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Chapter 7

Epilogue
In spite of the increasing incidence of non-communicable diseases, infectious diseases remain among the leading causes of human mortality and morbidity, accounting for more than 20% of the 57 million deaths worldwide in 2008 [1]. Contrary to predictions in the early 1960’s [2], our battle with infectious agents is far from reaching an end. With the old foes and newly emerging nuisances, it is just more realistic to accept the ever-continuing struggle with pathogens as a perpetual war. Among the emerging and reemerging infectious diseases, those caused by arthropod-borne viruses (arboviruses) display unique features, making them difficult to tackle. These viruses establish persistent infection in invertebrate vectors (e.g., mosquitoes, ticks, midges, sandflies), and can be transmitted to vertebrate hosts (humans, other mammals and birds) under favorable conditions [3;4]. Except for African swine fever virus, all arboviruses that infect vertebrates are RNA viruses [5], which is probably explained by the greater genetic plasticity and higher mutation rates exhibited by RNA viruses, which likely facilitate alternating replication in vertebrate and invertebrate hosts. Notorious arboviruses include members of the Bunyaviridae such as Rift Valley fever virus and Crimean-Congo hemorrhagic fever virus, as well as West Nile virus (WNV), Japanese encephalitis virus (JEV) and Dengue virus (DENV), which all belong to the Flavivirus genus within the Flaviviridae.

In order to curb the spread of diseases caused by arboviruses, a comprehensive understanding of the molecular characteristics of their replication, virus-host interactions and host adaptation is of great value. Compared to our understanding of DNA viruses, however, our knowledge of the molecular biology and biochemistry of RNA viruses was lagging behind in the early days, since most of the molecular biology techniques aimed at modifying nucleic acids available were directed at DNA substrates. The development of the so called “infectious cDNA clone technology”, however, greatly spurred RNA virus research, especially that of plus-strand RNA viruses. For these viruses, the genomic RNA not only serves as the genetic material, it is also directly translated into viral proteins. Therefore, the introduction of a plus-strand RNA virus genome into a cell can initiate the complete viral life cycle and result in the production of progeny virions that can start the next round of infection. Based on this principle, the infectious clone technology was developed and became widely used for RNA viruses like picornaviruses [6-15] and alphaviruses [16-19], yet this approach turned out to be more challenging when applied to flaviviruses, largely due to the genetic instability of full-length flavivirus cDNAs during propagation in E. coli [20-22]. Initially the instability problem was circumvented by using an in vitro ligation approach in which full-length templates for run-off RNA transcription were constructed by in vitro ligation of appropriate restriction fragments [20;21]. Alternative strategies that were subsequently developed to overcome the instability included: 1) the use of different bacterial strains for cDNA transformation and propagation [22]; 2) insertion of short introns to prevent the unwanted expression of ORFs present in the viral genome that are potentially
toxic to *E. coli* [23]; 3) exploiting low-copy number vectors for the assembly of full-length flavivirus cDNA clones [24;25].

A stable full-length clone for yellow fever virus strain 17D (YFV-17D) was successfully constructed in a low-copy number vector [25], which not only helped to deepen our insights into the molecular biology of this prototypic flavivirus [25], but also facilitated the design and construction of YFV-17D based recombinant vaccines [26-41]. Ever since its development in the 1930s [42], YFV-17D has stood as a paradigm for a successful live attenuated viral vaccine, with a great record of safety and efficacy, rendering it an appealing vector for genetically engineered recombinant vaccines against other diseases. In Chapter 2, YFV-17D was exploited to express the glycoprotein (GPC) sequence of Lassa virus (LASV). The recombinant virus was proposed as a bivalent vaccine candidate against both YFV and LASV that circulate in the same region in West Africa. While characterizing the recombinant YFV-17D expressing LASV GPC, genetic instability was encountered, as illustrated by the gradual and partial loss of the insert. Considering the duplication of the YFV NS1 signal peptide coding sequence flanking the GPC insert, we tried to stabilize the clone by changing the codons of the downstream signal peptide so as to minimize sequence similarity, which was postulated to induce homologous recombination and thus the loss of the insert. This strategy, however, turned out to be unsuccessful. Detailed analysis of recovered recombinant viruses strongly suggested that the large size of the insert was the major problem, which has been encountered and reported in other studies aimed at constructing recombinant flaviviruses [30;37]. Despite the lack of direct evidence for insert size limitations, most likely due to size restrictions of the icosahedral capsid, our success in stabilizing the viruses by using smaller inserts corresponding to the individual subunits GP1 and GP2 of the LASV GPC, provided support for this hypothesis. Therefore, it is advisable to take the length of the heterologous insert into consideration while constructing recombinant flaviviruses or RNA viruses with an icosahedral capsid structure in general.

Another approach for the construction of infectious clones is the so-called infectious DNA (iDNA) or DNA/RNA layered approach, by which +ssRNA viruses are recovered *in vivo* after transfection of plasmid DNA carrying the viral genomic cDNA under the transcriptional control of a eukaryotic promoter. Using iDNA, the procedure for recovering infectious virus particles can be simplified, since there is no need for *in vitro* transcription; moreover, DNA is much more stable at room temperature in comparison to the RNA genome or RNA viruses. The first infectious cDNA clone for an animal RNA virus was reported for poliovirus [43], and was later described for viruses belonging to other groups [44;45]. Attempts to make infectious YFV DNA were again hampered by the genetic instability of the plasmid in *E. coli*. The addition of the cytomegalovirus (CMV) promoter sequence at the 5’ end of the YFV-17D cDNA appeared to exacerbate the instability problem. Spurious transcription from eukaryotic promoters in bacteria were proposed to be the cause, and several strategies
to reduce the expression of toxic products were proposed, both at the transcriptional level or at the translational level [46]. In Chapter 3, we managed to stabilize the YFV-17D infectious DNA by replacing the low-copy number vector pACNR with a bacterial artificial chromosome (BAC), pBeloBAC11, which further reduced the copy number in *E. coli*. The iDNA was characterized in cell culture and was proven to be able to produce infectious progeny viruses.

As proof of concept, the immunogenicity of the YFV-17D iDNA was studied in mice. Unfortunately, mice are not ideal animal models for YFV, because they rapidly resolve the infection after subcutaneous or intravenous administration or develop neurotropic symptoms upon intracerebral inoculation, whereas human infection leads to viscerotropic diseases, instead of encephalitis or other neurological disorders. The mechanism behind the murine resistance to YFV, be it the vaccine strain 17D or the virulent YFV-Asibi strain [47], is not fully understood and was postulated to be related to the murine innate immune response towards YFV infection [47;48]. We did not detect any neutralizing antibodies in the sera of HLA-A2.1 transgenic C57BL/6 mice vaccinated with the infectious YFV-17D DNA, despite readily detectable YFV specific CD8+ T cell response (Chapter 3). It was not clear to what extent the genetic background of C57BL/6 mice could affect B cell activation by YFV antigens, yet the lack of viremia in the sera seemed to suggest quick and efficient clearance of the virus. During the course of this study, Meier *et al.* reported a study with interferon α/β receptor deficient mice (A129 mice), and proposed to use these mice as better small animal models for the study of YFV-Asibi infection and YFV-17D vaccination [47]. Therefore, we tested immunogenicity of the YFV-17D iDNA in both the interferon receptor knockout A129 mice and the congenic 129 mice. We were indeed able to detect humoral immune responses in A129 mice vaccinated with YFV17D iDNA, but not in the immuno-competent 129 mice, confirming the role of the murine innate immune system in the rapid clearance of YFV infection in mice. Due to time and funding constraints, however, our experiments were relatively limited, so larger scale vaccination-challenge experiments in A129 mice as well as in primates are needed to further evaluate the potential of the YFV-17D iDNA as a vaccine candidate, and as a platform for the development of DNA based recombinant YFV vaccines. To spare vaccine dose and to facilitate the application in human beings, more acceptable delivery routes (microneedle patch, for instance [49]) could be explored.

Despite the success of the YFV-17D vaccine [50-53] and the promise of the YFV-17D based chimeric vaccine platform [33;34;54-61], arthropod-borne (arbo-) flaviviruses still pose a great threat to human beings. A better understanding of viral-host interactions will provide valuable guidelines for the development of better vaccines and/or treatments. The host environment is by nature hostile towards any parasites, with no exception to viruses. The biological instinct is to either keep the pathogens at bay or to distinguish the non-self from self and to destroy the ‘invader’ upon their intrusion. During their evolution, mammals
have developed an armamentarium involving a network of pathways. The first line of
defense is the innate immune response, and it comes as no surprise that almost all animal
viruses have evolved strategies to evade or delay the host’s innate immune responses. In
fact, flavivirus-encoded proteins have been shown to inhibit both the interferon induction
pathway and the interferon signaling pathways (reviewed in [62;63]). Interestingly, the
recently identified subgenomic flavivirus RNA (sfRNA) has also been implicated as a viral
innate immune evasion strategy [64].

Whereas subgenomic RNA production is a well known characteristic for certain
+ssRNA families (e.g., coronaviruses and alphaviruses), the flavivirus subgenomic RNA that
is colinear with the distal part of the viral 3’-UTR was not discovered in cells or animals
infected with flavivirus until recently [65-68]. This unique small RNA species is clearly
different from subgenomic (m)RNAs produced by other plus-strand RNA viruses in that it
does not encode for any viral protein. Even more remarkably, sfRNA is not directly produced
by transcription involving the viral RdRP. Instead, sfRNAs were confirmed to be incomplete
5’→3’ degradation products of the viral genomes by the host exoribonuclease XRN-1 [68;69].

Though uncommon to RNA viruses that in general possess a compact genome, virus-derived
noncoding RNAs are, on the other hand, transcribed by many DNA viruses, which have been
found to interact with various cellular pathways and to serve distinct functions. For example,
Marek’s disease virus noncoding RNA was found to be a precursor in the production of
miRNA [70], whereas Herpesvirus saimiri noncoding RNA serves as an antagonist for cellular
miRNA [71]. A 2.7kb CMV-encoded RNA was reported to regulate mitochondria-induced cell
death [72]. In fact, WNV sfRNA was suggested to be further processed to yield an miRNA-like
small RNA that was identified in infected mosquito cells [73], although it cannot be ruled
out that the WNV genome RNA actually serves as the precursor for this small RNA, given
the sequence identity between the sfRNA and viral 3’-UTR. However, it is more likely that
viral genomic RNA, intertwined with viral as well as host proteins and wrapped in translation
and replication complexes, is inaccessible to the miRNA machinery, whereas sfRNA has
been found to localize to the P-bodies, where shRNA that is produced by Drosha in the
nucleus undergoes further processing and is eventually assembled into the RISC complex
[68]. The sfRNA has also been shown to interfere with RNAi in both mammalian and insect
cells [74], and was suggested to be correlated with viral cytopathogenicity and pathogenesis
[68;69]. Nevertheless, contrary to the CMV-encoded 2.7kb RNA that prevents cells from
entering apoptosis, flavivirus subgenomic RNA appeared to enhance cytopathic effect (CPE)
during viral infection, since mutants incapable of producing sfRNA were unable to induce
plaque formation in several mammalian cell lines, yielded relatively small infectious centers,
and were less pathogenic in small animal models compared to sfRNA-producing wildtype
viruses [68;69] (Chapters 4 and 5). It is paradoxical that killing instead of sustaining the host
could benefit the virus, or does the accumulation of sfRNA serve as a temporal switch from
replication to the release of progeny viruses that can potentially be facilitated by the onset of CPE?

In order to complete their replicative cycle and perpetuate, arboviruses also need to survive and thrive in arthropod vectors, where RNAi plays a major role in the host responses that combat infection. Just as plant and insect viruses, arboviruses have also evolved strategies to counteract this host response by expressing RNA interference suppressors (RSS). Strikingly, no flavivirus-encoded RSS proteins have been identified thus far, yet the noncoding sfRNA was proposed to (at least in part) fulfill the role of RNAi suppressor in insect cells [74].

Given the different experimental settings and approaches used for the study of sfRNA function, it is rather difficult to reach a consensus on sfRNA functionality. It is also hard to envision how these apparently different functions in immune modulation, RNAi suppression, miRNA production and inhibition of viral minus strand RNA synthesis and translation [64;73-75] are somehow connected to contribute to virus fitness. On the one hand, the conservation of sfRNA production throughout the Flavivirus genus seems to suggest a crucial function that has been preserved through evolution. Alternatively, sfRNA may interact with distinctive host factors to fulfill disparate roles in viruses that belong to different clusters and exhibit a unique host range. It is beneficial for arbo-flaviviruses to suppress the RNAi pathway in insects so as to reach high titers that will maximize their chances of transmission to a mammalian host. RNAi suppression would also benefit replication of insect-specific flaviviruses in insects. If this is the sole function of sfRNA, however, what would be the rationale of sfRNA production in NKV flaviviruses that do not require an arthropod vector for viral transmission. On the other hand, an immune evasion strategy in mammalian hosts will be beneficial for both the arbo- and NKV- flaviviruses, but does not provide an explanation for sfRNA production by insect flaviviruses. In this sense, there must have been either functional divergence during evolution or the use of a certain conserved pathway, such as RNAi, apoptosis, or RNA degradation pathways, which somehow contributes to viral fitness in disparate hosts.

In order to test the proposed sfRNA functions in an experimental setting independent from viral replication or viral protein expression, yet still pertaining to the intracellular physiological environment, mammalian and insect cell lines expressing sfRNA of flaviviruses belonging to various clusters could be very helpful. We have established mammalian cell lines harboring a gene cassette containing the complete 3’-UTR sequence of YFV-17D or WNV downstream of a reporter gene (eGFP) under the transcriptional control of a CMV promoter. These cell lines constitutively express authentic YFV-17D or WNV sfRNA. Our data showed that not only the YFV-17D sfRNA, but also the WNV sfRNA could complement the lack of cytopathogenic effect of an sfRNA-null YFV mutant (Chapter 6). This finding can be explained if conserved RNA elements within the 3’-UTR of mosquito-borne flaviviruses
interact with either host factors or viral components and promote cytopathogenicity. Alternatively, the inhibition of XRN-1 activity [76] per se might enhance cytopathology upon virus infection. We can test the latter hypothesis by using mammalian cell lines expressing XRN-1 stalling signals from other flaviviruses (e.g., NKV flaviviruses) with the trans-complementation approach. In addition, insect cell lines expressing sfRNA from insect-specific or arbo-flaviviruses can be established to study whether sfRNA by itself antagonizes cellular RNA interference pathways, and whether perturbation of this pathway contributes to the fitness of flaviviruses belonging to other clusters, or unrelated insect or arboviruses in general.

Although no consensus has been reached regarding sfRNA functionality, the production of sfRNA has been unambiguously confirmed to be driven by a host enzyme, XRN-1 [68;69;77;78]. XRN-1 is a well conserved and highly processive cellular exoribonuclease that functions as the key enzyme in the 5'→3' mRNA degradation pathway in eukaryotic cells. So far, only a few non-viral RNA structures like oligo(G) stretches and large thermodynamically favored stem-loop structures have been reported to be able to stall XRN-1, although the stalling efficiency of the stem-loop structures was much lower compared to that achieved by oligo(G) tracts or flavivirus stalling signals [79]. Some other structures of viral origin have also been reported to defy 5'→3' degradation, such as the long stem structure with four Gs buried at the bottom of the 20S RNA of Narnavirus [80], and a 58-nucleotide sequence containing stem-loop structures responsible for the accumulation of small viral non-coding RNA in plants and protoplasts infected with red clover necrotic mosaic virus (RCNMV) [81]. For the production of sfRNA, XRN-1 was proposed to be stalled upstream of a complex RNA structure involving a stem-loop structure that is stabilized by a pseudoknot interaction [69;77;78;82].

With their relatively small genome size and limited coding capacity, it is not uncommon for RNA viruses to usurp specific RNA motifs or versatile RNA structures to fulfill important roles in their replication. Pseudoknots, RNA tertiary structures involving at least two interacting stem-loop structures, are especially prevalent in RNA viruses [83] and have been identified as essential regulators in transcription and replication, inducers of ribosomal frameshifting, or structures that help to maintain RNA conformation for enzymatic function, as in ribozymes or telomerases (reviewed in [83]). A frameshift-inducing pseudoknot was recently identified in the NS1 coding sequence of flaviviruses belonging to the JEV serogroup. This pseudoknot is required for a frameshift that results in the production of a larger version of the viral NS1 protein [84]. Additional RNA pseudoknots were predicted to be formed in the 3'-UTR of the mosquito-borne flaviviruses, namely PSK1, PSK2 and PSK3 [85]. PSK1 and PSK2 were shown to be important for viral replication and/or translation [86] (Bredenbeek et al., unpublished data), whereas the more upstream pseudoknot (PSK3) was found to be nonessential for virus replication [69]. This XRN-1 stalling RNA pseudoknot structure exhibits
special features that distinguish it from classical H-type RNA pseudoknots. It does bear some structural resemblance to the RNase L antagonizing enterovirus RNA, which competitively inhibits the antiviral endoribonuclease domain of RNase L, thus protecting the viral genome from endoribonuclease cleavage [87]. However, in vitro studies did not provide convincing evidence for inhibition of RNase L activity by sfRNA [64]. Interestingly, the non-H-type RNA pseudoknot structure in sfRNA was found to inhibit the enzymatic activity of another cellular ribonuclease, i.e., XRN-1, such that cellular mRNA turnover is perturbed, resulting in a different gene expression profile in cells that are infected with wildtype WNV producing sfRNA versus cells infected with a mutant WNV that is unable to stall XRN-1 or uninfected cells [76]. The accumulation of uncapped mRNA could potentially affect other cellular pathways, such as apoptosis and the stress response, possibly explaining the observation that sfRNA-deficient WNV and YFV-17D mutants are less cytopathogenic in certain cells of mammalian origin.

Irrespective of their interaction partner, the function that RNA pseudoknot structures fulfill largely depends on the RNA conformation as well as its thermostability. It can be envisioned, therefore, that the change of a single nucleotide within the XRN-1 stalling structure may incapacitate XRN-1 stalling (Chapter 5). Despite our extensive mutagenesis studies of the XRN-1 stalling site of YFV-17D and MODV (Chapter 4 and Chapter 5), representing the mosquito-borne and NKV flaviviruses, a solid conclusion regarding the sequence/structure requirements for XRN-1 stalling is far from being reached. It is hard to envision how the loops and stems are oriented in space, or how XRN-1 binding might change the 3-D conformation, and we are not yet clear whether there is any specific nucleotide that is critical in the RNA-protein interaction. A better understanding of this structure probably requires additional data from in vivo biochemical probing analysis [88] and mutagenesis studies and would ideally involve structural biology (e.g., X-ray crystallography or N.M.R.).

Thanks to the versatile tools and novel techniques developed in molecular biology in the last two decades of the previous century, we have been able to construct infectious clones of flaviviruses, with which extensive knowledge has been gained on virus biology and which have been used to develop vaccines against pathogenic flaviviruses such as JEV [39;40]. Nonetheless, question marks still remain regarding the molecular determinants of host restriction, sfRNA function and the sequence/structure requirements for sfRNA production, etc. Studies aiming to address these fundamental questions will help fill our knowledge gap in the understanding of virus-host and/or virus-vector interaction, which will in turn point out directions for the design of better vaccines and antivirals to combat the (re) emerging flaviviruses and to limit the impact of these pathogens on society.
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