It is conceivable that such differences play major roles in determining pathogenesis, or the lack of it, when the same organism is infected with the same virus, but by different routes. It is likely that many of these viral-stress-inducible genes are induced by other infectious agents as well. Functionally, the products of these genes are probably designed to protect cells from many types of extracellular stresses. For limiting the spread of viral infection, they may directly block the synthesis of viral components or may cause premature apoptosis of the infected cell to abort virus replication. Future investigations will reveal the individual properties of these proteins and the evasive mechanisms that some viruses employ to counteract them.

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Part IV: Interferon-Induced Genes

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Structure and Function of the Protein Kinase R

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Abstract The protein kinase R (PKR) is an intracellular sensor of stress, exemplified by viral infection. Double-stranded (ds) RNA produced during viral replication activates PKR, which in turn arrests protein synthesis by phosphorylating the α subunit of the translation initiation factor eIF2. As well as dsRNA, two additional ligands, PACT and heparin, directly activate the kinase. These mediate the response of PKR to additional indirect stimuli, including bacterial lipopolysaccharides, ceramide and polyanionic molecules. This responsiveness to multiple stimuli advocates a broader role for PKR as a signalling molecule for diverse physiological stresses. Appropriately, a number of other protein substrates have been reported for PKR. These substrates support additional roles for PKR in the regulation of transcription and signal transduction in infected cells, as well as uninfected but diseased tissues, such as in tumorigenesis and neurodegenerative diseases. Finally, PKR plays a role in normal cell differentiation in platelet-derived growth factor signalling and in osteoblast-mediated calcification.

1 Abbreviations

ATD	Amino-terminal heparin-binding domain
CTD	Carboxy-terminal heparin-binding domain
dsRNA	Double-stranded RNA
FA	Fanconi anaemia
FADD	Fas-associated death domain
GCN2	General control non-de-repressible 2
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HIV	Human immunodeficiency virus
HRI	Heme-regulated inhibitor
IFN	Interferon
IRF	IFN regulatory factor
ISRE	Interferon stimulatory response element
KCS	Kinase conserved sequence element
LTR	Long terminal repeat
MEF	Mouse embryonic fibroblasts
NF-κB	Nuclear factor κ B
NF90	Nuclear factor 90
PDGF	Platelet-derived growth factor
PERK	PKR-like endoplasmic reticulum kinase
PKR	Protein kinase R
RBD	dsRNA-binding domain
RBMs	dsRNA-binding motifs
S-HDAg	Hepatitis D virus small delta antigen
siRNA	Small interfering RNA
SPNR	Spermatid perinuclear RNA-binding protein
TNFα	Tumour necrosis factor α
TRAF	TNF receptor-associated factor
TRBP	TAR RNA-binding protein

2 Introduction

Protein kinase R (PKR) belongs to a small family of protein kinases that respond to environmental stresses by phosphorylating the translation initiation factor eIF2. This phosphorylation event halts translation and allows cells

to reconfigure gene expression to effectively manage stress conditions. PKR and family members, PKR-like endoplasmic reticulum kinase (PERK), general control non-de-repressible 2 (GCN2) and heme-regulated inhibitor (HRI), share a conserved kinase domain but differ in their flanking regulatory domains. These different flanking domains allow tailored responses to different environmental stresses. Activating ligands for each of the kinases include viral double-stranded (ds) RNA for PKR, uncharged tRNA resulting from amino acid starvation for GCN2, accumulated unfolded proteins for PERK, and low heme concentrations for HRI. There is, however, a degree of redundancy in the response of these four kinases. This is in part due to coordinate induction of multiple signalling pathways, but also to a shared responsiveness of each kinase. The activation of GCN2 by amino acid starvation, for example, has clear application to the detection of intracellular parasites. It could be conjectured that pressure from viral pathogens has driven acquisition of motifs by a primordial kinase to enable earlier detection of infection. Double-stranded RNA is a primary product of viral replication and its detection is crucial for establishing effective immune defenses for multicellular organisms. While homologues of GCN2, PERK and HRI occur in lower eukaryotes, PKR appears to be confined to vertebrates. The significance of PKR in resistance to viral infection is emphasized by the numerous strategies viruses have elaborated to circumvent its activity. Cellular mediators of PKR have also been identified and this underscores a role for PKR in noninfectious diseases as well as normal cell differentiation. Accordingly, PKR has been ascribed roles in some disease processes as well as in normal cell differentiation. Regulation of these diverse processes requires complex protein interactions. This review addresses the function of PKR in these various roles, with particular emphasis on the considerable biochemical, mutagenic and structural data ascertained for this most studied eIF2α kinase. The current information offers a detailed insight into the function of this key enzyme.

3
Properties of PKR

3.1
Genetic Characterization

Human PKR is encoded at position 21–22 on the short arm of chromosome 2 (chromosome 17 in mouse) (Tanaka and Samuel 1994, 1995; Kuhen et al. 1996a, 1996b; Xu and Williams 1998). The gene has 17 exons dispersed within a 50-kb genomic region. It has been shown that alternative splicing of exon 2 gives rise to three 5′-untranslated exons of different length (Kawakubo et al.

1999). Splice variants have been reported to generate kinases of variable activity from interferon (IFN)-treated U cells. An alternatively spliced form of PKR has also been reported in human T cell leukaemia Jurkat cells in which exon 7 is deleted, resulting in a truncated protein that retains the amino terminus but lacks the catalytic domain (Li and Koromilas 2001; Hii et al. 2004). This isoform acts as a dominant negative. PKR is constitutively expressed in all tissues at a basal level and is induced by type I IFNs. The promoter regions of the human and mouse *pkr* genes contain the same regulatory elements but differ in their precise arrangement (Kuhen and Samuel 1997). Basal expression is driven from a unique 15-nucleotide kinase conserved sequence element (KCS; GGGAAGGCGGAGTCC) that functions in concert with an interferon stimulatory response element (ISRE; GAAAACGAAACT) for inducible expression (Kuhen and Samuel 1999; Ward and Samuel 2002). The transcription factors Sp1 and Sp3 mediate basal expression, while IFN-inducible expression is Sp3 independent, but STAT1 and JAK-1 dependent (Das et al. 2006). An additional 40-base pair negative regulatory domain occurs approximately 400 bases upstream of the KCS element that works in concert with the KCS element to suppress transcription. The additional transcription factors Sp1, Sp3, STAT1, STAT2, IFN regulatory factor 9 (IRF9), p127DDB1 and p48DDB2 immunoprecipitate with the 5′-untranslated region of *pkr* (Ward and Samuel 2003; Das et al. 2004). Other transcription factors have been ascribed putative roles from sequence analysis of the gene promoter. These include Ets, Myb, MyoD, E2F, nuclear factor κ B (NF-κB), and interleukin-6 activation factors (Tanaka and Samuel 1994). Together these regulatory elements distinguish a gene regulated by innate immune responses as well as cell growth and differentiation processes. There is also evidence that PKR expression is autoregulated in vivo at the level of translation (Thomis and Samuel 1992).

3.2 Protein Structure

Human PKR encodes a 551 amino acid (515 in mouse) protein consisting of two functionally distinct domains: an N-terminal regulatory dsRNA-binding domain (RBD) and a C-terminal catalytic kinase domain. The RBD (amino acids 1–170) contains two dsRNA-binding motifs (RBMs) of approximately 65 amino acid residues each, separated by a 20 amino acid linker (Feng et al. 1992). The RBM is found in diverse proteins across all animal orders (Saunders and Barber 2003). All RBMs tested bind dsRNA independent of sequence, but recognize a specific higher-ordered structure (Mellits and Mathews 1988; Roy et al. 1991; Eckmann and Jantsch 1997). The RBMs of PKR bind to any RNA containing sufficient A-form helical structure, regardless of non-Watson-Crick

base pairs or mismatches, with dissociation constants in the nanomolar range (Patel and Sen 1994; Bevilacqua et al. 1998). Point mutation and domain swapping experiments in the RBD have indicated that RBM1 is more important for dsRNA binding than RBM2, although both motifs are required for optimal binding (Green and Mathews 1992; McCormack et al. 1994; Schmedt et al. 1995). The N-terminal structure of PKR encompassing the RBD has been determined using NMR (Fig. 1A) (Nanduri et al. 1998b). The individual RBMs of PKR have identical secondary structures involving an α-β-β-β-α conformation. The 20 amino acid linker consists entirely of random coil conformation and likely affords flexibility for the RBD to wrap around the dsRNA helix for optimal interactions (Nanduri et al. 2000). The ternary conformation of the RBMs of PKR have been shown for diverse RBMs derived for *Drosophila*, *Xenopus* and *Escherichia coli* proteins (Bycroft et al. 1995a; Kharrat et al. 1995; Ryter and Schultz 1998). The mechanism of dsRNA-binding derived from the solution structure of the RBD from PKR, coupled with mutagenesis data, is reinforced by two high-resolution structures of RBMs, from the *Xenopus* Xlrbpa and *Drosophila* Staufin proteins, in complex with dsRNA (Patel et al. 1994; McMillan et al. 1995a; Ryter and Schultz 1998; Ramos et al. 2000). This data shows the motif spans two successive minor grooves and the intervening major groove of the nucleic acid helix.

The structures of RBMs derived from separate proteins are very similar. However, biochemical analysis tells us that there is differential specificity for variant RNA structures, as in the case of multiple RBMs on the Staufen protein (Ferrandon et al. 1994; Micklem et al. 2000). Furthermore, RBMs appear to have an additional role in mediating protein–protein interactions (Hitti et al. 2004). The second RBM of PKR interacts with the kinase domain to inhibit the enzyme in the absence of an activating ligand (Nanduri et al. 2000). Also, the RBD reconciles association between separate PKR monomers to generate the fully active dimeric enzyme. The mechanisms of these additional functions of RBMs are not revealed in the present structures of RBMs (Ramos et al. 2000; Ung et al. 2001). Interestingly, an orthologue of PKR has been identified in lower vertebrates in which Zα domains replace the N-terminal RBD. This domain binds to RNA and left-handed conformer DNA that is associated with negative supercoiling generated in actively transcribed genes. Intriguingly, the Vaccinia viral protein E3L also encodes a Zα domain at the N-terminus. This viral protein is required to overcome the host IFN response and its N-terminus has been shown to prevent eIF2α phosphorylation, suggesting it may interact with PKR (Kahmann et al. 2004; Langland and Jacobs 2004).

The C-terminus of PKR contains the catalytic domain. Eleven conserved subdomains are recognized, with residues in subdomains V–VII being descriptive of eIF2 kinases. Amino acids in subdomain VI (HRDLKP) are consistent

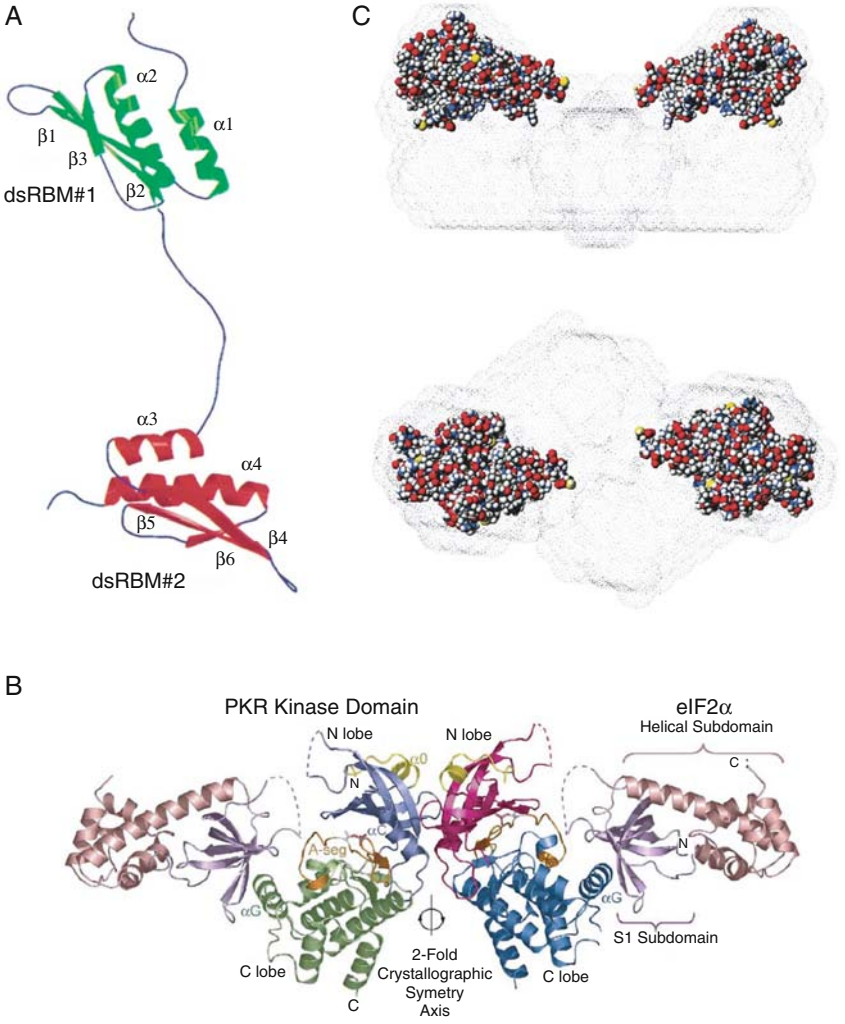

Fig. 1 **A–C** Structural analysis of PKR. The molecular structure of PKR deciphered by: **A** an NMR solution structure of the RBD of human PKR, comprising two tandem-linked RBMs both with an α–β–β–β–α fold separated by a flexible linker; **B** an x-ray crystal structure of two truncated kinases dimerized at the N-terminal lobe in complex with eIF2α, bound at the C-terminal lobe of the catalytic domain; or **C** a low-resolution solution structure determined by small-angle neutron scattering of the full-length PKR, revealing the relative orientation of the protein's RBD in the active dimer. **A** is reproduced from Nanduri et al. 1998b; **B** and **C** are reprinted with permission from Elsevier (Dar et al. 2005; Gabel et al. 2006)

with serine/threonine recognition by PKR (Hanks et al. 1988; Hanks and Hunter 1995). A distinctive feature of eIF2α kinases is an insert region between subdomains IV and V, although this varies greatly in length and in sequence between PKR, HRI, PERK and GCN2 (Chang et al. 1992; Wek 1994). A crystallographic structure of the active, dimeric kinase domain of PKR reveals that the insert region occurs in a cleft between two lobes of the domain (Fig. 1B). This cleft forms the active site of the kinase. The smaller N-terminal and larger C-terminal lobes form surfaces that separately regulate the interaction between each monomer and the substrate, respectively. In addition to the high-resolution structure of the truncated kinase domain, a low-resolution structure of the full-length active protein dimer has been generated (Fig. 1C). While the resolution of this data limits its informativeness, it is valuable to visualize the relative orientation of protein domains in the dimerized kinase. Interestingly, the orientation of the protein's RBDs in this structure is somewhat at odds with that predicted from the crystallographic structure of the truncated kinase domain. This apparent inconsistency will be resolved only when a full-length, high-resolution structure of PKR can be analyzed.

4
PKR Activity

4.1
Kinase Activation

Under normal circumstances, PKR is maintained as an inactive monomer that is converted into an active homodimer (Fig. 2). Activation requires concurrent events, autophosphorylation and dimerization. It has been proposed that PKR's second RBM interacts with residues within the insert region (328–335) to mask the enzyme's kinase domain (Li et al. 2006). This repression is released by activating ligands that elicit a conformational shift that permits binding of ATP within the kinase domain (between residues 273 and 296 in human PKR). The small molecular inhibitor 2-aminopurine also binds in the kinase's ATP-binding pocket (Jammi et al. 2003). Deletion of the amino terminus of the protein leads to relief of autoinhibition in vitro. However, this truncated kinase fails to inhibit translation in vivo, suggesting that the N-terminal sequences have an additional function. It is proposed that dimerization is partly mediated by the RBD (Patel et al. 1995). This is regulated either through direct protein–protein interactions or through dsRNA bridging the protein subunits (Patel et al. 1996). A deletion of the catalytic domain of PKR retained the ability to dimerize. In contrast, deletion of the RBD abolished dimerization (Cosentino et al. 1995). Recombinant PKR molecules with the RBD replaced by various

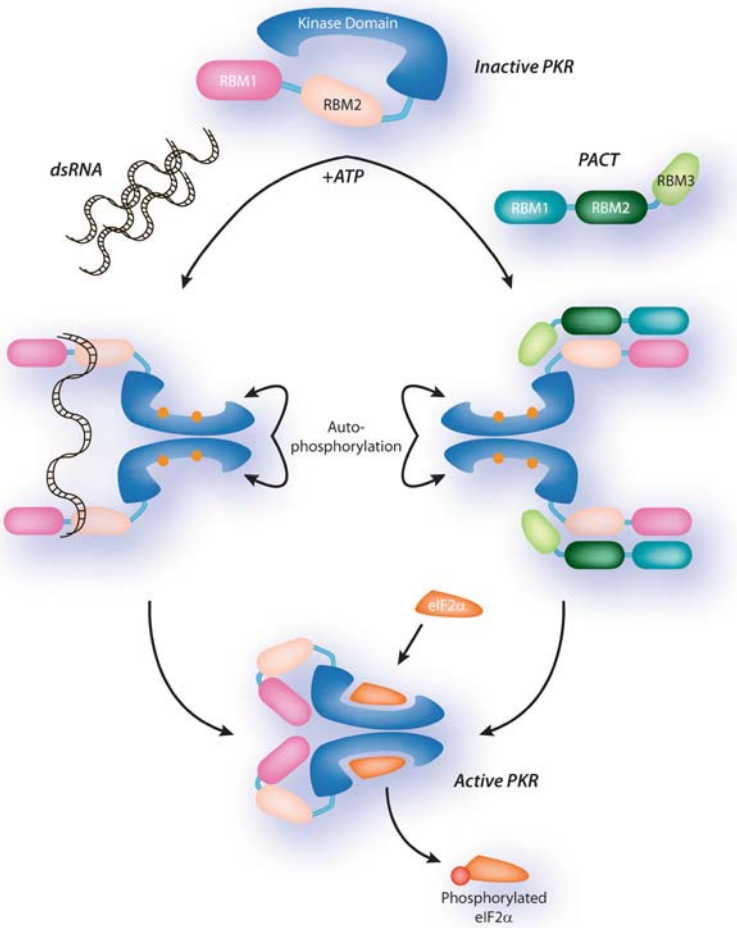

Fig. 2 Model of PKR activation. An inactive, unphosphorylated PKR monomer is activated by binding to dsRNA or protein ligands, such as the cellular regulator PACT. Binding of activating ligands at the N-terminus of the kinase disrupts an autoinhibitory conformation to enable association between monomers and binding of ATP within the catalytic site of the kinase domain. Each monomer is autophosphorylated to form the fully active, dimeric enzyme. The autophosphorylated dimer dissociates from the activating ligand and binds to and phosphorylates protein substrates, exemplified by eIF2α

heterologous dimerization domains, successfully dimerize, autophosphorylate and phosphorylate eIF2α in vivo, strongly suggesting that the RBDs contribute to dimerization by direct protein interaction (Galabru and Hovanessian 1987). However, NMR experiments have shown that the RBD is predominantly monomeric, even at very high (16 mg/ml) protein concentrations (Nanduri et al. 1998a). Similarly, single RBMs from the *Drosophila* Staufen and *E. coli* RNase III were also shown to have monomeric solution structures by NMR (Bycroft et al. 1995b; Kharrat et al. 1995). However, the third RBM from XLRBPA has been shown to mediate dimerization of this protein and heterodimers. As mentioned above, an ortholog of PKR, PKZ, has been isolated from Zebra fish and Crucian carp in which a Zα domain replaces the RBD. As PKZ functions efficiently, the RBD is not exclusive or sufficient to mediate PKR dimerization. Accordingly, residues within the C-terminal half of PKR are known to be required, and dimerization can be blocked by a peptide corresponding to amino acids 244–296 in the kinase domain (Tan et al. 1998). Multiple deletions within this region also interfere with dimerization (Romano et al. 1995). The high-resolution structure of the isolated kinase domain of PKR shows that residues on one face of the N-lobe in the kinase domain are critical in forming the interface between monomers in the active enzyme complex. This dimer interface orients the two kinase domains in a parallel, back-to-back attitude, with the enzyme's active sites facing outward. Surprisingly, this conformation of PKR differs from that determined for the dimerized kinase domains of GCN2. Although similar residues within the N-lobe of the kinase domain form the interacting interface, the two GCN2 monomers adopt an antiparallel orientation. The back-to-back conformation described for PKR makes transphosphorylation difficult, suggesting dimerization stimulates autokinase activity in cis or by another dimer. The high-resolution structure of the dimerized PKR kinase domains is formed in the absence of the RBD, so the contribution of this domain to dimer formation is not accounted for in this structure. As low-resolution images of full-length, autophosphorylated PKR dimer show an unexpected asymmetry, it remains a possibility that the formation of the fully active enzyme is strongly influenced by the protein's RBD. Therefore the variant orientation shown for the kinase domain of GCN2 may reflect flexibility of the association within the interacting interface between monomers.

The second, simultaneous process occurring during dimerization is autophosphorylation. The kinase dead PKR mutant (K296R) dimerizes weakly, suggesting autophosphorylation is required to form a stable protein dimer. This suggests that autophosphorylation and dimerization are mutually reinforced events. In this way, autophosphorylation and resulting molecular rearrangement may coordinate sequential formation of the fully active enzyme. Phosphopeptide analysis of PKR activated in vitro has identified 15 putative phosphorylation

sites. The consequence of each of the 15 predicted phosphorylation sites for kinase activity is not known. However, phosphorylation of residues within the activation loop is known to be essential for activity. Residues at position 446 and 451 lie within the activation loop between kinase subdomains VII and VIII. Substitution of threonine 451 with an alanine residue completely inactivated PKR, while a mutant with a threonine 446 to alanine substitution was partially active (Romano et al. 1998a). Identical phosphorylation sites within the homologous loop region have been identified in all eIF2α kinases. Mutation of a glutamic acid residue at 490 to glutamine partially restored the activity of the alanine 451 mutant, suggesting an interaction between the activation loop and this region (Romano et al. 1998a). Indeed, x-ray crystal analysis showed the lysine 79 and tyrosine 81 of eIF2α formed ionic and hydrophobic interactions with the glutamic acid at 490 (Dar et al. 2005; Dey et al. 2005). In addition, mutation of threonine 258 reduced, but did not eliminate, PKR activity, while mutations at another two residues, serine 242 and threonine 255, exacerbated this effect (Taylor et al. 1996).

4.2 Activating Ligands

PKR can be activated by dsRNA, polyanionic molecules such as dextran sulfate, chondroitin sulfate, poly(L-glutamine) and heparin, and protein activators (Bergeron et al. 2000). By nature of their inherent toxicity, few virally encoded protein activators have been identified. However, the capsid protein from the Semliki Forest virus has been shown to activate PKR. A variety of dsRNAs or highly structured single-stranded RNA molecules have been shown to activate PKR. Although RBMs have been shown to bind just 16 base pairs of RNA, longer RNA moieties are required to engage both motifs in the RBD. Consequently, RNA that is longer than 30 base pairs, or single-stranded RNA with duplexed regions of at least 16 base pairs with 10- to 15-nucleotide-long single-strand tails, activates PKR (Zheng and Bevilacqua 2004). As mentioned earlier, recognition is independent of nucleotide sequence and so PKR is broadly effective against dsRNA molecules produced during viral infection. Perhaps surprisingly, a number of cellular RNAs have been shown to activate PKR. These include highly structured mRNAs encoding tumour necrosis factor α (TNFα) and IFNγ. This appears to constitute an intrinsic mechanism by which these cytokines modulate their own expression. Five other cellular transcripts associated with abnormalities have been reported to activate PKR. These are mRNAs for tumorigenic p23/TCTP, the cytoskeletal muscle protein tropomyosin, stress-induced Alu RNA, and mutant forms of the Huntington's and myotonic dystrophy protein kinase (*DMPK*) genes (Chu et al. 1998; Tian et al. 2000; Peel et al. 2001; Bommer et al. 2002; Nussbaum et al. 2002).

Of the polyanionic activators, only the effects of heparin are well characterized. Heparin is a negatively charged polymer of regular disaccharide repeats with a high degree of sulfation. Heparin oligosaccharides with greater than eight sugars bind to PKRs to activate the kinase. Heparin binds within the kinase domain of the molecule. The amino-terminal heparin-binding domain (ATD, 279–318) overlaps with the ATP-binding site. The carboxy-terminal heparin-binding domain (CTD, 412–479) is located between the conserved kinase subdomains VII and VIII. Both domains function with equal efficiency, and independently, when removed from their natural context. However, in the context of the full-length kinase, the ATD has higher affinity for heparin. The mechanism by which heparin activates PKR is distinct from that of dsRNA. Unlike dsRNA-activated PKR, heparin-activated PKR cannot phosphorylate the K296R mutant, suggesting heparin stimulates intramolecular autophosphorylation and not intermolecular phosphorylation (George et al. 1996). Preincubation of PKR with heparin in the absence of ATP blocked subsequent autophosphorylation of PKR, by dsRNA or heparin in the presence of ATP, suggesting that the bound heparin may perturb dimerization, perhaps by molecular interference at the ATD. Despite the apparent different activation mechanism, PKR activated by heparin phosphorylates eIF2α. This is thought to be the process by which heparin instigates its antiproliferative effects in vascular smooth muscle cells when used to prevent atherosclerotic lesions after invasive surgical procedures (Clowes and Karnowsky 1977; Patel et al. 2002).

A number of cellular proteins have been shown to activate PKR. Most notable is the human PACT. PACT (RAX in mouse) has emerged as a regulatory molecule that responds to stress-inducing molecules such as calcium ionophores, sodium arsenite, H_2O_2 or lipopolysaccharides, as well as stress-induced cytokines, including IFNγ, interleukin-3, TNFα and ceramide (Patel and Sen 1998; Ito et al. 1999; Patel et al. 2000; Ruvolo et al. 2001; Bennett et al. 2006). PACT contains three RBMs that heterodimerize with PKR in the absence of dsRNA. The third RBM is crucial and sufficient to activate PKR in vitro (Huang et al. 2002). However, in vivo experiments demonstrate that interactions mediated by the first two RBMs of PACT are also required (Peters et al. 2001). PACT's third RBD interacts with residues within PKR's catalytic domain (328–335) to activate the kinase. A short peptide sequence (DGFDYD) within this region of PKR interacts with PACT's third RBM, as well as with the second RBM (Li et al. 2006). This interaction relieved the autoinhibition of PKR. Both PACT and RAX are substrates for PKR, and mutation of a demonstrated phosphoserine residue at position 18 (to alanine) reduced PACT/RAX's ability to activate PKR (Bennett et al. 2004). The mechanism of this interaction implies that other proteins that encode RBMs may also modulate the activity of PKR. In support of this supposition, the TAR RNA-binding protein (TRBP), which encodes three RBMs, also interacts with PKR (Cosentino et al. 1995). Rather than activating

PKR, however, TRBP inhibits the kinase (Park et al. 1994). Domain swapping experiments between PACT and TRBP show that the third RBM of each protein regulates the remarkable opposed effects on PKR (Gupta et al. 2003). A third protein that encodes RBMs, nuclear factor 90 (NF90), interacts with, and is phosphorylated by, PKR (Langland et al. 1999; Patel et al. 1999). NF90 has been claimed to modulate kinase activity (Parker et al. 2001). One further protein, the mouse spermatid perinuclear RNA-binding protein (SPNR), interacts with PKR, but did not appear to alter PKR activity. However, co-expression of the catalytically inactive mutant (K296) with PKR and SPNR was fatal for cells. Significantly, this mortality could be rescued by overexpressing eIF2α (Coolidge and Patton 2000). An additional protein that interacts with PKR is the tumour suppressor interleukin-24 (also called Mda7) (Pataer et al. 2002). This protein does not encode RBMs and the molecular nature of the protein association, and resulting activation of PKR, has not been deciphered.

Another mechanism of activation involves proteolytic cleavage of PKR. In response to apoptosis, activated caspase-3, caspase-7 or caspase-8 cleave PKR at asparagine residue 251, removing the inhibitory amino-terminal RBD (Saelens et al. 2001). The caspase-cleaved enzyme domain was shown to be constitutively active. Interestingly, biochemical analysis revealed the truncated kinase was autophosphorylated, and trans-phosphorylated intact PKR, as well as eIF2α in vivo. However, unlike the truncated PKR construct crystallized, the protein was not phosphorylated at key residues in the activation loop (451 and 446) and did not dimerize, but functioned as a constitutively active monomer (Wu and Kaufman 2004). This result reinforces the idea that the RBDs facilitate dimerization, full autophosphorylation and formation of the active kinase. As the truncation construct used to produce the crystallographic structure was autophosphorylated in the activation loop, it raises the possibility that in the absence of the RBD, protein substrates may facilitate formation of the active kinase dimer. This could be conferred by the higher order molecular interactions observed between eIF2α and PKR. In support of this, the informative crystallographic structures published by Dar et al. (2005) included a kinase dimer that bound only a single eIF2α molecule. The structure of the kinase domain that was not associated with the substrate showed considerable molecular disorder.

4.3 Inhibitors

Both viral as well as cellular inhibitors have been identified. The observation that cellular RNA can trigger PKR activation necessitates a mechanism whereby the cell can limit inappropriate activation of the kinase. Accordingly, a number of cellular proteins, such as the ribosomal protein L18 and the eIF2α-associated

glycoprotein p67, have been shown to repress PKR (Kumar et al. 1999; Gil et al. 2000b). Additional cellular repressors include nucleophosmin, which is overexpressed in a variety of human malignancies, and the oncolytic TRBP, as discussed above (Park et al. 1994; Pang et al. 2003). Three additional proteins, C114, HSP90 and P58IPK, with common protein–protein interacting motifs, one with the RBMs conserved in PKR and two with tetratricopeptide repeats, associate with and inhibit PKR (Melville et al. 1997; Donze et al. 2001; Yin et al. 2003). C114 is induced by interleukin-11 and is an example of a gp130 family cytokine modulator of PKR function (Yin et al. 2003). The Fanconi anaemia (FA) proteins, which regulate chromosome stability, reportedly associate with PKR to control its activity (Gunnery and Mathews 1998).

To circumvent the antiviral effects of IFN and to reduce an inflammatory reaction mediated by PKR, viruses have evolved elaborate mechanisms to inhibit PKR (Table 1). These include the synthesis of inhibitory dsRNAs, such as the internal ribosomal entry site from the hepatitis C virus, and the noncoding EBER-1 and -2 from the Epstein-Barr virus, as well as the adenoviral VAI RNA (Galabru et al. 1989; Sharp et al. 1993; Vyas et al. 2003). A wide variety of viral proteins inhibit PKR indirectly by sequester activating dsRNA. Examples include the reovirus sigma3 protein and the vaccinia viral E3L gene product (Davies et al. 1993; Yue and Shatkin 1997). Other proteins such as Us11 from the *Herpes simplex* virus and MC159L from the *Molluscum contagiosum* virus inhibit PKR indirectly (Poppers et al. 2000; Gil et al. 2001). At least one of these (Us11) does so by inhibiting PACT (Peters et al. 2002). The influenza virus recruits the cellular inhibitor P58IPK (Lee et al. 1994; Gale et al. 1996, 1998; Tan et al. 1998). Similarly, the *Herpes simplex* virus γ1 34.5 recruits the catalytic subunit of protein phosphatase 1α to dephosphorylate PKR (Chou et al. 1995). The poxvirus caspase-8 inhibitor, CrmA, inhibits PKR-mediated apoptosis (Ezelle et al. 2001). Other viral proteins, such as NS5A from the hepatitis C viral, vIRF-2 from *Herpes simplex* viral, vaccinias E3L and influenza NS1 proteins directly interact to inhibit PKR (Gale et al. 1997; Sharp et al. 1998; Tan and Katze 1998; Burysek and Pitha 2001). Still other inhibitors, such as the HIV TAT, vaccinia K3L and hepatitis D virus small delta antigen (S-HDAg), interact with PKR as substrates. In the instance of K3L from vaccinia virus, or C8L from swinepox virus, the proteins bind directly to PKR to block the substrate interaction sites (Carroll et al. 1993; Davies et al. 1993; Kawagishi-Kobayashi et al. 1997, 2000).

4.4 Protein Substrates

PKR is a serine and threonine protein kinase. The threonine at position 451 is diagnostic of the characterized function of PKR as a serine/threonine kinase.

Table 1 Viral inhibitors of PKR

Mechanism	Gene	Virus	Reference
Inhibitory dsRNA	IRES	Hepatitis C virus	Vyas et al. 2003
	EBER-1	Epstein-Barr virus	Clarke et al. 1990
	EBER-2	Epstein-Barr virus	Clarke et al. 1992
	VAI RNA	Adenovirus	Katze et al. 1986
	VAII RNA	Adenovirus	Ma and Mathews 1993
Binds dsRNA	σ3	Reovirus	Jacobs and Langland 1998
	E3L	Vaccinia virus	Chang et al. 1992
	NS1	Influenza virus	Lu et al. 1995
	MC159L	Poxvirus	Gil et al. 2001
	NSP3	Rotavirus group C	Langland et al. 1994
	NSP5	Rotavirus group A	Vende et al. 2002
	Us11	Herpes simplex virus	Khoo et al. 2002
	SM	Epstein-Barr virus	Poppers et al. 2003
	OVIFNR	Parapoxvirus	Haig et al. 1998
Recruits cellular proteins	NS1 (P58IPK)	Influenza virus	Tan and Katze 1998
	γ34.5	Herpes simplex virus	Mohr and Gluzman 1996
	CrmA	Poxvirus	Ezelle et al. 2001
	L(pro)	Foot-and-mouth disease virus	de Los Santos et al. 2006
	Us11	Herpes simplex virus	Peters et al. 2002
	E6	Papilloma virus	Kazemi et al. 2004
	Large-T antigen	SV-40	Rajan et al. 1995
	LANA2	Herpes virus	Esteban et al. 2003
	BILF1	Epstein-Barr virus	Beisser et al. 2005
Binds PKR	NS5A	Hepatitis C virus	Gale et al. 1997
	p58	Influenza virus	Lee et al. 1994
	SM	Epstein-Barr virus	Poppers et al. 2003
	vIRF-2	Human herpes virus-8	Burysek and Pitha 2001
	E3L	Vaccinia virus	Romano et al. 1998b
	NS1	Influenza virus	Lu et al. 1995
	PK2	Baculovirus	Dever et al. 1998
	TAT	HIV-1	McMillan et al. 1995b
	K3L	Vaccinia virus Iridoviridae	Carroll et al. 1993 Yu et al. 1999
	S-HDAg	Hepatitis D virus	Chen et al. 2002
	E2	Hepatitis C virus	Taylor et al. 1999
	C8L	Swinepox virus	Kawagishi-Kobayashi et al. 2000

However, structural features in the kinase domain suggest PKR could have broader activity. The confirmation of residues in the kinase activation region (448–452) approximates that of a tyrosine kinase (Dar et al. 2005). Some support for tyrosine kinase activity comes from an experiment that showed PKR (and also HRI) still phosphorylated eIF2α when the serine residue at position 51 was altered to a tyrosine (Lu et al. 1999). Moreover, in a recent report, tyrosine residues at position 101 and 162 in the RBD, as well as 293 in the kinase domain, were shown to be autophosphorylated (Su et al. 2006). However, the published structure of the kinase domain does not support phosphorylation of the tyrosine at position 293. The hydroxyl group of the residues at position 293 is inaccessible, and the introduction of a bulky phosphate at this position would be predicted to obstruct dimerization.

From the known phosphorylated residues, either autophosphorylation sites or residues within protein substrates, no conserved sequence motif is recognizable (Table 2). Consequently, PKR substrates have been recognized empirically. The most well-characterized PKR substrate is eIF2α (Huang et al. 2002; Dar et al. 2005). The phosphorylated serine 51 residue on eIF2α lies in a basic region and is flanked by four arginine residues on the C-terminal side (Colthurst et al. 1987; Nonato et al. 2002). The structure of the truncated PKR dimer in

Table 2 PKR has no recognizable phosphorylation motif

Protein	Phosphorylated residues	Peptide context
PKR	242	KAKR**S**LAPR
	255	DMKE**T**KYTV
	258	ETKY**T**VDKR
	446	DGKR**T**RSKG
	451	RSKG**T**LRY**M**
PACT	18	ERED**S**GTFS
eIF2α	51	LSEL**S**RRRI
TAT	62	AHQN**S**QTHQ
	64	QNSQ**T**HQAS
	68	THQA**S**LSKQ
S-HDAg	177	GVPE**S**PFSR
	180	RSPF**S**RTGE
	182	PFSR**T**GEGL

Amino acid sequences immediately adjacent to residues phosphorylated by PKR, either within the kinase itself, the protein activator PACT, the canonical substrate eIF2α, or two viral protein substrates of PKR from within the TAT protein of HIV and S-HDAg of hepatitis D virus**.** Phosphorylated residues are indicated in bold.

association with eIF2α shows a higher-order interaction between the two molecules (Dar et al. 2005). The interacting region on eIF2α appears to be unique in the human protein sequences database. This has been interpreted as meaning eIF2α can be the only substrate of PKR. This interpretation rebuts a number of previous observations. Residues within PKR itself are phosphorylated. This may not contradict the proposed specificity, as autophosphorylation is likely to be important in forming the final, active conformation of the kinase. Therefore, substrate specificity would increase during activation and autophosphorylation. A manifest challenge to such specificity is that a number of additional protein substrates have been reported. The PKR regulator, PERK, is phosphorylated by PKR. Moreover, this phosphorylation event is required for PERK-mediated activation of the kinase (Bennett et al. 2004). As with autophosphorylation of PKR, phosphorylation of PERK might be mediated by an immature conformation of PKR during activation of the kinase. However, two other cell proteins have also been identified that do not activate PKR and so do not conform to this model. These are the transcription factor NF90 and a regulatory subunit of PP2A, B56α (Patel et al. 1999; Xu and Williams 2000; Saunders et al. 2001). PKR also phosphorylates the guardian of the genome p53 at serine 392 (Cuddihy et al. 1999), although this may be indirect. In addition, a number of viral proteins have also been shown to be substrates for PKR. The HV-1 TAT protein is phosphorylated at multiple serine and threonine residues adjacent to the basic region important for TAR RNA binding and TAT function (McMillan et al. 1995b; Brand et al. 1997; Endo-Munoz et al. 2005). The hepatitis D viral S-HDAg protein is phosphorylated at serine residues at position 177 and 180, as well as at threonine residue 182 (Chen et al. 2002).

5
Processes Regulated by PKR

5.1
Cell Differentiation and Development

Phosphorylation of eIF2α mediates the antiviral and antiproliferative activities of PKR (Roberts et al. 1976; Farrell et al. 1977; Scorsone et al. 1987; de Haro et al. 1996). The consequence of phosphorylation by PKR of other substrates has not been well studied. Phosphorylation of B56α by PKR has been shown to affect the activity of downstream proteins. Regulatory B subunits associate with the core of the protein phosphatase, PP2A, to determine substrate specificity, catalytic activity and subcellular localization. When B56α is phosphorylated by PKR, the activity of PP2A is increased. B56α overexpression in cells increases protein synthesis, but this can be prevented by overexpressing PKR (Xu and

Williams 2000). The target for this regulation appears to be the translational control protein eIF4E, which is dephosphorylated by PP2A. Phosphorylation of eIF4E increases its efficiency of binding to capped mRNA, aiding translation initiation. PKR-dependent phosphorylation of B56α is proposed to increase PP2A activity, resulting in decreased eIF4E activity and reduced translation. Accordingly, PKR can regulate protein synthesis by either targeting eIF2α or eIF4E, through the regulation of the activity of PP2A. The consequence of PKR's phosphorylation of NF90 has not been delineated. Because NF90 is phosphorylated in the RBD, it would be envisaged that PKR has the potential to modulate the protein's association with dsRNA, or another demonstrated function of RBMs, such as the protein's association with ribosomes.

The effect of phosphorylation of eIF2α at serine residue 51 is well characterized. This phosphorylation event results in the inhibition of protein synthesis, which is a key protective response. Deletion of each of the different eIF2α kinases in mice emphasizes this protective response and reveals the specific activity of each kinase. GCN2-deficient mice, although viable under standard conditions, suffered significant prenatal and neonatal mortalities when mothers were reared on deficient diets during gestation (Zhang et al. 2002b). HRI deficiency, also not fatal in standard conditions, exacerbates erythropoietic protoporphyria and renders beta-thalassemia embryonically lethal (Han et al. 2001, 2005). The consequences of genetically deleting *perk* are more conspicuous, as the activating stimulus for this kinase is generated under normal physiological conditions. PERK deficiency is apparent in cells with high protein synthesis demands, such as secretory cells. Accordingly, cells such as the beta cells of the pancreas fail and mice genetically deficient for PERK display neonatal onset of insulin-dependent diabetes, as well as metabolic dysfunctions and growth retardation (Zhang et al. 2002a; Li et al. 2003).

The role of PKR has been investigated in mice using several transgenic models. Deletion mutations were targeted to both functional domains of the enzyme. The amino-terminal RBD region was ablated by deletion of exons 2 and 3, and the kinase domain of the protein was targeted by a deletion in exon 12 (Yang et al. 1995; Abraham et al. 1999). A third transgenic mouse defective in PKR activity was generated by expression of a trans-dominant negative mutant of PKR that is defective in kinase activity (K296R) (Scheuner et al. 2003). Finally, a transgenic mouse overexpressing wild type human PKR has been produced (Ladiges et al. 2002). Although the animal overexpressing wild type PKR displays a small body phenotype, there is no conspicuous phenotype in the other transgenic *pkr* mice. Mice that are genetically ablated for functional PKR appear to be compromised in the erythropoietin pathway in erythroid bone marrow precursors (Abraham et al. 1999). However, the demonstrated diminished response to erythropoietin has little physiological impact, since

hematocrit volumes from *pkr*-null animals appear normal. PKR has been shown to be required for the calcification of mouse osteoblastic MC3T3-E1 cells (Mundschau and Faller 1995; Yoshida et al. 2005). This might be expected to be apparent in murine models, as IFNβ induction is also impaired in *pkr*-null cells and IFNβ signalling has been established to be important for the regulation of the osteoclasts (Takayanagi et al. 2002; Coelho et al. 2005). However, there are no skeletal defects in *pkr*-null mice. Interestingly, *perk*-null mice also have defects in osteoblast function and exhibit skeletal dysplasia and growth retardation (Zhang et al. 2002a). Also, while expression of a dominant-negative PKR mutant (K296R) in murine C2C12 myogenic cells showed PKR was an essential component in the differentiation program of myogenic cells in vitro, there is no phenotype in the *pkr*-null mouse (Salzberg et al. 2000). The interpretation of data from the transgenic mice is complicated by contradictory findings on the observed defects in each of the mouse models. In particular, some cytokine signalling defects described in the *pkr*-null mice with a targeted N-terminal deletion are not observed in the mouse with a C-terminus deletion. Some of these irregularities have been ascribed to the different genetic backgrounds of the mice (C57B1/6J x SJL/J vs 129/terSv x BALB/c). It has also been contended that both engineered genetic deletions still express a truncated PKR product that may retain some biological activity (Baltzis et al. 2002), although in the case of the N-terminal deletion, residual kinase is not usually observed. Another complication is that any defect in murine gene deletion models is rescued, to some extent, by the other eIF2α kinase (Abraham et al. 1999). Regardless of shortcomings, the lack of conspicuous defects in any of these transgenic mice demonstrates that PKR's role is not exclusive or essential for development under normal conditions. This is as expected, as the constitutively expressed PKR is normally tightly regulated and is activated during a stress response.

5.2 Cell Signalling

As well as directly regulating proteins by phosphorylation, PKR evokes cellular responses by modulating cell-signalling pathways. The mechanisms by which PKR functions as a signalling molecule have not been fully delineated. However, PKR has been shown to mediate the response to stress stimuli such as dsRNA, IFNγ, TNFα, mitomycin C and serum deprivation by phosphorylating eIF2α, inducing degradation of IκB, IRF1 expression and indirectly mediating STAT1 phosphorylation. Effecter ligands activate PKR either directly or via protein activators such as PACT (RAX) (Bennett et al. 2006). Degradation of IκB activates the potent transcription activator NF-κB (Kumar et al. 1994; Bonnet et al. 2000; Gil et al. 2000a; Zamanian-Daryoush et al. 2000; Deb et al.

2001a; Takada et al. 2006). PKR also associates with the TNF receptor-associated factor (TRAF) to mediate activation of NF-κB (Gil et al. 2004). Regulation of NF-κB likely accounts for the diminished NOS_2 expression in *pkr*-null cells (Uetani et al. 2000; Auch et al. 2004). PKR regulates the induction of one of the principal immediate responses to viral infection, IFNβ, via NF-κB and IRF1 (Kumar et al. 1997). While PKR has been shown to be important for IFNβ induction, priming with IFN can restore this response (Gusella et al. 1995; Kirchhoff et al. 1995; Yang et al. 1995; Maggi et al. 2000). Consequently, it has been proposed that circulating endogenous IFN may prime immunity in the PKR knockout mice. While fibroblasts derived from the targeted N-terminal deletion show a defect in the induction of type I IFN and activation of NF-κB by dsRNA, no defect is apparent in the mice. PKR also physically interacts with STAT1 and STAT3 and there is a defect in IFN-induced phosphorylation of serine 727 on STAT1 in *pkr*-null cells (Wong et al. 1997, 2001; Lee et al. 2005; Wang et al. 2006). This phosphorylation of STAT1 is not mediated directly by PKR. Also, STAT1 levels are increased in cells null for *pkr* or expressing an inactive mutant PKR (Wong et al. 2001). Through this mechanism, PKR contributes to the increased expression of c-*fos* and other immediate early genes in cells exposed to platelet-derived growth factor (PDGF) (Mundschau and Faller 1995; Deb et al. 2001b). Because STAT1 phosphorylation on serine 727 is necessary for the basal expression of caspase-3 and sensitivity to apoptosis, PKR is implicated in apoptotic pathways (Deb et al. 2001b). Accordingly, *pkr*-null fibroblasts are variably resistant to apoptosis induced by different stimuli, including dsRNA, LPS and TNFα (Lee and Esteban 1994; Der et al. 1997). Conversely, overexpression of PKR in NIH3T3 fibroblasts sensitizes them to apoptosis induced not only by dsRNA or TNFα, but also by influenza virus (Gil and Esteban 2000). The transcription factor E2F-1 induces cell cycle progression at the G1/S checkpoint, and deregulation of E2F-1 provokes apoptosis in a wide variety of malignant cells. *pkr*-null, but not wild type mouse embryo fibroblasts, demonstrate significant resistance to E2F-1-induced apoptosis (Vorburger et al. 2002, 2005). Further support for a proapoptotic role comes from PKR's association with the apoptosis signal-regulating kinase ASK1 and the tumour suppressor p53 (Cuddihy et al. 1999; Takizawa et al. 2002). Although PKR-mediated apoptosis is strongly attributable to inhibition of translation through eIF2α phosphorylation, other mechanisms, including Fas-associated death domain (FADD)-mediated activation of caspase-8 are involved (Balachandran et al. 1998; Gil et al. 1999; Perl et al. 2005; Scheuner et al. 2006). Co-expression of PKR with a repressor form of IκBα (altered serine residues 32 and 36) also leads to the inhibition of apoptosis by abolishing NF-κB induction, while translation remains blocked (Gil et al. 1999). Resistance to apoptosis triggered by TNFα was not observed in mice from a different genetic background

carrying a targeted mutation in the PKR catalytic domain. Also, paradoxically, spleen cells from the transgenic mouse overexpressing wild type human PKR were resistant to apoptosis when treated with a genotoxic agent. Nevertheless, there are many independent reports describing a proapoptotic role for PKR (Lee and Esteban 1994; Der et al. 1997; Srivastava et al. 1998; Balachandran et al. 2000a, 2000b; Hsu et al. 2004).

5.3 Disease Processes

PKR plays a vital role in countering viral infection. Published phenotypes relating PKR dysfunction to other human disease are largely correlative, but they are supported by the established functions of the kinase or protein interaction data. PKR polymorphisms are associated with the outcome of hepatitis C virus infection (Knapp et al. 2003). While the permissiveness of *pkr*-null mice to viral infection is more limited than predicted, there is increased sensitivity to the normally innocuous Vesicular Stomatis virus (Lee et al. 1996; Durbin et al. 2002). Many other viral contagions are countered by PKR (see Table 3 for selected examples).

A notable departure from expectation was that *pkr*-null mice do not demonstrate increased tumour development. Overexpression of eIF2α, or a mutant form (S51A) that cannot be phosphorylated, is sufficient to cause malignant transformation (Donze et al. 1995). Expression of a functionally defective mutant of human PKR (K296R) in NIH 3T3 cells also resulted in malignant transformation, suggesting that PKR may function in tumorigenesis (Koromilas et al. 1992; Meurs et al. 1993). The failure to see conspicuous defects in the transgenic mice has been attributed to compensatory mechanisms and redundant cell signalling pathways. It is noted that neoplastic progression in human melanoma and colon cancer is associated with increased expression and activity of the kinase (Kim et al. 2002). PKR is activated in Fanconi anaemia (FA) patients, and the altered avidity of mutated FA proteins for PKR has been postulated as a pathogenic factor in this disorder (Pang et al. 2001). Furthermore, expression of a mutant RAX (S18A), which does not activate PKR, in Fanconi anaemia complementation group C-null mouse embryonic fibroblast cells not only prevents PKR activation but also blocks hypersensitivity to IFNγ/TNFα or mitomycin C, which results in enhanced apoptosis. PKR-mediated apoptosis in chronic fatigue syndrome may also contribute to the pathogenesis and fatigue symptoms associated with this immune disorder (Vojdani et al. 1997). The kinase is reportedly overexpressed in human breast carcinomas (Nussbaum et al. 2003). However, overexpressed PKR is often nonfunctional. Nonrandom chromosomal deletions of *pkr* have been found in acute leukaemia. Moreover, truncations and deletions of *pkr* have been observed in leukaemia cell lines

Table 3 PKR-mediated inhibition of viral replication

Virus	Model	Reference
Hepatitis C virus	*pkr* wt or null MEFs infected with a recombinant Vaccinia virus expressing structural and nonstructural HCV proteins, human hepatoma cells (Huh7) expressing a subgenomic HCV replicon and wt and mutant *pkr* constructs, or osteosarcoma (Saos-2) cells expressing the PKR inhibitor NS5A	Noguchi et al. 2001; Pflugheber et al. 2002; Rivas-Estilla et al. 2002; Gomez et al. 2005; Chang et al. 2006
West Nile virus	Wt and null RNase L or both RNase L and *pkr*-null mice (C57BL/6), primary bone marrow macrophages, and primary cortical neurons	Samuel et al. 2006
Human immunodeficiency virus type 1	Jurkat cells and astrocytes expressing wt or mutant PKR, or expressing an siRNA against *pkr*, latently infected promonocytic (U1) and lymphoblastoid (SupT1) cells expressing PKR regulated from the HIV-1 LTR	Nagai et al. 1997; Adelson et al. 1999; Muto et al. 1999; Ong et al. 2005
Vesicular stomatitis virus	*pkr* wt or null mice (BALB/c and 129SvEv), *pkr* wt or null MEFs, and cells expressing chicken *pkr* mRNA	Lee et al. 1996; Balachandran et al. 2000a; Stojdl et al. 2000; Durbin et al. 2002; Ko et al. 2004
Sindbis virus	*pkr* wt or null MEFs and NIH-3T3 cells expressing wt or mutant PKR	Gorchakov et al. 2004
Herpes simplex virus type 1	*pkr* wt or null MEFs, and mouse trigeminal ganglion cells transduced with an adenovirus expressing murine IFNβ	Khabar et al. 2000; Al-khatib et al. 2003
Influenza virus	*pkr* wt or null mice (BALB/c) infected with a mouse-adapted strain, as well as *pkr* wt or null mice (C57BL/6) infected with influenza virus lacking the nonstructural protein NS1	Balachandran et al. 2000a; Bergmann et al. 2000
Bunyamwera virus	*pkr* wt or null mice (129) infected with either wt or mutant virus lacking the nonstructural protein	Streitenfeld et al. 2003

(Continued)

Table 3 PKR-mediated inhibition of viral replication—cont'd.

Virus	Model	Reference
Hepatitis D virus	COS7 or HuH7 cells co-transfected with a HDV cDNA and plasmids expressing either wt or mutant PKR	Chen et al. 2002
Vaccinia virus	Infection of chicken fibroblasts or HeLa cells with recombinant Vaccinia virus encoding inducible wt or mutant PKR	Lee and Esteban 1993; Lee et al. 1996; Esteban and Patino 2000
Encephalomyocarditis virus	*pkr* wt or null MEFs, and promonocytic U937 cells with *pkr* repressed with an antisense construct	Yeung et al. 1999; Khabar et al. 2000
Foot-and-mouth disease virus	*pkr* wt or null MEFs infected with wt or mutant virus lacking the gene for L proteinase	Chinsangaram et al. 2001

HCV, hepatitis C virus; *HDV*, hepatitis D virus; *HIV*, human immunodeficiency virus; *LTR*, long terminal repeat; *MEFs* mouse embryonic fibroblasts; *siRNA*, small interfering RNA; *wt*, wild-type

and loss of PKR activity has been observed in chronic lymphocytic leukaemia (Hii et al. 2004). Interestingly, a point mutation was detected in the first RBM of PKR from a patient with acute lymphoblastic leukaemia (residue 17 changed from a tyrosine to a cysteine) (Murad et al. 2005). PKR expression levels were associated with disease recurrence and overall survival in lymph node-negative rectal cancer patients (Kwon et al. 2005). Also, the PKR-inhibitor ribosomal protein L18 is overexpressed in colorectal cancer tissue (Kumar et al. 1999). PKR has also been implicated as a disease factor in systemic lupus erythematosus by impairing the translational and proliferative response to mitogens by T cells (Grolleau et al. 2000). The known role of PKR in cytokine-induced signalling pathways, together with data showing cytokine regulation of PACT and PKR in chondrocytes, suggests PKR may be important in the pathogenesis of arthritic diseases (Gilbert et al. 2002).

A number of disorders of the central nervous system, such as Huntington's, Parkinson's and Alzheimer's disease, show PKR activation associated with neuritic plaques and pyramidal neurons in the hippocampus and neocortex, suggesting PKR is a potential pathogenic factor (Peel et al. 2001; Peel and Bredesen 2003; Bando et al. 2005; Paccalin et al. 2006). The pathogenic effect in Alzheimer's appears to be mediated by Aβ-triggered apoptosis in neurons (Onuki et al. 2004; Yu et al. 2006). Interestingly, the mice expressing

the trans-dominant negative mutant of PKR (K296R) did provide support for a role for PKR in modulating viral pathogenesis in intracerebral infection (Palma et al. 2003). In experiments with mouse-adapted poliovirus, PKR wild type mice had increased tissue damage in the central nervous system due to a deleterious host immune response as compared to mice expressing the K296R dominant negative kinase (Scheuner et al. 2003). Also, eIF2α phosphorylation, likely mediated by PKR, has been demonstrated to play a role during pilocarpine-induced epilepsy and subsequent neuronal death in mice (Cavalheiro et al. 1996; Carnevalli et al. 2004, 2006). These latter observations of pathologies in the brain may highlight a role for PKR at immune-privileged sites in the body.

6 Conclusions

PKR was originally discovered as a mediator of protein synthesis inhibition by dsRNA. The characterization of the response of *pkr*-null mice to different virus infections has confirmed its importance for mediating resistance to infection. However, it has become apparent that different viruses are differentially sensitive to the presence or absence of PKR. This is likely due to the nature of their replicative cycles and subsequent dsRNA production and their differing abilities to elaborate strategies to inhibit PKR activation or activity. The kinase also plays a role in pro-inflammatory gene expression in response to different stimuli by direct substrate phosphorylation and by indirect signal transduction. Further insight into its mechanism of activation, substrate specificity and physiological function awaits more detailed structural investigation and the production and analyses of mice with more subtle targeted mutations in PKR.

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Part V: Virus Defense

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Viral Regulation and Evasion of the Host Response

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Abstract The type I interferons (IFN) are cytokines produced by mammalian cells in response to virus infection or other stressors. IFNs exert antiviral function by binding to the IFNα/β receptors on the cell surface, with the major effect of triggering the JAK-STAT signaling cascade and inducing the expression of interferon-stimulated genes. The resulting innate antiviral response is a first line of immune defense against virus infection. Recent studies demonstrate that many viruses, including hepatitis C virus, direct processes to control the host response to infection. We provide a review of the virus/host processes involved in IFN signaling and regulation of innate immune defenses.

1 Introduction

The type I interferons (IFN), which include several IFNα subtypes and IFN-β are cytokines that are produced and secreted by most cell types in response to virus infection. The type I IFNs exert their antiviral function by binding specifically

to the IFNα/β receptors found on the cell surface of both infected and non-infected cells. This interaction initiates a signaling cascade that controls the expression of hundreds of interferon-stimulated genes (ISGs) that collectively alter the intracellular environment and modulate the immune response toward establishing an antiviral state (Honda et al. 2006). The resulting innate antiviral host response is our first line of defense against viruses. Without IFN defenses, cells become highly susceptible to viral infections. Viruses must overcome IFN actions to successfully infect the host cell. Recent studies demonstrate that most viruses have evolved a multitude of ways to control the host response. Here, we provide an overview of the virus/host processes involved in type I IFN signaling and discuss some of the recent advances made in understanding how viruses evade the innate antiviral response.

2
Viral Recognition by TLRs and DExD/H RNA Helicases Initiate IFN Production and Activation of the Innate Antiviral Response

Specialized pathogen recognition receptor (PRR) proteins signal the production of IFN when they recognize motifs termed pathogen associated molecular patterns (PAMPs) present within the virion or intermediate products of virus replication and infection. The two major classes of viral specific PRRs include Toll-like receptors (TLRs) and the family of DExD/H box RNA helicases (Meylan and Tschopp 2006). Once engaged by the appropriate PAMP ligand, PRRs initiate intracellular signaling cascades that result in the activation of latent transcription factors including IFN regulatory factor-3 (IRF-3) and nuclear factor κB (NF-κB) (Fig. 1). Once activated, IRF-3 and NF-κB accumulate in the host cell nucleus and bind to target promoters to coordinately induce the expression of IFN and other IFN responsive genes (ISGs). The products of many ISGs are thought to possess activities that can directly limit viral replication and ultimately facilitate virus clearance from host cells. As a consequence, PRR expression and distribution, which is known to vary in different tissues, dictates in large part the permissiveness of a particular cell type to virus infection. The specificity and affinity of PRRs for different PAMPs further ensures that mammalian cells are equipped with a battery of sensors to detect different viruses at each step of the viral life cycle.

Mammalian TLRs are a family of type I integral membrane glycoproteins that were first identified as homologs of the Toll genes involved in the *Drosophila* innate antifungal response (Meylan and Tschopp 2006). TLRs are expressed either on the cell surface or within endosomal vesicles of a variety of cell types. Of the human TLR members identified thus far, TLRs 2, 3, 4, 7, 8, and 9 have

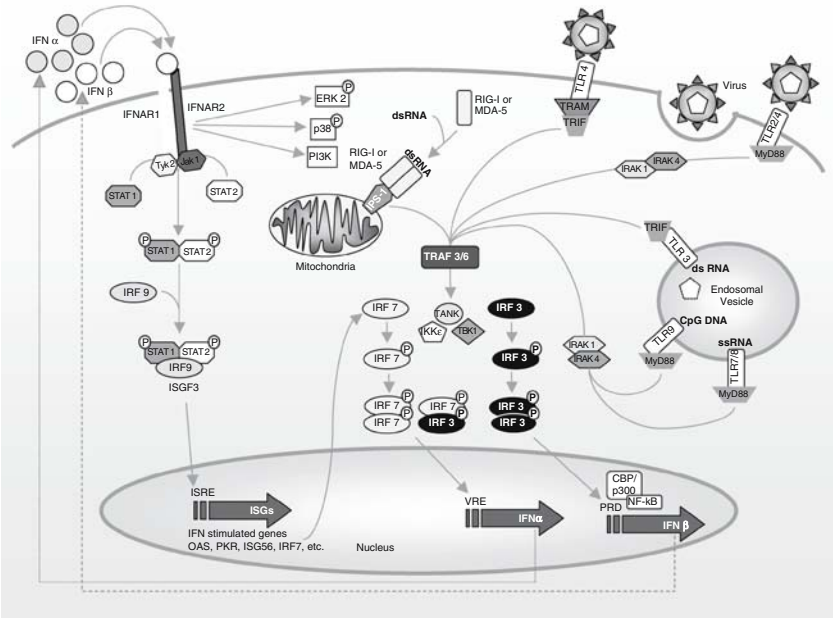

Fig. 1 Virus signaling and interferon production. Viral PAMPs are recognized by various cellular PRRs that signal the activation of transcription factors including IRF-3 to induce the expression of IFNβ. IFNβ is secreted into the extracellular space where it binds to the IFNα/β receptor and activates the JAK-STAT signaling pathway to drive the production of various IFNα subtypes and the expression of ISGs to establish an antiviral state within host cells. IFNα serves to further enhance the host response by amplifying the expression of an even broader collection of ISGs to limit virus replication and spread. Details are in the text

been suggested to recognize distinct viral components as PAMP ligands. Cell surface-expressed TLR2 and TLR4 have been shown to interact with viral envelope proteins, viral hemagglutinin, and inactive whole virus particles (Kawai and Akira 2006a, 2006b), and therefore may mediate the recognition of viral envelope glycoprotein or lipoprotein complexes during virus attachment and entry. TLRs 3, 7, 8, and 9 have been implicated in viral nucleic acid recognition. These TLRs are almost exclusively expressed within intracellular endosomal compartments, wherein they are ideally situated to detect viruses shortly after they uncoat within the host cell. TLR7 and TLR8 confer recognition of uridine-rich motifs in single-stranded RNA (ssRNA), and are implicated in the recognition of vesicular stomatitis virus, influenza virus, and human

immunodeficiency virus. TLR9 recognizes CpG DNA motifs and signals the host response to adenovirus and herpes simplex virus infections, whereas TLR3 has been implicated as a PRR for double-stranded RNA (dsRNA) motifs frequently present as secondary structures within viral RNA.

The compartmentalization of TLRs allows surveillance of both the extracellular space and endosomal lumen for viral PAMPs. TLRs recognize viral PAMPs via an extracellular domain that contains varying numbers of leucine-rich-repeat (LRR) motifs (Bell et al. 2003). Ligand binding by the LRR activates TLRs to facilitate recruitment of various Toll/IL-1 receptor (TIR) homology domain-containing adaptor molecules to the TLR cytoplasmic tail (Kawai and Akira 2006a). TLRs 2, 4, 7, 8, and 9 signal the production of type I IFN via myeloid differentiation primary response protein 88 (MyD88), which negotiates the activation of selective IL-1 receptor-associated kinases (IRAK-1 and IRAK-4), tumor necrosis factor receptor-associated factors (TRAF3 and/or TRAF6) and the TGF-β activated kinase (TAK1). In contrast, TLR3 signals via a MyD88-independent pathway involving the TIR adaptor Toll/IL-1 receptor domain-containing adapter-inducing IFNβ (TRIF). Similarly, TLR4 can also signal in a MyD88-independent manner through TRIF, but only when mediated by the TIR-related adaptor molecule, TRAM.

Retinoic acid inducible gene-I (*Rig-I*) and melanoma differentiation associated gene-5 (*Mda-5*) encode DExD/H box RNA helicases that comprise a second class of PRRs (Meylan and Tschopp 2006). Their expression in the cytoplasm enables these two receptors to specialize in the detection of viral RNA as they accumulate during viral genome replication in the cytosolic compartment. RIG-I and MDA-5 are unique among members of the RNA helicase superfamily because they contain tandem caspase activation and recruitment domains (CARDs) (Yoneyama et al. 2004). CARDs are known to direct interactions with other CARD-containing molecules and are common among caspase signaling components (Bouchier-Hayes and Martin 2002). Biochemical studies reveal that RIG-I and MDA-5 can engage various RNA ligands via their conserved helicase domains (Marques et al. 2006; Sumpter et al. 2005; Yoneyama et al. 2004). Interaction with an appropriate RNA PAMP is thought to promote the ATP-driven protein conformational change that facilitates CARD-dependent recruitment of a mitochondrial-bound adaptor molecule known as IPS-1, MAVS, CARDIF, and VISA essential for RIG-I and MDA-5 signaling of the host response (Johnson and Gale 2006). The interaction of RIG-I with IPS-1 promotes the activation of a TRAF3, TRAF6, IKKi/IKKε-containing macromolecular complex that signals the activation of IRF-3 and NF-κB, leading to IFN production (Saha et al. 2006; Xu et al. 2005).

Studies involving cells from mice with deletion of either the *Mda-5* or *Rig-I* genes demonstrate that MDA-5 is indispensable for the detection of

picornaviruses (Gitlin et al. 2006; Kato et al. 2006), whereas RIG-I appears to be the primary sensor for a variety of RNA viruses including influenza virus, vesicular stomatitis virus (VSV), New Castle disease virus, and Sendai virus (Kato et al. 2006). RIG-I is also essential for inducing the host response to hepatitis C virus infection (Sumpter et al. 2005). With the exception of plasmacytoid dendritic cells that are heavily dependent upon TLRs for signaling, cells from mice with *Ips-1* deletion failed to respond to RNA virus infections (Kumar et al. 2006; Sun et al. 2006). This demonstrates the essential role of IPS-1 as an adaptor for RIG-I and MDA-5 signaling, and further emphasizes the importance of RIG-I and MDA-5 in the recognition of RNA viruses in the host response.

Production of type I IFN is transcriptionally regulated and induced upon PRR recognition of viral PAMPs during infection. Transcription factors that are known to regulate type I IFN production include ATF-2/c-Jun, NF-κB, and the interferon regulatory factors (IRFs) (Honda et al. 2006). IRF-3 primarily regulates the transcription of IFNβ. Upon infection, PRR signaling results in IRF-3 phosphorylation by the TANK-binding kinase (TBK1) and/or the noncanonical IKB kinase IKKi/IKKε. Phosphorylated IRF-3 dimerizes and accumulates in the nucleus where along with other transcription factors and co-factors it coordinately binds to target promoters, drives the expression of IFN-β, and induces the expression of a subset of ISGs. As a result, IFNβ is secreted into the extracellular milieu where it binds to the cell surface IFNα/β receptors to signal the host response through the JAK-STAT pathway (Takaoka and Yanai 2006). Activation of the ISGF3 transcriptional complex specifically drives the expression of IRF-7 (Honda et al. 2006). IRF-7 is activated by TBK1 and/or IKKi/IKKε, and can dimerize with itself or heterodimerize with IRF-3 to drive the expression of the IFNα subtypes, thus amplifying the IFN signaling process. Excessive signaling of the host response is prevented by cellular negative regulators of JAK-STAT including members of protein inhibitors of STATs (PIAS1 and PIAS$_y$) (Shuai and Liu 2005) and suppressors of cytokine signaling (SOCS1 and SOCS3) (Yoshimura et al. 2005).

3 Viral Regulation of the Host Response

Viruses by necessity must evade or control the host response in order to replicate and spread. Many studies have revealed virus–host interactions that dysregulate or attenuate the host response to infection. In the following section, we will discuss some of the strategies utilized by viruses in their quest to regulate and evade the host response mediated by type I IFN (Fig. 2A and B).

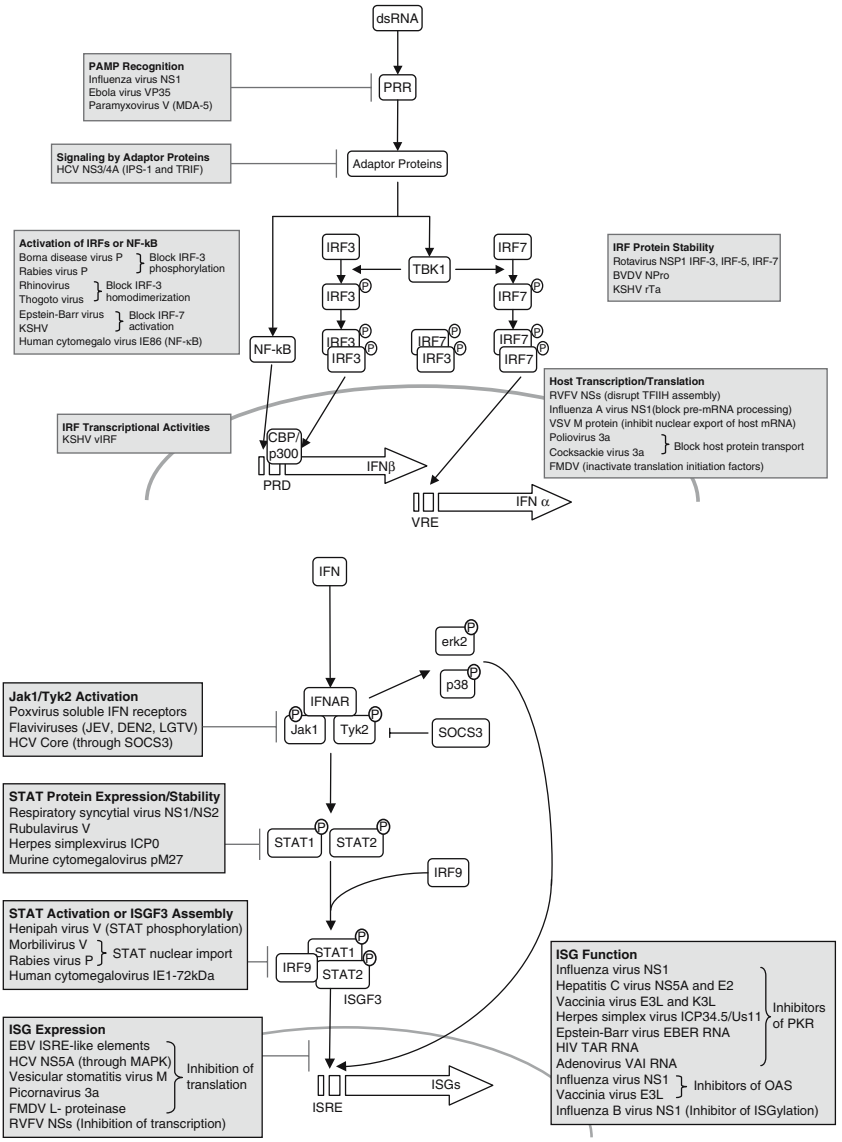

Fig. 2A, B Different strategies utilized by viruses as a means to evade or modulate the type I IFN-mediated host response. **A** Viruses can prevent PAMP detection or block the production and/or secretion of IFN to attenuate the innate antiviral response. **B** Viruses can modulate JAK-STAT signaling to control the IFN amplification loop and limit ISG expression.

3.1 Interference with PRR Activation

Viral interference of PAMP recognition and PRR activation essentially blinds the host cell from detecting or signaling the presence of invading viruses. The subsequent attenuation of the host response presents the virus with opportunities to escape and establish successful infection without the limitations imposed by IFN. Investigations into viral strategies that specifically interfere with PRR activation reveal that while some viruses conceal from PRR detection, others encode factors that disrupt PRR recognition of PAMPs or that block PRR activation downstream of PAMP recognition. For example, West Nile virus (WNV) triggers a potent host response that involves IRF-3 activation and ISG expression, but this response is delayed until late points in the viral replication cycle (Fredericksen et al. 2004). This host response delay is not due to the expression of a viral factor that actively blocks IRF-3 activation (Fredericksen and Gale 2006), but instead it is likely that WNV sequesters or conceals viral components from PRR detection to delay host response triggering until the replication cycle has been completed. In contrast, the paramyxovirus V protein binds to MDA-5 to block signaling of the host response (Andrejeva et al. 2004).

The influenza virus and Ebola virus encode dsRNA-binding proteins, respectively termed NS1 and VP35, that antagonize IFN production (Basler et al. 2000; Krug et al. 2003). Both are required for efficient viral amplification and virulence (Enterlein et al. 2006; Talon et al. 2000). The influenza virus NS1 protein attenuates type I IFN production in part by directing a global suppression of mRNA maturation (Krug et al. 2003) (discussed later) or disrupting the processes of PRR signaling of IRF-3 and NF-kB activation (Talon et al. 2000; Wang et al. 2000). Moreover, mutation of the two basic amino acid residues (R38 and K41) that are critical for dsRNA-binding also resulted in increased IFN production and attenuated pathogenicity in mice (Donelan et al. 2003), suggesting that the ability of NS1 to function as an IFN antagonist may be mediated in part by its ability to bind dsRNA. DsRNA binding activity of NS1 is also required for its inhibition of other cellular dsRNA-binding proteins (Hatada et al. 1999; Lu et al. 1995; Min and Krug 2006), implicating this activity as a common strategy used by influenza virus to control host processes.

The Ebola VP35 protein prevents type I IFN production by inhibiting the activation of IRF-3 (Basler et al. 2003). Mutational studies mapped the IFN antagonistic activity of VP35 to its C-terminus RNA-binding domain (Hartman et al. 2004). A basic amino acid motif within this domain is essential for blocking the host response (Hartman et al. 2006). This motif exhibits homology with

part of the dsRNA-binding domain of the influenza virus NS1 protein (Hartman et al. 2004). Recombinant viruses encoding VP35 mutants defective in dsRNA binding were partially impaired in their ability to block RIG-I signaling of type I IFN (Cardenas et al. 2006), suggesting that the inhibitory effects of VP35 are mediated by its ability to bind dsRNA and sequester it from activating RIG-I signaling.

3.2 Inactivation of the IFN Transcriptional Factors

The Kaposi's sarcoma-associated herpes virus (KSHV) and bovine viral diarrhea virus (BVDV) both encode proteins that direct the poly-ubiquitylation and degradation of IRFs (Hilton et al. 2006; Rezaee et al. 2006). KSHV encodes an ubiquitin E3 ligase (RTa) specific for IRF-7 (Rezaee et al. 2006), whereas the N-terminus protease fragment of the BVDV polyprotein (NPro) specifically targets IRF-3 for poly-ubiquitylation by directing it to a ubiquitylation complex (Hilton et al. 2006). The Rotavirus dsRNA-binding protein NSP1 also induces the rapid degradation of IRF-3, IRF-5, and IRF-7 (Barro and Patton 2005, 2007) that consequently attenuates IFN production. Expression of NSP1 directly correlates with increased virulence and spread, suggesting that NSP1 dysregulation of IRF-3 signaling contributes to virus fitness in evading the immune response. The various steps leading to activation of IRF-3 are also subject to viral regulation. Borna disease virus and rabies virus both express phosphoprotein P that blocks TBK1 phosphorylation and activation of IRF-3 (Brzozka et al. 2005; Unterstab et al. 2005). The Borna disease virus phosphoprotein P accomplishes this by acting as a viral decoy substrate that competes with IRF-3 for phosphorylation by TBK1 (Unterstab et al. 2005). In contrast, rhinoviruses and Thogoto virus inhibit the host response by blocking IRF-3 homodimerization (Jennings et al. 2005; Peng et al. 2006).

Members of the herpesvirus family have been shown to attenuate the host response by blocking the actions of transcription factors essential for IFN production. A unique strategy employed by KSHV is the expression of virally encoded IRF homologs (vIRFs) that are defective in signaling (Rezaee et al. 2006). These virally encoded IRFs (vIRFs) suppress the transcriptional activities of endogenous IRFs by interacting directly with their cellular counterparts, thus preventing them from binding to their target promoters. Moreover, like KSHV, Epstein-Barr virus also encodes viral factors that block IRF-7 activity (Hahn et al. 2005; Rezaee et al. 2006), whereas the human cytomegalovirus (CMV) IE86 protein attenuates NF-κB-directed cytokine and chemokine gene expression by blocking NF-κB binding to target promoters (Taylor and Bresnahan 2006). NF-κB destabilization

presents another common strategy of host response control that is also shared by poliovirus and other picornaviruses (Neznanov et al. 2005).

3.3 Abrogation of JAK-STAT Signaling

Viruses disrupt JAK-STAT signaling by directing the degradation, sequestration, or inactivation of the STAT proteins. The Rubulavirus V protein binds to STATs and directs their poly-ubiquitylation, resulting in STAT degradation within proteasomes (Horvath 2004). In contrast, the V protein from Henipaviruses functions by blocking the transcriptional activity of the STAT proteins. This is achieved by the V protein-dependent sequestration of STATs as high-molecular-mass complexes in the cytoplasm, thus preventing STAT activation. The V protein from measles virus blocks the nuclear import of activated STATs, thus preventing their transcriptional activity.

Herpes simplex virus (HSV) 1 ICP0 inhibits the host response by promoting the poly-ubiquitylation and degradation of STAT1 (Halford et al. 2006). HSV has further been implicated in blocking STAT transcriptional activities by inducing the expression of SOCS3 (Yokota et al. 2004). In contrast, CMV encodes an IFN antagonist that inhibits the transcriptional activity of STAT proteins. In this case, IE1-72kDa physically complexes with STAT1 and STAT2 to block ISGF3 complexes from interacting with target promoters (Paulus et al. 2006). The rabies virus phosphoprotein P targets the STAT proteins uniquely through a selective interaction with phosphorylated STAT1 and STAT2 (Brzozka et al. 2006). The phosphoprotein P-STAT interactions consequently retain activated STATs in the cytoplasm where they cannot initiate transcription of target genes.

Flaviviruses regulate IFN-induced signaling from the IFNα/β receptor by expressing proteins that prevent STAT activation. Dengue virus and the distantly related Japanese encephalitis virus (JEV) both impede IFN-induced tyrosine phosphorylation and activation of Tyk2 (Ho et al. 2005; Lin et al. 2006). In the case of JEV, this is mediated by the viral RNA-dependent RNA polymerase NS5 via a mechanism that requires the activity of a protein tyrosine phosphatase (Lin et al. 2006). Langat virus and WNV similarly attenuate IFN signaling by preventing the phosphorylation of IFN-receptor-bound kinases (Guo et al. 2005; Liu et al. 2005). In the case of WNV, this inhibition appears to be mediated primarily by a combination of viral nonstructural proteins (Liu et al. 2004, 2006). While WNV is generally associated with asymptomatic infections in regions where it is endemic, pathogenic strains that are associated with outbreaks of encephalitis and meningitis have emerged in Israel, Europe, and North America. A recent study comparing an endemic and an emergent strain of WNV provides evidence that successful modulation of the innate antiviral

response contributes to viral fitness and virulence of pathogenic WNV strains (Keller et al. 2006).

3.4 Viral Inhibition of Host Gene Expression

Viruses can evade the host response by selectively inhibiting cellular gene expression while simultaneously promoting transcription and translation of viral genes. The influenza A virus NS1 protein (NS1A) inhibits cellular translation processes by blocking nuclear export of mRNAs that contain 3′ poly(A) ends (Krug et al. 2003). NS1A regulation of cellular mRNA transport is twofold. NS1A binds to both the cellular cleavage and polyadenylation specificity factor (CPSF) and poly(A)-binding protein II (PABII) required for efficient 3′-end processing of cellular pre-mRNAs. Through these interactions, NS1A blocks cellular pre-mRNA processing and consequently retains mRNA in the nucleus where they are not accessible for translation. Infection of cells with a recombinant influenza virus that encodes a mutant NS1A incapable of binding CPSF induced robust IFN production and was substantially attenuated. Consistent with this, influenza B virus, which encode NS1 (NS1B) lacking the ability to bind CPSF, demonstrate no inhibition of cellular transcription and correspondingly failed to block IFN production during infection. These observations provide evidence that influenza virus NS1 regulation of cellular post-transcriptional mRNA processing blunts the innate antiviral response to virus infection.

Vesicular stomatitis virus (VSV) suppresses host gene expression by inhibiting nuclear export of mRNA through the actions of the matrix protein (M). The M protein inactivates components of the nuclear mRNA export machinery, Rae1 and Nup98 (Faria et al. 2005). Poliovirus, Coxsackie virus, and the foot and mouth disease virus (FMDV) all encode various factors that block protein transport and maturation through the secretory pathways (Choe et al. 2005; Moffat et al. 2005). In addition, FMDV exemplifies picornaviral strategies to block host protein synthesis: it encodes the leader-proteinase and 3C protease, which function to specifically cleave and inactivate the cellular translation initiation factors eIF4G and eIF4A within infected cells (Belsham et al. 2000), thus inhibiting cap-dependent cellular protein synthesis. Since picornaviral and FMDV translation is dependent on internal ribosome entry site (ISRE) elements, viral protein synthesis is not affected.

Viruses also evade the IFN-innate antiviral induced response by inhibiting global cellular transcriptional processes. For example, the NSs protein encoded by Rift Valley fever virus (RVFV) interacts with the basal transcription factor TFIIH and disrupts TFIIH assembly (Billecocq et al. 2004; Le May et al. 2004), thus inhibiting cellular gene expression. RVFV strains that express mutant NSs incapable of engaging TFIIH are robust inducers of IFN and exhibit an attenuated phenotype

due to the incapacity to control cellular transcription processes (Billecocq et al. 2004). These observations suggest that virus strains with attenuated properties of IFN signaling control could serve as platforms for the development of vaccines.

3.5 IFN Antagonists That Interfere with ISG Function

The activity of ISGs is also subject to viral regulation to effect immune evasion. PKR is a dsRNA activated, IFN-induced protein kinase that is expressed in most cell types. Upon binding to dsRNA or the cellular protein PACT, PKR is activated to phosphorylate the alpha subunit of the eukaryotic translation initiation factor-2 (eIF2α) to inhibit protein synthesis (Langland et al. 2006). Activation of PKR by PACT is regulated by the influenza NS1 protein through direct interactions (Krug et al. 2003). As a dsRNA-binding protein, NS1 may further sequester dsRNA activators from PKR (Hatada et al. 1999; Lu et al. 1995). Influenza virus also induces $P58^{IPK}$, a cellular PKR inhibitor, thus blocking the catalytic activity of PKR during late-stage infection (Melville et al. 1999). EBER and VA RNA encoded by Epstein-Barr virus and adenovirus, respectively, bind to PKR and prevent its activation (Langland et al. 2006). Moreover, Vaccinia virus encodes two PKR inhibitors, E3L and K3L (Haga and Bowie 2005). The former sequesters dsRNA, while the latter functions as a pseudo-substrate to block dsRNA-induced PKR activation.

The IFN inducible 2′-5′ oligoadenylate synthase (OAS) is an ISG product whose actions are regulated during virus infection. OAS is activated by dsRNA to catalyze the conversion of ATP into small 2′-5′ linked adenosine oligomers, which promotes the activation of a latent cellular endoribonuclease, RNAse L (Liang et al. 2006). Activated RNAse L functions to degrade both viral and cellular RNA nonspecifically and rapidly terminates all translation events within infected cells. Different OAS isoforms may further participate in apoptotic signaling or function to suppress virus replication. Catalytic activation of OAS is blocked by the influenza A virus NS1 protein (Min and Krug 2006) and Vaccinia virus E3L protein (Haga and Bowie 2005), which function to sequester dsRNA from activating OAS and RNase L.

ISG15 is an ISG that encodes a ubiquitin-like cellular protein whose expression is rapidly induced by IFN and virus infection (Langland et al. 2006). ISG15 is expressed as an inactive protein and is quickly processed and attached to various cellular proteins through a process termed ISGylation. This process of ISGylation has been implicated in the control of JAK-STAT signaling (Malakhova et al. 2003) and in protecting IFN responsive factors from cellular degradation (Lu et al. 2006), suggesting that ISG15 may function to promote the IFN innate antiviral response. The NS1 protein from influenza B virus but not that of

influenza A virus binds ISG15 and prevents its conjugation to cellular proteins during infection (Krug et al. 2003). Current evidence suggests that viral strain difference in ISG15 regulation may be due to the absence of an ISG15-interaction domain within the structure of NS1A.

4 Hepatitis C Virus: A Story of Immune Evasion and Persistence

Hepatitis C is a life-threatening disease that afflicts approximately 170 million people worldwide, and the most common cause of liver failure. The causative agent of this disease is the hepatitis C virus (HCV), a hepatotrophic virus of the *Flaviviridae* family (Major et al. 2001). HCV encodes a single-stranded RNA (ssRNA) genome of positive sense that is flanked on each side by a 3′ or 5′ nontranslated region (NTR). An internal ribosome entry site (IRES) located within the 5′ NTR directs the cap-independent translation of viral RNA to make a polyprotein that is post-translationally cleaved into ten individual structural (Core, E1, and E2) and nonstructural proteins (p7, NS2, 3, 4A, 4B, 5A, and 5B). The structural proteins are released by host signal peptidases, whereas the nonstructural proteins are processed by two virally encoded proteases, NS2 and NS3/4A.

Replication of the HCV genome takes place in the cytoplasm and is associated with intracellular membranes (Major et al. 2001). Structural analysis of the single-stranded RNA shows that it contains regions including the 3′ and 5′ UTRs that form extensive secondary structures (Brown et al. 1992; Tanaka et al. 1996). Biochemical studies reveal that there exist RNA motifs embedded within these regions that are recognized as PAMPs by PRRs, including RIG-I, and therefore are capable of engaging the host response to induce ISG expression and IFN production in cultured hepatocytes (Saito et al. 2007; Sumpter et al. 2005). Among the PRRs, RIG-I alone appears to be essential in hepatocytes for the recognition of HCV RNA, as induction of the host response is completely abolished in a cell line with intact MDA-5 but defective in RIG-I signaling (Sumpter et al. 2005). In general, the acute phase of HCV infection is associated with the induction of a robust host response, which is typified by the production of IFN and the expression of ISGs in infected liver (Gale and Foy 2005). Progression to a chronic state of disease is, however, accompanied by the suppression of IFN production and relative attenuation of ISG expression. Evasion of the IFN-induced immune response may therefore be a contributing factor that supports HCV persistence.

HCV encodes a number of proteins that have been implicated in regulating the various steps of the host response. The HCV NS3/4A protease cleaves the

adaptor proteins IPS-1 and TRIF to block signaling by RIG-I and TLR3, respectively (Johnson and Gale 2006; Li et al. 2005). IPS-1 cleavage at cysteine 508 releases it from its attachment to the mitochondria and disperses it throughout the cytoplasmic space where it cannot mediate signaling. In contrast, NS3/4A cleavage of TRIF leads to its rapid destabilization and degradation (Ferreon et al. 2005). As a consequence, HCV is able to abolish virus or dsRNA-dependent activation of IRF-3 and NF-κB, leading to the inhibition of IFN production. Liver biopsies of chronic infected patients demonstrate a direct correlation between NS3/4A cleavage of IPS-1 and suppression of ISG expression (Loo et al. 2006). Importantly, IFN signaling is rapidly restored during HCV infection by treatment with NS3/4A protease-specific inhibitors (Foy et al. 2003, 2005; Loo et al. 2006). These observations underscore the vital role of NS3/4A in HCV evasion of host defenses, and suggest that NS3/4A inhibitors will be useful as host response modifiers to treat HCV infection.

Gene expression analyses of HCV-infected liver tissue has shown that HCV infection is associated with an increase in the expression and activity of protein phosphatase 2A (PP2A) (Gale and Foy 2005; Thimme et al. 2006). The HCV core protein and the NS5A protein have been shown to stimulate PP2A expression or activity to dephosphorylate STAT1 (Georgopoulou et al. 2006). The core protein may further induce the expression of SOCS3 to prevent the IFN-induced assembly and transcription activity of ISGF3 (Lin et al. 2006). The HCV core and NS5A proteins have also been suggested to attenuate ISG expression by modulating MAPK and JNK signaling (Thimme et al. 2006). Moreover, the HCV E2 and NS5A proteins have been identified as PKR inhibitors to control the antiviral actions of PKR suppression of host translation (Gale and Foy 2005; Thimme et al. 2006). Taken together, these studies indicate that HCV employs multiple strategies to block PRR signaling and control ISG expression or function in order to establish a cellular environment that supports persistent infection.

5 Concluding Remarks

Viruses are uniquely adapted to their hosts and have evolved strategies to evade host immune defenses in order to efficiently replicate and spread. A detailed understanding of the viral recognition and signaling pathways that initiate host defenses and the viral processes that control and evade these defenses may lead to the design of more effective vaccines and therapies against virus infections.

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Pathogenic Viruses: Smart Manipulators of the Interferon System

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Abstract Vertebrate cells are equipped with specialized receptors that sense the presence of viral nucleic acids and other conserved molecular signatures of infecting viruses. These sensing receptors are collectively called pattern recognition receptors (PRRs) and trigger the production of type I (α/β) interferons (IFNs). IFNs are secreted and establish a local and systemic antiviral state in responsive cells. Viruses, in turn, have evolved multiple strategies to escape the IFN system. They try to avoid PRR activation, inhibit IFN synthesis, bind and inactivate secreted IFN molecules, block IFN-activated signaling, or disturb the action of IFN-induced antiviral proteins. Here, we summarize current knowledge in light of most recent findings on the intricate interactions of viruses with the IFN system.

1
Introduction: First Inklings of a Viral IFN Antagonist

The type I IFN system provides a powerful and universal intracellular defense mechanism against viruses. Knockout mice that are defective in IFN signaling (Muller et al. 1994) quickly succumb to viral infections of all sorts (Bouloy et al. 2001; Bray 2001; Grieder and Vogel 1999; Hwang et al. 1995; Muller et al. 1994; Ryman et al. 2000; van den Broek et al. 1995). Likewise, humans with genetic defects in interferon signaling die of viral disease at an early age (Dupuis et al. 2003).

IFNs-α/β are synthesized by virus-infected tissue and specialized immune cells. After secretion into the extracellular space, these cytokines circulate in the body and cause susceptible cells to express potent antiviral mechanisms, thus limiting viral spread. Pathogenic viruses, however, have learned to manipulate the IFN system for their own sake. They have evolved efficient escape strategies allowing them to suppress IFN production, to modulate IFN signaling, and to block the action of antiviral effector proteins. This facet of the virus life cycle is only now being fully appreciated. Nevertheless, the first inklings of an anti-IFN activity were noticed early on, soon after the discovery of interferons by Isaacs and Lindenmann in 1957 (Isaacs and Lindenmann 1957). Lindenmann himself made the surprising observation that infection of cells with a live virus inhibited the subsequent induction of IFN by an inactivated virus. This phenomenon was called inverse interference and was presumably the first description of a viral IFN-suppressive function (Lindenmann 1960). Since then, great progress has been made in our understanding of how cells recognize viral intruders and how viruses manage to survive in the face of the powerful IFN system (for reviews see Garcia-Sastre and Biron 2006; Goodbourn et al. 2000; Haller et al. 2006).

2
Host Cell Recognition of Invading Viruses: An Enigma of Self–Nonself Discrimination

It has become increasingly clear that conserved molecular signatures of viruses serve as danger signals that are recognized by specialized receptors of the host cell. These receptors are collectively called pattern recognition receptors (PRRs) because they recognize a diverse range of conserved pathogen-associated molecular patterns (PAMPs) found in infectious disease agents. The main PAMP of viruses appear to be nucleic acids, such as double-stranded RNA (dsRNA). The cellular PRRs designed to sense viruses can be divided into the extracellular/endosomal toll-like receptors (TLRs) (Akira and Takeda 2004;

Bowie and Haga 2005) and the intracellular receptors RIG-I, MDA-5, and PKR (Meylan et al. 2006). Signaling through these cellular sensors activates transcription of the IFN genes (Fig. 1). RIG-I and MDA-5 act trough the adaptor protein IPS-1/MAVS and the kinases TBK-1 and IKK-ε to activate the transcription factor IRF-3. A parallel pathway involves the dsRNA-binding kinase PKR, the TRAF adaptor molecules and the NF-κB kinase IKKα/β.

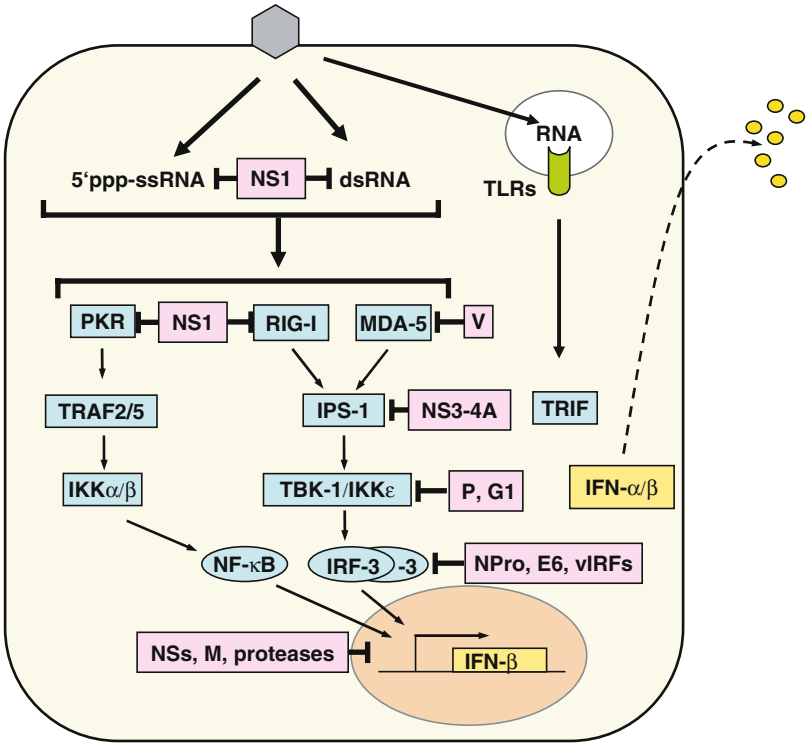

Fig. 1 Viral inhibition of IFN production. Intracellular recognition of 5′-triphosphorylated ssRNA or dsRNA by the intracellular receptors RIG-I, MDA-5, and PKR leads to activation of the transcription factors IRF-3 and NF-κB via several intermediate signaling factors. The kinases TBK-1 and IKKε phosphorylate and activate IRF-3. NF-κB is mainly activated by the PKR pathway. Examples of viral IFN antagonists interfering with different steps in the IFN induction pathways are shown (see text for details)

Until very recently, it was assumed that the only molecule that clearly distinguishes viruses from their host (i.e., self vs nonself) is dsRNA, which would act as a danger signal capable of activating the IFN system. This concept was supported by data showing that many RNA and DNA viruses express proteins that bind this key molecule to avoid both IFN induction and activation of dsRNA-dependent antiviral enzymes (Jacobs et al. 1998; Langland et al. 2006). Good examples are the NS1 protein of influenza A virus (Garcia-Sastre 2001 1998; Lu et al. 1995; Min and Krug 2006), the E3L protein of poxviruses (Hornemann et al. 2003; Xiang et al. 2002), the VP35 protein of Ebola virus (Cardenas et al. 2006; Hartman et al. 2006), the sigma3 protein of reoviruses (Jacobs and Langland 1998), and the US11 protein of herpes simplex virus (Mohr 2004; Poppers et al. 2000). It came therefore as a surprise when it was realized that some viruses do not produce detectable amounts of dsRNA at all (Weber et al. 2006). This unexpected finding indicated that cells must be able to sense other viral molecules important for IFN induction. Indeed, the cytoplasmic receptor RIG-I was subsequently found to bind to the 5′ end of certain viral ssRNA genomes provided they carried a 5′triphosphate group (Hornung et al. 2006; Pichlmair et al. 2006). Such 5′ triphosphate moieties are usually not present on host RNA species in the cytoplasm and appear to provide an ideal recognition pattern for nonself. In line with this, it was shown that the NS1 of influenza A virus can bind ssRNA as well, and is able to form complexes with RIG-I (Mibayashi et al. 2006; Pichlmair et al. 2006).

3 Viral Subversion of Host Cell Sensors and IFN Triggering: New Approaches

To subvert innate immunity, many viruses interfere with one or several steps in the IFN induction pathway. Figure 1 shows examples of viral antagonists that work at different levels of the signaling pathway. As mentioned above, the dsRNA-binding NS1 protein of influenza A virus binds to both dsRNA and ssRNA presumably by recognizing inter- or intramolecular dsRNA regions. Importantly, NS1 also associates with RIG-I in infected cells and seems to impair its signaling function (Mibayashi et al. 2006; Pichlmair et al. 2006). In contrast, the V protein of paramyxovirus SV5 has no apparent RNA-binding activity. It inhibits IFN induction by targeting the RIG-I-related RNA sensor MDA-5 (Andrejeva et al. 2004; Childs et al. 2006). Next in line is the adaptor protein IPS-1/MAVS, which connects the RNA sensors RIG-I and MDA5 with the IRF-3 kinases TBK-1/IKK-ε. It is specifically cleaved by the NS3-4A protease of hepatitis C virus (HCV) and additional flaviviruses (Chen et al. 2007; Lin et al. 2006; Meylan et al. 2005)

(see also chapter by M. Gale, this volume). Activation of IRF-3 by TBK-1 is prevented by the phosphoprotein P of rabies virus (Brzozka et al. 2005) and the G1 glycoprotein of the hantavirus NY-1 (Alff et al. 2006). IRF-3 itself is degraded by the NPro proteins of pestiviruses such as classical swine fever virus and of bovine viral diarrhea virus (Bauhofer et al. 2005; La Rocca et al. 2005; Ruggli et al. 2005) via the proteasomal pathway (Bauhofer et al. 2007; Hilton et al. 2006). Also, the E6 protein of human papilloma virus 16 binds and inactivates IRF-3 (Ronco et al. 1998). A sophisticated strategy to block IRF-3 is used by certain herpesviruses. Human herpes virus 8 (HHV-8), the causative agent of Kaposi sarcoma, expresses several IRF homologs, termed vIRFs, which exert a dominant-negative effect (Burysek et al. 1999a, 1999b; Fuld et al. 2006; Li et al. 1998; Lubyova et al. 2004; Lubyova and Pitha 2000; Zimring et al. 1998).

While these IFN subversion strategies show a degree of specificity and suggest an intimate co-evolution of viruses and their immunocompetent hosts, other and more basic mechanisms are also exploited by diverse viruses. For example, viruses with a lytic life cycle can afford to target the basic cellular transcription machinery and suppress IFN gene expression through a general shutoff of host gene transcription. For example, the nonstructural NSs proteins of bunyaviruses interfere with the basic cellular transcription machinery (Billecocq et al. 2004; Le May et al. 2004; Thomas et al. 2004). Although this strategy appears to be nonspecific, in vivo experiments with Rift Valley Fever virus (RVFV), Punta Toro virus, and Bunyamwera virus clearly demonstrated that the biological purpose of this broad-band shut-off is to inhibit IFN synthesis (Bouloy et al. 2001; Perrone et al. 2007; Weber et al. 2002). The matrix (M) protein of vesicular stomatitis virus (VSV) is also a potent host cell shutoff factor that inhibits basal transcription (Yuan et al. 1998), impairs nuclear-cytoplasmic transport of RNAs and proteins (Her et al. 1997), and inactivates translation factors (Connor and Lyles 2002). As in the case of bunyavirus NSs, the biological significance of M-mediated shutoff is to suppress IFN induction upon VSV infection (Ferran and Lucas-Lenard 1997; Stojdl et al. 2003). Likewise, proteinases of picornaviruses (e.g., foot and mouth disease virus, Theiler's virus, poliovirus) and pestiviruses (e.g., Classical Swine fever virus) cause a shutoff of the host cell metabolism to interfere with the IFN response (de Los Santos et al. 2006; Delhaye et al. 2004; Lyles 2000; Ruggli et al. 2003, 2005; van Pesch et al. 2001).

Finally, some viruses seem to use a stealth approach: they attempt to go undetected by the sensing machinery of the cell by either disguising or invading and replicating in hidden cellular compartments. SARS coronavirus and other members of the coronavirus family do not induce IFN in certain cell types (Cervantes-Barragan et al. 2006; Spiegel et al. 2005; Zhou and Perlman 2007) and are suspected to use such trickery (Stertz et al. 2007). In addition, SARS coronavirus expresses several proteins inhibiting IRF-3 and STAT1 (Kopecky-Bromberg et al. 2006).

4
Viral Downregulation of IFN Signaling: A Top-Down Strategy

IFN-β and the various IFN-α subspecies bind to and activate a common type I IFN receptor (IFNAR), which signals to the nucleus through the so-called JAK-STAT pathway. This pathway is well characterized (Levy and Darnell 2002) and will not be described here in detail. It should be noted, however, that IFN signaling is highly regulated by cellular factors to avoid overstimulation of the system and keep a physiological balance. Negative feedback regulation is mainly mediated by IFN-induced members of the suppressor of the cytokine signaling protein (SOCS) family and the protein inhibitor of the activated STAT (PIAS) family. Essentially, SOCS members inhibit JAK tyrosine kinase activity, while PIAS members work as small ubiquitin-like modifier (SUMO) E3 ligases and inhibit transcriptional activity of activated STAT in the nucleus.

It has become increasingly clear that the IFN signal transduction pathway is also targeted by numerous viruses (Fig. 2). Different approaches are used by different viruses according to their genetic capabilities.

A seemingly simple and highly preventive strategy is used by vaccinia and other poxviruses. They express soluble IFN-binding proteins to neutralize secreted IFN molecules (Alcami and Smith 1995; Alcami et al. 2000; Puehler et al. 1998; Symons et al. 1995). These so-called viroceptors prevent the establishment of an antiviral state as well as the autocrine IFN amplification loop, which normally leads to increased IFN production.

Most viruses cannot afford the luxury of encoding viroceptors. Instead they have evolved multifunctional proteins that specifically target select components of the IFN signaling cascade. In addition, some viruses exploit the cellular feedback loop to achieve the same result. A large number of viral proteins with anti-IFN properties have been described in the past few years, and we can discuss here only a few examples.

Members of the paramyxovirus family express up to three IFN-antagonistic proteins from the P gene (named P, C, and V) that interfere with JAK-STAT function. Depending on viral origin, these IFN antagonists act either by inhibiting the JAK kinases or by binding the STAT proteins, thereby sequestering them in high molecular mass complexes or inducing their proteasomal degradation (Andrejeva et al. 2002; Garcin et al. 2002; Gotoh et al. 2003; Nanda and Baron 2006; Palosaari et al. 2003; Parisien et al. 2001; Park et al. 2003; Rodriguez et al. 2003; Shaw et al. 2004, 2005; Takeuchi et al. 2001; Ulane et al. 2003; Yokota et al. 2003). The P protein of rabies virus (a rhabdovirus) binds to tyrosine-phosphorylated STAT1 and STAT2 and retains the activated transcription factors in the cytoplasm, thereby preventing STAT-dependent expression of

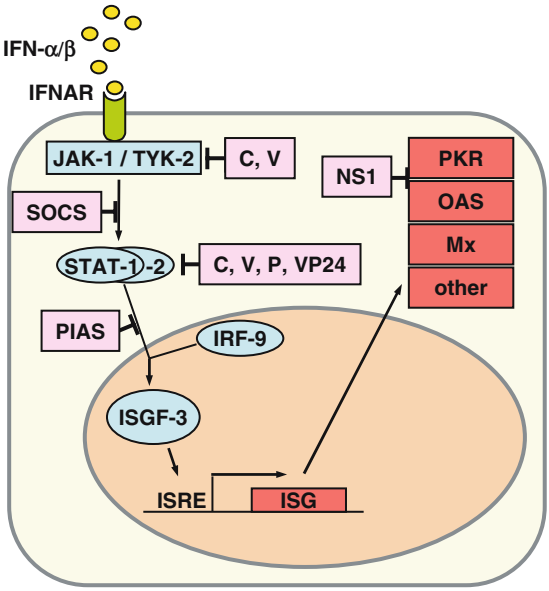

Fig. 2 Viral inhibition of IFN action. IFN-α and IFN-β bind to the type I IFN receptor (IFNAR) and activate the expression of numerous IFN-stimulated genes (ISGs) via the JAK/STAT pathway. Most viral antagonists described so far interfere on the level of either the JAK/TYK kinases or the STATs. Some also inhibit the activation and/or function of IFN-induced effector proteins (see text for details)

IFN-regulated genes (Brzozka et al. 2006). Interestingly, the paramyxoviral V protein as well as the rabies virus P protein have a dual anti-IFN function: they block both IFN induction (see above) and STAT signaling. Ebola virus, by contrast, uses a different protein, VP24, to block nuclear import of STAT by interacting with the transporter protein karyopherin alpha1 (Reid et al. 2006). STAT signaling is also disturbed by viruses causing persistent infections, such as HCV (François et al. 2000; Heim et al. 1999), herpes simplex virus (HSV) (Chee and Roizman 2004; Yokota et al. 2004), HHV-8 (Fuld et al. 2006), or cytomegalovirus (Khan et al. 2004; Zimmermann et al. 2005).

As mentioned above, some viruses exploit the cellular feedback loop to inhibit IFN signaling. HSV type 1 (HSV-1) induces SOCS-3 to downregulate JAK and STAT phosphorylation (Yokota et al. 2004). The core protein of HCV also appears to activate SOCS-3 (Bode et al. 2003), while the virulence factor NSs of RVFV activates SOCS-1 to suppress IFN action (M. Bouloy, personal communication). Again, NSs seems to have a dual function since it also inhibits IFN production by blocking IFN gene transcription (Billecocq et al. 2004).

5
Viral Inhibition of IFN Effector Proteins: A Testimony of Importance

An efficient way to escape the IFN response is to directly inhibit the specific antiviral proteins that mediate the antiviral state. The targeting of IFN-induced proteins by viral counterplayers is a telling case for the importance of these effector molecules in antiviral defense and virus–host evolution.

To date, the best studied antiviral pathways are the protein kinase R (PKR) system (Garcia et al. 2006; Williams 1999), the 2-5 OAS/RNaseL system (Silverman 1994), and the Mx system (Haller and Kochs 2002). Their importance for host survival following viral infections has been amply demonstrated (Arnheiter et al. 1996; Hefti et al. 1999; Zhou et al. 1999). Additional proteins with known antiviral activities are P56 (Guo et al. 2000; Hui et al. 2003), ISG20 (Espert et al. 2003), promyelocytic leukemia protein (PML) (Regad et al. 2001), guanylate-binding protein 1 (GBP-1) (Anderson et al. 1999), and RNA-specific adenosine deaminase 1 (ADAR1) (Samuel 2001). Mx protein expression is tightly controlled by type I IFNs, making Mx gene expression a useful marker for IFN action in clinical settings (Antonelli et al. 1999; Roers et al. 1994). In contrast, PKR and 2-5 OAS are constitutively expressed in a latent, inactive form in normal cells. Their expression is transcriptionally upregulated in IFN-treated cells. Importantly, these two enzymes need to be activated by viral dsRNA. This requirement makes them vulnerable to IFN antagonists found in many viruses. Indeed, viruses endowed with the capacity to sequester dsRNA by virtue of viral RNA-binding proteins are capable of preventing activation of PKR or the 2-5 OAS/RNaseL system (Antonelli et al. 1999; Roers et al. 1994; Weber et al. 2004). An alternative strategy used by several viruses is to encode small RNAs that compete with dsRNA for binding to PKR, thereby preventing activation. This is the case for adenoviruses (Mathews and Shenk 1991), HCV (Vyas et al. 2003), Epstein-Barr virus (EBV) (Elia et al. 1996), and HIV-1 (Gunnery et al. 1990). Some viruses express proteins that either directly bind to or otherwise inactivate PKR. For example, the γ34.5 protein of HSV-1 triggers the dephosphorylation of eIF-2α, thus reverting the translational block established by PKR (He et al. 1997). The E2 protein of HCV acts as pseudosubstrate for PKR (Taylor et al. 1999), as does the Tat protein of HIV-1 (Roy et al. 1990) or the K3L protein of vaccinia virus (Davies et al. 1992). Interestingly, FLUAV exploits a cellular pathway to block PKR in that it activates $p58^{IPK}$, a cellular inhibitor of PKR (Lee et al. 1990) and NS1 to block the 2-5 OAS/RNaseL system (Li et al. 2006; Min and Krug 2006). Poliovirus induces the degradation of PKR (Black et al. 1993). Many viruses also block the RNaseL pathway, either by expressing dsRNA-binding proteins (see above), or by other, more direct

means. Encephalomyocarditis virus as well as HIV-1 induce the synthesis of RLI, a cellular RNaseL inhibitor (Martinand et al. 1998, 1999). Infection with HSV-1 and HSV-2 activates the synthesis of 2′-5′-oligoadenylate derivatives, which bind and prevent RNaseL activation (Cayley et al. 1984). The antiviral effect of IFN is inhibited in cells infected with RSV (Atreya and Kulkarni 1999; Young et al. 2000), an effect most probably mediated by the viral NS1 and the NS2 proteins (Schlender et al. 2000; Spann et al. 2004; Wright et al. 2006).

Certain viruses induce the disruption of PML nuclear bodies (also called ND10) by proteasome-dependent degradation of PML and Sp100 (Moller and Schmitz 2003). In HSV-1 infected cells, viral ICP0 accumulates in ND10 and induces the degradation of PML and Sp100, an activity that requires the E3 ligase activity of ICP0 (Boutell et al. 2002; Van Sant et al. 2001). Similar disruptions of ND10 were observed in cells infected with CMV, EBV, HPV, and adenoviruses (Muller and Dejean 1999). It is conceivable that viruses disassemble these nuclear structures to get rid of antiviral components, but sufficient data supporting this view are not yet available.

6 The IFN Response Circuit: Inducing and Suppressing Amplification Loops

When considering the IFN-inducing and -suppressing activities of infecting viruses, it is important to keep in mind that the IFN response is generated in a cascade-like manner. As shown in Fig. 3, viral replication and genome amplification leads to accumulation of viral nucleic acids and other components that are sensed as danger signals or PAMPs. They activate the IFN induction pathway (left part of Fig. 3) via cellular sensors (RIG-I, MDA-5), adaptors (IPS-1/MAVS), protein kinases (TBK-1, IKK-ε), and transcription factors of the IFN regulatory factor (IRF) family (Honda and Taniguchi 2006). IFN gene expression depends on the basic cellular transcription machinery composed of the cellular RNA polymerase II (RNAPII) and essential co-factors, such as components of the transcription factor IIH (TFIIH). Secreted IFNs bind to their cognate receptors and activate the JAK-STAT signaling pathway (right part of Fig. 3), which induces the antiviral effector molecules. Most components of the IFN induction and signaling pathways are themselves IFN-inducible, representing a positive amplification loop. During viral replication, however, a number of viral IFN antagonists are produced (center part of Fig. 3) and interfere with the IFN response circuit. It is not unusual that a given virus displays more than one IFN-antagonistic protein and targets different parts of the IFN response pathway. Also, a single viral protein may inhibit quite different components of

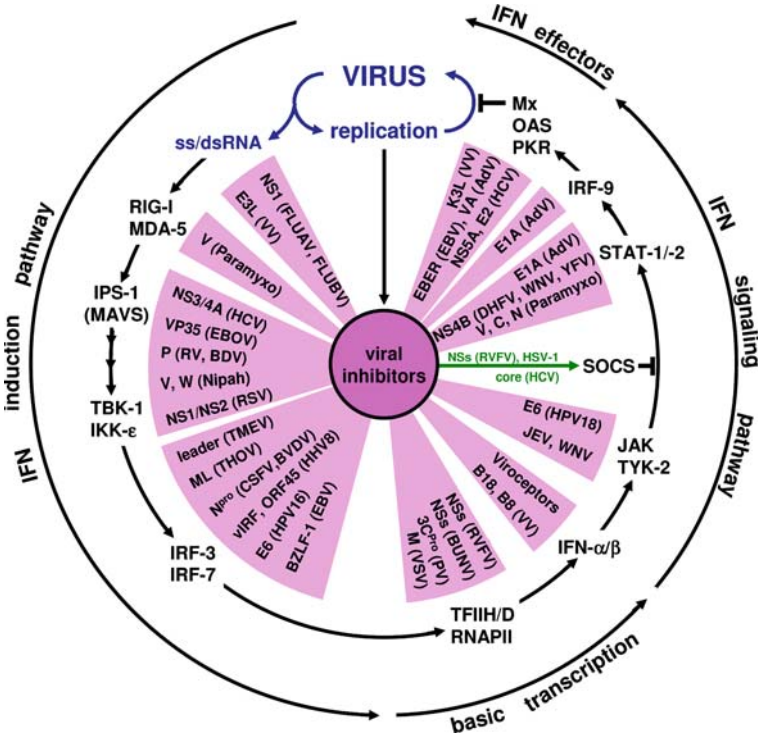

Fig. 3 Induction and suppression of the IFN response circuit. Viral gene products interfere with the IFN response circuit in a negative amplification loop, resulting in a balance between virus-promoting and virus-inhibiting factors. (see text for details). (Adapted from Haller et al. 2006, with permission)

the IFN induction and signaling cascade. Thus, viral dsRNA-binding proteins have the advantage of blocking both IFN production and action. Besides, the dsRNA- binding NS1 protein of influenza A virus has additional functions and impairs also the post-transcriptional processing and nuclear export of cellular pre-mRNAs (Chen et al. 1999; Fortes et al. 1994; Kim et al. 2002; Li et al. 2001; Noah et al. 2003). Since the IFN response is generated in a cascade-like manner, viral proteins blocking one component in this circuit also affect distant signaling or effector molecules, thereby amplifying the inhibitory effect. For example, JAK-STAT inhibitors suppress not only the production of antiviral proteins, but also the expression of RIG-I, MDA-5, IPS-1/MAVS, and IRFs, which are all IFN-inducible proteins. As a consequence, a negative amplification loop is produced, which further helps the virus to suppress the IFN system as a whole.

7 Concluding Remarks

Viruses are able to negatively influence the whole spectrum of the IFN response, often affecting different parts of the IFN circuit at the same time. The interplay between viruses and the IFN system, as described here, most likely results from an evolutionary race between the two genetic systems. The race is ongoing, as emerging viruses attempt transmission across species to new hosts. This is best illustrated by recent outbreaks of SARS coronavirus or the constant threat of avian influenza A viruses to invade the human population. Our present knowledge of the IFN system and viral countermeasures is still limited. Future research should provide better insight into the intricate interplay between viruses and the innate immune defenses of the host. This knowledge is important not only for a better understanding of viral pathogenesis, but also for designing novel vaccination strategies and therapeutic approaches.

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Part VI: Role of Interferon in Viral Infection and Autoimmunity

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Type I Interferon During Viral Infections: Multiple Triggers for a Multifunctional Mediator

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Abstract Type I interferons (IFN-I) orchestrate numerous biological and cellular processes and are essential elements during host antiviral defense. After recognition of highly conserved virus signatures, a complex network of signaling events is rapidly initiated and leads to IFN-I synthesis. These cytokines directly induce a strong antiviral state and exert several immune-regulatory actions aimed at preventing virus spread. On the other hand, viruses evolved to evade or subvert the IFN-I system for their own benefit. In the present article, we review selective aspects of IFN-I induction and functions during several viral infections and discuss the beneficial and detrimental roles of IFN-I illustrated during lymphocytic choriomeningitis virus (LCMV) infection in its natural host, the mouse.

1 Introduction

Type I interferons (IFN-I), which encompass twelve subtypes of IFN-α proteins and one IFN-β, represent a fundamental pillar of antiviral immunity. Although discovered half a century ago, novel aspects of these cytokines are

constantly being reported and updated. The understanding of their functions and regulation constitutes a major challenge in biomedical research. Outstanding work over the past 50 years contributed to the present view of IFN-I as the first line of antiviral defense and a critical link between innate and adaptive immunity. Recent interest in delineating the events involved in IFN-I induction uncovered a complex net of signaling pathways toward IFN-I transcription. In addition, remarkable progress has been made on unraveling the sophisticated strategies used by viruses to evade or divert the IFN-I effect. In the present article, we first review selective aspects of IFN-I induction and functions during several viral infections. In addition, in the last section of this review, we use infection with lymphocytic choriomeningitis virus (LCMV) in its natural host, the mouse, to illustrate the beneficial and detrimental roles of IFN-I during an in vivo natural viral infection.

2
Mechanisms of IFN-I Induction During Viral Infections

IFN-I is synthesized in response to pathogen associated molecular patterns (PAMPs) such as viral genomic DNA and RNA or double-stranded RNA (dsRNA) produced during viral replication. These pathogen-derived products interact with pathogen recognition receptors (PRR) to initiate a cascade of signaling events that lead to IFN-I transcription. This event is controlled by a limited number of transcription factors. In the case of IFN-β, these transcription factors are interferon regulatory factor (IRF) family members (IRF3 and IRF7 mainly), nuclear factor kappa β (NF-κβ) and AP-1 (Honda et al. 2006; Wathelet et al. 1998). There are two distinct signaling pathways that trigger IFN-I synthesis. One is operated by a subfamily of Toll-like receptors (TLRs) that detect nucleic acids in the endosome, while the other senses viral RNA in the cytosol in a TLR-independent fashion.

2.1
TLR-Dependent IFN-I Induction

TLRs recognize microbial PAMPs through their leucine-rich repeat (LLR) and transmit this danger signal to the intracellular compartment recruiting adaptor proteins by their cytoplasmic toll-interleukin-1 receptor (TIR) domain (Akira 2006; Beutler et al. 2006; Kopp and Medzhitov 2003). These receptors are differentially expressed in specific immune and nonimmune cells. Particularly, dendritic cells (DCs) express the broadest repertoire of TLRs, allowing them to recognize a plethora of microbial components and to bridge innate and adaptive immunity (Steinman and Hemmi 2006). Among TLRs, TLR-3, TLR-7,

TLR-8, and TLR-9 are critical to initiate IFN-I response during viral infections. They act in the endosomal compartment and are specialized in nucleic acid recognition.

Two major TLR signaling pathways are known to induce IFN-I (Asselin-Paturel et al. 2005; Barton and Medzhitov 2003; Theofilopoulos et al. 2005). The first exclusively takes place on plasmacytoid DCs (pDCs), which represent a unique DC subset specialized in producing copious amounts of IFN-I after stimulation with viral nucleic acids. IFN-I production in pDCs is initiated when TLR-7 or TLR-9 are activated by their specific ligands. TLR-7 and TLR-8 recognize uridine- or guanosine-rich single-stranded (ss) RNA present in mouse and human viruses including influenza virus, vesicular stomatitis virus, and HIV (Diebold et al. 2004; Heil et al. 2004; Lund et al. 2004). While TLR-7 specificity has clearly been demonstrated by several groups, the importance of TLR-7 signaling for antiviral response and elimination of ssRNA viruses during in vivo infections remains unclear and to be proven. TLR-9 is one of the TLRs most extensively studied. It is localized in the endoplasmic reticulum (ER) and rapidly appears in endosomes after stimulation (Latz et al. 2004). TLR-9 senses unmethylated 2′-deoxyribo (cytidine-phosphate-guanosine) (CpG) DNA motifs (Hemmi et al. 2000) present in DNA viruses and bacteria. Moreover, TLR-9 plays a critical role during host defense against DNA viruses such as murine cytomegaloviruses (Delale et al. 2005; Krug et al. 2004a; Tabeta et al. 2004) and herpes simplex virus (HSV) types I and II (Hochrein et al. 2004; Krug et al. 2004b; Lund et al. 2003). The strong immunostimulatory properties of TLR-9 ligands encouraged their use for immune intervention. To date, CPG treatment has been reported to confer protection against infectious diseases, allergy, and cancer in animal models and clinical trials have been initiated (Vollmer 2006; Wilson et al. 2006). Interestingly, both TLR-7 and TLR-9 can potentially bind self nucleic acids and their localization in endocytic vesicles is crucial to avoid their activation by self RNA or DNA and prevent autoimmunity (Barton et al. 2006; Diebold et al. 2004). After stimulation in the endocytic vesicles of pDCs, TLR-7 and TLR-9 rapidly recruit the adaptor molecule MyD88, forming a complex with interleukin-1 receptor-associated kinase (IRAK)-1 and interferon regulatory factor (IRF)-7, which are retained in the endosomal compartment (Kawai et al. 2004). Indeed, neither IRF-7 nor IRAK-1 knockout mice are able to produce IFN-I in response to TLR-7 or TLR-9 stimulation (Uematsu et al. 2005). Interestingly, Traf-6 (Kawai et al. 2004) and Traf-3 (Oganesyan et al. 2006) also associate with IRAK-1 and are essential for IRF-7 activation and IFN-I production. It was demonstrated that the duration of CPG retention in the endosome is critical in determining the final outcome of TLR-9 stimulation (Honda et al. 2005). IFN-I production

by DCs is only achieved when CPG is retained for long periods of time in the endosomal vesicles. Accordingly, pDCs exhibit a unique capacity to retain CPG in endosomes, in contrast to conventional DCs (cDCs), where CPG is rapidly transferred into lysosomal vesicles. Interestingly, the phosphoprotein osteopontin co-localizes with MyD88 and TLR-9 upon CPG stimulation and is essential for IFN-I production in pDCs (Shinohara et al. 2006). Finally, it is important to point out that TLR-7 and TLR-9 are expressed in cell types other than pDCs, such as cDCs and macrophages, in which their stimulation induces production of pro-inflammatory cytokines including IL-6, TNF-α, and IL-12 (but not IFN-I).

The second pathway mediating TLR-induced-IFN-I production is turned on upon TLR-3 or TLR-4 stimulation. Within the immune system, these TLRs are mainly expressed in cDCs and macrophages. TLR-3 localizes in endosomes, binds double-stranded (ds) RNA (Alexopoulou et al. 2001) generated as a byproduct of virus replication, and was found to play a role in the pathogenesis of West Nile virus (WNV) (Wang et al. 2004), Influenza A virus (Le Goffic et al. 2006) and Phlebovirus (Gowen et al. 2006) as well as in the innate response upon infection with murine cytomegalovirus (MCMV) (Tabeta et al. 2004). However, the participation of TLR-3 during MCMV infection has been questioned (Delale et al. 2005; Edelmann et al. 2004). Moreover, under conditions that used infections as stimuli, TLR-3 was not found to play a role in viral pathogenesis or the generation of adaptive antiviral responses to LCMV, vesicular stomatitis virus (VSV), MCMV, or even the double-stranded RNA reovirus (Edelmann et al. 2004). TLR-4 is expressed in the cell surface and was first identified as the receptor for bacterial lipopolysaccharide. However, recent studies demonstrated that TLR-4 also binds viral proteins such as mouse mammary tumor virus glycoprotein (GP) (Jude et al. 2003; Rassa et al. 2002) and VSV-GP (Beutler et al. 2006). TLR-3 and TLR-4 induce the production of IFN-I through MyD88 independent pathways. For that, TLR-3 and TLR-4 recruit the adaptor Trif (Hoebe et al. 2003; Yamamoto et al. 2003a), which activates Traf-3 and the kinases TANK binding kinase-1 (TBK-1) and IKKε, which phosphorylates IRF-3 (Fitzgerald et al. 2003a; Oganesyan et al. 2006; Fig. 1). In the case of TLR-4, another adaptor molecule named Trif related adaptor molecules (TRAM) is also recruited to the TLR complex to achieve IFN-I production (Fitzgerald et al. 2003b; Hoebe et al. 2003; Yamamoto et al. 2003b). Moreover, recent data showed that activation of IFN-stimulated genes by TLR-4 requires the production of reactive oxygen species, which leads to the activation of MAP kinases, which are essential for IRF-3 phosphorylation (Chiang et al. 2006).

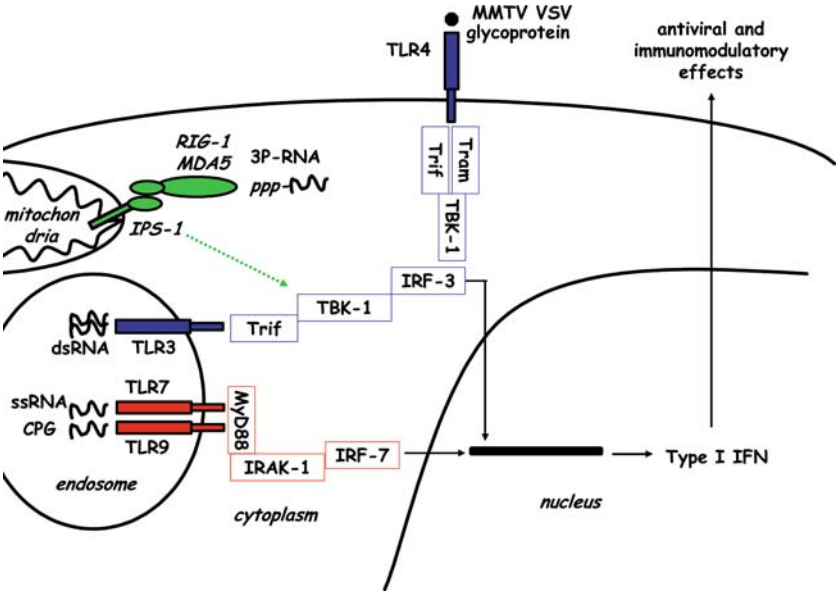

Fig. 1 IFN-I induction during viral infections. IFN-I is synthesized during viral infections in response to TLR-dependent and/or TLR-independent pathways. The two major TLR pathways that trigger IFN-I production during viral infections are those mediated by the adaptors MyD88 and Trif. Members of the RNA helicase family recognize viral RNA and trigger IFN-I response in a TLR-independent fashion

2.2 TLR-Independent IFN-I Induction

IFN-I production is induced by TLR-independent pathways in most cell types in response to cytosolic viral dsRNA or ssRNA. Recently, the RNA helicases cytoplasmic protein retinoic acid-inducible gene I (RIG-I) (Kato et al. 2005; Sumpter et al. 2005; Yoneyama et al. 2004) and the melanoma differentiation-associated gene 5 (MDa5) (Gitlin et al. 2006; Kato et al. 2006) have been reported to play a critical role in this response. They bind RNA through their helicase domain and transduce this danger signal through caspase-recruiting domain (CARD)-like domains initiating signaling events that lead to the activation of IRF-3 and NF-kβ. Although structurally related, these RNA helicases recognize specific groups of RNA viruses (Kato et al. 2006). RIG-I is essential for the production of interferons in response to RNA viruses including paramyxoviruses, influenza virus, and Japanese encephalitis virus, whereas MDA5 is critical for

picornavirus detection. Furthermore, RIG-I$^{-/-}$ and MDA5$^{-/-}$ mice are highly susceptible to infection with these respective RNA viruses compared to control mice (Gitlin et al. 2006; Kato et al. 2006). A recent report contributed to the understanding of RNA helicase specificity, demonstrating that uncapped 5′-triphosphate RNA is the ligand for RIG-I and is present in the viruses that it recognizes but not in picornaviruses, which are detected by MDA-5 (Hornung et al. 2006). Accordingly, RIG-I was also found to sense influenza virus single-stranded viral genomic RNA bearing 5′ phosphates (Pichlmair et al. 2006). Another member of this family, LGP2, lacks the CARD homology and functions as a negative regulator by interfering with the recognition of viral RNA by RIG-I and MDA5 (Komuro and Horvath 2006; Rothenfusser et al. 2005).

The adaptor protein linking RIG-1 and MDA-5 to downstream mediators has simultaneously been identified by several groups and is known as IFN-β promoter stimulator (IPS)-1, mitochondrial antiviral signaling protein (MAVS), virus-induced signaling adaptor (VISA) and CARD adaptor inducing IFN-β (Cardiff) (Kawai et al. 2005; Meylan et al. 2005; Seth et al. 2005; Xu et al. 2005). IPS-1 is anchored to the mitochondrial outer membrane and this localization is critical for its function, suggesting a participation of the mitochondria in antiviral immunity. IPS-1 interacts with MDA5 and RIG through the CARD-like domain and connects these RNA helicases to downstream signaling mediators including TANK-binding kinase-1 (TBK-1) and inducible inhibitor of kβ kinase (IKKi). In turn, TBK-1 and IKKi phosphorylate cytoplasmic IRF-3 and IRF-7, which then translocate into the nucleus to initiate transcription of IFN-I genes (Fitzgerald et al. 2003a; Hemmi et al. 2004; Sharma et al. 2003).

Another pathway that may contribute to TLR-independent-IFN-I production during viral infections is the recognition of apoptotic cells by lymphoid-related CD8$^+$ DCs. Although the signaling events mediating this pathways remain to be elucidated, it was clearly demonstrated that apoptotic cell-induced-IFN-I is critical for the CD8$^+$ T cell response (Janssen et al. 2006).

3 IFN-I Antiviral and Immunoregulatory Roles

Ever since virus-induced IFN-I were found to interfere with virus replication in 1957 (Isaacs and Lindenmann 1957), the anti-viral activity of IFN-I has been demonstrated in numerous systems. Creation of mice deficient in a gene coding for IFN-I receptor (Muller et al. 1994), molecules critical in the IFN-I synthesis pathway or Janus kinase (JAK)/STAT IFN-I signaling pathway (Meraz et al. 1996; Park et al. 2000), or IFN-I-induced antiviral proteins clearly demonstrated the role of IFN-I for host defense. Indeed, mice deficient in IFN-I receptor were

lacking expression of interferon stimulated genes (ISGs) and became highly susceptible to numerous viral infections including vesicular stomatitis virus (VSV), Semliki forest virus (SFV), and vaccinia virus. Upon IFN-I binding to its receptor, a cascade of signaling events is initiated (Aaronson and Horvath 2002). Activation of both STAT1 and STAT2 by Janus kinases leads to the formation of trimeric complex IFN-stimulated gene factor 3 (ISGF3) composed of STAT1, STAT2, and IRF9. ISGF3 transported into nucleus binds to specific IFN-stimulated response element (ISRE) and promotes serial synthesis of selected proteins that inhibit viral replication. Even though IFN-I are produced, if this JAK/STAT signaling pathway is disrupted by genetic modification, the antiviral state is impaired upon viral challenges (Durbin et al. 1996; Karaghiosoff et al. 2000; Meraz et al. 1996; Park et al. 2000). JAK/STAT integrity mediates ISRE-mediated transcriptional activation of hundreds of genes, which encode proteins that disturb viral amplification in infected cells and protects neighboring uninfected cells. Among them, dsRNA-activated protein kinase (PKR), 2′5′-oligoadenylated synthetase (2-5 OAS), and Mx proteins were known to exhibit antiviral activities. PKR binds to double-stranded (ds) RNA to mediate phosphorylation of eIF-2alpha, leading to inhibition of protein synthesis. Activation of 2-5 OAS enzyme yields multiple 2-5As, which activate RNase L, resulting in degradation of RNAs. Indeed, mice lacking PKR and RNase L were highly susceptible to subcutaneous WNV infection (Samuel et al. 2006). It needs to be further investigated why so many proteins are induced by virus-induced IFN-I, and whether all those proteins are required to maintain an antiviral state or specific proteins are destined for blocking spread of specific viruses.

Suppression of virus spread and replication appeared to be in part due to the apoptotic or anti-proliferative activity of IFN-I, which inhibits propagation of virus-infected cells, blocking amplification of virus progeny. However, IFN-I were recently shown to display anti-apoptotic activity and pro-proliferative activity as well (Gimeno et al. 2005; Tanabe et al. 2005; Yang et al. 2001). These results suggest that activities of IFN-I are influenced by other molecules and/or are operating in a cell type-specific manner. It remains to be clarified how IFN-I action promoting cell growth affects antiviral activity and if IFN-I differentially affect virus-infected and uninfected cells. It is possible that pattern recognition receptors such as TLR-3, TLR-7, and TLR-9 present inside cells detect viral components in infected cells to mark those cells as virus-containing cells to be taken care of by IFN-I.

Recent work on IFN-I revealed the complicated immunomodulatory roles of these cytokines. IFN-I was shown to activate NK cells, potentiate the clonal expansion of cytotoxic T lymphocytes (CTLs), inducing formation of effector and memory T cells, (Kolumam et al. 2005; Tough et al. 1996), and prolong the survival of activated T cells (Marrack et al. 1999). Moreover, IFN-I regulate

the function of DCs in a paradoxical way depending on the developmental status of this cell lineage. In fact, IFN-I enhance stimulatory capacity of committed DCs (Luft et al. 1998) and favor differentiation of bone marrow (BM) plasmacytoid DCs into CD11b+DCs, which are more specialized in antigen presentation (Zuniga et al. 2004). In this way, IFN-I contribute to T cell activation and favor the transition from innate into adaptive immunity. On the other hand, IFN-I act at the level of undifferentiated DC progenitors suppressing DC development, as is further explained in the following section (Hahm et al. 2005). Additionally, IFN-I control the host immune system indirectly by modulating production of multiple cytokines such as IL-12, IL-15, and TNF-α, which are important for host immune responses. For example, in the absence of IFN-I receptor or STAT1, TLR-mediated IL-12p70 synthesis was strongly inhibited (Gautier et al. 2005), demonstrating a critical role of IFN-I signaling for maximizing production of IL-12p70. In addition, IFN-I-dependent inhibition of IL-12 has also been reported under different experimental conditions, further emphasizing the double-edged profile of IFN-I (Cousens et al. 1997; Dalod et al. 2002). IFN-I were also found to induce IL-15, which should influence the function of NK cells and memory T cells (Nguyen et al. 2002). It is intriguing to find that amounts and activation of STATs are critical for regulating IFN-I activity on T cell function. Direct activation of STAT4 by IFN-I was required for type II IFN, IFN-gamma synthesis during viral infection (Nguyen et al. 2002). Antigen-specific CD8 T cells, but not CD4 T cells, were shown to express a low level of STAT1 protein and become less sensitive to anti-proliferative activity of IFN-I (Gil et al. 2006). It was not investigated whether this result is associated with the activity of IFN-I as an enhancer of T cell proliferation, which is increased in the absence of STAT1 or STAT2 (Gimeno et al. 2005). These recent data in collection suggest that the host may need to devise regulatory machinery at the level of STAT signaling to select specific function among numerous activities of IFN-I. It remains to be evaluated how diverse roles of IFN-I, including induction of lymphopenia and redistribution of lymphocytes (Kamphuis et al. 2006) and alteration of cellular differentiation (Verma et al. 2002; Vidalain et al. 2002) are regulated for host defense during viral invasion.

4 Two Sides of the Same Coin: Beneficial and Deleterious Roles of IFN-I During LCMV Infection

Viruses evolved a broad rage of maneuvers to interfere with IFN-I induction, signaling and functions and the understanding of these viral immune-evasive strategies has been the research focus of multiple laboratories (Garcia-Sastre

and Biron 2006). Notably, the study of IFN beneficial and deleterious roles during in vivo virus infections is limited by the lack of appropriate small animal models. For example, MV and HIV are important infectious viruses restricted to humans without a known intermediate animal host. To address this problem, we investigated IFN-I actions in a murine model of natural infection with LCMV.

LCMV infection of its natural host, the mouse, provides us with a powerful model system to study the virus–immune system interactions because of easy manipulation of the virus and host immune response, knowledge of immune genetics, and the availability of mice modified in several genes of interest. Indeed, the well-established parameters of immunity from MHC restriction, kinetics of generation expansion and contraction of virus-specific CD8 and CD4 T cells, CD4 T cell help for CD8 T cells, loss of T cell function during persistent infections, memory cell numbers, plasma cell migration, and residence and virus antigen–immune complexes were all first defined in the LCMV model and then translated to other viruses, bacteria, and parasite infections in humans and animals (Zinkernagel 2002). Furthermore**,** LCMV is the prototypic member of the family *Arenaviridae*, which includes important human pathogens that cause severe hemorrhagic fever, such as Lassa Fever and the South American hemorrhagic fever viruses, Junin, Guanarito, Machupo, and Sabia (Kunz et al. 2002; Kunz and de la Torre 2005). Thus the data obtained with LCMV have potential implications for the interplay between DCs and human pathogenic arenaviruses. Another advantage of the LCMV system is that depending on the isolate used, it can serve as a model for acute or chronic viral infection. Infection of mice with numerous strains of LCMV, including the prototypic ARM53b (ARM), results in a classical adaptive immune response highlighted by the proliferation and activation of highly effective $CD4^+$ and $CD8^+$ T cells. Importantly, both populations of activated T cells acquire effector functions, including production of antiviral cytokines such as IFN-γ ($CD4^+$ and $CD8^+$ T cells) as well as direct cytolytic activity ($CD8^+$ T cells) that participate in the clearance of virus from the host within 7–10 days postinfection (p.i.) (Fung-Leung et al. 1991; Ou et al. 2001; Tishon et al. 1995). However, only the LCMV-specific $CD8^+$ T cell response is required for viral clearance. Studies of mice persistently infected with LCMV-ARM since birth identified the emergence of viral variants that present an immunosuppressive phenotype (Ahmed et al. 1984, 1988). Of the 50 plus variants isolated and studied, LCMV Clone 13 (CL13) is a model LCMV variant that, in contrast to the parental virus (ARM), fails to be cleared from immunocompetent mice within 7–10 days and instead causes a persistent infection that can last up to 100 days. Virus-specific, activated $CD4^+$ and $CD8^+$ T cells are initially generated following CL13 infection to a level comparable to that

in mice infected with ARM during early stage of infection (5 days or less after infection). However, collapse of both $CD4^+$ and $CD8^+$ T cell function ensues after 5 days postinfection (Ahmed et al. 1984, 1988; Borrow et al. 1995; Sevilla et al. 2000). Interestingly, CL13 genetically differs from ARM by only five nucleotides, of which only two changes result in different residues in open reading frames (Dockter et al. 1996; Salvato et al. 1991). One change is in the viral glycoprotein at amino acid 260. This single amino acid change gives CL13 the advantage of binding more vigorously than ARM to α-dystroglycan (α-DG), a membrane glycoprotein identified as a common receptor for LCMV and several arenaviruses pathogenic in humans, including Lassa fever virus (Cao et al. 1998). Indeed, binding by CL13 is 2.5 logs stronger to α-DG than ARM binding. Among cells of the immune system, functional α-DG is restricted to DCs (Sevilla et al. 2000). By using α-DG as its primary receptor, CL13, but not ARM, is able to specifically target the DC population within both the spleen and BM (Sevilla et al. 2000). Thus, although ARM and CL13 replicates to similar titers within the spleen, replication of ARM is tightly restricted to the red pulp. In contrast, CL13 replicate in DCs within the marginal zone and in the white pulp (Borrow et al. 1995; Sevilla et al. 2000; Smelt et al. 2001).

DCs from CL13-infected mice are unable to stimulate allogeneic T cell responses, as observed by mixed lymphocyte reaction assays, whereas DCs from ARM-infected as well as uninfected mice readily stimulate T cell responses (Borrow et al. 1995; Sevilla et al. 2000, 2003). CL13 also inhibits DC accumulation within the spleen and BM, by both the elimination of mature DCs (Borrow et al. 1995) as well as the blockade of DC development (Hahm et al. 2005; Sevilla et al. 2004), thereby reducing the hosts' ability to stimulate adaptive immune responses.

ARM and CL13 viruses induce comparable levels of systemic IFN-I in serum, which peaks at day 1 or 3 after viral inoculation (probably depending on the dose and route of infection) and appears to be produced at least in part by pDCs (Montoya et al. 2005) (Fig. 2). This transient wave of high systemic IFN-I plays a beneficial role on committed DCs, favoring the generation of DCs with enhanced antigen-presenting capacity. Indeed, analysis of DC activation and maturation in mice unable to respond to IFN-I implicated these cytokines in driving activation of cDCs and their enhanced tendency to undergo apoptosis (Montoya et al. 2005). In addition, IFN-I released by day 3 after LCMV infection or following poly(I:C) injection participates in reprogramming a fraction of BM pDCs into cells that exhibit functional and phenotypic properties of $CD11b^+$cDCs (Zuniga et al. 2004). BM pDCs in this study were isolated from LCMV-infected or poly(I:C)-injected mice based on their expression of broadly accepted pDC markers, including expression of CD11c, B220, 120G8, and Ly6C. Moreover, the isolated BM pDCs from poly(I:C)-injected mice

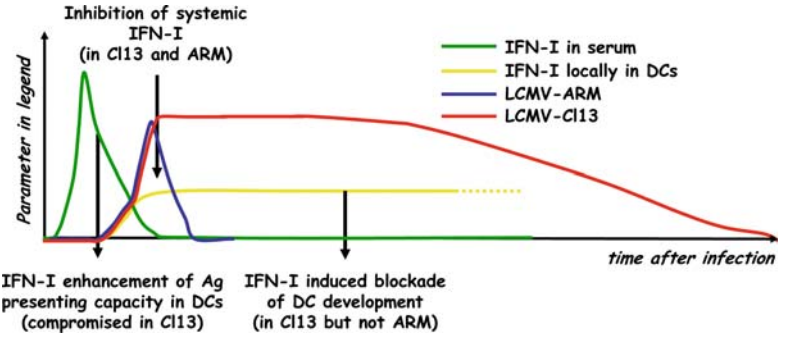

Fig. 2 Beneficial and detrimental roles of IFN-I during LCMV infection. A transient wave of high levels of systemic IFN-I is triggered early after acute and persistent LCMV infection and plays a beneficial role on committed DCs, favoring the generation of DCs with enhanced antigen-presenting capacity. This systemic IFN-I production is rapidly silenced and remains inhibited for the rest of the infection. This is probably a result of the host immunoregulatory mechanisms and LCMV direct inhibition of IFN-I synthesis. During infection with persistent LCMV strains that replicate within DCs, like CL13, DC maturation is compromised. In addition, local production of IFN-I by DCs is sustained chronically in spleen and BM. As a consequence, DC development from early undifferentiated progenitors is blocked, contributing to virus persistence and the immunosuppressive phenotype observed with CL13 infection

respond to TLR-7 and TLR-9 (but not TLR-4) stimulation by upregulating antigen-presenting machinery and secreting IFN-I. Further characterization of BM pDCs during LCMV infection is currently under investigation in light of the recently described pDC-specific antibodies such as 440c (Blasius et al. 2006) and Ly49Q Abs (Kamogawa-Schifter et al. 2005). It is likely that the differentiation of BM pDCs into $CD11b^{+}$cDCs with increased T cell stimulatory properties contributes to the IFN-I role as link between innate and adaptive immunity during viral infection.

Importantly, by 5 days after LCMV infection, systemic IFN-I in serum returns to basal levels and is undetectable throughout the course of the infection regardless of viral clearance or persistence (Dalod et al. 2002; E. Zuniga, unpublished data; Fig. 2). Moreover, in vivo stimulation with the synthetic dsRNA analog poly(I:C) after 5 days post-LCMV infection fails to enhance systemic IFN-l levels during either ARM or CL13 infection. These data suggest virus counterattack strategies to block IFN-I response, host immune regulatory mechanisms to avoid tissue damage due to excessive IFN-I production, or

both. Supporting virus-mediated IFN-I inhibition, recent work documented the ability of LCMV to interfere with IFN-β production in A549 cell cultures in response to different stimuli, including Sendai virus and liposome-mediated DNA transfection. Inhibition of IFN-I resulted from a rapid blockade of IRF-3 phosphorylation and nuclear translocation by LCMV nucleoprotein (Martinez-Sobrido et al. 2006).

A completely different picture is observed when local IFN-I production by $CD11c^+$DCs is analyzed in hematopoietic tissues such as spleen or BM, indicating differential regulation of systemic and local IFN-I synthesis during LCMV infection (Fig. 2). In contrast to mice infected with ARM, whose IFN-I levels within DCs begin to decrease within 3–5 days p.i., mice whose DCs support chronic infection by CL13 manifest a significantly increased and sustained production of IFNα/β from cDCs within the spleen and BM for at least 50 days p.i. (Diebold et al. 2003; Hahm et al. 2005). Based on the LCMV-NP IRF3 inhibition (Martinez-Sobrido et al. 2006), it is likely that IFN-I production in DCs is induced either through an IRF3-independent pathway or by the fraction of DCs that do not show viral replication, although this remains to be clarified. In any case, IFN-I production by DCs correlates with the ability of CL13 to block DC development from undifferentiated progenitors (Hahm et al. 2005; Sevilla et al. 2004). Because DC populations are very small fractions within cells of the BM and spleen, we utilized Flt3 ligand (Flt3L) stimulation of DC development to study the effects of LCMV infection on DCs. Flt3L is known to induce the expansion of undifferentiated progenitors into DCs within the spleen and BM (an approximate 20-fold increase) and to trigger DC maturation, both in mice and humans. Mice infected with ARM following or during treatment with Flt3L also display a dramatic increase in DCs and DC precursors (Hahm et al. 2005; Sevilla et al. 2004). In contrast, CL13-infected mice are refractory to the stimulatory effects of Flt3L. Indeed, the observed inhibition of DC development within CL13-infected mice is associated with infection of approximately 20% of plasmacytoid and myeloid DCs within the BM by 15 days p.i. (E. Zuniga, unpublished results). Experiments using IFNα/β receptor-deficient (IFN-α/$\beta R^{-/-}$) mice infected with CL13 surprisingly indicated that the impairment of DC development is dependent on these cytokines (Sevilla et al. 2004). Indeed, IFN-α/$\beta R^{-/-}$ mice regained sensitivity to Flt3L-mediated DC stimulation regardless of CL13 infection, suggesting that IFN-α/β production by CL13-infected DCs was critical for suppression of the DC developmental pathway. Further evidence that DC suppression was IFN-dependent was provided by treatment of mice in vivo with recombinant IFN-β, which resulted in an identical inhibition of DC development following Flt3L equivalent to that caused by CL13 infection (Hahm et al. 2005). Studies utilizing $STAT^{-/-}$ mice determined that the signaling cascade responsible for such inhibition was STAT2-dependent,

and STAT1, STAT4, and STAT6 independent, defining a novel signaling pathway in which IFN can signal directly through STAT2 to mediate the inhibition of DC development. Such findings indicate that an immunosuppressive virus can subvert the known antiviral effect of IFN-I to benefit its own survival. This notion is also supported by similar finding during measles virus infection in a transgenic mice and a recent report on the role of interferon regulating factor-2 (IRF-2) and IFN-α/β indicating that IFN-I negatively influences the generation of myeloid DCs (Honda et al. 2004; Ichikawa et al. 2004). Furthermore, a transient reduction in BM cellularity and the concomitant pancytopenia observed after LCMV infection failed to occur in the absence of IFN-α/β receptor (Binder et al. 1997). Transient aplasia within the BM as well as high serum levels of INF-α/β are common occurrences during infection with numerous viruses as well as variants of LCMV; however, the impairment of DC development occurs only during CL13 infection. This selective advantage associated with CL13 infection is likely related to its ability to directly infect DCs and trigger IFN α/β production to high concentrations within the BM selectively at the site of precursor development (Diebold et al. 2003; Hahm et al. 2005). Indeed, the depletion of $Flt3^+$, undifferentiated DC progenitors caused by CL13 infection, was not observed when the mice lacked IFN-α/β receptor or STAT2 molecules (Hahm et al. 2005). Consequently, IFN-mediated blockade of DC development is an important front of attack that immunosuppressive viruses can use to disable DCs' defenses and persist in the host.

As with many other paradigms of immune virology, is likely that the beneficial and detrimental roles of IFN-I described during LCMV infection would be applicable to other acute and persistent human viral diseases.

5 Concluding Remarks

After 50 years of challenging research on IFN-I, these cytokines continue to surprise us with their multitude of functions during infectious and noninfectious (autoimmune) diseases. Recent research findings have significantly improved our understanding of the events leading to IFN-I production and its consequences for host immune response and viral spread. However, major questions are still on the agenda. We foresee that the increasing study of these cytokines will lead to the discovery of additional sensors of virus-derived products that induce IFN-I secretion and the characterization of novel pathways. For instance, the critical molecules that uniquely enable pDCs to produce hundred of times more potent IFN-I production compare to other cell types will be relevant topics of future research. Also, the viral and/or host mechanisms that differentially

trigger and regulate local and systemic production of IFN-I will facilitate the analysis of the multiple IFN-I actions during in vivo viral infections. Moreover, as the effects of IFN-I on newly discovered paradigms on immune virology are tested, novel functional roles for these cytokines are sure to be uncovered. A major challenge will be to understand the specific effects of IFN-I in different cell types and in the same cellular lineage at different developmental stages. It would be important to elucidate how the relative abundance or phosphorylation status of particular STAT proteins determines the final outcome of IFN-I signaling. Finally, strategies used by both old and emerging viruses to disable IFN-I host defense will provide novel clues to combat viral infectious diseases as well as shedding light on regulatory and functional actions of IFN-I.

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Type I Interferon in Systemic Lupus Erythematosus

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Abstract Studies of the immunopathogenesis of systemic lupus erythematosus (SLE) have traditionally focused on the mechanisms of generation of the characteristic autoantibodies reactive with nucleic acid-containing intracellular particles and the contribution of autoantibody-autoantigen immune complexes to the inflammation and tissue damage that result in the clinical manifestations of lupus. The recent recognition of the central role of type I interferons (IFN) in this classic autoimmune disease has led to new understanding of the significant role of the innate immune system in the predisposition

to and amplification of autoimmunity and tissue damage. Ongoing studies are defining the genetic factors, immune stimuli, and molecular pathways that contribute to production of IFN and induction of its downstream targets in SLE. Investigations of lupus patients and murine lupus models suggest a primary role for type I IFNs in systemic autoimmunity and support the case for therapeutic inhibition of the IFN pathway in lupus and possibly other systemic autoimmune diseases.

1 Introduction

Studies of the immunopathogenesis of systemic lupus erythematosus (SLE) have focused predominantly on the mechanisms of generation of the characteristic autoantibodies, their specificity, and the contribution of autoantibody-containing immune complexes to the inflammation and tissue damage that result in the clinical manifestations of lupus. Characterization of T and B lymphocyte function and studies of impaired immune tolerance have contributed to the elucidation of the immune system mechanisms that underlie this prototype systemic autoimmune disease. But it is only with the recent recognition of the central role of type I interferons (IFN) in this disease that investigators have broken through to a new understanding of the significant role of innate immune system activation in the predisposition to and amplification of autoimmunity in SLE. Current investigations are defining the molecular pathways that account for IFN pathway activation in SLE. Detailed studies of lupus patients and murine lupus models, interpreted in the context of the decades-old observation of increased serum interferon in patients with active lupus and the insightful recent advances characterizing the molecular pathways triggered by conserved molecular structures expressed by microbial pathogens, suggest a central contribution of type I IFNs to systemic autoimmunity and support the case for therapeutic inhibition of the IFN pathway.

2 Systemic Lupus Erythematosus, the Prototype Systemic Autoimmune Disease

SLE is a multisystem autoimmune disease that results from immune system-mediated tissue damage. Manifestations of SLE can involve skin, joints, kidney, central nervous system, cardiovascular system, serosal membranes, and the hematologic and immune systems. The disease is highly heterogeneous, with

individual patients manifesting variable combinations of clinical features. In most SLE patients, the disease is characterized by a waxing and waning clinical course, although some demonstrate a pattern of chronic activity (Estes and Cjrostoa, 1971). A notable feature of SLE is that it occurs much more frequently in females than in males. Like Hashimoto's thyroiditis and Sjögren's syndrome, the female:male ratio is approximately 8–9:1 in adults, and most cases are diagnosed between the ages of 15 and 44. Between puberty and menopause, the female to male ratio may be as high as 15:1. In children and in women older than 55, the ratio is closer to 2:1. The prevalence of SLE is estimated to be approximately 124 per 100,000 in the United States, and the incidence of new cases is 1.8–7.6 per 100,000 per year. The prevalence, severity, and characteristics of disease differ in different ethnic groups, with SLE three to four times more frequent in African Americans than in Caucasians (Peschken and Esdaile 2000). The severity of disease is also greater in Hispanics than in Caucasians, although the data in Hispanic populations are less abundant. Although survival of patients holding a diagnosis of SLE is good, lupus remains a disease that is potentially fatal. SLE demonstrates a bimodal pattern of death, with deaths within the 1st year attributable to active lupus and infection, and late deaths attributable to atherosclerotic heart disease. Recent cohort studies have estimated 5-year survival at greater than 90% with improvement in medical management likely contributing to improved outcomes compared with earlier studies and 85% survival at 10 years (Trager and Ward 2001). However, once a diagnosis of SLE has been made, prolonged remission is rare (Urowitz et al. 2005). Therapeutic approaches generally involve immunosuppression, but no new therapies that specifically target the disease mechanisms have as yet been developed and no new therapies for SLE have been approved in the past 40 years.

The molecular triggers of the disease have not been defined, but the pathogenesis is known to involve production of multiple autoantibody specificities, with reactivity with nucleic acid-binding proteins a common feature. Immune complexes, along with immune system cells and soluble mediators, generate inflammation and tissue damage. Current understanding of lupus pathogenesis incorporates roles for genetic susceptibility, based on a threshold model involving multiple genes; environmental triggers, including microbial infection, sunlight, and certain drugs; and altered immune system function. Recent advances in immunology have focused attention on the mechanisms that account for innate immune system activation. At least some of the genetic and environmental contributions to lupus are likely to promote innate immune system activation and subsequent autoimmunity. Others may contribute to inflammation and tissue damage.

An important role for a genetic contribution to lupus susceptibility in humans is suggested by the high concordance of disease in monozygotic twins

(14%–57%). Genes that might account for increased lupus susceptibility or severity include those encoding components of the complement pathway, including C1q, C2, and C4A (Tsao 2004). Impaired production of these early complement components may decrease clearance of apoptotic cells, augmenting the pool of available autoantigens, or decrease solubility of immune complexes. Polymorphic variants in the interferon regulatory factor 5 (IRF5) and Tyk2 genes, both involved in the activation of the type I interferon pathway, have been associated with a diagnosis of SLE in some populations, although data indication altered expression or function of the associated gene products are limited (Graham et al. 2006, 2007; Sigurdsson et al. 2005). Association of SLE with the major histocompatibility complex (MHC) class II alleles human leukocyte antigen (HLA)-DR2 and -DR3 has been documented in many studies and is most striking in patients expressing particular autoantibody specificities (van der Linden et al. 2001). Polymorphisms in the Fc receptor genes *FCGR2A* and *FCGR3A* have been associated with SLE nephritis, possibly based on altered clearance of immune complexes. Variants of the *PDCD1* and *PTPN22* genes, encoding proteins that negatively regulate T cell activation, are also associated with SLE in some populations. Genetic variants of tumor necrosis factor (TNF) and possibly other cytokine genes may alter immune effector function and inflammatory responses (Morita et al. 2001; Ollier 2004; Schotte et al. 2005; Suarez et al. 2005). The available data suggest that a common theme among the genes that have been associated with lupus is that they confer either increased activation or impaired regulation of the innate or adaptive immune responses.

Several classes of potential environmental triggers for lupus have been studied. Although the female predominance of SLE implies a role for hormonal factors in the disease, recent concepts describe a possible contribution of epigenetic modification or dosage effects of the X chromosome rather than hormonal effects per se as accounting for at least some of the sex skewing (Pisitkun et al. 2006; Siegal et al. 1999). A role for microbial triggers, particularly virus infection, has been postulated for many years, consistent with the constitutional symptoms that often characterize the earliest stage of the disease. Epstein-Barr virus has garnered particular interest among investigators as evidence of previous infection among SLE patients is significantly higher than in the general population (99% vs 94%) (James et al. 2001). Evidence of exposure to other viruses, including cytomegalovirus, is equivalent between SLE patients and healthy control subjects. Ultraviolet light exposure is a well-described trigger of lupus flares. Possible mechanisms that account for that observation include DNA damage and induction of apoptosis of skin cells, resulting in concentration of nucleic acids and associated proteins in cell membrane blebs and increased availability of those self-antigens for processing by antigen-presenting cells (Herrmann et al. 1998). Recent data also support

an association between current tobacco use and anti-double-stranded DNA antibodies and lupus disease activity. Certain drugs, including procainamide and hydralazine, can induce a lupus-like syndrome, but the symptoms usually abate after discontinuing the drug. These agents may promote demethylation of DNA, increasing the availability of immunostimulatory DNA. Sulfa antibiotics have been reported to induce lupus flare in some patients. Administration of recombinant interferon-alpha (IFNα) to patients with hematologic malignancies or hepatitis C infection has been associated with induction of a lupus-like syndrome, a point that will be elaborated in this article (Gota and Calabrese 2003; Pittau et al. 1997; Ronnblom et al. 1990; Schilling et al. 1991; Wandl et al. 1992; Zhang et al. 1997). In addition, anti-tumor necrosis factor agents have induced lupus autoantibodies and occasionally clinical lupus in rheumatoid arthritis patients.

Genetic and environmental factors that increase the probability of developing SLE are likely to act upon the immune system to induce autoimmunity and consequent tissue inflammation and damage. In parallel to the events that account for effective immune responses directed at exogenous microbes, the autoimmunity that occurs in SLE patients requires activation of both innate and adaptive immune responses. The innate immune response is first activated by common molecular patterns expressed on the microbe, resulting in augmented antigen-presenting cell capacity and successful generation of an antigen-specific adaptive immune response. The recent description of the Toll-like receptor (TLR) family of pattern recognition receptors has provided new understanding of the mechanisms through which the innate immune system is activated by exogenous and endogenous stimuli and has led to new understanding of the important role that adjuvant-like factors that stimulate the innate immune response play in inducing a successful adaptive immune response (Janeway and Medzhitov 1999). Type I IFNs, particularly IFNα, are essential products of the innate immune response to viral triggers and have recently taken center stage as important pathogenic mediators of SLE.

3 Type I Interferons

Productive infection of host cells by a virus, leading to synthesis of RNA or DNA molecules of viral origin, induces production of host proteins, including the IFNs (Isaacs and Lindenmann 1957; Vilcek 2006). The function of these proteins is to inhibit viral replication and to modulate the immune response to the virus, with the aim of controlling infection. The type I IFN locus on chromosome 9p21 comprises genes encoding 13 IFNα isoforms, as well as

IFN-beta, IFN-omega, IFN-kappa, and IFN-epsilon, the latter mostly restricted to trophoblast cells and produced early in pregnancy (Fountan et al. 1992; Martal et al. 1998). The IFNα gene complex is likely to have been generated by repeated gene duplications and recombinations. While the need for and function of each of the IFNα genes is not clear, specific virus infections are associated with induction of one or another IFNα isoform (Barnes et al. 2001; Lin et al. 2000). Recent data from two groups have identified additional IFNs that are encoded by a gene family related to the classic type I IFNs (Bandyopadhyay et al. 1995; Barnes et al. 2003; Sheppard et al. 2003). IFN-lambdas (IL-28 and IL-29) have only moderate sequence similarity to IFNα, bind to a distinct receptor, yet induce genes similar to those induced by IFNα. The relative functional roles of IFNλ and the chromosome 9p-encoded IFNs are under study (Coccia et al. 2004).

IFNα can probably be produced by all leukocytes, but plasmacytoid dendritic cells (pDC) are the most active producers. Rapid progress in the study of type I IFN regulation indicates that cell type (plasmacytoid dendritic cells, pDC vs fibroblast), stimulus (double-stranded RNA, dsRNA; single-stranded RNA, ssRNA; DNA), and signaling pathway activated all contribute to determining the specific IFN isoforms that are produced (Bandyopadhyay et al. 1995; Barnes et al. 2002, 2003a, 2003b, 2004; Coccia et al. 2004; Daly and Reich 1995; Greenway et al. 1995; Juang et al. 1998; Kawai et al. 2004; Nguyen et al. 1997; Schoenemeyer et al. 2005; Takaoka et al. 2005). The TLR family of innate immune system receptors and their downstream signaling components play a central role in mediating activation of type I IFN gene transcription. The details of these pathways are now being elucidated; TLR3 is triggered by dsRNA, TLR7 and 8 are triggered by ssRNA, and TLR9 is triggered by demethylated CpG DNA (Beutler 2005). TLRs 7, 8, and 9 signal through the MyD88 adaptor. IFN regulatory factors and additional transcription factors, including NF-κB and ATF-2, bind to and activate an IFN-stimulated response element (ISRE) present in the IFNα and IFNβ gene promoters (Bandyopadhyay et al. 1995; Daly and Reich 1995; Nguyen et al. 1997). TLR-independent pathways have also been implicated in the induction of type I IFN transcription as well as production of IFN target genes (Ishii and Akira 2006; Sanjuan et al. 2006). RIG-1 and MDA-5 have been identified as cytosolic proteins that mediate type I IFN production induced by viral RNAs and synthetic dsRNA. Similarly, recent data demonstrate the capacity for dsDNA to activate the IFN pathway in a TLR-independent manner through uncharacterized cytosolic receptors. Although the details of these complex pathways are being modified on a weekly basis, with new publications providing new insights into the complex regulation of the IFN system, what is clear is that tracking the specific intracellular factors that mediate transcription of specific IFN isoforms can provide

clues to the innate immune system receptors and the relevant triggers that drive production of those IFNs.

4 Functional Role of IFNs in Immune System Activation

Type I IFN production represents the first line of defense in response to viral infection. Following invasion of the host by a virus, IFNα is secreted by pDC, along with other immune system cells, and binds its receptor on many target cells, resulting in engagement of intracellular signaling molecules and induction of a gene transcription program (Belardelli and Ferrantini 2002). The IFNs were used as model cytokines when Darnell and collaborators defined the requirements for cytokine-mediated signal transduction (Darnell et al. 1994; Reich and Darnell 1989; Veals et al. 1992). Binding of IFNα to its cell surface receptor was shown to activate Jak-1 and then STAT1. Subsequently, it was shown that Tyk-2, also a Jak kinase, is constitutively associated with the α subunit of the type I IFN receptor (IFNAR), while Jak-1 is associated with the β subunit of the receptor. Cytokine binding leads to activation of Tyk-2 and Jak-1 and phosphorylation of the α receptor subunit and part of the β subunit. Subsequent events include activation of STAT1, 2, and 3, the insulin receptor substrate proteins 1 and 2 (IRS-1 and IRS-2) and vav (Uddin et al. 1997). STAT1:STAT1 and STAT1:STAT2 dimers bind to the pIRE element and ISGF3, including STAT1, STAT2, and a third protein, p48, binds the ISRE element (Daly and Reich 1995; Veals et al. 1992). The Jak-STAT pathway seems to be sufficient to mediate the antiviral effect of IFNα, while the IRS proteins, as well as other factors, are also required for the anti-proliferative effect of IFNα (Uddin et al. 1997).

Activation of the type I IFN pathway has diverse and numerous functional effects on immune system cells (Garcia-Sastre and Biron 2006). IFNα matures dendritic cells by inducing ICAM-1, CD86, MHC class I, and IL-12p70 expression (Luft etal. 1998; Radvanyi et al. 1999). IFNα also promotes expression of some T cell activation molecules and it preferentially promotes Th1 responses, by decreasing IL-4 and increasing IFN-γ secretion (Aman et al. 1996; Brinkmann et al. 1993; Chakrabarti et al. 1996; Lauagalo et al. 1999; Lift et al. 1998; Radvanyi et al. 1999). IFNα leads to increased NK and T cell-mediated cytotoxicity (Djeu et al. 1982; Kirou et al. 2000; Trinchieri and Santoli 1978). This effect on CTL function has been exploited in the treatment of several malignancies with IFNα in order to augment tumor lysis, although the mechanism that accounts for the increased killing has not been elucidated fully. At least one such mechanism is the induction of FasL expression on NK cells and increased

Fas-mediated apoptosis (Kirou et al. 2000). IFNα has anti-proliferative effects on T cells, and it is generally described as a suppressor of T cell immune activity. In the setting of culture of $CD4^+$ T cells with anti-CD3 and anti-CD28 monoclonal antibodies, IFNα augments IL-10 production, generally considered an anti-inflammatory cytokine (Aman et al. 1996; Ding and Shevach 1992; Hermann et al. 1998; Malefyt et al. 1993; Taga et al. 1993). IFNγ does not have these effects and in fact inhibits IL-10 production. Taken together, studies of the impact of type I IFN on T cell function demonstrate a complex pattern that cannot be simply characterized. Regarding B cell functions, IFNα has been shown to promote B cell proliferation and immunoglobulin (Ig) class switching (Le Bon et al. 2001). At least some of this effect might be attributable to the increased IL-10 induced by IFNα, which can augment B cell proliferation and differentiation (Itoh and Hirohata 1995; Malisan et al. 1996). In addition, IFNα induces expression of B cell-activating factor (BAFF), a mediator that promotes B cell survival and Ig class switching and represents an additional mechanism that might account for amplification of pathogenic antibody production by IFNα (Ittah et al. 2006; Jego et al. 2003; Le Bon et al. 2001). Recent demonstrations of enrichment of autoreactive B cells among bone marrow emigrants suggest that effects of IFNα on central B cell tolerance mechanisms might be a fruitful area for investigation (Bekeredjian-Ding et al. 2005; Yurasov et al. 2005).

5 Type I IFN Effects on Inflammation

IFNα can also promote an inflammatory response. Among IFNα-inducible gene targets are several chemokines, soluble mediators that attract lymphocytes and inflammatory cells to tissues (Crow and Wohlgemuth 2003; Crow 2003; Der et al. 1998). As examples, CCL7 (also called monocyte chemotactic protein 3, MCP3) and CCL8 (MCP2) can be produced by skin fibroblasts and promote monocyte recruitment to sites of inflammation. In addition to proinflammatory gene products directly induced by type I IFNs, many of the molecular stimuli that result in type I IFN production are potent triggers of hundreds of pro-inflammatory gene products. These include interleukin-8 (IL-8), pre-B cell colony stimulating factor (PBEF), and many others (Barnes et al. 2003a). Additionally, type I IFN can facilitate the expression of type II IFN, IFNγ, a classic pro-inflammatory factor that amplifies monocyte responses and promotes production of pathogenic Ig subclasses (Garcia-Sastre and Biron 2006) In brief summary, IFNα helps to initiate an adaptive immune response by promoting maturation of antigen-presenting cells (APCs),

increases cytotoxic T and NK cell activity, increases antibody production, but decreases T cell proliferation. IFNα also contributes to amplification of inflammatory responses. Many of these immune system effects are reminiscent of those observed in patients with SLE.

6 Type I IFNs in SLE

Several sets of compelling data suggest an important pathogenic role for IFNs in SLE (Crow 2003; Crow and Kirou 2004; Ronnblom and Alm 2001; Vilcek 1984). Papers published as early as 1979 described increased serum levels of IFN in patients with SLE, particularly those with active disease (Hooks et al. 1979, 1982; Preble et al. 1982; Shi et al. 1987; Yee et al. 1990). At that time, the distinct type I and type II IFNs had not yet been documented, but within several years, IFNα was cloned and it became clear that IFNα was present in particularly high levels in SLE blood. This IFN was said to be acid-labile, a characteristic that is still not fully understood but may relate to its glycosylation state (Boumpas et al. 1985; Capobianchi et al. 1992; Yee et al. 1990). Soon after, it was observed that tubuloreticular-like structures in the renal endothelial cells of SLE patients and in murine lupus models were associated with IFNα and that in vitro culture of cell line cells with IFNα induced similar intracellular structures (Rich 1981). These observations suggested that IFNα was not only increased in concentration in SLE blood but also that it might have a functional impact on cells and perhaps contribute to disease. Another key observation was first reported in 1990 and has been noted many times subsequently. Therapeutic administration of IFNα to patients with viral infection or malignancy occasionally results in induction of typical lupus autoantibodies and, in some cases, clinical lupus (Pittau et al. 1997; Ronnblom et al. 1990; Schilling et al. 1991; Wandl et al. 1992; Zhang et al. 1997). This demonstration of induction by IFNα of SLE in some individuals indicated that given the appropriate genetic background and perhaps in the setting of concurrent stimuli, SLE could be induced by IFNα. In one report, twenty patients, 80% of those treated with IFNα, were noted to develop autoantibodies specific for thyroid or nuclear antigens, including anti-DNA autoantibodies (Gota and Calabrese 2003). Clinically apparent disorders include autoimmune thyroiditis, inflammatory arthritis, and SLE. Hints regarding possible mechanisms of these IFNα toxicities come from an animal model of autoimmune diabetes (Chakrabarti et al. 1996). Expression of IFNα by pancreatic islets correlates with development of type I diabetes, and transgenic mice overexpressing IFNα acquire diabetes. These mice develop autoreactive CD4 T cells that are Th1 and can kill islet cells.

The view that IFNα might play a central pathogenic role in SLE has only recently gained momentum with the completion of several large-scale studies of gene expression profiling using microarray technology (Baechler et al. 2003; Bennett et al. 2003). At least four groups have used this powerful technology to demonstrate that mRNAs encoded by IFN-regulated genes are among the most prominent observed in peripheral blood cells of lupus patients (Baechler et al. 2003; Bennett et al. 2003; Crow and Wohlgemuth 2003; Crow et al. 2003; Han et al. 2003). Several previous reports documented increased expression of IFNα-induced genes in SLE, including dsRNA-dependent protein kinase (PRKR) and oligoadenylate synthase (OAS), as well as Mx1, present in lupus-involved skin (Grolleau et al. 2000; Preble et al. 1983). Recently, microarray studies have reproducibly demonstrated that in SLE, IFN-induced genes are the most significantly overexpressed of all those assayed on the microarray (Baechler et al. 2003; Bennett et al. 2003; Crow and Wohlgemuth 2003; Crow et al. 2003; Han et al. 2003). While these data could have initially been interpreted as attributable to either type I IFN (IFNα) or type II IFN (IFNγ), our experiments have used quantitative real-time PCR analysis of SLE PBMC to show that those genes that are increased in expression in SLE are those that are preferentially induced by IFNα, not those induced by IFNγ (Kirou et al. 2004, 2005). With the description of the new type III IFN gene family (IFNλ), its gene products can also be considered candidate inducers of the genes overexpressed in SLE. High expression of IFN-inducible genes and plasma type I IFN activity is seen in approximately 40% of adult SLE patients. These patients are characterized by autoantibodies to RNA-binding proteins (Ro, La, Sm, and RNP), increased disease activity, and frequent renal involvement (Feng et al. 2006; Hua et al. 2006; Kirou et al. 2005).

Additional data have proposed an important functional role for IFNα in the induction of autoimmunity. Blanco and colleagues have shown that IFNα is one component in lupus serum that can promote maturation of blood monocytes to generate antigen-presenting activity (Blanco et al. 2001). These data are consistent with the demonstration that IFNα is one of several maturation factors for immature dendritic cells, permitting efficient antigen-presenting function to T cells, and the authors propose a central role for the activated dendritic cell in the induction of an immune response directed at self-antigens (Luft et al. 1998; Radvanyi et al. 1999). Alternatively, Yan and colleagues present data identifying the activated B cell as the first APC to capture and effectively present self-proteins to T cells, with dendritic cells coming into play secondarily (Yan et al. 2006). Regardless of the identity of the specific APCs that initiate autoimmunity, generation by IFNα of an APC competent for activation of autoantigen-specific T cells could be an important immune mechanism that incorporates a role for innate immune system activation in the initiation of autoimmunity characteristic of SLE (Crow 2003; Ronnblom and Alm 2001).

In retrospect, it is apparent that convincing data implicating IFNα as a key pathogenic mediator in SLE have been available for more than 25 years based on studies of lupus patients (Vilcek 2006).

Murine studies have supported a role for type I IFN in SLE (Nacionales et al. 2006). Both New Zealand Black (NZB) and B6/lpr lupus-susceptible mice deficient in the IFN-α/β receptor show significantly less severe manifestations of autoimmunity as well as decreased renal disease and improved survival (Braun et al. 2003; Santiago-Raber et al. 2003). Administration of an adenoviral vector encoding murine IFNα, resulting in prolonged expression of the cytokine, accelerated development of autoantibodies, nephritis and death in NZB x New Zealand White (NZB/NZW) F1 mice, but not in control mice, while increased levels of B lymphocyte stimulator (BLyS; BAFF) were observed in both lupus-susceptible and control mice (Mathian et al. 2005). Results in a distinct murine lupus model, the MRL/lpr strain, have provided different results, suggestive of a protective role for IFNα (Hron and Peng 2004). The variable results from one murine strain to another indicate the need for caution in extrapolating murine data to the human situation. Overall, data from some murine studies support a more significant relevant role for IFNγ compared to IFNα in mouse lupus, although both IFNα and IFNγ can contribute to various aspects of disease, such as autoimmunity vs inflammation (Theofilopoulos et al. 2005).

7
Mechanisms of Induction of IFN Pathway Activation

7.1
Receptor Pathways

Recent studies support a contribution of signals through TLRs to the activation of the innate immune response in lupus (Barrat 2005; Berland et al. 2006; Capobianchi et al. 1992; Christensen et al. 2006; Hoffman et al. 2004; Jakymiw et al. 2006; Kelly et al. 2006; Lartigue et al. 2006; Lau et al. 2005; Leadbetter et al. 2002; Lovgren et al. 2004; Magnusson et al. 2001; Means et al. 2005; Pisitkun et al. 2006; Savarese et al. 2006; Subramanian 2006; Vollmer et al. 2005; Wu and Peng 2006; Yu et al. 2006; Zhuang et al. 2006). Among the documented triggers relevant to SLE are immune complexes containing DNA or RNA along with specific antibodies. A consequence of TLR ligation is production of type I IFN, predominantly IFNα, that then mediates numerous functional effects on immune system cells. pDCs, a rare cell type that is enriched in skin lesions of lupus patients, are presumed to be active producers of IFNα (Bave et al.

2001; Blomberg et al. 2001, 2003; Farkas et al. 2001; Ronnblom and Alm 2001; Scheinecker et al. 2001; Siegal et al. 1999; Svensson et al. 1996; Vallin et al. 1999a).

The rules guiding innate immune system activation and targeting of specific nucleic acid-associated antigens by autoantibodies in human lupus are beginning to take shape. In fact, the mechanisms responsible for induction of type I IFN in SLE have been elucidated mainly through studies in human lupus patients, with murine studies coming later. In the early 1990s, the major cellular source of IFNα had not yet been identified, but Ronnblom, Alm and colleagues were able to demonstrate that immune complexes containing lupus autoantibodies and cellular material, including nucleic acids, could induce production of IFNα by peripheral blood mononuclear cells in vitro (Bave et al. 2000, 2001; Magnusson et al. 2001; Vallin et al. 1999a, 1999b). In view of the apparent contribution of nucleic acids to these stimulatory complexes as well as the role of DNA and RNA-binding proteins such as histones or Ro as autoantigens in SLE, TLRs triggered by DNA or RNA became prime candidates for the cell receptors mediating the induction of type I IFN gene transcription and synthesis. TLR9, the receptor for demethylated CpG-rich DNA, was an initial top candidate given the well-documented association between anti-DNA antibodies and lupus disease activity. In addition, a publication from Mean et al. showed that DNase treatment of immune complexes isolated from SLE sera ablated the capacity of those complexes to induce downstream gene activation by pDCs (Means et al. 2005). That group did not systematically study the impact of RNase treatment on unfractionated SLE serum or total isolated immune complexes. With the assignment of pDCs as the major source of IFNα, lupus immune complexes were shown to be active inducers of IFNα by those cells, while additional recent data implicate TLR9 and FcγRIIa in the induction of IFNα by some of those complexes (Blomberg et al. 2003; Ronnblom and Alm 2001; Siegal et al. 1999; Svensson et al. 1996).

Additional studies in murine lupus models are elucidating the relative roles of distinct TLR pathways and their ligands in triggering particular autoantibody specificities as well as disease. Murine studies using animals deficient in TLR9, TLR3, or TLR7 have been particularly informative in identifying the top candidate for an innate immune system pathway that mediates type I IFN production in SLE. The most striking insight from the work initiated by Ronnblom and Alm and pursued by Marshak-Rothstein, Shlomchik and others, is that the specificity of autoantibodies produced in SLE appears to be determined by the specificity of the TLR that mediates the innate immune response to the relevant antigen. For example, the production of anti-DNA autoantibodies depends on the presence of TLR9, the TLR that recognizes immunostimulatory DNA sequences, including demethylated CpG-rich DNA, and the production of autoantibodies specific for RNA-associated proteins, and perhaps for RNA, is dependent on the presence of TLR7, the TLR that recognizes ssRNA.

These requirements are most clearly illustrated in recent papers from the Shlomchik and Imanishi-Kari laboratories (Berland et al. 2006; Christensen et al. 2006). Deficiency of TLR9 in the MRL/lpr mouse resulted in absence of antinucleosome antibodies, loss of the diffuse antinuclear antibody staining pattern, and increased IFNα production. Most surprisingly, TLR9 deficiency was associated with more severe disease, consistent with an unexpected protective effect of TLR9 pathway activation and/or the associated production of antinucleosome antibodies. In contrast, loss of TLR7 maintained anti-DNA antibodies but decreased levels of anti-Sm antibodies and IFNα. The interpretation of these interesting results is not obvious. Possibilities include a role for some anti-DNA antibodies in detection and clearance of autoantigen-rich apoptotic cells or microparticles or a situation of competition or interaction between the signaling pathways linked to TLR9 and TLR7. As the level of anti-DNA antibodies detected by ELISA was not different in TLR9-deficient and TLR9-intact lupus mice, it is also possible that TLR-independent pathways contribute to production of additional anti-DNA species beyond those mediated through the TLR9 pathway and that those TLR-independent pathways, presumably maintained in the TLR9-deficient mice, contribute to inflammation and disease. Such TLR-independent pathways have recently been described and are responsive to both dsRNA and dsDNA, although the intracellular molecules mediating the DNA response have not yet been characterized (Ishii and Akira 2006). The possibility that anti-DNA antibodies might be heterogeneous and serve both protective and pathogenic roles is not entirely new but is newly emphasized and provocative. The data from the murine system could also be interpreted to suggest that signaling through TLR9, presumably mediated by DNA ligands, protects from lupus pathology based on transcription of distinct downstream targets of the TLR9 compared to the TLR7 pathways. Understanding these new results will be important for development of strategies to therapeutically modulate innate immune system activation, autoantibody production, and tissue damage.

The suggested role for the ssRNA-responsive TLR7 pathway in induction of type I IFN and SLE pathogenesis was predicted by human studies that demonstrated the RNase sensitivity of stimulatory immune complexes that induce IFNα production in vivo, an association between the presence of autoantibodies reactive with RNA-binding proteins and type I IFN inducible gene expression in peripheral blood mononuclear cells, as well as a significant correlation between anti-RNA-binding protein autoantibody titers and plasma type I IFN functional activity (Hua et al. 2006; Kirou et al. 2004, 2005; Lovgren et al. 2004). Moreover, recent genetic studies of a murine lupus model are providing additional strong support for an essential role for the TLR7 pathway in development of autoimmunity and disease in some lupus mice (Pisitkun et al. 2006; Subramanian 2006).

The Y-linked autoimmune accelerator (Yaa) locus of the male-predominant BXSB murine strain, a model characterized by expansion of the monocyte and dendritic cell populations as well as autoimmunity, has been defined as the translocation of a 4-megabase segment of the pseudoautosomal region of the X chromosome, including the TLR7 and several other less characterized genes, onto the Y chromosome. The effect of this duplication is increased expression of TLR7 mRNA and protein, at a level approximately twice that observed in nonautoimmune mice, along with a shift in the specificity of the autoantibodies toward a nucleolar, RNA-associated, pattern. A provocative study of the response of human cells to TLR7 and TLR9 ligands indicated an increased capacity of female cells to produce IFNα after TLR7 stimulation compared to male cells, while the response to TLR9 stimulation was equivalent (Berghofer et al. 2006). If confirmed, those data suggest a possible role for hormonal regulation of any of a number of components in the TLR7 pathway and might point to a mechanism that would explain the highly skewed female production in SLE.

In summary, current data support the TLR7 pathway, triggered typically by ssRNA, as the most important innate immune system molecular pathway responsible for induction of excessive type I IFN production in both murine and human lupus. Of additional interest, TLR7 itself is a target of transcriptional regulation by IFNα, providing a likely positive amplification loop for innate immune system activation. Our own data indicate that TLR7 expression is increased at sites of organ involvement (K. Kirou et al., unpublished observations). Taken together, recent data from both mouse and human systems implicate TLR7 as an important and possibly central innate immune response receptor and pathway that drives IFNα production in SLE. The complex contribution of TLR9 to lupus pathogenesis will require further study.

7.2 Composition of Stimulatory Immune Complexes

While work aimed at identifying the most relevant innate immune receptors that mediate IFNα production in SLE is underway, studies to further elucidate the components of the immunostimulatory immune complexes are in progress. Initial demonstrations of the capacity of isolated immune complexes from SLE sera to mediate activation of pDCs and expression of mRNA encoding IFNα and other proinflammatory cytokines have been followed by efforts to more specifically define those components that activate the IFN pathway. Newly constituted immune complexes including either U1RNA (typically associated with Sm or RNP proteins in the spliceosome particle) or hYRNAs (typically associated with Ro or La proteins) have been shown to activate pDCs (Hoffman et al. 2004; Kelly et al. 2006; Savarese et al. 2006; Vollmer et al. 2005). In addition to these well-characterized RNA components of particles relevant to SLE, an interesting

class of possible ligands is suggested by studies showing that some siRNAs, or miRNAs, can activate signaling pathways through TLR7. An intriguing report identifying argonaute 2 (Ago2), a protein component of the RNA-induced silencing complex (RISC), as a target of lupus autoantibodies raises the possibility that this particle could be another relevant nucleic acid-containing complex that triggers immune activation through an RNA-sensing TLR (Jakymiw et al. 2006). Our current data (J. Hua et al., unpublished observations), investigating the capacity of plasma from SLE patients expressing either anti-DNA autoantibody or anti-RNA binding protein autoantibodies, along with data from Lovgren et al., support RNA-associated molecular complexes as the most active for induction of IFNα production by mononuclear cells or isolated pDCs (Lovgren et al. 2004). The mechanisms that contribute to the described shift in the specificities of autoantigens targeted over time, from Ro and La early in the predisease course, to DNA, to Sm and RNP concurrent with onset of clinical manifestations, have not yet been elucidated (Arbuckle et al. 2003). Understanding the basis of this shift in targeting of the immune response may come with further characterization of the details of TLR pathway activation.

New data indicate that additional components of immune complexes may be required for induction of IFNα. High mobility group box 1 (HMGB1) is a ubiquitous DNA-binding protein that is translocated from nucleus to the extracellular environment during cell death (Dumitriou et al. 2005). HMGB1 can then act as a cytokine, bind to its cell surface receptor, the receptor for advanced glycation end products (RAGE), and generate proinflammatory signals in target cells or induce dendritic cell maturation. In collaboration with Anthony Coyle and colleagues at MedImmune, we have demonstrated that antibody-mediated inhibition of HMGB1 inhibits induction of type I IFN and IFN-inducible gene expression by peripheral blood mononuclear cells cultured with SLE plasma containing anti-DNA or anti-RNA-binding protein specific autoantibodies (Tian et al., 2007). Additional experiments indicated association of RAGE, HMGB1, and TLR9 after cell stimulation with CpG-HMGB1 molecular complexes. In a manner similar to Fc receptors, which have been shown to contribute to internalization of DNA or RNA-containing immune complexes, HMGB1 may mediate internalization of nucleic acid-containing immune complexes or target the complex to a TLR-containing intracellular compartment. These immune complexes, then, serve as both adjuvant and antigen for initiation of an autoimmune response.

7.3
Activation of Additional Molecular Pathways by Lupus Immune Complexes

In addition to induction of IFN and its direct targets, immune complex-mediated innate immune cell activation induces numerous pro-inflammatory

mediators as well as other cellular functions that are likely to contribute to autoimmunity, altered immune regulation, and tissue inflammation and damage. The nature of the inflammatory products induced by these complexes may be as important as the specificity of the autoantibodies induced in determining the character of disease in one patient vs another.

While the investigator community has gained important understanding of the contribution of TLR pathways to initiation of innate immune activation, a role for TLR-independent pathways remains unexplored. In addition, the intracellular mechanisms that link an internalized immune complex to the antigen presentation system are not known. A clue is provided by the recent identification of UNC93B1, a protein localized to the endoplasmic reticulum and apparently required for induction of antibody responses initiated through TLR3, 7, 8, and 9 (Tabeta et al. 2006). This protein may be a requirement for directing components of the autoantigen-containing complex from the intracellular TLR compartment to a site where the complex is either digested or associates with MHC molecules. Finally, the full consequence of signaling through either TLR-dependent or TLR-independent pathways, as well as signaling through the IFN receptor, for regulation of gene expression has not been characterized. A TLR9-independent pathway of immune system activation has identified an interaction between CpG oligonucleotides and an uncharacterized cell surface molecule that results in activation of tyrosine kinases, cell adhesion and increased motility and also intersects the MyD88 pathway (Ishii and Akira 2006). This TLR9-independent pathway is not inhibited by chloroquine. The somewhat limited efficacy of hydroxychloroquine as a therapeutic agent in SLE may be consistent with the resistance of this TLR-independent pathway or the involvement of other signaling systems that are triggered by SLE immune complexes that do not necessarily activate the IFN pathway.

8
Genetic Contributions to Increased Type I IFN Production in SLE

Genetic contributions to variability among individuals in production and signaling of IFN have been suggested by recent investigations. Patients from Sweden were studied to identify single nucleotide polymorphisms in a group of IFN pathway genes (Sigurdsson et al. 2005). Statistically significant associations with a diagnosis of SLE were found for interferon regulatory factor 5 (IRF5), a gene encoding a transcription factor that has been implicated in TLR signaling, and Tyk-2, a member of the Jak family of kinases that transduces signals through the type I IFN receptor (Takaoka et al. 2005). The IRF5 association has been confirmed in subsequent studies (Graham et al. 2006, 2007).

The complexity of the IRF5 gene structure and expression is being investigated, and differential transcription of distinct mRNA products based on variations in a splice site conferred by the SLE-associated polymorphism provides a clue to possible mechanisms of this genetic association (Graham et al. 2006). A recent report of an association of trisomy of the region of chromosome 9p that includes the type I IFN locus, encoding all subtypes of IFNα, with increased IFNα expression, autoantibody production, and clinical manifestations of SLE further supports a pathogenic role for type I IFNs in SLE (Zhuang et al. 2006).

These observations draw attention to the pathways that utilize IRF5, the TLR7 and 8 pathways, but also suggest that variations in additional genes encoding products involved in production of or response to type I IFN, including those that regulate the TLR7 pathway, could potentially impact the efficiency of activation of this cytokine system (Barnes et al. 2004; Fanzo et al. 2006; Honma et al. 2005; Negishi et al. 2005).

9 Model for Type I IFN Expression and Pathogenic Role in SLE

As described, production of IFNα and overexpression of the gene targets of type I IFN are central features of the altered immune system regulation that characterizes SLE, and a role for nucleic acid-containing immune complexes in the activation of the IFN pathway is strongly supported (Fig. 1). A more significant issue is the potential role of IFNα as a primary etiologic factor in SLE. That is, is the overexpression of IFNα a primary abnormality contributing to development of disease or is it produced only after autoantibodies and immune complexes have formed? If the latter were true, IFNα would contribute to the amplification of autoimmunity and inflammation, but it might not be primary to the disease process. New data from our laboratory are providing an answer to this question. Results from a study of healthy family members of lupus patients are documenting elevated levels of IFNα activity in the absence of any detectable serum autoantibodies (Niewold et al., 2007). Increased IFN activity is preferentially seen in those healthy individuals who have a family member with SLE and high serum IFN levels. These data strongly suggest that increased IFNα is a susceptibility factor for development of SLE. Based on these data, we propose that IFNα likely acts at two points in the series of events that result in the development of SLE. First, based on genetic factors, elevated constitutive expression of type I IFN primes the immune system to become more readily activated by either endogenous or environmental innate immune system triggers. Innate immune system activation by self-antigens, as in apoptotic debris, or by a virus, would then promote low-level secretion of self-directed autoantibodies

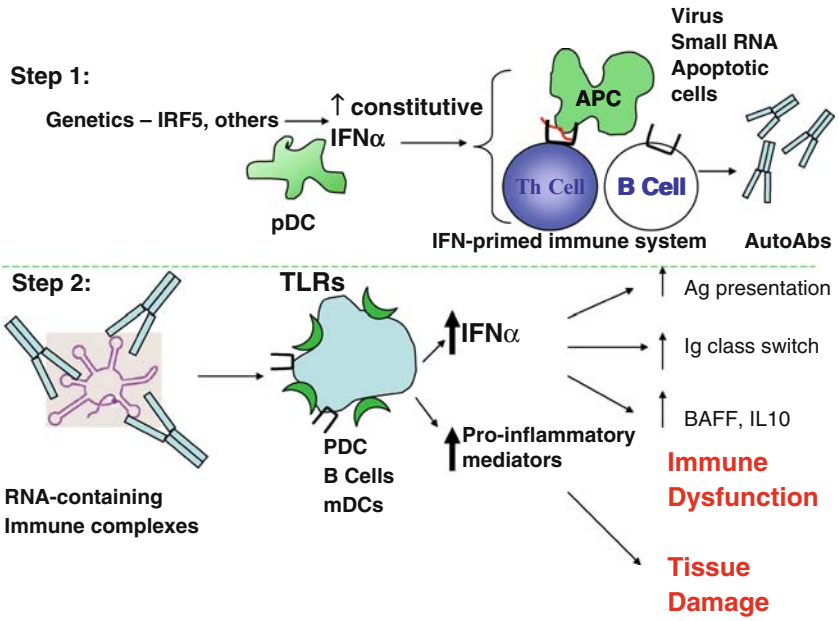

Fig. 1 Model for type I IFN expression and pathogenic role in SLE (see text). Genetic susceptibility factors result in constitutive expression of IFNα in some individuals. This low level of IFNα confers a primed state on immune system cells, making them more reactive to endogenous or microbial immune stimuli. In some susceptible individuals, low levels of autoantibodies reactive with RNA-binding proteins form. These events complete step 1 in the development of SLE. When sufficient levels of autoantibodies have formed in the presence of sufficient self-antigen, likely provided by apoptotic debris, immune complex-mediated activation of TLR pathways results in a further increase in IFNα expression as well as production of pro-inflammatory mediators. These events complete step 2 in the series of pathogenic events that comprise development of SLE, and clinical disease and tissue damage are present

that form nucleic acid-containing immune complexes. Second, those immune complexes would amplify the production of IFNα through activation of TLR pathways. Effective immune system activation by immunostimulatory immune complexes would not only stimulate the IFN pathway but also generate transcription and production of pro-inflammatory gene products that are responsible for promoting inflammation and tissue damage.

As our data demonstrating a primary role for high constitutive expression of IFNα in susceptibility to SLE suggest, this important cytokine may serve as an effective biomarker for predicting those who might develop systemic autoimmune disease as well as serving as a target for therapy in those diagnosed with SLE. It is conceivable that disease might even be preventable through modulation of the type I IFN pathway.

Once IFNα production is sufficiently established such that immunostimulatory autoantibody-containing complexes are available to the immune system, a spectrum of pathogenic mechanisms come into play, some mediated by IFNα itself and others mediated by distinct gene products induced by the immunostimulatory complexes. It is likely that IFNα itself is responsible for many of the altered immune functions that have been described in SLE patients. These include altered antigen-presenting cell capacity, increased Ig class switching, possibly altered central B cell tolerance resulting in a pro-autoimmune repertoire, inhibition of T cell proliferation, and increased production of pro-inflammatory cytokines and chemokines, including IL-8, PBEF, and others. Determinant spreading of the autoimmune response to include the classic SLE autoantibody specificities and complement activation might result.

IFNα might also contribute to clinically important disease manifestations that are not obviously related to immune system function. We propose that IFNα might alter the metabolism of cells in the central nervous system and contribute to cognitive dysfunction or depression, as has been observed in some patients with hepatitis C infection who have been treated with recombinant IFNα (Reichenberg et al. 2005). The IFN pathway might also contribute to the development of premature atherosclerosis, as supported by our preliminary data derived from SLE patients with rapid progression of carotid plaque (Kirou et al. 2006; Roman et al. 2003). Our current studies are documenting the local expression of IFNα in renal tissue from patients with class IV glomerulonephritis. In addition to the direct contribution of IFNα to disease, additional downstream targets of immune complex-mediated cell activation will include products triggered through other signaling pathways, including reactive oxygen intermediates in addition to cytokines. Our data indicate that IL-8 and IL-1 are also associated with rapid progression of atherosclerosis in SLE patients (Kirou et al. 2006). Further definition of the genetic factors, including IRF5, that contribute to constitutive production of IFNα in some individuals and lead to initial manifestations of autoimmunity, the pathways that amplify immune system activation and immune complex formation, and the gene products induced by those complexes will permit more precise understanding of the immunopathogenesis of SLE.

10 Therapeutic Approaches to Type I IFN Inhibition

Given all of the described observations, there is strong support for the hypothesis that inhibition of the type I IFN pathway may benefit lupus patients, particularly those with increased expression of IFN-inducible genes (Crow 2003). However, IFN pathway blockade might weaken the innate and adaptive immune responses to viral infection. Potential approaches to inhibit the type I IFN pathway could include antibodies specific for the IFNα receptor or for one or more of the various IFN subtypes noted above. Other approaches might include inhibition of upstream (e.g., TLR pathways) or downstream (e.g., Jaks or STATs) signaling molecules (Barrat 2005; Crow and Kirou 2004). Humanized monoclonal antibodies to IFNα are currently available and clinical studies have been initiated (Stewart 2003). While acknowledging the compelling case for a primary and central pathogenic role for IFNα in SLE, it must be considered that some of the immunologic consequences of IFNα activity may serve to control inappropriate immune system activation. A clear view of the potential for IFNα blockade in the treatment of SLE and other autoimmune diseases will await data from the clinical trials. Until then, we continue to follow the important advances in unraveling the key mechanisms of SLE in which IFNα plays a central role and maintain our enthusiasm for investigating the impact of manipulating this important viral defense pathway.

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