Vaccine 34 (2016) 4827–4834

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Increased attenuation but decreased immunogenicity by deletion of multiple vaccinia virus immunomodulators

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ARTICLE INFO

Article history:
Received 25 April 2016
Received in revised form 30 July 2016
Accepted 1 August 2016
Available online 17 August 2016

Keywords:
Vaccinia virus
Immunogenicity
NF-κB inhibitor
IRF3 inhibitor
Vaccine efficacy

ABSTRACT

Vaccinia virus (VACV)-derived vectors are popular candidates for vaccination against diseases such as HIV-1, malaria and tuberculosis. However, their genomes encode a multitude of proteins with immunomodulatory functions, several of which reduce the immunogenicity of these vectors. Hitherto only limited studies have investigated whether the removal of these immunomodulatory genes in combination can increase vaccine efficacy further. To this end we constructed viruses based on VACV strain Western Reserve (WR) lacking up to three intracellular innate immunomodulators (N1, C6 and K7). These genes were selected because the encoded proteins had known functions in innate immunity and the deletion of each gene individually had caused a decrease in virus virulence in the murine intranasal and intradermal models of infection and an increase in immunogenicity. Data presented here demonstrate that deletion of two, or three of these genes in combination attenuated the virus further in an incremental manner. However, when vaccinated mice were challenged with VACV WR the double and triple gene deletion viruses provided weaker protection against challenge. This was accompanied by inferior memory $CD8^+$ T cell responses and lower neutralising antibody titres. This study indicates that, at least for the three genes studied in the context of VACV WR, the single gene deletion viruses are the best vaccine vectors, and that increased attenuation induced by deletion of additional genes decreased immunogenicity. These data highlight the fine balance and complex relationship between viral attenuation and immunogenicity. Given that the proteins encoded by the genes examined in this study are known to affect specific aspects of innate immunity, the set of viruses constructed here are interesting tools to probe the role of the innate immune response in influencing immune memory and vaccine efficacy.

1. Introduction

The poxvirus family is a diverse group of dsDNA viruses that replicate in the cytoplasm of infected cells. The potential use of this family, particularly the well-characterised vaccinia virus (VACV) strains, as vaccine vectors has been under investigation since the 1980s [1]. VACV has many features that make it attractive for this use; a large capacity for foreign gene expression, relative ease of manipulation, and, importantly, induction of strong innate and memory immune responses, including both cellular and humoral arms [2]. Much research has focused on modified virus Ankara (MVA) and NY-VAC because of their severe attenuation, and, at least for MVA, the inability to replicate in most mammalian cells. These vectors are safe even in immunocompromised hosts [3,4], which is of particular importance for vaccination against diseases such as HIV-1, malaria and tuberculosis [2]. There is still a need to improve the immunogenicity of these vectors, particularly because large viral doses, repeated vaccination or prime-boost regimes are required to achieve adequate correlates of protection [5].

The host antiviral innate immune response provides a strong selective pressure and, consequently, viruses have evolved a plethora of mechanisms to counteract its effects. Engagement of viral components with innate immune receptors activates transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and interferon (IFN) regulated factors (IRF)-3/7 that coordinate the production of pro-inflammatory cytokines, chemokines and type I IFNs (reviewed in [6,7]). Importantly this pro-inflammatory milieu attracts professional antigen presenting cells to the site of infection, providing an important link to the adaptive immune response and the subsequent establishment of immune memory.

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http://dx.doi.org/10.1016/j.vaccine.2016.08.002
0264-410X/

VACV dedicates between one third and one half of its genome to dampening host innate responses (reviewed in [6]). For example, nine intracellular NF-κB inhibitors have been identified and there is evidence that more remain to be discovered [8]. In addition, VACV encodes numerous IRF-3/7 inhibitors and multiple mechanisms to counteract the actions of IFNs [6]. The mechanisms by which innate immunity impacts successful vaccine design remain incompletely understood. Importantly, recent data have demonstrated that the deletion of several VACV immunomodulatory genes individually enhances the immunogenicity of these vectors (reviewed in [6,9]). Examples of such genes include the NF-κB inhibitor N1 [10], the IRF-3/7 inhibitor C6 [11,12] and the dual NF-κB and IRF-3/7 inhibitor K7 ([13] and Ren et al., unpublished data). Proteins N1 and K7 have Bcl-2 folds [14,15] and C6 is predicted to do so [16] and all are virulence factors [17–19].

Some studies have investigated the effect of deleting multiple immunomodulatory genes from VACV vectors but so far have not included an in-depth comparison of single gene deletions in isolation versus deletion of genes in combination [13,20,21]. These comparisons have also not always included challenge experiments, instead measuring aspects of immunological memory that may correlate with immune protection. This study therefore tested whether the immunogenicity of VACV could be improved further by deleting three intracellular innate immunomodulators in combination (encoded by genes *N1L*, *C6L* and *K7R*) from VACV strain Western Reserve (WR). These immunomodulators were selected because their function in innate immunity was known, and because deletion of each of these genes in isolation from VACV WR increased immunogenicity, but decreased virulence. This study shows that deletion of these three genes did not affect VACV growth *in vitro*, but led to sequential attenuation of the virus in two *in vivo* models. In challenge models, despite each individual gene deletion enhancing immunogenicity, a virus lacking all three genes was a poorer vaccine, accompanied by inferior memory T cell responses and lower neutralising antibody titres. This illustrates how the design of vaccines for optimal immunogenicity must consider how the degree of attenuation impacts on the induction of immunological memory.

2. Materials and methods

2.1. Cells, viruses and mice

BSC-1 and CV-1 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% (v/v) foetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, USA) and penicillin/streptomycin (P/S, 50 μg/ml, Gibco, NY, USA). EL-4 cells were grown in Roswell Park Memorial Institute medium (RPMI, GIBCO, Paisley, UK) supplemented with 10% (v/v) FBS and 50 μg/ml P/S. The deletion and revertant VACVs for *N1L* [17], *C6L* [18] and *K7R* [19] were described previously. Female mice Balb/c and C57/B6 (6–8 weeks old) were purchased from Harlan (Blackthorn, United Kingdom).

2.2. Construction of Western Reserve recombinant viruses

Viruses were constructed using the transient dominant selection method [22] as described [18], using vΔN1 [17] as a starting point and plasmids Z11ΔC6 [18] and pSJH7-ΔK7 [19] to delete *C6L* and *K7R* respectively. Z11 is a pCI-derived plasmid containing the *Escherichia coli guanylphosphoribosyl transferase* (*Ecogpt*) gene fused in-frame with the *enhanced green fluorescent protein* (*EGFP*) gene under the control of the VACV 7.5 K promoter. Revertant viruses were constructed by replacing the deleted genes in their natural loci using plasmids Z11C6Rev [18] and pSJH7-K7 [19]. The genotype of resolved viruses was analysed by PCR following proteinase K-treatment of infected BSC-1 cells using primers that anneal to the flanking regions of *N1L*, *C6L* and *K7R* [17–19]. Infectious virus titres were determined by plaque assay on BSC-1 cells.

2.3. SDS-PAGE and immunoblotting

Infected BSC-1 cells were lysed in a cell lysis buffer containing 50 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X100, 0.05% (v/v) NP40 supplemented with protease inhibitors (Roche). Samples were boiled for 5 min and then subjected to SDS-PAGE. Primary antibodies were from the following sources: mouse anti-α-tubulin (Upstate Biotech), mouse anti-D8 mAb AB1.1 [23], rabbit-anti-N1 polyclonal antiserum [17], rabbit-anti-C6 polyclonal antiserum [18] and rabbit-anti-K7 polyclonal antiserum [24]. Primary antibodies were detected with goat-anti-mouse/rabbit IRdye 800CW infrared dye secondary antibodies and membranes were imaged using an Odyssey Infrared Imager (LI-COR Biosciences).

2.4. Plaque size analysis

BSC-1 cells were inoculated at approximately 50 plaque forming units (p.f.u.) per well of a 6-well plate and stained with crystal violet 3 days later [18]. The sizes of 20 plaques per well were measured using Axiovision acquisition software and a Zeiss AxioVert. A1 inverted microscope as described [25].

2.5. Murine intranasal and intradermal models of infection

Female BALB/c mice (n = 5, 6–8 weeks old) were infected intranasally (i.n.) with 5×10^3 p.f.u. of purified VACV strains. VACV was purified from cytoplasmic extracts of infected cells by two rounds of sedimentation through 36% (w/v) sucrose at 32,900*g* for 80 min. Virus was resuspended in 10 mM Tris-HCl pH 9. Virus used for infections was diluted in phosphate-buffered saline containing 1% bovine serum albumin and the titre of the diluted virus that was used to infect mice was determined by plaque assay on the day of infection. Mice were monitored daily to record body weight and signs of illness as described [26,27]. Female C57BL/6 mice (n = 5, 6–8 weeks old) were inoculated intradermally (i.d.) in both ear pinnae with 10^4 p.f.u. and the resulting lesions were measured daily as described [28,29]. For the challenge experiments, mice that had been inoculated i.n. were challenged 6 weeks later and mice that had been inoculated i.d. were challenged 4 weeks later, i.n., with 5×10^6 p.f.u. of wild-type VACV WR.

2.6. Intracellular cytokine staining

Splenocytes were prepared as described [10] and incubated for 4 h with a C57BL/6-specific $CD8^+$ VACV peptide $B8_{20–27}$ [30] or a negative control $CD8^+$ VACV peptide specific for BALB/c mice, $E3_{140–148}$ [31] at a final concentration of 0.1 μg/ml. After 1 h Golgi stop (BD Biosciences) was added and the cells were incubated for a further 3 h. Cells were then stained for CD8 and either IFNγ or TNFα and analysed by flow cytometry [10].

2.7. ^{51}Cr release cytotoxic assay

Cytotoxic T lymphocyte (CTL) activity was assayed with a standard ^{51}Cr-release assay using VACV-infected EL-4 cells as targets, as described [32]. The percentage of specific ^{51}Cr-release was calculated as specific lysis = [(experimental release − spontaneous release)/(total detergent release − spontaneous release)] × 100. The spontaneous release values were always <5% of total lysis.