UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
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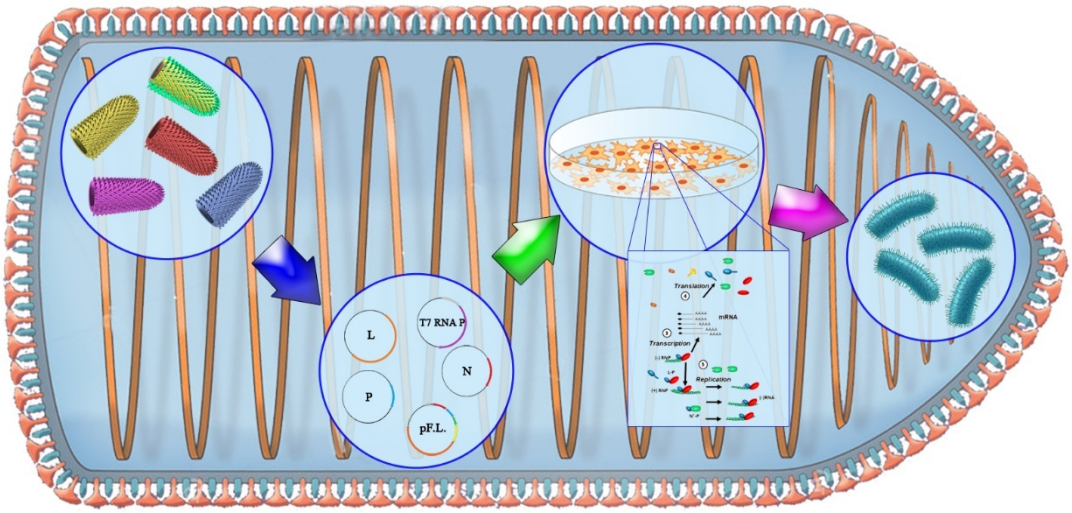
XXXII CYCLE

Giuseppina Miselli

THE JURONA RHABDOVIRUS AS A NEW VIRAL VECTOR PLATFORM



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THE JURONA RHABDOVIRUS AS A NEW VIRAL VECTOR PLATFORM

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LIST OF ABBREVIATIONS

Ads Adenoviruses
APCs Antigens processing cells
ATCC American type culture collection
CD Cluster of differentiation
CEF Chicken embryo fibroblasts
CMV Cytomegalovirus promoters
CPE Cytopathic effect
CXCL11 C-X-C Motif Chemokine Ligand 11
DC Dendritic Cells
DMEM Dulbecco's Modified Essential Medium
eGFP Enhanced green fluorescent protein
EGFR Epidermal growth factor receptor
EMA European Medicine Agency
FBS Fetal bovine serum
FDA Food and Drug Administration
GM-CSF Granulocyte colony stimulating factor
hDCT Dopachrome tautomerase
HDV Biosafety level 2
HDV Hepatitis delta virus
HIV Human Immunodeficiency Virus
HPV Human papillomavirus.
HSV Herpes simplex virus
ICs Immune checkpoint inhibitor
IRES Encephalomyocarditis ribosome entry site
MAGE-A3 Melanoma-associated antigen A3.
MOI Multiplicity of infection
MVA Modified vaccinia Ankara
NHp Non-human primates
NK Natural killer
OV Oncolytic virus
OVs Oncolytic viruses
PAMP Pathogen-associated molecular patterns
PD-L1 Programmed death receptor ligan-1
PSMA Prostate-specific membrane antigen
RVs Rhabdoviruses
SIV Simian immunodeficiency virus

T7 RNAP T7 RNA polymerase
TAA Tumor associated antigens
TK Thymidine kinase
TLR Toll-like receptor
VSV Vesicular Stomatitis virus
VV-T7 Vaccinia virus

ABSTRACT

Recombinant viral vectors represent a powerful emerging technology for the development of novel classes of biologics to prevent or treat human diseases. Replication competent viral vectors with natural or engineered tumor selectivity (Oncolytic Viruses, OV) have reached different levels of clinical development with one product (Talimogene Laherparepvec) being approved for the treatment of Melanoma. Viral vectors have also been shown to mediate efficient *in vivo* transduction of therapeutic genes, thus paving the way to the development of a number of gene therapy products or genetic vaccines aimed at eliciting specific immune responses against the encoded proteins.

In this PhD project, we aimed at generating a new recombinant viral vector platform from a virus of the Rhabdoviridae family that could be used as oncolytic virus, or as a vectored vaccine as well as being amenable to large scale manufacturing using an industrial process.

For this purpose, we screened five Rhabdoviruses from the American Type Culture Collection (ATCC) for their growing properties on Vero cells. Our screening identified the Jurona virus as the best candidate for vector construction. We established a reverse genetic system based on the T7 RNA polymerase (T7 RNAP) expression helper virus-free method to recover Jurona recombinant viral particles from a cDNA clone of the viral genome. We designed and cloned five plasmids by using various cloning methods: the full Jurona anti-genome, the T7 RNAP, and three helper viral proteins (Nucleocapsid (N), Phosphoprotein (P), and Large polymerase (L)). Following initial failure to rescue the Jurona vector, we used an end-joining-RT PCR sequencing strategy to identify a previously unknown leader sequence in the Jurona genome. By introducing this new sequence in our Jurona full-length constructs, we successfully rescued the Jurona vectors using the reverse genetic system. We showed that Jurona is capable of efficient expression of a heterologous gene upon infection of target cells and of potent oncolytic activity *in vitro*. Finally, we demonstrated that the Jurona vector could be efficiently produced in Vero cells, a validated cell line for vaccine production, and that it could be purified without losing infectivity.

Thus, the Jurona vector platform may represent a novel valuable option for oncolytic virus and genetic vaccine development, to be used alone or in combination with other compounds for the development of highly effective immunotherapeutic treatments.

1. INTRODUCTION

1.1 Recombinant viral vectors

Recombinant viral vectors represent a flexible technology for the development of novel therapeutics to prevent or cure diseases. This technology either exploits live replicating (but often attenuated) viruses to be used for oncolytic virotherapy (Breitbach 2016), or non- replicating viruses as vectors for delivering genetic material within the infected cells, thereby inducing or potentiating immune responses against cancer or infectious diseases (i.e.: genetic vaccines; Sivanandam 2019).

Recombinant viral vectors can express high levels of the encoded antigens *in vivo*, thereby eliciting potent and broad cytotoxic T cell immune responses that traditional vaccine strategies are not able to achieve. Indeed, unlike other vaccination methods, they have the advantage of being capable of stimulating the innate immune response without the use of an adjuvant, leading to the production of interferons and inflammatory cytokines, along with diverse pathogen-associated molecular patterns (PAMP). Furthermore, their capacity to infect cells and shed the encoded antigen into the extracellular milieu or to direct it to the intracellular processing pathway ensures a highly efficient induction of both humoral and cytotoxic (CD8+) T cell responses.

One of the main limitations of this technology is that the transgene-specific response can be affected by pre-existing or de-novo adaptive immunity against the vector itself that can limit the possibility to use the same viral vector for a second efficient immunization (i.e.: boosting). Strategies to overcome this limitation include the use of vectors based on viruses that are rare in the population, and of heterologous prime-boost vaccination regimens (Katie 2016). Indeed, this immunization protocol has proven to be more effective in stimulating a specific immune response against the encoded antigen than that based on the use of a single viral vector (Hill 2010).

Recently recombinant viral vectors have been proposed for oncolytic virotherapy. This emerging anti-cancer approach uses viruses that preferentially infect, replicate in, and kill cancer cells. They are aimed at killing tumor cells through direct cytopathic effect and at enhancing the immune system through the release of tumor antigens, thereby acting as *in situ* anti-tumor vaccines. Many viruses have shown to naturally infect and propagate in tumor cells, acting as natural oncolytic vectors. Furthermore, tumor antigens proved to be more immunogenic when delivered as transgenes in a viral vector, as they can be expressed in the contest of an active infection, thus remodeling the tumor microenvironment.

Several technological platforms based on recombinant viral vectors were derived from human and animal viruses and have been evaluated for their efficacy for oncolytic virotherapy and genetic vaccination in preclinical and clinical studies. Among the best characterized viral vectors are Adenoviruses, Poxviruses (e.g., Modified vaccinia Ankara, MVA) Herpetic viruses (HSV), and Rhabdoviruses (VSV, Maraba). The oncolytic activity as well as the potency and quality of the induced immune response critically depends on the route of administration and regimen used, but, most importantly on the type of viral vector used. In fact, each viral vector platform has its own advantages and disadvantages that rely on the properties of the virus from which the vector derives.

1.2 Recombinant viral vectors for oncolytic virotherapy

Oncolytic virotherapy emerged as a novel anti-cancer therapeutic approach after the approval of the Herpes Simplex Virus (HSV) T-VEC (Talimogene Laherparepvec) by the US Food and Drug Administration (FDA) and European Medicine Agency (EMA) for the treatment of advanced melanoma (Pol 2016).

Oncolytic viruses (OVs) are replication-competent viruses that naturally target, replicate in, and ultimately destroy cancer cells. These viruses have a multi-mechanistic mode of action depending upon the virus platform considered and the clinical indication. Indeed, OV activity reflects the biology of the virus from which it was originated and its host-virus interaction. They are classified into two main classes. The first class includes viruses that are non-pathogenic in humans and that replicate only in cancer cells either because they are blocked in normal cells by innate immunity or because they have been engineered to selectively replicate in cancer cells (replication conditional oncolytic viruses). The second class includes those viruses that have engineered tropism for cancer cells. Thus, the tumor selectivity of each OV relies on the specific natural or engineered replication or infection mechanism (Lin 2018). The viral replication in the tumor can be controlled by using gene promoters that are activated only in tumor cells (Howells 2017) or by mutating viral genes required for virus survival in normal cells but not in malignant cells (Fukuda 2009). Selective entry in tumor cells can be obtained by retargeting the virus to cancer receptors (Campadelli-Fiume 2016) that are overexpressed in a wide range of tumors (e.g., EGFR,) (Coughlan 2009) or limited to particular cancers (e.g., HER2/breast cancer, PSMA/prostate cancer) (Menotti 2009; Liu 2009).

Specific tumor-killing can be achieved in different ways. OVs can target tumor cells directly by promoting several forms of immunogenic cell death (ICD), including necrosis, necroptosis, pyroptosis, autophagic cell death and immunogenic apoptosis as

a consequence of lytic viral replication (e.g., Adenovirus, HSV). Indeed, some viral proteins can activate apoptosis or necrosis, such as the adenoviral proteins E3 (Heise 2000). Indirectly, they can replicate and express proteins that are toxic to cell survival, increasing oncolysis. The cell lysis releases danger-associated molecular patterns (DAMPs) and various pathogen-associated molecular patterns (PAMPs) that are recognized by pattern recognition receptors on innate immune cells such as DCs and NK cells and function as 'danger' and 'eat me' signals. This signaling attracts more DCs to the tumor microenvironment, which in turn leads to increased recruitment and maturation of tumor-specific T cells.

Moreover, OVs can act as in situ vaccine as the virus-mediated oncolysis of tumor cells releases tumor-associated antigens and neoantigens (TAAs and TANs), which can be captured and processed by tumor-infiltrating antigen-presenting cells (in particular, DCs), ultimately leading to a tumor-specific T cell response against a broad spectrum of the released antigens. The consequent production of cytokines and danger signals determines changes inside the tumor bed that repolarize the tumor microenvironment making it more responsive towards a less immunosuppressive phenotype.

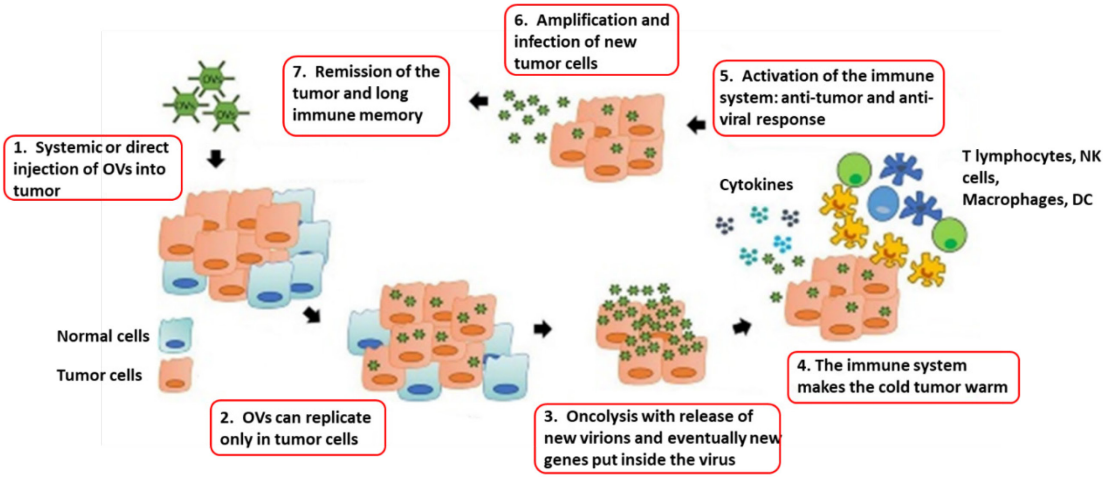


Figure 1. Anti-tumor activity by oncolytic virus (OV) therapy: OVs selectively target transformed cells. Upon replication and tumor cell killing, they stimulate the immune system which is recruited into the tumor, skewing the neoplastic mass from an immuno-suppressive environment to an inflammatory site. Macrophages and T lymphocytes are key players in this process, producing cytokines that can recruit other immune cells and actively destroy cancer cells. This action generates an immunological memory that avoids cancer recurrence and synergizes with the oncolytic action of the viruses, potentially leading to tumor remission.

1.3 Oncolytic viruses as vectors expressing immunomodulators and as genetic vaccines

Talimogene laherparepvec (T-VEC) is the first oncolytic vector approved for use in metastatic melanoma and contains key deletions, to maximize tumor-specific replication while minimizing neurovirulence. In addition, it encodes the transgene for GM-CSF (Andtbacka 2015). In the T-VEC clinical trial, Andtbacka (2015) demonstrated that local injection of T-VEC in metastatic melanoma lesions could stimulate sufficient antitumor immune responses locally, providing systemic, long-lasting, cancer-killing immune responses in advanced cancer patients. However, OVs that can be delivered by intravenous administration are preferred as metastatic cancer remains, for the most part, incurable, and thus, a therapeutic agent that can reach and attack all sites of cancer growth would be highly desirable. According to Breibach (2011), an oncolytic vaccinia virus could be delivered by intravenous infusion and was able to spread within the tumor bed. Also, Russell (2011) has demonstrated in phase 1 clinical study that an oncolytic measles virus could be delivered to multiple metastatic lesions of melanoma patients leading to a complete and durable response in one patient after intravenous injection. Although several trials have demonstrated the ability of OV to reduce tumor burden, their killing activity alone is not enough to produce a high therapeutic effect. Therefore, to further improve the immune-stimulatory properties of the OVs, many researchers have introduced cytokines, chemokines, and other immunomodulators as transgenes into OVs. In this way, the antigens processing cells ((APCs) (Dendritic Cells (DC)), macrophages, neutrophils), and lymphocytes (NK, T, and B cells) could be recruited and activated locally, enhancing the immune system response against the tumor. One of the most used immunomodulators is the Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) which is encoded in T-VEC. Also Heo (2013) engineered an oncolytic VV called JX-594 by deleting the viral thymidine kinase gene (TK) and introducing human GM-CSF (Senzer 2009). Oncolytic viruses have also been modified to encode tumor-associated antigens, thus introducing the concept of the oncolytic vaccine (Diaz 2007). Oncolytic viruses may represent a next-generation of genetic vaccines that engage a more robust immune response towards tumors. Indeed, replicating in cancer cells, they activate a cryptic population of T cells in the tumor microenvironment to break down immuno-tolerance. Also, by using oncolytic viruses encoding Tumor-Associated Antigens (TAA), it has been possible to directly destroy cancer cells and induce a specific immune response against the expressed TAA transgene in the tumor bed. Vaccinia virus and VSV, engineered to express model TAAs, have been used in an attempt to reduce tumor burden effectively (Zhang 2010). Combining the benefits of viral oncolysis with that of genetic vaccination has led to substantially enhanced therapeutic benefit in animal

models (Pol 2014). Also, oncolytic viruses and immune checkpoint inhibitors could be employed in combination to create a more responsive tumor microenvironment and to intensify immune responses against tumors (Ribas 2017).

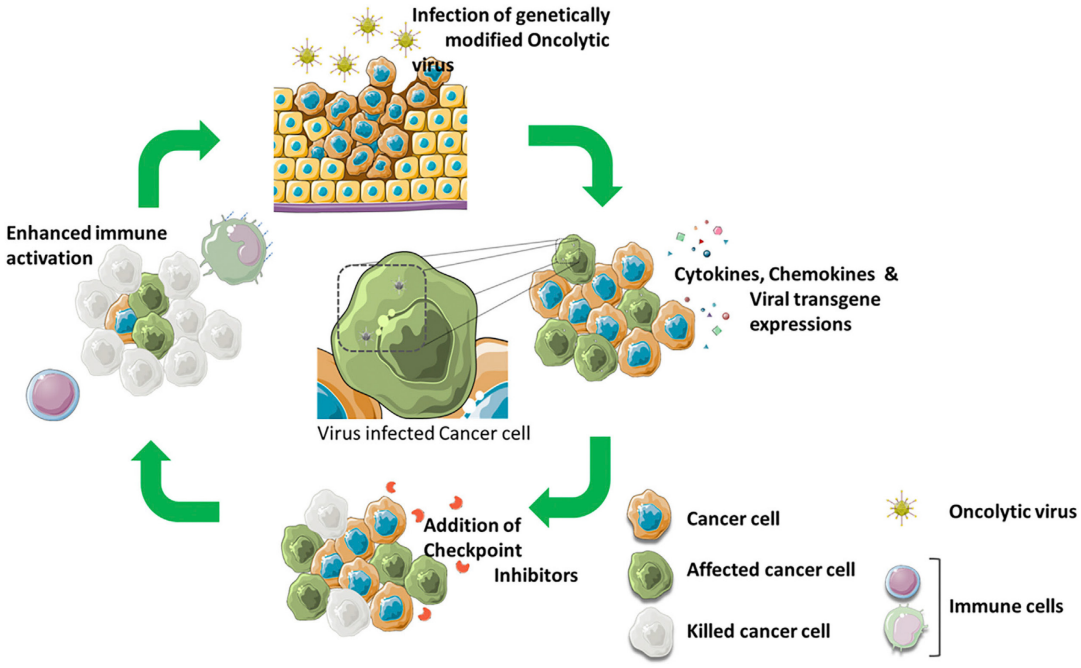


Figure 2. Oncolytic Viruses Stimulate the Tumor Microenvironment and Synergize with Immune Checkpoint Inhibitors (ICIs). Schematic representation of different stages of tumor suppression using combination therapy involving oncolytic viruses (OVs) and immune checkpoint inhibitors. Initially, tumor cells are infected with OVs resulting in the release of cytokines, chemokines, and viral transgene which triggers immune responses. A further treatment of those infected cells with immune checkpoint inhibitors mounts additional immune responses, resulting in the killing of most cancer cells (Sivanandam 2019).

1.4 Recombinant viral vectors as genetic vaccines: key considerations

New advances in immunology and basic virology have highlighted the molecular and cellular mechanisms by which vaccines should stimulate both arms of the immune response. Indeed, Pulendran (2011) reported that to protect against complex pathogens that escape the adaptive immunity, such as HIV, Hepatitis C, malaria, tuberculosis (TB), it might be necessary to elicit along with humoral response also CD8+ T cells of sufficient magnitude and effector function to recognize and rapidly clear infected cells before the pathogen establishes a chronic infection. Other pathogens that cause important outbreaks such as Pandemic influenza, Ebola, SARS, MERS and, more recently, Zika and CoV2019 virus, represent a global threat and have highlighted the need for more rapid vaccine discovery and development strategies. Conventional approaches have failed in the past to provide for and effective and rapid vaccine platform to combat these diseases, while recombinant viral vectors may represent an effective approach for vaccine development against chronic infectious diseases as well as against variable pathogens and those that cause rapid outbreaks.

Viral vectors present several features that make them a promising platform technology for the development of novel vaccines. Among them are: i) their ability to induce robust CD8 responses along with humoral immunity; ii) their ease of rapid generation in response to emerging infections; iii) their manufacturability on a large scale, with their production process being the same for all vaccines; iv) their safety.

1.5 Recombinant viral vector platforms

Among the viral vector platforms, those based on Adenoviruses, Poxviruses, Herpesviruses, and Rhabdoviruses are the most studied and employed as oncolytic viruses and genetic vaccines for prophylactic or therapeutic use. Here we will highlight the properties of each viral vector platform and describe their applications and the clinical experience for each of them.

1.5.1 Adenoviruses

Replication defective Adenoviruses (Ads) have been first developed as vectors for gene therapy applications. Subsequently, Ads have become one of the most exploited vector system for genetic vaccine development. The significant advantages of using Ads as a vaccine platform include their ability to infect a broad range of hosts and to

express high levels of transgene without integrating the viral genes into the host genome. Importantly, thanks to their ability to grow to high titers in cell culture, Ads can be effectively manufactured to large scale (Choi 2013). Adenoviral vectors can stimulate innate immune responses via Toll-like receptor-dependent and Toll-like receptor-independent pathways. Moreover, by infecting dendritic cells (DCs), Ads can induce significant up-regulation of costimulatory molecules accompanied by increased cytokine and chemokine production that contribute to more effective antigen presentation to the immune cells (Banchereau 1998). Such intrinsic ability of adenoviral vectors to stimulate the innate immune response and to enhance the process of antigen presentation can provide the adjuvant-like effect, thereby promoting the development of potent humoral and cell-mediated immune responses against the encoded antigen which would otherwise be less immunogenic. Furthermore, as Ads also possess tropism for epithelial cells, adenoviral vectors can be administered to target both mucosal and systemic immunity. To that end, various recombinant adenoviral vectors have been engineered and tested for vaccine applications for a wide range of diseases, including cancer, human immunodeficiency virus (HIV), HCV, RSV and malaria (Gabitzsch 2009; Shott 2008; Barnes 2012; Pierantoni 2015).

Adenoviral vectors can induce potent antibody as well as T cell responses with different immune response rates depending on the serotype employed. Replication-deficient Ad5, is one of the most widely used adenoviral vectors, and it can elicit robust cellular and antibody responses. However, the pre-existing immunity to this virus in the human population can seriously inhibit specific immune response against the encoded transgene, thus hampering its clinical use. A first alternative approach to overcome this issue has been to select rare serotypes that have low prevalence in humans, such as Ad26 or Ad35, but they resulted in lower levels of cellular immunity overall. A more successful strategy has been the development of adenoviral vectors of non-human origin, such as the chimpanzee virus-derived vector ChAd63 that is rarely neutralized by antibodies present in humans (Colloca 2012; O'Hara 2012). Furthermore, chimpanzee virus-derived vectors (ChAd63, ChAd3, PanAd3, ChAdOx1) have shown extremely potent immune response when used in heterologous prime/boost regimens with Modified Vaccinia Ankara (MVA) in human clinical trials for HIV-1, hepatitis C virus (HCV), influenza and respiratory syncytial virus (RSV) immunogens (Green 2015; Ewer 2016; Swadling 2014; Hartnell 2019). Protection was correlated with high frequencies of antigen-specific CD8+ T cells (Ewer 2016). Moreover, Adenoviruses derived from non-human Great Apes (GAd) encoding multiple neo-antigens have been tested alone or in combination with anti-PD-1, as prophylactic or therapeutic cancer vaccines. This approach has demonstrated to elicit strong and effective T cell-mediated immunity and to control tumor growth in mice (D'Alise 2019).

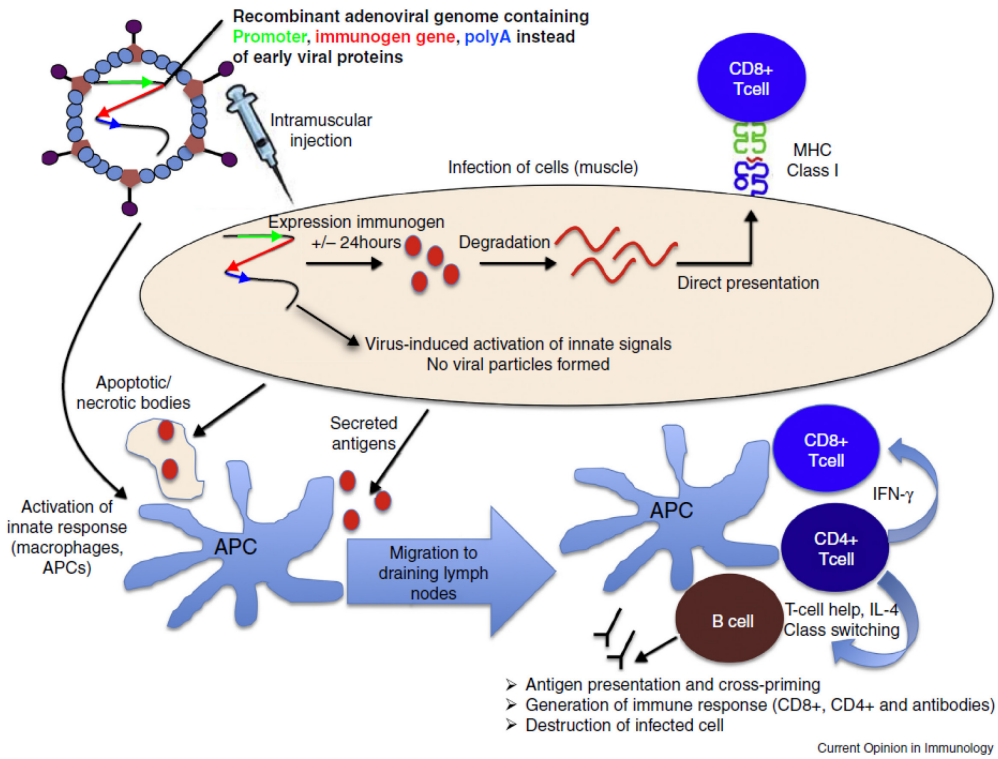


Figure 3. Mechanism of induction of transgene-specific cellular and antibody responses by replication-defective viral vector vaccines. Administration of a recombinant adenovirus vaccine by intramuscular injection results in infection of muscle cells and other cells in draining lymph nodes (non-productive in the case of replication-defective viral vectors) followed by expression of the transgene within 24 hours, together with triggering of innate immune responses via interactions between viral nucleic acids and pathogen recognition receptors. Expressed proteins undergo proteasomal degradation and presentation to CD8+ T cells in association with MHC class I molecules or may be secreted and taken up by professional antigen presenting cells (APC). APC may also acquire vaccine antigens as apoptotic or necrotic bodies or may be directly activated by interaction with the viral vector. Antigen-loaded APC also migrate to draining lymph nodes where they are able to prime CD8+, CD4+ T cells and B cells (Ewer 2016).

Ads have also been used as a potential anti-cancer agent. Oncolytic Ads have been engineered to express tumor-specific antigens for prophylactic or therapeutic antitumor treatments (Choi 2013). Moreover, such recombinant oncolytic Ads have been specifically designed to target and replicate in cancer cells but not in normal cells. For example, the ONYX-015 has been the first replication-selective oncolytic virus tested in humans for cancer therapy (Kirn 2001). ONYX-015 has been modified to lack the gene that encodes the p53-inactivating protein, E1B. This deletion has enabled this viral vector to selectively replicate in tumor cells (Nemunaitis 2000). Numerous clinical trials tested ONYX-015 for the treatment of various forms of cancers such as prostate, ovarian, colorectal, head, and neck, hepatocellular, and pancreatic carcinomas (Alemany 2007). These trials have demonstrated that although ONYX-015 intratumoral administration seemed to be well tolerated in recipients during phase I and II clinical trials, the overall efficacy achieved has been relatively low (Hermiston 2002). Hence, in order to overcome this critical problem, Shashkova (2007) has evaluated oncolytic Ads expressing cytokine and costimulatory molecule-expressing to improve antitumor efficacy. An example of this type of vector is ONCOS-102, an adenovirus serotype 5 expressing GM-CSF and carrying a 24 bp deletion in the E1A region and a chimeric fiber knob region. This vector is currently in clinical testing in combination with immune checkpoint inhibitors.

1.5.2 Poxviruses

One of the most studied viral vector systems stems from the poxviridae family and has been extensively employed as an oncolytic and vaccine vector for infectious diseases and cancer. In particular, the poxvirus vaccinia virus (VV) was used for a worldwide vaccination campaign against smallpox, leading to the eradication of the disease in 1978.

Poxviruses are large, enveloped double-stranded DNA viruses. They have a series of advantages, including: i) large cloning capacity for inserts of foreign DNA, ii) broad tropism of infection in mammalian cells; iii) cytoplasmic lifecycle; iv) possibility to administer it by different routes; v) good levels of transgene expression; vi) ability to induce both humoral and cellular immune responses.

Among the reasons to use vaccinia as an oncolytic virus for cancer immunotherapy are i) its stability allowing intravenous delivery; ii) its enhanced replication ability in cancer cells; iii) its extensive safety history as a live vaccine (Thorne 2014). Heo (2013) engineered an oncolytic VV called JX-594 by deleting the viral thymidine kinase gene (TK) and expressing transgenes encoding human granulocyte-macrophage colony-stimulating factor (GM-CSF) and beta-galactosidase. To date, Pexa-Vec (JX-594) is the most clinically advanced oncolytic VV. This oncolytic virus has shown to mediate

tumor cell death via the induction of innate and adaptive immune responses. In different phase I clinical trials following intra-tumoral and systemic delivery, this OV agent has induced complete responses of bulky tumors and systemic efficacy at the highest dosage in patients suffering from HBV associated hepatocellular carcinoma.

Another oncolytic vaccinia virus that has been evaluated in a clinical trial is (JX-929) named vvDD. Downs-Canner (2016) has genetically engineered a tumor-selective, oncolysis-potent Western Reserve (WR) strain VV through the deletion of viral genes encoding vaccinia growth factor (VGF) and thymidine kinase (TK). This vector has been evaluated in a phase 1 study after intravenous injection on 11 patients with standard treatment-refractory advanced colorectal or other solid cancers, demonstrating an excellent safety profile and clinical regression of some metastatic lesions. Further studies to assess the efficacy of this vector, in combination with other immunomodulators, are still ongoing. Also, another Western Reserve oncolytic vaccinia virus has been engineered by Kleinpeter (2016), by introducing three different forms of a murine PD-1 binder into the virus. In multiple in vitro and in vivo models, it has demonstrated that the vector could induce a long-lasting and high level of anti-PD-1 antibody expression. Moreover, Lui (2017) has combined the use of an oncolytic vaccinia virus expressing C-X-C Motif Chemokine Ligand 11 (CXCL11) with an anti-PD-l1 antibody in the colon and ovarian, peritoneal carcinomatosis model, thus demonstrating an improved survival compared to the monotherapy.

As a vaccine vector, a modified vaccinia virus Ankara (MVA) has been developed to improve safety for the development of prophylactic genetic vaccine vectors to be used in healthy individuals. MVA has been produced by over 500 serial passages in chicken embryo fibroblasts (CEF), resulting in approximately 15% loss of its original genome as an examination of the MVA genome revealed six major deletions totaling approximately 31 Kb. MVA can infect mammalian cells and express transgenes. On the contrary, it cannot produce infectious viral particles in humans as the replication cycle is blocked at a late stage of morphogenesis in mammalian cells. Moreover, marker transfer experiments have indicated that multiple gene defects need to be corrected for the efficient replication of MVA in mammalian cells.

MVA has been extensively evaluated in infants, young children and adolescents as a candidate vaccine in prime/boost regimens for P. falciparum malaria, tuberculosis, and HIV-1, with no associated safety concerns (Bejon 2007).

Ogwang (2015) has shown that prime/boost immunizations with ChAd63-vectored and MVA vectored Plasmodium falciparum malaria vaccines targeting the pre-erythrocytic stage conferred significant protection against both controlled human malaria infection, in naıve volunteers and natural infection in malaria-exposed adults. Protection has been associated with high frequencies of antigen-specific CD8+ T cells. These results demonstrated that MVA is an excellent vector for boosting specific primary immune responses.

In conclusion, Modified Vaccinia virus Ankara (MVA) is licensed as a third-generation vaccinia type vaccine against smallpox, and it serves as a potent vector system for the development of new candidate vaccines against a range of infectious diseases, including those caused by emerging pathogens.

1.5.3 HSV

Herpes simplex virus 1 (HSV) is a member of the Herpesviridae family. It has a DNA-containing, icosahedral capsid, surrounded by a layer termed the tegument, which in turn is surrounded by a protein-containing lipid bilayer, the envelope. Its genome is a double-stranded, linear-DNA genome of approximately 160 kb. Replication competent HSV vectors can accommodate large foreign gene inserts, as they usually have one or more nonessential genes deleted, in some cases combined with the insertion of a therapeutic transgene (Coffin 2015). Deleted or mutated genes in HSV oncolytic viruses include ICP34.5 (the neuro-virulence factor), thymidine kinase (necessary for sensitivity to acyclovir and for efficient replication in vivo), ICP6 (the large subunit of ribonucleotide reductase), and ICP47 (which blocks antigen presentation by the transporter associated with antigen processing).

Replication competent HSV vectors are mostly used as oncolytic viruses (Grinde 2013) also delivering potentiating factors or immunostimulatory proteins such as the suicide gene thymidine kinase (TK) gene or other cancer therapeutic genes including p53, interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Kim 2000; Toda 2000). Talimogene laherparepvec (T-VEC) was the first oncolytic vector approved for cancer therapy and has key deletions, to maximize tumor-specific replication while minimizing neurovirulence. In addition, it encodes the transgene for GM-CSF (Andtbacka 2015). This vector has demonstrated a durable overall response rate. Recently, its therapeutic efficacy has been improved by using it in combination with an immune checkpoint inhibitor (ICs) (Ribas 2017).

As genetic vaccine vector, HSV presents some advantages such as the ability to induce mucosal immune responses and to induce pro-inflammatory cytokines and type I interferons through Toll like receptor 8 (TLR8) and TLR9 (Watanabe 2007). Both replication-competent and incompetent vectors have been evaluated in a simian immunodeficiency virus (SIV) rhesus macaque model for protective efficacy. Following immunization, weak but persistent anti-SIV envelope antibodies were elicited, along with relatively weak and sporadic cellular immune responses. Nevertheless, following the intrarectal challenge with pathogenic SIVmac239, two of seven immunized macaques were strongly protected, and a third showed diminished chronic viremia, providing a basis for the continued development of the herpesvirus vector system (Guroff 2007).

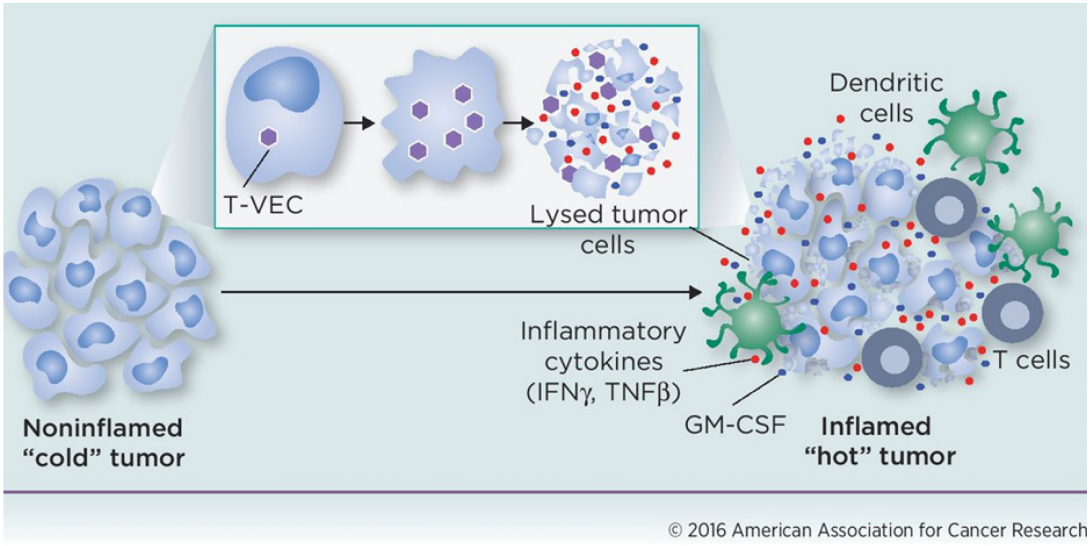


Figure 4. In situ vaccination effect of T-VEC, potentially converting a non–T-cell-inflamed tumor to a T-cell–inflamed tumor. Replication of T-VEC in tumor cells leads to their lysis with release of tumor antigens, viral antigens and pathogen-associated molecular patterns (viral DNA, RNA, and proteins), and GM-CSF. This process results in the recruitment and maturation of antigen-presenting cells, including dendritic cells, which present tumor antigens to cytotoxic CD8 T cells (Ott 2016).

1.6 Rhabdoviruses as a compelling viral vector platform

Rhabdoviridae is one of the virus families that has shown important features for being considered as a genetic vaccine and oncolytic virus.

Rhabdoviruses (RVs) are a large family of membrane-enveloped, negative sense, non-segmented, single-stranded RNA viruses. They are grouped in six genera, among the most important are lyssavirus (such as Rabies virus) and Vesiculo-virus (like Vesicular Stomatitis virus VSV). The negative single-stranded (ss) RNA genome is about 11–16 kb in length, and the virions range in size between 100 to 430 × 45 to 100 nm. The essential genome organization shared by all Rhabdoviruses includes five canonical genes encoding (from 3'to 5') the nucleoprotein (or nucleocapsid protein, N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and large protein (L, RNA-dependent RNA polymerase). The viral genome is flanked by regulatory 3'leader and 5'trailer sequences that have terminal complementarity and contain promoter sequences to initiate replication. Each viral gene junction has conserved transcription stop and start signals separated by short un-transcribed intergenic sequences. The infectious ribonucleoprotein (RNP) complex, which is active in transcription and replication,

consists of the genomic RNA that is always associated with N protein, together with P and L proteins. M protein enables the RNP complex to assemble virions at the host plasma membrane, and the transmembrane spike protein G ensures efficient virion budding, and host cell entry. The replication pathway occurs in the cytoplasm of infected cells and follows (i) cell entry, facilitated by clathrin-mediated or receptor-binding endocytosis; (ii) uncoating; (iii) transcription and translation of the viral proteins; (iv) genome replication and encapsidation; and (v) assembly and release (budding) (Dietzgen 2017).

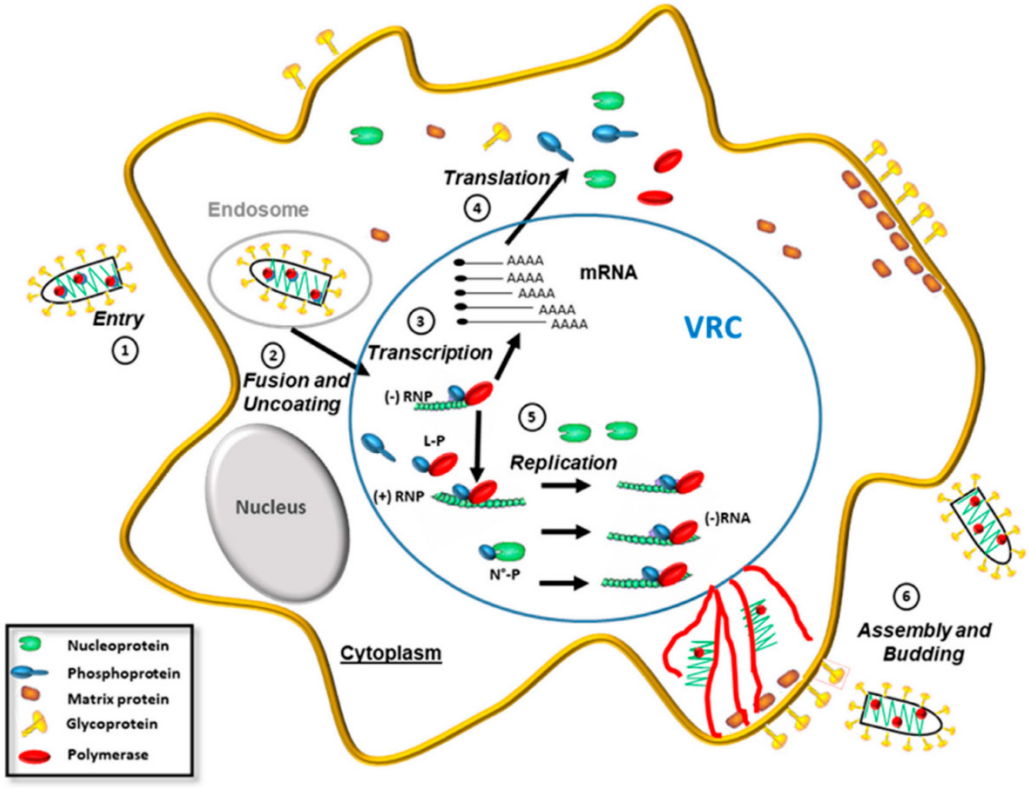


Figure 5. Rhabdoviruses life cycle. Several steps can be observed in the cytoplasm: 1. Entry phase involving the binding of viral particles to receptors. 2. Endocytosis followed by membrane fusion and RNPs release in the cytosol. 3. Transcription of viral mRNAs. 4. Translation of viral mRNAs by the cell machinery. 5. Replication of the viral genome and 6. transport of viral RNP by microtubules to membrane virus assembly and progeny virus budding. Viral transcription and replication occur in viral replication compartments (VRC), which are phase-separated liquid compartments. (Viruses 2018, 10(12), 686;)

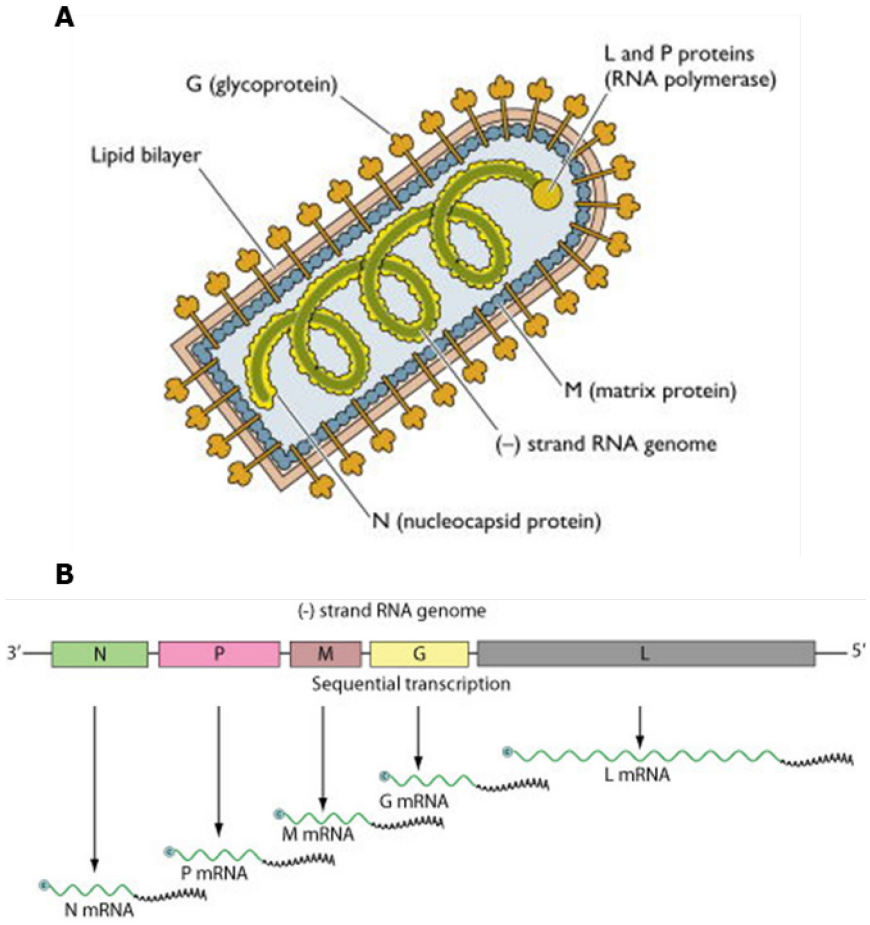


Figure 6. A - Rhabdovirus viral particle description. The nucleoprotein (N) coats the RNA a forming a nucleocapsid with helical symmetry. Associated with this ribonucleoprotein are the minor virion proteins P (phosphoprotein) and L (large). The helical ribonucleoprotein is coated with a helical layer of M (matrix) protein, forming a layer between the nucleocapsid and the envelope. Trimers of G (glycoprotein) form spikes that protrude from the envelope. B - Rhabdovirus genome organization and gene expression strategy. The N, P, M, G and L mRNAs are then sequentially produced, capped and poly-adenylated by the L protein during synthesis.

Several properties of Rhabdoviruses have provided a strong rationale to explore them as a vector platform. First, they can infect many species, but are rarely pathogenic in humans. Most of them are insect or animal viruses, with transmission to human populations and subsequent seroconversion uncommon, therefore they do not present

pre-existing immunity in humans. Moreover, as the family is large and diverse, different Rhabdovirus strains can be employed in a heterologous 'prime-boost' vaccine regimen, enabling repeated vector delivery.

Many RVs are pantropic (e.g., VSV, Maraba), entering cells through receptor-mediated endocytosis and replicating entirely within the cytoplasm. Also, they proliferate and express their gene products to high levels.

RVs are highly immunogenic and elicit effective humoral and cellular immune responses against expressed foreign antigens while simultaneously functioning as a potent immune system adjuvant (Bridle 2009; Kim 2017). They can be engineered using reverse genetics (Lawson 1995) allowing the generation of recombinant RV strains, which are pseudo-typed with pathogen glycoproteins (e.g., rVSV with Ebola GP-protein). Moreover, the structural and regulatory nature of the RV genome allows for transgene insertion upstream of the N gene, between genes, or downstream of the L gene. Finally, with a cloning capacity of about 6 kb, multiple epitopes can be expressed from a single, multivalent RV vaccine.

Reverse genetics of RVs has also enabled the engineering of attenuated RV strains that are safer than the wild type virus. This aspect has been especially crucial for neurotropic RVs such as VSV. Over the past two decades, numerous attenuation strategies have been evaluated in preclinical model systems, including Non-human primates (NHPs). These include limiting viral replication by using single-cycle pseudotyped G-deleted viruses; shuffling the order of the viral genes (e.g., moving the N gene from position 1 to 4 in the genome); preventing virus maturation (and thereby attenuating budding of mature virions) by truncating the C terminus of the G protein (e.g., from 29 to 1 amino acids); allowing for a faster host antiviral response to attenuate virus replication, production and spread, by mutating the M protein (e.g., deleting amino acid 51) (Zempa 2018).

As the RV life cycle is entirely cytoplasmic, RVs do not integrate into host genomes reducing the risk of insertional mutagenesis. Moreover, they have a small size and grow to high titer in mammalian cells, thus enabling the virus to be quickly produced under good manufacturing practice. All these features have led to RVs development and study as genetic vaccine and oncolytic virus platforms for the past two decades.

1.7 Rhabdoviruses as vector platform for infectious diseases and cancer

Since its first rescue by Lawson in 1995 using the reverse genetic system, rVSV has been intensively studied as a vector platform for vaccine development. However, only recently, rVSV has come up as a valuable replication-competent vector platform. In fact, during the 2014/15 Ebola outbreak in the Democratic Republic of Congo, an rVSV

Ebola vaccine (rVSV-ZEBOV) was shown to control Ebola spread with over 90% efficacy. rVSV-ZEBOV contains a wild type rVSV backbone pseudotyped with Ebola virus GP (Zempa 2018).

This vaccine has demonstrated to be safe, immunogenic, and, most importantly, effective at protecting against the viral spread. Preclinical studies in mice, guinea pigs, and hamsters have shown that rVSV-ZEBOV was safe and could generate strong immune reactivity and protection against pathogenic Ebola virus inoculated after or soon before vaccine administration (Geisbert & Feldmann 2011). In NHPs, vaccination 7 or 31 days before high-dose Ebola virus challenge have protected animals completely from disease, whereas vaccination between three days pre- and one-day post-exposure has been partially protective (Dahlke 2017). Rapid innate immunity, as well as neutralizing antibody production, have been thought to play significant roles in the mechanism of protection (Dahlke 2017). The preclinical success of rVSV-ZEBOV has prompted to evaluate the vaccine during the recent outbreak. An initial placebo-controlled, double-blind phase 1/2 trial in 2015 has reported the rVSV-ZEBOV vaccine to be immunogenic. However, significant vector-associated reactogenicity has also been found, from arthritis and dermatitis to vasculitis (Huttner 2015). At least three other trials have since been reported, showing high antibody titers within a week post-vaccination, which were partially maintained for 6–12 months.

Moreover, a third open-label, cluster-randomized, ring vaccination trial also reported that rVSV-ZEBOV was safe, well-tolerated, and immunogenic (Henao-Restrepo 2017). Also, this trial has reported for the first time vaccine efficacy. A single IM dose of 2 × 107 plaque-forming units (PFU) was been administered to individuals in direct contact with Ebola-infected patients and found to be over 90% protective against Ebola virus infection if given as soon as possible. Despite the great results, vaccine safety has been improved by using a more attenuated strain. Therefore, a highly attenuated rVSVN4CT1 strain expressing the Ebola GP antigen from the first position of the rVSVN4CT1 vector has been generated. Subsequent preclinical data has reported the new vaccine to be safe and elicit robust neutralizing antibodies towards the Ebola virus antigens, and to protect against a lethal Ebola virus challenge in mice, guinea pigs, and monkeys (Dahlke 2017).

rVSV has also been investigated as a vector platform for a vaccine against HIV. Early studies have proved that an rVSV encoding HIV env and gag genes was immunogenic and could protect mice against HIV challenge when delivered as a prophylactic vaccine in homologous prime-boost vaccination regimen. In this approach, VSV has been pseudotyped using a glycoprotein arising from different VSV serotypes to avoid antibody-mediated vector neutralization (Schell 2009).

Subsequently, NHp have been vaccinated by using a VSV prime and an MVA boost regimens to maximize T cell activation against HIV. However, although the results have been promising, the safety test in Non-human primates has shown unacceptable

toxicity. Indeed, after intrathalamic inoculation, the brain of macaques has shown evidence of neuropathology, even if those viruses have not caused clinical disease (Johnson 2007). Engineered strains have been developed, harboring multiple attenuating mutations to slow viral replication, and promote safety (Dahlke 2017). These highly attenuated rVSV strains have been as immunogenic as their replication-competent counterparts, at least when delivered intramuscularly (IM). Indeed, immunogenicity after IM inoculation has been found to be more tightly associated with antigen expression levels than viral replication. These findings have led to regulatory approval of a first in man rVSV-based HIV vaccine trial in 2011.

This trial has tested the safety and immunogenicity of dose-escalated rVSVN4CT-1gag1 delivered IM into healthy volunteers (Fuchs 2015). The vaccine has been safe, with only mild-moderate adverse events. In conclusion, these studies have proved the safety of the rVSVN4CT1gag1 vaccine providing evidence of its immunogenicity, particularly when the vaccine has been used as a boosting agent.

Rhabdoviruses as a vector for cancer immunotherapy are particularly attractive. As they are oncolytic vectors, they can combine the ability to deliver and express antigens to secondary lymphoid organs for T and B lymphocyte functioning as immune system adjuvant and the ability to directly kill cancer cells. As oncolytic vector rVSV has been employed because of its broad tropism. Moreover, its sensitivity to type 1 INFs in normal cells but not in tumor cells has enabled the virus to be naturally replicating only in the tumor, thus increasing the safety. One of the vector currently in phase II clinical trial contains a sodium-iodine symporter, which facilitates the uptake of radioisotopes and non-invasive visualization of viral replication and spread in treated patients. However, the most promising result has been achieved when the oncolytic vector has been used in combination with checkpoint inhibitors (Shen 2016).

Other clinical trials are currently testing the Maraba virus. Maraba virus is a member of the Rhabdoviridae family closely related to VSV but antigenically distinct.

Brun (2010) demonstrated that Maraba had good cytolytic activity against tumor cell lines, rapid virus production, and large burst size. Moreover, Maraba was shown to be effective at treating both human xenograft tumors and in an immunocompetent syngeneic tumor model by systemic delivery as it achieved significant tumor regression in both tumor models. Pol (2014) has found that a genetically modified Maraba strain (MG1) harboring two point mutations had along with oncolytic activity also a remarkable capacity to boost adaptive antitumor immunity. The therapeutic activity of Maraba MG1 resulted from a multimodal mechanism: i) selective direct oncolysis obtained by systemic administration of MG1 to the tumor bed, and ii) reprogramming of the tumor microenvironment after infection of tumor cells by MG1 leading to the recruitment and activation of innate and adaptive mediators of antitumor immunity. Also, circulating MG1 access secondary lymphoid organs and rapidly engages the central memory compartment thereby being able to act as a boosting vector to

potentiate anti-viral or anti-cancer adaptive immunity (Pol 2014). rMaraba vaccine vectors expressing human dopachrome tautomerase (hDCT) was shown to induce T-cell mediated tumor clearance when used as a boosting vector vaccine (Pol 2014).

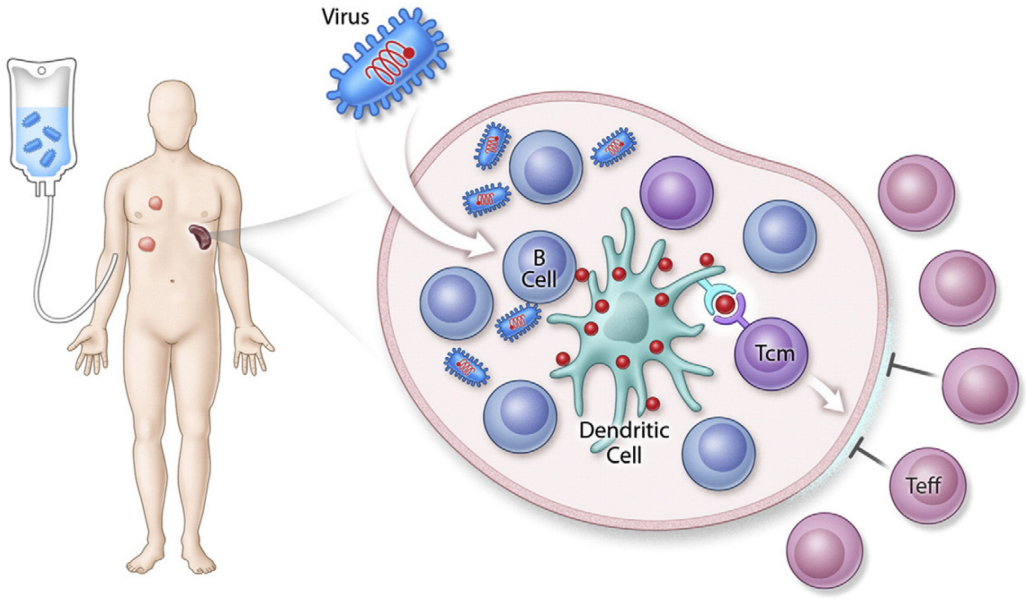


Figure 7. Mechanism of MG1 immune boost. The oncolytic virus MG1 expressing a tumor associated antigen transiently infects B cells in the splenic follicle. Antigens expressed on these cells are transferred to follicular dendritic cells that present antigen to tumor microenvironment cells. The splenic follicles are immune privileged sites as T effector cells cannot enter. Therefore, follicular dendritic cells are protected from T effector cell killing, a negative feedback loop. This provides a mechanism for the large anti-tumor immune responses observed following treatment with MG1 expressing a tumor antigen (Breitbach 2016).

These proof-of-principle studies have laid the development of RV vaccines against more clinically relevant endogenous tumor antigens. Pol (2018) has developed an MG1 strain oncolytic Maraba virus expressing the melanoma-associated antigen A3 (MAGE-A3). In combination with a replication-deficient adenovirus encoding the same MAGE-A3 antigen (Ad-MAGEA3), the MG1 vector has been widely tested in a prime/boost strategy in preclinical models, showing a significant expansion in the population of MAGEA3 antigen-specific CD4+ and CD8+ T cells. Currently, the

Maraba MG1-MAGEA3 virus (along with Ad-MAGEA3) is being tested in clinical trials with pembrolizumab in patients with non-small-cell lung cancer as well as those with metastatic melanoma or cutaneous squamous cell carcinoma.

Vectors based on rVSV and rMaraba have also been developed as vaccines encoding the E6 and E7 early proteins of human papillomavirus (HPV). They have been tested to treat HPV+ tumors caused by oncogenic viruses. These vaccines have been quite successful in preclinical tumor models: for instance, therapeutic vaccination of papilloma with E6-expressing rVSV caused rapid tumor clearance in rabbits (Brandsma 2007). Similarly, rMaraba expressing E6/E7 antigens promoted T-cell dependent tumor regression of HPV+ mouse tumors when delivered as a boosting vaccine (Atherton 2017). These studies demonstrate the potential of RV vaccines to treat HPV+ cancers and have supported their clinical trials.

1.8 Reverse genetic system for negative stranded RNA viruses rescue

Reverse genetic approaches are indispensable tools to generate recombinant viral particles for negative-stranded RNA viruses and have revolutionized the biology of Rhabdoviruses and other negative-stranded RNA viruses by allowing for the first time to genetically manipulate their genome and opening the way for their use as vectors for vaccine development. Conzelman (1994) reported the first successful recovery of a recombinant rabies virus using a reverse genetic approach. Since then, many other negative-stranded RNA viruses have been produced using the same strategy.

A unique methodology is required to rescue the recombinant negative-stranded RNA virus from cloned DNAs. Unlike the positive-sense RNA virus, the genome of the negative sense viruses is not infectious by itself and cannot initiate the replication cycle when transfected into permissive cells. The genome must be associated with the nucleocapsid proteins to form the active template for the RNA-dependent RNA polymerase.

The selection of promoters that drive viral RNA transcription is based on whether viral transcription occurs in the nucleus or the cytoplasm of the infected cells during the normal viral replication. T7-dependent systems are usually used for viruses that replicate RNA within the cytoplasm, while polymerase II systems are suitable for viral replication cycles that involve transcription in the nucleus. Cellular RNA polymerases such as polymerase I could also be used. Indeed, Groseth (2005) reported the successful recovery of Ebola virus from cloned cDNA by the use of cellular RNA polymerases I (polI). The role of polI is to transcribe ribosomal RNAs without the addition of 5'caps and 3' poly-A tails (Paule 2000). Therefore, it could be a proper host enzyme for the processing of viral RNA molecules generating well-defined vRNA 3' and 5' termini.

However, the interaction between RNA polI and its promoter is species-specific, therefore, the promoter sequence has to be carefully selected to suit the cell line destined for virus rescue. RNA polII cytomegalovirus promoters have also been employed to initiate the transcription of viral messenger RNA for Influenza virus rescue systems (Hoffmann 2000). However, as polII transcripts may be spliced and poly-adenylated, the use of polII for virus rescue of other negative-sense RNA viruses needs to be further investigated (Edenborough 2014).

T7 polymerase has been particularly useful for the recovery of negative-sense RNA viruses, which undergo transcription in the cytoplasm, including the Ebola virus, Marburg and VSV (Edenborough 2014).

One of the disadvantages of T7 systems is the necessity to have autocatalytic ribozymes to cleave non-viral terminal nucleotides added during transcription. Early rescue systems introduced an adjacent Hepatitis delta virus (HDV) sequence before the T7 terminator sequence to determine the correct processing of the RNA 5' ends, or trailer sequence. On the other hand, T7-dependent systems allow the use of different transfectable cell lines of several species for virus recovery. Indeed, the choice of the cell to use for viral rescue is crucial for viral vectors intended for human use. In early reverse genetics systems, cytoplasmic T7 was supplied by the addition of a recombinant vaccinia virus. However, its cytopathic effects have been found to hamper virus recovery and require plaque purification for the removal of vaccinia from the virus culture. To overcome these issues, the use of modified vaccinia Ankara strains was introduced. Lawson (1995) reported the first rescue of a recombinant Vesicular virus by using a reverse genetic system based on T7 RNA Polymerase. In this way, the cytoplasmic expression of the transfected DNAs encoding the RV genome under the control of the T7 polymerase is supplied by infection with a recombinant poxvirus that expresses the phage polymerase, such as the vaccinia virus (VV-T7) or MVA. However, more practical systems are now available such as T7 expression plasmids that can be transiently transfected or the use of a stably transfected into permissive cell lines (Edenborough 2014).

Although reverse genetic systems proved to allow recovering of negative-stranded RNA viruses, they must be adapted for each virus to be generated and often require further optimization.

2. AIM

This PhD project aims at generating a new viral vector platform based on a virus of the Rhabdoviridae family that could be used to generate new oncolytic virus for cancer immunotherapy and genetic vaccines encoding antigens or immune-modulators, thus extending the platforms currently available for therapeutic use. The new viral vector could be employed alone or in combination with other viral vectors to produce a strong specific immune response against the encoded cargo, thus developing new vaccination strategies. It should also be manufacturable by an industrial process.

The Rhabdoviridae family presents several compelling properties for viral vector generation. To date, several Rhabdoviruses-based vaccine platforms have already been developed. In particular, those based on VSV and Maraba viruses are currently in clinical trials as vaccines for cancer immunotherapy and against infectious diseases. They have shown to elicit robust humoral and cellular immune responses as well as efficacy in preclinical studies. Most importantly, during the 2014 Ebola outbreak in the Democratic Republic of Congo, a recombinant VSV vector expressing the Glycoprotein of Ebola Zaire (rVSV-ZEBOV) was tested in phase III clinical trial in humans giving rise to effective protection against viral spread. Moreover, a recombinant Maraba vector expressing the melanoma-associated antigen 3 (MAGE-A3) is now being evaluated in a series of clinical trials in combination with PD-1 monoclonal antibodies and has already shown impressive result in preclinical studies. Also, these type of vectors have the additional advantage of an easy manufacturing process on a large scale, thanks to their ability to grow to high titers in cell culture and to the small size of viral particles that facilitates the purification process.

Although significant efficacy was demonstrated by Rhabdovirus vectors, safety concerns have remained. Toxicology studies in NHPs showed that rVSV can induce toxicity after intrathalamic inoculation even without signs of clinical disease. Also, during a double-blind ½ clinical trial, high rVSV vector associated reactogenicity was found (Huttuner 2015).

Of note, Maraba has been shown to be useful to elicit a strong CD 8 T cells response only when employed in combination with adenoviral vectors as boosting vaccine or with other therapeutic agents (Pol 2014).

Hence, developing a new viral vector based on the family Rhabdoviruses would give us the possibility to extend the platform available for vaccine and oncolytic virus production.

3. MATERIALS AND METHODS

3.1 Bacterial strains and culture media

Chemically competent *E.Coli* DH5α (Invitrogen) and *E.Coli* DH10B cells were used to amplify and to transform all the plasmids.

Luria-Bertani (LB) culture medium, prepared as described by Sambrook, was used for bacteria's growth. The selection of transformants was done by adding 100 µg/ml ampicillin (Applichem, Darmstadt, Germany) to LB medium, both liquid and semisolid.

3.2 Cell culture and viruses

The Hek 293 T cell line was cultured in Dulbecco's Modified Essential Medium (DMEM) plus 10% (GibcoBRL) supplemented with 10% Fetal Bovine Serum (FBS) (heat inactivated 56°C 30 min Hyclone), 2mM L-glutamine (GibcoBRL) and penicillin /streptomycin (100 g/ml) and maintained at 37°C in a 5% CO2/95% air atmosphere. whereas Vero cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% Fetal Bovine Serum (FBS) (heat inactivated 56°C 30 min Hyclone and penicillin /streptomycin (100 g/ml) and maintained at 37°C in 8% CO_2/95% air atmosphere.

All the viruses used for the screening were purchased at the American Type Culture Collection (ATCC).

3.3 Viral RNA Isolation and cDNA synthesis.

Virus RNA was extracted from the supernatant of infected cells by using the Kit QIAmp Viral RNA mini-kit (Qiagen) following manufacturer's protocol. The RNA was recovered in 50 µL of RNase-free water. The synthesis of cDNA from viral RNA was performed using Superscript III First-Strand Synthesis System (Invitrogen) and a gene-specific primer annealing on the first nucleotides position of the viral genome. (5' GCAATCAAGATGTCCTCCATAG-3'). Five µL of viral RNA were reverse

transcribed with 200 Units of Superscript III RT, 2 μM gene-specific primer (GSP), 10 mM dNTP mix, 0.1 M DTT, 20U RNAse-OUT and RNase-free water. The reaction was incubated at 55ºC for 1 h, followed by enzyme inactivation at 85ºC for 5 min. RNA removal was performed by treating the samples with *E. coli* RNase H (2 U/μL) (Invitrogen) for 20 min at 37°C. The cDNA samples were stored at -20ºC until processed or used for PCR amplification by using Phusion High Fidelity DNA polymerase (Invitrogen).

3.4 RNA ligation and RT-PCR sequencing.

To determine the terminal sequences of Jurona genomes, the viral RNA genome isolated from the supernatant virions was circularized by T4 RNA Ligase in a two steps reaction. To remove the 5′ -triphosphate residues from genomic RNAs prior to RNA ligation, 1 μg RNA was digested with 5 units of 5′ Pyrophosphohydrolase (RppH; New England Biolabs) in 20 μL of reaction buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl2, 1 mM DTT, pH 7.9) and was then purified (RNeasy mini kit, Qiagen) according to the manufacturer's protocols 400 ng of RppH digested genomic RNA were ligated with 10 units T4 RNA Ligase (New England Biolabs) in 20 μL reaction buffer (50 mM Tris-HCl, 10 mM MgCl2, 1 mM DTT, 1 mM ATP, pH 7.5) for 1 h at 37 °C. The reaction was stopped by adding EDTA to a final concentration of 5 mM and 15 min incubation at 65 °C. Five μL of the ligation sample was directly used as a template for RT-PCR with Superscript III reverse transcriptase (Invitrogen) and Phusion DNA polymerase (Invitrogen) according to the manufacturer's protocols with gene specific oligonucleotides. PCR product was gel purified (Promega Wizard SV gel and PCR Clean Up system) and sequenced using the same oligonucleotides used for PCR amplification.

3.5 Gibson assembly cloning

The plasmid expressing the full complementary anti-genome (pGem was constructed from three cDNA fragments by using Gibson assembly cloning. (NeBuilder HiFI DNA assembly cloning Kit (New England BioLabs). The fragments (F1, F2 and F3) were PCR amplified by using Phusion High Fidelity DNA polymerase (Invitrogen). The primers used to amplify the fragments, described in the Table n.1 were designed to have two sequence components: an overlap sequence, required for the assembly of adjacent fragments; a gene-specific sequence, required for template priming during PCR amplification.

name	Sequence
F1 fw	**GAATTGGGCCCGACGTGCAATCAAGATGTCCTCCATAG**
F1 rev	**GGGACATATAATTGTTGTTGATTTATCTCCAATGACGTTGATTATTTG**
F2 fw	**CGTCATTGGAGATAAATCAACAACAATTATATGTCCC**
F2 rev	**TGTTTAAACTTGTCGGGTCCTCTAAGAGTTTGTCTATATG**
F3 fw	**GACAAACTCTTAGAGGACCCGACAAGTTTAAACATTGCAATG**
F3 rev	**CATGGCGGCCGGGAGCATGACGAAGAAAAACAAACCATATCTACTC**

Table n.1. Oligonucleotides for PCR amplification of the cDNA fragments for assembly of the full length anti-genome. The underling sequences represent the overlap introduced within each adjacent fragment as homology arms.

A linearized pGem plasmid was prepared by restriction cut digestion using AatII and SphI unique restriction sites. The linearized vector and the PCR fragments were gel purified (Promega Wizard SV gel and PCR Clean Up system) and the concentration determined using Nanodrop instrument. The assembly reaction was set up following manufacturer's protocol. Briefly 0.2 ρmoles of total amount of DNA fragments (0.05 ρmoles for each fragment) were mixed with 10 µL of Gibson Assembly Master Mix (2x) in a 20 µL final reaction volume. A positive control of the assembly was also included. The samples were incubated in a thermo-cycler at 50° C for 60 min. Then, 5 µL of assembly reaction were transformed in NEB 5-alpha Competent *E. coli* (New England Biolabs). Fifteen colonies were screened by restriction enzyme digestions and two positive clone were finally sequenced.

The new leader sequence of Jurona virus was introduced into the full length anti-genome plasmid using a mutagenesis protocol of Gibson assembly cloning. A new F1 fragment was amplified from the viral cDNA. A new forward primer was designed to contain the leader sequence between the overlap and the gene specific sequences.

To construct the plasmid expressing the full length anti-genome sequence including the additional eGFP marker, a slightly different protocol of Gibson assembly cloning was applied. Five total fragments were included in the assembly mix, instead of four. The eGFP coding sequence was amplified by PCR from the plasmid pIRES2-eGFP (ClonTech). The primers used were as follow: Forward 5' GCTGTTTCTTATTGACTATATGTGACTCGAGTATGAAAAAAACCAACAGT CATCGCCACCATGGTGAGCAAGGGCGAGG-3'; reverse 5'GATGACTGTTGGTTTTTTTCATAGCTAGCTTACTTGTACAGCTCGTCCAT G-3'. The mix reaction and the assembly conditions used were the same as previously described.

3.6 Support plasmids construction

The cDNA for the N, P and L proteins of Jurona virus were sub-cloned into a previously modified form of pGem plasmid. The IRES sequence and T7 termination signal were introduced into the pGem vector in two steps of cloning. The IRES sequence was amplified by PCR from the plasmid pIRES-eGFP2 (Clone-Tech). The primers used for the amplification were: Forward 5' TTACTCGATGACGTCAATTCTGCAGTCGACGGTAC-3'; Reverse 5'TACTTACTACGGCCGTGTGGCCATATTATCATCGTG-3', and contained the AatII and EagI linkers to allow the sub-cloning in the AatII EagI sites of the pGem plasmid. The T7 terminator signal was amplified by PCR from the plasmid p17ADJXQC (GeneArt). The primers used for the amplification were: Forward 5'TTACTCGATACGCGTCTGCTAACAAAGCCCGAAAG 3' and Reverse 5'TACTTACTAATGCATTCCGGATATAGTTCCTCCTTTC-3') and contained MluI and NsI linkers to facilitate sub-cloning in the pGem-IRES plasmid. The resulting plasmid (pGemIRES-T7Term) was digested with Mlu and EagI and the the N, P, and L coding sequence were sub-cloned by ligation reaction (Rapid DNA ligation kit; Roche) using recommendation's protocol.

To construct the plasmid expressing the T7 RNAP (pVJhCMT7 RNA pol) the T7 RNAP coding sequence was amplified by PCR from the plasmid pAR1219 (Sigma-Aldrich) using the following primers: forward 5' TTACTCGATGATATCGATTTACTAACTGGAAGAGGCAC-3'; reverse 5'TACTTACTAGAATTCGAGTCGTATTGATTTGGCGTTAC-3'. These primers included linkers for EcoRI and EcoRV restriction enzyme to allow cloning in the unique EcoRI and EcoRV sites of the plasmid pvjhCMV empty, by ligase reaction. The final plasmid obtained contained the T7 RNAP cloned between the hCMV promoter and the BGH poly-A signal.

To generate helper plasmids expressing the N, P and L viral proteins controlled by the hCMV promoter, their coding sequences were introduced into the plasmid pvjhCMV by Gibson assembly cloning. The N, P and L coding sequences were PCR amplify respectively from the plasmid pGemN, pGemP and pGemL. The primers included overlap sequences. The pvjhCMV plasmid was linearized by restriction enzyme digestion using unique EcoRI and EcoRV restriction sites. The assembly reaction was performed by using 0.2 ρmoles of total amount of DNA fragments (0.15 ρmoles of each insert and 0.05 ρmoles of linearized vector). The reaction was incubated at 50°C for 60 min and 5 µL of the assembly reaction was transformed in in NEB 5-alpha Competent *E. coli* (New England Biolabs).
The positive colonies were checked by restriction digestions and sequencing.

3.7 Jurona vector rescue on Vero cells

Virus rescue was first performed on Vero cell line. The cells were seeded in 6 well plates at 5E+05 cells/well using DMEM %5 FBS. 24h later each well was co-transfected with 2 µg of Jurona anti-genome full length plasmids (pGem JurV. w.t. and pGem F.L. mut), 2 µg of pvjT7 RNA pol plasmid together with pGem constructs encoding the proteins N (0.75 µg), P (1.25 µg) and L (0.25 µg) with 14 µL/well of Lipofectamine 2000 (Invitrogen), according with protocol instruction. After 6 hours the transfection media was removed and replaced with complete DMEM containing 5% FBS. 72 h post transfection the supernatant was collected and centrifuged at 1000 rpm for 5 min. 1 ml of the transfection supernatant (P0 passage) was used to infect new Vero cells, seeded at 5E+05 cells/well in 6 well plate to be confluent (90%) on the day of the infection. After the infection, the cells were incubated at 37°C 5% CO2 for 6 days. Six days later, the infections were collected and subjected to three round of freeze-thawing (-80°C, 37°C). 1 ml of cell lysate first passage (P1) was used to infect 3E+06 fresh Vero cells in T25 flask. The same procedure was performed up to three serial passages on Vero cells.

3.8 Jurona empty and Jurona-eGFP vector rescue on Hek293T cells

The rescue of the Jurona empty and Jurona-eGFP vectors was performed on Hek293T cells. The cells were seeded 5E+05 cells/ml in T25 flasks to be 90% confluent on the day of transfection using DMEM 10%FBS without antibiotics. 24h later co-transfection mix were prepared according to the ratio amounts indicated in the Table n.2.

MIX	pJurona-empty	pJurona-eGFP	pCMV-T7	pT7-N	pT7-P	pT7-L
B	2 µg	___	2 µg	1.25 µg	1.75 µg	1 µg
C	___	2 µg	2 µg	1.25 µg	1.75 µg	1 µg
MIX	**pJurona-empty**	**pJurona-eGFP**	**pCMV-T7**	**pCMV-T7**	**pCMV-P**	**pCMV-L**
D	2 µg	___	2 µg	1.25 µg	1.75 µg	1 µg
E	___	2 µg	2 µg	1.25 µg	1.75 µg	1 µg

Table n. 2. Co-transfection mix and ratios used for rescue of Jurona vectors. Jurona-empty vector and Jurona-eGFP vector were both co-transfected with two different set of helper plasmids: i) one encoding the helper proteins from a T7 promoter and ii) the other encoding the helper proteins from a CMV promoter

The transfection was performed by using 20 µL/flask of Lipofectamine 2000 CD (Invitrogen) following protocol instructions in OptiMem medium. After 6 hours two mL of complete DMEM containing 10% FBS was added to each flask, without removing the transfection media. After 48 h incubation at 37°C 5%CO_2, cells were detached and subjected to three rounds of freeze-thawing (-80°C, 37°C). The cells were centrifuged at 1500 rpm for 10 min and 5 mL of the cells lysate (P0) were used to infect 5E+06 Hek 293 T cells on T25 Flask. 11 day-post infection for Jurona empty vector and 19 day-post infection for Jurona-eGFP vector the cell bulk was collected at full CPE (P1).

3.9 $TCID_{50}$ assay for infectious titer determination

The infectious titer of the virus was determined by a standard $TCID_{50}$ assay.
One day before the infection, Vero cells were seeded in 96 well plates at $4x10^4$ cell/well in DMEM 5% FBS and grown to 90% confluency. The day after, purified vector or virus infected samples were tenfold serially diluted (from 1e2 to 1e9) and used to infect the cells. Ten replicates of each dilution were done. The cells were incubated at 37°C 5% CO_2 and monitored daily for CPE. A positive well was a well where a clear cytopathic effect was visible.
The $TCID_{50}$ was calculated by the Spearman-Karber method taking into account factors such as pre-dilution, serial dilution and initial volume. The titer expressed as $TCID_{50}$ /ml was converted as Focus Forming Unit (FFU) by multiplying the titer 0,7.

3.10 Digital droplets PCR for quantification of viral genome

The viral genome titer was calculated by Digital droplets PCR by using a protocol previously published (Veach 2015). Briefly, 140 µL of sample (undiluted clarified harvest) was added to a 1.5 mL tube. 560 µL of Buffer AVL (viral lysis buffer containing guanidine thiocyanate) was added, mixed by pulse-vortexing and incubated at room temperature (15–25°C) for 10 min. Then, 560 µL of ethanol (96–100%) was added to the sample and mixed by pulse-vortexing for 15 s. After, the lysed virus/extracted nucleic acid was diluted serially (1e2–1e8) in 1x PBS. and used directly in the reaction or frozen at -20°C for an application at a later date. RNA quantification of 'non lysed' sample was used to evaluate 'free RNA' (RNA originated from lysed infected cells) which was taken into account in the final RNA quantification. A volume of PBS equal to the combined volume of Buffer AVL and Ethanol was added to the sample (e.g. for 140 µL of sample, 1120 µL of 1x PBS was added). Droplets were

generated with the QX100 Droplet Generator and the emulsion generated was transferred to a 96 well semi-skirted plate for PCR.

The primers used for the amplification were as follow: Forward primer 5' GTATCTGATTGACCTGGGGC-3'; reverse 5'AATAACTGAGCGGCTTTGGT-3'; probe: 5' FAM TCCATTTCTGGGGACAATTGACCGCGCT-3'TAMRA. Cycling program with T100 Thermal Cycler (Bio-Rad): RT step: 50 °C, 60 minutes. Taq activation: 95°C, 10 minutes. 40 PCR cycles (Denaturation: 95°C, 30 seconds. Annealing and extension: 60°C, 60 seconds). Taq de-activation: 98°C, 10 minutes. Hold at 4°C. After thermal cycling, the sealed 96-well plate was placed in the QX100 with wavelength settings - HEX/FAM. After data acquisition, the same threshold was applied to all samples and absolute concentration was reported in copies per µL of RNA in the final 1x ddPCR reaction. For every sample, concentration of the 'free RNA' was subtracted from the total RNA (lysed sample). Dilutions for lysis, serial dilutions, PCR mix were accounted for to determine final concentration: 9x dilution in lysis (140 µL µL in 1260 final), 1eX dilutions of sample (serial dilutions), 5x dilution in PCR mix (4 µL in 20 final).

3.11 Western blot analysis

The supernatant, pellet and bulk from Jurona rescue samples were diluted with 4x LDS Sample Buffer (NuPage Invitrogen) containing 20% β-mercapto-ethanol and heated at 99°C for 5 min. After loading on 4-12% Sodium dodecyl sulfate-polyacrylamide gel (NuPAGE Bis-Tris gel Invitrogen) the proteins were transferred to a nitrocellulose membrane using the I blot 2 DRY Blotting System (Invitrogen). After a blocking overnight at 4°C in PBS with 5% powered milk 0.01% Tween 20, the membrane was probed with anti N protein (mouse polyclonal antibody anti Nucleocapsid of Jurona virus, 1:1000 dilution) and anti G protein (mouse polyclonal antibody anti Glycoprotein of Jurona virus, 1:1000 dilution). Secondary staining was performed by using a peroxidase-conjugated anti-mouse (A3682, Sigma-Aldrich, 1:5000). Imaging was performed using ECL (SuperSignal West Pico Chemiluminescent substrate (Thermo scientific) and a ChemiDoc Imagers (Bio-Rad).

3.12 Jurona vectors production and iodixanol purification

Jurona-eGFP vector was produced in Vero cells. The cells were seeded at 1x 104 cells per cm2 in DMEM supplemented with 5% FBS in 150 mm culture petri dishes. At 90% confluency, the cells were infected with Jurona-eGFP vector cell lysate at 0.01 (Multiplicity of Infection-vp/cell) MOI. The cells lysate was diluted in 50 ml of serum-

free DMEM. 5 ml of diluted virus was added to each dish and incubated for 1 h at 37°C 5% CO_2. After the incubation, 20 mL of complete DMEM supplemented with 5% FBS was added to each dish and the infected cultures were placed at 37°C 5% CO_2. 48 h later, the supernatant was collected and centrifuged at 1000 rpm for 15 min. Cleared supernatant was filtered through 0.2 µm membranes (Millipore) and further purified using a iodixanol gradient. The cleared supernatant was ultra-centrifuged at 25.000 rpm for 3 h at 4°C by using SW28 Swinging Bucket rotor on the top of a 50% iodixanol working solution (OptiPrep 50% w/v) (5 volumes of Optiprep 60% and 1 Volume of OptiPrep diluent: 0.15 M NaCl, 6 mM EDTA, 0.3 M TrisHCl pH 7.4). After the first centrifugation, 6 ml were recovered from each tube. They were re-suspended in the suspension medium (0.1 M NaCl, 0.5mM EDTA, 50mM EDTA, 50 mM Tris HCl pH 7.4) and further centrifuged at 50.000 rpm for 16h at 4°C by using rotor SWT55i.

4. RESULTS

4.1 Screening of different Rhabdovirus strains

To develop a new vector platform based on a novel member of the Rhabdoviridae family, different Rhabdoviruses were chosen among those available from the American Type Culture Collection (ATCC). The viruses were selected to be Biosafety Level class II, to facilitate their manipulation in conventional research laboratories. Five Rhabdoviruses named Marco, Chaco, Jurona, Aruac e Timbo were used as starting material for the initial screening, in order to identify the best candidate virus for vector construction (Table 3).

Classification	Strain	BSL	Culture method	Sequence	Source	Growth condition	Collection (d.p.i.)
Timbo (unclassified RV)	Be An 41787	2	BHK-21(C-13) cells	partial cds	Caiman Lizard Brazil	30°C 5% CO_2	13 d.p.i.
Jurona (Vescicolovirus)	Be AR 40578	2	mouse	complete genome	Mosquitos Brazil	30°C 5% CO_2	3 d.p.i.
Chaco (unclassified RV)	Be An 42217	2	NA	partial cds	Lizard Brazil	30°C 5% CO_2	11 d.p.i
Marco (unclassified RV)	Be An 40290	2	suckling mouse	partial cds	Lizard Brazil	30°C 5% CO_2	8 d.p.i.
Aruac (unclassified RV)	TRVL 9223	2	suckling mouse	partial cds	Mosquitos	30°C 5% CO_2	7 d.p.i.

Table 3: Summary of the Rhabdoviruses selected from the ATCC collection. Each Rhabdovirus was classified on information available in the literature. All the viruses were Biosafety level 2 (BSL2). Most of them were initially isolated from Mosquitos and

Lizards. The sequence was entirely available for the Jurona virus and partially available for the others. 3E+06 cells were infected with a 1:100 dilution of each virus stock and cultured at 30°C 5% CO_2. Infections were collected when over 70% CPE was visible for Jurona, Chaco, and Marco viruses. No CPE was visible in cultures infected with Timbo and Aruac Viruses. Abbreviations: RV, Rhabdoviruses; cds, Coding sequence; d.p.i., days post-infection.

The viruses were isolated from lizards and mosquitos in Brazil (Monath 1978) and collected at the ATCC. Initially, a validated cell line was used to screen the capacity of each of the above-listed viruses to replicate and to generate a progeny in culture. The Vero cell line, established from an African green monkey (non-human primate; NHP), has already been employed and validated for viral vector production (Barrett 2009). Therefore, we infected Vero cells with a 1:100 dilution of each virus stock in the same culture conditions, and the cultures were monitored for viral replication. Among the viruses tested, Jurona, Marco, and Chaco viruses demonstrated to efficiently replicate in the target cell line, giving rise to a clear cytopathic effect (CPE). On the contrary, Aruac and Timbo viruses did not show an evident lytic activity in the same culture conditions, even after a further passage of a lysate from infected Vero on fresh cells. Among the viruses able to replicate, the Jurona virus showed the most rapid replication, as it reached a CPE with a complete clearance of the cells within three days from the infection, whereas Marco and Chaco viruses developed a complete CPE only at 8 and 11 days post-infection. So, thanks to its ability to rapidly replicate in culture, we chose the Jurona virus as our candidate for further characterizations.

4.2 Jurona oncolytic activity

As previously reported by Pol (2010), several Rhabdoviruses are very efficient at killing cancer cells and represent potent oncolytic agents. However, differences in the killing activity are evident across the members of the family. Among all the Rhabdoviruses, VSV and Maraba have shown the most potent oncolytic activity on cancer cells. As the Jurona virus is strictly related to VSV and Maraba, we thought that it could behave in the same manner. Therefore, we sought to preliminary test the Jurona oncolytic activity on different tumor cell lines. As Hek 293 and Hela cells are immortalized tumorigenic cell lines, we used them as our model for testing the Jurona killing activity. To this end, we infected 3E+06 of Hek-293 and Hela cells with 1:100 dilution of Jurona virus stock, and we checked for oncolysis and cell death at different time points. As shown in the figure 8, Jurona had a robust killing activity on both cell lines as it induced cell cytolysis leading to a substantial decrease of cell vitality within three days post-infection. This result preliminary demonstrated that, like the other

members of the Rhabdovirus family, Jurona has a strong oncolytic activity and that it could be employed as an oncolytic vector.

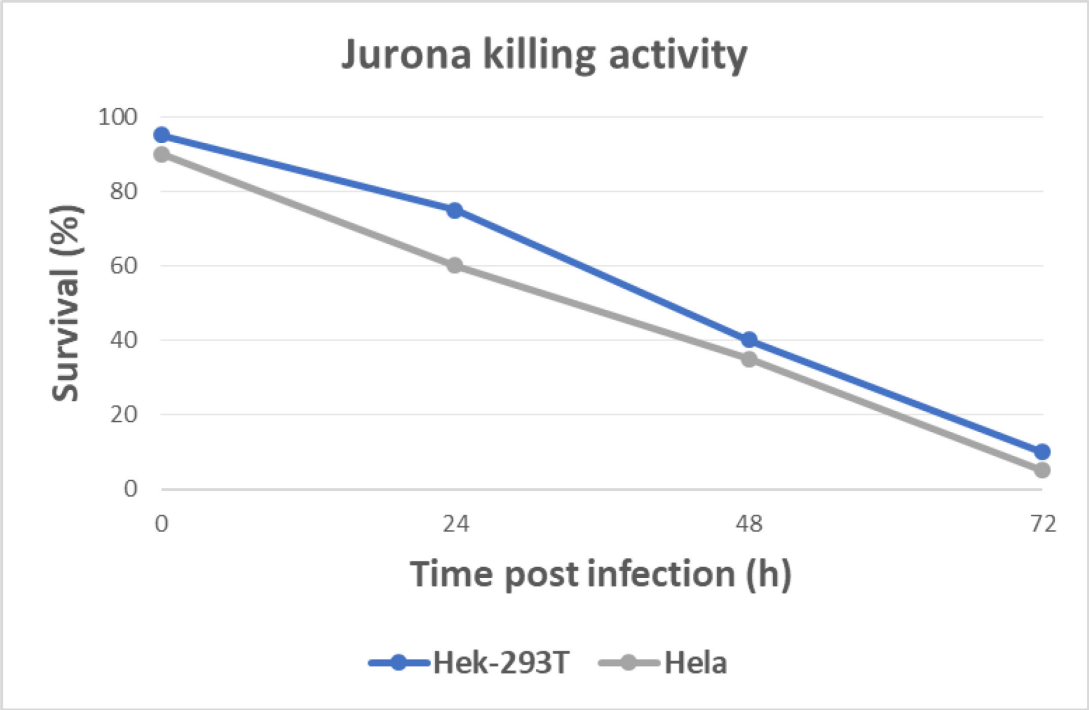


Figure 8. Jurona Killing activity on tumor cell lines. 3E+06 cells were infected with a 1:100 dilution of Jurona virus. The cell survival was evaluated at 0, 24, 48, and 72 h post-infection for each cell line as the percentage of the living cells on the total number of infected cells.

4.3 Construction of full length and helper plasmids for Jurona vector rescue

The recovery system of negative-stranded RNA viruses, like the Jurona virus, requires a reverse genetic strategy to obtain infectious particles from full-length cDNA clones. Indeed, unlike positive RNA virus, the viral genome of the negative single-stranded RNA virus is not infectious by itself, and it is not sufficient to start an infectious cycle in the host cells. The genome must be enrolled by the Nucleocapsid protein to form a ribonucleoprotein complex (RNP) that serves as a template for recognition, transcription, and replication by the complex of the viral RNA polymerase. This complex is formed by the Large RNA polymerase (L) and the Phosphoprotein (P),

which serves as a cofactor for transcription and replication. Once formed, the RNP complex will direct the synthesis of capped and poly-adenylated mRNAs encoded by the viral genome, completing the replication cycle and resulting in the release of infectious virus. Thus, as result of this mechanism, to obtain viral particles from negative-stranded RNA virus, the entire anti-genomic sequence of the virus, the Nucleocapsid (N), the Phosphoprotein (P) and the Large RNA polymerase have to be introduced into the host cells in trans, to express the essential components required to start the replication cycle (Figure 9).

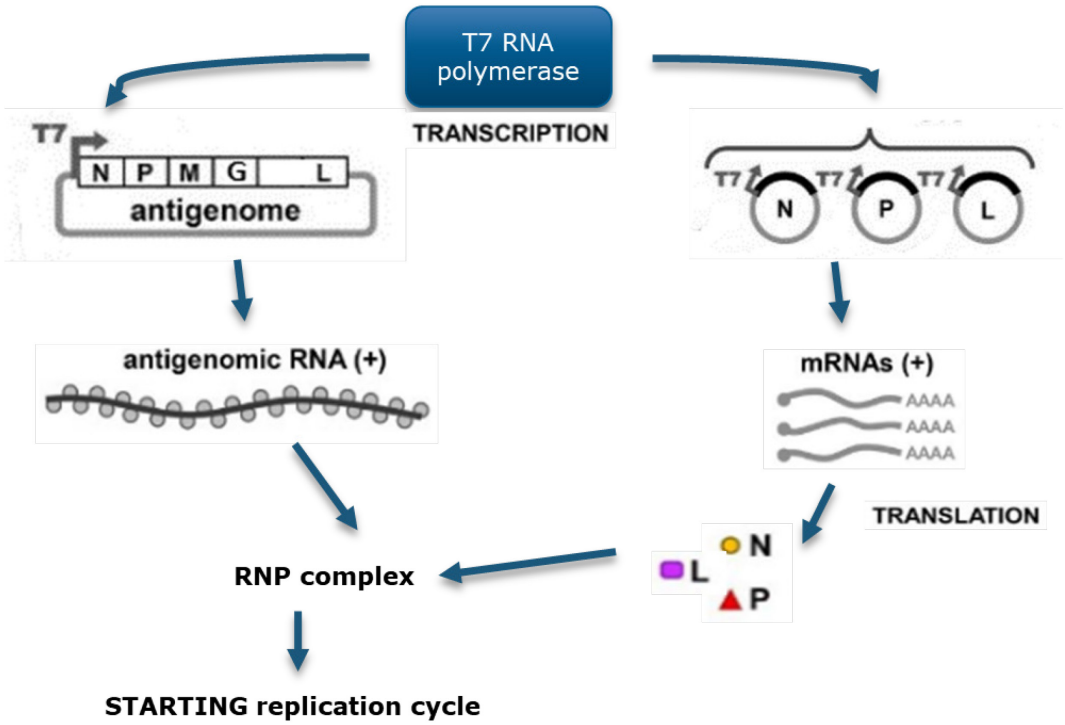


Figure 9. Schematic representation of a Reverse genetic system for negative-strand RNA viruses. The plasmids expressing the helper viral proteins and full-length anti-genome are under the control of the T7 promoter T7 RNAP. T7 RNAP leads the expression of both helper proteins and the full-length genome. Once the RNP complex has formed, viral replication can start leading to a complete infectious cycle.

Typically, the viral anti-genomic RNA, as well as the helper viral proteins, are produced in the cytoplasm from plasmids where the bacteriophage T7 RNAP drives expression of the encoded functions. The T7 polymerase is a bacteriophage DNA-dependent RNA Polymerase that localizes into the cytoplasm when expressed ectopically. It is usually employed in reverse genetic systems to produce a viral RNA

transcript without a cap and with correct viral ends within the cytoplasm. Indeed, it is essential to generate correct viral genome ends to enable the N protein to recognize essential cis-acting sequences for the encapsidation. Several strategies have been employed to transduce the T7 RNAP: i) by infecting cells with a recombinant vaccinia virus expressing bacteriophage T7 RNAP (Fuerst 1986); ii) by using cells stably expressing T7 polymerase (BSR-T7; Harty 2001), or iii) by transfecting the cells with a plasmid that expresses T7 polymerase (pC-T7; Neumann 2002). To implement the reverse genetic strategy and obtain recombinant viral particles based on Jurona virus, five constructs were generated using the Jurona Genebank sequence as reference (KM204996.1): a plasmid expressing the complementary anti-genome sequence, three helper plasmids expressing the N, P, and L viral proteins, and a plasmid expressing the bacteriophage T7 RNAP.

The Jurona genome is a negative single-stranded RNA genome of about 11 kb, which encodes five proteins: The Nucleocapsid (N), the Phosphoprotein (P), the Matrix protein (M), the Glycoprotein (G), and the Large polymerase (L). The corresponding genes are expressed from a single 3' promoter as distinct transcriptional units on a gradient from highest (N; position 1) to lowest (L; position 5) level of expression (Dietzgen 2017). The complementary anti-genome is usually used instead of the genome because it is crucial to avoid the hybridization of genomic RNA to the mRNAs transcribed from the plasmids expressing N, P, and L proteins.

Initially, the plasmid encoding the full viral complementary anti-genome sequence was designed to have a T7 promoter upstream of the 5′-antigenomic leader sequence and a modified hepatitis delta virus (HDV) ribozyme followed by a T7 polymerase termination signal sequence immediately downstream of the 3′-end. The HDV ribozyme, a self-cleaving ribozyme, was required to cut a precise terminal region at the 3’ whereas the T7 promoter produced a 5’ end with the addition of three G nucleotides (Roberts 1999).

As the viral anti-genome was difficult to be amplified and cloned as a full-length sequence, we used the Gibson assembly cloning method to construct the plasmid expressing the complementary genome. Gibson assembly efficiently joins multiple overlapping DNA fragments simultaneously in a single-tube isothermal reaction that includes three different enzymatic activities: the exonuclease that creates single-stranded 3' overhangs; the DNA polymerase that fills in gaps within each annealed fragment; the DNA ligase that seals nicks in the assembled DNA (Gibson 2009). So, we first split the anti-genome in three fragments of 4642 bp, 3184 bp, and 3826 bp. Then, the fragments were PCR amplified from the viral cDNA by using primers designed to introduce overlapping sequences and a high fidelity DNA polymerase. Then, the three purified DNA fragments and the acceptor plasmid (pGem) were incubated in the Gibson Assembly Master Mix reaction, and the assembly product was

subsequently transformed into E. coli. Sequencing of positive clones revealed that the viral genome was correctly assembled into the acceptor vector.

The Jurona vector was designed to be replication-competent, therefore, in order to improve its safety, we thought to attenuate the vector for its replication. A variety of strategies can be employed to attenuate the replication such as: i) introducing mutations into the M protein; ii) deleting small portions of the G protein or entirely the G protein; iii) shuffling the regular order of the genes within the genome; or iv) introducing non-viral genes into the viral genome (Van den Pol 2013). Brun (2010) previously demonstrated that by mutating the lysine in position 123 of the M protein and the tryptophan in position 242 of the G protein of Maraba vector, it was possible to obtain a phenotype of the vector that was attenuated in normal cells while showing improved killing activity in tumor cells. The mechanism underlying this observation is still not well understood. As Jurona is closely related to the Maraba virus, we hypothesized that the same mutations would produce the same effect on the Jurona vector. Thus, we first aligned the M and G protein sequences of Jurona with those of VSV and Maraba, and then we identified L123 and N242 in the M and G proteins, respectively, as the corresponding positions in the Jurona sequence where we should introduce the attenuating mutations. Therefore, we introduced the L123W and N242R point mutations in our synthetic construct.

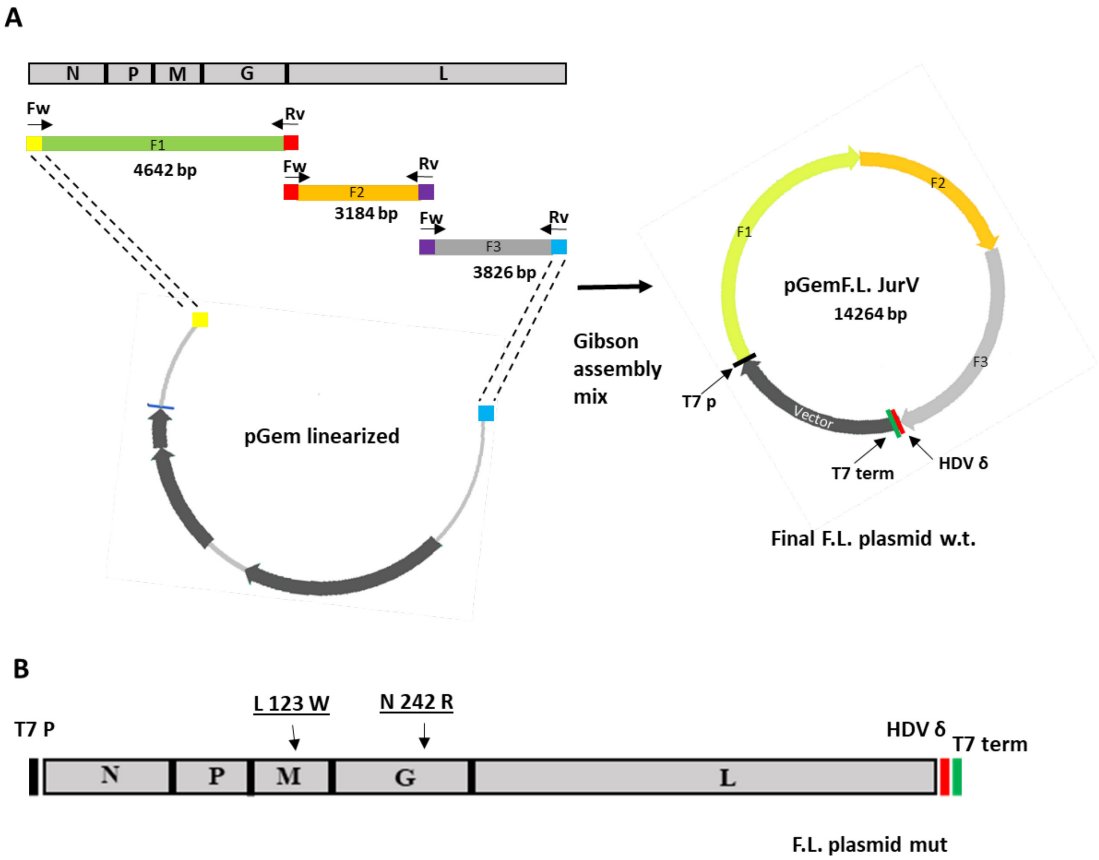


Figure 10: Schematic representation of the full-length plasmid cloning strategies. (A)The Gibson assembly strategy was used to clone the Jurona complementary genome. Three fragments (F1, F2 and F3) were assembled in the acceptor vector (pGem linearized) using Gibson assembly mix. (B) L123W and N242R represent the two-point mutations introduced in the M and G proteins, respectively, to attenuate virus replication.

To construct the helper plasmids expressing the viral N, P, and L proteins, we PCR amplified the corresponding protein-coding sequences from viral cDNA and cloned them in the pGem T easy plasmid under the control of a T7 promoter, using classical enzyme digestion and ligation reactions. The pGemT easy vector was previously modified to carry an Internal Ribosome Entry Site sequence (IRES) derived from Encephalo-myocarditis virus, between the T7 promoter and the coding sequence to ensure translation of viral mRNA, since no cap structure was added at 5' end of T7

RNAP transcripts produced in the cytoplasm. Furthermore, a T7 termination signal was introduced downstream of the coding sequence, to guarantee transcription termination by the T7 RNAP.

Lastly, to provide for a source of T7 RNAP within the cytoplasm without the need to use a helper virus or a stable cell line, an additional plasmid was constructed with the T7 RNAP coding sequence under the transcriptional control of the strong human CMV promoter and a BGH (bovine growth hormone) poly-A signal, using classical enzyme digestion and ligation reactions. A schematic representation of all six plasmids generated to rescue the Jurona vector is shown in Figure 11 (A, B).

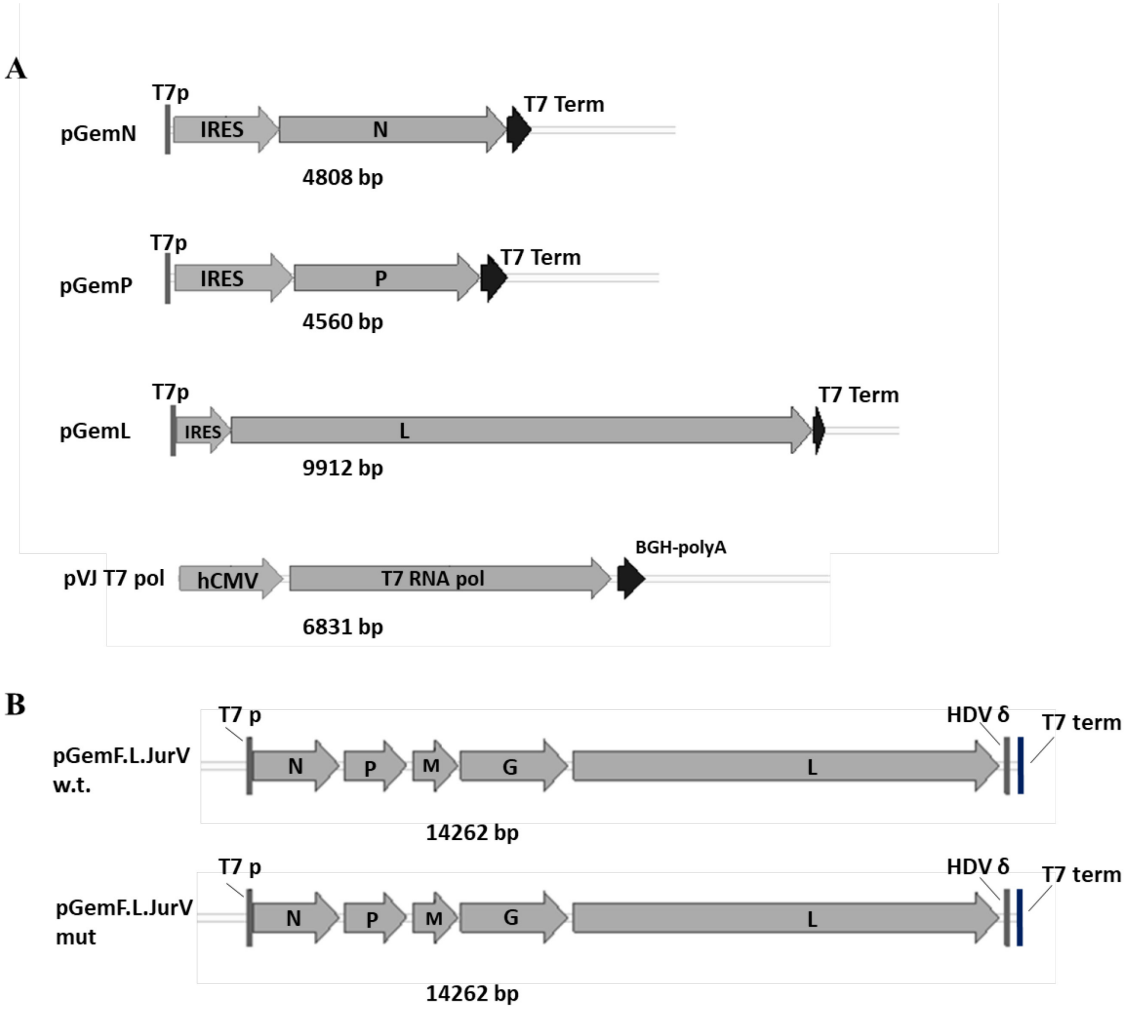


Figure 11: Schematic representation of the six plasmids generated to rescue the Jurona vector by reverse genetics. (A) In the expression plasmids encoding the viral helper proteins N, P, and L, transcription is under the control of the T7 promoter and an IRES element preceding the coding sequences was introduced to promote translation. (B) Map of the plasmids encoding the full-length anti-genome. The T7 RNA pol promoter at the 5' leads the synthesis of the complementary anti-genome, and the T7 terminator promotes the termination of the transcript. The HDV ribozyme cleaves the transcript to generate the correct 3' end. From top to bottom: i) plasmid pGem N encoding the N protein of Jurona virus ii) plasmid pGem P encoding the P phosphoprotein protein iii) plasmid pGem L encoding the L polymerase iv) pVJT7 pol plasmid encoding the T7 RNAP under the hCMV promoter v) plasmid pGemJurV w.t. encoding the full-length wild type Jurona anti-genome. vi) plasmid pGemF.L. mut encoding the full-length Jurona anti-genome along with two point mutations in the M and G proteins.

4.4 Setting up of the Jurona vector rescue system

To rescue the vector based on the Jurona virus by the reverse genetic system, we decided to use Vero cells. This choice was based on previous experience with most of the reverse genetic systems developed up to date for VSV and other negative-stranded RNA viruses, which used Bhk-21 or Vero cell lines as host for the recovery of the recombinant viral particles. One of the protocol previously published for VSV recovery (Whitt 2016) was based on the cell transfection with N, P and L expression plasmid mixture that had a ratio of 3:5:1 µg of each helper plasmid, respectively, together with 2 µg of the plasmid encoding the full-length virus anti-genome. In this protocol, the source of cytoplasmic T7 RNAP was a helper vaccinia virus (vvT7) expressing T7 RNAP. Since we did not want to use a helper virus in our system, we decided to use a T7 expression plasmid, thus introducing a fifth plasmid in the reverse genetic system. We slightly adapted the transfection protocol by seeding the cells in six-well plates and co-transfecting them with an N, P and L expression plasmid mixture that had a ratio of 3:5:1 plus 2 µg of the plasmid encoding the full-length virus anti-genome and 2 µg of a plasmid expressing T7 RNAP.

After the transfection, the cells were passaged on fresh Vero cells and monitored daily for the development of cytopathic effect (CPE) for a total of 6 days, as a sign of correct viral particle formation. Since no CPE was observed in the transfected culture, three additional passages of cell lysates were performed on fresh cells. Once again, no CPE was observed. To confirm that no viral particles were produced, the supernatant from each passage was analyzed for the presence of viral genomes by measuring the total amount of RNA genomes with a quantitative One-step RT-digital droplets qPCR. As shown in Figure 12, the total amount of viral genomes in P1, P2, and P3 passages gradually decreased as a result of the dilution of the contaminating original plasmid DNA containing the anti-genome used for the transfection and of its transcripts. Furthermore, the genome titer was about four logs lower than that of the control supernatant obtained by infection of Vero cells with the original wild type Jurona virus (Jurona w.t.). These results conclusively demonstrated that no rescue events were initiated in the transfected cells and that the genome copies detected in the analysis came up from the amplification of the transfected plasmid and of its transcripts.

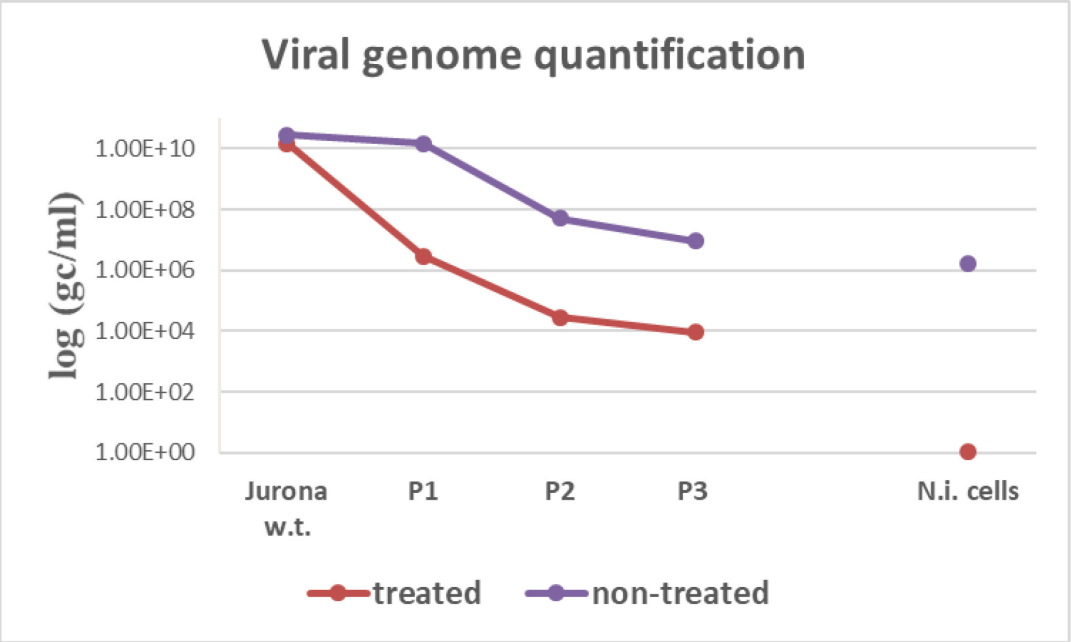


Figure 12: Viral particles quantification on Jurona P1, P2, and P3 cell lysates. The viral RNA was extracted from the supernatant of the infected cells. Samples were treated (DNase+) and not-treated (DNase-) with DNase enzyme to remove plasmid contamination. The RNA was directly amplified in the One-Step RT-ddQPCR. The result is expressed as gc/ml and was calculated considering the average of three independent viral dilutions loaded in duplicate. N.i. cells: non- infected cells, Jurona w.t. is the original Jurona virus used as positive control.

4.5 Viral ends sequencing: leader determination

To understand why the rescue system did not work, we went back to analyze the integrity of the Jurona genome extracted from the amplified virus. No difference was observed between the nucleotide sequence of the rescued genome and the Jurona sequence annotated in the Gene Bank. We then performed a multiple sequence alignment between the Jurona genome and that of other Rhabdoviruses like VSV and Maraba. This analysis showed that Jurona had a shorter terminal region at the 3'end of the genome (Figure 13). This finding was somewhat surprising because despite the Rhabdoviridae family is very large with several non-classified viruses, all have a similar modular structure, with five cistrons encoding for five viral proteins, separated

by the transcriptional start/stop sequences. Also the 3' end of the genome is very similar, and it was reported to be essential to regulate the transcription and replication by the viral RNA polymerase (Dietzgen 2017). Thus, a lack of the complete viral end in the reference sequence, and therefore in our full-length viral genome plasmid could have explained the failure of the viral rescue. In fact, the generation of the Jurona cDNA 3' end was done using a primer matching the shorter 3' end of the Jurona sequence annotated in the Gene Bank.

To confirm the lack of a complete 3' terminal end in the reference genome, the sequence of the 3' and 5' ends of the Jurona RNA extracted from the wild type virus preparation was determined using an RNA end-joining strategy. As Jurona has a single negative-stranded RNA genome, to obtain the 3' viral end, the viral RNA was first circularized by using the enzymatic activities of an RNA 5′-Pyrophosphohydrolase (RppH) and of a T4 RNA Ligase in a two steps reaction. Then, the ligated trailer and leader sequences were RT-PCR amplified using specific primers designed to anneal upstream and downstream of the regions of interest. The resulting PCR product of about 500 bp was subsequently sequenced (Figure 14A).

As a result of our strategy, 55 additional nucleotides (Figure 14B) were identified in the RNA genome extracted from the wild type virus preparation that were not previously known and were not included in the sequence published in the nucleotide database, thus confirming a lack of a complete 3' leader end in the published sequence and in our full-length genome plasmid.

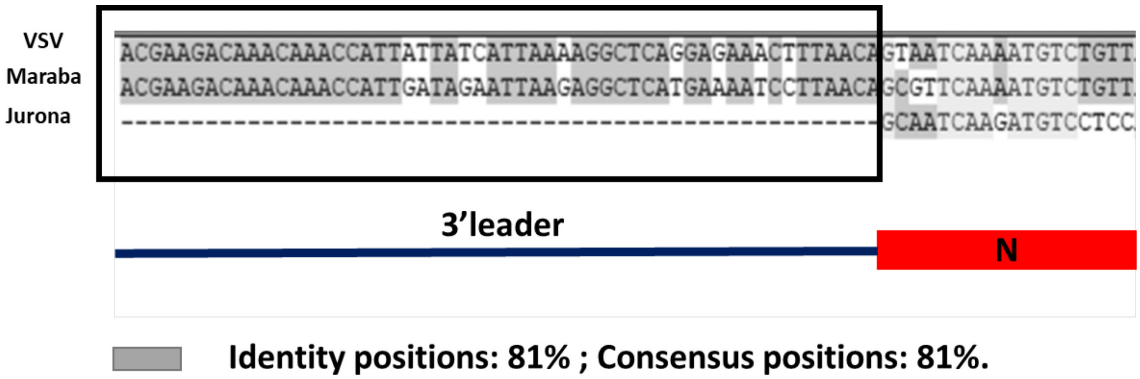


Figure 13. Sequence alignment of VSV, Maraba, and Jurona viruses. The box in black highlighted the leader sequence. The text marked in grey describe the identity and the consensus positions in the alignment. VSV and Maraba viruses shared an 81% of identity and consensus positions; the dashed lines described the lack of a complete leader in the Jurona virus sequence.

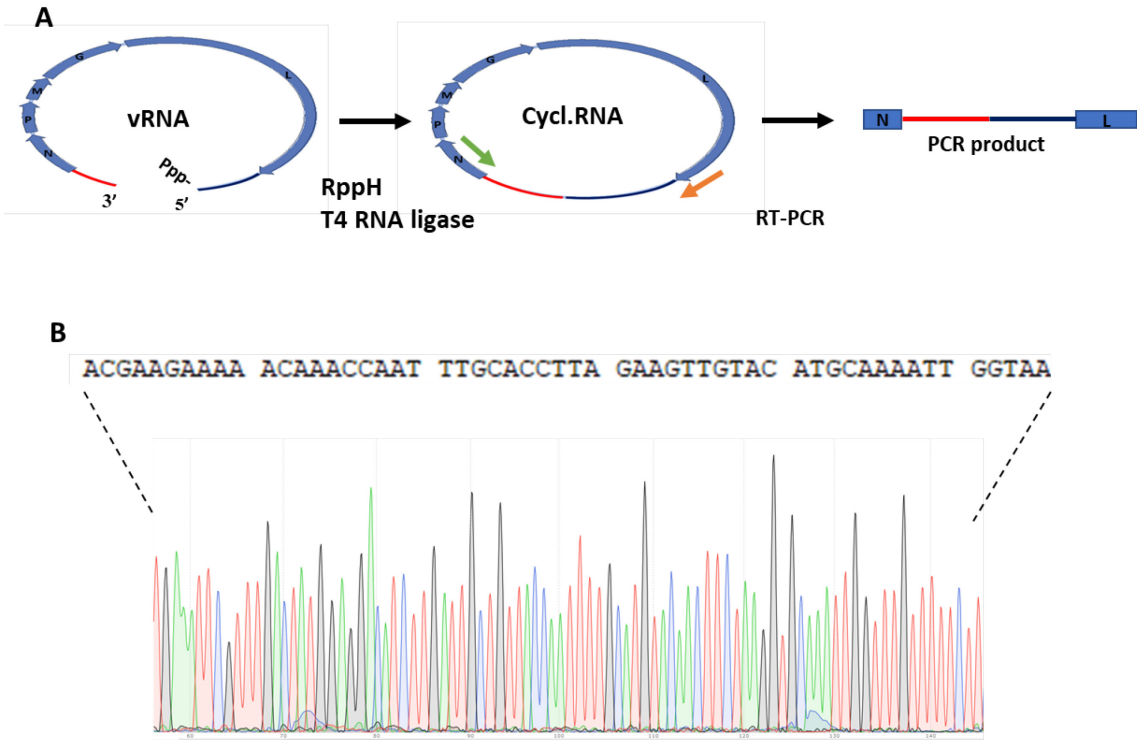


Figure 14: (A) Schematic presentation of T4-RNA-mediated RNA end-joining for the determination of virus end-sequences. (B) 3' terminal sequence identified after the ends sequencing.

4.6 Introduction of the new leader fragment and the eGFP marker gene in the full-length vector plasmids

The 55 nucleotides-long new leader sequence was introduced in the full-length plasmid expressing the complementary anti-genome. Two new plasmids were generated: a plasmid with the Jurona leader sequence cloned immediately downstream of the T7 promoter and a second plasmid in which the eGFP coding sequence was introduced as an additional transcriptional unit within the viral genome, in order to follow viral replication and spread *in vitro* using fluorescence microscopy.

The transcription of negative stranded RNA viruses is based on a start and stop mechanism that occurs progressively trough the genome starting at the 3'end from the highest level in the first position to the lowest in the last one (for example, N > P > M > G > L). This is governed by a transcriptase complex that recognizes cis-acting

sequences such as gene transcription termination polyadenylation (TTP) signal at the end of each viral gene, thus regulating the relative abundance of each viral mRNA and of each protein. As previously demonstrated (Van den Pol 2013), introducing an additional gene within the viral genome attenuates viral replication. The attenuation level depends on where the gene is introduced and it is higher when the gene is placed upstream of all the viral genes. Therefore, the eGFP marker was cloned into the full-length anti-genome plasmid in the intergenic region between the G and L proteins, in order to minimize the attenuating effect on viral replication and to express a right level of eGFP gene marker.

The primers used to amplify the eGFP coding sequence were also designed to carry: i) a copy of the viral promoter to start the transcription; ii) a Kozak sequence upstream the ATG, to enhance the eGFP expression, and iii) two restriction enzyme sequences flanking the open reading frame, to easily replace the eGFP with others marker genes. Two new plasmids were finally generated using the Gibson assembly strategy, and they were finally employed for the rescue experiments: a pGemF.L.JurV leader w.t. and a pGemF.L.JurV leader-eGFP. The schematic representation of the plasmids is shown in Figure 15.

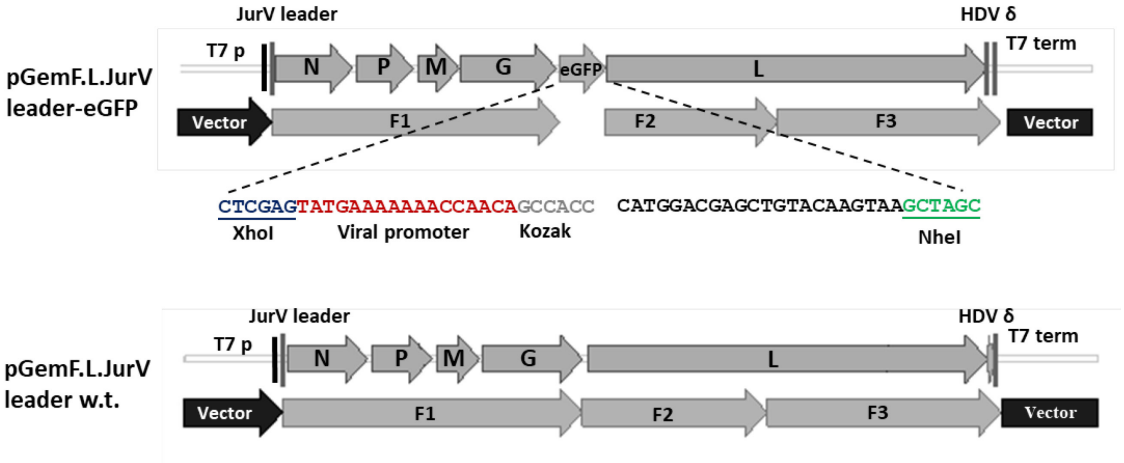


Figure 15: Schematic representation of the final full-length plasmids. The map showed the plasmids encoding Jurona eGFP vectors (pGemF.L.JurV leader eGFP and pGemF.L.JurVleader w.t.) The eGFP marker was introduced between G and L proteins in the intergenic region. The colored sequences indicate in blue and green the restriction sites, in red the intergenic viral promoter and in grey the Kozak sequence. The futures mentioned above were introduced within the primers used to amplify the eGFP. Both plasmids had the new discovered Jurona leader immediately downstream of the T7 promoter.

Moreover, in order to ensure that a high amount of proteins would be expressed during transfection and to make the production independent from the T7 RNAP, the virus helper proteins were also sub-cloned in a new vector under the control of the potent human CMV promoter by using Gibson cloning. The schematic map of the CMV plasmids encoding the viral N, P and L proteins is shown in Figure 16.

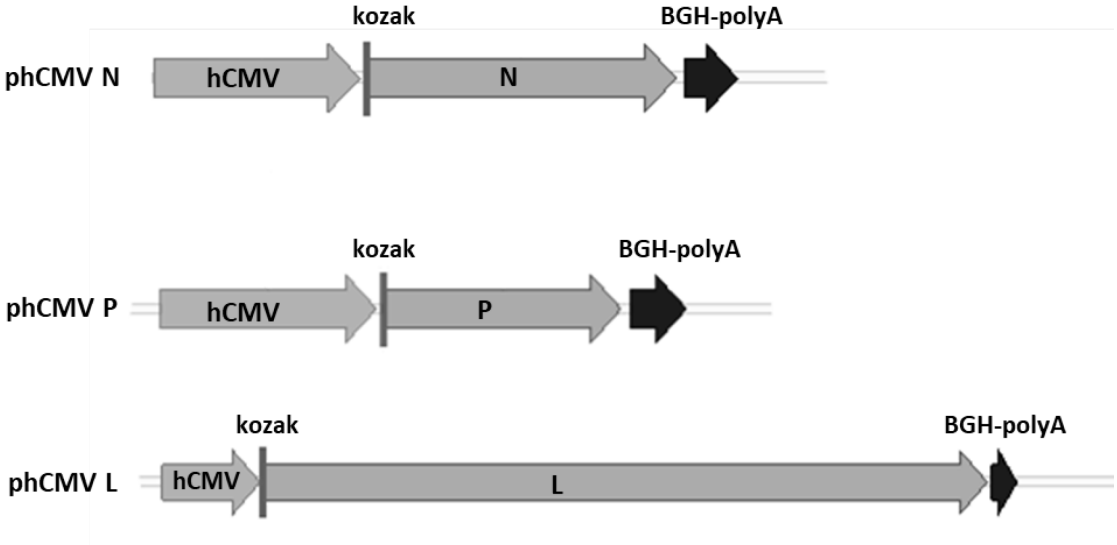


Figure 16. Schematic representation of the helper plasmids cloned under the CMV promoter. The plasmids encode the N protein, the P protein, and the L protein under the control of the human CMV promoter. A Kozak sequence was introduced to allow translation. A BGH polyA was introduced as a poly-adenylation signal.

4.7 Rescue of the Jurona vectors

Co-transfection experiments were performed using the newly generated full-length plasmids. While in the preliminary rescue experiments, we used Vero cells as host for the rescue procedure, in these new experiments, we employed Hek 293 T cells. This choice was supported by the high transfection efficiency required for a five plasmid co-transfection. Although Vero cells have been used in other rescue protocols, in our hands, they did not show good transfection efficiency. Furthermore, in some

preliminary experiments, we already showed that the Jurona virus could replicate in Hek 293. Different transfection conditions were explored to increase the probability of a rescue event (described in the Materials and Methods Section). We simultaneously tested two different types of helper plasmids: T7 promoter helper plasmids and the hCMV promoter helper plasmids to figure out which one would be more effective in producing the right amount of helper viral proteins. Cell lysates obtained 48 h after the transfection were used to infect fresh cells, and cultures were checked daily for CPE and eGFP expression. As shown in Figure 17 (B), 6 days after the infection, a clear cytopathic effect became visible in the cell monolayer infected with Jurona empty vector (pGemF.L.JurV leader w.t.), as a result of infectious viral particles formation and spread. Similarly, a clear CPE and eGFP expression were visible in the cell monolayer infected with the cell lysate obtained from cells transfected with the Jurona eGFP construct (Figure 17 (C, D)). In this case, viral vector rescue was observed a week later than the empty viral vector, presumably due to some attenuation of the virus replication caused by transgene insertion within the genome. Furthermore, for both wild type and the eGFP Jurona viral vectors, the CPE was visible in the samples in which the CMV helper plasmids were used to express the viral proteins. In contrast, no viral particles were produced in the samples in which T7 promoter plasmids were employed, even after longer incubation time in culture, suggesting that in our experimental conditions only the helper plasmids whose expression was led by the CMV promoter, were efficiently able to produce the correct amount of helper proteins required to form the RNP complex.

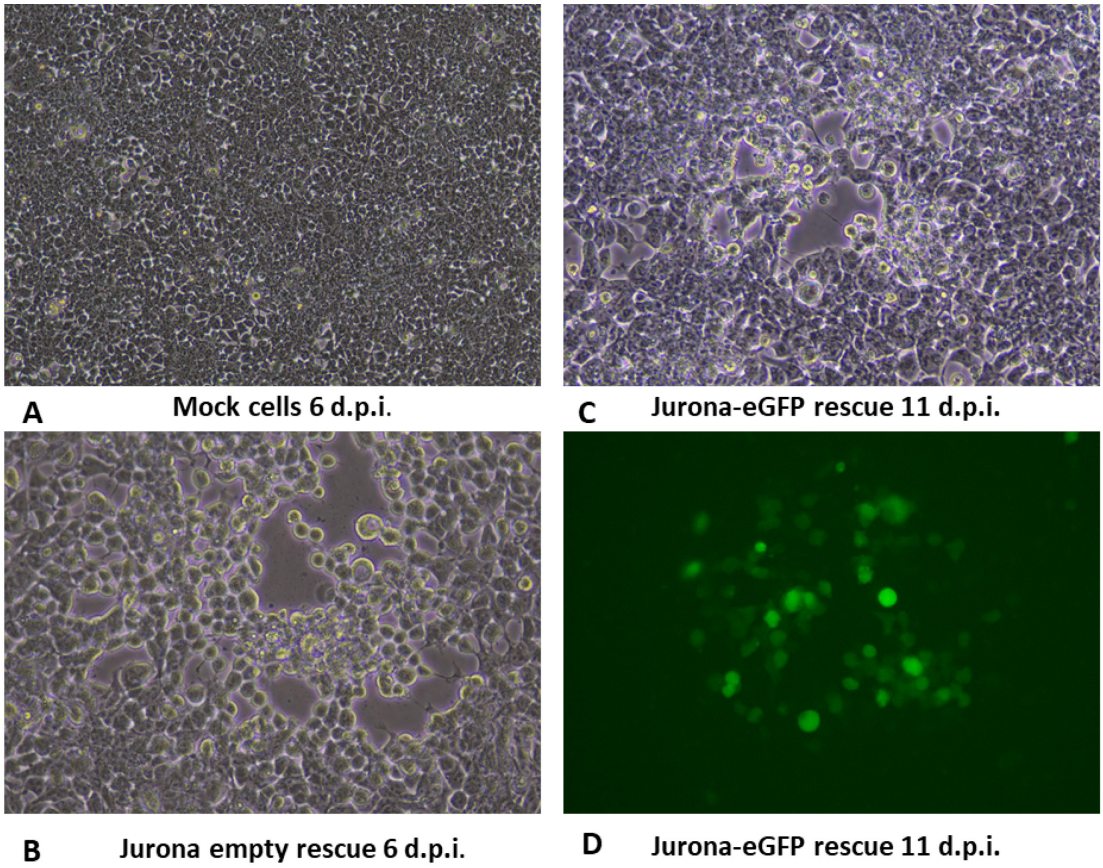


Figure 17: Jurona empty (pGemF.LJurVleader w.t.) and Jurona-eGFP vectors (pGemF.LJurVleader-eGFP) rescue in Hek-293T cells. 3E+06 cells were infected with Jurona empty, and Jurona-eGFP P0 cells lysates. (A) Mock infected cells. (B) CPE induced by Jurona empty vector 6 after the infection. (C, D) CPE and eGFP expression of Jurona eGFP vector 11 days after the infection.

4.8 Jurona vector characterization: infection assay, immunoblot, and sequencing

To characterize the vectors produced by the rescue system, we performed a western blot analysis. Jurona wild type and Jurona-eGFP vector infection mixtures were collected 11 and 19 days after the infection, respectively, when full CPE was observed. Then cells were lysed by freeze/thaw, and the bulk, the pellet, and the supernatant were tested for the presence of the Jurona Nucleocapsid protein by Western blotting using a

mouse polyclonal antibody. A cell lysate from wild type virus infected cells was used as control. As shown in Figure 18, a band migrating with the expected molecular weight of 46 kDa was detected in both Jurona-empty and Jurona-eGFP vector preparations. Most importantly, the blot showed that the virus is mainly extracellular as the band of the N protein was detected in the bulk and the supernatant, whereas it was absent or barely detectable in the cell pellet samples of both vectors.

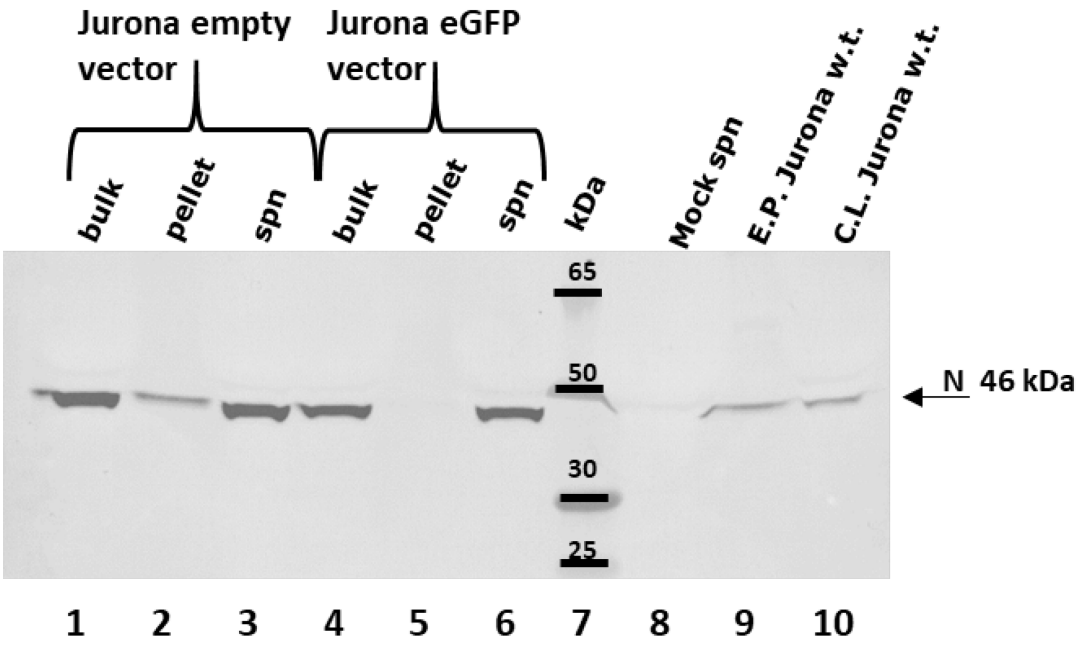


Figure 18: Western blot analysis of Jurona virus vector rescue samples. The bulk, the pellet, and the supernatant (spn) were loaded on a NuPage 4-12% Bis-Tris gel. The samples were analyzed using an anti N protein polyclonal antibody and anti-mouse-HRP. Lane 8: Mock spn, no-infected supernatant; lane 9: E.P., protein extract of cells infected with the Jurona original w.t. virus; lane 10: C.L., cells lysate of cells infected with the Jurona original w.t. virus. lanes 9-10 were used as positive controls.

Subsequently, we aimed at testing and comparing the productivity of the Jurona-eGFP vector in Hek 293T and in Vero cells. To this end, the Jurona-eGFP virus obtained from the rescue was passaged on Hek 293T and on Vero cells. The cells were infected with the same amount of cell lysate, and the ability to replicate and spread was evaluated. A CPE was already visible in culture 24h after the infection, and the eGFP transgene was expressed in both cell lines, as confirmed by fluorescence microscopy

(Figure 19). As shown in Figure 19, the number of eGFP expressing cells appeared to be higher in the Vero infected culture, suggesting that Vero cells have a higher competence for the Jurona-eGFP vector production.

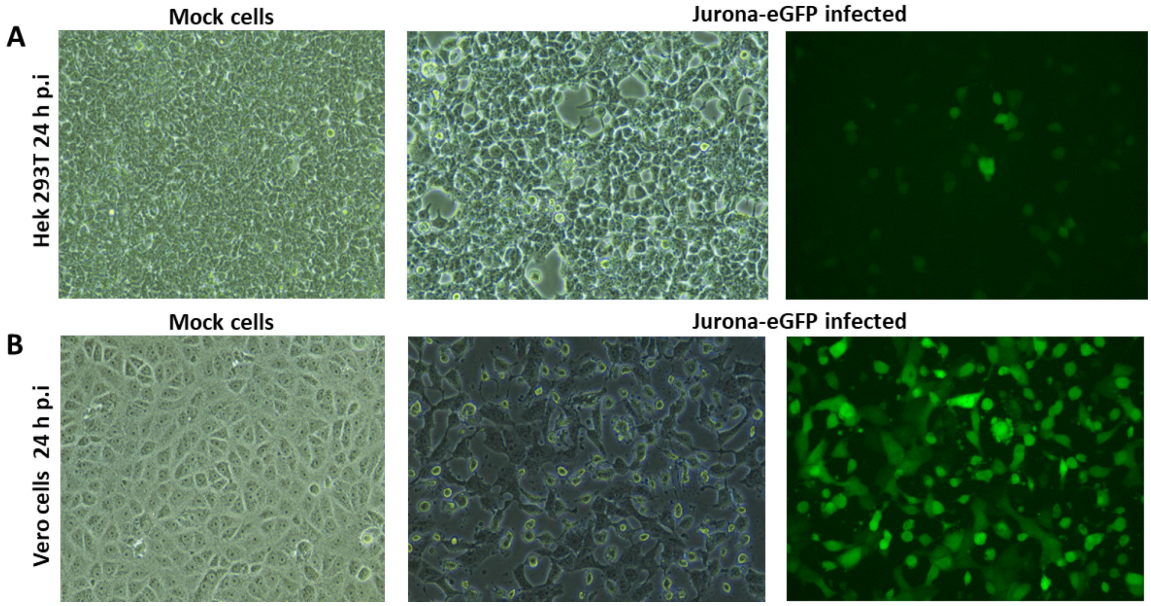


Figure 19. Jurona-eGFP infected cells. 3E+06 cells of Hek293T cells and Vero cells were infected with Jurona-eGFP cell lysate (P1) at no-controlled MOI. The cells were checked 24h later and the induced CPE and the eGFP expression evaluated by microscopy.

To compare the physical viral titer produced in both cell lines, we extracted the viral RNA from the supernatant of infected cells and analyzed by One Step RT ddQ-PCR by using a Taq-man probe designed on the N protein-coding sequence. As shown in Figure 20, the concentration of Jurona-eGFP viral particles (vp/ml) produced on Vero cells and on Hek 293T cells was almost the same, with no significant difference between the two cell lines. However, that productivity was reached on Vero cells a day before than Hek 293 as the infections were collected at full CPE 48h and 72h respectively for Vero and Hek293T cells. These data confirmed that Vero cells can support a faster and better replication of Jurona vector, leading to a rapid spread of the virus and complete clearance of the cells in 48 h.

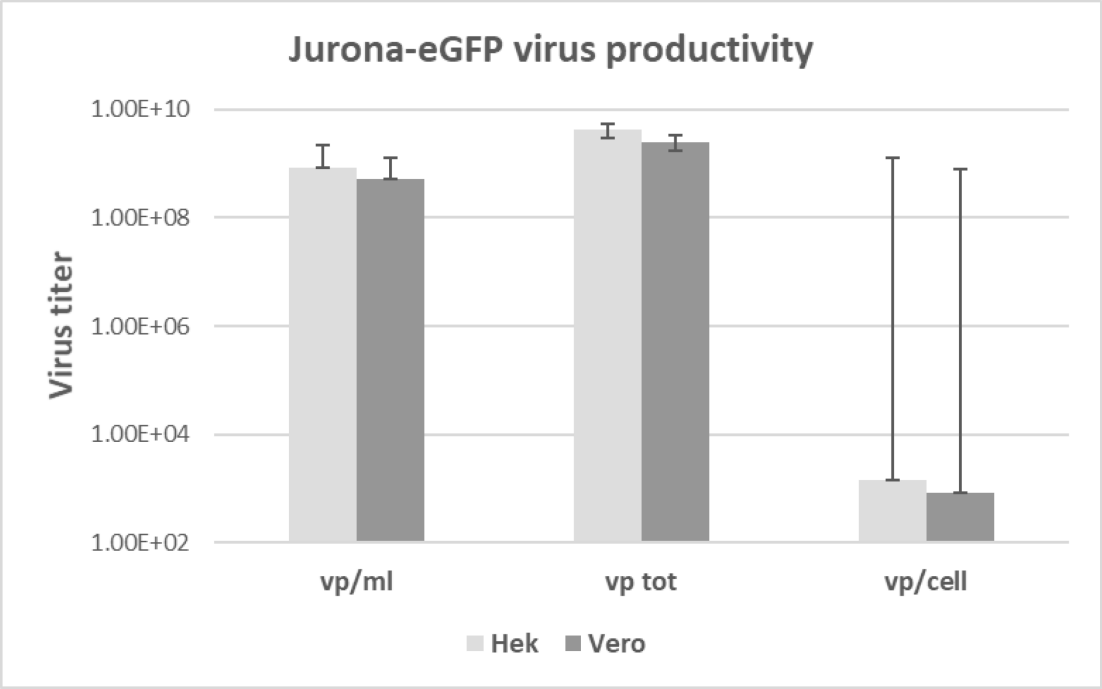


Figure 20. Quantification of viral vector titer of Jurona-eGFP virus in cell culture. Replication of Jurona-eGFP virus in Hek 293T and Vero cell lines. The viral vector titer (vp/ml) was analyzed by a One-step RT digital droplets qPCR and expressed in vp/ml, total vp, and vp per infected cells. The viral RNA was extracted from the supernatant of infected cells and analyzed. The vp/ml was the result of the average of the viral particles obtained from three independent viral vector dilutions loaded in duplicate.

Finally, to confirm the identity of the viral vector generated during the rescue, the Jurona eGFP genome was analyzed by Next Generation Sequencing (NGS). The viral RNA was extracted from a purified virus stock and treated with DNase I, in order to eliminate the genomic DNA contamination, that would have affected the sequencing result. Then, the extracted RNA genome was RT-PCR amplified using a genome-specific primer, and the purified products were analyzed.

The sequencing results showed that the vector produced from the rescue had the same sequence introduced in the full-length plasmids, and no additional mutations were found. This result confirmed that the viral vector generated during the rescue remained stable even after multiple passages in culture.

4.9 Viral vector production, purification, and titration

To set up a purification method for the Jurona vectors, we produced a small scale of the Jurona-eGFP vector by using Vero cells, which proved to be the most efficient cell substrate. Cells were grown in adherence in 150 dishes and infected at 0.01 MOI, using DMEM supplemented with 5% FBS. According to Diallo (2012), by using this MOI, the possibility to produce defective interfering particles (DI) was remarkably reduced in purified preparations of recombinant VSV vectors. DI particles were discovered to be the main product resulting from infections at a high multiplicity of infection. They can reduce the yield of infectious virus thereby interfering with normal virus replication. The virus was collected from the supernatant 48 h after the infection at 90% CPE and subsequently filtered on a 0.22 filter and then purified on a Iodixanol gradient, to eliminate cellular contaminants and further centrifuged to increase concentration (see Materials and Methods). In the end, samples from each production step were collected and analyzed to estimate the productivity, the yield, and the quantity of the total proteins. The data reported in Table 4 showed that the titer of the purified virus was higher than in the supernatant, as a result of the virus concentration. Moreover, although the total yield of the process was not highly efficient (11% in viral particles (vp) and 17% in Focus forming unit (FFU), most of the virus produced and purified was infectious, as shown by the infectivity ratio (vp/FFU).

A

Samples	vp/ml	vp tot	vp/cells	yield vp %	Volume (ml)
Filtered	2.10E+09	3.41E+11	2.38E+03	100%	162
Concen	5.53E+09	1.99E+11	1.39E+03	58%	36
Purified	1.64E+10	3.61E+10	2.53E+02	11%	2

B

Samples	FFU/ml	FFU tot	FFU/cells	yield FFU %	vp/ifu
Filtered	8.67E+08	1.40E+11	9.82E+02	100%	2.4
Concen	3.45E+09	1.24E+11	8.69E+02	88%	1.6
Purified	1.09E+10	2.40E+10	1.68E+02	17%	1.5

Table 4: Productivity and purification of the Jurona-eGFP vector. The titer was evaluated by RT-dd-QPCR(A) and by TCID50 (B) on samples from the purification process. The vp/ml was the result of the average of the viral particles obtained from

three independent viral dilutions loaded in duplicate. The Spearman & Kärber algorithm calculated the FFU/ml.

To analyze the degree of the vector purification from the cellular proteins, we analyzed the final product on an SDS-page. 5E+07 viral particles from each sample were loaded on a 4-12% polyacrylamide gel, and the gel was stained with a Coomassie brilliant blue. As reported in Figure 21 (A), there was a decreased staining of the total cell proteins in the purified virus compared to the bulk sample, indicating that most of the cellular proteins were eliminated during the purification. Furthermore, the western blot analysis made with anti-N and anti-G proteins on the same samples showed the presence of bands of the viral proteins migrating with the expected molecular weight (Figure 21 B).

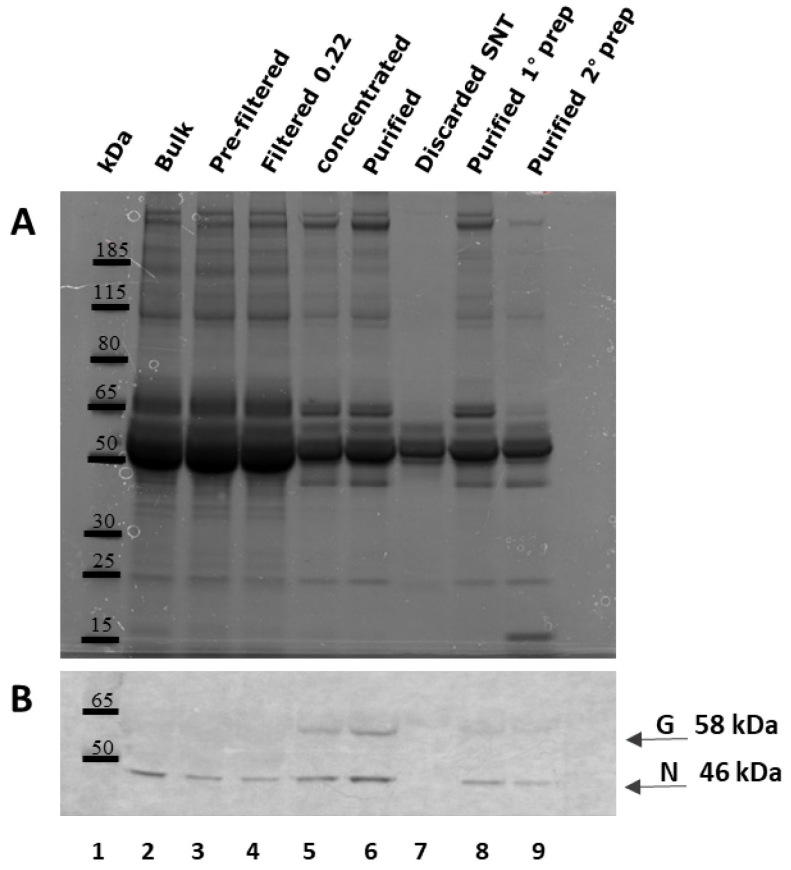
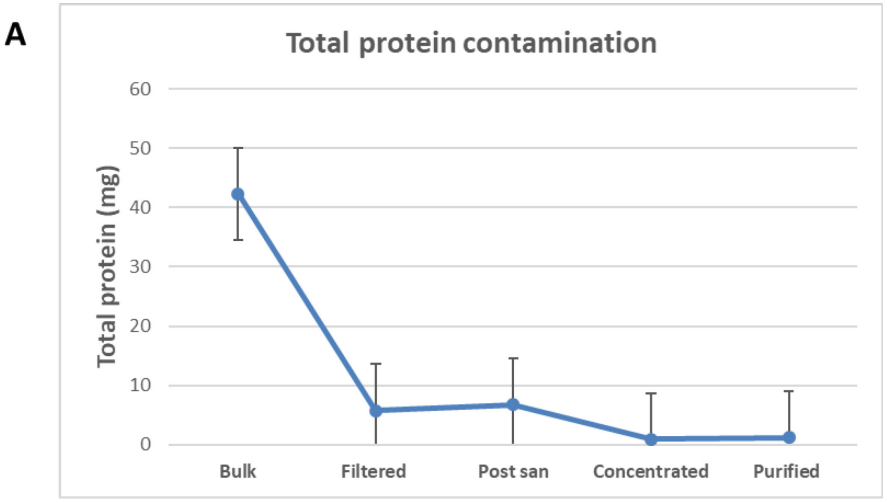


Figure 21: Analysis of the total proteins in purified samples of the Jurona eGFP vector. A) 5E+07 vp for each sample were loaded on sodium dodecyl sulfate-polyacrylamide gel (NuPage 4-12% BisTris). The gel was stained with Coomassie

Brilliant Blue. B) Western blot analysis of the same samples with anti N and anti G proteins of Jurona virus.

To improve the quality of the purification process, we aimed at performing viral vector infection in the absence of serum. To this end, Vero cells were sequentially adapted to grow in adherence without serum in VP-SFM, an ultra-low protein (5 µg/ml) medium designed for the growth of Vero cells for virus production (Frazzati-Gallina 2001). First, the cells were sub-cultured in conventional medium with 5% serum into a 25:75 ratios of complete VP-SFM to the original media. Next, as the growth rate was not affected, the ratio of VP-SFM to the original medium was increased to a 50:50, followed by a 75:25 and by 90:10 until the cells were transferred into 100% VP-SFM medium. After the cells were wholly adapted, they were used to produce a new small scale of the Jurona-eGFP vector. A step of host DNA digestion was also introduced in the purification process. Finally, to analyze the improvement performed on the vector production, the quantity of the total proteins at each step of the process was again evaluated by using an SDS page and a Bradford assay. As shown in Figure 22 (B), the proteins in each sample were significantly reduced, as the staining on the gel was very low. Moreover, the proteins were better detected in the concentrated and purified samples, where the vector and consequently, the proteins were more concentrated as a result of the purification process. Also, the measurement of the total proteins by a Bradford assay expressed as total mg (Figure 22 (A) confirmed a robust total protein reduction in the final purified samples compared to the bulk. These results demonstrate that the Jurona-eGFP vector can be produced efficiently in medium without serum, maintaining high productivity and with a reduced level of host cell protein contamination.



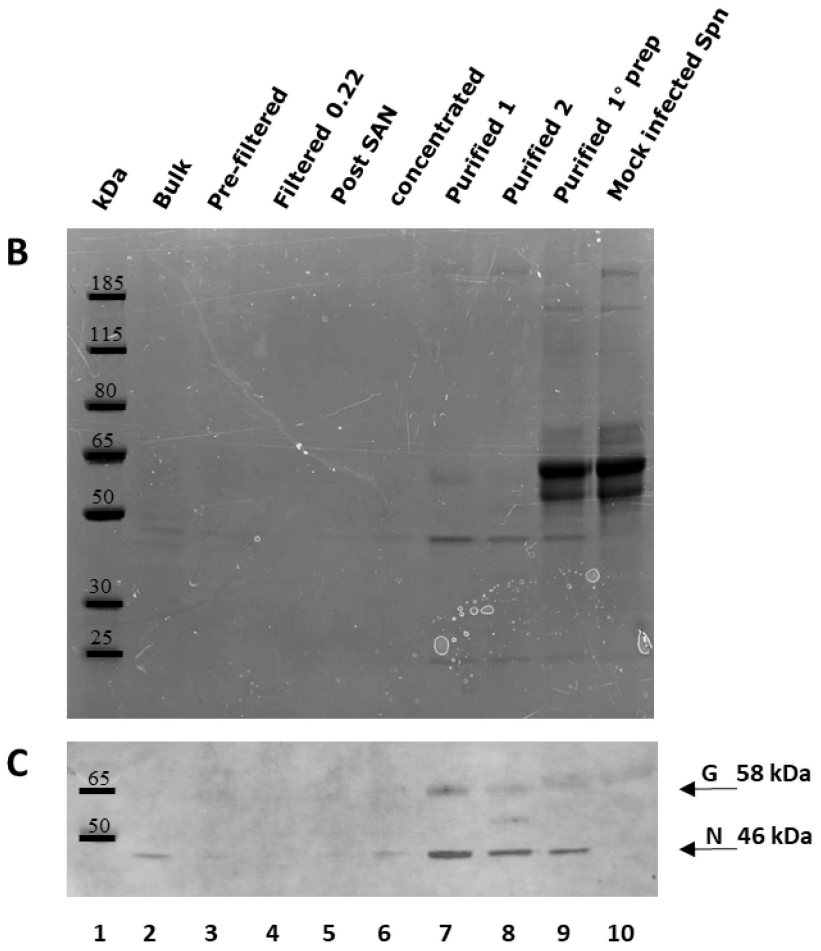


Figure 22: Total proteins in samples of Jurona-eGFP vector preparation. A) Bradford assay on samples of the Jurona-eGFP vector purification. The protein concentration (mg) was calculated by a Bradford assay applying a BSA standard curve and multiply the concentration obtained for the volume of each sample. Aliquots of the bulk, filtered, post-SAN treated, concentrated, and purified virus from different samples from Jurona-eGFP vector preparations were quantified. B) 5E+07 vp for each sample were loaded on sodium dodecyl sulfate-polyacrylamide gel (NuPage 4-12% BisTris). The gel was stained with Coomassie Brilliant Blue. C) Western blot analysis of the same samples with anti N and anti G proteins of the Jurona virus.

5. DISCUSSION

Recombinant viral vectors are a powerful tool for the development of novel therapeutics (i.e.: oncolytic viruses and genetic vaccines) as they could target several cell types, express heterologous proteins *in situ* thereby inducing potent humoral and cellular immune responses, as well as infect and kill tumor cells.

One of the limitations of this technology is the presence of pre-existing immunity for those vectors that are derived from viruses circulating in the human population. Furthermore, anti-vector immunity may hamper re-administration of the same vector to improve therapeutic efficacy. Strategies to overcome these limitations include the use of viruses of non-human origin and the use of different vectors carrying the same cargo in heterologous regimens of administration. To this end, new viral vector platforms are needed to achieve more effective treatments.

Rhabdoviridae represents a new source of viruses for vector development because they are easy to manipulate, they grow to high titers and have a cytoplasmic lifecycle without host genome integration. Furthermore, many of them are not pathogenic to humans and being of animal origin, there is no pre-existing immunity against them in the human population.

In this study, we have described the generation and initial characterization of a new recombinant viral vector platform based on the Jurona virus, a member of the Rhabdoviridae family, that could be employed as an oncolytic virus, as a genetic vaccine or as a vector encoding therapeutic proteins.

Our first aim was to select a virus that was able to replicate to high titers in a validated cell line for the production of biologic materials for human use, thus facilitating the transition to a drug development phase. Since Rhabdoviruses replicate rapidly and to high titer in mammalian cells, we selected five Rhabdoviruses from the ATCC collection and tested them for their growth properties in Vero cells, a validated cell line for production of biological products. Among those viruses able to replicate and to induce an evident CPE, Jurona was the fastest, giving rise to a full CPE within three days from the infection. As Jurona was classified as vesiculovirus by a previously serological test in early identification, we thought that it could share the same properties of the related VSV and Maraba vesiculoviruses. For these reasons, we chose the Jurona virus as our candidate for vector construction with the expectation that it would perform well as an oncolytic virus and/or as a genetic vaccine carrier. Therefore, we preliminarily characterized the oncolytic activity of the Jurona virus by infecting two tumorigenic cell lines: Hela and Hek293T. As we expected, Jurona appeared to be very

efficient at killing the cells that we used as a tumor model, as it was able to induce a strong oncolysis with decreased cell survival after three days post-infection, thus demonstrating that we might use it as a possible oncolytic vector candidate. After that, we established a reverse genetic system based on the T7 RNAP expression strategy for the recovery of the Jurona vector in culture using five DNA plasmids. Most of the reverse genetic systems developed up to date for negative-stranded RNA viruses use a helper vaccinia virus to express the T7 RNAP in the production cells. However, introducing a helper virus to rescue a recombinant viral vector is not ideal as it would add complexity to the subsequent downstream purification process. Furthermore, the extensive CPE induced by the vaccinia virus during the rescue would limit the possibility of maintaining the transfected cells in culture for more prolonged incubation, thus potentially affecting the recovery of the recombinant vector. Hence, to deliver T7 RNAP intracellularly during the rescue, we designed and constructed a plasmid expressing the T7 polymerase under the control of the strong human CMV promoter, which can achieve high levels of T7 RNAP in the host cells.

To express the full-length Jurona anti-genome, we first generated two plasmids: one expressing the wild type anti-genome and the second including two point mutations in the M and G proteins (Fig. 10 (A, B)). Both plasmids had the viral anti-genome cloned downstream the T7 promoter and upstream the HDV ribozyme sequences and T7 terminator signal. By including these features we sought to produce a viral transcript into the cytoplasm with correct viral ends, an absolute requirement for efficient encapsidation of the genome.

The construction of the full-length plasmid initially presented several issues. The first report that described the construction of a plasmid for recovering of recombinant VSV vector (Rose 1996) adopted a nine-step cloning procedure using standard ligation reactions. Tobias (2016) recently proposed a straightforward strategy for the reverse genetic of Rabies virus, that introduced the full-length anti-genome directly into the cloning plasmid by amplifying the full-length anti-genome from the cDNA and by recombination cloning. Unexpectedly, by adopting this strategy, we could not amplify the full-length anti-genome from the viral cDNA presumably because of insufficient polymerase efficiency leading to low viral cDNA quality. Therefore, we subsequently adopted a new cloning strategy based on the Gibson assembly method. This protocol has the advantage of joining multiple adjacent fragments in a unique isothermal reaction without the need to use restriction enzyme sites. By applying this method, we successfully produced a full-length plasmid expressing the complete Jurona anti-genome starting from three overlapping fragments (Fig. 10 A).

We introduced two point-mutations in the M and G proteins to generate a better replicating virus in tumor cells while being attenuated in normal cells, thereby resulting in a form of the vector with improved safety. Brun (2010) reported that both L123W in the M protein and H242R (Q242R in Maraba) in the G protein were individually

able to increase VSV replication in BHK-21 cells. While each of these two mutations individually had no significant effect on the efficiency of replication of Maraba virus, the combined presence of both mutations did improve the replicative activity of the virus in tumor cell lines, but did not increase the cytolysis of primary human fibroblast cells. Therefore, by aligning the amino-acid sequences of the M and G proteins with those of VSV and Maraba viruses, we identified the mutations L123W in the M protein and N242R in the G protein as the potential candidates for generating Jurona variant with a cancer selective replication. Finally, we also generated the plasmids expressing the Jurona helper viral proteins (N, P, and L) required for vector rescue. The original rescue system applied for VSV by Lawson (1995) used the T7 promoter to direct the expression of the viral proteins in pBluscript plasmids. To follow the same successful strategy, we cloned the cDNA of the Jurona helper proteins under the control of the T7 promoter in pGem plasmids. However, since we decided not to use a vaccinia virus that can add cap and methylation to the transcripts within the cytoplasm, and to guarantee the translation of the proteins from the uncapped transcripts, we introduced an IRES sequence upstream of the coding sequence to enable translation even without the 5'cap.

Although several papers reported successful rescue using plasmids encoding the helper proteins under the control of a T7 promoter, they always used as a source of T7 RNAP a vaccinia helper virus that notoriously expresses a high level of the transgene in the infected cells. In the T7 RNAP based reverse genetics system, the expression level of the helper proteins relies on the effective production of the polymerase into the cytoplasm. As we could not predict if the levels of helper proteins obtained by this system would be sufficient for the rescue of the Jurona vectors, we also produced other three plasmids where the Jurona helper proteins N, P, and L are under the control of the potent human CMV promoter.

We then set up a co-transfection protocol to rescue recombinant viral vectors based on the Jurona virus. Several protocols were already published that used the Vero cell as the primary host for the recovery procedure of the related VSV. Thus, we adapted the standard protocol for VSV recovery to our conditions using Vero cells as the primary host for transfection, as we already demonstrated that Jurona replicates very well in this cell line. Our initial rescue experiments were utterly unsuccessful. Indeed, we could not produce any recombinant vector either using the full-length plasmid expressing the wild type Jurona anti-genome or the attenuated cancer-selective form. By analyzing the viral RNA genome copies, we demonstrated that no rescue events were initiated in the transfected cells and that the genome copies detected in the analysis came from the amplification of the transfected plasmid and its transcripts.

Successful rescue of viral vectors by reverse genetics requires several events to concurrently occur within the cytoplasm to guarantee the start of the replication cycle from plasmid DNA: i) high efficiency of transfection; ii) high expression of T7 RNAP;

iii) high and correct expression ratios of helper proteins, and iv) correct viral ends formation. Even by assuming that all these events did not efficiently occur in our system, we should have expected at least one recombinant viral particle capable of spreading in culture.

To find an alternative explanation, we went back to analyze our strategy, starting from the analysis of the Jurona Genebank reference sequence used to construct all our plasmids. By multiple alignment of the Jurona reference sequence with those of other Rhabdoviruses such as VSV and Maraba, we found that the annotated Jurona sequence had a shorter 3'terminal region. We therefore hypothesized that the annotated sequence was lacking an essential element for replication thereby explaining our failure to rescue the Jurona vector. In fact, in the standard Rhabdovirus genome organization the 3'terminal region usually contains sequences essential for viral replication and transcription. By using an end-joining RT-PCR sequencing strategy, we could demonstrate that the original Jurona wild type virus had an additional 55 nucleotides at the 3'end that were not previously known and were not present in the Genebank reference that we used to synthesize the complementary anti-genome.

We therefore introduced the new leader sequence in the full-length plasmid. To have a marker allowing the easy monitoring of viral replication and spread during the rescue, we also introduced in the full-length plasmid the eGFP as an additional open reading frame in the intergenic region of G and L viral proteins. According to Wollmann (2010), the insertion of a heterologous gene into the viral genome can attenuate viral replication. This effect is higher if the heterologous gene is placed upstream of all the viral genes. Thus, by introducing the eGFP in an intermediate genome position within the intergenic region of the G and L proteins, we sought to produce an attenuation of the vector still preserving good productivity and functional expression of the encoded gene marker.

We then used the full-length plasmids, carrying the newly identified Jurona leader to rescue the Jurona vectors. In the preliminary rescue experiments performed on Vero cells, along with no viral particles formation we also observed a low transfection efficiency, even using a single plasmid transfection (data not reported). Although Vero cells have always been used for the primary recovery of other negative-stranded RNA viruses, in our hands by using Lipofectamine they did not efficiently support the rescue based on a five plasmids co-transfection. Therefore, to increase the efficiency of a rescue event, we performed the rescue experiments using Hek293T cells instead of Vero cells. We had preliminary confirmed that Jurona could replicate in Hek 293T cells (data not shown), moreover, as an improvement of the system, we tested in the same experiments the two different helper plasmids previously generated: T7 helper plasmids and CMV helper plasmids. We observed a clear CPE in culture for the Jurona-empty vector six-days post transfection, witnessing the generation of viral particles and their spread. As expected, for the Jurona-eGFP vector, we observed the CPE and the

eGFP expression a week later than the empty vector. This delay confirmed the attenuation that we hypothesized to be induced by the introduction of an additional gene within the genome.
The successful rescue obtained for Jurona-empty and Jurona eGFP vectors definitively demonstrated that the leader is a crucial sequence for virus replication, as the lack of the entire region in our full-length plasmid did not enable correct viral particles formation in preliminary rescue experiments.
Importantly, the CPE and consequently, the viral particle formation occurred only in those cultures were the helper viral proteins were expressed from the CMV promoter. This observation indicated that only the helper plasmids with the CMV promoter were effectively able to produce the right amount of viral proteins required to start the virus replication cycle, indicating that the level of the helper proteins within the cells was another critical factor that could seriously affect the rescue procedure.
We could also detect good levels of eGFP expression by the Jurona eGFP vector indicating that the Jurona system could be used to deliver therapeutic proteins *in vivo*.

By a western blot analysis, we showed correct expression of the Nucleocapsid protein by the recombinant Jurona vectors. By the same analysis we could also observe that the virus localized almost entirely in the supernatant. This property allows the virus to be purified from the supernatant without completely lysing the infected cells and releasing immature viral particles.

By an infection assay and by physical viral particle quantification, we identified the best cell line for Jurona vector production. Although the Jurona-eGFP vector could replicate and spread in both Vero and Hek 293T, we found that Vero cells could support a faster replication of the vector, thus reducing the production time. Furthermore, by Next Generation Sequencing, we could confirm that the recombinant Jurona-vector had the same nucleotide sequence of the original plasmid employed for the rescue procedure, highlighting the stability of our vector even after different passages in culture.

We established a protocol to produce and purify a small scale preparation of the Jurona-eGFP vector. By infecting Vero cells in adherence at low MOI (0.01) and by using a two-step of Iodixanol gradient ultracentrifugation, we obtained a purified preparation of Jurona-eGFP vector. Interestingly, by analyzing the physical viral titer and the infection titer we observed that the vector produced by this process was highly infectious as the ratio of the total viral particles produced and the infectious particles (ratio vp/FFU) remained quite low.

By loading the samples of each step of the purification on an SDS gel, we noticed that despite the purified sample presented a lower level of total proteins than the bulk, several host proteins remained in the purified sample. Therefore, we transitioned to a production process based on Vero cells previously adapted to grow in a virus production medium without serum (VP-SFM). By analyzing the total amount of

proteins in bulk and purified samples, we observed a substantial reduction in the level of total proteins in the purified sample as compared to the bulk. Although we produced the Jurona-eGFP vector without FBS, we could achieve a vector preparation that was infectious and had a reduced level of cellular contaminants. These preliminary data suggested that we could quickly produce the Jurona-eGFP vector without serum, allowing the use of suspension cultures.

6. CONCLUSIONS

In this study, we generated a new vector platform based on the Jurona virus, a member of the Rhabdoviridae family, closely related to VSV and Maraba viruses. By adopting this new recombinant viral vector, we will be able to design new oncolytic viruses and genetic vaccines encoding antigens or immune-modulators, thus extending the platforms currently available for therapeutic use. This new vector has the advantage of having a small genome of about 11 kb that can be easily manipulated using molecular cloning and rapidly manufactured using methods applicable to an industrial process. Our reverse genetic strategy employed the T7 RNAP to direct the expression of a cDNA clone of the Jurona viral anti-genome within the cytoplasm. In particular, a T7expressing plasmid fulfilled the function of the T7 RNAP in a helper virus-free manner. We established a 5-plasmid co-transfection method. We proved that this system could successfully produce recombinant viral vectors based on the Jurona virus from the transfected cell when all the critical components are provided within the cells in the correct ratios. Furthermore, we identified a previously unknown leader region in the Jurona virus that is essential for replication and allowed us to recover recombinant Jurona-empty and Jurona-eGFP vectors by using the reverse genetic system that we have developed. We also confirmed that the vector could be produced in Vero cells to high titer and that the virus can be purified, maintaining high infectious capability. Finally, our preliminary data in HEK 293 and HeLa cells hold the promise for this vector to represent a suitable candidate to develop a tumor selective lytic virus.

To consider the Jurona vector as a candidate for further development, we need to continue to analyze its properties. Therefore, the next objectives of our work will be a detailed characterization of the oncolytic activity of the Jurona vector with and without attenuating mutations. We will also make further efforts to prove the effectiveness of the Jurona vector at inducing antigen-specific immune response against a model antigen, by using different immunization protocols alone or in combination with other vectors to develop new effective vaccination strategies. Moreover, although we could demonstrate that a reverse genetic rescue system works, the current protocol is still not optimized for the large scale manufacturing of a product for human use. Therefore, different strategies will be adopted to improve the rescue system including a novel complementing cell line expressing the helper functions.

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