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Deletion of the vaccinia virus F13L gene results in a highly attenuated virus that mounts a protective immune response against subsequent vaccinia virus challenge

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ABSTRACT

Vaccinia virus F13L encodes the envelope protein p37, which is the target of the anti-pox virus drug ST-246 (Yang et al., 2005) and that is required for production of extracellular vaccinia virus. The F13L (p37)-deleted (and ST-246 resistant) vaccinia virus recombinant (Vac-ΔF13L) produced smaller plaques than the wild-type vaccinia (Western Reserve vaccinia). In addition, Vac-ΔF13L proved, when inoculated either intravenously or intracutaneously in both immunocompetent and immunodeficient (athymic nude or SCID) mice, to be severely attenuated. Intravenous or intracutaneous inoculation of immunocompetent mice with the ΔF13L virus efficiently protected against a subsequent intravenous, intracutaneous or intranasal challenge with vaccinia WR (Western Reserve). This was corroborated by the observation that Vac-ΔF13L induced a humoral immune response against vaccinia following either intravenous or intracutaneous challenge. In conclusion, F13L-deleted vaccinia virus may have the potential to be developed as a smallpox vaccine.

1. Introduction

In 1980, successful global immunization campaigns with the vaccinia virus led by the World Health Organization, resulted in the eradication of smallpox and vaccination programs were consequently suspended (Geddes, 2006). The etiological agent of smallpox, variola virus, like vaccinia virus, belongs to the family of the orthopoxviridae (Moss, 2001). Use of vaccinia virus may be associated with serious adverse events and rare fatal reactions, particularly in immunodeficient patients and patients with atopic dermatitis (Bray, 2003; Lane and Goldstein, 2003). In light of the potential use of the variola virus as a bioweapon it is important to have a safer vaccine at hand.

Several vaccinia viruses with attenuated properties that mount a protective immunity against smallpox have been reported (Gurt et al., 2006; Jentarra et al., 2008; Vijaysri et al., 2008). Vaccinia mutants with deletions in the E3 interferon resistance gene (Jentarra et al., 2008) have been proven to be highly attenuated in both immunocompetent and immunodeficient mice following either intranasal or intracranial infection and induced a protective immune response following intranasal vaccination (Jentarra et al., 2008). Furthermore, vaccination by scarification resulted in a potent cell mediated and humoral immunity. Gurt et al. (2006) isolated two mutants of the Western Reserve (WR) strain of vaccinia virus, that carried mutations in A33R or B5R in genes coding for two proteins of the outer membrane of the extracellular enveloped virus (EEV) (Katz et al., 2002, 2003). Whereas a high degree of attenuation in mice was achieved with the A33R mutant, the B5R mutant remained somewhat pathogenic. Mice intranasally infected with one of both mutant viruses were all protected against a subsequent challenge of wild-type virus (Gurt et al., 2006).

We here report that vaccinia virus, from which the F13L has been deleted may have the potential to be further developed as a potential poxvirus vaccine. The F13L gene encodes a 37-kDa palmitylated peripheral membrane protein required for extracellular virus particle formation (Grosenbach and Hruby, 1998; Husain and Moss, 2001, 2002) and p37 is the target of the anti-pox virus drug ST-246 (Yang et al., 2005; Duraffour et al., 2008).

2. Materials and methods

2.1. Cells and viruses

BSC-40 (CRL-2761) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The origin of vaccinia virus strain Western Reserve (WR) has been described

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before (Neyts et al., 2004; Yang et al., 2005). The vaccinia virus recombinant was derived from strain WR and expressed green fluorescent protein (GFP) from the F13L locus (Vac-ΔF13L-GFP).

Vac-ΔF13L-GFP: To construct Vac-ΔF13L-GFP, vaccinia virus F13L was amplified by PCR using primers Vac39831 (5′-CAT CCA TCC AAA TAA CCC TAG-3′) and Vac42787 (5′-AGA TAC TCC TAG ATA CAT ACC ATC-3′). Primers were designed based upon the published sequence information for vaccinia virus strain WR (GenBank Accession No. AY243312). The resulting PCR product (2956 bp) was cloned into pCR2.1 (Invitrogen, CA), to generate plasmid pF13L-KO1. Plasmid pF13L-KO1 was digested with Hind III and Bam HI restriction enzymes and the overhanging nucleotides were removed with Klenow fragment. The construct was religated to generate plasmid pF13L-KO1-ΔKpnI which resulted in the removal of sequences in the vector that included a KpnI site. The EGFP2 gene was amplified by PCR from plasmid pEGFP2 using primers EGFP2_kpn1 (5′-GGT ACC GAG TAA AGG AGA AG-3′) and EGFP2_BglII (5′-AGA TCT TTA TTT GTA TAG TTC ATC C-3′) and cloned into pCR2.1 to generate pEGFP2-KO2. This plasmid was digested with KpnI and EcoRV to release the EGFP2 gene that was subsequently cloned into the KpnI-PmlI site of pF13L-KO1-ΔKpnI where all but the first six amino acids of F13L were deleted to generate pEGFP2-ΔF13L-KO4. This plasmid contained the EGFP2 gene fused in frame to the first six amino terminal amino acids of F13L. The EGFP2-ΔF13L gene was amplified by PCR using primers Vac39831 and Vac42787, and the PCR product was gel purified using QIAquick purification kit (Qiagen, MA). The PCR product was transferred into the wild-type vaccinia virus strain WR genome by marker transfer (Yao and Evans, 2003). Virus recombinants were identified by fluorescence microscopy of infected BSC40 cell monolayers, and virus from small plaques that expressed GFP was isolated. The recombinant virus was plaque-purified three times prior to large scale stock preparation. The genotype of the recombinant was verified by DNA sequencing of a PCR product amplified from purified Vac-ΔF13L-GFP DNA.

Virus stocks were propagated in BSC40 cells. BSC40 cell cultures were grown at 37 °C in a humid incubator containing 5% CO_2 in Dulbecco's modified minimal essential medium containing (D-MEM), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal bovine serum (Invitrogen). Vaccinia WR, and Vac-ΔF13L-GFP stocks were cultured using standard methods. Briefly, BSC40 cells were seeded at a density of 1×10^7 cells per 15 cm diameter dish. The cultures were infected at a multiplicity of infection (MOI) of 0.01 PFU vaccinia WR/cell or 0.1 PFU Vac-ΔF13L-GFP/cell for efficient virus propagation. The cultures were incubated with the viral inoculum for 1 h at 37 °C in 5 ml medium with constant gentle rocking. The inoculum was removed and fresh culture medium was added to the dishes. The cultures were incubated 2–3 days or until extensive CPE was observed. The cultures were harvested and cell associated virus was released by sonication at 40% power in a Misonex Sonicator® 3000 (Farmingdale, NY) using a cup horn attachment. The cell debris was removed by centrifugation 1000*g* for 10 min at 4 °C and the crude virus suspension was stored at −80 °C. Subsequently virus stocks were purified by velocity sedimentation on a sucrose gradient (Hruby et al., 1979). Vaccinia titers were determined by plaque assay on BSC40 cell monolayers.

2.2. Mice and inoculation strategies

SCID (Severe Combined Immune Deficient) mice (C.B.-14 *scid/scid* inbred strain) were bred at the Rega Institute under germ-free conditions and were housed under specific-pathogen free conditions during the experiments. Female immunocompetent outbred NMRI (RjHan:NMRI, Naval Medical Research Institute) mice were obtained from the Animal Production Centre of the University of Leuven. Hairless (FVB/NRj-hr^{rh}) outbred mice were bred in house by backcrossing and intercrossing of the homozygous parents. Both mouse strains were housed under conventional conditions during the experiments. Female athymic outbred nude mice (Rj:NMRI-nu) were obtained from Elevage Janvier (Le Genest Saint Isle, France). Athymic nude mice (in which the development and differentiation of hair is severely impaired) lack functional T-cell immunity. Animals were placed in group housing and did not receive antibiotics.

NMRI, hairless, SCID and athymic nude mice were infected intravenously with 2×10^5 PFU (Table 1) or 2×10^4 PFU of virus (Tables 2 and 3). An inoculum of 2×10^5 PFU for intravenous inoculation of mice with WR was selected since this inoculum resulted in a countable number of tail lesions in NMRI mice but did otherwise not result in morbidity and mortality. For challenge (vaccination) studies whereby mice were inoculated twice, a 10-fold lower inoculum (2×10^4 PFU) was chosen.

In some experiments hairless mice, SCID and athymic nude mice were inoculated intracutaneously by means of scarification at the lumbosacral area (5×10^5 PFU). This inoculum was selected because it resulted in a reproducible induction of cutaneous lesions. Prior to scarification, animals received light ether anesthesia and were immobilized manually by one person; scarifications were made by a second person. A 50-μl droplet of the viral inoculum was placed at the lumbosacral area. A sterile stainless steel blood lancet (Maersk Medical, Sheffield, United Kingdom) was used to

Table 1
Effect of intravenous or intracutaneous inoculation of immunocompetent and immunodeficient mice with vaccinia WR or Vac-ΔF13L-GFP on virus-induced lesion formation and mortality.

Mouse strains	Virus	Inoculation [a]	# (death/total)	# Mice with lesions	Mean lesion number ± SD[b]	MTD ± SD[c]
NMRI	WR	i.v.	0/4	4/4	59 ± 11	>50
	Vac-ΔF13L-GFP	i.v.	0/4	0/4	0*	>50
Hairless	WR	i.cut.	0/13	13/13		>50
	Vac-ΔF13L-GFP	i.cut.	0/15	0/15		>50
Athymic nude	WR	i.v.	4/4	4/4	37 ± 7	9 ± 0.5
	Vac-ΔF13L-GFP	i.v.	4/4	0/4	0*	22 ± 1*
	WR	i.cut.	5/5	5/5		11 ± 1
	Vac-ΔF13L-GFP	i.cut.	0/15	0/15		>50
SCID	WR	i.v.	4/4	4/4	20 ± 8	11 ± 1
	Vac-ΔF13L-GFP	i.v.	0/4	0/4	0*	>50
	WR	i.cut.	5/5	5/5		18 ± 2
	Vac-ΔF13L-GFP	i.cut.	0/5	5/5		>50

[a] i.cut.: intracutaneous infection with 5×10^5 PFU – lesions on the back, i.v.: intravenous infection with 2×10^5 PFU – lesions on the tail.
[b] Lesion score as determined on day 7 post-WR infection.
[c] MTD: mean time to death.
* $p < 0.001$.

Table 2
Vac-ΔF13L-GFP induces a protective immunity against subsequent vaccinia WR challenge.

Mouse strain	Vaccination			Infection			# Mice with lesions	Mean lesion number ± SD[b]
	Day	Virus	Inoculation[a]	Day	Virus	Inoculation[a]		
Hairless	0	Vac-ΔF13L-GFP	i.cut.	7	WR	i.cut.	0/5	
		Mock					5/5	
Hairless	0	Vac-ΔF13L-GFP	i.cut.	7	WR	i.v.	0/8	0[$]
		Mock					8/8	15 ± 4
NMRI	0	Vac-ΔF13L-GFP	i.v.	7	WR	i.v.	5/5	1 ± 0.8[*]
		Mock					5/5	11 ± 8
Hairless	0	Vac-ΔF13L-GFP	i.cut.	21	WR	i.cut.	0/5	
		Mock					5/5	
Hairless	0	Vac-ΔF13L-GFP	i.cut.	42	WR	i.cut.	0/5	
		Mock					5/5	

All mice survived.
[a] i.cut.: intracutaneous infection with 5×10^5 PFU – lesions on the back, i.v.: intravenous infection with 2×10^4 PFU – lesions on the tail.
[b] Lesion score as determined on day 7 post-WR infection.
[$] $p < 0.001$.
[*] $p < 0.05$.

Table 3
Induction of a humoral immune response following intracutaneous or intravenous inoculation with Vac-ΔF13L-GFP or vaccinia WR in hairless, NMRI mice or athymic-nude mice.

Mouse strain	Inoculation [a]	Sample day p.i.	Antibody titer (fold increase) relative to naive mice	
			Vac-ΔF13L-GFP	WR
Hairless	i.cut.	2	1.2 ± 0.6[b]	1.1 ± 0.7[b]
		4	1.1 ± 0.2[b]	1.8 ± 0.3[b]
		7	50 ± 39[b]	65 ± 57[b]
		11	81 ± 77[b]	124 ± 117[b]
NMRI	i.v.	2	0.9 ± 0.2[c]	1.7 ± 0.4[c]
		4	1.0 ± 0.1[c]	2.5 ± 2.1[c]
		7	1.6 ± 1.0[c]	118 ± 97[c]
		11	41 ± 37[c]	182 ± 105[c]
Athymic nude	i.cut.	2	0.7 ± 0.43[b]	n.d.
		4	1.2 ± 0.1[b]	n.d.
		7	2.0 ± 0.7[b]	n.d.
		11	0.9 ± 0.4[b]	n.d.

n.d.: not determined.
$n = 3$ per day per mouse strain.
[a] i.cut.: intracutaneous infection with 5×10^5 PFU – lesions on the back, i.v.: intravenous infection with 2×10^4 PFU – lesions on the tail.
[b] Relative to naive hairless mice.
[c] Relative to naive NMRI mice.

produce a light scarification of ≈0.5 cm^2. In a last experiment, female hairless mice anesthetized with pentobarbital (Nembutal®) were challenged intranasally with 10^4 PFU/20 μl vaccinia WR. This challenge was chosen to overcome premature mortality (earlier than 4 days post-infection); 2 of 3 mice were severely ill 11 days after inoculation with 10^5 PFU/20 μl.

2.3. Vaccination protocols

NMRI mice were inoculated intravenously with Vac-ΔF13L-GFP or were not inoculated (experiment 1). Hairless mice were inoculated intracutaneously by means of scarification at the lumbosacral area with Vac-ΔF13L-GFP (experiments 2, 3, 4, 5). One week later, animals were infected intravenously with 2×10^4 PFU of vaccinia WR (experiments 1, 2) or intracutaneously at the same area of skin of the cutaneous vaccination with 5×10^5 PFU of vaccinia WR (experiment 3). In experiments 4 and 5, intracutaneous vaccinia WR infection was performed at, respectively 3 or 6 weeks post-Vac-ΔF13L-GFP inoculation. Pox tail lesions were counted or pox lesions at the lumbosacral area were monitored 1 week post-vaccinia WR infection. Mortality was monitored up to 50 days post-vaccinia WR infection. Athymic nude mice were inoculated intracutaneously with Vac-ΔF13L-GFP by means of scarification and 1 week later mice were infected intravenously with 2×10^4 PFU of vaccinia WR. One week post-vaccinia WR infection poxtail lesions were counted. Female hairless mice were challenged intranasally with vaccinia WR virus 1 week post-Vac-ΔF13L-GFP scarification. Age-matched non-immunized mice served as controls in the protection protocols. At 4 and 7 days post-vaccinia WR infection, mice were sacrificed and liver and lung samples were isolated and snap frozen for later determination of total vaccinia DNA copy number. All experiments were approved by the Ethical Committee on animal vertebrate experiments of the University of Leuven.

2.4. Vaccinia ELISA

Ninety-six well plates (Nunc Maxisorp) were coated with 50 μl Wyeth Dryvax at 1×10^7 PFU/ml in PBS. Plates were UV-irradiated five times with 125 m J, covered and stored at 4 °C for about 4 days. At the start of the ELISA procedure, coating solution was removed and plates were washed four times with 300 μl of 1 × PBS, 1% Brij, 0.02% Sodium Azide. Block solution [1 × PBS, 5% Casein Hammerstein, 1% Brij, 0.06% Kathon (antimicrobial); 200 μL/well] was added and the plates were incubated for 1 h at 37 °C. Serial dilutions (1:5) of sera prepared in blocking solution were added in duplicate to the assay plate. Samples were incubated for 3 h at

37 °C. Serial dilutions of the pooled sera of mice challenged intranasally with 2×10^5 PFU of vaccinia WR served as a positive control. After washing, goat anti-mouse IgG (H + L)-HRP (Bio Rad) was added for 2 h at 37 °C. Before adding the TMB substrate (Pierce), wells were washed again. The reaction was stopped after 20 min with 100 μl/well of 2 M sulfuric acid and plates were read on a Tecan Spectrafluor Plus (absorbance 450 nm, reference 620 nm).

2.5. Vaccinia real-time qPCR

Liver and lung fragments were incubated in ATL lysis buffer (5.6% w/v, QIAamp DNA Mini kit, Qiagen Benelux B.V., Venlo, the Netherlands) in 2 ml tubes containing ceramic beads that were shaken at a high frequency in an automatic homogenizer (Precellys24, Bertin, France). Homogenization was performed at 6500 rpm for three cycles of 5 s, with intervals of 5 s. Next, the tubes were centrifugated for 15 min at 4 °C/13,000 rpm and 180 μl of the supernatant (10 mg tissue) was used for total DNA extraction using the QIAamp DNA Mini kit according to the Manufacturer (elution in 100 μl). Real-time quantitative PCR (Q-PCR) was performed on 2 μl DNA extract in a total reaction volume of 25 μl and using the TaqMan Universal PCR Master Mix kit (Applied Biosystems, Branchburg, NJ) to quantitate the amount of viral DNA: reverse primer (5′-TGA CTA CGT TGT TAT GAG TGC TTG GTA-3′; final concentration: 300 nM; positioned in gene A56R nt120–146), forward primer (5′-AGA TCA TCG TAT GGA GAG TCG TAA GAT-3′; final concentration: 300 nM; positioned in gene A56R nt225–251), Taqman probe (6-FAM-ATC AAA ATA CAA GAC GTC GCT TTT AGC AGC TAA AAG AA-TAMRA; final concentration 200 nM; positioned in gene A56R nt167–204) using a SDS 7000 (Applied Biosystems, Foster City, CA). Plasmid DNA containing the amplified insert was used to prepare the standard curve and to quantitate the amount of viral DNA.

2.6. Statistical analysis

Data are expressed as mean ± SD. One-sided parametric analysis were performed with the Student's *t*-test to determine statistical significant differences between vaccinia WR and Vac-ΔF13L-GFP infected groups.

3. Results

3.1. ΔF13L-deleted vaccinia is attenuated in vitro and in vivo

Deleting the F13L gene (the target of ST-246) from the vaccinia virus genome resulted in a virus that is replication-competent in cell culture but that produces markedly smaller plaques than the corresponding wild-type virus (data not shown). This is in accordance with previously published data (Blasco and Moss, 1991, 1992; Grosenbach and Hruby, 1998; Borrego et al., 1999; Honeychurch et al., 2007). Intravenous (i.v.) inoculation of NMRI mice with vaccinia WR resulted in 59 ± 11 pox tail lesions per mouse (Table 1). The same inoculum of Vac-ΔF13L-GFP did not cause any lesions ($p < 0.001$). Intracutaneous inoculation of hairless mice at the lumbosacral area with vaccinia WR resulted in the formation of typical pox lesions in all mouse strains at the site of scarification (Table 1). No lesions were formed in mice that had been infected with the same inoculum of Vac-ΔF13L-GFP. Intravenous inoculation of athymic nude or SCID mice with Vac-ΔF13L-GFP did not result in the development of pox tail lesions. SCID mice remained healthy until the end of the experiment (50 days). Intravenously inoculated athymic nude mice died although significantly later (13 days) than mice that had been infected with vaccinia WR despite the fact that no visible lesions developed (Table 1). Intracutaneous inoculation of athymic nude mice with Vac-ΔF13L-GFP did not result in the formation of pox lesions and animals remained healthy, whereas the same inoculum of vaccinia WR resulted in the formation of lesions and in mortality in all mice (Table 1). Intracutaneous infection of SCID mice with either vaccinia WR or Vac-ΔF13L-GFP resulted in the formation of pox lesions, although these were markedly less severe in mice scarified with Vac-ΔF13L-GFP (Table 1). Although intracutaneously Vac-ΔF13L-GFP inoculated SCID mice developed minor lesions, the animals, unlike the vaccinia WR-infected animals, remained healthy until the end of the experiment (50 days).

3.2. Vaccination experiments with Vac-ΔF13L-GFP

In a first experiment (Table 2), hairless mice were infected intracutaneously at the lumbosacral area with Vac-ΔF13L-GFP. None of the infected mice developed any visible lesion at the site of inoculation. One week later, animals were challenged intracutaneously with vaccinia WR. All mock-infected animals, but none of the Vac-ΔF13L-GFP inoculated animals developed poxvirus lesions (Fig. 1.).

In a second experiment (Table 2), hairless mice were inoculated intracutaneously with Vac-ΔF13L-GFP and were challenged 1 week later with vaccinia WR by means of intravenous inoculation. Non-vaccinated animals developed on average 15 ± 4 pox tail lesions; whereas no lesions developed in the Vac-ΔF13L-GFP vaccinated animals ($p < 0.001$).

In a third experiment (Table 2), NMRI mice were inoculated intravenously with Vac-ΔF13L-GFP. One week later, animals were challenged intravenously with vaccinia WR. The non-vaccinated group developed on average 11 ± 8 lesions; the Vac-ΔF13L-GFP vaccinated mice developed 1 ± 0.8 ($p < 0.05$) lesions.

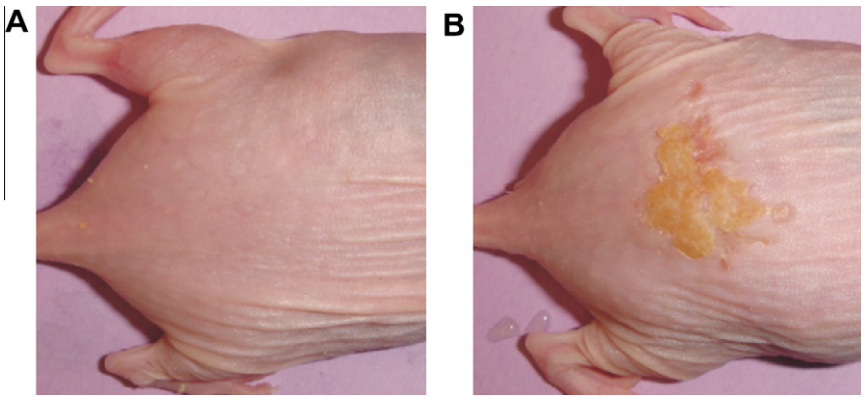

Fig. 1. Hairless mice were inoculated intracutaneously with (A) mock or (B) Vac-ΔF13L-GFP and were 7 days later infected intracutaneously at the lumbosacral area with vaccinia WR. Pictures of representative mice of each group were taken at day 7 post-vaccinia WR infection.

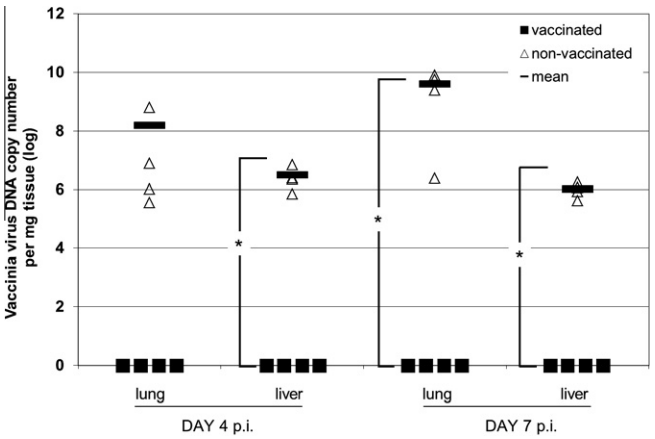

Fig. 2. DNA copy numbers of vaccinia virus in liver and lung of vaccinated and non-vaccinated hairless mice that have been challenged with vaccinia WR via intranasal inoculation. Copy numbers were measured at 4 and 7 days post-WR challenge. Mice were infected with WR 1 week post-Vac-ΔF13L-GFP scarification. $N = 4$/group. $^{*}p < 0.05$.

To study whether Vac-ΔF13L-GFP vaccination results in a prolonged protective effect, hairless mice were vaccinated intracutaneously with Vac-ΔF13L-GFP and were 3 or 6 weeks later challenged intracutaneously with vaccinia WR (Table 2). All non-vaccinated animals, but none of the Vac-ΔF13L-GFP developed poxvirus lesions.

As expected, because of the lack of a functional immune system, intracutaneous inoculation with Vac-ΔF13L-GFP did not protect athymic nude mice against a subsequent intravenous challenge with vaccinia WR [15 ± 6 lesions; data not shown].

To mimic more closely vaccinia virus infections in man, hairless mice (that had been vaccinated by intracutaneous inoculation with Vac-ΔF13L-GFP) were intranasally challenged with vaccinia WR. Liver and lung tissues were collected at 4 or 7 days post-vaccinia WR challenge after which the viral DNA content was quantified. No viral DNA was detectable in livers or lungs of vaccinated mice by means of quantitative PCR, whereas high copy numbers were measured in organs of unvaccinated mice (Fig. 2.).

3.3. Immune responses to vaccination with Vac-ΔF13L-GFP

Total anti-vaccinia antibody titers were determined in the sera of hairless mice or in NMRI mice (at 2, 4, 7 and 11 days post-inoculation) that had been inoculated through respectively the intracutaneous or the intravenous route with Vac-ΔF13L-GFP (Table 3). The construct readily induced a humoral immune response. As expected, athymic nude mice did not develop anti-vaccinia antibodies following Vac-ΔF13L-GFP vaccination (Table 3).

4. Discussion

The F13L gene of vaccinia virus encodes for a 37-kDa palmitylated peripheral membrane protein (p37) required for extracellular virus particle formation (Blasco and Moss, 1991; Grosenbach and Hruby, 1998). This protein participates in the envelopment of the intracellular mature virus (IMV) particles in virus-modified membranes derived from the *trans*-Golgi and early-endosome compartments to produce an egress competent form of the virus particle (Blasco and Moss, 1991). p37 is highly conserved amongst orthopoxviruses and has been shown to be the target of the antiviral molecule ST-246 (Tecovirimat™) (Yang et al., 2005; Duraffour et al., 2008). ST-246 is a small molecular weight compound, recently developed by SIGA Technologies, Inc. (Corvallis, OR), to treat pathogenic orthopoxvirus infections in humans (Jordan et al., 2008).

Vaccinia virus lacking proteins involved in extracellular virus particle formation exhibit altered plaque phenotypes. For example, mutations in the A33R, A34R and A36R genes that interfere with the formation of actin-containing microvilli result in a small-plaque phenotype and reduced virulence (Wolffe et al., 1997, 1998; Roper et al., 1998). In addition, similar defects in plaque formation are described for F13L (p37) mutated vaccinia virus (Blasco and Moss, 1991, 1992; Grosenbach and Hruby, 1998; Borrego et al., 1999; Sanchez-Puig and Blasco, 2005; Honeychurch et al., 2007). Deletion of the F13L gene resulted in an attenuated replication-competent virus that produced markedly smaller plaques than the corresponding wild-type virus.

Although the above described vaccinia mutants are all small plaque-forming deletion mutants showing reduced virulence, they differ in the release of extracellular enveloped virus (EEV). Vaccinia virus spreads efficiently by direct cell-to-cell transfer of the cell-associated enveloped virus (CEV) (Blasco and Moss, 1991, 1992) and by long-range spread accomplished by EEV released into the medium (Boulter and Appleyard, 1973; Payne, 1980). WR vaccinia produces low amounts of EEV and virions are greatly retained at the cell surface (Blasco and Moss, 1992). Replacing the F13L gene from WR results in a virus that is defective in EEV and CEV generation and plaque formation but that produces normal amounts of intracellular naked virus (Blasco and Moss, 1991, 1992). In contrast, mutations in the WR vaccinia virus envelope proteins A33R (C-terminal truncations) or B5R (a single point mutation) result in an enhanced release of EEV. Large numbers of virions of these variants attach to the cell surface but fail to spread from cell-to-cell (Katz et al., 2003).

Today there are four vaccinia virus vaccine types (Jacobs et al., 2009). The first are the wild-type vaccinia viruses manufactured on the skin of live animals that were employed during the smallpox eradication program (e.g. Dryvax, Lister, Copenhagen). The second generation consists of newer and safer (e.g. no-microbial contaminations from livestock) tissue-culture adapted wild-type strains (ACAM2000) and vaccinia virus prepared in embryonated chicken eggs (standard commercial vaccine of Israel). Vaccines attenuated by passaging wt viruses in tissue culture cells from alternative hosts form the third generation of vaccines (e.g. MVA, LC16m8, DIs). The last generation consists of vaccines attenuated through deletion of genes but still inducing a potent immune response e.g. NYVAC, VACVvD4-ZG and VACVΔE3L. In addition, targeted mutations of E3L A33R or B5R also result in attenuated versions of the wild type strain and still induce a protective immune response (Gurt et al., 2006; Jentarra et al., 2008; Vijaysri et al., 2008).

It was our aim to study whether poxviruses from which this F13L gene has been deleted may have, in addition to a reduced replication capacity *in vitro*, (i) a reduced *in vivo* pathogenicity in mice and if so (ii) could protect against a challenge with wild-type vaccinia and thus serve as a potential novel and safe vaccine candidate. In accordance with an earlier study (Grosenbach et al., 2011), the ΔF13L-vaccinia virus was markedly attenuated for replication in mice. No evidence of infection-related disease or lesion development was observed in immunocompetent mice following either intravenous or intracutaneous inoculation. The virus proved also to be markedly attenuated in immunodeficient athymic nude mice (that lack T-cell immunity) and SCID mice (that lack both functional T- and B-cell immunity). None of the inoculated SCID mice developed disease or succumbed. Athymic-nude mice intravenously challenged developed a fatal infection (despite the fact that no visible lesions were observed) but died much later than animals infected with wild-type virus. Intracutaneous inoculation of athymic-nude mice with the ΔF13L-virus, however, did not lead to disease or mortality.

A remarkable observation was that none of the SCID mice intravenously inoculated with the F13L-deleted vaccinia virus developed disease whereas athymic nude mice, while not developing pox tail lesions following intravenous inoculation, ultimately died as a result of intravenous infection with the F13L-deleted virus. Yet, compared to the wild-type virus, disease progression developed at a slower rate in mice inoculated with the deletion mutant. A possible explanation for this observation is that the F13L-deleted virus is less efficient at seeding endothelial cells in the tail vein and therefore is less efficient in tail lesion formation. The virus may also spread more readily to internal sites when mice are inoculated by tail vein injection. In athymic mice the attenuated virus is capable of limited replication in internal organs ultimately causing a lethal infection. This scenario is repeated in SCID mice, however, replication of the attenuated virus is restricted by host-factors allowing for clearance of the virus in most cases. These host factors may be related to the inherent genetic differences in susceptibility of mouse strains to orthopoxvirus infection which has been best studied in systems using ectromelia virus (Esteban and Buller, 2005). Further investigations will be ensued to determine why athymic-nude mice are more susceptible to Vac-ΔF13L-GFP infection than the more severely immunodeficient SCID mice.

Having established the highly attenuated *in vivo* character of the ΔF13L-virus, a set of different vaccination studies was performed to assess the potential protective activity against subsequent vaccinia virus challenge in mice. Following either intravenous or intracutaneous inoculation, the ΔF13L-virus induced an efficient humoral immune response (when used at a similar dose as the parent wild-type WR strain) that protected against a subsequent challenge with wild-type virus (either an intravenous or intracutaneous challenge). Since smallpox is transmitted by aerosol and the traditional smallpox vaccine was/is administered through scarification of the skin (Fenner et al., 1988), an experiment was conducted in which mice were vaccinated by intracutaneous inoculation and subsequently challenged via the nose with wild type virus. The F13L-deleted virus provided complete protection in this experimental set-up. ΔF13L vaccinia virus may thus form the basis for a safe vaccine that efficiently protects against infections with variola virus.

While deletion of F13L from the genome reduces susceptibility to ST-246 (Yang et al., 2005; Duraffour et al., 2008), the attenuated phenotype reduces the risk of adverse events due to excessive viral replication at a distance from the site of inoculate. In the case of an adverse event with this vaccine candidate, the antiviral drug (CMX001) which is in clinical development and targets poxvirus replication by a different mechanism i.e. inhibition of the viral polymerase could be used (Hostetler, 2010). Moreover, Vaccinia Immune Globulin (VIG), which is used to treat vaccine related adverse events (Xiao and Isaacs, 2010), would be expected to be effective against this putative vaccine candidate. In the case of reversion of the attenuated phenotype to the wild-type phenotype through recombination with other poxvirus, which is a general concern in vaccinia virus-infected cells (Fenner, 1959; Evans et al., 1988), the resulting wild-type virus will be susceptible to ST-246 (Yang et al., 2005; Grosenbach et al., 2011). In addition, as F13L-deleted virus readily induces a humoral immune response subsequent poxvirus challenge will most likely be cleared before recombination might even happen.

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