6 RNA Viruses and RNA-Based Drugs

A Perfect Match for RNA Delivery and the Identification of Candidate Therapeutic Target Inflammatory Molecules

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CONTENTS

6.1 INTRODUCTION

Viruses have an unmatched genius at infiltrating cells and manipulating the expression of host genes that influence viral replication and survival. This complexity of virus–host interaction presents significant challenges to contemporary medical science, with viral diseases continuing to cause mortality and morbidity worldwide. Viruses of current major concern to human health show the majority to be those comprising an RNA genome (e.g., HIV, influenza, dengue) [1]. Such RNA virus capacity in terms of the manipulation of host-cell function, in terms of potential RNA-based gene therapies and vaccines, does have positive dimensions:

1. The ability of such viruses to infiltrate cell membranes and deliver genetic material to the cell interior, and thereafter exploit the cell's molecular machinery to replicate viral genes
2. The complexities of the virus–host relationship that emphasize the crucial host molecules/pathways responsible for antiviral defense, as well as inflammation control.

This chapter focuses on two aspects learned from RNA viruses:

1. A review of RNA viral vectors (e.g., alphaviral "replicons") and their potential to deliver therapeutic RNA molecules to individuals suffering from a disease
2. The identification of host inflammatory pathways and immune responses that, because of their potent antiviral activities, are targeted by viruses for disruption, evasion, or sabotage.

The combination of knowledge in gene delivery and in RNA virus-manipulated host defense responses provides an exciting future arena for RNA-based drug targets, as well as for gene-therapy vector designs derived from engineering RNA virus genomes.

6.2 RNA VIRUS VECTORS AND REPLICON TECHNOLOGIES

Since the demonstration by Racaniello and Baltimore in 1981 that RNA from a molecularly cloned poliovirus was infectious [2], there has been significant interest in utilizing RNA viruses as vectors for vaccination or gene therapy. For many years, much of this work was limited to positive-sense RNA viruses, such as the picornaviruses, togaviruses, and flaviviruses, due to the development of infectious cDNA clones for many of these viruses [2–4]. However, in recent years, infectious cDNA clones have been generated for negative-sense RNA viruses, as well as the large coronaviruses, raising the possibility of generating expression vectors based on many different virus types.

RNA virus vectors, which for the purpose of this discussion are viruses or their derivatives that express heterologous genetic material that has been engineered into the viral genome, can be classified either as replication-competent viral vectors or replication-defective "replicon vectors." Replication-competent vectors are

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6.2.1 Replication-Competent Viral Vectors

Replication-competent viral expression vectors have been constructed for a number of virus families, including togaviruses and coronaviruses [5, 6]. The major use, or intended use, of these vectors is in vaccination, where the engineered virus expressed heterologous nucleic acid sequences ranging in size from T- and B-cell epitopes to entire open reading frames. Three basic strategies have been employed to express heterologous genetic material from replication-competent vectors. The first involves the insertion of short genetic sequences into viral genes that will tolerate the additional sequence. This approach has been successfully applied to alphaviruses, where antigenic sequences from heterologous pathogens have been engineered into the glycoproteins of Sindbis virus [7]. A second approach is to replace a viral gene that is nonessential for viral replication with a heterologous sequence. For example, for coronaviruses, the transmissible gastroenteritis virus (TGEV) genome has been engineered to express heterologous genes in place of the nonessential 3a and 3b open reading frames [8], while in the mouse hepatitis virus genome, the nonessential gene 4 can be replaced with a heterologous gene [9]. Finally, a genetic sequence can be expressed from the viral genome by engineering in a new viral promoter to drive expression of the heterologous sequence. This approach has been used extensively with alphaviruses, where additional copies of the 26S subgenomic promoter can be introduced at the beginning of the viral 3′ UTR (untranslated region). This additional subgenomic promoter will then drive high-level expression of the heterologous sequence in infected cells [10].

There are several advantages and disadvantages to using replication-competent expression vectors. One major advantage with replication-competent vectors is that they can be propagated in culture by simply allowing the vector to grow, which greatly decreases the cost of producing/packaging the vector. The ability of the vector to spread and replicate within the inoculated host may also result in the generation of more robust immune responses against the vector and the protein derived

from the heterologous sequence. However, though many virus-based expression vectors are derived from existing live attenuated vaccines, such as the 17D strain of yellow fever virus [4], there are obviously some safety concerns whenever a replication-competent vector is used, especially in highly susceptible populations, such as immunocompromised individuals. Many vectors also have limitations with respect to how large a sequence can be inserted, with some vectors limited to the coding sequence for one or a few B- or T-cell epitopes. Finally, vector stability is also an issue, where many recombinant viruses lose transgene expression after a few rounds of replication [11].

6.2.2 Propagation-Defective Viral Vectors

In addition to replication-competent viral vectors, propagation-defective viral vectors, or "replicons," have been developed for a number of RNA viruses. Replicon vectors are generally made by removing one or more of the viral structural genes from the viral genome, which results in a virus that can initiate viral RNA synthesis and gene expression, but is unable to produce infectious progeny virions. The missing gene or genes can then be provided in *trans* to package the viral RNA, with the resulting "single-hit" particle (replicon particle) able to infect a new target cell and initiate viral RNA synthesis and gene expression, but not produce progeny virion due to the lack of virally encoded structural genes. This approach has been used effectively with a number of positive-sense RNA viruses, including picornaviruses, togaviruses, flaviviruses, and coronaviruses, as well as negative-sense RNA viruses such as vesicular stomatitis virus (VSV) [12–15].

Alphavirus vectors provide an excellent example of the power of replicon vectors for gene delivery. Though the genome is a single-stranded RNA of positive-sense polarity, the genome can readily be broken down into two functional units. The 5′ two-thirds of the viral genome encodes the viral nonstructural genes (nSP), which are essential for viral RNA synthesis, while the 3′ one-third of the viral genome encodes the viral structural genes, which are expressed from a highly active subgenomic promoter. Therefore, the structural genes, which are made up of a capsid gene, two major glycoproteins (E2 and E1), as well as two additional proteins (E3 and 6K) that serve as signal sequences, can be removed from the genome without affecting viral RNA synthesis. A heterologous gene can then be expressed from the subgenomic promoter in place of the structural genes. The resulting replicon RNA will initiate viral RNA synthesis if introduced into a permissive cell, and drive high-level expression of the heterologous gene, though no progeny virions will be produced [16]. The replicon RNA can be packaged by providing helper RNA, which contains the alphavirus 5′ and 3′ ends, as well as the subgenomic promoter driving expression of the viral structural genes, but which have deletion of the viral nonstructural genes [17]. If this helper RNA is introduced into the same cell as the replicon RNA, the replicon-derived nonstructural proteins will amplify the helper RNA in *trans*, and drive expression of the viral structural genes from the helper. The structural proteins will then package the replicon RNA to produce replicon particles, while the helper RNA, which lacks an RNA packaging signal [16], will not be packaged, or will be packaged inefficiently. In order to decrease the likelihood of a recombination event

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between the helper and the replicon RNA that would generate a replication-competent virus, a split helper system, where the viral capsid is expressed from one helper RNA and the viral glycoproteins are expressed from a second helper, can also be used [17]. Variations on this theme, including packaging cell lines that stably express the alphavirus structural genes [17], have also been used to produce alphavirus replicon particles. Vectors based on several different alphaviruses have been used for vaccine delivery or in short-term gene therapy applications.

Single-hit vectors have also been constructed for a number of negative-sense RNA viruses, including VSV. Defective VSV vectors can be generated by deletion of the viral G protein, which is essential for the production of infectious viruses [15]. Heterologous genes can be inserted in place of G within the VSV genome, and the viral G protein then provided in *trans* using a packaging cell line transduced with a mammalian expression vector driving VSV G expression. One advantage of these vectors is the fact that the defective VSV particles can be pseudotyped with glycoproteins from a wide range of viruses, which will allow the vectors to be directed to specific cell types, depending on which viral glycoprotein is used for packaging [15].

6.3 RNA VIRUS EVASION OF THE INTERFERON SYSTEM

It is widely published that DNA viruses utilize a number of strategies to evade the antiviral immune responses of the host [18]. DNA viruses are thought to have "stolen" genes from the host that were modified to benefit virus survival [18]. However, fewer examples exist for smaller-genome RNA viruses, and information on immune evasion strategies employed by this class of viruses is emerging [1]. Several important immune evasion strategies employed by RNA viruses are discussed below for the human immunodeficiency virus (HIV), influenza virus, and Ross River virus (RRV), with a special focus on the interferon (IFN) system. The type I interferons are crucial in exerting antiviral effects against RNA viruses. Understanding the various IFN evasion mechanisms employed by these viruses will assist in the development of appropriate drugs or other therapeutic strategies in combating these and other virus pathogens.

The activation of host proteins such as IFNs, following viral infection, appears to correlate with early defense mechanisms, since IFNs act to inhibit both DNA and RNA viruses. Because of this, viruses often target IFNs in their attempt to modify or neutralize their antiviral potency [19]. The antiviral effects of IFNs are mediated through various intracellular pathways and are initiated by their binding to cellular receptors, which are present on most cells [20] (Figure 6.1). For instance, transcription factors such as STAT (signal transducer and activator of transcription) complexes become phosphorylated following binding of type I IFNs to its receptor. This is followed by the translocation of phosphorylated STAT complexes (consisting of STAT-1 and STAT-2 heterodimers) to the nucleus, where they bind to transcription elements of several antiviral genes (IFN-β, RNA-dependent protein kinase [PKR]), 2′ 5′ oligoadenylate synthetase [OAS], nitric oxide [NO], as well as secondary transcription factors [interferon-regulatory factor 1 [IRF-1], IRF-3, IRF-7]]. Genes such as IRF-1, IRF-3, and IRF-7, which become activated, also play important roles in the transcription of various other downstream antiviral genes. The production of these antiviral proteins

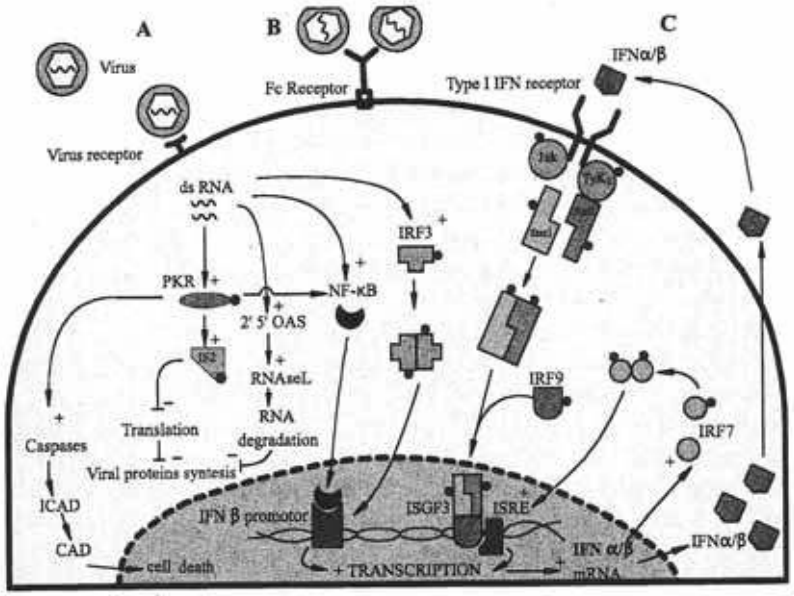

FIGURE 6.1 Virus induction of Type I IFNs. A and B represent primary events. C represents host response. Arrows (→) indicate stimulation or induction. Dashed lines (–) indicate suppression or inhibition. Virus infection and double-stranded RNA (dsRNA) will trigger the early activation of different cell proteins (PKR, NF-κB, 2′5′ OAS, IRF3) that mediate the primary expression of IFN alpha/beta. Type I IFN interaction with the specific cell receptor stimulates Jak1 and Tyk2 proteins. The activation of STAT-1 and STAT-2 is followed by the formation of an ISGF3 (interferon-stimulated gene factor-3) complex. ISGF3 will bind to the interferon receptor ISRE, promoting IFN synthesis and IRF-7 induction.

can directly disrupt the regulation of viral and cellular macromolecular synthesis and degradation. In recent years, many human RNA viruses have been shown to interrupt/disrupt the IFN system by targeting various proteins in the signaling cascade, such as STAT complexes, IRF proteins, 2′5′ OAS, and PKR [1, 19, 20].

6.3.1 Human Immunodeficiency Virus

Human Immunodeficiency virus (HIV) is of major concern worldwide, with devastating health impacts seen particularly in the developing world. HIV infection leads to the condition known as acquired immunodeficiency syndrome (AIDS), and is transmitted primarily through infected blood or semen. HIV is extremely successful in modulating various compartments of the host immune responses to its benefit. In the past 10 years, studies have shown that several HIV proteins, such as Nef, Env, and Vpu, can cripple the host immune responses. These proteins are shown to abolish CD4 surface protein from infected cells [21], resulting in the inability of T cells to engage with antigen-presenting cells and mount an immune response. Additionally, the Nef protein can trigger the down-regulation of MHC-I molecules, suppress apoptosis of infected cells by inhibiting the apoptosis-associated kinase 1 (ASK 1), as well as stimulate the expression of Fas ligands on T cells, inducing their death [22].

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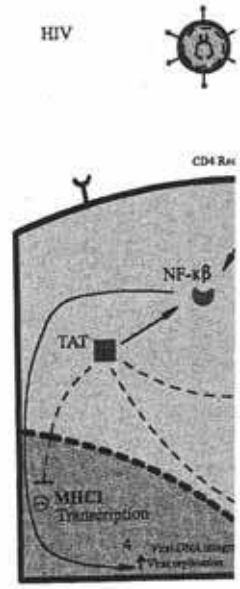

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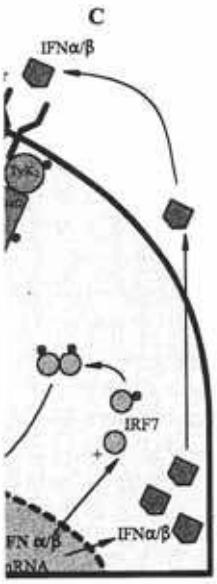

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In addition, immune regulatory processes may also be manipulated through the HIV-1 Tat protein, a potent monocyte chemoattractant expressed early in the life cycle of this virus [23]. Tat has been shown to facilitate infection by the recruitment of mononuclear cells toward HIV-producing cells, possible through its expression of conserved amino acid sequences that correspond to critical chemokine sequences. Furthermore, it is possible that Tat plays a role in the down-regulation of both HLA class I and II molecules, although there is much controversy present within the literature [24–26].

The binding of the virus to a specific cell receptor, viral cellular membrane fusion, and dsRNA activates NF-κB, resulting in a strong transcriptional stimulation of several early viral genes. Tat has also been shown to inhibit HIV-1 Tar (transactivation response) RNA binding and activation of 2′-5′ OAS. The HIV-1 Tat and Tar RNA binding proteins were shown to inhibit dsRNA-mediated activation of PKR [27]. Tar-RNA forms an inactive heterodimer with PKR. Tat, on the other hand, inhibits autophosphorylation of PKR and competes with eIF2α [28, 29] (Figure 6.2).

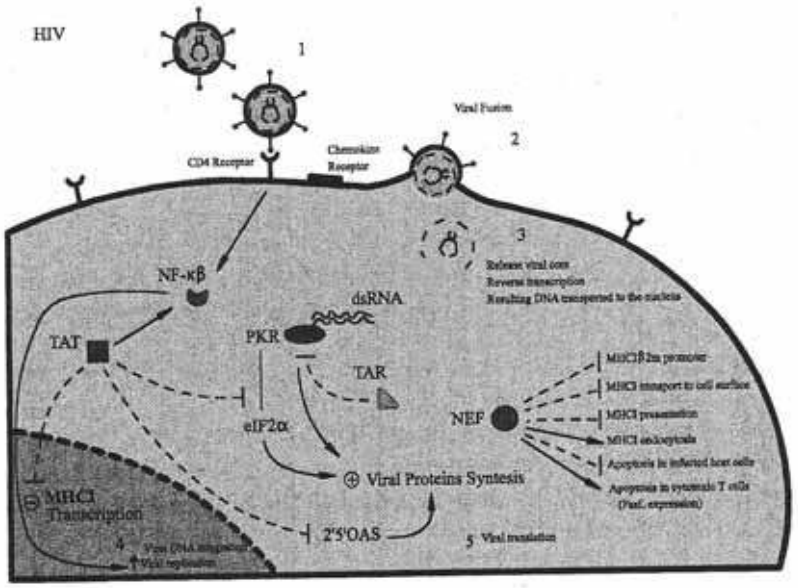


FIGURE 6.2 Early events after HIV infection of a host cell. After internalization and uncoating, reverse transcription results in the production of double-stranded DNA. The viral DNA is integrated into the host genome. Transcription results in various viral mRNAs encoding the regulatory and structural proteins to be translated in the cytosol (steps 1–5). The binding of the virus to a specific cell receptor, viral cellular membrane fusion, and dsRNA activates NF-κB, resulting in a strong transcriptional stimulation of several early viral genes. TAR and TAT are known to down-regulate PKR function. TAR-RNA forms an inactive heterodimer with PKR. TAT inhibits autophosphorylation of PKR and competes with eIF2α. NEF induces down-regulation of MHC I molecules and inhibits apoptosis of the infected cells by inhibiting the apoptosis-associated kinase 1 (ASK 1), but stimulates the expression of Fas ligands on the surface of T cells, inducing their death.

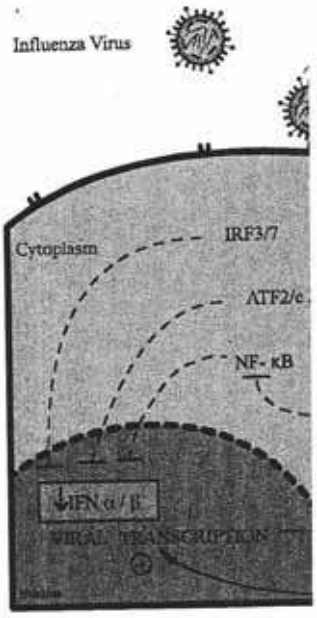
Other research has demonstrated the ability of HIV to manipulate the chemokine system. It was shown that chemokine receptors were utilized as co-receptors to facilitate entry into $CD4^+$ cells [30]. Activity of cells expressing CD4 as well as CCR5 and CXCR4 may be influenced by the HIV structural protein, gp120, and benefited by its interaction with cellular receptors for viral entry. Such binding activity by gp120 can elicit not only apoptosis in $CD4^+$ T cells and endothelial cells, but other outcomes affecting function, including neurodegenerative effects and dysregulated lymphocyte homing [31].

6.3.2 Influenza Virus

The influenza virus continues to pose significant global health problems, causing morbidity and mortality worldwide. In the United States, mortality rates over the last 30 years have ranged between 30,000 to 70,000 cases per year, with the elderly and those who are immunocompromised most at risk of death after influenza virus infection [32].

Insights into influenza pathogenesis have been helped over the past decade by studies into the modulation of the host type I IFN response via influenza proteins postinfection. The nonstructural protein of influenza A, NS1, has been shown to have a role in inhibiting the host activation of type I IFNs [33, 34], associated with the disruption of PKR activation by NS1 binding to dsRNA [35]. NF-κB and other transcription factors play a role in the transactivation of the IFN-β promoter, and in addition to the capacity for influenza to block the PKR response, NS1 can inhibit the activation of both NF-κB and IRF-3 [36, 37], thus compromising transcriptional activation pathways associated with IFN expression (see Figure 6.3). Therefore, it is evident that NS1 has an immune evasion role in addition to its polymerase activity, suggesting that viral proteins are capable of executing diverse functions within the viral life cycle. For these and other studies, an influenza virus was engineered with a deleted NS1 gene (delNS1), and early results showed that the delNS1 influenza virus could only replicate in IFN-deficient cells [33]. In IFN-intact cells, delNS1 influenza virus infection stimulated increased type I IFN mRNA transcription [33, 34], as well as high levels of NF-κB activation, in comparison to the NS1-competent parent virus [37], which suggests that the growth inhibition of delNS1 influenza was a result of the robust IFN response postinfection.

Subsequent work on influenza virus interaction with the type I IFN response has also revealed some of the subtle mechanisms employed by the virus to disrupt the IFN antiviral effect. As discussed earlier, influenza infection disrupts PKR activity in the infected host cell via the viral NS1 protein, allowing enhanced influenza virus replication [35]. The early IFN response to influenza infection has found a critical role for the host PKR inhibitory protein, $P58^{IPK}$, which, through binding to the PKR kinase domain, leads to the suppression of PKR-mediated phosphorylation of eIF2α [38, 39], a key intracellular protein required for both host and viral protein translation. $P58^{IPK}$ is bound to I-$P58^{IPK}$ as an inactive complex under conditions free of cell stress, such as infection. However, following infection with influenza virus, this inactive $P58^{IPK}$/I-$P58^{IPK}$ complex becomes disrupted, and $P58^{IPK}$ is released to interact with PKR and inhibit its kinase activity. Therefore, with decreased phosphorylation



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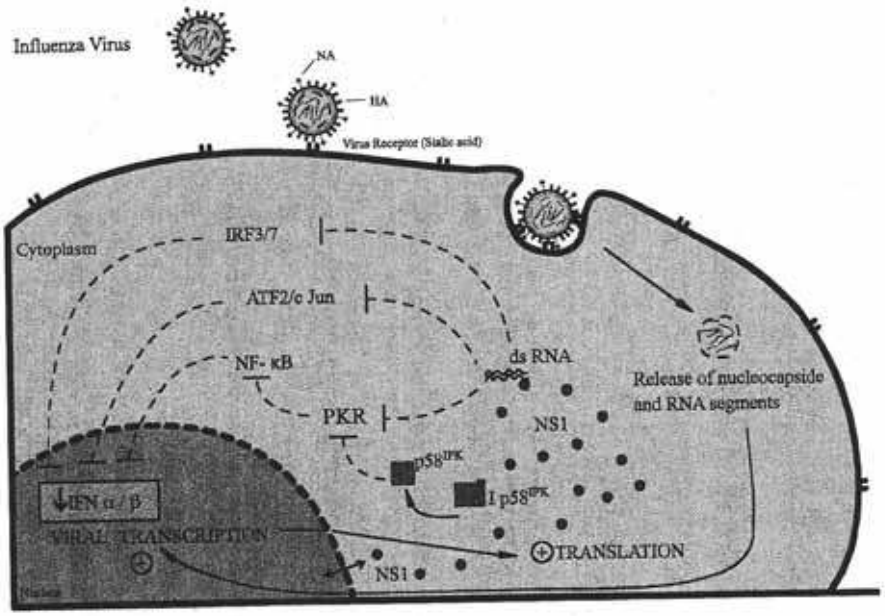

FIGURE 6.3 Interference of the interferon pathway by the influenza virus. Influenza virus infection results in expression of NS1 protein. NS1-dsRNA binding has the ability to prevent IRF3/7, ATF2/cJUN, NF-κB, and PKR activation, resulting in the inhibition of IFN α/β. The virus mobilizes a cellular protein ($p58^{IPK}$), which is released from its own inhibitor I-$p58^{IPK}$ to repress PKR activity. Full lines indicate induction; dotted lines indicate inhibition.

of eIF2α by PKR, the translation of influenza virus proteins is increased, leading to the enhanced production of progeny influenza virus by the infected cell [38].

6.3.3 Ross River Virus (RRV)

Ross River virus (RRV) is an endemic Australian alphavirus and the agent responsible for the greatest incidence of arboviral disease in Australia. Disease resulting from infection is not fatal, but involves a syndrome of symptoms that includes arthritis/arthralgia, myalgia, lethargy, and/or rash, which can persist for several weeks to months [40]. RRV is closely related to the reemergent Chikungunya virus (CHIK; *Alphaviridae*, Semliki Forest virus clade) [41], which has recently caused dramatic disease outbreaks in the French territories of the West Indian Ocean, as well as in southern India [42]. RRV and CHIK infections have similar disease manifestations in humans (arthritis, lethargy, etc.), and along with other relevant members of the Alphavirus family are broadly referred to as "arthrogenic/arthritogenic alphaviruses" [40]. Macrophage and monocyte infiltrates have been associated with human disease [43], and monocyte/macrophage ($F4/80^+$) cells have been identified as the cellular agent of severe muscle damage in RRV-infected mice [44]. Further studies in the mouse model have also detected inflammatory infiltrates in the joint and bone tissue of RRV-infected mice. Again, the infiltrate was predominantly $F4/80^+$

cells, with significant populations of $CD4^+$ T cells and NK cells detected via flow cytometric analysis [45].

RRV replicates in human and murine macrophages after cell uptake directly via a "natural" cellular receptor, or through Fc-γ receptor involving the "antibody-dependent enhancement" (ADE) mechanism of infection [46]. Since its discovery and implication as an important infection strategy, ADE has become a subject of significant scrutiny by many interested in human disease viruses. Essentially, the mechanism of ADE involves antibody-facilitated virus entry into macrophages or monocytes via Fc-γ receptors, resulting in a subsequent enhanced growth of RRV or other ADE viruses [46–48]. The first observation of RRV-ADE was reported in 1996 by Linn and colleagues [46], with the RRV-ADE and macrophage infection model leading to novel insights into the viral control of early immune and inflammatory host-gene expression postinfection.

An intriguing observation associated with RRV-ADE was the early suppression or ablation of macrophage antiviral responses, mediated by the disruption of TNF and NOS2 expression (mRNA and protein) [49]. In experiments involving pre-infection lipopolysaccharide (LPS)-stimulated antiviral activity for macrophage cultures (through enhanced pro-inflammatory cytokine and IFN expression), it was found that RRV was able to undergo unrestricted replication where infection was facilitated by polyclonal anti-RRV antibodies at a subneutralizing titer [49]. Conditions of considerable antiviral activity induced by LPS in macrophages did not allow RRV to grow when infection occurs through non-ADE (direct) infection [49]. The decrease in expression of TNF and NOS2 after RRV-ADE infection was associated with the disruption of the transcription factors interferon regulatory factor-1 (IRF-1) and nuclear factor-κB (NF-κB) [49, 50]. Significantly, the transcription and translation of non-antiviral *de novo* cellular proteins were unaffected by RRV-ADE [49]. Additional investigations showed that RRV-ADE infection stimulated increased interleukin-10 (IL-10) expression in macrophage cultures [50], explaining the global suppression of inflammatory/antiviral proteins after RRV-ADE infection (see Figure 6.4). This study also showed the suppression of IFN-associated STAT transcriptional protein complexes (e.g., ISGF-3), while the IL-10 associated transcription factor, Sp-1, was not affected by RRV-ADE infection [50].

The increased expression of IL-10 after RRV-ADE infection, at the same time as the suppression/ablation of TNF, NOS2, and type I IFN, suggests that viral infection mediated by ADE enables a sophisticated manipulation of host defense responses involving antiviral cytokines and immune protein expression. It is not simply the case that infection results in either a general suppression or general enhancement of host-gene expression postinfection. This ability of viruses to manipulate the cellular response through their interaction with transcriptional pathways that regulate key defense gene expression will provide important clues to the future design of antiviral and antiinflammatory genetic drugs, for example, the identification of gene candidates for targeting, either to augment or ablate downstream expression, during a given virus infection. The desired outcome for such a strategy is, on one hand, to enhance host immunity for optimum virus clearance, while also reducing host pathology and associated patient side effects. Also, with the above example of TNF/IL-10 expression for RRV-ADE, future antiviral genetic drug design will benefit

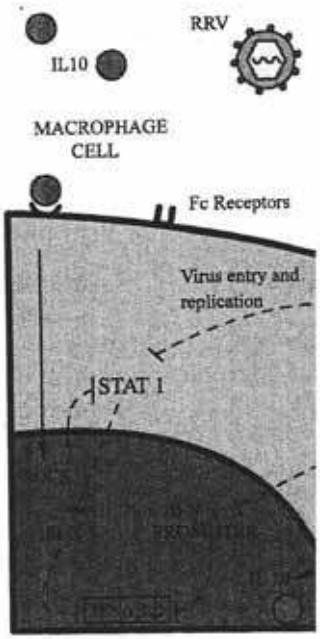

FIGURE 6.4 Suppression of replication results in the suppre the production of IFN α/β is inl response through IL-10 mediate regulation of SOCS3.

from transcriptional profilin filing of individual patients giving clues to the essential therapeutic targeting.

6.4 CONCLUDING RE

With the exciting developme and anti-sense technologies ment, it is essential that lea questions associated with p new drug development. The delivery and the developmen ing disease protection, do n realm of new gene-based dr the above challenges can be section 6.1, viruses, through in penetrating cells and del sion. Furthermore, if constr the virus will effectively de

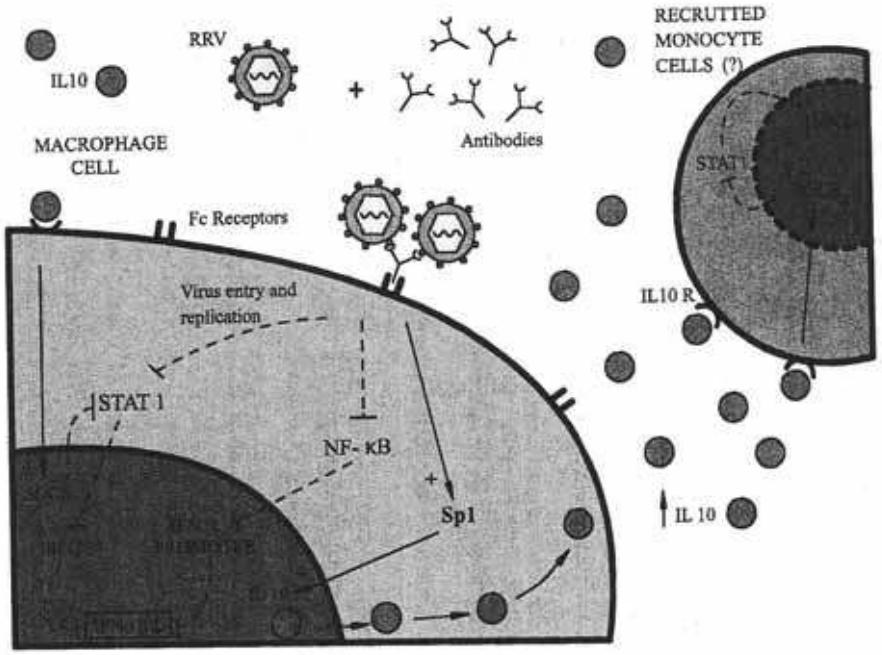

FIGURE 6.4 Suppression of antiviral response by RRV-ADE. RRV-ADE infection and replication results in the suppression of STAT-1 and NF-κB, but not Sp-1. As a consequence, the production of IFN α/β is inhibited while IL-10 is increased. The suppression of antiviral response through IL-10 mediated activity in monocyte cells is most likely mediated via up-regulation of SOCS3.

from transcriptional profiling of host genes during infection, allowing both the profiling of individual patients to determine disease severity and outcome, while also giving clues to the essential defense gene/protein interactions during infection for therapeutic targeting.

6.4 CONCLUDING REMARKS

With the exciting development of RNA-based gene technologies (for example, RNAi and anti-sense technologies) promising a revolution in drug and vaccine development, it is essential that leaders in such fields take pause to consider fundamental questions associated with pathogen-host interaction and disease mechanism with new drug development. The key questions involve the challenges of effective drug delivery and the development of drugs that, while curing a health disorder or providing disease protection, do not induce deleterious side effects in the patient. In this realm of new gene-based drug technologies, RNA viruses offer enormous hope that the above challenges can be met and overcome. As outlined in the introduction in section 6.1, viruses, through their fundamental nature and life cycle, have a genius in penetrating cells and delivering genetic material to the cell interior for expression. Furthermore, if constructed correctly through recombinant DNA technology, the virus will effectively deliver, replicate, and express therapeutic genes in target

cells, leading to the subsequent modification of a biological response to ameliorate disease. In addition to these properties, and in the context of broader disease processes like inflammation, RNA viruses have proven to be outstanding tools in the elucidation of key host intracellular pathways that control aberrant immune and/or inflammatory response. In this regard, we have focused on the lessons learned from HIV and influenza virus, as well as Ross River virus antibody-dependent enhancement (RRV-ADE), which has exposed how the involvement of host-immunity can assist in the viral manipulation of the early response to infection.

In terms of the first challenge suggested above, viruses as natural gene delivery vectors provide a way to specifically target cells and tissues associated with a disease process. In terms of unwanted side effects, our knowledge of virus–host interaction will assist in the identification of key molecules and intracellular pathways that contribute to nonspecific inflammation and overzealous immune activity that can result from infection or other biological stimuli, and thereafter enhance or exacerbate disease. The delivery of exogenous genes via therapeutic virus infection will be used to block the overexpression of "disease" genes while also, where necessary, reconstituting critical host-gene activation pathways after specific suppression by viral proteins or nucleic acids.

The application of knowledge from the discipline of virology, both engineering RNA virus genomes and the molecular biology of virus–host interaction, is tailor-made to accelerate developments in modern drug design using gene profiles from both the infected eukaryotic cell and the infecting virus itself.

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