



REVIEW ARTICLE

Strategies for the Modification of Vaccinia Virus towards a Better Vaccine Vector

Hao Sun^{1,2}, Xinwen Chen^{1,3,*} and Yun Wang^{1,*}

Abstract

Vaccinia virus (VACV) confers cross-protective immunity against variola virus, the causative agent of smallpox, and has therefore been extensively exploited as a preventive vaccine. Despite the global eradication of smallpox in the 1980s, research efforts involving VACV have continued due to the potential threat of variola virus being used as a bioweapon, which poses grave risks to human health. VACV has emerged as a promising viral vector for vaccine development beyond the traditional application of VACV in smallpox prophylaxis, which has been attributed to its capacity for efficient foreign gene expression. Leveraging strategies, such as serial passage in alternative hosts and genetic engineering techniques, have generated highly attenuated VACV vaccine vectors by deleting specific viral genes, most notably those encoding immunomodulatory proteins. The recent emergence of pathogenic orthopoxvirus outbreaks, exemplified by monkeypox, has reignited scientific interest in VACV research. This review delineates the commonly targeted viral genes for attenuation during VACV vector modification and provides an overview of the progress in VACV-vectored vaccine development.

Keywords: Vaccinia virus, Monkeypox, Smallpox, Attenuated vaccine vector

*Corresponding authors:

E-mail: chen_xinwen@gzlab.ac.cn (XC); wangyun@wh.iov.cn (YW)

¹State Key Laboratory of Virology, Center for Biosafety Mega-Science, Wuhan Institute of Virology, Chinese Academy of Sciences, 44 Hongshancelu Avenue, Wuhan 430071, China

²University of Chinese Academy of Sciences, No. 19(A) Yuquan Road, Shijingshan District, Beijing 100049, China

³Guangzhou National Laboratory, State Key Laboratory of Respiratory Disease, Guangzhou Medical University, Guangzhou 510320, China

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INTRODUCTION

The Poxviridae family is comprised of a group of enveloped double-stranded DNA (dsDNA) viruses with a brick-shaped structure, measuring 200–400 nm in size and possessing a genome size ranging from 130–375 kb. These viruses infect a wide range of hosts, including mammals, birds, reptiles, fish, and insects. Poxviridae is divided into two subfamilies (Entomopoxvirinae and Chordopoxvirinae) and further classified into 22 genera [1]. Among these, the *Orthopoxvirus* genus has been extensively studied due to its significant pathogenicity in humans. Members of the *Orthopoxvirus* genus include variola virus (VARV), cowpox virus (CPXV), vaccinia virus

(VACV), and monkeypox virus [MPXV] [2]. Smallpox, one of the most devastating epidemics in human history, has had severe societal impacts during multiple outbreaks [3–5]. VACV was used as the vaccine for smallpox eradication and remains the most representative member for molecular and cellular research. In recent years VACV and MPXV have continued to infect both wild and domestic animals, leading to zoonotic outbreaks in humans and posing a serious threat to public health [6].

In 1967 the World Health Organization (WHO) initiated a global smallpox eradication campaign (SEC), which was ultimately declared successful in 1980 after a 14-year endeavor [7–11]. This milestone victory over one

of the most formidable contagions for humanity was achieved through robust public health measures, including mass vaccination and effective surveillance. While the successful eradication of smallpox marked the end of a pivotal era in infectious disease control, the residual threat posed by VARV as a potential bioweapon has sustained interest in VACV research. As a result, the focus has shifted from eradication efforts to leveraging VACV as a versatile vector for contemporary vaccine development, particularly in response to emerging threats, such as monkeypox.

The eradication of smallpox and the emergence of recombinant poxviruses engineered from VACV have demonstrated the feasibility of utilizing VACV to express protective antigens from specific pathogens, conferring immunity against those pathogens [1,12]. VACV retains residual virulence despite widespread use that may elicit adverse reactions in certain populations, including immunocompromised individuals [13]. Consequently, there is a pressing need for scientists to develop attenuated VACV strains through strategic gene deletions to enhance safety profiles while maintaining immunogenic potency. Various approaches have yielded numerous attenuated VACV strains, such as modified vaccinia Ankara (MVA) and New York vaccinia virus (NYVAC), which have been shown to improve the safety profile as smallpox vaccines [14–16].

In recent years the threat of orthopoxviruses has once again drawn widespread attention with respect to global public health. Notably, since the outbreak of monkeypox in 2022, the virus has emerged in multiple countries, leading to large-scale cross-border transmission. Although MPXV is not as deadly as smallpox, the diverse transmission routes and the threat MPXV poses to immunocompromised populations have placed significant pressure on public health systems. Additionally, with the acceleration of globalization and changes in the ecologic environment, the risk of other orthopoxviruses infecting humans is also increasing. Given the broad applicability of VACV in immunology and molecular biology research, VACV is considered an ideal platform for developing new vaccines and antiviral strategies. Therefore, research on VACV is not only crucial for developing a universal viral vector for gene delivery, but also provides a scientific basis for addressing potential future orthopoxvirus threats.

ORIGIN AND BIOLOGICAL CHARACTERISTICS OF VACV

VACV and CPXV are closely related [2,17]. VACV and CPXV possess highly homologous genomic sequences, indicating a close evolutionary relationship [18]. Hypotheses regarding the origin of VACV can be broadly categorized into two main theories (natural evolution and laboratory escape). The natural evolution hypothesis encompasses several possibilities, including VACV

representing a hybrid between CPXV and VARV [19], VACV evolving from CPXV through human passages [4], and a perspective suggesting that VACV is more likely to have originated from horses [20]. The laboratory escape hypothesis speculates that VACV might be the result of laboratory manipulations. However, this theory currently lacks substantial supporting evidence.

Overall, the likelihood of VACV originating from a laboratory is relatively low because there is insufficient evidence to suggest VACV was generated in a laboratory setting. The limited laboratory conditions in the 18th–19th centuries made it improbable to deliberately engineer viruses and VACV does not appear to be a simple laboratory hybrid.

VACV is a large, complex enveloped virus belonging to the Poxviridae family along with MPXV and VARV. VACV possesses a linear double-stranded DNA genome approximately 190 kb in length and encoding approximately 250 genes. Viral particles are approximately 360×270×250 nm in size and weigh 5–10 fg [21–23]. Analysis of different VACV strains has revealed that the central region of the genome is conserved, primarily encoding late proteins with a few early proteins, while the terminal regions are variable, mainly encoding non-essential early proteins [24]. VACV particles encode an array of enzymes and proteins, including DNA-dependent RNA polymerases, transcription factors, capping and methylating enzymes, and poly(A) polymerases [25]. Through these enzymes and transcription factors, VACV can transcribe its own mRNAs independent of the host RNA polymerase II [25]. Gene expression in VACV is tightly regulated with continuous coding sequences for VACV genes. These genes can be categorized into early, intermediate, and late genes based on the temporal sequence of expression. Early genes, possibly involved in DNA replication initiation, are transcribed prior to DNA replication but are not translated, while intermediate and late genes are transcribed and translated during viral replication [26,27]. VACV gene expression exhibits a cascading pattern with enzymes and transcription factors required for each subsequent stage encoded by the preceding stage [14].

Poxvirus replication occurs entirely within the cytoplasm of infected cells [28,29]. Virus particles enter cells through direct fusion of viral and cellular membranes or via macro-pinocytosis, followed by release from endosomal compartments [30,31]. Upon cellular entry, lateral bodies disassemble while the core remains intact and is transported to the perinuclear region via the cellular microtubule network. There, the core undergoes morphologic changes and is activated at early transcription sites. Messenger RNAs (encoding early proteins), produced by viral DNA-dependent RNA polymerase, are released from the core into the cytoplasm, where translation occurs on cellular polyribosomes. Following early gene expression and a complex, poorly understood uncoating process, the core wall disintegrates, releasing the DNA genome. This genome immediately associates with

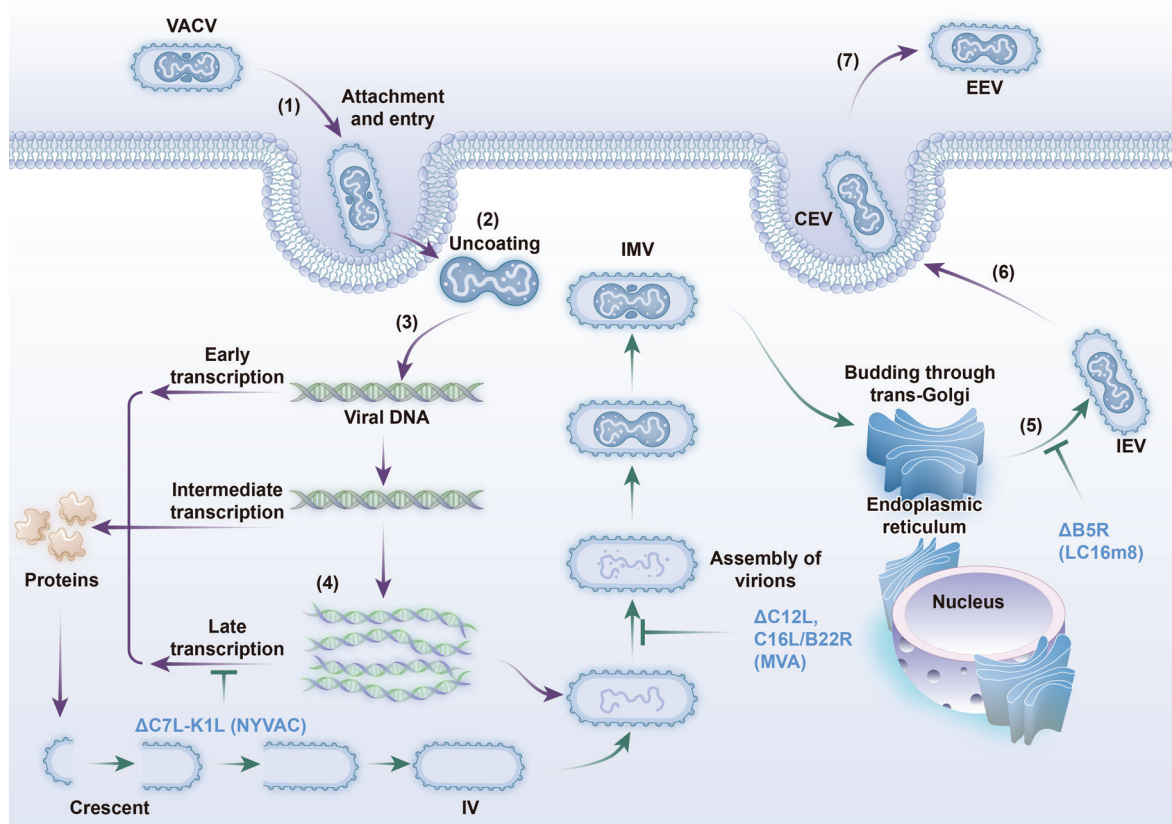


FIGURE 1 | The schematic illustration of the VACV life cycle and key viral proteins in various VACV strains (MVA, NYVAC, LC16m8) that overcome host restriction barriers. (1) Viral cell entry occurs through membrane fusion or endosomal uptake. (2) The core is released in the cytoplasm through the uncoating step. (3) Early gene expression produces non-structural proteins mediating the synthesis of viral double-stranded DNA (dsDNA). (4) The transcription and translation of intermediate genes lead to the expression of enzymes and structural proteins for the assembly of virions and the production of IMVs in viral factories. (5) IMVs travel to the Golgi compartment and undergo envelopment to produce IEVs. (6) IEVs are propelled to the cell surface and fuse with the plasma membrane to form CEVs, which remain attached to the cell. (7) CEVs are then released from the cell and form EEVs. CEVs, cell-associated enveloped virions; EEVs, extracellular enveloped virions; IEVs, intracellular enveloped virions; IMVs, intracellular mature virions; IVs, immature virions; VACV, vaccinia virus.

endoplasmic reticulum membranes and several early viral proteins, forming pre-replication centers. As DNA synthesis progresses, these pre-replication foci expand, leading to the formation of viral factories [32]. After DNA replication and intermediate and late gene expression, viral genomes are packaged to form immature virions, which subsequently mature into intracellular mature viruses (IMVs), representing the first infectious form. IMVs can be released upon cell lysis or acquire a second lipid bilayer from the Golgi network to form wrapped virions (also known as intracellular enveloped virions) and bud to become extracellular enveloped viruses [EVs] [33]. IMVs and EVs differ in their antigenic composition and have distinct roles in host cell invasion and infection cycles. EVs lack some non-structural proteins compared to IMVs [34] but contain additional surface membrane protein components [35]. These characteristics enable EVs to protect the virus against antibody neutralization, facilitating widespread dissemination within the host [34]. Simultaneously, EVs induce the production of host chemokines, recruiting more target cells to facilitate infection [36]. Fig. 1 shows the VACV replication cycle.

DEVELOPMENT HISTORY OF SMALLPOX VACCINES BASED ON VACV

Variolation and first-generation vaccines

The practice of exposing healthy individuals to small amounts of infectious smallpox material for prevention has been documented in both China and Europe since the 15th century [37]. Jenner conducted the first human trial on an 8-year-old boy in 1796 using a CPXV inoculation to prevent smallpox. He subsequently verified the efficacy of a CPXV inoculation in multiple volunteers. Jenner discovered that a CPXV inoculation induces immunity against VARV, thereby preventing smallpox infection [38–40], marking the beginning of the vaccination era. Table 1 provides a comprehensive description of the different generations of smallpox vaccines, illustrating the evolution from early variolation practices to modern genetically attenuated vaccines.

Various viral strains for vaccine inoculation spread worldwide in the years following Jenner's discovery [1,41,42]. These viral strains were widely used in various

TABLE 1 | Description of smallpox vaccine generations.

Generation	Vaccine characteristics	Examples	Advantages	Challenges	Safety Concerns	References*
1 st	Wild-type VACVs, produced from live animals	Dryvax (USA), Parisstrain (France), Bernstrain (Switzerland), Ankara strain (Turkey)	High efficacy in eradicating smallpox; strong and long-lasting immune response	Relied on production from live animals	Higher risk of adverse reactions, such as post-vaccination encephalitis; Not suitable for immunocompromised individuals; severe side effects in some cases	19563829
2 nd	Novel wild-type VACVs, produced using embryonated eggs and cell lines	ACAM2000™	Easier to produce and scale up compared to live animal methods; suitable for mass vaccination	The vaccine is replication-competent	Can cause severe adverse events, such as myopericarditis; contraindicated in individuals with certain skin conditions	18363519
3 rd	Derived through extensive serial passaging in alternative hosts	MVA, LC16m8, Dis, KAVC103	Significantly improved safety, especially for immunocompromised populations; reduced replication in human cells	Lower immunogenicity, often requiring booster doses or adjuvants for enhanced efficacy	Lower immune response in some cases; requires additional research for optimal dosing	5109577 17052815 13918925 34334254
4 th	Attenuated through genetic engineering	NYVAC, NTV, SCV	Precise genetic control allows targeting specific antigens; enhanced safety and broader applicability	High cost of genetic engineering; challenges in regulatory approval due to novel modifications	Long-term safety data is limited; potential concerns regarding unexpected genetic interactions	1566575 32266167 28720468

*Indicates the PMID number of the reference.

countries during the late 18th and early 19th centuries, significantly reducing smallpox morbidity and mortality [5]. The most common VACV strains used to manufacture the first-generation smallpox vaccines (FGSVs) include the New York City Board of Health (NYCBH), which was used for the production of Dryvax [43,44], and Lister and EM-63 [37] vaccines, which were used for the production of Elstree [45] and Lancy-Vaxina Berna vaccines [46]. Other VACV strains that were widely used in Europe, China, or the Union of Soviet Socialist Republics (USSR) were Ankara, Copenhagen, Bern, TianTan, and Tashkent. However, FGSVs still had a risk of severe adverse reactions in vaccinees [47]. Most FGSVs were produced on the live skin of animals, such as cows, sheep, and rabbits, resulting in inconsistent quality and efficacy between vaccine batches. Moreover, the vaccine-induced immunity waned over time [47]. began developing safer and more effective second-generation vaccines after the 1950s with advances in virologic theories.

The major contribution of FGSVs was the validation of the vaccine concept, which protected millions worldwide from smallpox [48] while demonstrating that attenuated strains of pathogens could serve as vaccines. This design approach inspired the development of numerous subsequent vaccines, such as those for influenza and measles [19]. The application of smallpox vaccines propelled large-scale public health immunization campaigns, accelerating the development of public health initiatives. The research and application of smallpox vaccines pioneered modern immunology and vaccinology, profoundly influencing both medical theory and practice.

Second-generation vaccines

Although the FGSVs successfully controlled the epidemic, FGSVs had issues, such as challenging production processes and suboptimal safety [37]. The successful cultivation of VACV in cell cultures replaced the traditional animal inoculation method in the 1950s, which improved vaccine quality [49]. Animal models were also established to evaluate the safety and immunogenicity of second-generation smallpox vaccines [50].

Second-generation VACV vaccines are novel wild-type VACV strains, such as ACAM2000. Due to quality concerns, like microbial contamination associated with using live animals for vaccine production, researchers adopted tissue culture systems or embryonated eggs to produce “second-generation” VACV vaccines. ACAM2000 is a representative second-generation live attenuated smallpox vaccine. ACAM2000 is a clonal derivative of the Dryvax, which was purified by plaque assay in Vero cell cultures [42]. ACAM2000 received FDA approval in 2007 [51], replacing the previous Dryvax vaccine and becoming the current smallpox vaccine in the United States. Compared to first-generation smallpox vaccines, ACAM2000 is produced more consistently

with less batch variability, offering improved safety and inducing stronger, more durable immune responses [52]. The reduced inoculation volume and attenuated virulence mitigate adverse reactions post-vaccination [53]. A single dose of ACAM2000 confers lifelong immunity, demonstrating remarkable protective efficacy against both smallpox virus and MPXV [54]. However, risks of inoculation site infections, systemic vaccinia, and cardiac complications exist [55,56].

Given the safety concerns with second-generation smallpox vaccines, scientists have pursued the development of third-generation vaccines with improved safety profiles while maintaining or enhancing immunogenicity.

Third-generation vaccines

Third-generation smallpox vaccines are primarily produced through classical attenuation of parental VACV strains via multiple passages in alternative tissue culture cells. During this process, many gene deletions and random mutations are introduced into the viral genome, leading to VACV strains showing compromised replication competence in human cells.

MVA was developed through serial passaging in chicken embryo fibroblasts and used as a safer vaccine in the final years of the SEC. Genomic research has indicated that

the parental chorioallantois vaccinia virus Ankara (CVA) strain experienced a genome reduction of approximately 15% [57], decreasing the genome size from 208 kb in CVA to 177 kb in MVA [14]. Six significant genomic deletions were identified, varying in size from 2.6 kb to 10.2 kb, along with numerous shorter deletions, insertions, and point mutations, resulting in the fragmentation, truncation, or deletion of open reading frames [ORFs] [58]. Fig. 2 provides a detailed comparison of the genomes of MVA, NTV, NYVAC, KVAC103, and DIs strains with the Copenhagen strain, highlighting the specific genomic modifications that contributed to VACV attenuation. Consequently, MVA no longer encodes many virulence factors, including virulence factors that inhibit the immune response to the vaccinia virus, such as viral receptors for γ -interferon, α/β interferons, and CC chemokines [59]. The progenitor virus was the Ankara vaccinia strain, which was initially propagated on the skin of calves and donkeys at the Turkish Vaccine Institute in Ankara for smallpox vaccine production [58]. MVA exhibits high attenuation in mouse models [57]. As a live attenuated smallpox vaccine, the primary advantage of MVA over first- and second-generation vaccines is the improved safety profile, including relative safety for immunocompromised individuals. MVA encodes early, intermediate, and late proteins in

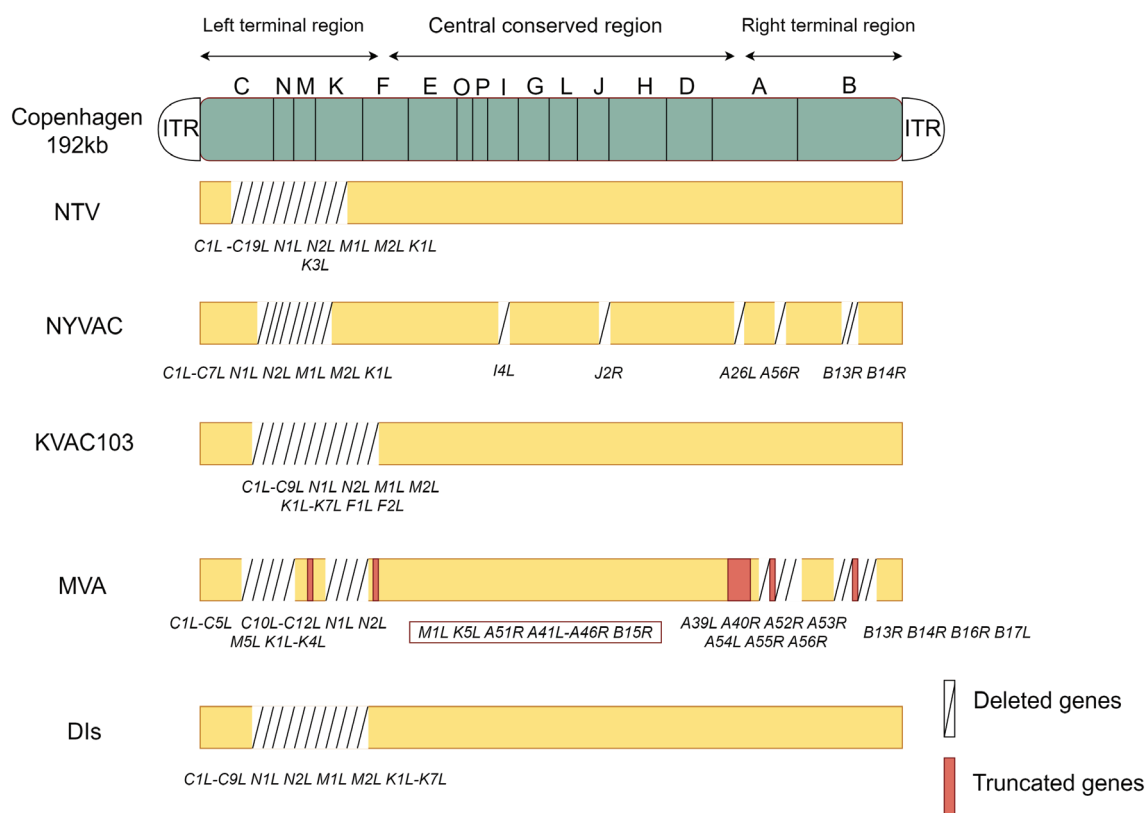


FIGURE 2 | Genomic deletions and truncations in attenuated vaccinia virus strains compared to the Copenhagen strain. This schematic representation illustrates the genomic regions of the Copenhagen vaccinia virus strain (192 kb) and its derivative strains NTV, NYVAC, KVAC103, MVA, and DIs. Yellow bars indicate retained genomic regions in each derivative strain, while diagonal lines represent deleted regions, and red bars denote truncated genes.

most cells but cannot form infectious viral particles [60], which minimizes the risk of severe adverse events [61]. Additionally, MVA is highly immunogenic, inducing robust immune responses at low doses [62]. MVA has the ability to elicit protective immunity against MPXV in non-human primates [63]. Preclinical studies and phase I and II trials have shown MVA to be safe, immunogenic, and relatively safe for immunocompromised individuals, although two doses are required to achieve antibody levels comparable to Dryvax [57]. In recent years improved MVA vaccines have exhibited enhanced immunogenicity in clinical trials [64–66]. Overall, MVA represents a promising third-generation smallpox vaccine candidate, potentially playing a crucial role in responding to bioterrorist attacks or natural outbreaks involving VARV. Research involving the application of MVA in immunocompromised populations indicates that MVA has good safety and immunogenicity. Vaccination with the MVA vaccine elicits specific immune responses with minimal adverse reactions in HIV-positive individuals [67]. Clinical trial results show that the MVA vaccine can induce effective immune responses in patients with impaired immune function, highlighting the potential of MVA as a vaccine vector [68]. Although MVA induces a level of immune response in immunocompromised patients, the degree of immunosuppression may affect the efficacy of vaccination, particularly in patients receiving strong immunosuppressive therapy [69]. Therefore, developing personalized vaccination strategies is crucial for optimizing immune responses. Overall, research involving MVA vaccines in immunocompromised populations shows promising prospects but further clinical studies are needed to confirm the efficacy and safety.

The LC16m8 vaccine was developed by Japanese researchers in 1975 through serial passaging of the Lister strain on rabbit kidney epithelial cells at low temperatures (30°C). The LC16m8 clone exhibited reduced replication capacity in monkey kidney cells after multiple rounds of cloning and screening and formed smaller plaque sizes across various cell lines [70]. LC16m8 demonstrated a similar *in vivo* infection rate compared to the Lister strain but restricted replication, a narrower host range, and significantly reduced adverse effects [70]. LC16m8 exhibited favorable safety profiles in animal studies, while inducing neutralizing antibodies and cell-mediated immune responses [71]. The safety of LC16m8 is attributed to a single nucleotide deletion in the *B5R* gene, impairing the formation of EVs. LC16m8 underwent small-scale phase I/II clinical trials in Japan in the 1970s, supporting its protective efficacy. Human trials in the early 21st century with larger cohorts confirmed the favorable safety and potent immunogenicity of LC16m8, which had overall superiority to second-generation vaccines [72].

The Dairen I strain (DIs) vaccine was derived through serial passaging in 1-day-old embryonated eggs [73]. The DIs harbors a 15.4 kb deletion in the left terminal region (from C9 to K5L) of the VACV genome [74],

which includes the well-characterized host range genes, *K1L* and *C7L*, as well as the interferon resistance gene, *K3L*. The *C7L* gene is associated with the formation of the viral envelope [75], while the *K1L* gene is involved in immune evasion [76]. *C7L* and *K1L* have important roles in the pathogenicity of VACV. Consequently, DIs exhibits a highly restricted host range and its replication cannot be readily rescued by complementing *K1L* and *C7L*. Notably, while similar to the deletion region in the fourth-generation engineered smallpox vaccine, NYVAC, the extent of the DIs host range deletion is more substantial. The DIs was widely used in public immunization programs in the 1950s across the Soviet Union and Eastern European nations and exported to many developing countries. Post-vaccination adverse reactions were relatively mild with the DIs strain but the immunogenicity was slightly lower compared to other VACV strains, like EM-63. Furthermore, the DIs did not provide long-lasting immunity, necessitating booster vaccinations.

KVAC103 is a novel third-generation vaccine derived from the Lancy-Vaxina Berna vaccine after 103 passages in Vero cells. *In vivo* studies demonstrated the safety profile of KVAC103, showing no neurotoxic symptoms compared to the parental or Western-reserve (WR) strains. This attenuated phenotype is likely attributed to two major deletions in the parental strain, resulting in the loss of 23 genes and truncation of 4 genes [77]. Recently, a vaccine candidate based on KVAC103 (designated KVAC103-thPA), which expresses a major component of anthrax toxin (protective antigen [PA]), has been shown to induce binding and neutralizing antibodies against PA in a murine model [78]. KVAC103 represents a promising new third-generation vaccine candidate against smallpox and potentially other infectious diseases, warranting further investigation to elucidate its full potential.

Fourth-generation vaccines

With advances in genetic engineering enabling the insertion, deletion, and disruption of genes at specific genomic loci, targeted attenuation of viruses has become a feasible objective. Fourth-generation vaccines involve modifying the VACV genome to engineer non-replicating or highly attenuated VACV strains that retain immunogenicity against smallpox [79]. Herein we focused on two specific attenuated VACV strains (NYVAC and the recently developed Sementis Copenhagen Vector [SCV]).

NYVAC is a highly attenuated strain of VACV derived from the Copenhagen strain. NYVAC was developed in the 1980s through the targeted deletion of 18 ORFs associated with host range and virulence factors [16,80,81]. These deletions encompass genes encoding thymidine kinase (which restricts viral replication in non-dividing cells), hemagglutinin, complement control protein, and other virulence and host range genes [16,81]. Specifically, the deleted genes include ORFs [*C7L* to *K1L*, *J2R*, *B13R*, *B14R*, *A26L*, *A56R*, and *I4L*] [80,82]. Due to the deletion

of the host range region (from *C7L* to *K1L*), NYVAC exhibits highly attenuated characteristics in most mammalian cell lines, including human cells [83]. Upon entry into human cells, NYVAC can only initiate early replication processes and is unable to complete a full replication cycle, thereby serving as a safer alternative to traditional VACV strains [82]. Despite NYVAC attenuation, NYVAC maintains robust immunogenicity and is capable of eliciting both humoral and cellular immune responses. NYVAC can express high levels of heterologous antigens and induce antigen-specific immune responses [84]. This characteristic makes NYVAC an attractive vector for developing recombinant vaccines against various infectious diseases and cancers. Initially considered as a safer alternative for smallpox vaccination, the genetic stability of NYVAC, large capacity for foreign gene insertion, and favorable safety profile have led to its extensive application in clinical trials for vaccines against HIV, malaria, and various types of cancer [85]. These attributes position NYVAC as a significant contributor to the development of next-generation smallpox vaccines and as a versatile vector for other vaccine applications.

The SCV is a replication-deficient virus generated through the targeted deletion of the viral assembly gene, *D13L*, in the VACV Copenhagen strain. The *D13L* gene product is essential for viral replication. As a result, recombinant SCV vectors require production in modified Chinese hamster ovary (CHO) cells expressing D13 and the host range protein, CP77. This vector has demonstrated complete attenuation in immunodeficient mice while inducing robust and long-lasting cellular and humoral immunity in preclinical models when expressing antigens from Zika virus, Chikungunya virus, or SARS-CoV-2 [86–88]. NYVAC and SCV are promising vectors for vaccine development but NYVAC and SCV exhibit notable differences in immunogenicity and safety profiles. NYVAC has been widely studied and is considered a well-established vector with extensive data supporting its safety and moderate immunogenicity, particularly in the context of HIV, malaria, and other infectious disease vaccines. NYVAC is known to be safe for use in immunocompromised individuals and has few adverse reactions reported in clinical trials. However, the replication capacity of NYVAC is highly restricted, which can limit the strength of the immune response, necessitating the use of adjuvants or booster doses to achieve optimal efficacy. In contrast, SCV (a newer platform) has demonstrated potentially higher immunogenicity in early preclinical trials, particularly for emerging infectious diseases, like SARS-CoV-2. The SCV design allows for the targeted deletion of specific genes to enhance safety, while maintaining a moderate level of replication competence, which may contribute to a stronger immune response. However, due to its recent development, SCV has less extensive clinical data compared to NYVAC and the long-term safety profile remains to be fully characterized.

The non-replicating vaccinia virus, TianTan strain (NTV), is derived from the VACV Tian Tan strain. Genetic attenuation of NTV was achieved through deletion of 21,243 nucleotides encompassing 26 VACV genes associated with host range and virulence [84,89]. This deletion spans the genomic region from *C7L* to *K1L*, rendering NTV incapable of replication in most human cell lines while maintaining its replicative capacity in chicken embryo fibroblasts (CEFs). The replication deficiency of NTV is attributed to the translational shutdown of late viral protein synthesis [89]. Preclinical studies conducted in murine models using recombinant viruses based on NTV have demonstrated favorable safety and immunogenicity profiles [90,91].

The development of third- and fourth-generation vaccines, such as MVA and NYVAC, has brought significant advances in terms of safety. However, these vaccines have also encountered several development challenges. One critical issue is the balance between achieving sufficient attenuation and maintaining strong immunogenicity. For example, extensive passaging of MVA has successfully reduced the replication capacity, making MVA safer for use in immunocompromised individuals, yet this process can sometimes result in diminished immune responses requiring higher doses or booster vaccinations to achieve optimal efficacy [58]. While MVA is generally well-tolerated, mild-to-moderate adverse events, such as local injection site reactions and transient fevers, have been noted, particularly at higher doses [92]. In the case of NYVAC, which incorporates targeted gene deletions for enhanced attenuation, the primary safety concern relates to the long-term genetic stability and potential for unexpected immune responses, especially given the reduced replication capacity [93]. Clinical studies have shown NYVAC to be immunogenic and safe in immunocompromised populations, yet ongoing monitoring is essential to evaluate the stability and efficacy over time.

VACV AS A VACCINE CANDIDATE AGAINST EMERGING AND TRADITIONAL VIRAL DISEASES

The emergence of a novel coronavirus in late December 2019 precipitated a severe pandemic that persists to the present day with profound impact across all of humanity. Since that time the etiologic agent of the COVID-19 pandemic, severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2] [94], has resulted in the deaths of >70 million individuals and infected >700 million globally (<https://covid19.who.int/>). MVA, the most widely-used VACV strain, has been extensively used by research laboratories and pharmaceutical companies globally for the development of MVA-based SARS-CoV-2 vaccine candidates. While the majority of these MVA-based candidates utilize wild-type MVA strains as parental vectors [95–97], alternative MVA vectors have been explored, including vectors with targeted deletions in immunomodulatory

genes, such as *C6L* and *K7R* [98,99]. The MVA vector system demonstrates remarkable capacity for stable expression of not only the full-length SARS-CoV-2 spike (S) protein [100,101], but also additional SARS-CoV-2 antigens, most notably the nucleocapsid (N) protein, thereby expanding the breadth of immune responses against SARS-CoV-2 [96,102]. Investigation has revealed the induction of sustained humoral and cellular responses persisting for a minimum of 6–8 months post-immunization [103]. Various MVA-based vaccine candidates have exhibited substantial immunogenicity across three primary animal models, eliciting robust SARS-CoV-2-specific humoral and cellular immune responses [98,104,105]. Furthermore, MVA has been extensively investigated as a vaccine vector against other emerging viral pathogens, including Ebola virus [106], Marburg virus [107], and MERS-CoV [108], demonstrating promising efficacy in each instance.

The acquired immune deficiency syndrome (AIDS), which is caused by the human immunodeficiency virus (HIV)-1, has become a pandemic that has spread worldwide, critically affecting human health. Attenuated poxvirus vectors expressing HIV-1 antigens are considered promising HIV/AIDS vaccine candidates. For example, one study reported that VACV, as a vaccine vector, can effectively activate the host cellular immune responses and has specificity for HIV antigens [109]. Another study assessing the MVA vaccine showed that this modified VACV enhances the HIV-specific immune response and demonstrates good safety and immunogenicity in animal models [110]. By deleting the *A40R* gene from VACV, researchers further improved the immunogenicity of the MVA-B vaccine, providing new insights for HIV vaccine development [111]. These results indicated that the potential of VACV as a vector for HIV vaccines continues to be validated and future studies should continue to optimize genetic modification strategies and evaluate more efficient immunization protocols.

However, using MVA to tackle other zoonoses does present some critical challenges. First, the development and optimization of MVA vaccines require precise design of specific antigens for different pathogens to ensure effective induction of protective immune responses. This customization process demands substantial preliminary research and validation. Second, in the case of some viruses, the immunogenicity of MVA may be insufficient, necessitating the combination with other vaccine platforms or adjuvants to enhance efficacy. Additionally, the pre-existed immunity against MVA after orthopoxvirus infection or smallpox vaccine inoculation could impair MVA-based vaccine efficacy.

VACV ATTENUATION TARGETS

Genes involved in EV formation and transport

VACV must replicate and disseminate to adjacent or distant cells for propagation within a host, while evading elimination by the host immune response. Modifying

genes associated with EV release to alter the intrinsic EV release characteristics of VACV may impact viral infection, release, and distribution. This approach could facilitate the screening of novel recombinant VACV vectors with potentially enhanced exogenous antigen expression and improved vector vaccine immunogenicity, thereby providing feasible modification strategies for constructing safe and efficacious recombinant VACV vectors.

ST-246, a small molecule drug developed by SIGA Technologies (New York, State of New York, USA) as a potential smallpox treatment, has demonstrated efficacy in reducing morbidity and mortality caused by lethal poxvirus infections in various animal models, including MPXV and VARV infections in non-human primates [112,113]. The mechanism of action involves inhibiting poxvirus cell-to-cell spread [114]. ST-246 targets the p37 protein of poxviruses (encoded by *F13L*), which is crucial for EV formation. By preventing the interaction between p37 and host cell proteins, such as Rab9 and TIP47), which are necessary for EV formation, ST-246 effectively inhibits viral envelope acquisition and release from cells, thereby limiting viral dissemination within the host and mitigating virus-induced symptoms. IMCBH, another inhibitor of VACV release, functions by preventing p37 localization to envelope structures, thus blocking MV envelopment to form EVs and consequently inhibiting virus release [115].

Deletion of the *F13L* gene significantly attenuates viral spread, substantially reducing viral dissemination between hosts. Inhibition of EV formation impairs efficient virus propagation from one host cell to another. This reduced spreading capacity renders *F13L*-deficient VACV highly attenuated. Due to limited viral spread, *F13L* gene knockout VACV can serve as a safer live vaccine, inducing potent immune responses without posing significant risks to recipients. This vaccine can provide effective protection against smallpox and other orthopoxvirus infections, while mitigating the adverse reactions associated with the wild-type VACVs [113,116,117].

Involvement of *B5R*- and *F13L*-encoded proteins is essential for MV acquisition of a double membrane at the trans-Golgi network (TGN) or endosomes [118,119]. The p37 protein function requires acylation modification. Mutation of cysteine residues at positions 185 and 186 to serine prevents protein palmitoylation, resulting in free p37 that does not associate with membrane structures, thereby inhibiting EV formation [120,121]. The B5R protein, a transmembrane protein typically localized to EV envelopes, contains a signal peptide in its cytoplasmic domain that targets B5R to the Golgi network and endosomes. This targeting allows B5R transfer to EVs during the subsequent process of MV acquiring Golgi/endosomal membranes [122]. Deletion of the *B5R* gene significantly impairs the efficiency of MV packaging into EVs, reducing EV release to 10%–20% of the original level [119]. Paradoxically, partial deletion of the B5R protein can lead to increased EV release. For example, deletion of one or more of the four conserved repeat sequences in *B5R* can

markedly enhance EV production [123]. Even a single amino acid mutation (proline-to-serine at position 189) significantly increases EV release [124,125]. The development of LC16m8 resulted from a single base deletion that caused alterations in the *B5R* gene product, thus leading to impaired EV formation, reduced viral pathogenicity, and consequently inhibited viral spread *in vivo* [70].

Well-characterized VACV EV genes include *B5R* and *F13L*. Both *B5R* and *F13L* serve as targets related to EV formation. In the future, genes associated with EV transport also hold potential as targets for therapeutic and vaccine development.

Immunomodulatory and virulence genes

One of the primary objectives in studying the VACV virulence and immunogenicity genes is the development of safer and more efficacious attenuated vaccine vectors. Researchers aim to reduce the pathogenicity of VACV through the deletion or modification of specific virulence and immunogenicity genes, while maintaining or even enhancing VACV capacity to induce immune responses. Table 2 lists the identified viral genes within the VACV genome that can be safely deleted while preserving viral replicative competence. These non-essential viral genes are often deleted during vaccine vectors development to attenuate vector virulence. However, deletion of these viral genes is not without potential drawbacks. One of the primary concerns is the risk of reduced immunogenicity. While some gene deletions may attenuate the virus, deletions can also decrease robustness of the immune response, necessitating higher doses or booster vaccinations to achieve protective immunity. Additionally, unintended genetic interactions may occur, potentially leading to unexpected pathogenicity or altered host immune responses. For example, some deletions might interfere with viral replication dynamics in a way that could enhance the ability of the virus to persist in tissues or evade immune detection [126,127]. Addressing these risks requires careful balancing of safety and immunogenicity during the design and testing phases of modified VACV strains. As research progresses it will be crucial to continue balancing the attenuation of viral virulence with the preservation of immunogenicity. This delicate equilibrium is key to creating vaccine vectors that can be safely administered while effectively eliciting robust immune responses against target antigens.

Orthopoxviruses have evolved multiple molecular mechanisms during co-evolution with susceptible hosts to suppress different stages of the innate and adaptive immune responses. Targeted inactivation of immunomodulatory protein genes typically results in attenuation of the vaccine strain in *in vivo* systems, thereby enhancing the safety profile [128].

The *E3L* gene is a non-essential gene for VACV replication, encoding a virulence factor that suppresses the antiviral activity of interferon. The *E3L* gene is also associated with host range determinants, host defense modulators, and cell death regulators [129]. The *E3L* gene encodes the E3 protein, a double-stranded RNA (dsRNA) binding

protein that competitively binds to cellular dsRNA, inhibiting the interferon-mediated antiviral pathways activated by dsRNA [the protein kinase R {PKR} and 2'-5' oligoadenylate synthetase/RNase L pathways] [130]. PKR activation leads to translational arrest, while RNase L activation degrades viral RNA. Thus, E3 is crucial for poxvirus replication by suppressing these pathways. Typical E3 proteins balance the inhibition of the PKR and RNase L pathways through a high-affinity dsRNA binding domain, ensuring viral evasion of the interferon-mediated immune response [131]. However, if the *E3L* gene undergoes critical point mutations, the reduced affinity of the encoded E3 protein would lead to excessive RNase L activation while failing to inhibit the PKR pathway, thereby attenuating viral replication [131]. The *E3L* gene facilitates viral evasion of the innate immune response, particularly the PKR pathway, by suppressing the interferon-mediated antiviral response in host cells. Deletion of the *E3L* gene significantly impairs VACV replication in interferon-competent host cells [132].

The VACV genes, *A46*, *A52*, *B15*, *K7*, and *N1*, interfere with TLR signaling pathways at various levels. *A46* contains a putative Toll/interleukin-1 receptor (TIR) domain and targets several TIR adaptors, including MyD88, MAL (TIRAP), TRIF, and TRAM [133,134], thereby blocking MAP kinase activation and TRIF-mediated IRF3 activation. *A52* targets IRAK2 and TRAF6 and has a more pronounced effect on inhibiting NF- κ B activation compared to *A46* [135]. Notably, *A52* has also been reported to activate the p38 MAPK and enhance LPS-induced IL-10 [136]. The sequence relationship between *A52* and *N1* proteins led to experiments associating *N1* with inhibition of NF- κ B activation through several signaling pathways [137]. The VACV *N1L* gene encodes an intracellular homodimer with early/late expression characteristics. This protein has been demonstrated to associate with multiple components of the IKK complex and TANK-binding kinase 1 (TBK1), thereby inhibiting activation of NF- κ B and IRF3, respectively [138]. Although *N1L* is not essential for *in vitro* replication, *N1L* has a crucial role in virulence *in vivo*. The *N1L* protein belongs to the Bcl-2-like protein family [139] and has the ability to inhibit apoptosis and modulate the function of the nuclear factor κ B pathway [140]. Compared to wild-type virus infection, intradermal infection with VACV lacking the *N1L* gene results in smaller skin lesions [141]. This finding indicates that *N1L* contributes significantly to the pathogenicity of VACV *in vivo*.

VACV encodes several virulence-associated genes. Among these genes, key virulence genes include *N1L*, *A56R*, *J2R*, *I4L*, and *F4L* [142,143]. These genes have crucial roles in viral pathogenicity, immune evasion, and host adaptation. Researchers have conducted in-depth functional analyses of these genes. For example, experiments involving mutations or insertional inactivation of the *F4L* and *I4L* genes demonstrated that these alterations do not affect normal replication of VACV [144,145]. These

TABLE 2 | Specific immunogenic genes within the VACV genome can be ablated without compromising viral replicative competence.

Gene	Function of gene	Gene length (bp)	Change after gene deletion	References*
B2R	Inhibition of innate immunity	660	Enhanced innate immune response	37016144
B8R	Inhibition of innate immunity	819	Reduced virulence, retains immunogenicity	11119568
B19R	Inhibition of innate immunity	1062	Increased immune response	25609807
C6L	Inhibition of innate immunity	456	Enhanced memory T cell response	21909386
C7L	Inhibition of innate immunity	453	Increased antiviral capacity	19656868
E3L	Inhibition of innate immunity	573	Improved response to interferons	11967338
E5R	Inhibition of innate immunity	996	Heightened immune response	37217469
K1L	Inhibition of innate immunity	855	Increased antiviral response	19656868
N2L	Inhibition of innate immunity	528	Enhanced immune response	23761407
A41L	Inhibition of cytokines	660	Increased antibody response	16361415
B7R	Inhibition of cytokines	549	Enhanced immune response	29280955
B16R	Inhibition of cytokines	873	Augmented immune response	22973033
C12L	Inhibition of cytokines	1062	Improved immunogenicity	22384183
A46R	Inhibition of TLR	645	Increased immune response	24069354
A52R	Inhibition of TLR	573	Heightened immune response	29280955
B15R	Inhibition of TLR	450	Augmented immune response	29280955
K7R	Inhibition of TLR	450	Enhanced immunogenicity	23826170
A26L	Inhibition of apoptosis and other functions	969	Increased cell apoptosis, reduced virulence	31220181 1566575
A35R	Inhibition of apoptosis and other functions	531	Enhanced immune response, increased apoptosis	21352940
A44L	Inhibition of apoptosis and other functions	1041	Increased apoptosis sensitivity	27223301
A56R	Inhibition of apoptosis and other functions	948	Enhanced apoptosis susceptibility	18353946
B6R	Inhibition of apoptosis and other functions	522	Reduced cell survival	29280955
B9R	Inhibition of apoptosis and other functions	234	Increased apoptosis	29280955
B10R	Inhibition of apoptosis and other functions	501	Heightened sensitivity to apoptosis	29280955
B12R	Inhibition of apoptosis and other functions	852	Increased apoptotic response	8277291
B13R	Inhibition of apoptosis and other functions	351	Increased apoptosis	1566575
B14R	Inhibition of apoptosis and other functions	669	Increased apoptosis sensitivity	1566575
E4L	Inhibition of apoptosis and other functions	780	Enhanced cell survival	26821204
F1L	Inhibition of apoptosis and other functions	681	Increased cell survival	31428674
F11L	Inhibition of apoptosis and other functions	1065	Reduced apoptosis, promotes viral survival	20041165
J2R	Inhibition of apoptosis and other functions	534	Increased susceptibility to apoptosis	1566575
I4L	Inhibition of apoptosis and other functions	2361	Increased viral virulence	1566575
A40R	Related to viral virulence	507	Increased viral infectivity	32041218

*Indicates the PMID number of the reference.

findings suggest that these genes may not directly participate in the basic life cycle of the virus but rather influence viral virulence through other mechanisms. Notably, an I4L insertion mutant did not exhibit significant attenuation in a rabbit model but showed slightly reduced neurovirulence

in a mouse model. This result implies that the *I4L* gene might be associated with the neurovirulence of VACV, providing insight for further investigation into the neuroinvasive mechanisms of VACV [144]. VACV also possesses an A-type inclusion (*ATI*) protein repeat gene, which is

highly homologous to its counterpart in VARV but partially deleted in VACV. The status of the *ATI* gene varies among different VACV strains. For example, the WR strain completely lacks the *ATI* gene and is replaced by a sequence encoding a C-terminally truncated 94 kDa protein [146]. In contrast, the Copenhagen strain retains only a small portion of the 3' end of the *ATI* sequence [147]. This variability among strains suggests that *A26L* (the gene associated with *ATI*) may not be a critical virulence factor because the virus maintains essential functions even when absent.

Individual deletions of *N1L*, *C6L*, and *K7L* genes lead to reduced virulence and increased immunogenicity in murine intranasal and intradermal infection models [148]. The VACV *B8R* gene encodes a secreted glycoprotein that exists as a homodimer in infected cells during early infection. This protein is homologous to the extracellular domain of the IFN- γ receptor and can inhibit IFN- γ activity in various mammalian models [149]. Deletion of the *B8R* gene in murine infection studies results in an attenuated phenotype compared to wild-type strains [150], which causes only mild disease, even at high doses [151]. The *C3L* gene encodes a secreted complement binding protein (CBP) in infected cells during early infection [152] that inactivates complement by interacting with C3b and C4b [153,154]. CBP suppresses inflammatory responses [155] and inhibits antibody-dependent neutralization of VACV virions with the assistance of complement proteins [156]. CBP-negative mutant VACV strains exhibit reduced virulence in animal experiments [154,156]. The early/late gene, *A56R*, encodes a surface hemagglutinin protein that mediates viral attachment to host cells, inhibits fusion of infected cells, and promotes proteolytic activation of infectivity. Deletion of the *A56R* gene from the NYCBH VACV strain resulted in a 40-fold reduction in the LD₅₀ compared to the parental strain when inoculated intracerebrally and intranasally in mice [157]. Inactivation of the *HA* gene in the VACV WR strain leads to significant attenuation [158]. The early VACV *J2R* gene expresses viral thymidine kinase. *J2R* mutant viruses exhibit reduced virulence *in vivo* [159,160].

Beyond their roles in VACV, similar genes involved in EV formation and immune modulation are also present in other orthopoxviruses, such as MPXV and CPXV.

These viruses harbor genes analogous to those found in VACV, such as *B5R* and *F13L*, which have crucial roles in viral dissemination and immune evasion. For example, which encodes a key membrane protein involved in EV formation, exhibits >90% sequence homology in VARV (smallpox virus) and MPXV, underscoring the functional importance of *F13L* across these viruses. Similarly, the *B5R* gene, which contributes to EV formation and release in VACV, shows a high homology range of 85%–90% in MPXV and other orthopoxviruses, indicating a potentially similar function in viral survival and propagation. The conservation of these mechanisms suggests that attenuation strategies could be applied to other orthopoxviruses, providing new avenues for developing broad-spectrum antiviral approaches. These findings highlight the potential for extending research on the functions of these genes in VACV to other members of the *Orthopoxvirus* genus, thus offering a broader perspective on possible immunomodulatory strategies.

Host range genes

Host restriction factors pose a significant barrier to viral replication and viruses have evolved corresponding countermeasures [161]. EVs typically enter cells through membrane fusion or endocytosis with MVs capable of utilizing both mechanisms [162,163]. Table 3 provides a detailed list of host range genes in VACV, highlighting the role in viral replication and potential as targets for genetic modification to create safer vaccine vectors. While poxviruses can effectively bind and enter cells from various animal species [164], successful replication after entry depends on the “host range” genes [79,165,166]. Many poxviruses have relatively narrow host ranges [79]. Host-restricted poxviruses have been used as safe vectors for vaccines, gene therapy, or oncolytic virus therapy, although the host restriction mechanisms remain largely unclear [166]. Currently, no single host range gene has been identified as present in all poxviruses [79]. The earliest identified VACV host range genes are *C7L* and *K1L* [167,168]. Studies have shown that deletion of these genes causes premature termination of viral replication before late gene expression [169]. Most mammalian poxviruses

TABLE 3 | VACV host range genes.

Gene	Function of Protein	Cultured cells with defects in virus host range	References*
B5R	Membrane glycoprotein	Vero, CEF, PK-15 and quail (QT-6)	9201221
C7L	Inhibition of apoptosis	RK13, hamster Dede cells	16731942
C12L	May inhibit caspase-independent pathway of apoptosis	PK15 cells, A549	7494278
E3L	Inhibit IFN responses	HeLa, Vero, murine dendritic cell line, CEF (MVA-E3L)	12167348
K1L	Inhibit NF- κ B activation	RK13 cells	15016878
K3L	Pseudosubstrate inhibitor of PKR	BHK cells, mouse L929	12167348
C16L/B22R	Promotes the processing of core protein A3 and enhances virion assembly	A549, MRC-5	32019881

*Indicates the PMID number of the reference.

possess a functional orthologue of the VACV *C7L*. The VACV *C7L* complements *K1L* by counteracting the dsRNA-activated protein kinase pathway through inhibition of eIF2 α phosphorylation [79]. Attenuated VACV strains retaining the *K1L* and *C7L* ORFs have confirmed the anti-apoptotic function of *C7L* [170]. Furthermore, deletion of *K1L* has revealed the ability of *C7L* to counteract the interferon-mediated antiviral response [171].

Through prolonged evolution, most viruses have acquired the ability to inhibit the antiviral PKR pathway [172]. A *K3L*-deficient VACV exhibited growth defects in murine L929 and BHK cells but replicated normally in HeLa and rabbit RK13 cells [173]. Cell infection experiments showed that targeted *E3L* deletion impairs VACV replication in HeLa, Vero, and L929 cells but not in BHK or RK13 cells. Additionally, the host range of an *E3L*-deficient VACV was broader than a *K3L* mutant, suggesting differences in their roles in determining host range. Scientists have also attempted to reintroduce deleted genes into different VACV strains to determine if the deleted genes are host range factors. One study found that reintroducing the *C12L* gene into MVA increased virus spread in human diploid cell lines by >100-fold [174]. Ultimately, the dynamic interplay between viral host range factors and cellular antiviral pathways determines the outcome of a specific infection. A better understanding of the roles played by host range factors in manipulating signaling pathways in target host cells is needed before we can fully comprehend the complex interactions between viral and cellular proteins.

The *C16L/B22R* gene is highly conserved across most orthopoxviruses, typically with one copy located at each terminus of the genome. *C16L* is severely truncated in MVA, while *B22R* harbors a 45-bp deletion, rendering the gene non-functional. *C16L/B22R* is a crucial host range gene, playing a vital role in MVA replication within human cells. Restoration of *C16L/B22R* functionality has been shown to enhance MVA replication capacity across diverse human cell lines [175].

MVTT_{2-GFP} lacks a fragment containing the *M1L*, *M2L*, *K1L*, and *K2L* genes. When inoculated into mice via the intracerebral route, this mutant virus was attenuated 340-fold compared to the wild-type virus. The virus is unable to replicate in several cell lines, including HeLa (human), RK-13 (rabbit), and Vero (African green monkey). The deletion of the host range gene, *K1L*, reduces replication in RK-13 cells and absence of *K1L* along with the serine protease inhibitor, *K2L*, the ankyrin-like protein, *M1L*, and the early gene, *M2L*, further diminishes replication *in vitro* [176].

CONCLUSION

Vaccines have had a pivotal role in preventing numerous infectious diseases. The eradication of smallpox using VACV and the development of the hepatitis B vaccine

represent two of greatest achievements in scientifically combating infectious diseases. Since the 20th century, frequent outbreaks of infectious diseases have posed unprecedented challenges to mankind. In particular, the outbreak of the novel coronavirus pneumonia in 2019 rang the alarm bell and the widespread availability of vaccines accelerated the end of the COVID-19 pandemic. This has also led to the realization that vaccines are the best solution to combat emerging infectious diseases. To successfully develop a new vaccine, a clear understanding of the pathogenesis and immune mechanisms underlying the disease is paramount, followed by breakthroughs in enhancing vaccine immunogenicity and selection of the most appropriate vaccine type and expression system.

VACV has become a promising gene expression vector with broad application prospects due to its long history of implementation in the eradication of smallpox. To date, numerous exogenous genes have been successfully expressed in VACV, with the majority exhibiting biological activities and physicochemical properties like their natural counterparts and some antigens displaying excellent immunogenicity. One of the greatest advantages of VACV in vaccine research is the application of VACV in multivalent genetic engineering vaccines, owing to the large genome capacity, which allows for the insertion of up to 25 kb of exogenous genes without compromising replication, even without gene deletion [177]. Another core advantage of VACV in vaccine research is cytoplasmic expression with DNA remaining outside the nucleus, rendering VACV safer than nuclear-replicating vaccine vectors, such as adenoviruses [25,164]. However, as a live recombinant vaccine vector, the potential for strong host immune reactions and severe adverse effects poses challenges for VACV-based vaccine development. Therefore, while achieving effective immunity, ensuring safety is also crucial.

To further advance the development and application of genetically engineered vector-based vaccines, several key strategies warrant consideration. First, the development of robust vaccine production systems is critical. Leveraging more widely available or simpler production platforms, such as cell culture systems that do not require high-containment facilities, can significantly reduce production costs and increase scalability. It is possible to develop special cell lines that support robust viral replication to further increase viral titers. Alternatively, by altering the ratio of EV:MV particles, the virus can be more abundantly released into the supernatant, thereby reducing the complexity of viral purification. Second, improving methods for safety assessments is vital to ensure the long-term application of these vaccines. This includes comprehensive long-term monitoring for potential issues, such as reversed virulence or unexpected immune responses, that may arise over time. Developing standardized protocols for monitoring viral genetic stability, immune profiling, and conducting longitudinal studies on vaccinated populations,

especially immunocompromised populations, can provide critical insights into the long-term safety profile of these vaccines. By addressing these areas, the development and application of genetically modified vector-based vaccines can be significantly enhanced, leading to safer and more effective vaccination strategies.

VACV has many advantages as an expression vector. This virus is easy to propagate and has a broad host range in cultured cells and experimental animals. Transcription occurs in the cytoplasm. Avoiding the steps of mRNA processing and nuclear-cytoplasmic transport allows for high levels of gene expression and efficient recombination promotes gene insertion. The basic strategy for constructing recombinant vaccinia virus using plasmid transfer vectors was described >40 years ago and remains one of the most popular methods today. An alternative strategy is to prepare DNA through polymerase chain reaction (PCR) without the need for cloning in bacteria. With the development of synthetic biology technologies in recent years, significant progress has been made in assembling virus genomes through synthetic approaches. Viruses, such as SARS-CoV-2, baculoviruses, and herpes simplex viruses, have constructed viral reverse genetics systems or recombinant viruses through synthetic biology techniques [178–180]. Regarding poxviruses, Noyce et al. [181] assembled poxvirus genes into multiple overlapping DNA fragments that covered the entire genome. These fragments are then transfected into cells and under conditions where helper viruses provide replication and transcription machinery, the fragments are rescued as infectious viral particles [96,181]. Synthetic biology builds viruses in a “building block” manner, allowing for custom modules of the virus composition that offers new ideas for the development of VACV vectors. The choice of delivery route depends on the characteristics of the vaccine and the desired immune response. For example, intranasal administration is an attractive method for vaccines targeting respiratory infections because intranasal administration can induce both mucosal and systemic immune responses [182,183]. Poxvirus vectors can also explore various immunization routes, such as through the skin (<https://www.cdc.gov/smallpox/vaccine-basics>), inhalation [184], or even oral delivery [185,186], based on different needs.

Based on these findings, the present article reviews the genetic modification strategies for VACV host range genes, virulence genes, and other aspects, with the hope that this virus, which made a tremendous contribution to the eradication of smallpox, can also make new contributions in the field of genetic engineering vaccine research.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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