Trying to treat the untreatable: Experimental approaches to clear rabies virus infection from the CNS

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# Abstract

Rabies virus causes an invariably fatal encephalitis following the onset of clinical disease. Despite the availability of safe and effective vaccines, the clinical stages of rabies encephalitis remain untreatable with few survivors being documented. A principal obstacle to the treatment of rabies is the neurotropic nature of the virus, with the blood-brain barrier size exclusion limit rendering the delivery of antiviral drugs and molecules to the central nervous system inherently problematic. This review focuses on efforts to try and overcome barriers to molecule delivery to treat clinical rabies and overviews current progress in the development of experimental live rabies-virus vaccines as treatments for clinical rabies, including the attenuation of rabies virus vectors through either the duplication or mutation of existing genes or the incorporation of non-viral elements within the genome. Rabies ‘Post-Infection Treatment’ (PIT) remains the holy grail of rabies research.

# Introduction

Rabies, caused by all members of the lyssavirus genus, is a significant zoonosis affecting both human and animal populations globally with an estimated 60,000 human deaths annually ([1](#_ENREF_1), [2](#_ENREF_2)). The lyssavirus genus belongs to the *Rhabdoviridae* family of viruses*,* and as such, lyssaviruses contain a negative-sense single-stranded RNA genome encoding five viral genes: N (nucleoprotein), P (phosphoprotein), M (matrix), G (glycoprotein), and L (large) (See Figure 1) ([3](#_ENREF_3)). Rabies virus (RABV) is the prototypic virus of the lyssavirus genus with well-defined epidemiology in both volant and non-volant species. However, the epidemiology of the remaining lyssaviruses, 16 distinct viral species that have been most often reported from bat species, remain largely undefined with often only single isolates being available. Furthermore, although a handful of human fatalities have been attributed to lyssavirus infection other than RABV, the primary diagnostic assay for detection of virus antigen does not discriminate between lyssavirus species, meaning infection with other lyssaviruses is likely often underestimated.

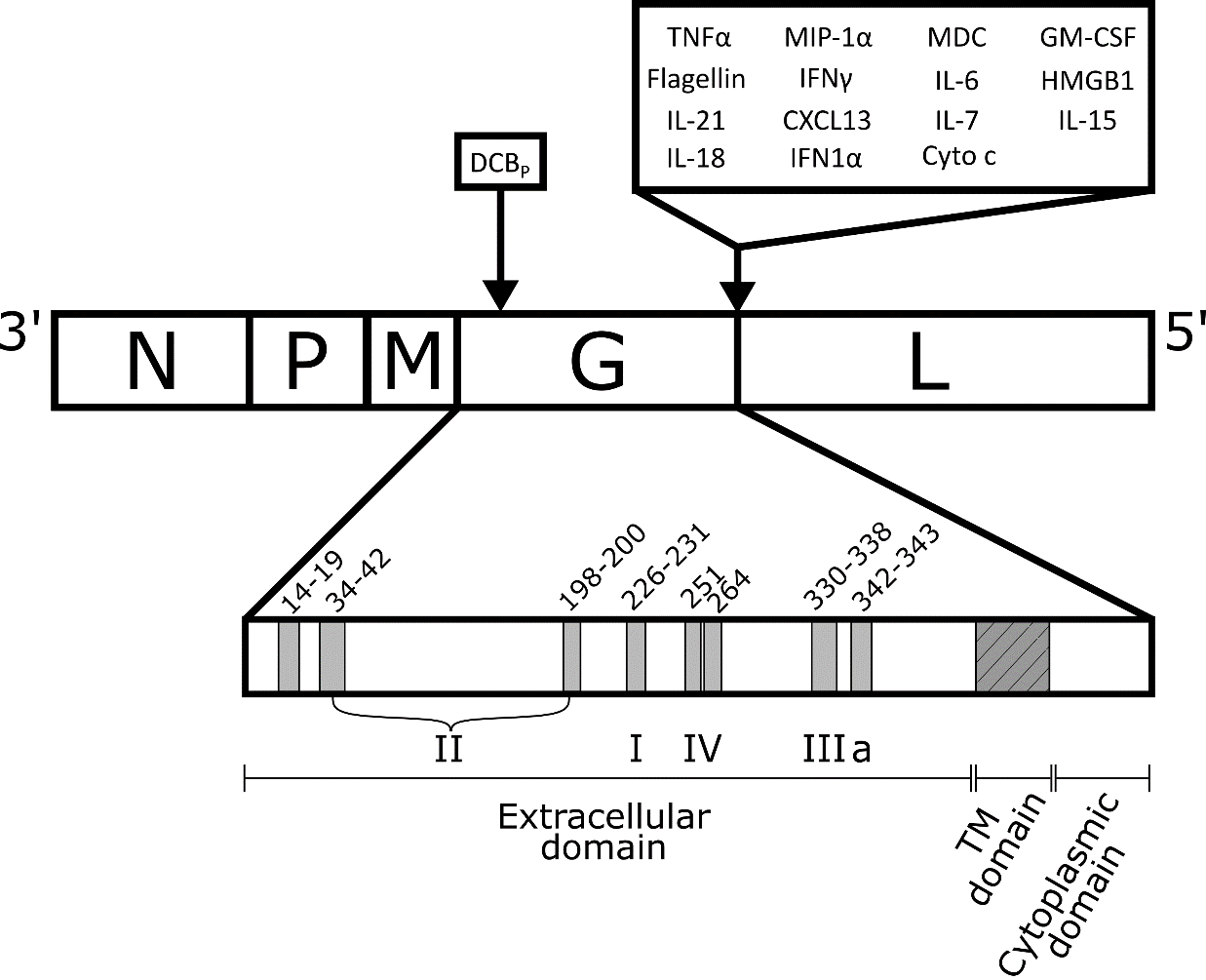


Figure 1: A diagram of the rabies virus genome, including a schematic illustrating the various domains and antigenic sites of the G protein, and details of the insertion sites various non-viral elements.

Although RABV causes thousands of human deaths annually, safe and effective RABV vaccines that confer effective neutralising antibody responses for both pre-immunisation (PrEP) and post-exposure prophylaxis (PEP) have been available for decades ([4](#_ENREF_4), [5](#_ENREF_5)). For human use, these vaccines consist of an inactivated preparation of whole virus antigen that stimulates a neutralising antibody response to the viral glycoprotein, the major viral antigen processed by the host to generate neutralising antibodies. Live attenuated virus vaccines (LAVVs) have not been licensed for human use through concerns of insufficient inactivation and reversion to virulence. Despite this, live attenuated virus preparations have been used extensively in vaccination of wildlife, although occasional occurrences of clinical disease have been reported ([6-8](#_ENREF_6)). PrEP with inactivated vaccines is practised for individuals considered to be at high risk from infection in either an occupational capacity or through travel to endemic regions ([9](#_ENREF_9)). Due to the observation that in endemic regions, children under the age of 14 constitute up to 40% of recorded fatalities, the utility of rabies vaccination in paediatric regimens has been proposed ([10](#_ENREF_10)). Although PrEP is not practised as a general recommendation in endemic areas. PEP is recommended following potential virus exposure and, if administered promptly, is 100% effective in preventing the development of clinical disease ([11](#_ENREF_11)). PEP consists of a regulated regimen defined by the World Health Organisation (WHO) that depends on the degree of exposure ([12](#_ENREF_12)). PEP often consists of vaccination alone where potential exposures through breaches of the dermal barrier are minimal, although in more severe exposures (category III exposures), PEP consists of both infiltration of rabies immunoglobulin (RIG), either human (HRIG) or equine (ERIG), in and around the wound and vaccination at a site distant to the wound ([13](#_ENREF_13)). However, while PEP is available there are restrictions around its utilisation; 1) PEP is only effective if given before the onset of clinical disease ([14](#_ENREF_14)); 2) PEP is often not immediately available in endemic areas; 3) A lack of awareness of rabies virus and the fatal disease it causes can mean that PEP is not sought following exposure; and 4) Even where PEP is sought, it is often administered incorrectly. Furthermore, costs associated with vaccine and RIG can be prohibitively high. For example, despite the lower cost of ERIG ($25-50 /dose) compared to HRIG ($100-250 /dose), ERIG is still unaffordable in many countries ([13](#_ENREF_13), [15](#_ENREF_15), [16](#_ENREF_16)). A proposed alternative to RIG is RABV G specific monoclonal antibodies (MAb). Suggested MAbs are highly potent and able to neutralise a wide range of lyssaviruses. In an effort to overcome the expense and short supply of RIG, in 2002, the WHO recommended several MAbs for investigation for inclusion in MAb cocktails ([17](#_ENREF_17)). These MAb cocktails were broadly neutralising with results comparable to HRIG ([18](#_ENREF_18), [19](#_ENREF_19)). Further studies have indicated that the MAb-based approach can be improved through reduction in production cost through the use of novel plant-based expression systems such as *Nicotiana benthamiana,* which can be biotechnologically up-scaled at little cost ([20](#_ENREF_20), [21](#_ENREF_21)).

Despite the tools available to prevent the onset of disease, rabies ‘post-infection treatment’ (PIT) remains problematic. Where human survival has been reported only a small number of cases, associated with either dog or bat rabies, have been described ([22](#_ENREF_22)), although often following syndromic assessment rather than ante-mortem diagnosis using defined diagnostic techniques that are required for confirmatory diagnosis ([23-32](#_ENREF_23)). In 2004, a potential breakthrough life-saving treatment for patients exhibiting clinical rabies was announced, entitled the “Milwaukee protocol” ([23](#_ENREF_23)). This led to the survival of a young patient through induction of a therapeutic coma, although numerous attempts to employ this approach since have failed and as such this protocol is no longer recommended for rabies PIT ([33](#_ENREF_33), [34](#_ENREF_34)).

Regardless of PEP administration, almost all cases require long-term rehabilitation. Of interest, factors thought to influence a positive outcome in such treatments include the age of the affected individual, immunocompetence, the early detection of neutralising antibodies in cerebral spinal fluid and serum, infection with a bat rabies virus variant, and diagnosing the disease in the early stages where only a mild neurological illness has developed ([35](#_ENREF_35)). Numerous experimental studies have since attempted to assess different molecules in their ability to prevent rabies replication in vivo but with little success in experimental models for rabies PIT ([36-38](#_ENREF_36)). No alternatives have shown significant value as rabies PIT and as such, the outlook for those that have developed clinical rabies remains very poor.

Consequently, efforts continue to be made to develop novel therapies to improve survival prospects for those that have progressed to the clinical stages of disease. Here, the major obstacles to treating rabies virus replication within the nervous system are discussed, in addition to highlighting potential options for post-symptomatic treatment, and areas of research that may be of future use as PIT for rabies.

# Why is rabies so difficult to treat?

The fundamental barrier to treating infection with RABV lies in its tropism for cells of the nervous system. Indeed, for decades it has been proposed that the replication of RABV within the nervous system enables the virus to evade the myriad of host responses triggered during non-neuronal infection. Lethal RABV strains actively evade the immune system by both preventing the apoptosis of host neurons and promoting the apoptosis of infiltrating T cells ([39](#_ENREF_39)). Infection can only initiate following virus exposure when delivered across the dermal barrier, either through the mechanistic action of a bite or more rarely, via the exposure of exposed mucus membranes to live virus. Primary infection of cells occurs within the immediate vicinity of the virus entry point with lyssaviruses being able to enter a wide range of host cells ([40](#_ENREF_40)). At this stage, it is unclear what localised immune activity the virus evades to establish a productive infection although it must be assumed that at this stage the virus may, in the absence of neuronal infection, be cleared by cell-mediated immune responses. Certainly, the observation of unvaccinated individuals serologically positive for bat rabies in the Amazon suggests that viral clearance following replication in the periphery can occur ([41](#_ENREF_41)). However, due to the invariably fatal nature of the disease, it is recommended that any suspected exposure involving lyssaviruses be treated with the relevant PEP regimen. This anxiety of fatal disease is the basis for the 20 million post-exposure treatments that are sought each year ([14](#_ENREF_14)).

Once the virus has entered the host, a period of low-level replication may occur in sub-optimal cellular environments before peripheral nerve infection and the establishment of infection. This period of low-level replication may contribute to inordinately long prodromal periods following exposure, although a variety of factors are thought to be involved and virus activity in the host during pre-clinical incubation periods remains unclear ([42](#_ENREF_42), [43](#_ENREF_43)). Furthermore, as these pre-clinical stages are asymptomatic, a significant number of rabies cases will go undiagnosed and untreated until clincal symptoms develop where, at some point post-infection, the virus will enter a peripheral nerve and a productive infection may initiate (Figure 2). Unfortunately, once clinical symptoms develop, rabies is invariably fatal. Lyssaviruses have a single envelope glycoprotein that mediates attachment and entry, with infection occurring via G protein-mediated binding to host cell receptors. For RABV, three primary receptors have been defined for cellular entry, including nicotinic acetylcholine receptors (nAchR), neuronal cell adhesion molecules (NCAM), and p75 neurotrophin receptor (p75NTR) (Figure 2) ([1](#_ENREF_1)). However, mechanisms of neuronal entry of RABV at peripheral inoculation sites remains undefined and utilisation of other host receptors is likely ([44](#_ENREF_44)). Following receptor binding, the virus likely enters most efficiently through clathrin-mediated endocytosis on motor neuron end-plates ([45](#_ENREF_45), [46](#_ENREF_46)). Then, once into the peripheral nervous system (PNS), virions spread transynaptically in a retrograde fashion towards the CNS ([46](#_ENREF_46), [47](#_ENREF_47)).

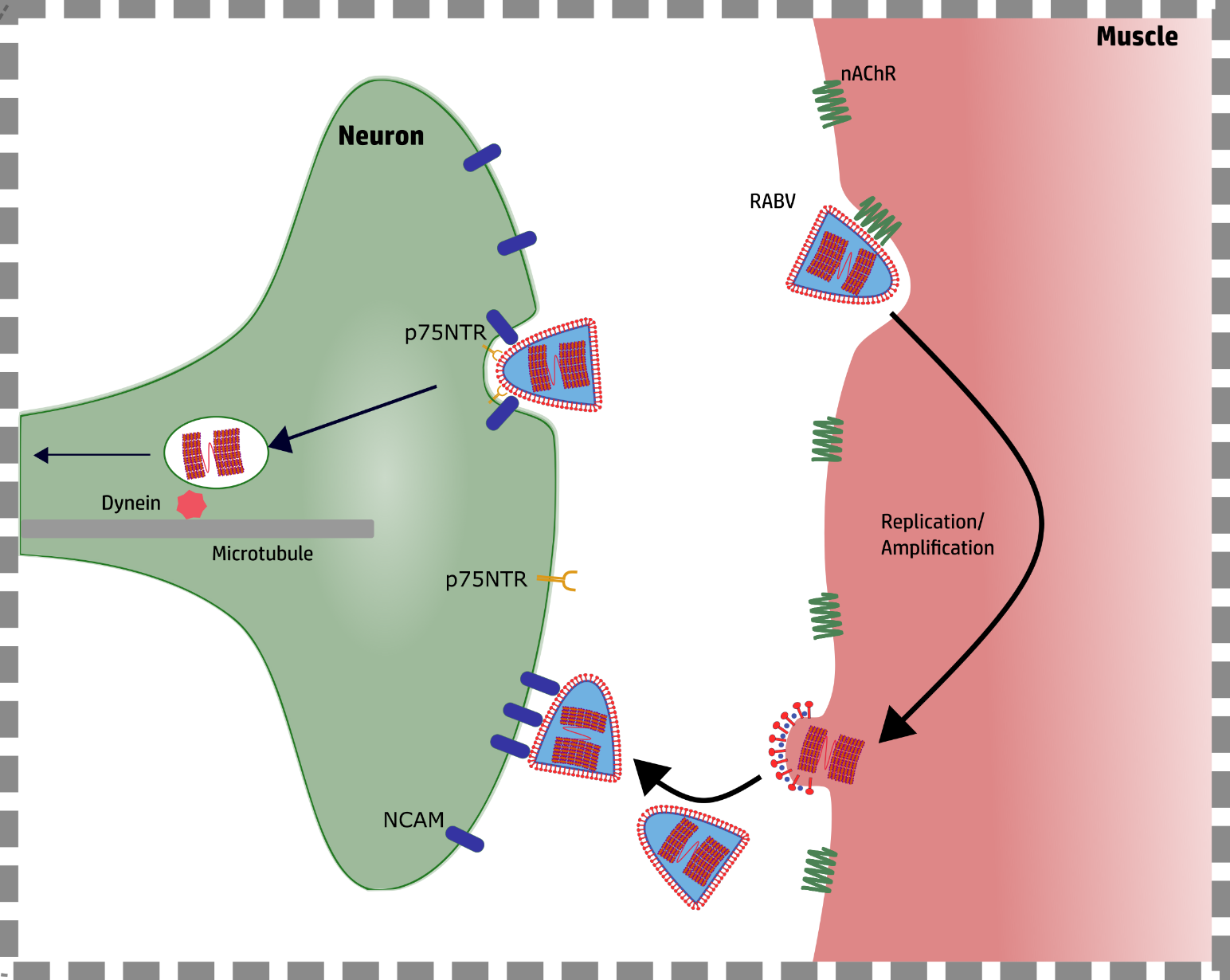


Figure 2: Showing the timeline of the virus life-cycle from muscle to peripheral nerve cells. nAChR=nicotinic acetylcholine receptor. RABV=rabies virus. NCAM= neuronal cell adhesion molecules. mGLuR2=metabotropic glutamate receptor subtype 2. p75NTR=p75 neurotrophin receptor. Adapted from Fooks et al, 2017.([1](#_ENREF_1))

Once RABV reaches the brain, viral replication leads to a range of non-specific symptoms, including fever, headache, paralysis, and death with often either a furious or paralytic form of the disease developing ([42](#_ENREF_42), [48](#_ENREF_48)). No clinical outcomes are considered pathognomonic for RABV infection, with only laboratory diagnosis, either *ante*- or *post-mortem*, able to determine lyssavirus infection. This inability to conclusively diagnose rabies infection without laboratory confirmation can be, alongside cost and availability of PEP, a major factor in cases of delayed treatment. One key feature demonstrated to influence the outcome of rabies in experimental models is the permeability of the blood-brain barrier (BBB) and PIT. The BBB is a feature of the neurovasculature which acts to separate the brain from macromolecules in the blood. The BBB also plays major roles in infection, forming a physical barrier to protect the brain ([49](#_ENREF_49)). While the permanent opening of the BBB is associated with many pathologies, a transient increase in permeability has been shown to be a major factor in CNS clearance of RABV, as this facilitates infiltration of both antibodies and immune cells ([50-54](#_ENREF_50)). However, wildtype street strains of RABV have been shown to employ various mechanisms to counteract the opening of the BBB, and the subsequent infiltration of immune cells. For example, during experimental infection with street rabies strains the BBB becomes refractory to permeabilizing signals and CNS inflammation does not occur ([52](#_ENREF_52), [55](#_ENREF_55)). When immune cells do infiltrate past the BBB, rabies can actively promote apoptosis through both caspase-dependent and independent mechanisms (Figure 3) ([39](#_ENREF_39), [56](#_ENREF_56)), or downregulate antigen presentation by inducing the production of Calcitonin and Vasoactive Intestinal Peptide (VIP) from infected cells ([57](#_ENREF_57), [58](#_ENREF_58)). Furthermore, the impermeability of the BBB during clinical rabies infection is also proposed to be a major hurdle in PIT using antibody-based PEP. The BBB has also proved to be a major factor in the ineffectiveness of antiviral drugs in PIT of rabies. Antiviral drugs, including amantadine, favipiravir, and ribavirin, have been successful in in vitro studies, where treatment of cell culture has effectively preveted or reduced RABV replication. However, these results have not translated well into in vivo studies ([42-46](#_ENREF_42)). Furthermore, while RNA based approaches (small-interfering RNA and microRNAs) have had success in demonstrating supression of viral replication in vitro ([59](#_ENREF_59), [60](#_ENREF_60)), these approaches have not been as successul as traditional PEP in the protection of mice against mortality after lethal challenge ([61](#_ENREF_61), [62](#_ENREF_62)). Certainly, from a therapeutic standpoint the ability to manipulate the BBB and allow entry of appropriate effector molecules would seem to be beneficial for rabies PIT.

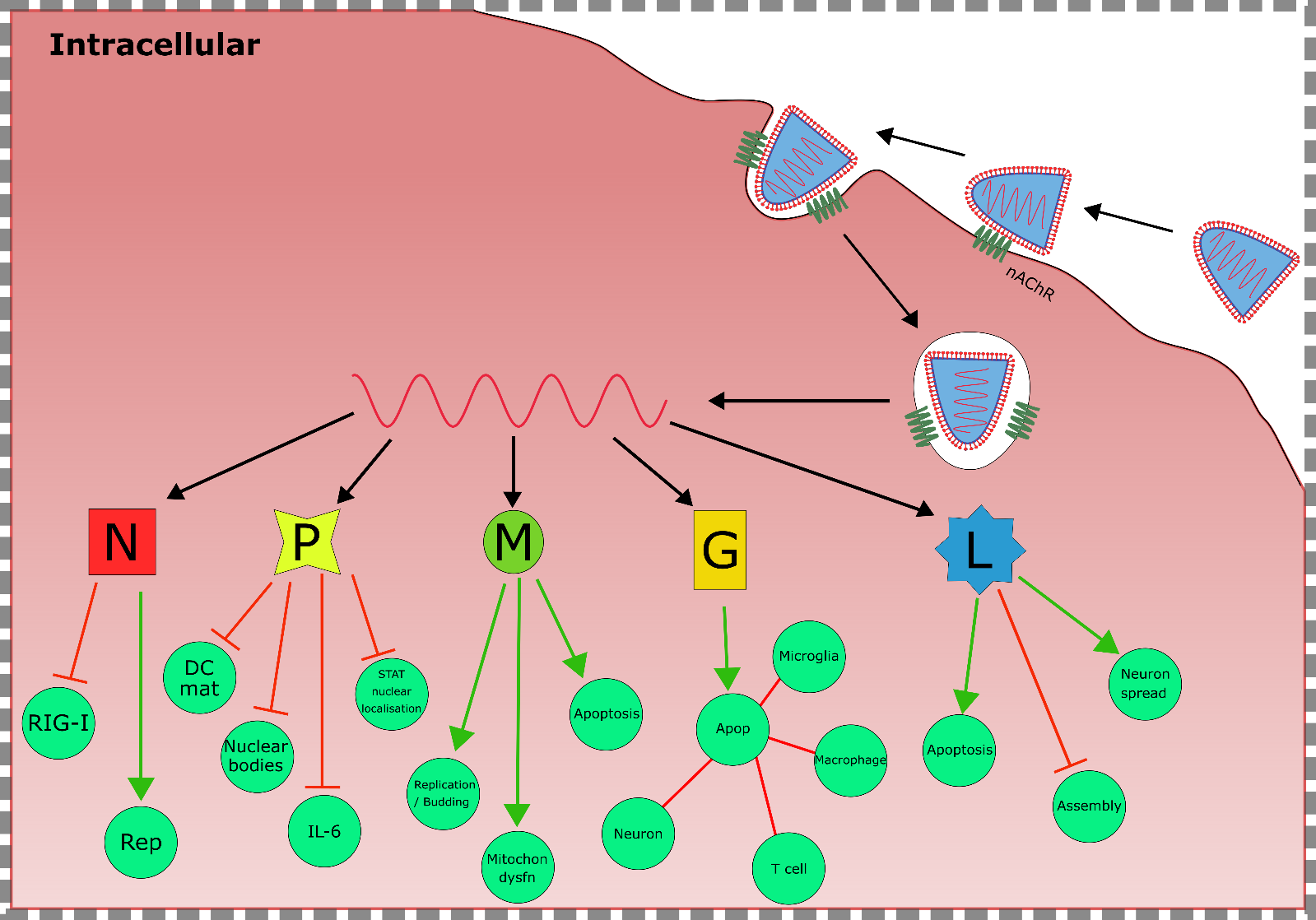


Figure 3: Interactions between viral proteins and the host. Red lines indicate inhibition and green lines indicate upregulation. RIG-I=retinoic acid-inducible gene I. Rep=Replication. DC mat=Dendritic Cell maturation. IL-6=Interleukin-6. STAT 1/2/3=signal transducer and activator of transcription 1–3. Mitochon dysfn=Mitochondrial dysfunction. Apop=apoptosis

# EXPERIMENTAL VACCINES and potential future options

Inactivated rabies vaccines for human use have, since the development of cell culture-derived preparations, remained unchanged for decades. Such vaccines are safe, highly immunogenic, and induce sufficient neutralising antibody profiles, although multiple clinic visits are required to provide long-lasting protection in line with WHO vaccination recommendations ([63-65](#_ENREF_63)). To counter this, optimised regimens have been developed to reduce vaccine use and ensure available supplies are utilised most appropriately ([16](#_ENREF_16), [63](#_ENREF_63), [66](#_ENREF_66)). Despite their utility in pre- and post-exposure immunisation, inactivated rabies vaccines are unable to prevent rabies replication following clinical disease onset ([50](#_ENREF_50), [67-71](#_ENREF_67)). Whilst inactivated vaccines have been shown to increase serum antibody titers post-vaccination, antibody titres peak after the transient increase in BBB permeability and as such, with the BBB’s molecular exclusion limit for molecules >400 daltons ([72](#_ENREF_72)), it is likely that a significant proportion of virus-neutralizing antibodies (VNA) or immune cells are unable to pass through the BBB and counter virus replication in the CNS ([54](#_ENREF_54)). Whilst experimental models have shown that the infiltration of CD3 positive T cells may aid the control of infection, only the presence of VNAs (either from the blood or infiltrating B cells) can clear infection ([42](#_ENREF_42), [50](#_ENREF_50), [52](#_ENREF_52), [69](#_ENREF_69)). Transient opening of the BBB is known to occur when inflammatory responses are triggered in the CNS ([73](#_ENREF_73)), however, while this successfully demonstrated viral clearance in a mouse strain more susceptible to BBB permeability changes in response to infection ([51](#_ENREF_51)), inducing CNS inflammation during human infection is likely counterproductive due to associated pathologies ([74](#_ENREF_74)).

While inactivated vaccines promote an inadequate increase in BBB permeability to enable a sufficient CNS immune infiltrate, advances in the development of LAVVs have improved opportunities to utilize this pathway to deliver effector molecules to the CNS. The immune response elicited in the CNS following vaccination with a live-attenuated virus is not associated with any significant pathology ([50](#_ENREF_50)) and live-attenuated rabies vaccines have consistently been shown in experimental models to facilitate both an increase in BBB permeability and to protect against or clear lethal virus infection ([67](#_ENREF_67), [68](#_ENREF_68)). These features of LAVVs indicate their potential for therapeutic use. Certainly, a LAVV that can effectively increase the permeability of the BBB and promote sufficient immune cell infiltration to clear virus infection during the clinical stages of disease is considered to be a high priority for rabies PIT research.

# Developing live virus vaccines and their applicability for human use

Historically, the first successful LAVV was used in the eradication of smallpox in 1980. Since then, various LAVVs have been developed to combat diseases such as poliovirus, yellow fever virus, rinderpest virus, measles virus, and mumps virus ([75](#_ENREF_75)). Live attenuated rabies vaccines are currently only used for the protection of animals. To vaccinate a large population of wildlife, an effective method is the use of a liquid virus containing sachet incorporated into the animal’s bait ([76](#_ENREF_76)), as intact virus either replicating locally in the oral mucosae or following ingestion and exposure in the intestinal tract is sufficient to cause an immune response and subsequent protection ([77](#_ENREF_77), [78](#_ENREF_78)). The attenuated strains used in the live vaccination of animals are descended from the parental SAD-Bern strain (a derivative of Street Alabama Dufferin (SAD) strain) ([8](#_ENREF_8)). These include SAD B19, SAD Bern, SAD P5/88, SAG1/2, SAD VA1, V-RG, ERA G333, KMIEV-94 and RV 97 ([8](#_ENREF_8), [79](#_ENREF_79), [80](#_ENREF_80)). With the exception of Arg333 in RABV G protein, the majority of the antigenic sites in N, P, and G are conserved within SAD-strain derivatives. However, mutations within the highly conserved blocks (1, 2, 4, and 5) of L have been described in both SAD Bern and SAD B19 strains. These blocks are responsible for the enzymatic function of L, including mRNA capping and N protein binding ([8](#_ENREF_8), [81](#_ENREF_81), [82](#_ENREF_82)).

The use of liquid sachets to deliver LAVVs to animal populations throughout Europe has been widely successful reducing human infection, following the bite from terrestrial carnivores, by up to 96% in some countries ([83](#_ENREF_83)). Several cases of vaccine-induced rabies have been described globally in either immunocompromised foxes or non-target species such as skunks ([84-86](#_ENREF_84)). With the majority occurring in Germany and Austria, these have been attributed to reversion mutations (post-bait consumption) ([79](#_ENREF_79), [84](#_ENREF_84)). Alongside rare reversion to virulence, some SAD Bern-based LAVVs are ineffective in the vaccination of some species, such as striped skunks (*Mephitis mephitis*) ([87](#_ENREF_87)). The basis for differential responses in different species is unclear but is key to delivery of vaccine baits to different wildlife species.

Although the concerns regarding incomplete attenuation and reversion to virulence exist for LAVVs there are many benefits to their potential use in humans. Certainly, where fatality is inevitable, as it is considered to be following the onset of clinical rabies, risk of reversion versus potential survival is hard to assess. Alongside their ability to induce transient BBB permeability, there are considerable cost benefits to the production of LAVVs, as production methods can be scaled rapidly and viruses can be attenuated by a range of mechanisms. These important differences allow attenuated viruses to activate a more vigorous and protective immune response. Furthermore, LAVVs have been shown to be effective in experimental models at both preventing live rabies infection and clearing virus if administered soon after lethal challenge ([88-90](#_ENREF_88)). Therefore, due to their ability to permeabilise the BBB and possible cost-effective production methods, LAVVs could have a significant future role in human PEP and PIT.

However, live vaccines are not without problems. Studies investigating a live ERA vaccine revealed severe side effects in animals if given i.c. ([76](#_ENREF_76)). Furthermore, as with all live vaccines, there is also a concern of reversion. Active viral shedding has also been shown not to occur after oral vaccination, with no live virus found in saliva sections after 24 hours ([91](#_ENREF_91)). However, combined with close surveillance, many studies have demonstrated the safety of live vaccines used in wildlife ([92-94](#_ENREF_92)), and shown that outbreaks in new areas have been due to animal migration/trafficking rather than vaccine reversion ([95](#_ENREF_95)).

## DETERMINANTS OF VIRAL VIRULENCE

Determining key elements that lead to viral attenuation is vital to generating a safe and effective vaccine. Seminal studies investigating mechanisms behind RABV attenuation demonstrated that rabies pathogenicity could be significantly reduced through a single amino acid substitution in the G protein at position 333 whereby substituting the native arginine with a glutamine or a glycine attenuated the resulting virus ([96](#_ENREF_96), [97](#_ENREF_97)).

Arg333 is located in G proteins antigenic site III, a conformational epitope linked to viral pathogenicity and neuroinvasiveness common across all lyssaviruses ([96](#_ENREF_96)). However, due to a high mutation rate, escape mutants have been described after just 5 passages in suckling mice, where a rabies vector (SPBNG) containing the Arg333 substitution reverted back to a pathogenic phenotype after a single Asn to Lys mutation at residue 194 ([98](#_ENREF_98)). Therefore, to develop a safe LAVV, multiple mutations are required. To further this, various studies have investigated the effect of incorporating multiple attenuated G proteins into a pSPBN vector (developed from the SAD strain) ([88](#_ENREF_88), [97](#_ENREF_97), [99](#_ENREF_99), [100](#_ENREF_100)). Increased G protein expression induced markedly higher VNA titres than the parent pSPBN plasmid, and upregulated immune cell infiltration (preferentially B cells) to protect against lethal infection (either by the intramuscular (i.m.) or intracranial (i.c.) routes) if administered before or shortly after exposure ([88](#_ENREF_88)). Ultimately, uncontrolled G expression can both activate an effective immune response triggering viral destruction and severely limit RABV CNS infection ([39](#_ENREF_39), [53](#_ENREF_53), [99-102](#_ENREF_99)). Further studies assessing overexpression of G demonstrated that triplicating the G protein (pSPBNGAS-GAS-GAS) elicited longer lasting protection than viruses containing two, or just one copy of G. The triGAS vaccines have also been investigated as a potential alternative form of PEP. Mice treated with 107 FFU of i.c. triGAS four hours post infection (p.i.) with a lethal dose of a street rabies isolate did not develop a clinical disease, whilst those treated 48 hours p.i. developed hind limb paralysis although none developed terminal disease. Furthermore, upon i.m. administration of 108 FFU of triGAS vaccine at 4 hours p.i. with 106 FFU of street RABV, a 0% mortality rate was observed. If given at 16, 24, 48, and 72 hours p.i., mice displayed a 90%, 55%, 40%, and 30% survival rates, respectively ([88](#_ENREF_88)).

However, the attenuation of LAVVs should not depend entirely on mutations within G. This was explored during the construction of the (ERA-N273/394) Ni-CE strain, containing two additional N protein mutations: 273/394 (Phe-Leu and Tyr-His respectively). Upon i.c. Ni-CE challenge, a 0% death rate was observed in mice. The Ni-CE strain has now been further attenuated by including a G333 mutation ([70](#_ENREF_70), [103](#_ENREF_103), [104](#_ENREF_104)).

# Stimulating an adaptive response whilst controlling host inflammation

While the role of the immune system in viral clearance and pathogenicity has not yet been fully described, its importance is fully appreciated. Infection causes a wide range of cytokine/chemokine upregulation, with functions ranging from upregulating MHC expression to an increase in BBB permeability ([58](#_ENREF_58), [105](#_ENREF_105)). While some vaccines aim to only activate the immune system through the presentation of attenuated G proteins, there has recently been a focus on the expression of non-viral elements as a way of supplementing the immune response. A number of studies have since addressed the incorporation of immune stimulatory or regulatory molecules (some of which have been highlighted for their role in BBB permeabilisation ([58](#_ENREF_58), [106-108](#_ENREF_106))) into the rabies genome (Table 1), with the majority of novel recombinant vaccines demonstrating over 90% protection (Table 2).

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| --- | --- | --- | --- | --- |
| **Non-viral element** | **Function** | **Produced by** |  | **Effect** |
| MIP-1α/CCL3\* ([109-111](#_ENREF_109)) | An inflammatory chemokine, promotes and activates monocyte/macrophage/neutrophils, and DC-progenitor cells. | Lymphocytes, fibroblasts and epithelial cells | Increased survivorship (VNA meditated protection) | Local cytokine production increase, T/B cell recruitment, DC/B cell activation ([112](#_ENREF_112)) |
| GM-CSF ([113](#_ENREF_113)) | Pro-inflammatory cytokine. Aids the development of DCs and recruits/enhances myeloid cell survival and activation. | Fibroblasts, endothelial cells, macrophages, DCs, T cells, neutrophils, eosinophils. | Maturation and activation of BMDCs, recruitment/activation of DC/B/T cells ([112](#_ENREF_112)) |
| MDC/CCL22 ([114](#_ENREF_114)) | Migration of activated T, Treg, NK cells, monocytes, and monocyte-derived DCs | Macrophages, monocyte-derived DC’s, and activated NK / T cells, keratinocytes. |
| Flagellin ([115](#_ENREF_115)) | Filamentous bacterial protein. Induces the migration and maturation of DC and neutrophils. | Bacteria | DC/B cell activation/maturation ([116](#_ENREF_116)) |
| Cytochro-me c ([117](#_ENREF_117)) | Intramembrane protein involved in the transport of electrons from complex III to IV within the electron transport chain. Upon release from membrane has roles in caspase-dependent apoptosis initiation. | All cells undergoing cellular respiration | Increased apoptosis, development of N protein inclusion bodies in neuronal cell body ([118](#_ENREF_118)) |
| CXCL13 ([119-121](#_ENREF_119)) | Promotes B and Tfh cell recruitment to B cell follicles. | Stromal cells, follicular DC (FDC), Tfh cells, mature B cells. | Tfh/GC B cell recruitment, GC formation ([122](#_ENREF_122)) |
| IL-21 ([123-125](#_ENREF_123)) | Activation and differentiation of NK, NK-T, B, and CD8 cells. | T and NK T cells | Generation of activated DCs/Tfh/GC B cells ([126](#_ENREF_126)) |
| IL-15 ([127-130](#_ENREF_127)) | Similar functions to IL-21 in the development of T, B, NK, and NK T cells | Monocytes, macrophages, and DC’s. | Increased generation of Tfh/GC B/PCs and DC maturation ([131](#_ENREF_131)) |
| IL-7 \* ([132](#_ENREF_132)) | Proliferation and maintenance of T/B cells, generation of Tfh and GC B cells, and promotes survival of CD4/8 T cells | Stromal cells present in adult bone marrow | Increased GC B cell presence in inguinal lymph nodes, increased Tfh population ([132](#_ENREF_132)) |
| IL-18 ([133](#_ENREF_133)) | Pro-inflammatory cytokine able to induce IFNγ in a variety of immune cells (CD4/8, NK cells, and macrophages), activates NK cells, and upregulates cytotoxicity in NK/CD8 cells. | Monocytes, macrophages, DC’s, endothelial cells, keratinocytes and intestinal epithelial cells | Increased levels of CD4/8 T cells, IFNγ-secreting CD4/8 T cells, and activated B cells in blood/lymph nodes ([134](#_ENREF_134)) |
| IL-6 \* ([135](#_ENREF_135)) | Stimulates PC development and T cell proliferation/development, with major roles in mucosal IgA antibody responses | Various cell types, primary sources being monocytes and macrophages | Increased BBB permeability, increased recruitment of B/T cells to lymph nodes, increased chemokine production in CNS ([135](#_ENREF_135)) |
| TNF-α \* ([136-139](#_ENREF_136)) | A pro-inflammatory cytokine expressed as either a soluble or transmembrane protein with roles in cellular differentiation, proliferation, and death. | TNF-R1 is expressed in most tissues, while TNF-R2 is found in immune system cells. |  | T cell infiltration to brain tissue and increased reactive microgliosis ([140](#_ENREF_140)) |
| IFNγ \* ([141](#_ENREF_141), [142](#_ENREF_142)) | A pleiotropic cytokine involved in the Th1 immune response, including the activation of macrophages and the production of chemokines. | Initially expressed by NK and NK-T cells. Then later by CD4/8 cells. |  | Increased survivorship (not VNA mediated), no increase in BBB permeability, type I IFN induction ([143](#_ENREF_143)) |
| IFN1α/IFNα ([144](#_ENREF_144)) | A type 1 IFN, able to induce antimicrobial states within neighbouring cells, module innate immune responses for antigen presentation, and promote immunological memory. | Predominantly haematopoietic cells, especially plasmacytoid DC cells | Increased survivorship (VNA meditated protection) | Stronger innate immune response, no increased apoptosis, increased levels of activated B cells and CD8 T cells in lymph nodes and peripheral blood respectively ([145](#_ENREF_145)). |
| HMGB1 ([146](#_ENREF_146), [147](#_ENREF_147)) | While usually intracellular, acts as a pro-inflammatory cytokine upon release, promoting DC maturation, activation, and migration into lymph nodes. | Actively secreted by DCs, NK cells, monocytes, and macrophages. Passively secreted by necrotic or damaged cells. | Increased Tfh/GC B/PC recruitment and activation ([146](#_ENREF_146)) |
| DCBp ([148](#_ENREF_148), [149](#_ENREF_149)) | N/A | N/A – Extracted from a peptide library | Increased DC binding/activation and Tfh/GC B cell generation ([148](#_ENREF_148)). |

Table 1: Various non-viral elements have been incorporated into the genome of LAVVs (Live Attenuated Virus Vaccines). Those marked with an asterix (\*) are those which have been previously suggested as molecules which are involved in the permeabilisation of the BBB. MIP-1α=Macrophage inflammatory protein α. CCL3=Chemokine (C-C motif) ligand 3. GM-CSF=Granulocyte-macrophage colony-stimulating factor. BMDC=Bone marrow derived dendritic cell MDC=Macrophage-derived chemokine. CCL22=Chemokine (C-C motif) ligand-22. NK=Natural killer. CXCL13=chemokine (C-X-C motif) ligand-13. Tfh=T follicular helper cell. GC B=Germinal centre B cells. IL-21=Interleukin-21. IL-15=Interleukin-15. IL-2=Interleukin-2. PC=Plasma cell. IL-6=Interleukin-6. TNF-α=Tumour necrosis factor-α. IFN-γ=Interferon-γ. HMGB1=High mobility group box 1. DCBp=Dendritic cell binding peptide.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Non-viral element** | **Backbone** | **PrEP/PEP** | **Dose (FFU)** | **Route** | **Challenge Virus** | **Challenge route and d.p.i** | | **Parent virus infection survival (control)** | | **Survival post-vaccination** |
| MIP-1α/CCL3 ([112](#_ENREF_112)) | LBNSE | PrEP | 106 | i.m | 50 LD50 CVS-24 | i.c  21 d.p.i | | 50% | | 80% |
| GM-CSF ([112](#_ENREF_112)) | LBNSE | PrEP | 106 | i.m | 50 LD50 CVS-24 | i.c  21 d.p.i | | 50% | | 90% |
| MDC/CCL22 ([112](#_ENREF_112)) | LBNSE | PrEP | 106 | i.m | 50 LD50 CVS-24 | i.c  21 d.p.i | | 50% | | 90% |
| Flagellin ([116](#_ENREF_116)) | LBNSE | PrEP | 106 | i.m | 50 LD50 CVS-24 | i.c  21 d.p.i | | 60% | | 80% |
| LBNSE | PrEP | 107 (again for 21 d.p.i booster) | Oral | 50 LD50 CVS-24 | i.c  7 days (post-booster) | | 50% | | 90% |
| CXCL13 ([122](#_ENREF_122)) | LBNSE | PrEP | 106 | i.m | 50 LD50 CVS-24 | i.c  14 d.p.i | | 45% | | 95% |
| IL-21 ([126](#_ENREF_126)) | LBNSE | PrEP | 106 | i.m | 50 LD50 CVS-24 | i.c  21 d.p.i | | 68% | | 92% |
| IL-15 ([131](#_ENREF_131)) | LBNSE | PrEP | 106 | i.m | 50 LD50 CVS-24 | i.c  21 d.p.i | | 68% | | 84% |
| IL-6 ([135](#_ENREF_135)) | rHEP | PrEP | 104 | i.m | 50 LD50 CVS-24 | i.c  23 d.p.i | | 40% | | 80% |
| 105 | 60% | | 90% |
| IL-7 ([132](#_ENREF_132)) | LBNSE | PrEP | 106 | i.m | 50 LD50 CVS-24 | i.c | 360 d.p.i | 42% | | 78% |
| 102 | 49 d.p.i | 0% | | 15% |
| 103 | 49 d.p.i | 25% | | 75% |
| 104 | 49 d.p.i | 40% | | 75% |
| 105 | 49 d.p.i | 50% | | 75% |
| 106 | 49 d.p.i | 58% | | 100% |
| TNF-α ([140](#_ENREF_140)) | SPBN | Not tested as PrEP or PEP | | | | | | | | |
| IFNγ ([71](#_ENREF_71)) | GAS | PrEP | 102 | i.m | 103 FFU DRV4 | i.c  13 d.p.i | | N/A | | 20% |
| 103 | N/A | | 90% |
| 104 | N/A | | 100% |
| 105 | N/A | | 100% |
| HMGB1 ([146](#_ENREF_146)) | LBNSE | PrEP | 106 | i.m | 50 LD50 CVS-24 | i.c  14 d.p.i | | 45% | | 85% (HMGB1mut) |
| 65% (HMGB1wt) |
| DCBp ([148](#_ENREF_148)) | LBNSE | PrEP | 106 | i.m | 50 LD50 CVS-24 | i.c  21 d.p.i | | 54.55% | | 90.91% |
| IL-18 ([134](#_ENREF_134)) | rHEP | PrEP | 105 | i.m | 100 IMLD50  HuNPB3 ([150](#_ENREF_150), [151](#_ENREF_151)) | i.m | | 60% | | 100% |
| IFNα1/IFNα ([145](#_ENREF_145)) | rHEP | PrEP | 5x102 | i.m | 50 LD50 CVS-24 | i.c  23 d.p.i | | 20% | | 60% |
| 5x103 | 30% | | 70% |
| 5x104 | 60% | | 80% |
| Cyto c ([118](#_ENREF_118)) | SPBN | PrEP | 3x102 | i.m | 100 LD50 CVS-N2c | i.c  10 d.p.i | | 20% | (SPBN-Cyto c(-)) | 30% |
| 3x103 | 30% | 80% |
| 3x104 | 40% | 80% |
| 3x105 | 80% | 90% |
| 3x106 | 90% | 90% |

Table 2: A summary of *in* vivo viral challenge results of mice immunised with recombinant live viruses.

Following studies into post-vaccination immunological responses and to further interrogate the effects of non-viral elements post-infection, a number of challenge studies have been reported. These include; GM-CSF, flagellin, HMGB1, DCBp, CXCL13, cytochrome c, common γ-chain cytokine family members IL-15, IL-21, IL-6, IL-18, and IL-7, TNF-α, IFNα1, and IFNγ ([71](#_ENREF_71), [116](#_ENREF_116), [118](#_ENREF_118), [122](#_ENREF_122), [126](#_ENREF_126), [131](#_ENREF_131), [132](#_ENREF_132), [134](#_ENREF_134), [135](#_ENREF_135), [140](#_ENREF_140), [143](#_ENREF_143), [145](#_ENREF_145), [146](#_ENREF_146), [148](#_ENREF_148)). The majority of these elements have been cloned into the vaccine strain LBNSE, between the G and L genes, while some (DCBp) have been inserted within the G protein itself (Figure 1) ([148](#_ENREF_148)).

The effect of incorporating various dendritic cell (DC)-activating molecules into a pLBNSE vector (based on the SAD B19 strain with two G protein mutations) has been demonstrated ([112](#_ENREF_112), [152](#_ENREF_152)). Activated DCs play a major role in the immune response to RABV infection, such as activation of naïve B cells and promotion of appropriate class switching ([153](#_ENREF_153)). B cell activation is vital to RABV clearance, as while T cells may control certain features of infection, it is VNA production by B cells which infiltrate the CNS through the BBB that enables virus clearance ([50](#_ENREF_50)). The immunization of mice with LBNSE-vectors expressing GM-CSF, MDC, or MIP-1α ([112](#_ENREF_112)) (Table 2) revealed significant increases in the local production of cytokines and chemokines (such as MIP-1α, IFNγ, and IL-4). Furthermore, survivorship after i.c. challenge with CVS-24 was shown to increase when compared with inoculation of parent LBNSE virus (Table 2). Immunization also increased T and B cell recruitment, while facilitating VNA induction through increased DC and B cell activation. Furthermore, GM-CSF, MDC, or MIP-1α overexpression had few adverse effects upon mouse infection, save for negligible weight loss (5-10% at low viral doses and 10-15% with high doses) ([112](#_ENREF_112)). However, the overexpression of a chemokine is not necessarily beneficial ([154](#_ENREF_154)). For example, studies using rHEP (high-egg passage) to express IL-10 or RANTES showed significant decrease in survivorship after i.c. challenge ([109](#_ENREF_109)).

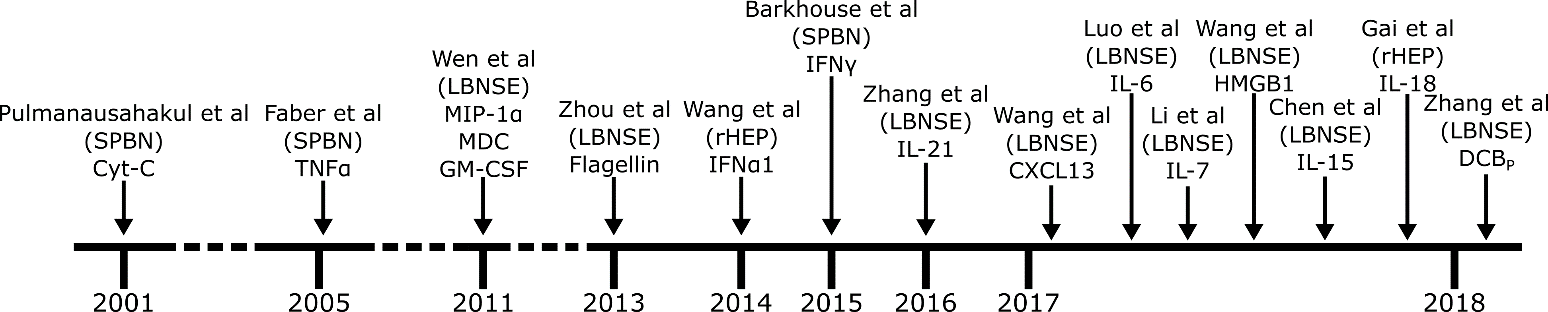


Figure 4: A timeline of research papers publishing details of non-viral elements being inserted into a RABV genome. First authors are named, followed by the vector, and details of the non-viral element inserted. See Table 1 for information of non-viral elements, and Figure 1 for insertion location.

While not directly able to activate DCs, the mitochondrion protein cytochrome c is able to efficiently promote host cell apoptosis (Table 1) ([117](#_ENREF_117)). Normally localised to the mitochondrial membrane, upon cellular stress cytochrome c is released into the cytosol where it can bind protease activating factor-1 (Apaf-1) to enable the formation of the apoptosome, eventually leading to apoptosis of the host cell, which may then promote DC maturation {Sarmento, 2006 #198}([56](#_ENREF_56), [155](#_ENREF_155), [156](#_ENREF_156)). The immunisation of mice challenged with an SPBN-vector ([118](#_ENREF_118)) expressing cytochrome c elicited a three-fold increase in VNA titres compared to a negative control (SPBN-cyto c(-), a SPBN vector containing an inactive cytochrome c gene) regardless of the route of infection. This indicates that that caspase-dependent apoptosis of infected cells is a significant contributor to antibody responses. Furthermore, survivorship after lethal challenge with CVS-N2c was greatly increased at low doses compared to a negative control (SPBN-cyto c(-)) (Table 2) ([118](#_ENREF_118)). Immunization of mice (i.m, oral, or i.m with an oral booster) with an LBNSE vector expressing either GM-CSF or flagellin ([116](#_ENREF_116)), resulted in increased recruitment and activation of DC and B cells in draining lymph nodes. This resulted in VNA-mediated protection against i.c. CVS-24 (50 LD50) challenge 21 d.p.i., with LBNSE-flagellin and LBNSE-GM-CSF survival rates reaching 80% (or 90% when given with an oral booster) and 90%, respectively.

While the importance of B cells in clearing rabies infection is well documented, T cell involvement has also been shown to be of importance. Tfh (follicular helper) cells differentiate from naïve CD4+ve T cells upon DC activation in secondary lymph nodes ([157](#_ENREF_157)). These are vital in establishing germinal centres (GC), where GC derived B cells (GC B cells) may undergo further differentiation into plasma cells to reside in the bone marrow ([120](#_ENREF_120), [121](#_ENREF_121), [158](#_ENREF_158)). CXCL13, a homeostatic chemokine known to recruit and activate and recruit various immune cells, (Table 1) has been inserted into an LBNSE vector to enhance the humoral response to RABV infection ([122](#_ENREF_122)). Intramuscular immunization of 1x106 FFU LBNSE-CXCL13 resulted in Tfh and GC derived B cell recruitment, while also promoting GC formation. CXCL13 expression resulted in minimal weight loss but protected against i.c. challenge with 50 LD50 CVS-24 14 d.p.i. (95% survivorship).

Various members of the common γ-chain cytokine family have also been inserted into the LBNSE vector. Among the first were IL-21 and IL-15 (Figure 4) ([126](#_ENREF_126), [131](#_ENREF_131)), cytokines well documented for their interactions with Tfh cells, GC B cells, DCs, and plasma cells (Table 1) ([123-125](#_ENREF_123), [128-130](#_ENREF_128)). Furthermore, various studies have also investigated IL-15 as a possible vaccine adjuvant due to its ability to induce DC maturation and enhance antibody titers ([159](#_ENREF_159), [160](#_ENREF_160)). Mice immunized (i.m.) with either LBNSE-IL-21 or LBNSE-IL-15 displayed significantly enhanced germinal centre B cells and Tfh cells development and infiltration into both the infection site and spleen, but did not affect DC activation ([131](#_ENREF_131)). Immunization with LBNSE-IL-21 or LBNSE-IL-15 also resulted in minimal weight change relative to parental LBNSE virus infection. Both IL-21 and IL-15 were taken forward to challenge studies, where 21 d.p.i. mice were i.c. challenged with 50 LD50 of CVS-24, resulting in survival rates increasing from 68% to 92% and 84% for LBNSE-IL-21 and IL-15, respectively ([126](#_ENREF_126), [131](#_ENREF_131)).

Another common γ-chain cytokine family member, IL-7, has also been utilised as an adjuvant in HSV (herpes simplex virus), tetanus, and influenza vaccines ([161](#_ENREF_161), [162](#_ENREF_162)) due to its effects on B cell expansion and development (Table 1) ([163](#_ENREF_163), [164](#_ENREF_164)). To investigate the effect of IL-7 expression in the context of rabies vaccination, murine IL-7 was inserted into the LBNSE vector (LBNSE-IL-7) ([132](#_ENREF_132)). Intramuscular inoculation of 106 FFU of LBNSE-IL-7 resulted in significantly more Tfh and GC derived B cells in draining lymph nodes, leading to raised VNA (IgG, IgG1, IgG2a/b) titres. Upon i.c. challenge of 50 LD50 of CVS-24 at 360 d.p.i., immunization with LBNSE-IL-7 significantly increased survival rates when compared to vaccination with the parent LBNSE vector ([132](#_ENREF_132)). Canine IL-6 has also been taken forward and expressed in a recombinant rabies virus vector rHEP (rHEP-CaIL6) ([135](#_ENREF_135)). After establishing the safety of rHEP-CaIL6 in BALB/c mice (upon 3.3×104 FFU i.c. inoculation, mice showed less severe clinical signs and recovered quicker compared to rHEP inoculation), rHEP-CaIL6 was taken forward to challenge experiments. Here, rHEP-CaIL6 vaccination promoted immunity at 7 days d.p.i., with a significant increase in survivorship after i.c. challenge of 50 LD50 CVS-24 at 21 d.p.i with 1x104 FFU of rHEP-CaIL6 ([135](#_ENREF_135)).

IL-18 is not part of the common γ-chain cytokine family, rather the IL-1 superfamily, but has major roles across both innate and adaptive immunity, in addition to promoting high amounts of IFNγ production (Table 1) ([133](#_ENREF_133)){Kaplanski, 2018 #371}. Upon incorporation of IL-18 into a HEP-Flury cDNA backbone (rHEP-IL18) pathogenicity within mice was significantly decreased with any weight loss recovered by 21 days post-infection and no clinical symptoms observed ([134](#_ENREF_134)). Upon i.m infection with rHEP-IL18, significantly more CD4/8 T cells were present in peripheral blood samples, alongside increased levels of activated B cells, IFNγ, and IFNγ-secreting CD4/8 T cells. Upon viral challenge with HuNPB3, 100% of mice immunised with rHEP-IL18 survived challenge ([134](#_ENREF_134)).

TNF-α, a pro-inflammatory cytokine existing as either a 26 kDa transmembrane or 17 kDa soluble protein, has effects extending into cellular differentiation, proliferation, and death (Table 1) ([139](#_ENREF_139)). To investigate the effect of overexpression in rabies infection, both soluble and membrane-bound TNF-α were cloned into an SPBN vector (pSPBN-TNF-α and pSPBN-TNF-α(mem)) ([140](#_ENREF_140)). Upon intranasal (i.n.) infection with 109 FFU, mice displayed increased infiltration of T cells into brain parenchyma (pSPBN-TNF-α 45% more than pSPBN-TNF-α(mem)), enhanced microglial activation, and reactive microgliosis. Lastly, survival rates of TNF-α knockout (KO) mice infected i.n. with 105 FFU of pSPBN- TNF-α(mem) and pSPBN- TNF-α remained at 80% and 100% respectively ([140](#_ENREF_140)).

IFNγ is another pro-inflammatory cytokine, heavily involved in various antiviral mechanisms (Table 1). Similar to previous studies, IFNγ was inserted into the pSPBN vector between genes G and L (SPBNγ) ([71](#_ENREF_71), [143](#_ENREF_143)). Upon IFNγ KO mouse infection i.n. with 105 FFU of SPBNγ, IFNγ mRNA levels were approximately 10x greater than mice infected with the parent SPBN virus. However, viral attenuation was not due to immune cell or VNA infiltration, but the early activation of both type I and II IFN expression which aided the early innate response to protect mice from mortality ([143](#_ENREF_143)). While SPBNγ has not been advanced to viral challenge studies, a similar vector expressing IFNγ utilising the GAS backbone (GASγ) has been shown to be effective as a pre-exposure vaccination ([71](#_ENREF_71)). Here, mice were challenged i.c. with 103 FFU of lethal DRV4 rabies virus 13 days after i.m. vaccination with GASγ, with survival rates increasing from 20% to 100% depending on the vaccination dose given (102–105 FFU) ([71](#_ENREF_71)). Furthermore, a HEP-Flury vector expressing canine IFN1α (rHEP-CaIFN1α) has been. IFN1α (aka IFNα), has various roles within both adaptive and innate immunity (Table 1) ([145](#_ENREF_145)). Upon infection of mice with rHEP-CaIFN1α, no clinical signs of infection presented, and any weight loss was regained within 16 days after infection. Furthermore, flow cytometry performed on both peripheral blood and lymph nodes revealed increased levels of CD3/4-positive T and activated B (CD19/40-positive) cells. Upon challenge of mice with CVS-24, significantly more mice immunised with rHEP-CaIFN1α survived compared to those immunised with the parent virus ([145](#_ENREF_145)).

The high mobility group box 1 (HMGB1) protein has also been investigated as a possible contender for inclusion in a rabies vaccine ([146](#_ENREF_146)). Although normally bound to nuclear DNA, HMBG1 can also be released from macrophages or monocytes upon activation to act as a pro-inflammatory cytokine ([146](#_ENREF_146), [147](#_ENREF_147)). To prevent nuclear localisation, a mutated version of HMGB1 (HMGB1mut) was cloned into an LBNSE vector. After establishing HMGB1mut could activate bone marrow-derived dendritic cells (BMDC) *in vitro*, mice were i.m. immunized with 106 FFU of LBNSE- HMGB1mut. Flow cytometry analysis of spleen, inguinal lymph nodes, and peripheral blood samples revealed increased recruitment of DC, Tfh, GC derived B cells, and increased plasma cell generation. Furthermore, peripheral blood samples revealed that upon i.c. challenge with 50 LD50 of CVS-24 at 14 d.p.i., LBNSE- HMGB1mut immunization elicited VNA protection as soon as 7 d.p.i ([146](#_ENREF_146)).

# ConclusionS

Pre- or post-exposure vaccination with inactivated rabies vaccines, alongside the administration of RIG, are currently the only options available in the prevention of the development of clinical rabies and human mortality. However, following the onset of clinical disease, they are of no utility in preventing disease progression and death and PIT options are urgently needed. Experimental approaches with LAVVs have demonstrated that future developments in PIT may rely on a LAVV approach in the absence of any efficacious antiviral drug to counter virus replication in the brain. LAVVs have already been used extensively in the eradication of rabies in natural wildlife reservoirs ([165](#_ENREF_165), [166](#_ENREF_166)), however, the ethical debate around the use of LAVVs in human infection is controversial, even where experimental data in animal models has been reported. Furthermore, the severity of rabies infection means that studies in non-human primates are increasingly difficult to justify in the assessment of possible PITs.

Fundamentally, the lack of post clinical survival from experimental rabies following attempts with antiviral molecules *in vivo*, despite apparent *in vitro* successes, requires that different approaches are considered. Antivirals including ribavirin, amantadine, and favipiravir have all demonstrated an antiviral effect *in vitro* ([167-169](#_ENREF_167)), but these successes have failed to translate into viable options *in vivo* ([167-170](#_ENREF_167)). The potentiating effect of chemokine and cytokine expression through LAVV delivery has gained considerable interest within the field and may be of use in future rabies PIT options. Centred on expression of host molecules to stimulate or modulate different immunological responses to infection, expression of heterologous genes from a rabies LAVV have yielded promising results although studies are often limited to the mouse model ([71](#_ENREF_71), [112](#_ENREF_112), [116](#_ENREF_116), [122](#_ENREF_122), [126](#_ENREF_126), [131](#_ENREF_131), [140](#_ENREF_140), [146](#_ENREF_146)). The success of these LAVVs must be further investigated to try and offer hope for an appropriate treatment for clinical rabies. A combination of approaches whereby both multiple copies of highly immunostimulatory G proteins are expressed alongside relevant foreign genes from a live virus may enable future development of promising LAVVs. In the absence of effective antiviral molecules, LAVVs may represent the future for clinical rabies treatment.

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## Search Strategy and Selection Criteria

We identified references through searches of both PubMed and Google Scholar with terms including “rabies”, “rabies virus attenuation”, “live rabies virus vaccine”, and “recombinant rabies virus”. The search was not restricted by either language or publication date. In total, 170 references from these searches (and relevant references cited within those articles) were reviewed.

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