Gene Therapy for Viral Infections

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To Pamela

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CHAPTER 1

Essentials of Viruses and their Suitability for Treatment Using Gene Therapy

1.1 GENE THERAPY

The term "gene therapy" was coined in 1972 to explain the use of procedures that are intended to treat or alleviate disease by genetically modifying the cells of a patient [1]. The concept of gene therapy was developed after publication of the first reports demonstrating that it was possible to alter gene expression in cultured cells. These early studies showed that gene expression in murine or human cells could be modified by transfection with DNA expressing herpes simplex virus thymidine kinase [2] or hexose-1-phosphate uridylyltransferase [3], respectively. Initial definitions of gene therapy referred exclusively to the use of genes to treat disease, but the meaning has now become broader [4–6]. Currently, gene therapy is defined by the use of nucleic acids, which may include DNA, RNA, or chemically modified derivatives, to alter gene function and treat disease. Because abnormalities of gene function underlie many disease processes, including those caused by viral infections, interventions using gene therapy potentially have wide-ranging applicability.

Gene therapy has many applications and may be used for restoring the health of diseased cells, killing of malignant tissue, and induction of immune responses to gene-encoded proteins. To treat diseased cells, gene therapy may entail repairing damaged genes or silencing "rogue" genetic elements that are expressed by viral pathogens. With the advent of recombinant DNA technology, polymerase chain reaction, and sophisticated nucleic acid sequencing procedures, insights into molecular biology and the fundamental mechanisms causing disease processes have greatly progressed. These developments have had a profound enabling effect on the rational design of gene therapy approaches.

Different methods of therapeutic inhibition of gene function have been used to counter viral infections. These include silencing of virus-encoded genes (Chapter 2) and introduction of targeted disabling mutations into viral genes or host factors (HFs) (Chapter 3). Using gene transfer to augment patients' immune responses to virus infections has been another way of achieving preventative or therapeutic antiviral therapeutic effects (Chapter 11).

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Exploiting gene therapy to counter virus replication has advanced considerably, and several viruses have now been shown to be candidates for treatment using this approach. However, there is no universal method of using gene therapy for viral infections. Individual viruses have particular characteristics, and this necessitates that viral gene therapy be tailored to specific infections. In developing viral gene therapy, tissue tropism, the acute or chronic nature of an infection, and the efficiency with which antiviral sequences can be delivered to infected tissues are important considerations (Table 1.1). Both synthetic nucleic acids and DNA expression cassettes are being developed for viral gene therapy. Expressed antiviral sequences may be more useful for countering chronic viral infection whereas synthetic antiviral nucleic acids are better suited to inhibiting acute viral infections. Preventing viral escape from gene therapy is important, and overcoming this problem by simultaneous targeting of multiple viral sites or suppressing HFs has shown promise.

1.2 ESSENTIALS OF VIRUSES

Viruses are the simplest life forms; not surprisingly, they are also very plentiful. Estimations place the number of viral particles in the biosphere to be between 10^{31} and 10^{32} [7–9]. In natural waters of the Earth, they are estimated to outnumber bacteria by an order of magnitude. Viruses are a major cause of disease and constitute a reservoir of enormous genetic diversity. They are highly varied with respect to their structure, genome replication mechanisms, and modes of interacting with their host organisms. Common and interrelated defining features of viruses are the following:

- They are obligate intracellular parasites that only reproduce within host cells and are incapable of independent replication.
- Viruses do not have the machinery required for translation of proteins. They use host protein synthesis mechanisms, with their own genetic material as template, to produce the components constituting intact infectious viral particles (virions).
- Viruses lack the mechanisms for generating the energy required to drive the biochemical processes required for their existence.

Viruses may essentially be considered as nucleic acid parasites that use virions to introduce their own genetic material into cells. Thereafter, the host cellular machinery is reprogramed for copying the viral genome and the formation of more virions to result in completion of the viral replication cycle. Viruses have evolved efficient mechanisms for introducing DNA or RNA genomes into cells, and this property has been exploited for development of viral gene therapy vectors. Ironically, in some cases these recombinant vectors are being developed as therapeutics to counter virus infections.

Table 1.1 Viral Characteristics That Influence the Gene Therapy Strategy

Viral characteristics	Implications for gene therapy of viral infections
DNA or RNA virus	Both DNA and RNA viruses are susceptible to RNA silencing mechanisms (e.g., by RNAi activators). DNA targeting, such as by sequence-specific nucleases, is only possible with DNA viruses.
Virus tropism	Delivery of gene therapies remains a challenging task for successfully implementing gene therapy of viral infections. Therefore, accessibility of infected tissues to gene therapy vectors is an important factor that currently influences success. For example, delivery of small interfering RNAs targeting respiratory syncytial virus after inhalation is easier than delivery to hepatocytes after systemic administration of gene therapy formulations.
Acute or chronic nature of infection	Acute infection may require a single dose of a therapeutic whereas chronic infection may require repeated administrations. Approaches that achieve sustained inhibition of viral replication may also be necessary for treating chronic infections.
Viral replication rate	Very high replication rate may overwhelm efficacy of antiviral gene therapy. Moreover, viral dormancy may also evade nucleic acid antivirals.
Predisposition to mutation	RNA viruses or viruses that use reverse transcription during genome duplication are prone to error and may introduce mutations that evade sequence-specific antivirals. Combinatorial approaches or host factor targeting may be necessary to provide a higher barrier to resistance.
Host immune response to virus	Host immunity may augment efficacy of antivirals. Although generally undesirable, induction of the innate immune response by viral gene therapies may augment their antiviral efficacy (e.g., 5′ triphosphate-containing small interfering RNAs against HBV infection).
Reliance on host factors (HFs) that may be silenced	Disabling HFs (e.g., CCR5 for HIV-1 infection and micro RNA 122 for HCV replication) that are required for viral replication may be used to inhibit viral replication. Successful use of this approach requires that inhibition of HF function is not toxic to cells.
Virus-encoded mechanisms of countering gene silencing	Viruses that are capable of inhibiting the RNAi pathway (e.g., adenovirus virus-associated RNAs) may result in attenuated inhibition of silencing efficacy.
Similarity of viral targets to host cellular sequences	Sequence homology between viral targets and cellular sequences may result in unintended harmful off-target effects of a gene therapy.

Whether viruses meet the basic requirements of what constitutes life has been a subject of debate. Definitions of life are themselves fraught [10], but viruses display at least some of the traits that characterize living entities. Key attributes of living entities include the ability to reproduce and evolve in response to

external influences. Because viruses reproduce, albeit in a parasitic manner, they may be considered as living organisms. Moreover, adaptation of viruses to their environments through evolution, an important and clinically relevant property, is another argument in favor of viruses being classed as living entities. However, because viruses are obligate intracellular parasites that are incapable of independent replication, some have argued that they do not qualify as living organisms.

Defining what makes a virus a virus has also been the subject of some debate [11–13]. It has been argued that the disappearance and appearance of virions (disintegration and reconstitution) is the fundamental characteristic feature of viruses [13]. Disappearance of the virion occurs when the particle breaks down to release the viral genome into the cell, then reappearance happens when the intracellular machinery is used to propagate virion progeny. Although understanding the phenomenon of disappearance and appearance of viruses is useful, defining viruses on the basis of such phenotypic features may be problematic because they may not be particular to viruses. Therefore, the specifics of genome coding capacity have been preferred as the essential attribute of viruses [11,12]. Raoult and Forterre proposed the existence of genes that encode a viral capsid as the unique feature of viruses, and a paraphrased version of their widely accepted definition of viruses is the following: "Viruses are capsid-encoding organisms that are composed of proteins and nucleic acids. They self-assemble in nucleocapsids and use ribosome-encoding organisms for the completion of their life cycles." According to this description, all living organisms may be classified as either capsid-encoding viruses or organisms that have translational capacity (Bacteria, Archaea, and Eukarya). The definition of capsids themselves becomes important for distinguishing viruses from other organisms. It is well known that capsid-encoding genes may be integrated into other organisms. A good example is the existence in human immunodeficiency virus (HIV)-1-infected humans of capsid-encoding sequences located in the integrated provirus. Raoult and Forterre cleverly address the possible designation of host organisms as viruses by classifying a capsid as a "structure that is used to disseminate a genome that encodes the capsid proteins" [11].

1.2.1 Viral Origins

Identifying the origin of viruses has been contentious. The dependence of viruses on cellular life forms led to the idea that cellular life preceded the evolution of viruses. However, there is some evidence that viruses co-evolved with their cellular hosts. A particularly interesting recent insight into virus evolution has come from structural analysis of virion architecture and coat topology (reviewed in refs [14,15]). Unexpected similarities were found in viruses that infect organisms of different kingdoms (Bacteria, Archea, and Eukarya), which

was interpreted as indicating a common ancestry to all viruses. The three main propositions to explain the origins of viruses are the following [16]:

1. The *progressive hypothesis* purports that mobile genetic elements gained an ability to be transmitted between cells and then gave rise to viruses. The similarity between eukaryotic retrotransposons, commonly found in eukaryotic cells [17], and retroviruses gives support to this notion. Among other common features, retroviruses and retrotransposons possess flanking long terminal repeats and encode reverse transcriptase and integrase. Both entities convert RNA into DNA, which is then integrated into the host genome by similar mechanisms. Although the progressive hypothesis postulates that retrotransposons gave rise to retroviruses, it is also possible that the converse is true: Infection of host cells with retroviruses gave rise to infection-deficient retrotransposons.
2. The *regressive hypothesis* states that viruses may have evolved from free-living, more complex intracellular parasites. According to this theory, an initial symbiotic relationship between the virus precursors and their hosts turned parasitic during evolution of the viruses. Characteristics of the nucleocytoplasmic large DNA viruses (NCLDVs) back this hypothesis. NCLDVs are large and have complex genomes. The Mimivirus member of this family, which is also the largest known virus, has a genome size of 1.18 million base pairs. Sequencing of the genome interestingly revealed evidence of remnants of translational machinery [18]. These included amino acyl transfer RNA synthetases, translation factors, and tRNA-encoding sequences. These observations suggest that NCLDVs lost translational capabilities as they evolved into obligate intracellular parasites.
3. The *virus-first hypothesis* proposes that viruses were the first replicating entities that gave rise to cellular life [19,20]. According to this theory, self-replicating viral units acquired membranes that gave rise to bacterial, archaeal, and eukaryotic cells. Furthermore, while parasitizing their cellular descendants, the hypothesis proposes that ancestral viruses evolved into present day viruses. Detailed analysis of the DNA polymerase genes of phycodnaviruses and other organisms supported this idea. Reconstruction of a phylogenetic tree demonstrated that the viral genes are exclusively located at the root of the clade containing all eukaryotic DNA polymerase delta genes [20]. The observation was reasonably interpreted as indicating that the polarity of the flow of genetic information was from viruses to primitive eukaryotic hosts, and not the reverse. An important underlying concept of this theory is that simpler viral genomes evolve more rapidly than their more complex host genomes. Thus, the resultant enhanced capacity for genetic novelty would be capable of providing a rich source of molecular complexity to the host.

There are compelling arguments in support of very different hypotheses to explain the origin of viruses. A possibility is that various underlying processes gave rise to different viruses, and that these events may also have taken place several times during the evolution of viruses. However, this notion may not reconcile with the structural evidence supporting a common viral origin [14].

1.2.2 Basics of Virus Replication

There is considerable variation in the molecular mechanisms that viruses use for their propagation. However, because all viruses are parasitic and their replication involves disintegration and reconstitution of virions after infection of host cells, there are some essential features that are common to all virus life cycles (Figure 1.1):

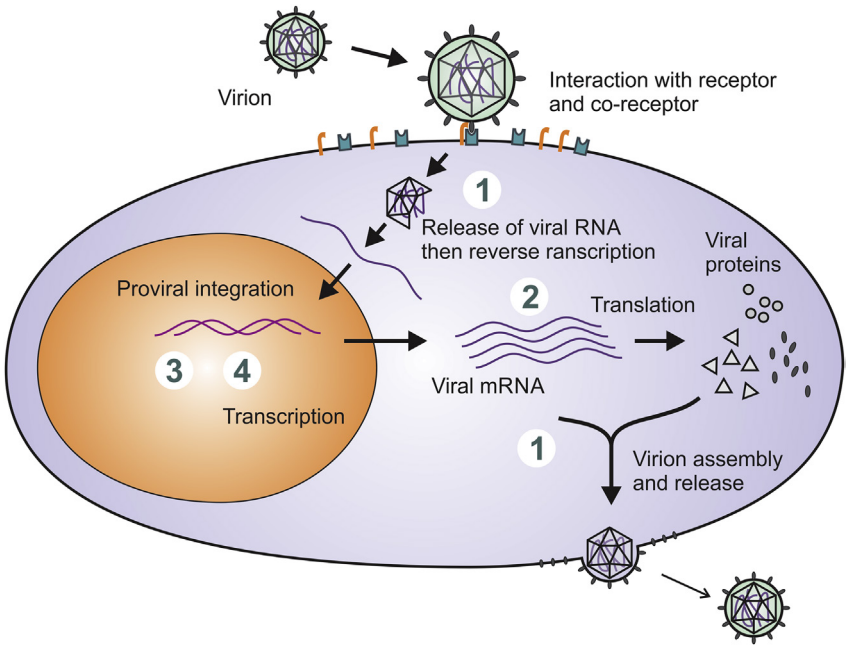

FIGURE 1.1 Schematic illustration of infection of a cell by a retrovirus with potential targets for gene therapy.

Attachment of the virion to the target cell involves interaction with a receptor (e.g., CD4) and co-receptor (e.g., CCR5). Release of viral RNA is followed by reverse transcription and integration of proviral DNA into the host genome. Transcription and then translation of viral mRNA result in the formation of viral proteins. Release of the newly formed virions involves assembly of the capsid, incorporation of viral genomic RNA, and budding from the cell membrane to form the viral envelope. Examples of potential mechanisms of gene therapy are (1) suppression of expression of the viral co-receptor and other host factors, (2) post-transcriptional gene silencing, (3) mutation of viral DNA, and (4) transcriptional silencing of viral genes.

1. The initial step is typically considered to be the interaction of virions with the host cell membrane. This involves binding by molecules on the surface of virions to receptors on the recipient cells. The specificity of this interaction is a major factor that defines the tissue tropism and the particular species that a virus infects. Some viruses have broad tropism and infect different species or several different tissues (e.g., cytomegalovirus). Others may be limited to particular cell types in one organism (e.g., hepatitis B virus (HBV) infection of hepatocytes of humans, certain primates, and the Asian tree shrew). In addition to susceptibility to virus infection conferred by interaction of virion and cell surface receptors, target cell infection range is also determined by permissiveness to infection. This permissiveness involves specific interactions of viral and HFs that are required for viral replication during subsequent stages of the virus infection cycle. Inhibiting the function of these HFs is an approach that is being used to counter viral replication using gene therapy. An important example is inactivation of the C–C chemokine receptor 5 (CCR5), an HIV-1 co-receptor, to make CD4 cells resistant to infection with the virus. Another example is the use of chemically modified oligonucleotides to hybridize and inhibit the function of micro RNA-122, a hepatitis C virus (HCV) HF. Using this method to treat HCV infection has reached an advanced stage of clinical testing [21].
2. After interaction with cell surface receptors, viruses penetrate target cells. The process may involve translocation; endocytosis; or, in the case of enveloped viruses, fusion of the viral envelope with the target cell membrane. Internalization of the particles is followed by breakdown of the viral particles and release of their genetic material.
3. Production of viral proteins follows, and this essential step exploits the host cell translational machinery. Viral protein production may involve direct use of virion-released nucleic acid, and an example is the use of poliovirus plus strand viral RNA as translation template (reviewed in ref. [14]). Processing of released viral nucleic acid may be required before translation of viral proteins occurs. An example is the reverse transcription of the HIV-1 RNA to form proviral DNA, which is in turn transcribed before being translated into viral proteins. Viruses have also evolved noncanonical mechanisms to ensure efficient protein expression in host cells. Three examples are the following:
   a. Use of an internal ribosomal entry site by viruses such as HCV and poliovirus to guide translation initiation in the absence of a 5′ cap on viral mRNA [22].
   b. Production of precursor polyproteins that form mature viral proteins after proteolysis. An example is the HCV polyprotein that generates 10 structural and functional protein elements of the virus (reviewed in ref. [23]; Chapter 7).

   c. Exploiting RNA secondary structures to induce reading frame shifts. HIV-1 utilizes this mechanism to increase mRNA protein coding versatility and allow formation of required ratios of Gag and Pol protein components from a single mRNA template.
4. Interaction of viral proteins with host cellular factors leads to replication of viral genomes and assembly of new viral particles. This step is complex and highly variable among different viruses. It is important to note that it is largely the way in which cellular functions are subverted that determines whether a virus infection is manifested as disease. For example, the killing of $CD4^+$ T helper cells that is effected by HIV-1 results in compromised immunity and attendant complications leading to the acquired immunodeficiency syndrome (AIDS).
5. Release of newly formed virions is required to spread the infection between cells from the same individual or from one person to another. The process of releasing nascent virions is also variable and depends on properties of the host and viral elements. Viral capsid assembly and packaging of the genome is an essential step before release of newly formed virions. This process occurs by different mechanisms, but it generally requires interaction of a packaging signal on the viral genome with viral and cellular proteins to introduce the viral nucleic acid into the capsid particle. Enveloped viruses, such as HIV-1, may be budded from a cell and carry the cell membrane with them to form the envelope. Nonenveloped viruses (e.g., poliovirus) lack an outer membranous layer and may be released after cell death and lysis. After release from cells, virions are free to start a new round of infection by interacting with receptors on the surface of susceptible cells.

1.2.3 Virus Structures and Classification

The classification of viruses is based on their genome composition, size, shape, host range, and mode of replication [24]. Descriptive nomenclature has been helpful to assist workers in understanding pathogenic and structural properties of groups of viruses. For example, the Hepadnaviridae family, of which HBV is a member, includes viruses that have DNA genomes and infects the liver to cause hepatitis.

Viral genomes may be RNA or DNA, single or double stranded, linear or circular, and monopartite or multipartite. In addition, the genomes of single-stranded RNA viruses may have a sense (+) or antisense (−) orientation. The sense-stranded RNA genomes may serve as translational templates. However, antisense single-stranded viruses require conversion to plus stand sequences before translation is possible. Ambisense translation is also possible with some RNA viruses and is an interesting mechanism of increasing coding capacity.

With these viruses, protein translation is possible from genomic RNA or its complement. The term is derived from the ambiguous coding properties of each RNA strand. That is, each strand may have both sense and antisense polarity. Examples of viruses with ambisense genomes are found in the members of the Bunyaviridae family, such as Rift Valley Fever virus [25]. DNA viruses generally have less variability in their genome structures. Most of them contain a single DNA molecule that may be single stranded (e.g., parvovirus), double stranded (e.g., adenovirus), or circular (e.g., HBV). More viruses have RNA genomes than DNA genomes. Because the error rate of RNA replication is high, these viruses frequently generate mutations, which in turn may provide fitness advantages or disadvantages to the viruses.

Capsids, which provide a protective shell for viral genomes, are generally classified as being either helical or icosahedral in structure, although complex and pleomorphic alternatives may also occur. An example of a capsid with variant helical structure is that of HIV-1. This viral component has been described as a "fullerene cone." It comprises a helical arrangement of hexamers of capsid proteins that is sealed by 12 pentamers of the capsid protein [26–28]. Capsids themselves may be surrounded by a lipid membrane that is derived from the host cells. Enveloped virions typically have glycoproteins embedded in the lipid bilayer. These function as ligands for the receptors on target cells and serve as viral antigenic determinants.

1.2.4 Spread of Virus Infections

Modes of viral transmission, seasonal variation of infections, incubation periods, and phases of communicability are important to understand the epidemiology of infections and to implement measures to prevent spread. When transmission occurs between individuals it is referred to as "horizontal." Vertical transmission results from a mother infecting her unborn baby. Some viruses (e.g., HBV) may be passed from mother to baby during childbirth. In such cases in which transplacental transmission of the virus is rare, virus transmission is referred to as "perinatal." Horizontal transmission may occur across epithelial surfaces, which include intestinal, respiratory, genitourinary, conjunctival mucosa, and the skin. In addition, blood–blood (parenteral) contact between individuals and zoonotic spread between arthropods and different species may cause viral spread.

1.3 VIRAL PATHOGENESIS OF DISEASE

Because viruses are obligate intracellular parasites, it is not surprising that some disruption to cellular functions occurs during their propagation. Virulence, or the capacity of viruses to cause pathology, varies and is dependent on several host- and virus-derived factors. It is important to note that the

severe cytotoxicity associated with high virulence potentially incapacitates virus propagation; therefore, it would be an evolutionary disadvantage to a virus. Consequently, viruses that have less virulent effects are more common. Disruptive effects may vary considerably and are largely responsible for the pathogenic effects of virus-related disease. Direct cytotoxic effects of viruses may result from disruption to essential host cellular functions, such as the maintenance of normal cell membrane ion permeability and synthesis of macromolecules. So-called "genotoxic effects" may occur after mutational effects of viruses on host genomes. Indirect toxicity is another potentially serious consequence of viral infection and typically results from effects of the host's immune response to a virus infection. HBV is an example of a virus that causes indirect toxicity. The virus has minimal direct cytopathic effects and symptoms of acute infection occur as a result of cell-mediated attack on infected hepatocytes. Ironically, severe symptoms indicate a good long-term prognosis with low risk for chronic infection. Conversely, asymptomatic infection indicates a poor immune response and high risk for viral persistence. Cytopathic effects of viruses manifest in different ways, such as the induction of programmed cell death, formation of inclusion bodies, changes to cell morphology, and syncytium formation.

Persistent infection occurs when a virus is not cleared and remains in infected cells. Naturally persistent virus infections have been characterized as being latent or chronic [29]. An example of a latent virus infection is that caused by herpes viruses (Chapter 11). The replication occurs during bouts of disease manifestation, but the viral genome lies dormant between such episodes. HBV and HCV may cause chronic infections, and these viruses are detectable during the periods of persistence (Chapters 6 and 7). Inadequacy of host immune responses to the infections plays a major part in determining the persistence of these virus infections [30]. However, latent and chronic viral infections are not mutually exclusive. For example, HIV-1 manifests latent and chronic characteristics. This has important implications for therapy of the infection. Current antiretrovirals are capable of suppressing HIV-1 replication, but they do not eradicate the reservoir of quiescent proviral integrants.

The mechanisms by which viruses become persistent are largely a result of immune- or gene expression-related effects. Viruses may have immunoevasive or immunomodulatory effects to limit or attenuate efficacy of host immune responses. Variation in viral antigens by HIV-1 is the classic example of avoidance of neutralizing effects of a host immune response. Reduced expression of major histocompatibility complex (MHC) class I molecules by cytomegalovirus [31] and modulation of monocytes and macrophages by Epstein Barr virus [32] are other examples of modulatory effects that viruses use to evade host immune responses. Stability and quiescence of viral replication intermediates may also add to persistence of viral infections. The enduring nature of

integrated proviral DNA of HIV-1 together with suppressed viral gene expression are clinically important examples of viral mechanisms that enable avoidance of immunodetection by a host's antiviral immune response.

1.4 IMMUNE RESPONSES TO VIRUS INFECTIONS

Viral infections in vivo result in the stimulation of innate and adaptive immune responses. The innate response is activated during the initial stages of an infection and is triggered by pattern recognition receptors (PRRs) that distinguish particular pathogen-associated molecular patterns (PAMPs) that are found in various microbial pathogens such as viruses [33–36]. The adaptive immune response, with humoral and cell-mediated arms, occurs later during a virus infection and is a more recent evolutionary development. The innate system uses germ-line-encoded PRRs that identify groups of viral pathogens whereas the adaptive immune response entails selection of clonally expressed pathogen-specific receptors. Adequate stimulation of the innate immune response contributes significantly to the effectiveness of the adaptive response. Mounting of an antiviral immune response is understandably critically important for elimination of viral infections. Many antiviral therapies, including candidate viral gene therapies, require augmentation from the host's pathogen-specific immune response to be effective. Furthermore, because some antiviral gene therapies are based on use of recombinant viral vectors, antiviral immunity may also be a factor that affects the efficiency of delivery of antiviral sequences. In addition, some antiviral nucleic acids, particularly activators of RNA interference (RNAi), may be perceived as foreign; therefore, they induce an immunostimulatory effect. Therefore, the antiviral immune response has an important influence on the efficacy of gene therapy at several levels. The essentials of antiviral immune responses are described below. For comprehensive accounts of the topic, the reader is referred to the many excellent reviews in the field.

1.4.1 Innate Immunity

The components that are recognized by PRRs include viral double-stranded RNA, single-stranded RNA, RNA with 5′ triphosphates, proteins, and DNA [36]. PRRs comprise three main groups:

1. Retinoic acid-induced gene I (RIG-I)-like proteins (RLPs),
2. Toll-like receptor (TLR) proteins, and
3. Nucleotide oligomerization domain (NOD)-like receptors.

Each group is responsible for recognizing particular viral motifs. In addition, the downstream activation of pathways that counter viral replication differs. Activation of TLR and RLP pathways are schematically illustrated in Figure 1.2. RLPs, which are essential for mounting an innate immune response to RNA