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# Improved immune response against HIV-1 Env antigen by enhancing EEV production via a K151E mutation in the A34R gene of replication-competent vaccinia virus Tiantan

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ABSTRACT

The development of an effective HIV-1 vaccine is still a global priority. In recent years, vaccinia virus (VV) has been widely used as an HIV-1 vaccine vector, but its immune efficacy against HIV-1 antigens needs to be optimized. The extracellular enveloped virus (EEV) of VV is capable of faster entry, earlier release, and long-range dissemination. We hypothesized that an improvement in EEV formation by the manipulation of VV genes involved in the EEV release would consequently cause an improved expression of the VV carrying HIV-1 Env antigen and a subsequent enhanced immune response. To this end, an A34R K151E mutant (rVTT-A34R$_{mut}$) from VV Tiantan strain (VTT) with robustly increased EEV release was selected to serve as an optimized vaccine vector. The results were consistent with our hypothesis: the A34R mutant-based HIV-1 vaccine candidate rVTT-A34R$_{mut}$-Env produced more HIV-1 Env antigen *in vitro* and *in vivo*, and thus led to an improved HIV-1 Env-specific T cell immune response, binding antibody, and even the neutralizing antibody response in mice without increased virulence. Meanwhile, the application of the A34R mutation on another VV-based HIV-1 vaccine candidate, VTKgpe, also exhibited a similar immune enhancement effect with no enhanced virulence. The results in this study suggested that rVTT-A34R$_{mut}$ is a potentially improved vaccine vector candidate for human application. In addition, the improvement of the EEV formation via the A34R gene mutation may also be potent in other poxvirus vector-based vaccines against HIV-1 or other pathogens and even cancer in the future.

## 1. Introduction

Human immunodeficiency virus-1 (HIV-1) infections are still a major global health problem. To date, HIV-1 has resulted in more than 60 million infected individuals, and nearly half of the patients have died (Lelievre and Levy, 2016). A preventive vaccine is urgently needed as it is the most efficient method to control the epidemic of HIV-1 infection. Numerous efforts have been devoted to the development of available prophylactic vaccines (Buchbinder et al., 2008; Flynn et al., 2005; Hammer et al., 2013); however, there is no effective vaccine that is currently available. Most of the previous clinical trials experienced disappointing results; only the RV144 trial that used an ALVAC-HIV prime and AIDSVAX B/E boost regimen showed a modest HIV-1 protective effect (31.2%) (Rerks-Ngarm et al., 2009). Researchers believe that the higher quality of immune responses induced by RV144 may lead to improved efficacy and guide the way for HIV-1 vaccine development (Haynes et al., 2014).

Modified vaccinia virus (VV) has been widely used as a vector for prophylactic and therapeutic vaccination against various infectious diseases as well as oncolytic therapy in recent years (Verardi et al., 2012). Its wide mammalian host range and the natural tropism for tumor cells allows its use as a promising oncolytic vector: A well designed oncolytic virus Pexa-Vec (JX-594) has been demonstrated promising anti-tumor effects and stepped into clinical trial for several kinds of cancers including hepatocellular carcinoma, neuroblastoma and Colorectal Cancer (Breitbach et al., 2015; Cripe et al., 2015; Park et al., 2015). Likewise, its excellent safety profiles, high stability, significant immunogenicity against foreign antigens, and its ability to induce protective immune responses make it an excellent vaccine vector. While the predominant non-replicating VV vectors showed outstanding safety profiles, their replication-competent counterparts had the advantage in eliciting more robust immune responses (Kibler et al., 2011). Several

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**Abbreviations**

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|---|---|
| HIV | human immunodeficiency virus |
| VV | vaccinia virus |
| VTT | vaccinia virus strain Tiantan |
| EEV | extracellular enveloped virus |
| hpi | hours post infection |
| IMV | intracellular matured virus |
| NAb | neutralizing antibody |
| IM | intramuscular |
| MOI | multiplicity of infection |

replication-competent vaccine vector-based HIV-1 vaccines have shown certain efficacy in non-human primates, and they are currently in various stages of clinical trials (Garcia-Arriaza et al., 2017; Liu et al., 2015; Zurawski et al., 2017). The vaccinia virus Tiantan (VTT) strain has played a critical role in the eradication of smallpox in China, and it has been applied as a vaccine vector against HIV and influenza virus, for example (Liu et al., 2015; Xiao et al., 2013). Efforts have been made to develop a balance between immunogenicity and safety of VTT vector-based vaccines; most were focused on decreasing the virulence (Dai et al., 2008; Li et al., 2016; Wu et al., 2013) but less on improving both immunogenicity and safety simultaneously.

There are multiple approaches for vaccine improvement of VV vectors, including the widely used heterologous prime/boost immunization regime (Draper et al., 2013; Gomez et al., 2012; Pantaleo et al., 2010), the combined use of adjuvants (Gomez et al., 2009; Liu et al., 2008a), the optimization of carrying target antigens (Barouch et al., 2013; Santra et al., 2010), or the direct manipulation of the VV vector backbone (Garber et al., 2012; Garcia-Arriaza et al., 2013, 2014). Another potent way is through increasing vector replication capacity to improve the duration and level of target antigen expression in the host (Kibler et al., 2011; Quakkelaar et al., 2011). VV contains two distinct infectious forms: intracellular mature virus (IMV) and extracellular enveloped virus (EEV) (Meiser et al., 2003). In comparison to IMV, which remains within the infected cells until lysis and represents the majority of infectious progeny, EEV-the minority (< 1%) of infectious progeny, is capable of faster entry (Locker et al., 2000), earlier release (Smith and Vanderplasschen, 1998), and long-range dissemination (Roberts and Smith, 2008; Smith and Vanderplasschen, 1998). Therefore, the use of early-release EEV will enable a larger amount of cells to be infected sooner, and subsequently, there will be more infected cells and progeny viruses.

Several VV envelope proteins have been proven to play important roles in the formation of EEV at different stages (Smith et al., 2002). Firstly, the IMV wrapping to form enveloped virus requires B5R and F13L proteins (Blasco and Moss, 1991; Engelstad and Smith, 1993); then, the movement of enveloped virus to cell membrane requires A36R and F12L proteins (Herrero-Martinez et al., 2005); finally, the release of cell-associated enveloped virus (CEV) to form EEV requires A33R, A34R and B5R proteins (Duncan and Smith, 1992; Roper et al., 1998). Deletion of these genes can certainly affect the EEV formation. The A33R and A34R knock-out mutant exhibited increased EEV production, while deletion of the other genes (including A36R, B5R, F12L, and F13L) showed decreased EEV production (Smith et al., 2002). Remarkably, even single point mutation in certain envelope proteins affects the EEV formation. Previous reports showed that a P189S point mutation on B5R is sufficient to improve the EEV formation, in a mechanism of loosening the contact between extracellular virus and host membrane during release (Horsington et al., 2013; Katz et al., 2002). The A34R K151E point mutation is also reported to improve EEV formation, but works in a different manner from B5R mutant (Horsington et al., 2013; McNulty et al., 2011).

We hypothesized that an improvement in EEV formation through the manipulation of virus genes involved in the viral release would consequently cause an improved expression of the VTT carrying HIV-1 antigens in timing and/or in quantity, and thus making them potent to elicit an enhanced immune response in an animal model. Here, we present the results of a study of an optimized VTT-vectored HIV-1 vaccine candidate based on EEV enhancement via manipulation of the A34R gene.

## 2. Materials and methods

### 2.1. Cell lines and viruses

The cell lines, including BHK-21, Vero, Caco2, Hela, MDCK, 293-T, and mouse skeletal muscle cell C2C12, were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS (Gibco). The parental wild-type VTT and HIV-1 vaccine candidate VTKgpe were obtained from the Chinese Center for Disease Control and Prevention (China CDC). The viral stocks were prepared as previously described (Zhang et al., 2011).

### 2.2. Recombinant virus/viral replication assay

Homologous recombination was used to obtain recombinant VTT (rVTT) viruses as previously described (Zhu et al., 2007). Briefly, the shutter vectors pCDNA-A34R$_{mut}$-GFP/pCDNA-A34R$_{mut}$ harboring an A34R gene fragment with a K151E point mutation were used to generate the recombinant virus rVTT-A34R$_{mut}$, and shutter vector pCDNA-Env-GFP containing the HIV-1 clade B' envelope gene was utilized to construct the vaccine candidates rVTT-Env and rVTT-A34R$_{mut}$-Env (as illustrated in Fig. 2b). Target clones were screened with a GFP marker by plaque purification under a fluorescence microscope. All of the envelope genes that participate in the EEV formation, including A33R, A34R, A36R, B5R, F12L, F13L were sequenced. These genes were identical in recombinant virus rVTT-Env and rVTT-A34R$_{mut}$-Env except for the desired point mutation in A34R gene (data not shown).

Viral replication capability was evaluated in Vero, BHK-21, and/or C2C12 cells. Briefly, the cells that had been cultured in 24-well plates were infected with the indicated viruses at the MOI of 0.05 or 1. The culture medium with non-adsorbed viruses was washed away 90 min later and replaced with 500 μl fresh medium (DMEM plus 3% FBS, 1% penicillin and streptomycin). Twenty-four hours later, the extracellular virus in supernatants (Ext., Contains mostly the EEV virions, representing the EEV production) and cell-associated virus in cell lysates (Cel., contains mostly the IMV virions, representing the IMV production) were collected and determined by plaque assay on Vero cells, respectively.

### 2.3. Quantitative real-time PCR

The samples, including cell lysates and tissue homogenates of mice muscle, were resolved in 1 ml of TRI REAGENT (Molecular Research Center, Inc., USA) for the extraction of total RNA. Quantitative real-time PCR was performed in a Bio-Rad real-time PCR cycler using SYBR QPCR master mix (Vazyme Biotech, China) and the conditions were: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 52 °C for 30 s, and 72 °C for 15 s. Primers were designed by the Primer 5 program, and their sequences and the product size are listed in Supplementary Table S1. The transcript of beta-actin was used for internal normalization and the data are displayed in every $10^6$ actin copies.