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ARTICLE

A marker-free system for highly efficient construction of vaccinia virus vectors using CRISPR Cas9

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The current method for creation of vaccinia virus (VACV) vectors involves using a selection and purification marker, however inclusion of a gene without therapeutic value in the resulting vector is not desirable for clinical use. The Cre-LoxP system has been used to make marker-free Poxviruses, but the efficiency was very low. To obtain a marker-free VACV vector, we developed marker gene excision systems to modify the thymidine kinase (TK) region and N1L regions using Cre-Loxp and Flp-FRET systems respectively. CRISPR-Cas9 system significantly resulted in a high efficiency (~90%) in generation of marker gene-positive TK-mutant VACV vector. The marker gene (RFP) could be excised from the recombinant virus using Cre recombinase. To make a marker-free VV vector with double gene deletions targeting the TK and N1L gene, we constructed a donor repair vector targeting the N1L gene, which can carry a therapeutic gene and the marker (RFP) that could be excised from the recombinant virus using Flp recombinase. The marker-free system developed here can be used to efficiently construct VACV vectors armed with any therapeutic genes in the TK region or N1L region without marker genes. Our marker-free system platform has significant potential for development of new marker-free VACV vectors for clinical application.

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INTRODUCTION

Since the eradication of smallpox, vaccinia virus (VACV) has been utilized as a vector for development of vaccines for the prevention of infectious diseases as well as in cancer immunotherapies,[1–7] and oncolytic therapies.[8,9] The renewed interest in VACV has driven a number of vaccine and therapeutic candidates to clinical trials, showing encouraging results for cancer treatment.[10,11] To exploit VACV to best effect, it needs to be modified by deletion of gene(s) or arming with a therapeutic gene.[8,12] The thymidine kinase (TK) is the gene commonly targeted, but other genes can also be replaced. The traditional method for modification of VACV is based on homologous recombination (HR). However, the frequency of recombination is typically less than 0.1%,[13] and the purification of recombinant virus is tedious and time-consuming. To purify the recombinant virus, a number of selection methods have been described including selection for TK-positive or -negative phenotypes,[14] and resistance to neomycin[15] or mycophenolic acid.[16] In addition, β-galactosidase,[17] β-glucuronidase,[18] and fluorescent reporter constructs[19] have also been used. However, the marker gene in the resultant mutant virus is not desirable in clinical use, and the Cre/loxP system has been used to remove the marker gene from the recombinant virus,[20] however the efficiency of recovery of the recombinant marker-free vector was still very low.

The CRISPR (clustered regularly interspaced short palindromic repeat)-Cas system is a natural microbial immune mechanism against invading viruses and other genetic elements.[21–23] There are three CRISPR-Cas systems in a range of microbial species.[24] The type II CRISPR-Cas system consisting of the RNA-guided Cas9 endonuclease (from Streptococcus pyogenes), a single guide RNA (sgRNA) and the trans-activating crRNA (tracrRNA), has been developed for genome editing in eukaryotic cells.[25,26] The Cas9 system can be designed to cleave any sequence preceding a 5′-NGG-3′ PAM sequence in mammalian cells.[25,26] It has been a huge success in efficient generation of genetically modified cells and animal models.[25–34] It has also been used to modify adenovirus and type I herpes simplex virus.[35] Recently, we developed an efficient method to edit the VACV genome using the CRISPR Cas9 system.[36]

Here we developed a marker-free system for efficiently and rapidly making VACV vectors by combining the CRISPR Cas9 and two repair donor vectors targeting the TK and N1L genes respectively. The RFP marker gene in the resultant TK-deleted VACA vector can be excised using Cre recombinase, and the RFP marker gene in the resultant N1L-deleted VACA vector can be excised using Flp recombinase. The marker-free system platform developed in this study has significant potential for development of new marker-free VACV vectors for clinical application.

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RESULTS

Construction of a homologous recombination repair donor vector with an excisable RFP gene targeting the VACV TK region

The TK gene in VACV is commonly deleted to engineer a safer vector and to insert therapeutic or reporter genes. Therefore, we first constructed a repair donor vector to target the TK region for homologous recombination. The cassette for HR is cloned between the *Eco*RI sites of the pGEMT-easy vector (Figure 1a). RFP or GFP have frequently been used as plaque purification markers to purify engineered mutant VACV. However, these genes are not desirable features for the virus to possess when moving towards clinical trials as they may impair the efficacy of the vector and compromise the safety of the treatment. In order to remove RFP in the mutant VACV vector subsequently, we adopted the Cre-Loxp system to make RFP excisable after a pure TK-deleted VACV vector was obtained. The RFP gene is flanked by LoxP sites in the repair donor vector (Figure 1a).

To make the TK repair donor vector more versatile for inserting therapeutic genes, we included a multiple cloning site (MCS, with *PacI*, *SmaI*, *PmeI*, *NheI* and *MIUI* as unique sites) downstream of the VV H5 promoter, (Figure 1a). The resulting TK region repair donor vector is designated as pTK Loxp-RFP.

Efficient editing of the VACV TK region by gRNA-guided Cas9

Previously, we have shown that homologous recombination of VACV can be efficiently mediated by gRNA-guided Cas9 in the N1L and A46R regions.[36] To gain highly efficient HR in making TK-mutant VACV using the TK region repair donor vector pTK Loxp-RFP, we designed gRNAs targeting the TK gene and constructed three gRNA vectors targeting this region (Figure 1b) (designated as TKgRNA1, TKgRNA2, and TKgRNA3 hereafter). We observed successful HR events using all three gRNA-guided Cas9, with rates for TKgRNA1, TKgRNA2, and TKgRNA3 being 93, 94, and 67%, respectively (Supplementary Figure S1 and Supplementary Table S1). The time to complete the whole process of obtaining pure mutant VACV is approximately 10–12 days. The resulting mutant VACV was designated vTK-Loxp-RFP (Supplementary Table S2).

RFP is excisable in VTK-Loxp-RFP using Cre recombinase

To remove the RFP selection marker from the resulting TK-deleted VACV vector, we employed the Cre-LoxP system (Figure 1a; Figure 2a). RFP was removed in about 10% of plaques after vTK-Loxp-RFP infection of CV-1 cells expressing Cre recombinase (Figure 2b). The RFP-negative plaques were purified until the RFP-positive plaques were completely diluted out upon a few rounds of infection of CV-1 cells by RFP-negative purification. The deletion of RFP in the final mutant VACV vector was also confirmed by polymerase chain reaction (PCR) (Figure 2c). The resulting mutant VACV was designated vTK-RFPnull (Supplementary Table S2).

Generation of TK-deleted VACV vector armed with the therapeutic transgene human IL12 without RFP

To test the potential of the TK region repair donor pTK Loxp-RFP for introducing therapeutic genes into the TK-mutant VACV, we cloned human Interleukin-12 (hIL-12) into the MCS of the vector,

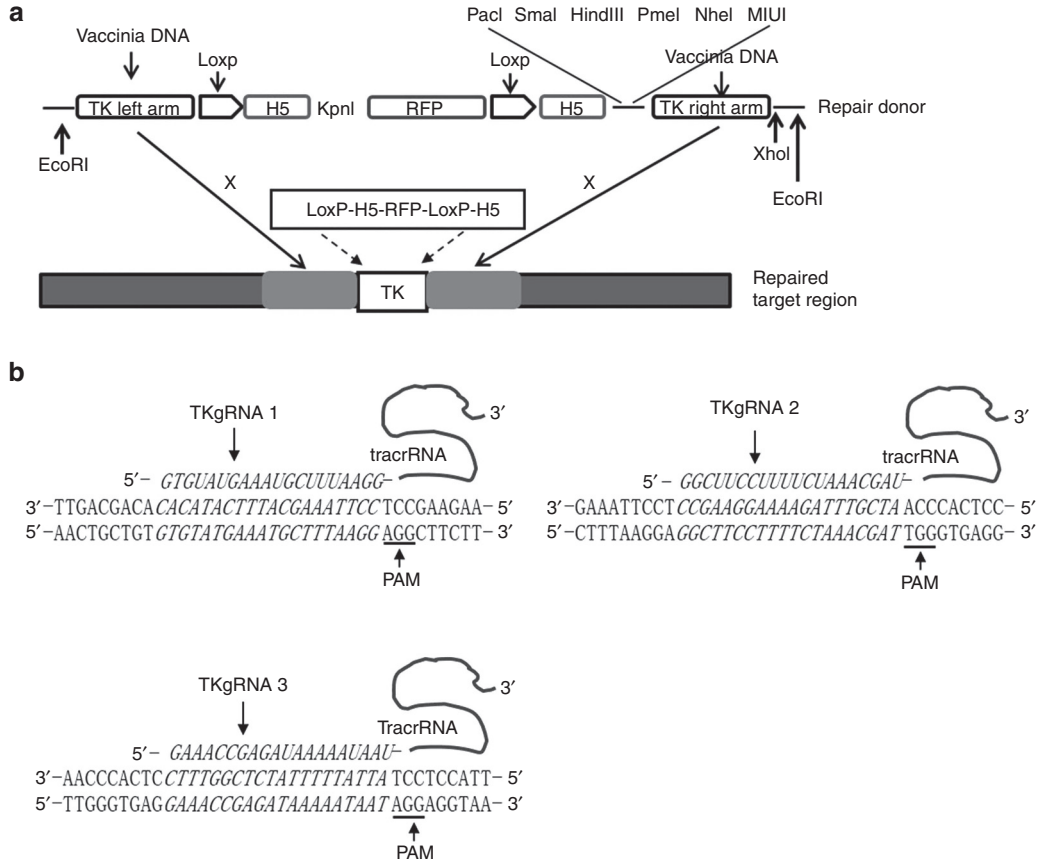

Figure 1 gRNA-guided Cas9 induces homologous recombination in the TK region of VACV. (**a**) Schematic of the shuttle vector pTK Loxp-RFP (TK gene repair donor vector) cassette and repaired target region on the VACV genome. H5 is the promoter driving gene expression. RFP refers to red fluorescent protein. (**b**) The sequence of TK gRNA1, 2 and 3 and their alignment on the TK gene. PAM is underlined. TK, thymidine kinase; VACV, vaccinia virus.

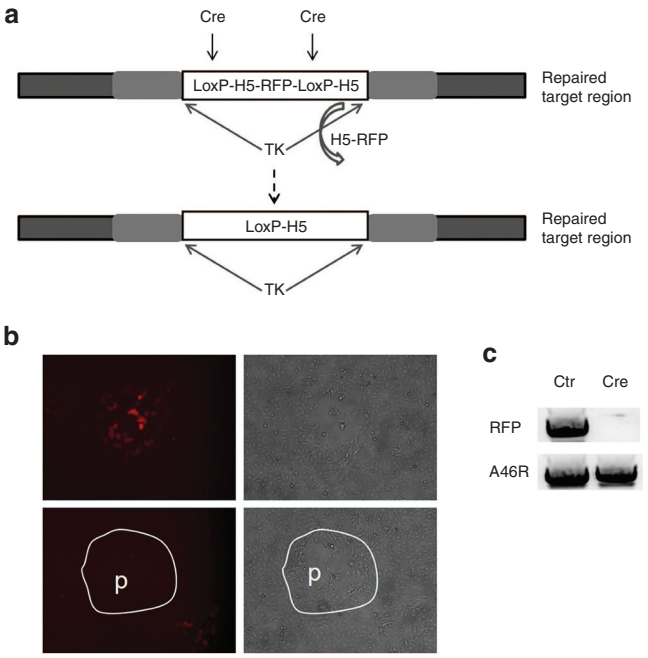

Figure 2 The RFP selection marker in modified VACV is excisable by Cre recombinase. (**a**) Schematics of Cre targeting Loxp sites spanning RFP in VACV before and after RFP is removed by Cre. (**b**) Images of plaques of TK-LoxP-RFP "in" (lower panel) or "not in" (upper panel) presence of Cre expression. Pure plaques of TK-LoxP-RFP were used to infect CV-1 cells with or without Cre expression. The RFP-negative plaque (indicated with p) was picked up and purified further. (**c**) RFP gene deletion was verified by PCR in pure mutant VACV obtained from vTK LoxP-RFP virus after RFP was excised by Cre in CV-1 cells. A46R gene amplification was used as a DNA control. Ctr is the control sample from the TK-LoxP-RFP virus, Cre is the sample from a pure plaque after RFP was removed by Cre from the TK-LoxP-RFP virus. RFP, red fluorescent protein; TK, thymidine kinase; VACV, vaccinia virus.

the expression of hIL-12 was driven by the H5 promoter (Figure 3a) (designated as pTK Loxp-RFP-hIL12). TK gRNA1 and Cas9 plasmids were cotransfected into CV-1 cells to facilitate HR using pTK Loxp-RFP-hIL12. The rate of successful HR in RFP-positive plaques was 80% (8/10) (Figure 3b), and the resulting mutant VACV was designated vTK-hIL2-RFP (Supplementary Table S2). hIL-12 was expressed efficiently in the mutant VACV (Figure 3c). To remove RFP from the mutant VACV, pCAG-Cre was transfected into the CV-1 cells 24 hours before infection with the mutant VACV (Figure 4a). RFP was successfully removed from numerous plaques (Figure 4b). One RFP-negative plaque was purified further by infection of CV-1 cells until no RFP-positive plaques were present. The deletion of RFP in the mutant VACV VTK-hIL12 was confirmed by amplification of RFP using PCR (Figure 4c), and hIL12 expression was maintained after RFP excision (Figure 4d). The resulting mutant VACV was designated vTK- hIL2-RFPnull (Supplementary Table S2).

Construction of a homologous recombination repair donor vector for the VACV N1L region with excisable RFP

To make VACV a more potent oncolytic virus, we further engineered a TK-deleted VACV by deletion of the virulence factor N1L gene, and the double gene-deleted VACV vector shows better selectivity and antitumour potency compared to the VACV with only TK deletion (unpublished data). Since we had exploited the Cre-Loxp system in removing the RFP gene from the TK-deleted virus, we in turn used the Flp-FRT system incorporated into the N1L region in the repair donor vector to excise the RFP from the mutant N1L VACA, in which the cassette for HR is cloned between the *HindIII and KpnI* sites of the pUC19 vector with the RFP gene flanked by FRT sites in the repair donor vector (Figure 5a).

To make the N1L repair donor vector more versatile for carrying therapeutic genes, we included a MCS downstream of the H5 promoter. *SnaB*I, *Sal*I, *EcoR*V, *Pme*I, *Bgl*II, *Nhe*I, and *Pac*I are unique sites in the MCS, which can be used for cloning of any therapeutic genes. The H5 promoter was used to drive RFP and potential transgene expression in the MCS of the vector (Figure 5a). The resulting N1L region repair donor vector is designated as pN1L FRT-RFP.

RFP is excisable from mutant VACV using Flp recombinase

We previously showed that HR in the N1L region was efficiently achieved using the gRNA vector N1L gRNA2-guided Cas9.[36] N1L gRNA2 and Cas9 were cotransfected into CV-1 cells to facilitate HR using pN1L FRT-RFP (the backbone virus used for HR was vTK-RFPnull). The rate of successful HR in RFP-positive plaques was about 78% (11/14) (Figure 5b), and the resulting mutant VACV was designated vTK-N1L-RFP (Supplementary Table S2). To remove the RFP selection marker from the resulting Flp-deleted VACV, we employed the Flp-FRT system (Figure 5a, Figure 6a). RFP was removed in about 10% of plaques after vN1L-FRT-RFP infection of CV-1 cells expressing Flp recombinase (Figure 6b). The RFP-negative plaques were purified until the RFP-positive plaques were completely diluted out upon a few rounds of infection of CV-1 cells by RFP-negative selection. The deletion of RFP in the final mutant VACV was also confirmed by PCR (Figure 6c). The resulting mutant VACV was designated vTK-N1L-RFPnull (Supplementary Table S2).

Generation of N1L-mutant VACV armed with the therapeutic transgene human IL-21 without RFP

To test the potential of the N1L region repair donor pN1L Flp-RFP for introducing therapeutic genes into the N1L-mutant VACV, we cloned human IL-21 (hIL-21) into the MCS of the vector under the H5 promoter (Figure 7a) (designated as pN1L FRT-RFP-hIL21). N1L gRNA2 and Cas9 were cotransfected into CV-1 cells to facilitate HR using pN1L Flp-RFP-hIL21, and the backbone virus used for HR was vTK-RFPnull. The rate of successful HR in RFP-positive plaques was 88.9% (8/9) (Figure 7b), and the resulting mutant VACV was designated vTK-N1L-hIL21-RFP (Supplementary Table S2). hIL-21 was expressed efficiently in the mutant VACV (Figure 7c). To remove RFP from the mutant VACV, pCAG-Flp was transfected into the CV-1 cells 24 hours before infection using the mutant VACV (Figure 8a). RFP was successfully removed from numerous plaques (Figure 8b). One RFP-negative plaque was purified further until no RFP-positive plaques were present. The deletion of RFP in the mutant VACV VN1L-hIL-21 was confirmed by amplification of RFP using PCR (Figure 8c), and hIL-21 expression was maintained after RFP removal (Figure 8d). The resulting mutant VACV was designated vTK-N1L-hIL21-RFPnull (Supplementary Table S2). In addition, using the pN1L FRT-RFP vector system, we successfully made the vTK-N1L-mIL21-RFPnull virus (Supplementary Result, Supplementary Figures S2 and S3, Supplementary Table S2).

DISCUSSION

The efficiency of the traditional HR method for constructing mutant VACV is less than 0.1%.[13] Recently we showed that the efficiency of mutant VACV construction can be improved significantly using gRNA-guided Cas9 system, by which we generated mutant VACV

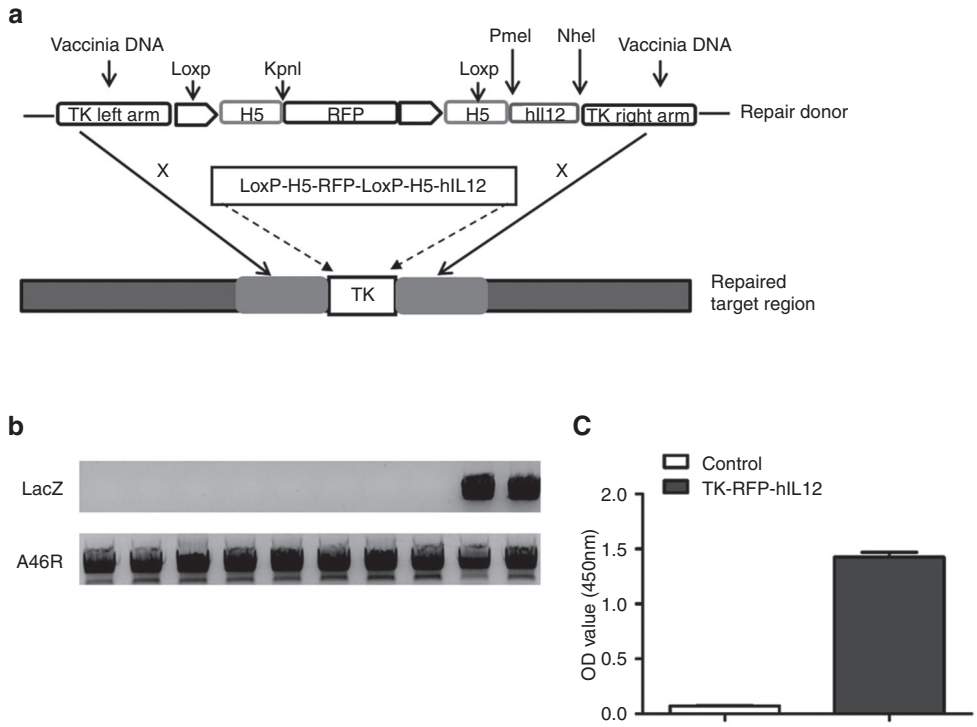

Figure 3 gRNA-guided Cas9 induces homologous recombination in the TK region with a repair donor vector expressing hIL12. (**a**) Schematic of the homologous recombination cassette of the shuttle vector (TK gene repair donor vector) pTK LoxP-RFP-hIL12 expressing hIL12 and repaired target region on the VACV genome. (**b**) TK gene deletion was verified by polymerase chain reaction (PCR) in pure plaques of mutant VACV obtained from TKgRNA1-guided Cas9-induced homologous recombination. Partial LacZ gene was amplified by PCR to confirm the HR in TK region, A46R gene amplification was used as a DNA control. 8/10 plaques show TK deletion. (**c**) hIL12 expression was detected by enzyme-linked immunosorbent assay from the supernatant of pure vTK-Loxp-RFP-hIL12 virus-infected CV-1 cells. HR, homologous recombination; RFP, red fluorescent protein; TK, thymidine kinase; VACV, vaccinia virus.

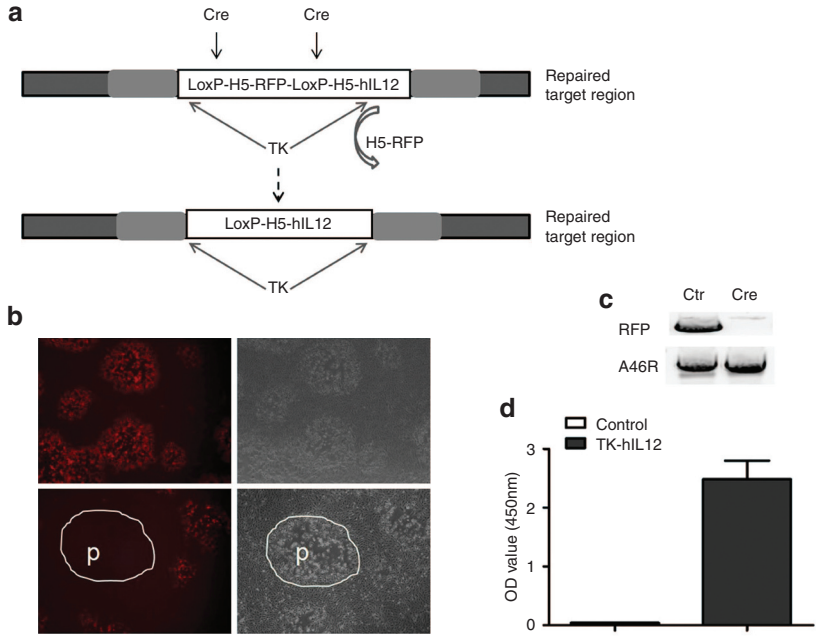

Figure 4 The RFP selection marker in mutant vTK-Loxp-RFP-hIL12 is excisable by Cre recombinase. (**a**) Schematic of Cre targeting LoxP sites spanning RFP in VACV before and after RFP was removed by Cre. (**b**) Images of plaques of vTK-Loxp-RFP-hIL12 "in" (lower panel) or "not in" (upper panel) the presence of Cre expression in CV-1 cells. A pure plaque of vTK-LoxP-RFP-hIL12 was used to infect CV-1 cells with or without Cre expression. The RFP-negative plaque (indicated with p) was picked up and purified further. (**c**) RFP gene deletion was verified by polymerase chain reaction in pure mutant VACV obtained from vTK-LoxP-RFP-hIL12 virus after RFP was excised by Cre in CV1 cells. A46R gene amplification was used as a DNA control. Ctr is the control sample from vTK-LoxP-RFP-hIL12 virus; Cre is the sample from pure plaque after RFP was removed by Cre from vTK-LoxP-RFP-hIL12 virus. (**d**) hIL12 expression detected from the supernatant of CV-1 cells infected with pure vTK-hIL12 virus by enzyme-linked immunosorbent assay after RFP was removed by Cre. RFP, red fluorescent protein; TK, thymidine kinase; VACV, vaccinia virus.

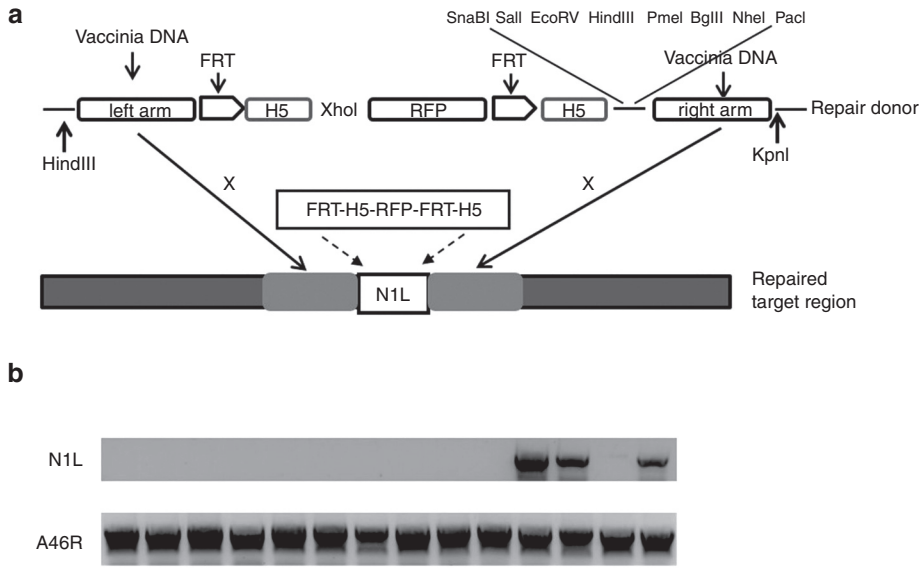

Figure 5 gRNA-guided Cas9 induces homologous recombination in the N1L region of VACV. (**a**) Schematic of the shuttle vector pN1L FRT-RFP (N1L gene repair donor vector) cassette and repaired target region on the VACV genome. (**b**) N1L gene deletion was verified by polymerase chain reaction (PCR) in pure plaques of mutant VACV obtained from N1L gRNA2 guided Cas9-induced HR. Partial L026 gene and Partial N1L gene was amplified by PCR to confirm the HR in N1L region. A46R gene amplification was used as a DNA control. 11/14 plaques show HR in the N1L region. HR, homologous recombination; RFP, red fluorescent protein; VACV, vaccinia virus.

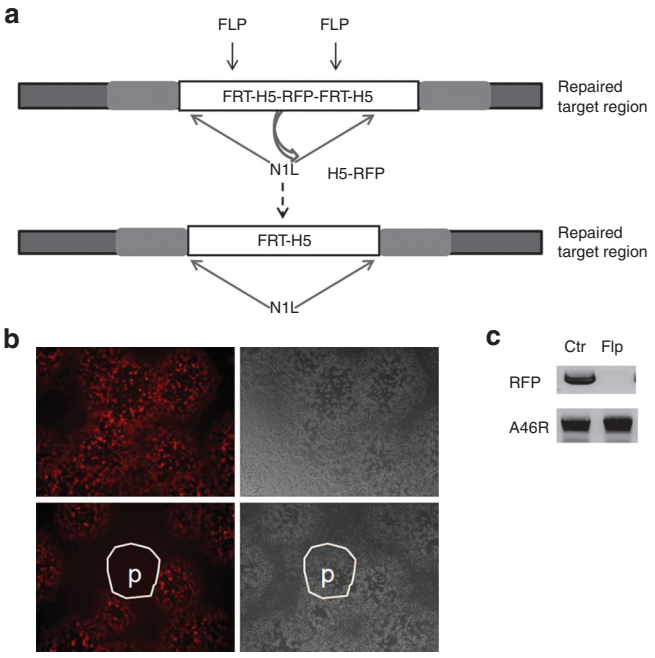

Figure 6 The RFP selection marker in modified VACV is excisable by Flp recombinase. (**a**) Schematics of Flp targeting FRT sites spanning RFP in VACV before and after RFP is removed by Flp. (**b**) Images of plaques of N1L-LoxP-RFP "in" (lower panel) or "not in" (upper panel) presence of Flp expression. Pure plaques of N1L-FRT-RFP were used to infect CV-1 cells with or without Flp expression. The RFP-negative plaque (indicated with p) was picked up and purified further. (**c**) RFP gene deletion was verified by PCR in pure mutant VACV obtained from vN1L LoxP-RFP virus after RFP was excised by Flp in CV-1 cells. A46R gene amplification was used as a DNA control. Ctr is the control sample from the N1L-LoxP-RFP virus; FlP is the sample from a pure plaque after RFP was removed by Flp from the N1L-LoxP-RFP virus. RFP, red fluorescent protein; VACV, vaccinia virus.

with deletion of the N1L or A46R regions.[36] We used the gRNA-guided Cas9 system to achieve high HR efficiency in making mutant VACA with repair donor vectors with excisable marker genes.

VACV is usually modified by gene deletion to create a more selective virus for infection and lysis of tumour cells. This improves the efficacy of the treatment as well as its safety. Deletion of the TK region is one of the most common modifications to the VACV genome to achieve its selectivity.[8,9,12,37] We created the TK region repair donor vector pTK Loxp-RFP, which has the features of an excisable RFP selection marker flanked by LoxP sites, and unique restriction enzyme sites that can accommodate any therapeutic gene in the MCS, under control of an endogenous, stable H5 promoter. Combination of this donor vector with TK gRNA-guided Cas9 system was used to efficiently and rapidly produce TK-deleted VACV armed with a therapeutic gene, hIL-12 as an exemplar. Comparing three TK gRNAs, TKgRNA1- and TKgRNA2-guided Cas9 can induce more than 90% HR, subsequently, one of these two TK gRNA vectors was used for TK region HR. After construction of the TK-deleted VACV using the donor vector pTK Loxp-RFP or pTK Loxp-RFP-hIL-12, RFP was successfully removed. The removal of RFP using Cre recombinase system does not alter the expression of hIL-12 in vTK-hIL-12. Within just 10–12 days, a mutant VACA was made using the above method.

As the MCS of the TK donor vector pTK Loxp-RFP has the potential to accommodate any therapeutic gene, combination of this donor vector with TK gRNA1- (or TKgRNA2)-guided Cas9 would efficiently engineer a TK-mutant VACA armed with a therapeutic gene free of the plaque purification marker RFP.

VACV has distinctive features for development as a tumor-targeted oncolytic virus for cancer treatment,[5,38–40] TK-deleted Lister strain VACV is an attractive platform for development of the next generation of oncolytic viruses.[39] The next generation of tumor-targeted oncolytic VACV should be safer and more potent compared to TK-deleted VACV. The VV N1L gene (called L025 in the VVL strain)

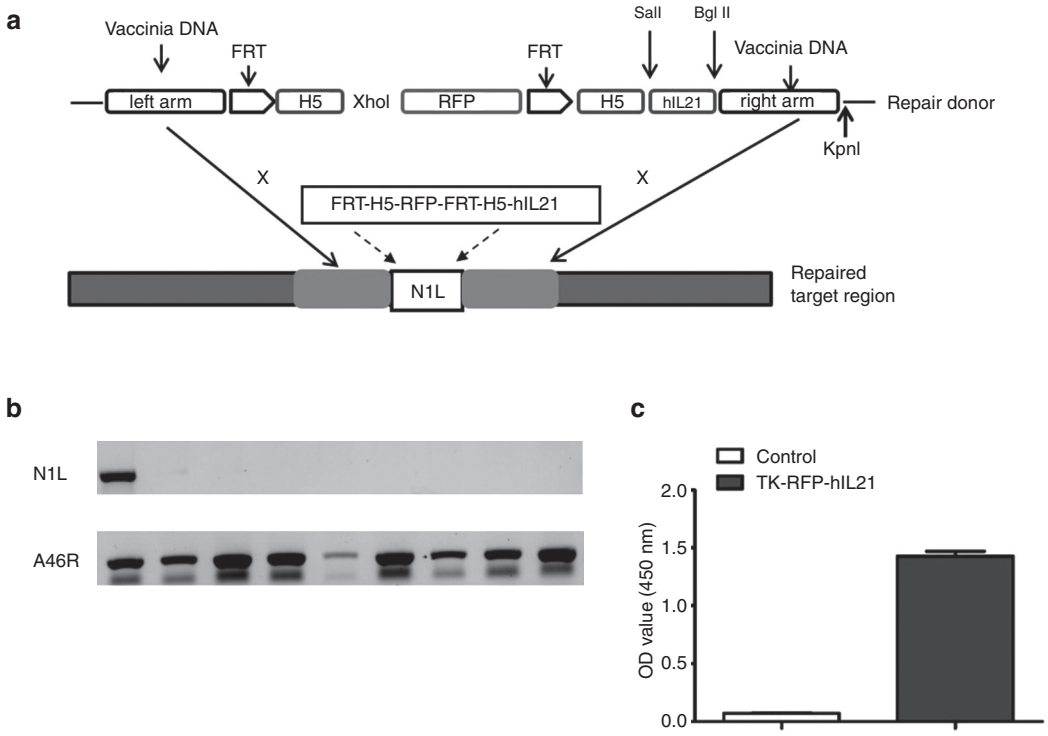

Figure 7 gRNA-guided Cas9 induces homologous recombination in the N1L region with a repair donor vector expressing hIL21. (**a**) Schematic of the homologous recombination cassette of the shuttle vector (N1L repair donor vector) pN1L FRT-RFP-hIL21 expressing hIL21 and repaired target region on the VACV genome. (**b**) N1L gene deletion was verified by PCR in pure plaques of mutant VACV obtained from N1L gRNA2-guided Cas9-induced homologous recombination. Partial N1L and Partial L026 gene amplification was used to confirm the HR in N1L region, A46R gene amplification was used as a DNA control. Eight out of nine plaques show N1L deletion. (**c**) hIL21 expression was detected by enzyme-linked immunosorbent assay from the supernatant of pure vN1L-Flp-RFP-hIL21 virus-infected CV-1 cells. HR, homologous recombination; RFP, red fluorescent protein; TK, thymidine kinase; VACV, vaccinia virus.

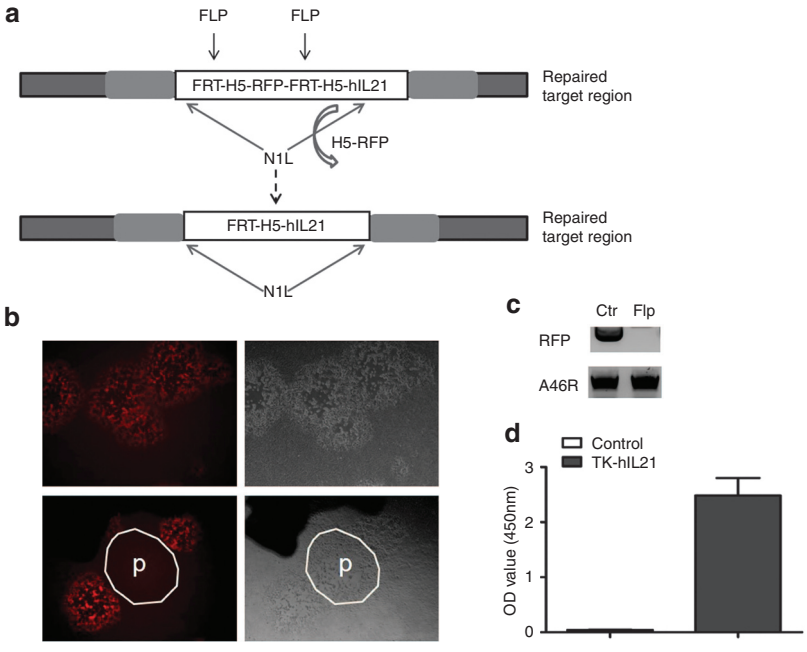

Figure 8 The RFP selection marker in mutant vN1L-Flp-RFP-hIL21 is excisable by Flp. (**a**) Schematic of Flp targeting FRT sites spanning RFP in VACV before and after RFP was removed by Flp. (**b**) Images of plaques of vN1L-Loxp-RFP-hIL21 "in" (lower panel) or "not in" (upper panel) the presence of Flp expression in CV-1 cells. A pure plaque of vN1L-LoxP-RFP-hIL21 was used to infect CV-1 cells with or without Flp expression. The RFP-negative plaque (indicated with p) was picked up and purified further. (**c**) RFP gene deletion was verified by PCR in pure mutant VACV obtained from vN1L-Flp-RFP-hIL21 virus after RFP was excised by Flp in CV-1 cells. A46R gene amplification was used as a DNA control. Ctr is the control sample from vN1L-FRT-RFP-hIL21 virus, Flp is the sample from pure plaque after RFP was removed by Flp from vN1L-Flp-RFP-hIL21 virus. (**d**) hIL21 expression detected by enzyme-linked immunosorbent assay from the supernatant of CV-1 cells infected with pure vN1L-hIL21 virus after RFP was removed by Flp. RFP, red fluorescent protein; TK, thymidine kinase; VACV, vaccinia virus.

is a major virulence gene that when disrupted,[41,42] was shown to significantly reduce pulmonary toxicity following various routes of administration into animal models. N1L gene disruption led to a significant reduction in the neurovirulence of VACV, with reduced viral titres in the brains of mice that had been directly inoculated.[43] Recently, we demonstrated that deletion of N1L gene can significantly enhance antitumour immunity by oncolytic VACV (unpublished data). Therefore, N1L gene is another potential region that can be deleted to insert therapeutic genes. We have shown here that the Flp-FRT system is another site-specific recombination system to efficiently excise reporter genes.[44] The VTK-N1L-RFPnull, VTK-N1L-hIL21-RFPnull, and VTK-N1L-hIL12-RFPnull viruses could be useful in the construction of vectors for the clinic.

MATERIALS AND METHODS

Cell culture and transfection

CV-1 (Monkey kidney fibroblast) cells were maintained in Dulbecco's Eagle's medium (Life Technologies, Paisley, UK) supplemented with 5% fetal bovine serum (Hyclone, Northumberland, UK), 100 U/m; penicillin, and 100 µg/ml streptomycin at 37 °C with 5% CO_2. CV-1 cells were transfected using Effectene (Qiagen, Manchester, UK) according to the manufacturer's instructions.

Restriction enzymes

All restriction enzymes were bought from New England Biolabs (Hitchin, UK).

Plasmids

pCAG-Cre (Addgene,#13775), pCAG-Flpe (Addgene, #13787), and pGEM-Teasy (Promega, UK) were purchased commercially.

Plasmid purification

Plasmids were purified using the Qiagen miniprep kit (Qiagen, Manchester, UK) according to the manufacturer's instructions.

Cloning of gRNAs

The target RNA sequences for the TK region of VACV were selected following the principle stated previously.[27] gRNA oligos with extra ends for cloning were synthesized (Sigma, UK) and cloned into the gRNA cloning vector PB-gRNA-*Bsa*1 vector[45] as follows:

PB-gRNA-*Bsa*I vector was digested with *Bsa*I to produce sticky ends on the backbone as 3-GAAC-5 and 3-GTTT-5. After annealing and ligation, a 20 bp sequence will replace the 400 bp unrelated sequence in the backbone, which was checked by *Xho*I-*Bgl*II digestion (If ligation works, 0.5k + 1.7K + 3.9K bands will be seen; if not, 0.9K + 1.7K + 3.9K bands will be seen). The sequences of the gRNA oligos are shown in the Supplementary Figure S1 and Table S1. The individual gRNA coding sequences in the resulting vector were confirmed by Sanger sequencing. The resulting plasmids were designated TKgRNA1, TKgRNA2, and TKgRNA3.

Construction of a TK-directed shuttle vector containing RFP flanked by LoxP sites for homologous recombination

The left arm for TK region (TK left arm) homologous recombination was amplified by PCR from Lister strain of VACV DNA with forward primer 5′-TCAAACATCTGCGTTATCA-3′ and reverse primer 5′-*CTCGAG GGTACCTATTT ATGATTATTTCTCGCTTTCAATTTAACACAACCCTCAAGAACCTTTGTATTTATTTTCA ATTTTT*ATA **ACTTCGTATAGCATACATTATACGAAGTTAT**CGTTCATGATGACAA TAAAGA-3′ (the elements of this primer are as follows: *Xho*I and *Kpn*I are underlined; *H5 promoter*; **LoxP**; and reverse primer sequence to amplify VACV gene). The right arm for the TK region (TK right arm) homologous recombination was amplified by PCR from Lister strain of VACV DNA with forward primer 5′-*AAGCTTGTTTAAACGCTAGCACGCGT*GTAGAAAGTG TTACATCGACTC A-3′ (*Hin*dIII, *Pac*I, *Nhe*I and *Mlu*I are underlined) and reverse primer 5′-*CTCGA G*ATTTCAGCTGAATATGAAGGA-3′ (*Xho*I is underlined). RFP was amplified by PCR using dsRED (Clonetech) as the template with forward primer 5′-*GGTACC*GCTACCG GACTCAGATCCA-3′ (*Kpn*I is underlined) and reverse primer 5′-*AAGCTTCCCGGGTTAAT TAATATTTATGATTATTTCTCGCTTTCAATTTAAC ACAACCCTCAAGAACCTTTGTATTTATTTTCAATTTT***ATAACTTCGTATAGCATAC ATTATACGAAGTTAT**CGCCTTAAGATACATTGATGAG-3′ (*Hin*dIII, *Sma*I and *Pac*I are underlined; *H5 promoter;* **LoxP**; and reverse primer sequence to amplify the RFP gene). Extensor Long PCR ReddyMix Master Mix (Thermo Scientific, UK) was used for all PCR reactions. The PCR products were resolved on agarose gel and purified from the gel using a gel purification kit (Qiagen). All PCR fragments were cloned into the pGEMT easy vector; the sequence of individual cloned fragments was verified by Sanger sequencing. The RFP-H5 fragment was released by *Kpn*I and *Hin*dIII digest from the pGEMT-easy RFP-H5 plasmid. The TK right arm was released by *Hin*dIII and *Xho*I digest from the pGEMT-easy TK right arm plasmid. The RFP-H5 and TK right arm were cloned into the *Kpn*I and *Xho*I sites of pGEMT-easy-TK left arm-H5 plasmid. The resulting plasmid was the TK gene repair donor vector pGEMT-easy-TK left arm-Loxp-H5-RFP-Loxp-H5-TK right arm, which is the shuttle vector for the homologous recombination into the TK region, and is referred to as TK Loxp RFP vector hereafter. The sequences of individual cloned fragments were verified by Sanger sequencing.

Construction of a shuttle vector TK-Loxp-RFP-hIL-12

Human IL-12 (hIL-12) was released by *Sna*BI and *Nhe*I restriction digestion from pORF hIL-12 (Invivogen, UK). The released hIL-12 was cloned into the *Pme*I and *Nhe*I sites of the TK Loxp RFP vector. The resulting plasmid is TK Loxp RFP-hIL-12.

Construction of an N1L-directed shuttle vector containing RFP flanked by FRT sites for homologous recombination

The left arm for N1L region (N1L left arm) homologous recombination was amplified by PCR from Lister strain of VACV DNA with forward primer 5′-*AAGCTT*GTCCTATCGTAGGCGATAGA-3′ (*Hin*dIII is underlined) and reverse primer 5′-*GGTACC* GAATTCGAATTC*CTCGAGTATTTATGATTATTTCTCG CTTTCAATTTAACACAACCCTCAA GAACCTTTGTATTTATTTTCAATTTTT***GAAGTT CCTATACTTTCTAGAGAATAGGAACTTC**GATCTAATGATTGATCTATATGGTG-3′ (the elements of this primer are as follows: *Xho*I and *Kpn*I are underlined; *H5 promoter;* **FRT site**; and reverse primer sequence to amplify VACV gene). The right arm for N1L region (N1L right arm) homologous recombination was amplified by PCR from Lister strain of VACV DNA with forward primer 5′-*AAGCTT GTTTAAAC AGATCT GCTAGC TTAATTAA* ATCTAATAAGTAGAGTCCT-CAT GCT-3′ (*Hin*dIII *Pme*I, *Bgl*II, *Nhe*I and *Pac*I are underlined) and reverse primer 5′-GGTACCCGGAAGGTAGTAGCATGGA-3′ (*Kpn*I is underlined). RFP was amplified by PCR using dsRED (Clonetech) as the template with forward primer 5′-*CTCGAG* GCTACCG GACTCAGATCCA-3′ (*Xho*I is underlined) and reverse primer *5′-AAGCTT GATATC GTCGACTACGTA*TATTTATGATTATTTCTCG CTTTCAATTTAACACAACCCTCAAGAACC TTTGTATTTATTTTCAATTTTTGAAGT TCCTATACTTTCTAGAGAATAGGAACTTCCGCCTTAAGATACATTGATGAG-3′(the elements of this primer are as follows: *Hin*dIII, *Eco*RV, *Sal*I, and *Sna*BI are underlined; H5 promoter; **FRT site**; and reverse primer sequence to amplify the RFP gene). Extensor Long PCR ReddyMix Master Mix was used for all PCR reactions. The PCR products were resolved on agarose gel and purified from the gel using a gel purification kit. All PCR fragments were cloned into the pGEMT easy vector, and the sequence of individual cloned fragments was verified by Sanger sequencing. The RFP-H5 fragment was released by *Xho*I and *Hin*dIII digestion from the pGEMT-easy RFP-H5 plasmid. The N1L right arm was released by *Hin*dIII and *Kpn*I digestion from the pGEMT-easy N1L right arm plasmid. The RFP-H5 and N1L right arm were cloned into the *Kpn*I and *Xho*I sites of the pGEMT-easy-N1L left arm-H5 plasmid. The resulting plasmid was the N1L gene repair donor vector pGEMT-easy-N1L left arm-FRT-H5-RFP-FRT-H5-N1L right arm, which is the shuttle vector for the homologous recombination into the N1L region, and is referred to as N1L Loxp RFP vector hereafter. The sequences of individual cloned fragments were verified by Sanger sequencing.

Construction of shuttle vector N1L-FRT-RFP-hIL21

Human IL-21 (hIL-21) was released by *Sal*I and *Bgl*II restriction digest from pORF hIL-21 (Invivogen). The released hIL21 was cloned into the *Sal*I and *Bgl*II sites of the N1L FRT RFP vector. The resulting plasmid is N1L FRT RFP-hIL-21.

Construction of shuttle vector N1L-FRT-RFP-mIL-21

Mouse IL-21 (mIL-21) was released by *Sal*I and *Bgl*II restriction digestion from pORF mIL-21 (Invivogen). The released mIL-21 was cloned into the *Sal*I and *Bgl*II sites of the N1L FRT RFP vector. The resulting plasmid is N1L FRT RFP-mIL-21.

Virus production and titration

VACV was produced and titrated as described previously using CV-1 cells to propagate the virus.[5]

Cas9-mediated homologous recombination

2×10^5 CV-1 cells were seeded into each well of a six-well plate the day before transfection. gRNA vector was cotransfected with Cas9 into CV-1 cells in the six-well plate. The next day, the transfected well was infected with 0.01 pfu/cell of backbone virus. The repair donor vector for homologous recombination was transfected into infected wells 2 hours after virus infection. Cells were harvested 24 hours later, and frozen at −80 °C for plaque purification (Supplementary Figure S4).

Plaque purification of mutant VACV

3×10^5 CV-1 cells were seeded into each well of a six-well plate the day before virus infection. 0.5 µl of lysate was used to infect one six-well plate of CV-1 cells, six 6-well plates were infected for each recombinant virus (first round of virus purification). After 2 days of infection, RFP-positive plaques were picked up under fluorescence microscopy using a 20× objective lens (Olympus). After one freeze-thaw cycle, the lysate was added into two six-well plates of 80% confluent CV-1 cells. The following day, one RFP-positive plaque was picked from each of the first round plaque-infected six-well plates, and put into one cryovial containing 200 µl serum-free Dulbecco's Eagle's medium. After one freeze-thaw cycle, the lysate was added into two six-well plates with CV-1 cells seeded the day before infection (second round plaque purification). Plaque purification continued as above for three to five more rounds until the plaques were pure.

Verification of mutant TK VACV

CV-1 Cells were infected with purified plaques. Infected cells were harvested after 2 days of infection. VACV DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. The VVL15 virus (TK-deleted VACV with LacZ inserted into TK region) was used as the backbone virus for HR. To verify the deletion of LacZ gene in the TK region, part of LacZ gene was amplified by PCR using forward primer: 5′-TCGGCTTACGGCGGTGA-3′ and reverse primer: 5′-GCAAGTGTATCTGCCGT-3′. A control DNA fragment spanning the A46R and A47L genes was amplified by PCR using forward primer: 5′-TTGGCTATTAAACAGTA TGGA-3′ and reverse primer: 5′-GGATCCCGATAACAAATG-3′. Extensor Long PCR ReddyMix Master Mix was used for all PCR reactions. The PCR products were analyzed by 1% agarose gel electrophoresis.

Verification of mutant N1L VACV

CV-1 Cells were infected with purified plaques. Infected cells were harvested after 2 days of infection. VACV DNA was extracted using DNeasy Blood & Tissue Kit according to the manufacturer's protocol. To verify the deletion of the N1L gene, a DNA fragment spanning the N1L gene and the L026 gene was amplified by PCR using forward primer: 5′-TATCTAGCAATGGACCGT-3′ (within the N1L gene) and reverse primer: 5′-CCGAAGGTAGTAGCATGGA (within the L026 gene). A control DNA fragment spanning the A46R and A47L genes was amplified by PCR using forward primer: 5′-TTGGCTATTAAACAGTA TGGA-3′ and reverse primer: 5′-GGATCCCGATAACAAATG-3′. Extensor Long PCR ReddyMix Master Mix was used for all PCR reactions. The PCR products were analyzed by 1% agarose gel electrophoresis.

Excision of RFP using Cre recombinase

pCAG-Cre was transfected into CV-1 cells in one well of six-well plate. Twenty-four hours after transfection with pCAG-Cre, CV-1 cells were infected with 100–200 pfu of Cre-RFP VACV. Two days later, RFP-negative plaques were picked (Supplementary Figure S5), and used to infect CV-1 cells in six-well plates to purify RFP-negative plaques. Then RFP-negative plaques were picked and CV-1 cells were infected until no RFP-positive plaques were seen under fluorescence microscopy every 2 days (Supplementary Figure S6). The excision of RFP from the virus by Cre recombinase was tested by PCR of RFP gene.

Excision of RFP using Flp recombinase

pCAG-Flpe was transfected in CV-1 cells in one well of six-well plate. Twenty-four hours after transfection with pCAG-Flpe, CV-1 cells were infected with 100–200 pfu of Flp-RFP VACV. Two days later, RFP-negative plaques were picked (Supplementary Figure S5), and used to infect CV-1 cells in six-well plates to purify RFP-negative plaques. Then RFP-negative plaques were picked and CV-1 cells were infected until no RFP-positive plaques were seen under fluorescence microscopy every 2 days (Supplementary Figure S6). The excision of RFP from the virus by Flp recombinase was tested by PCR of RFP gene.

Verification of RFP-deleted mutant VACV

After purification of RFP-negative plaques upon the excision of RFP by Cre or Flp recombinase (RFP was amplified by PCR using forward primer 5′-GCTACCGGACTCAGATCCA-3′and reverseprimer5′-CGCCTTAAGATACATTG ATGAG-3′ to verify the deletion of RFP in the RFP-negative mutant VACV, A control DNA fragment spanning the A46R and A47L genes was amplified by PCR using forward primer: 5′-TTGGCTATTAAACAGTATGGA-3′ and reverse primer: 5′-GGATCCCGATAACAA ATG-3′.

Enzyme-linked immunosorbent assay

The expression of hIL-12 was detected by enzyme-linked immunosorbent assay (eBioscience, UK) following the manufacturer's instructions. The expression level of hIL1-2 was recorded as OD value. The expression of hIL-21 was detected by enzyme-linked immunosorbent assay (eBioscience) following the manufacturer's instructions. The expression level of hIL-21 was recorded as OD value.

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