Herstellung neuer Orf Virus- (*Parapoxvirus*) Rekombinanten und Analyse deren protektiven und immunrelevanten Eigenschaften

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Dipl.-Agr. Biol. Jörg Rohde

aus Villingen-Schwenningen

Tübingen

2016

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	08.05.2017
Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	Prof. Dr. Hans-Georg Rammensee
2. Berichterstatter:	Prof. Dr. Friedrich Götz

Inhaltsverzeichnis

I. Abkürzungsverzeichnis

AHSV	Afrikanische Pferdepest *(African Horse Sickness Virus)*
AIV	Aviäres Influenzavirus
BPSV	Bovines Papuläres Stomatitis-Virus
BRV	Bovines Rotavirus
BTV	Blauzungenvirus *(Bluetongue Virus)*
CBP	Chemokin-Binde Protein
CPE	Zytopathischer Effekt
CTL	Zytotoxische T-Lymphozyten
DC	Dendritische Zellen
ECTV	Ektromelie-Virus (Mäusepocken-Virus)
ELISA	*Enzym Linked Immunoassay*
ER	Endoplasmatische Retikulum
FMDV	Maul-und-Klauenseuche-Virus *(Foot-and-Mouth disease virus)*
G-CSF	Granulozyten-Kolonie-stimulierender Faktor
GIF	Orf Virus kodierter GM-CSF inhibitorischer Faktor
GM-CSF	Granulozyten-Makrophagen Kolonie- Stimulierungsfaktor
HA	Hemagglutinin
HBV	Hepatitis B Virus
HIV	Humanes Immundefizienz-Virus
HPAIV	Hochpathogene aviäre Influenzaviren
HRV-1A	humanes Rhinovirus 1A

IFN	Interferon
IL	Interleukin
i.m.	intramuskulär
i.n.	intranasal
i.p.	intraperitoneal
IPMA	Immunperoxidase Monolayer Assay
i.v.	intravenös
LCMV	Lymphocytic Choriomeningitis Virus; WE Stamm
MERS-CoV	Nahost-Atemwegssyndrom-Coronavirus *(Middle East respiratory syndrome coronavirus)*
MHC I	*Major Histocampatibility Complex class I*
MKS	Maul- und Klauenseuche
MLD50	Maus-letale-Dosis 50%
moi	Multiplizität der Infektion *(multiplicity of infection)*
MVA	Modified Vaccinia Virus Ankara
NA	Neuraminidase
NF-κB	*Nuclear factor kappa B*
NK	Natürliche Killerzellen
NYVAC	New York Vaccinia Virus
ORFV	Orf Virus
ORFV IFNR	Orf Virus Interferon Resistenz-Gen
PAMP	Pathogen-assoziierte molekulare Muster
PBMC	mononukleäre Zellen des peripheren Blutes

PBS	Phosphat gepufferte Saline *(Phosphate-buffered saline)*
PCPV	Pseudokuhpockenvirus
pfu	Plaque formende Einheit *(plaque forming unit)*
PRR	*pattern-recognition receptors*
PRV	Pseudorabiesvirus *(Suid Herpesvirus 1)*
PVNZ	Parapoxvirus des Rotwildes in Neuseeland
RHD	hämorrhagische Krankheit der Kaninchen
RHDV	*Rabbit hemorrhagic disease virus*
RIG-I	*Retinoic acid inducible gene I*
TAA	Tumorassoziierte Antigene
TAP	Transporter der Antigenprozessierung
TGN	*trans*-Golgi Netzwerk
TH	T-Helferzelle
TLR	Toll-like Rezeptor
TNF-α	Tumornekrosefaktor-α
VEGF	*vascular endothelial growth factor*
VSV	Vesikuläres Stomatitis-Virus
VV	Vaccinia Virus
ZNS	Zentrales Nervensystem

1 Auflistung der Publikationen und Ziele der Arbeit

1.1 Auflistung der Publikationen und Anteil der Arbeit

Folgende Liste gibt Aufschluss über die benutzten Veröffentlichungen zur kumulativen Dissertation und deren persönlichen Arbeitsanteil. Die Auflistung erfolgt in der Reihenfolge, wie sie im Kapitel 3. Ergebnisse und Diskussion erscheinen.

(1) Generation and Selection of Orf Virus (ORFV) Recombinants
Rziha HJ, **Rohde J**, Amann R; Alejandro Brun (ed.), *Vaccine Technologies for Veterinary Viral Diseases: Methods and Protocols*, Methods in Molecular Biology, vol. 1349, DOI 10.1007/978-1-4939-3008-1_12, Springer Science+Business Media New York 2016

Die Verbesserung und Optimierung der Methoden zur Herstellung und Selektion von ORFV-Rekombinanten wurde in Zusammenarbeit mit R. Amann erreicht. Dabei handelte es sich im Speziellen um die Optimierung der Transfektionen, der fluoreszenzbasierten Selektion mittels eines Markergens, der DNA Isolation aus Virus infizierten Zelllysaten und der damit verbundenen Selektion durch PCR und Virustitrationen. Entscheidenden Anteil hatte meine Arbeit in der Verbesserung der Nachweise von Rekombinanten mittels spezifischer Antikörper.

(2) A new recombinant Orf virus (ORFV, Parapoxvirus) protects rabbits against lethal infection with rabbit hemorrhagic disease virus (RHDV)
Rohde J, Schirrmeier H, Granzow H, Rziha HJ; Vaccine 29 (2011) 9256-9264

Die neue Rekombinante D1701-V-VP1 wurde von mir hergestellt und selektioniert. Alle Versuche wie IPMA, Western Blot und Immunfluoreszenz, die die Expression des Antigens demonstrierten, wurden von mir vorgenommen. Experimente der RNA Isolation und Northern Blot Hybridisierungen wurden von mir mit Unterstützung von Frau B. Bauer durchgeführt. Die Wachstumseigenschaften der neuen Virus-Rekombinanten wie auch die Zytokinsekretion im Serum der Tiere wurden von mir analysiert.

(3) New Orf Virus (Parapoxvirus) Recombinant Expressing H5 Hemagglutinin Protects Mice against H5N1 and H1N1 Influenza A Virus
Rohde J, Amann R, Rziha HJ; PLoS ONE 8 (12) (2013): e83802

Die beiden Rekombinanten D1701-V-NPh5 und D1701-V-HAh5n wurden von mir hergestellt und selektioniert. Alle Versuche, die die ORFV-Rekombinanten charakterisieren und deren Proteinexpression darstellen, wurden von mir durchgeführt. Alle Tierversuche inklusive der Immunisierungen, Zelldepletionen und deren Kontrolle mit durchflusszytometrischen Messungen wie auch die Messungen der induzierten Antikörperantworten in den immunisierten Tieren habe ich vorgenommen. Lediglich bei anfänglichen Belastungsinfektionen mit Influenzavirus wurde mir von A. Vogel und E. Haasbach assistiert. Darüber hinaus wurde der erste schriftliche Entwurf der Veröffentlichung von mir ausgearbeitet.

(4) New baculovirus recombinants expressing Pseudorabies virus (PRV) glycol-proteins protect mice against lethal challenge infection
Grabowska AK, Lipińska AD, **Rohde J**, Szewczyk B, Bienkowska-Szewczyk K, Rziha HJ; Vaccine. 2009 Jun 2;27(27):3584-91

Die Immunisierungen mit Baculovirus-Rekombinanten und die PRV Infektionsversuche sowie die Entnahme der Splenozyten und deren Aufbereitung wurden zusammen mit A. K. Grabowska durchgeführt. Die Zytotoxizitätstests mit radioaktiv markiertem Chrom wurden von mir durchgeführt.

(5) Orf virus interferes with MHC class I surface expression by targeting vesicular transport and Golgi
Rohde J, Emschermann F, Knittler MR, Rziha HJ; BMC Veterinary Research 2012, 8:114

Experimente zur Messung der Oberflächenexpression mittels Durchflusszytometrie wurden von F. Emschermann und mir durchgeführt. Alle anderen Studien wie die semi-quantitative reverse Transkriptions PCR, Immunfluoreszenz, Markierung von Proteinen mit radioaktivem Schwefel, Immunpräzipitationen, Western Blot Analysen und statistische Auswertungen wurden von mir durchgeführt. Ein erster schriftlicher Entwurf für die Veröffentlichung wurde von mir verfasst.

Es ergab sich folgende weitere Publikation, die nicht Teil der Dissertation ist, hier aber erwähnt sein soll:

A new rabies vaccine based on a recombinant ORF virus (parapoxvirus) expressing the rabiesvirus glycoprotein
Amann R, **Rohde J**, Wulle U, Conlee D, Raue R, Martinon O, Rziha HJ; J Virol. 2013 Feb;87(3):1618-30

1.2 Ziele der Arbeit

Das Ziel der Dissertation war die Generierung neuer ORFV- (*Parapoxvirus*) Rekombinanten, die eine protektive Immunität gegen virale Infektionskrankheiten vermitteln. Zunächst sollten mittels molekularbiologischer Techniken Fremdgene in das Genom von ORFV inseriert werden, um anschließend die neuen Rekombinanten zu selektionieren und die Expression dieser eingefügten Gene zu testen. Die protektiven Eigenschaften der ORFV-Rekombinanten sollten anschließend in geeigneten Tierversuchen evaluiert und die beteiligten immunologischen Mechanismen, die zu einem Schutz beitragen, analysiert werden.

Mit bereits existierenden ORFV-Rekombinanten welche Glykoproteine von PRV exprimieren sollten in Untersuchungen von Mäusen die immunrelevanten Vorgänge genauer aufgeklärt werden, die zur Immunität führen. Vorangegangene Untersuchungen mit diesen ORFV-Rekombinanten ergaben, dass Mäuse welche Gendefekte für die Produktion von T- und B-Zellen wie auch für Perforin besaßen, trotzdem einen effektiven Immunschutz aufwiesen (1). Messungen der Aktivität von relevanten Immunzellen, Rezeptoren und Zytokinen in ORFV immunisierten Mäusen sollten Aufschluss über Funktionen und Anteil der angeborenen wie auch der erworbenen Immunantwort geben.

Zudem sollten die immunmodulatorischen Eigenschaften von ORFV *in vitro* charakterisiert werden. Pockenviren besitzen eine Vielzahl an Genen, die es dem Virus ermöglichen der Immunantwort des Wirtsorganismus zu entgehen. Einige dieser Mechanismen sind auch für das *Parapoxvirus* beschrieben. Die Wechselwirkungen zwischen dem hochattenuierten ORFV Stamm D1701-V und seinem Wirtsorganismus sowie mögliche Evasionsmechanismen von ORFV sollten durch Infektionsstudien in Vero Zellen und anschließenden Untersuchungen des vesikulären Peptidtransports näher analysiert werden.

2 Einleitung

2.1 Viren als Vektor-Impfstoffe

Das Auftreten von neuen hochpathogenen Erregern innerhalb der letzten Jahrzehnte erfordert neue Strategien für die Entwicklung von modernen Vakzinen. Virus-Vektoren stellen im Einsatz als Impfstoff gegen Infektionserkrankungen eine relativ neue und äußerst vielversprechende Impfstrategie dar. Die Infektionsraten etlicher Pathogene konnten in der Vergangenheit mit klassischen Impfstoffen erfolgreich gesenkt werden. Bei der Anwendung dieser Impfstoffe ergaben sich jedoch Grenzen der Protektivität gegen bestimmte Infektionserkrankungen wie HIV, nicht auf einer Infektion beruhende Erkrankungen oder gegen Tumor- und Autoimmunerkrankungen bei denen es kein bestimmtes Ziel-Antigen gibt [Übersichtsartikel (2)]. Für die Entwicklung gut funktionierender Impfstoffe gegen Infektions- wie auch solche speziellen Erkrankungen ist es notwendig, die pathogenen Eigenschaften zu kennen. Des Weiteren ist es von Vorteil zu wissen, welche Bereiche des Immunsystems erforderlich sind, um eine protektive Immunität aufzubauen [Übersichtsartikel (2, 3)]. Genügt die Induktion einer humoralen Immunantwort für den Schutz, so werden zum Beispiel inaktivierte Erreger oder Untereinheit-(subunit) Impfstoffe eingesetzt [Übersichtsartikel (4)]. Da attenuierte Viren die zelluläre wie auch die humorale Immunantwort mit dazugehörender Sekretion von funktionellen Zytokinen aus CD4-positiven und CD8-positiven Zellen und teilweise auch die T-Gedächtniszellen induzieren, weisen sie sich als sehr effektive Impfstoffe aus [Übersichtsartikel (2)] (5). Ein bedeutender Vorteil von Vektor-Impfstoffen ist, dass sie CTL stimulieren können, indem sie ihre exprimierten Fremdantigene in die Peptidprozessierung der Wirtszelle einbringen, die somit effektiv über MHC I präsentiert werden. Dies ist bei Vakzinen, die auf Basis von Proteinen beruhen, nicht der Fall [Übersichtsartikel (6, 7)]. Die Induktion der zellulären Immunantwort ist unter anderem für den Schutz gegen virale Infektionen wie auch gegen Krebserkrankungen ein entscheidender Faktor [Übersichtsartikel (2)]. Impfstoffe, die beide Arme der Immunantwort aktivieren können, wirken auch gegen Pathogene, die keine neutralisierenden Antikörper induzieren. Diese Wirkung wird unterstützt durch CTLs, da diese auch die Fähigkeit zur Kreuzreaktivität gegen verschiedene Virustypen wie zum Beispiel gegenüber neuen Subtypen von Influenzaviren besitzen (8). Die durch virale Vektoren stimulierte zelluläre Immunantwort umfasst auch T-Gedächtniszellen, welche aus CTLs

hervorgegangen sind, die ebenfalls gegen verschiedene Stämme von Influenzaviren gerichtet sein können (9). Im Gegensatz zu attenuierten Impfstoffen aus viralem oder bakteriellem Ursprung liefern virale Vektoren nur diejenigen Antigene des Erregers, welche für den Aufbau eines Immunschutzes gegen das Pathogen notwendig sind. Das gezielte Einbringen ausgewählter Gene birgt gewisse Vorteile. Die Virämie des Pathogens wird verhindert, da nur bestimmte Gene des Erregers verwendet werden und dieser dadurch nicht vermehrungsfähig ist. Zusätzlich können bestimmte Gene des Erregers eine kreuzreaktive Immunität gegen eine Vielzahl von Virusstämmen induzieren (10). Die ebenso durch den viralen Vektor induzierten T-Helferzellen beeinflussen nicht nur die humorale und/oder CTL Immunantwort, sondern können auch das Fortschreiten der eigentlichen Erkrankung wie zum Beispiel bei HIV-1 mindern, da diese T-Helferzellen das Hauptangriffsziel von HIV-1 darstellen [Übersichtsartikel (11)]. Dieses breite Spektrum von viralen Vektoren bietet den potentiellen Einsatz nicht nur gegen Infektionskrankheiten, sondern auch gegen beispielsweise Asthma und Diabetes [Übersichtsartikel (6)]. Ein weiterer Aspekt der Entwicklung von Vektoren ist, dass Viren spezielle Zelltypen infizieren und diese Zellspezifität genutzt werden kann, um virale Vektoren zu optimieren. Um gezielt den Tropismus von Vektoren für spezielle Zelltypen zu ändern, wurden zum Beispiel Gen-Deletionen oder Additionen bei Adenoviren vorgenommen. Adenoviren, die durch diese Mutationen nicht mehr fähig waren an ihre Rezeptoren wie auch an Integrine der Zelle zu binden, verloren hierdurch ihren sehr variablen natürlichen Zelltropismus (12). Auch wurden DNA-basierende Impfstoffe bei der Herstellung in spezielle Partikel (kationische Mikropartikel aus biodegradierbaren Polymeren) verpackt, um die Aufnahme durch Antigenpräsentierende Zellen zu erhöhen (13).

2.1.1 Eigenschaften von viralen Vektor-Impfstoffen

Virale Vektoren entstammen ursprünglich zumeist pathogenen Viren, beinhalten ein oder mehrere definierte Fremdgene und überbringen diese als eine Art Liefersystem dem zu impfenden Wirt. Im Unterschied zum klassischen Impfstoff muss die Expression der inserierten Gene im geimpften Wirt gewährleistet sein, wohingegen bei herkömmlichen Vakzinen der gesamte attenuierte oder abgetötete Erreger appliziert wird. Erfolgversprechende schutzinduzierende virale Vektoren sollten folgende Eigenschaften aufweisen (i) möglichst einfache Manipulierbarkeit des

Vektors, (ii) Aufnahmekapazität für große Gene, (iii) biologische Sicherheit als Vakzine wie zum Beispiel keine Integration von genetischem Material in das Genom der Zelle, (iv) Induktion einer humoralen, zellulären sowie lang-anhaltenden Immunantwort, (v) genetische Stabilität der inserierten Gene, (vi) geringe oder keine anti-Vektor Präimmunität, (vii) die Möglichkeit zur Herstellung der Vakzine im industriellen Maßstab und (viii) die Erfüllung von regulatorischen Vorgaben wie der gleichbleibenden Wirksamkeit sowie Effektivität und der medizinischen Unbedenklichkeit der Vakzine [Übersichtsartikel (3, 7, 14)]. Ein weiterer Vorteil von viralen Vektoren ist, dass die Vakzinierung einer viralen Infektion gleichkommt und das Fremdgen hierbei exprimiert wird, was zu einer gesteigerten Immunogenität führt, da das komplette Repertoire des Immunsystems aktiviert werden kann. Als Nachweis einer Immunantwort nach Applikation eines viralen Vektors sind die Reaktionen der angeborenen und humoralen Immunantwort gegen den Vektor, sowie die daraufhin induzierte Immunantwort gegen das inserierte Fremdantigen maßgeblich [Übersichtsartikel (2)].

Das Auftreten einer anti-Vektor Immunität des Impflings gegen zum Beispiel Adenoviren, Polioviren, manchen Pockenviren (VV) und Masernviren und die damit verbundenen Virus-Vektor neutralisierenden Antikörper können ein Nachteil für deren Nutzen als Vakzine sein [Übersichtsartikel (15-17)]. Die vorhandenen Antikörper sind in der Lage den Virus-Vektor zu neutralisieren und reduzieren dadurch die Wirksamkeit des Impfstoffs. In diesem Zusammenhang wird diskutiert, dass eine Applikation des viralen Impfstoffs über die Schleimhäute eine anti-vektorielle Immunität umgehen könnte [Übersichtsartikel (3)]. Abhängig vom Zelltropismus des genutzten Virus-Vektors und von der technischen Machbarkeit ist dies jedoch nur für spezielle Impfstrategien anwendbar. Virale Vektoren, die ihre Fähigkeit zur Replikation behalten haben, eignen sich aufgrund von Sicherheitsaspekten weniger für die Anwendung als Vakzine. Werden solche Impfvektoren jedoch zum Beispiel durch genetische Manipulation soweit attenuiert, dass sie nicht mehr replizieren können, besitzen sie häufig nur noch eine reduzierte Fähigkeit zur Immuninduktion [Übersichtsartikel (18)]. Dies trifft auch bei den Vektor-Impfstoffen der Vogelpockenviren (Kanarienpockenvirus und Hühnerpockenvirus) zu. Diese sind nicht fähig zur Replikation in Säugerzellen und weisen im Menschen nur ein geringes Potential zur Induktion von CD4-positiven T-Zellen auf (19, 20).

Weitere Vektoren wie beispielsweise Lentiviren oder auch Herpesviren weisen ebenso ein höheres Gefahrenpotential bei der Anwendung im Menschen auf, da sie sich in das Wirtsgenom integrieren oder persistierend bzw. latent im vakzinierten Wirt verbleiben können [Übersichtsartikel (14)]. Eine andere Art von Impfstoffen sind subunit-Vakzine. Diese gelten als sicher, sind aber nur schwach immunogen, da sie nicht über PAMPs erkannt werden und die angeborene Immunantwort somit nicht vollständig in Gang gesetzt werden kann [Übersichtsartikel (21)]. Virale Vektoren dagegen besitzen solche PAMPs als natürliche Adjuvants-Eigenschaft, die über PRR des angeborenen Immunsystems erkannt werden und somit eine schnelle, umfassende Immunantwort unterstützen. Die Ausgewogenheit zwischen biologischen Sicherheitsansprüchen und verbleibender Immunogenität der attenuierten viralen Vektor-Vakzine ist mitentscheidend bei der Entwicklung des Impfstoffs.

Um die maximale Wirksamkeit viraler Vektor-Vakzinen zu erzielen muss auch deren Applikationsweg und die Anzahl der Immunisierungen berücksichtigt werden. Der Impfweg ist mitentscheidend für die Erzeugung einer hohen Protektivität. So wurde beispielsweise für Adenoviren bei der Vakzinierung über die Mukosa festgestellt, dass dies einer natürlichen Adenovirusinfektion sehr nahe kommt und zu erfolgreichen Immunisierungen gegen verschiedene Erreger führen konnte [Übersichtsartikel (7, 22, 23)]. Eine aussichtsreiche Impfstrategie beinhaltet heterologe Prime / Boost Immunisierungen wie etwa eine erste Vakzinierung mit dem viralen Vektor und als zweite (Boost) Immunisierung die Gabe einer DNA Vakzine [Übersichtsartikel (24)]. Dies kann zu einer verstärkten Immunantwort des Wirts führen. Der Grund dafür ist unklar, jedoch könnte das Fehlen von zusätzlichen viralen Vektor-Antigenen bei der zweiten Vakzinierung die Immunabwehr auf das inserierte Fremdgen konzentrieren (25).

Aufgrund ihrer hohen Immuninduktion sind virale Vektoren auch als Vakzine gegen Krebserkrankungen geeignet. Durch Integration von TAA in den Vektor, kann eine gezielte krebsspezifische Immunantwort ausgelöst werden. Im Vergleich zu Tumorimpfstoffen auf der Basis von RNA, DNA oder Tumorproteinen, sind Virus-Vektoren aus der Familie der *Adenoviridae*, *Poxviridae* und *Herpesviridae,* welche TAA exprimieren, Gegenstand der Entwicklung von prophylaktischen oder immuntherapeutischen Vakzinen gegen Tumore [Übersichtsartikel (26, 27)].

2.1.2 Pockenviren als virale Vektor-Vakzine

Zu den ersten rekombinanten Virusimpfstoffen zählen Pockenviren aus der Gattung *Orthopoxvirus*. Ursprünglich wurden Stämme von VV in den 70er Jahren des 20. Jahrhunderts für die Ausrottung des humanpathogenen Variolavirus verwendet. Abkömmlinge und Varianten hiervon sind die heutige Grundlage für neuartige Virus-Vektoren. Pockenviren eignen sich als Impfvektoren, da sie Fremd-DNA von mindestens 25 Kilobasenpaare aufnehmen können (28), einen breiten Tropismus für Säugetierzellen aufweisen und im Zytoplasma replizieren, wodurch die Gefahr der Genintegration in das Wirtsgenom unterbleibt wie es bei Retroviren vorkommen kann [Übersichtsartikel (29)]. Zu diesen Vektoren zählt unter anderem der replikationsdefiziente Virus-Vektor MVA und der attenuierte VV Stamm NYVAC [Übersichtsartikel (3)](30). Die Entwicklung von MVA erfolgte über mehr als 570 Passagen des VV Stammes Ankara auf embryonalen Hühnerfibroblasten (31). Hieraus resultierte eine weitaus geringere Infektiosität für Saugetiere und auch Säugetierzellen. Dies hatte jedoch den Nachteil, dass auch die Immunogenität von MVA nicht mehr so ausgeprägt war wie bei parentalen VV Stämmen [Übersichtsartikel (32)]. Der Stamm NYVAC entstand durch die Deletion von 18 für das Virus nicht-essentiellen Genen (33, 34) und besitzt eine zu MVA vergleichbare Immunogenität gegen Human- wie auch relevante Tierpathogene. Beispielsweise konnten die immuninduzierenden Fähigkeiten beider Vektoren gegen HIV bei Untersuchungen in der Maus miteinander verglichen werden (35). Weitere replikationsdefiziente virale Vektoren sind das Kanarienpockenvirus (canarypox, ALVAC) und das Hühnerpockenvirus (fowlpox, FPV), die ausführlich in ihrer Schutzwirkung gegen verschiedenste Human- und Tier-Pathogene charakterisiert wurden (33, 36, 37). Sie eignen sich besonders für die Anwendung beim Menschen, da es gegen diese Vogelpockenviren keine präexistierende Immunität gibt und somit multiple Vakzinierungen möglich sind [Übersichtsartikel (38)]. Ein auf ALVAC basierender Vektor, welcher die HIV-1 Gene Gag, Protease und Env exprimiert, konnte bei 31,2% von über 16.000 beobachteten Personen vor einer HIV-1 Infektion schützen (39). Auch zeigte ein rekombinanter Schimpansen-Adenovirus-Vektor in Kombination mit einem auf MVA basierenden Vektor erfolgreichen Schutz in Makaken gegen das Ebolavirus (40). Eine weitere MVA-Vakzine, die das Oberflächenprotein von MERS-CoV exprimiert, konnte zudem erfolgreich die Ausscheidung von MERS-CoV in Dromedaren signifikant senken und damit den

Infektionsdruck für den Menschen reduzieren (41). In jüngerer Zeit wurde beschrieben, dass MVA wie auch NYVAC auf Grund der Vielzahl deletierter Gene, insbesondere sogenannter Immunevasionsgene eine reduzierte Immunogenität und nur ein suboptimales Immunogenitätsprofil besitzen. In der Literatur wurde gezeigt, dass dies durch Rückinsertion mancher Gene wieder verbessert werden kann. Beispielsweise wurde nach Immunisierungen in der Maus gezeigt, dass MVA-Rekombinanten, die das Zytokin GM-CSF oder das Makrophagen-inflammatorische Protein 3α (CCL20) exprimieren, eine zwei- bis vierfach verstärkte zelluläre Immunantwort gegen das inserierte Fremdantigen, sowie einen sechs bis 17-fachen erhöhten MVA-spezifischen Antikörper Titer aufwiesen, verglichen mit MVA-Rekombinanten, die nur das Fremdantigen beinhalteten (42). Durch die Insertion der humanen Wirtsgene K1L und C7L sowie der Deletion des immunmodulatorisch wirksamen viralen Gens B19 in NYVAC, konnte die Antigenexpression in infizierten Zellen verstärkt, die Virusreplikation in humanen Keratinozyten und Fibroblasten ermöglicht, die Signaltransduktion in Wirtszellen aktiviert und eine verringerte Virusverbreitung in Organen des Wirts ermöglicht werden (43).

2.1.3 Adenoviren als virale Vektor-Vakzine

Adenoviren aus der Familie der *Adenoviridae* sind unbehüllte dsDNA Viren und weisen eine ikosaedrische Struktur auf. Diejenigen Adenoviren, die als virale Vektoren in Betracht gezogen werden, weisen eine Deletion im E1 Gen auf. Hieraus ergibt sich eine Replikationsdefizienz der Adenoviren in Zellen und eine erhöhte Sicherheit beim Einsatz als Vakzine [Übersichtsartikel (44)]. Das Virus kann zum Beispiel in speziellen HEK 293 oder PER.C6 Zellen vermehrt werden, die dabei das für das Wachstum essentielle E1 Gen liefern (45). Zudem liegt meistens auch eine Deletion im E3 Gen vor, was eine Fremdgeninsertion von bis zu 8,5 kb ermöglicht [Übersichtsartikel (46)]. Rekombinante Adenoviren des Serotyps 5 sind in der Lage eine gesteigerte Immunantwort durch Rekrutierung von CTL auszulösen (47). Der Nachteil dieses Serotyps 5 ist die weitverbreitete Präimmunität im Menschen. Dies ist für diesen Vektor ein kritischer Faktor, der die Immunantwort gegenüber den Antigenen der Vakzine reduziert (48). Abhängig von der geographischen Region und vom Alter werden neutralisierende Antikörper bei bis zu 90% von Erwachsenen nachgewiesen. Es besteht die Strategie, weniger verbreitete Adenoviren als

Vektoren einzusetzen wie beispielsweise die Serotypen 11 oder 35. Diese lösen allerdings auch eine weniger starke Immunantwort aus (49). Eine weitere Möglichkeit die anti-vektorielle Immunantwort zu umgehen besteht darin, mit einer DNA-Vakzine (oder eines Impstoffs anderen Ursprungs) die erste Immunisierung und mit Rekombinanten von Adenovirus 5 die zweite Impfung durchzuführen. Die einmalige Immunisierung mit dem Virus soll hierbei die Induktion von neutralisierenden Antikörpern gegen das Adenovirus reduzieren (50). Vielversprechende Ansätze für die Entwicklung von Vakzinen gibt es auch mit adenoviralen Stämmen, die aus Schimpansen (*Pan*) oder Bonobos (*Pan paniscus*) isoliert wurden und ausgeprägte immunogene Eigenschaften besitzen (51, 52).

2.1.4 Andere Virusfamilien als virale Vektor-Impfstoffe

Weitere Klassen von Viren wurden untersucht, um sie als rekombinante virale Vektoren einsetzen zu können. Vektoren basierend auf Viren der Familie *Herpesviridae* werden nicht nur als reine Gen-Lieferanten in Betracht gezogen, sondern auf Grund ihres Zelltropismus auch für Immuntherapien gegen Hirntumoren und als Therapeutika Lieferant für das ZNS evaluiert [Übersichtsartikel (53)].

Das VSV gehört zur Familie der *Rhabdoviridae* und ist ein negatives ssRNA Virus. Es infiziert domestizierte Tiere wie Schweine, Pferde und Rinder und erzeugt nach Infektion im Menschen nur eine geringe Symptomatik [Übersichtsartikel (54, 55)]. Für die Herstellung und spätere Verwendung als Vektor-Impfstoff ist wichtig zu wissen, dass Gene am 5‘ Ende des viralen Genoms stärker exprimiert werden als Gene am 3‘ Ende und Fremdgene entsprechend inseriert werden müssen. Als Vakzine ist es attraktiv, da es zu hohen Titern angezogen werden kann und nach Fremdgeninsertion genetisch stabil ist [Übersichtsartikel (14)]. Es wird im Menschen nur eine geringe Vektorimmunität aufgebaut, da neutralisierende Antikörper gegen VSV selten sind. Zudem wurde gezeigt, dass VSV DC infiziert und dabei eine ausgeprägte anti-tumorale Immunantwort bei der Verwendung als Vektor-Vakzine gewährleistet (56). Außerdem zeigten kürzlich veröffentlichte Studien vielversprechende Ergebnisse einer anti-Ebola Immunantwort von rekombinanten VSV-Vektoren (57, 58).

Viren der Gattung Flavivirus besitzen ein (+)ssRNA Genom und eine ikosaedrische Struktur. Das Gelbfieber Virus dieser Gattung, basierend auf dem Stamm YF-17D, ist als attenuierte, Lebendvakzine äußerst vielversprechend. Diese Vakzine stimuliert zunächst die angeborene Immunantwort über die TLR 2, 7, 8 und 9 (59). Der eigentliche Schutz beruht dann auf der Induktion von neutralisierenden Antikörpern wie auch auf CD8-positiven T-Zellen. Gene aus der Virushülle können gegen Gene anderer Flaviviren, wie dem Japan-Enzephalitis-Virus, Dengue-Virus oder West-Nil-Virus ausgetauscht werden und anschließend als Impfstoff dienen [Übersichtsartikel (60)].

Die dsDNA Baculoviren der Familie *Baculoviridae* infizieren Insekten und sind häufig genutzte Vektor-Vakzinen, da sie für den Menschen nicht pathogen sind und somit eine hohe biologische Sicherheit aufweisen [Übersichtsartikel (61)]. Zudem sind sie relativ einfach herzustellen und es können auch größere Gene (>20 Kilobasenpaare) in das Genom inseriert werden [Übersichtsartikel (61, 62)]. Der Nachteil von Baculoviren als rekombinanter Impfstoff sind die häufig gezeigten geringen immunogenen Eigenschaften, wie am Beispiel gegen das Japan-Enzephalitis-Virus beschrieben wurde (63).

Eine weitere Möglichkeit Viren als Impfstoffe einzusetzen besteht durch die Technologie der zielgerichteten Mutagenese, die bei RNA Viren auch als ‚reverse genetics' bezeichnet wird. Es wird die Aktivität RNA-abhängiger RNA Polymerasen für die Vervielfältigung genomischer Information, die Unabhängigkeit der RNA Virus Replikation vom Zellkern des Wirts sowie die Eigenschaft, dass bei (+)RNA Viren das Genom direkt zu viralen Proteinprodukten translatiert wird genutzt [Übersichtsartikel (64, 65)]. Diese Technik wurde unter anderem für das Rabiesvirus angewendet und führte zu einer reduzierten Virulenz und hierdurch zu einer erhöhten biologischen Sicherheit von Vakzinen, die eine Kreuzreaktivität gegen verschiedene Varianten von Rabiesvirus aufwiesen (66). Auf diese Art hergestellte Rabiesvirus-Vektoren waren zudem fähig eine spezifische Antikörperantwort gegen das Ebolavirus zu induzieren und erzeugten einen 100%igen Schutz in Makaken (67). Initiale Arbeiten dieser Technik erfolgten für das Rabiesvirus durch die Insertion der bakteriellen Enzyme Chloramphenicol-Acetyltransferase und β-Galaktosidase in das Virusgenom (68, 69). Auch für Influenzaviren existieren so hergestellte Vakzine wie zum Beispiel das

pandemische H1N1 Virus aus dem Jahr 2009, welches verändert wurde und nun chimäres HA und NA in deutlich größeren Mengen enthält (70).

2.1.5 Nicht virale Impfstoff-Vektoren

Attenuierte bakterielle Vektoren bieten die Möglichkeit zur Vakzinierung über den oralen Weg, was einer natürlichen Infektion wie bei *Salmonella* oder *Mycobacterium bovis* entspricht (71) [Übersichtsartikel (72)]. Der immunologische Vorteil von bakteriellen Impfvektoren besteht wie auch bei viralen Vektoren darin, dass sowohl eine humorale wie auch zelluläre Immunantwort induziert werden kann, da intrazelluläre Bakterien das heterologe Antigen oder Plasmid direkt in den intrazellulären MHC I vermittelten Antigen Prozess einbringen können [Übersichtsartikel (2)]. Mit Blick auf den Sicherheitsaspekt ist zu erwähnen, dass die DNA Plasmide genetisch eher instabil sind und dass sich zudem das auf einem Plasmid befindliche Fremdgen auf andere Bakterien übertragen werden kann (73) [Übersichtsartikel (74)].

2.1.5.1 DNA und RNA-basierende Impfstoffe

Im Gegensatz zu Viren besitzen DNA und RNA keine speziellen Strukturen und Mechanismen, um eine Zelle infizieren zu können. Trotzdem konnte gezeigt werden, dass auch ein DNA Plasmid erfolgreich in Muskelzellen der Maus transfiziert werden konnte und dies zur Expression des inserierten Gens führte (75). DNA-Vakzinen bestehen generell aus einem Plasmid, in dem das entsprechende Antigen inseriert ist und unter Kontrolle eines eukaryotischen Promoters steht. Des Weiteren stammen Plasmide ursprünglich von Bakterien und beinhalten daher CpG Motive, welche für die Stimulation von TLR9 bekannt sind (76, 77). Diese Aktivierung der angeborenen Immunantwort, die sich direkt gegen das bakterielle Plasmid richtet, führt zu einer generell verstärkten Immunantwort gegen das Gerüst der DNA-Vakzine. DNA Impfstoffe zeichnen sich als einfache Lieferanten von multiplen Fremdgenen aus und induzieren sowohl eine humorale wie auch zelluläre Immunantwort [Übersichtsartikel (78)]. Zudem sind diese Impfstoffe sicher und gut verträglich [Übersichtsartikel (79)]. Jedoch zeigte sich, dass diese Vakzinen im Vergleich zu anderen Impfstoffarten wie

Peptid-Impfstoffen, RNA und viralen Vektor-Vakzinen eine geringere immunogene Wirkung aufweisen [Übersichtsartikel (78)].

RNA-Vakzine haben im Gegensatz zu DNA basierenden Impfstoffen den Vorteil, dass sie für die Translation nur in das Zytoplasma und nicht in den Zellkern gelangen müssen und RNA zudem nicht in das Zellgenom integrieren kann [Übersichtsartikel (78)]. RNA stimuliert außerdem direkt das Immunsystem über die TLR3, TLR7 und TLR8 [Übersichtsartikel (80)]. Die eukaryotische mRNA besteht aus einem kodierenden Bereich, welcher von einer nicht-kodierenden Region flankiert wird, einer 5‘ 7-Methylguanosin Triphosphat Cap Struktur sowie einem Poly(A) Schwanz. Die Schwachpunkte für Stabilität und Translation der mRNA sind dabei der nicht-kodierende Bereich, die Cap Struktur, welche die mRNA vor 5‘-3‘ Exonukleasen schützt und der Poly(A) Schwanz (81, 82) [Übersichtsartikel (83)]. Da RNAs, welche keine Cap Struktur besitzen, äußerst instabil sind und nicht effizient translatiert werden, können für Impfstoffe posttranskriptional Cap Strukturen an die RNA angefügt werden [Übersichtsartikel (78)]. Die Vakzinierung erfolgt als „nackte“ mRNA, wobei diese ohne Trägersubstanz direkt appliziert wird (75). Auch konnte gezeigt werden, dass diese Form der Vakzinierung zur Induktion von Antigen-spezifischen Antikörpen und T-Zell vermittelter Immunreaktion führte (84, 85). Jedoch sind allgegenwärtige RNasen verantwortlich für die Degradierung von extrazellulärer „nackter“ mRNA und bedingen daher eine relative kurze Halbwertszeit dieser RNA [Übersichtsartikel (86)]. Eine weitere Möglichkeit mRNA zu applizieren besteht durch eine „Gene Gun“. Hierbei wird die mRNA direkt in das Zytoplasma der Zelle gebracht, wodurch die Exposition gegenüber extrazellulären Nukleasen minimiert wird [Übersichtsartikel (78)]. Eine weitere Darreichungsform stellt auch die Kondensierung der mRNA durch Protamin dar, wodurch die Stabilität gegenüber Nukleasen erhöht wird [Übersichtsartikel (87)]. Um zusätzlich die exprimierte Proteinmenge und auch die Dauer der Expression zu erhöhen, können die kodierenden und nicht-kodierenden Bereiche der RNA molekularbiologisch verändert werden [Übersichtsartikel (87)]. Die Vakzinierung mit der modifizierten RNA aktiviert zusätzlich die MyD88, TLR7 und TLR8 abhängigen Stoffwechselwege und der Impfstoff wirkt somit zusätzlich als Adjuvants (88, 89) [Übersichtsartikel (87)]. RNA-basierende Impfstoffe wurden in den letzten Jahren gegen eine Vielzahl von

Pathogenen wie auch gegen Tumore auf ihre immunstimulierenden Eigenschaften hin getestet (84, 90, 91) [Übersichtsartikel (92)].

2.2 Das Orf Virus (ORFV)

Das Orf Virus (ORFV), wie auch das BPSV, das PCPV, das PVNZ und das Parapoxvirus des Seehundes, ist eine Art des Genus *Parapoxvirus*, welches zu den *Chordopoxvirinae*, einer Subfamilie der *Poxviridae* gehört.

ORFV ist relativ temperaturstabil und nicht onkogen. Wie bei allen Pockenviren findet die Replikation im Zytoplasma der Zelle statt und es gibt keine Hinweise auf Integration viraler Gene in das Wirtsgenom. Das Virion des ORFV besitzt eine ovoide Form, eine wollknäuel-artige Struktur auf der Oberfläche und das Genom weist einen hohen Guanin und Cytosin Anteil (63-64%) auf. Dieses Genom besteht aus einer linearen, etwa 135 kb großen dsDNA und ist am Ende kovalent als Haarnadelstruktur geschlossen (93) [Übersichtsartikel (94)].

2.2.1 Die Reifung der Pockenviren

Um die unterschiedlichen Reifungsformen von Pockenviren zu beschreiben wird im Folgenden die hierfür gültige neue Nomenklatur verwendet [Übersichtsartikel (95)]. Von VV gibt es zwei infektiöse Formen zum Einen das MV (mature virion) und zum Anderen das EV (extracellular virion). Das EV besitzt im Gegensatz zu MV eine zusätzliche Hüllmembran. Beide Formen geben nach dem Zelleintritt ihr Genom in das Zytoplasma der infizierten Zelle ab. Das virale Genom befindet sich in einem ‚core-Kompartiment' und es wird vermutet, dass sich dieser ‚core' entlang des mikrotubulären Netzwerks der infizierten Zelle bewegt (96). Im Bereich des ER kommt es dann zu einer Akkumulation der ‚core-Kompartimente' (97). Diese Virus-Kompartimente enthalten zusätzlich zum viralen Genom etwa 100 virale mRNAs. Diese RNAs werden nun ATP-abhängig in das Zytoplasma der infizierten Zelle transportiert, wo sie anschließend translatiert werden können. Diese frühe Proteinbiosyntese hat in erster Linie den Zweck den ‚core' des Virions zu öffnen, die virale DNA frei zu setzen und Replikation des viralen Genoms zu initiieren [Übersichtsartikel (98)]. Die intermediäre und späte DNA Replikation von VV wie

auch von ORFV findet in einer Art „Virus Fabrik“ im Zytoplasma der Zelle statt. Die Replikationseinheiten sind mit einer Membran umgeben, welche bei VV aus dem rauen ER entstammt. Die Umhüllung der Replikationseinheit ist ein zeitlich begrenzter Prozess. Hierfür verantwortlich ist das frühe VV Protein E8R, welches zunächst die Umhüllung initiiert. Nachfolgend wird es phosphoryliert, wodurch die Bindung an die DNA abnimmt und die Membran des ER von der Replikationseinheit dissoziiert (99). Die Biogenese der Virionen beginnt mit der Bildung einer sichelförmigen Membran in IVs (immature virions) in der Nähe der DNA-Replikationseinheit. Nach Aufnahme von DNA reifen die IVs in backsteinförmige MVs. Ein geringerer Anteil der MVs entwickelt sich zu EVs weiter, indem sie von dem TGN oder den Endosomen der Zelle eine weitere Membran erhalten. Am mikrotubulären Netzwerk entlang gelingt der Transport der EVs zur Plasmamembran, wo die äußere EV Membran mit der Plasmamembran fusioniert. Die hierdurch entstandenen Virionen werden so im Anschluss aus der Zelle entlassen [Übersichtsartikel (98)].

2.2.2 Replikation und Transkription des Genoms der Pockenviren

Poxviridae sind die einzigen DNA Viren bei denen die DNA Replikation im Zytoplasma anstatt im Nukleus von infizierten Zellen stattfindet. Die Virus Replikation wie auch der Lebenszyklus ist bei VV als Modelvirus der Pockenviren intensiv untersucht worden. Das Genom, virale Enzyme und andere Faktoren, welche unabdingbar für die Transkription von frühen VV Genen sind, befinden sich im ‚core-Kompartiment‘ von infektiösen Viruspartikeln [Übersichtsartikel (100)]. Dahingegen sind DNA Replikationsproteine nicht in das ‚core-Kompartiment‘ verpackt, sondern werden von frühen viralen mRNAs translatiert [Übersichtsartikel (101)]. Die Replikation der viralen DNA erfolgt nachdem das Genom das ‚core-Kompartiment‘ verlassen hat. Hier dienen dann DNA Tochterprodukte als Schablonen für intermediale und späte Gentranskription (102). Die Koordination der Genomreplikation und der Zusammenbau der Virionen von Pockenviren sind durch zeitlichen Versatz der Expression einzelner viraler Gene geregelt. Proteine, die bei der DNA Replikation, der Nukleotid-Synthese und der intermediären Transkription beteiligt sind, gehören zur Gruppe der frühen-Genklasse; Proteine, die bei der Morphogenese und Zusammenbau des Virions beteiligt sind, zählen zur Gruppe der

post-replikativen, intermediären und späten-Genklasse [Übersichtsartikel (103)] (104). Pockenviren kodieren eine DNA Polymerase, deren katalytische Untereinheit homolog zu anderen viralen wie auch eukaryotischen α und δ DNA Polymerasen ist [Übersichtsartikel (101)]. Die replizierte DNA liegt dann in Form eines unverzweigten Konkatemers vor [Übersichtsartikel (101)]. Etwa die Hälfte der VV Gene gehört zur Klasse der frühen Gene und die Initiation der Synthese dieser frühen Gene benötigt nur eine geringe Anzahl an Transkriptionsfaktoren sowie Adenosintriphosphat als Energiequelle [Übersichtsartikel (103)]. Neuere Untersuchungen charakterisierten die Transkription von Genen der Pockenviren, ob diese durch frühe, intermediäre oder späte Promotoren reguliert werden (102, 105). Die frühe Expression von Genen der Pockenviren oder auch der inserierten Fremdgene benötigt transkriptionelle Promotoren, welche durch den RNA Polymerase Komplex der Pockenviren erkannt werden können (106). Die frühen Promotoren von Pockenviren weisen die Konsensussequenz 5‘ AAAAAAGTGAAAAAAT/A 3‘ auf (107). Studien zeigten, dass die Motive von frühen ORFV Promotoren große Ähnlichkeiten mit denen des VV aufweisen (108, 109). Eine größere Effizienz von frühen VV spezifische Promotoren im Vergleich zu herkömmlichen und bei Vektor-Vakzinen verbreiteten p7,5 und pS Promotoren konnte erreicht werden, indem verschiedene Tandem-Elemente in die Sequenz der Promotoren eingefügt wurden (110-112). Auch konnte für MVA die frühe Genexpression, durch verlängerte ‚spacer‘ Sequenzen zwischen Promotor und Fremdgen, verstärkt werden (113). Die intermediäre Gentranskription wiederum wird durch Promotoren gesteuert, welche über ein Initiationselement am Transkriptionsstart und eine vorgeschaltete A-T reiche Region verfügen (114). Des Weiteren sind hierfür mehrere virale Proteine notwendig, möglicherweise auch die virale RNA Polymerase [Übersichtsartikel (103)]. Neu synthetisierte RNA Polymerase ist unabdingbar für die Transkription von Genen der späten Genklasse (115). Die späte Gentranskription wird häufig durch das wenig variantenreiche TAAAT oder TAATG Element initiiert [Übersichtsartikel (103)] (116). Hierbei wird die Translation der RNA durch das vorhandene Trinukleotid ATG bestimmt, welches später zu Methionin translatiert wird (104). Bei Pockenviren wird die frühe Gentranskription durch eine Terminationssequenz beendet; dies geschieht etwa 30-50 Nukleotide Strang abwärts des Signals auf der DNA [Übersichtsartikel (103)]. Dieses Signal besteht aus der Abfolge 5’TTTTTNT3‘, wobei N jedes mögliche Nukleotid sein kann (117). Die Termination der intermediären und späten Gentranskription bedarf keiner

spezifischen Signalsequenz, jedoch gibt es Hinweise auf einen aktiven Mechanismus durch virale Proteine, der die Transkription der intermediären und späten Gene von VV beendet [Übersichtsartikel (118)]. Mutationen oder das Fehlen des VV Protein A18R führten zu deutlich längeren Transkripten, wobei Mutationen innerhalb des G2R Proteins zu verkürzten Transkriptionsprodukten führte [Übersichtsartikel (118)] (119).

2.2.3 Immunantwort des Wirts und Immunmodulation durch das Orf Virus

Das natürliche Wirtsspektrum von ORFV sind kleine Wiederkäuer wie Schafe und Ziegen. Ansonsten ist der Wirtsbereich auch *in vitro* auf wenige Zellspezies eng begrenzt, was wiederum ein hervorzuhebender Sicherheitsaspekt für die Verwendung als Vektor-Impfstoff ist. Das ORFV infiziert die epidermalen Keratinozyten der Tiere. Im Verlauf der Infektion kommt es vor allem an den Lippen, den Mundschleimhäuten und Nüstern zu kontagiösen, pustulösen und proliferativen Läsionen der Haut. Auch innere Bereiche des Mauls wie die Zunge können betroffen sein. Zudem bildet sich Wundschorf und das Auftreten von Eiter ist keine Seltenheit. Eine Schädigung des äußeren Epithels ist Voraussetzung für die Infektion mit ORFV, wobei immer nur lokale Bereiche betroffen sind und sich die ORFV Infektion nicht systemisch ausbreitet [Übersichtsartikel (94, 120)].

Das virulente Feldvirus ist auch zur zoonotischen Infektion des Menschen in der Lage. Betroffen sind hauptsächlich Personen aus den Bereichen der Landwirtschaft, Fleischverarbeitung und Veterinärmedizin. In seltenen Fällen, vor allem bei immundefizienten beziehungsweise immunsupprimierten Personen, kann es zur Ausbildung einer atypischen, stark proliferierenden Läsion, dem sogenannten ‚Giant Orf', kommen (121, 122).

ORFV besitzt die Eigenschaft mit antigenpräsentierenden Zellen, wie DC und Langerhans-Zellen der Haut, zu interagieren und löst hierdurch Reaktionen des angeborenen und adaptiven Immunsystems aus [Übersichtsartikel (123)]. Es wird vermutet, dass das ORFV sowohl Monozyten wie auch DC über den CD14/Toll-like Rezeptor Signalweg aktivieren kann und es zur Ausschüttung von pro-inflamatorischen Zytokinen, wie auch der TH-1 Zytokine IL-12 und IL-18 kommt [Übersichtsartikel (124)].

Das ORFV besitzt Gene für verschiedene Virulenz- und Evasions-Faktoren, die dem Virus die Möglichkeit bieten die Immunantwort des Wirts zu umgehen [Übersichtsartikel (125, 126)]. Hierzu gehört das ORFV IFNR (ORF020), das CBP (ORF112) und der GIF (ORF117) (127, 128) [Übersichtsartikel (123)]. Des Weiteren kodiert ORFV für ein Heparin-Binde-Protein (F1L, ORF059), ein IL-10 Homolog (vIL-10, ORF127), das Virulenzgen vegf-e (ORF132) und das Protein ORF125 welches ein Bcl-2 ähnliches Protein ist und die mitochondrielle Apoptose von Zellen hemmt (129, 130). Als weitere Faktoren wurden im Jahr 2010 das Gen ORF024 identifiziert, das für einen Aktivierungsinhibitor von NF-κB kodiert sowie im Jahr 2011 ORF002, welches spezifische Reaktionen innerhalb des Zellkerns inhibiert, dies wiederum bewirkt eine reduzierte Transkription von NF-κB und dem Protein ORF121, welches ein weiterer NF-κB Inhibitor ist (131-133). Die Einflussnahme von ORFV auf die Transkriptionsfaktoren der NF-κB Familie beeinträchtigt dadurch direkt die Expression von zellulären Genen die bei der angeborenen Immunität, Entzündungsreaktionen oder Zellproliferation von Bedeutung sind [Übersichtsartikel (134, 135)].

Das zuvor angeführte ORFV IFNR ist ein Resistenzfaktor gegen IFN, der fähig ist die RNA-abhängige Proteinkinase auf eine ähnliche Weise zu inhibieren wie das E3L Genprodukt von VV (136). Das von ORFV kodierte vIL-10 ist ein Virulenz-Faktor mit einer hohen Homologie zu IL-10 anderer Säugetiere (137). Die immunsupprimierende Wirkung auf den Wirt zeigt sich dadurch, dass es die Synthese von Zytokinen und die Reifung von murinen sowie humanen DC hemmt (138-140). Außerdem wurde gezeigt, dass das virale vIL-10 wie auch das IL-10 von Säugetieren Entzündungsreaktionen reduzieren und die Wundheilung beschleunigen kann (141). Zudem führten Infektionen mit ORFV Mutanten, welche vIL-10 nicht exprimierten, zu höheren IFN-γ Mengen im Gewebe von Schafen verglichen zu ORFV mit vIL-10 (142). In der viralen Pathogenese besitzt das von ORFV exprimierte VEGF-E eine zentrale Rolle (143). Es verstärkt die Proliferation von Endothelzellen und die Angiogenese in Läsionen im Schaf wie auch beim Menschen (144-146). Ein möglicherweise weiterer Virulenzfaktor ist die von ORFV kodierte, für das Viruswachstum nicht essentielle aber funktionelle UTPase (ORF007) (147). Da das Fehlen dieses Enzyms zur Attenuierung von mutanten Alphaherpesviren führte,

wurde vermutet, dass es sich um ein Protein handelt, welches die Virulenz beeinflussen könnte (148, 149).

Durch die frühe Expression dieser immunmodulatorischen Proteine hat das ORFV die Fähigkeit in die Prozesse der Immunabwehr des Wirtes einzugreifen, was wiederum die Bildung infektiöser Viruspartikel begünstigen kann. Diese Mechanismen ermöglichen die Replikation des ORFV, bevor der Wirt seine Immunabwehr gegen das Virus gebildet hat. ORFV besitzt die Fähigkeit nach natürlichen Infektionen seine Wirte wiederholt zu infizieren, wobei dies zu einer verstärkten inflammatorischen Immunantwort des Wirts führt [Übersichtsartikel (123, 150)]. Eine maßgebliche Beteiligung der humoralen Immunantwort und somit die Bildung von neutralisierenden Antikörpern gegen das ORFV scheinen nicht vorzuliegen (151). Die beschriebenen Evasionsmechanismen, welche das ORFV entwickelt hat, könnten die Möglichkeit der Reinfektion seiner Wirte erlauben [Übersichtsartikel (94)].

Für das ORFV wurde auch die Eigenschaft beschrieben in inaktivierter Form immunmodulatorisch wirken zu können, indem es anti-inflammatorische und TH-1 Zytokine induziert und darauf folgend auch TH-2 Zytokine anregt [Übersichtsartikel (152)]. Beispielsweise wurde beschrieben, dass inaktiviertes ORFV *in vitro* porcine PBMCs aktiviert oder beim Hund die Proliferation von T-Zellen sowie Monozyten induziert (153, 154). Modulierende Interaktionen von inaktiviertem ORFV mit dem Immunsystem wurden auch in verschiedenen weiteren *in vivo* Modellen beschrieben. Die Applikation von inaktiviertem ORFV reduzierte in der Maus die Replikation von HBV und konnte auch die klinischen Symptome nach Infektion mit Herpes Simplex Virus reduzieren (155) [Übersichtsartikel (152)]. Interessanter Weise konnte auch eine anti-tumorale Wirkung sowohl von inaktiviertem als auch von lebendem ORFV in verschiedenen Modellen der Maus gezeigt werden (156, 157). Eine wichtige Rolle wurde hierbei den NK zugeschrieben (157). Weitgehend unklar ist, welche Faktoren des inaktivierten ORFV für diesen Effekt der Immunmodulation verantwortlich sind. Ein löslicher Faktor, der von Zellen nach der Infektion mit ORFV produziert wird, konnte hierfür ausgeschlossen werden, da virusfreie Überstände von infizierten Zellen keine immunstimulierende Eigenschaften aufwiesen (158). Nachdem durch Untersuchungen mit Proteinase K ausgeschlossen werden konnte, dass DNA für den

immunmodulatorischen Effekt verantwortlich ist, liegt die Vermutung nahe, dass der Effekt Proteinen von Viruspartikeln zuzuschreiben ist (158).

2.2.4 ORFV Stamm D1701 als neuer viraler Vektor

D1701 ist ein hochattenuierter ORFV Stamm, der ursprünglich aus einem Virusisolat eines Schafs gewonnen wurde (159, 160). Der auf BKKL-3A Zellen (bovine Nierenzellen) adaptierte ORFV Stamm D1701 eignet sich unter anderem durch eine erhöhte Attenuierung für den Einsatz als Vakzine und wurde zeitweise gegen Ecthyma contagiosum im Schaf eingesetzt (159). Eine weitere Adaption zum Wachstum in Vero Zellen (Affennierenzellen, *Chlorocebus*) führte zu Deletionen in drei Regionen im Genom des ORFV Stamm D1701, welche zu einer weiteren Attenuierung des D1701 führte und selbst die Pathogenität im natürlichen Wirt (Schaf) stoppte [Übersichtsartikel (124, 161)] (162). Die stabile Insertion von fremden, viralen Genen unter der Kontrolle des frühen vegf-e Promotors führte zu sehr guten Genexpressionen und Protektionsergebnissen insbesondere in Tieren, in denen ORFV sich nicht vermehrt (1, 163-169). Bemerkenswerterweise konnte mit rekombinanten ORFV ein Schutz gegen PRV im Schwein induziert werden (164, 167). Dies ist im Schwein, dem natürlichen Wirt von PRV, meist nur mit Impfstoffen möglich, die alle PRV spezifischen Antigene beinhalten [Übersichtsartikel (170)]. Zudem konnte bei diesen PRV Infektionsversuchen auch ein Schutz durch heterologe Prime (DNA-Vakzine) / Boost (ORFV-Rekombinante) Immunisierungen induziert werden (164). Auch wurde eine langanhaltende und schützende Immunität durch eine ORFV-Rekombinante beschrieben, welche in der Ratte gegen das Bornavirus untersucht wurde (163).

Des Weiteren wurden andere ORFV-Rekombinanten entwickelt, die das Peptid EG95 exprimieren und im Schaf auf ihre Immunogenität gegen den Parasiten *Echinococcus granulosus* untersucht wurden (106, 171). Da diese Rekombinanten jedoch mit dem virulenten ORFV Stamm NZ2 hergestellt wurden, bleibt zu klären ob sie den Ansprüchen der biologischen Sicherheit genügen können. Außerdem wurde hier nur die Fremdgen-spezifische Antikörperantwort und kein Schutz gegen eine Belastungsinfektion getestet. Zudem wurde kürzlich eine ORFV-Rekombinante beschrieben, in der das ‚spike'-Protein von PEDV (Virus der epizootischen

Virusdiarrhoe des Schweins) in den ORFV121 Genort des ORFV Stamm IA82 inseriert wurde (172). Nach der Immunisierung von Schweinen und einer anschließenden Belastungsinfektion konnte diese Rekombinante die klinische Symptomatik der epizootischen Virusdiarrhoe verhindern sowie die Virusverbreitung über den Kot der Tiere verringern (172). Generierte ORFV-D1701-Rekombinanten zeigten nach i.m. Immunisierung gute Erfolge bezüglich des Schutzes der Tiere gegenüber viralen Pathogenen. Die frühe Genexpression der inserierten Fremdgene ist hierbei unabhängig von der ORFV Replikation. Somit müssen keine neuen Viruspartikel entstehen, um eine protektive Immunität zu erzeugen. Dies gewährleistet eine Expression des inserierten Fremdgens im permissiven wie auch nicht-permissiven Wirt. Eine i.m. Immunisierung führt möglicherweise zu einem Depoteffekt der ORFV-Rekombinanten und zu einer Immunstimulierung der regionalen Lymphknoten.

Der virale Vektor auf Basis des ORFV Stamm D1701 bietet durch seine Attenuierung und Apathogenität, der möglichen *in vitro* Vermehrung und Produktion auf Vero Zellen, der reduzierten anti-vektoriellen Immunität und der Fähigkeit zur Induktion einer humoralen und zellulären Immunantwort gegen die inserierten Fremdgene optimale Voraussetzungen für die Entwicklung weiterer Impfstoffe nicht nur gegen eine Vielzahl von Pathogenen.

3 Ergebnisse und Diskussion

3.1 Methoden der Herstellung und Selektion neuer ORFV-Rekombinanten

3.1.1 Optimierung der Herstellung von ORFV-Rekombinanten

Die Generierung von ORFV-Rekombinanten ist aufgrund des großen dsDNA Genoms, der Tatsache, dass die Pockenvirus DNA nicht infektiös ist und der relativ geringen Wachstumsgeschwindigkeit von ORFV ein komplexer und zeitintensiver Vorgang. Die Herstellung von neuen Rekombinanten erfolgt durch homologe Rekombination zwischen dem Genom des parentalen Ausgangsvirus und einem Transferplasmid, in dem das gewünschte Antigen von zu ORFV homologen Sequenzen flankiert wird. Das Transferplasmid enthält zusätzlich in 5‘ Richtung vor dem Insert den frühen vegf-e Promotor des ORFV. Dieser Promotor ermöglicht die Transkription der inserierten Fremdgene zu einem sehr frühen Zeitpunkt unabhängig von der Replikation der ORFV DNA. Das parentale Ausgangsvirus D1701-VrV besitzt anstelle des vegf-e Gens die Selektionskassette LacZ/Xgpt, wobei das LacZ Gen β-Galaktosidase exprimiert und bei erfolgreicher Rekombination die Selektionsgene durch das Fremdgen ausgetauscht werden (1, 162, 163). Bei dieser Vorgehensweise ergibt sich, dass bei einer Überschichtung mit Agarose-Substrat Plaques der neuen ORFV-Rekombinanten farblos erscheinen, während Plaques, die vom elterlichen Virus, β-Galaktosidase exprimierenden Virus erzeugt werden, bläulich gefärbt sind. Die isolierten Plaques von potentiell neuen Rekombinanten wurden daraufhin durch einen IPMA mit Hilfe von spezifischen Antikörpern gegen ORFV sowie gegen das inserierte Antigen getestet (173). Virusisolate, die im IPMA Test sowohl positiv für die Expression von ORFV Proteinen und des neuen Inserts waren, wurden für weitere Reinigungsschritte herangezogen. Diese Art der negativen Selektion ist relativ zeitaufwendig, da ein einzelner Schritt der Virusplaque Reinigung mit anschließendem IPMA Test etwa eine Wochen in Anspruch nimmt. Zudem besteht die Notwendigkeit, dass die gewählten Antikörper die Expression des Fremdproteins auch sicher nachweisen können. Dies stellte sich bei der Selektion von verschiedenen Rekombinanten oft als limitierender Faktor dar.

Um zum Einen die Effizienz der Erzeugung von ORFV-Rekombinanten zu erhöhen und zum Anderen die Selektion von neuen Rekombinanten zu vereinfachen und zu beschleunigen wurden mehrere Arbeitsschritte optimiert. Für die Optimierung der

Transfektionen von ORFV infizierten Zellen wurden Zeitdauer und Virustiter sowie die Menge an Transferplasmid DNA variiert. Zusätzlich wurden verschiedene Transfektions-Reagenzien und Techniken getestet. Die Kombination von geringen Infektionsdosen (moi 0,01 bis 0,2) sowie einer auf Elektroporation beruhenden Nukleofektion ermöglichte stabile, reproduzierbare und äußerst effiziente Transfektionen, wie sie auch bei Transfektionen mit microRNA in Neuronen von Säugetieren beschrieben wurden (174).

Die Aufreinigung der neu generierten ORFV-Rekombinanten konnte dahingehend verbessert werden, dass statt dem auf Antikörper basierenden IPMA eine PCR für das jeweilige inserierte Gen etabliert wurde. Für die praktische Umsetzung bedeutete dies, dass nun DNA direkt aus den isolierten Virusplaques extrahiert und auf positive Insertion des Fremdgens getestet werden konnte. Abbildung 3.1.1. zeigt exemplarisch die Analyse mittels PCR von rekombinanten Viren nach der fünften Virusplaquereinigung für die ORFV-Rekombinante D1701-V-HAh5n, in welche das Gen HA von Influenza Virus A H5N1 inseriert wurde. Bis auf zwei Virusisolate (15 und 18) war durch die HA spezifische PCR das inserierte Gen in allen Isolaten nachweisbar. In einer zweiten PCR wurde durch Detektion des parentalen LacZ Gens die Reinheit der isolierten Rekombinante überprüft. In den Isolaten 13, 16, 17 und 21 konnte das Ausgangsvirus D1701-VrV nicht mehr nachgewiesen werden, da das LacZ Gens in der PCR nicht mehr nachweisbar war (Abb. 3.1.1).

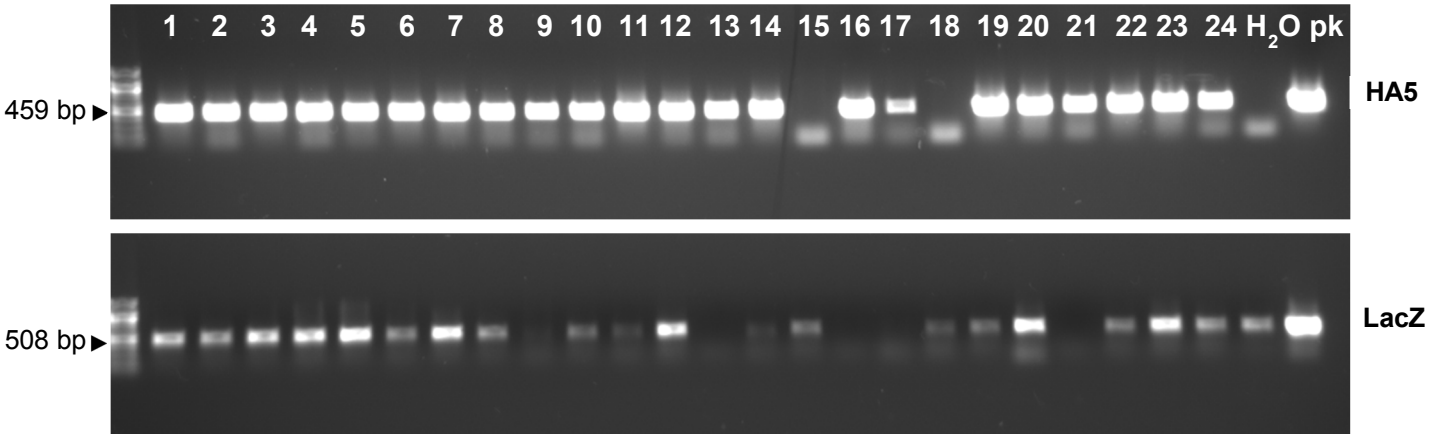

Abbildung 3.1.1. Selektion von D1701-V-HAh5n durch PCR.
Horizontale, 0,8%ige Gelelektrophorese der fünften Virusplaquereinigung von 24 Isolaten der ORFV-Rekombinanten D1701-V-HAh5n. PCR wurde mit DNA der Isolate und spezifischen Primern für HA5 und LacZ durchgeführt. Das inserierte Fremdgen HA5 (459 bp) ist bis auf zwei Proben (15 und 18) in allen Isolaten nachweisbar. Das elterliche LacZ (508 bp) Gen ist bei den Proben 13, 16, 17 und 21 nicht mehr durch PCR detektierbar.

Zusätzliche Zeitersparnis und erleichterte Selektion erbrachte die Umstellung auf ein parentales ORFV, welches anstatt des LacZ Gens das Fluoreszenzgen AcGFP (*Aequorea coerulescens* green-fluorescent protein) exprimierte. Diese Variante des Fluoreszenzproteins GFP enthält eine chromophore Mutation und ist auf Aminosäurenbasis zu 94% homolog zu EGFP (Enhanced GFP). Zudem ist es codon-optimiert, was in Säugetierzellen in einer 35-fach helleren Fluoreszenz mit sehr ähnlichen Extinktions- und Emissions-Maxima resultiert (175).

Die Selektion rekombinanter Virusplaques konnte daraufhin direkt *in vitro* durch Titrationen und Fluoreszenzmikroskopie durchgeführt werden und machte die Überschichtung der Zellen mit Agarose entbehrlich. Zudem bedeutete dies, dass für ein homogenes Viruslysat weniger Virusplaquereinigungen notwendig waren. Bei optimalem Titrationsverlauf und dem mikroskopischen Nachweis der neuen Rekombinanten sollte ein einzelner Virusplaque nach einer Titration isolierbar sein. Verunreinigungen mit parentalem Ausgangsvirus sollten in der darauffolgenden Titration dann vollständig entfernt werden können. Dass die fluoreszenzvermittelte Selektion bei der Herstellung neuer Rekombinanten zu Zeitersparnis führte, konnte auch für den virulenten ORFV Stamm IA82 gezeigt werden (176).

Die optimierte Methodik zur Herstellung und Selektion der ORFV-Rekombinanten wurde in folgender Veröffentlichung publiziert:

Generation and Selection of Orf Virus (ORFV) Recombinants
Rziha HJ, **Rohde J**, Amann R
Alejandro Brun (ed.), *Vaccine Technologies for Veterinary Viral Diseases: Methods and Protocols*, Methods in Molecular Biology, vol. 1349, DOI 10.1007/978-1-4939-3008-1_12, Springer Science+Business Media New York 2016

3.1.2 Übersicht der Herstellung nicht publizierter ORFV-Rekombinanten

Im Folgenden wird die Generierung einer Auswahl von verschiedenen ORFV-Rekombinanten beschrieben, die Gene von unterschiedlichen Viren mit veterinär-medizinischer Relevanz im ORFV Genom enthalten. Dafür wurde wie zuvor beschrieben der vegf-e Gen Locus des ORFV für die Insertion der Fremdgene genutzt, wobei deren Transkription durch den frühen vegf-e Promoter kontrolliert wird.

Das Gruppe A Bovine Rotavirus (BRV) ist weltweit ursächlich für Gastroenteritis bei Kälbern und mitverantwortlich für eine hohe neonatale Mortalitätsrate (177). Erhöhte Morbiditätsraten, geringere Wachstumsraten und daraus folgende hohe Behandlungskosten und wirtschaftliche Verluste, welche durch diese Virusinfektion verursacht werden, spiegeln die Notwendigkeit von effektiven Vakzinen wieder. Die humane Rotavirus Infektion führt bei Kleinkindern zu Durchfallerkrankungen, die vor allem in Entwicklungsländern mit einer hohen Sterblichkeitsrate einhergehen [Übersichtsartikel (178)]. Das BRV könnte als Modellvirus die Grundlage für die Entwicklung von Impfstoffen gegen das humane Rotavirus darstellen (179). Das Rotavirus wird genetisch in sieben unterschiedliche Gruppen von A-G eingeteilt, wobei die Grundlage hierfür die beiden immunogenen Oberflächenproteine VP4 und VP7 sind [Übersichtsartikel (180)]. Das ikodsaedrische, nicht umhüllte dsRNA Virus gehört zur Familie der *Reoviridae* und das Genom kodiert jeweils sechs Struktur- und Nichtstrukturporteine [Übersichtsartikel (178)]. Gegen die beiden äußeren Kapsidproteine VP4 und VP7 können unabhängig voneinander neutralisierende Antikörper gebildet werden. Zudem wird VP4 durch Trypsin in die Proteine VP8 und VP5 gespalten, was für die Infektiosität des Virus notwendig ist (181, 182). Die mittlere Schicht des Viruspartikels besteht aus dem Strukturprotein VP6, welches ebenso immunogen wirkt, aber keine neutralisierenden Antikörper induzieren kann. Eine vielversprechende Impfstrategie besteht darin, durch Impfstoffe rotavirus-spezifische Antikörper zu induzieren, welche von trächtigen Kühen *in utero* auf ihre ungeborenen sowie postnatal an die neugeborenen Kälber über die Milch übertragen werden (183). Eine Vakzinierung gegen BRV kann generell die Morbidität, die Diarrhoe bedingte Mortalität sowie die Verbreitung von Rotaviren durch infizierte Kälber verringern [Übersichtsartikel (184)]. Außerdem sind Antikörper gegen VP6 kreuzreaktiv gegen Gruppe A Rotaviren und somit könnte diese Vakzinierung einen heterologen Schutz bewirken (185). Auf Grundlage der beschriebenen immunogenen Eigenschaften der BRV Proteine, wurden die entsprechenden Gene zur Insertion in das ORFV ausgewählt (Tabelle 3.1.2). Die protektiven Fähigkeiten der neu hergestellten Rekombinanten gegen BRV konnten bisher nicht im Tierversuch getestet werden.

Neben ORFV-Rekombinanten, welche Antigene des BRV exprimieren, wurden auch Rekombinanten mit Fremdgenen vom Blauzungenvirus (BTV) des Serotyps 8

generiert. BTV ist eine weitere Gattung (*Orbivirus*) der *Reoviridae* und verursacht bei Wiederkäuern die Blauzungenkrankheit. Diese Erkrankung betrifft vor allem Schafe und Rinder und zeichnet sich bei schweren Krankheitsverläufen durch hämorrhagische und nekrotische Läsionen des oberen Gastrointestinaltrakts, Ödeme und hämorrhagische Lymphknoten aus [Übersichtsartikel (186)]. Übertragen wird das Virus durch Vektoren der Stechmückengattung *Culicoides* [Übersichtsartikel (186)]. BTV breitete sich in den letzten Jahren geographisch über weite Teile der Erde aus, vor allem auch in Nordeuropa, wo BTV zuvor nicht nachgewiesen wurde (187). Das segmentierte Genom kodiert sieben Strukturproteine (VP1 bis VP7), wobei die äußeren Kapsidproteine VP2 und VP5 verantwortlich für Zelladhäsion und Zelleintritt des Viruspartikels sind. Es wird VP2 zur Charakterisierung der bisher 26 identifizierten Serotypen von BTV herangezogen, da eine Korrelation von Serumneutralisationstests (serotypspezifisch) und der unterschiedlichen Aminosäurensequenzen des VP2 besteht (188). Das Protein VP2 ist hauptverantwortlich für die Aktivierung von neutralisierenden Antikörpern [Übersichtsartikel (189)]. Der Serotyp 8 des BTV wurde als erster Stamm identifiziert, der sich 2006 vom Mittelmeerraum bis nach Nordeuropa ausbreitete [Übersichtsartikel (186)]. Um eine Ausbreitung dieses Virus zu verhindern wurden inaktivierte Impfstoffe verwendet, die den Vorteil einer höheren biologischen Sicherheit gegenüber lebend attenuierten Impfstoffen besitzen [Übersichtsartikel (190)]. Diese sind jedoch teurer in der Produktion und zudem sind mehrere Wiederholungsimpfungen notwendig, um einen ausreichenden Schutz zu induzieren [Übersichtsartikel (190)]. Ein weiterer wichtiger Bestandteil für die Herstellung einer erfolgreichen Vakzine scheint VP7 zu sein. Es konnten in der Maus T-Zell Epitope des VP7 identifiziert werden, welche auch im Schaf erkannt wurden (191). Des Weiteren wurde beschrieben, dass CTL auch gegen Nichtstrukturproteine wie NS1 gerichtet sind (192). Sowohl virusneutralisierende Antikörper wie auch CTL sind notwendig beim Aufbau einer protektiven Immunität gegen BTV (193, 194).

Weitere ORFV-Rekombinanten wurden mit Fremdgenen des Erregers der Afrikanischen Pferdepest (AHSV), das ebenfalls der Gattung *Orbivirus* zugehörig ist, hergestellt. Der vorrangige Zweck war die Induktion einer auf CTL beruhenden Immunantwort in Pferden. AHSV verursacht die Afrikanische Pferdepest bei Maultieren, Eseln und Pferden wie auch bei Zebras, hier jedoch mit milderen

klinischen Symptomen [Übersichtsartikel (195)]. Die Erkrankung zeichnet sich durch Fieber, Appetitlosigkeit und Läsionen aus und zudem sind respiratorische sowie vaskuläre Funktionen der Lunge durch die Bildung von Ödemen beeinträchtigt [Übersichtsartikel (196)]. Das AHSV ist endemisch im tropischen Afrika, zudem kommt es sporadisch in Teilen Nordafrikas und dem Mittleren Osten vor und wurde außerdem auf der Iberischen Halbinsel nachgewiesen (197). Aktuell gibt es die Besorgnis, dass sich dieses Virus durch *Culicoides* Arten weiter innerhalb Europas ausbreiten könnte. Das AHSV Genom kodiert die Strukturproteine (VP1-VP7) wie auch Nichtstrukturproteine (NS1-NS3) und es konnten bisher neun verschiedene Serogruppen für das Virus identifiziert werden (197). Die Proteine VP5 und VP7 befinden sich auf der Oberfläche des Viruspartikels, beziehungsweise des Kapsids und weisen die höchste Variabilität von AHSV auf. Da es gegen die Afrikanische Pferdepest aktuell keine Behandlungsmöglichkeit gibt, stellen effektive Impfstrategien die vielversprechendsten Ansätze zur Bekämpfung der Krankheit dar. Lebend attenuierte AHSV-Impfstoffe sind kommerziell verfügbar und werden beispielsweise in Südafrika gegen die meisten Serotypen eingesetzt [Übersichtsartikel (198)]. Problematisch anzusehen ist jedoch, dass die Möglichkeit einer Vermischung von Genen zwischen dem lebend attenuierten Impfstamm und dem Wildtyp Virus besteht und so neue, gefährlichere Varianten entstehen können. Ansätze zur Entwicklung von neuartigen Impfstoffen gibt es derzeit im Bereich von inaktivierten Impfstoffen, DNA- sowie Vektor-Vakzinen (197). Auch die Entwicklung von ‚core-like' Partikeln, die aus den Kapsid Proteinen VP3 und VP7 bestehen, können die Grundlage für neue Impfstoffe gegen AHSV sein (199). Zudem wird durch die Technik ‚reverse genetics' und dem darauf beruhendem spezifischen Genaustausch von VP2 und der Deletion von NS3/NS3a, die Herstellung von Vakzinen gegen alle Serotypen von AHSV ermöglicht (200).

Ein weiteres Ziel für neue ORFV Vektor-Vakzinen war die Maul- und Klauenseuche (MKS). MKS ist eine hochansteckende Erkrankung bei Paarhufern wie Rindern, Büffeln, Schweinen, Schafen und Wildtieren. Der Erreger ist das Maul-und-Klauenseuche-Virus (FMDV), ein ssRNA Virus der Familie *Picornaviridae* [Übersichtsartikel (201)]. Das FMDV kommt so gut wie überall auf der Welt vor, ist aber in den wirtschaftlich höher entwickelten Ländern weitestgehend ausgerottet [Übersichtsartikel (202, 203)] (204). Eine Infektion kann in der Landwirtschaft zu

beträchtlichen ökonomischen Verlusten führen. Mit dem Virus infizierte Tiere entwickeln Fieber sowie Aphten im Bereich der Mundschleimhaut und Klauen [Übersichtsartikel (205, 206)]. Die dominanten Immunogene sind die viralen Oberflächenproteinen VP1, VP2 und VP3. Ein weiteres immunogenes Strukturprotein (VP4) befindet sich dagegen nicht auf der Oberfläche des Viruspartikels. Diese Proteine entstehen jedoch erst durch die Prozessierung des vorläufigen Kapsidproteins (P1-2A) durch die FMDV kodierte 3C Protease zu den Nachfolger-Proteinen VP0, VP3 sowie VP1 [Übersichtsartikel (201)]. Nachfolgend wird das VP0 im Verlauf der Auflösung des Kapsids zu VP4 und VP2 gespalten [Übersichtsartikel (201)]. Dies stellt auch die Entwicklung von rekombinanten Vektor-Impfstoffen vor Schwierigkeiten, da gewährleistet werden muss, dass die 3C Protease korrekt und in ausreichender Menge exprimiert wird. Bei der Herstellung der neuen ORFV-Rekombinanten wurde die Expression der Nachfolger-Proteine dadurch gewährleistet, dass die 3C Protease direkt an das Kapsidprotein fusioniert wurde. Die Kontrolle der Expression der gesamten Genkassette erfolgte auch hier durch den vegf-e Promoter von ORFV. Von FMDV existieren sieben serologisch unterschiedliche Subtypen wie zum Beispiel die Serotypen O, A und Asia1 [Übersichtsartikel (207)] (208). Existierende inaktivierte Impfstoffe haben den Nachteil, dass sie nur unzureichend gegen die spezifischen Serotypen des FMDV schützen [Übersichtsartikel (209)]. Des Weiteren gibt es Untersuchungen zu Peptid-, DNA-, lebend attenuierten- und anderen Vektor-Vakzinen [Übersichtsartikel (209, 210)]. Beispielsweise induzierte eine Vektor-Vakzine, basierend auf dem humanen und replikationsdefizienten Adenovirus, in Rindern und Schweinen einen kompletten Schutz gegen FMDV (211). Vielversprechende Ergebnisse wurden auch für eine Peptid-Vakzine beschrieben, welche in Form von Dendrimeren die Induktion von FMDV neutralisierenden Antikörpern eine spezifische T-Zell Antwort und einen Schutz in Schweinen bewirkte (212).

Die Tabelle 3.1.2. gibt einen Überblick der generierten ORFV-Rekombinanten und zeigt, welche Gene der beschriebenen Erreger in das ORFV Genom erfolgreich inseriert wurden.

Tabelle 3.1.2. Generierte ORFV-Rekombinanten

Virus	Inseriertes Gen	Nachweis Gen/Protein	ORFV-Rekombinante	Parentales Virus
Bovines Rotavirus[a]	VP4	+/+	D1701-V-BR-VP4	D1701-VrV
Bovines Rotavirus	VP5	+/+	D1701-V-BR-VP5	D1701-VrV
Bovines Rotavirus	VP6	+/+	D1701-V-BR-VP6	D1701-VrV
Bovines Rotavirus	VP7	+/+	D1701-V-BR-VP7	D1701-VrV
BTV-8[b]	VP2	+/+	D1701-V-BTV8-VP2	D1701-V-AcGFP
BTV-8	VP7	+/+	D1701-V-BTV8-VP7	D1701-V-AcGFP
BTV-8	NS1	+/ng[e]	D1701-V-BTV8-NS1	D1701-V-AcGFP
AHSV-9[c]	VP2	+/+	D1701-V-AH9-VP2	D1701-V-AcGFP
AHSV-9	VP5	+/+	D1701-V-AH9-VP5	D1701-V-AcGFP
AHSV-9	VP7	+/ng	D1701-V-AH9-VP7	D1701-V-AcGFP
AHSV-9	NS1	ng/ng	D1701-V-AH9-NS1	D1701-V-AcGFP
FMDV-O[d]	2A	+/+	D1701-V-FMD-O-2A	D1701-V-AcGFP
FMDV-O	2A+2B	+/+	D1701-V-FMD-O-2B	D1701-V-AcGFP
FMDV-A	2A	+/ng	D1701-V-FMD-A-2A	D1701-V-AcGFP
FMDV-A	2A+2B	+/ng	D1701-V-FMD-A-2B	D1701-V-AcGFP
FMDV-Asia1	2A	ng/ng	D1701-V-FMD-AS1-2A	D1701-V-AcGFP

[a]In Zusammenarbeit mit T. Vahlenkamp, Universität Leipzig
[b]In Zusammenarbeit mit C. Pinoni et al., IZSAM, Teramo, Italien und M. Cabana et al., Pfizer, Olot, Spanien
[c]In Zusammenarbeit mit M. Cabana et al., Pfizer, Olot, Spanien
[d]In Zusammenarbeit mit M. Hosamani et al., Indian Veterinary Research Institute (IVRI), Bangalore, Indien
[e]nicht-getestet

Die neu hergestellten Rekombinanten wurden durch verschiedene Nachweismethoden auf ihre Fremdgenexpression untersucht. Auf genomischer Ebene wurden die in das ORFV Genom eingefügten Gene durch PCR Nachweise sowie Analysen mittels Restriktionsenzymen und Northern Blot charakterisiert. Der Nachweis der Proteinexpression erfolgte durch Methoden wie Western Blot, Immunfluoreszenz oder IPMA.

Für die Charakterisierung der einzelnen Rekombinanten ist exemplarisch in Abbildung 3.1.2.1. die Expression des VP2 in D1701-V-BTV8-VP2 infizierten Vero

Zellen mittels Western Blot (168) nach 4, 8, 12, 24 und 33 Stunden nachgewiesen. Nach Auftrennung durch SDS-PAGE und Western Blot Analyse mit einem spezifischen Kaninchen-Antiserum gegen BTV-8 konnte bereits nach 12 sowie nach 24 und 33 Stunden nach Virusinfektion das etwa 111 kDa große VP2 Protein des BTV-8 nachgewiesen werden, dessen Spezifität in der positiven Kontrolle (pk, infizierte Vero Zellen) bestätigt wurde. Die negativen Kontrollen, wie nicht-infizierte Zellen (ni) und mit parentalem D1701-V infizierte Vero Zellen (V) blieben negativ (Abb. 3.1.2.1. Spuren ni und V).

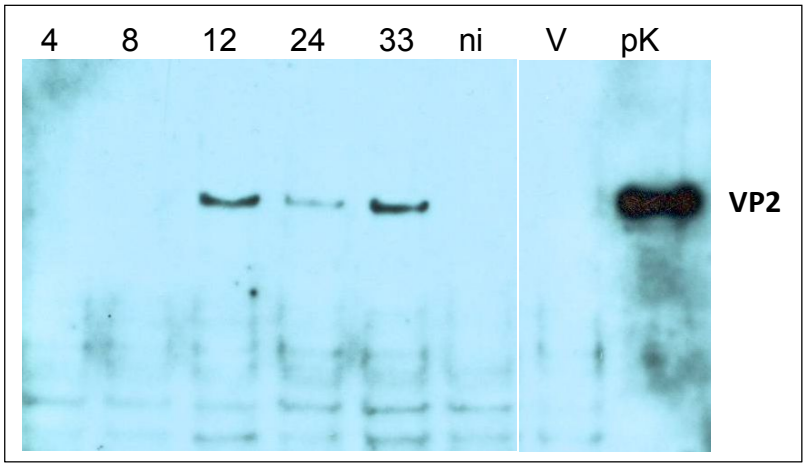

Abbildung 3.1.2.1. VP2 Nachweis des D1701-V-BTV8-VP2 durch Western Blot
Proteinlysate wurden nach 4, 8, 24 und 33 Stunden aus D1701-V-BTV8-VP2 infizierten (moi 3) Vero Zellen (etwa $2x10^6$ Zellen/Lysat) hergestellt. Als Kontrollen dienten nicht-infizierte Zellen (ni), Lysate infiziert mit D1701-V (V) sowie eine positive Kontrolle (pK) für den VP2 Nachweis. Die Proben wurden durch eine SDS-PAGE (Natriumdodecylsulfat-Polyacrylamid-gelelektrophorese) aufgetrennt und auf eine PVDF (Polyvinylidenfluorid) Membran übertragen. Die VP2 Detektion erfolgte mit spezifischem Kaninchen-Antiserum und war 12 Stunden nach Virusinfektion nachweisbar.

Als weiteres Beispiel einer Detektion der Fremdgenexpression von ORFV-Rekombinanten ist in Abbildung 3.1.2.2. der Nachweis des AHSV-9 Protein VP5 durch IPMA (169) beschrieben. Nach der Titration von D1701-V-AH9-VP5 in Vero Zellen wurden diese Zellen vier Tage (sichtbarer CPE) später fixiert und mit einem VP5 spezifischen monoklonalen Antikörper (10AE12, Ingenasa, Spanien) in einer 1:30 Verdünnung markiert. Die braun-rötliche Färbung im Bereich der Plaquebildung

weist VP5 in infizierten (Abb. 3.1.2.2. A) aber nicht in nicht-infizierten Zellen (Abb. 3.1.2.2 B) nach.

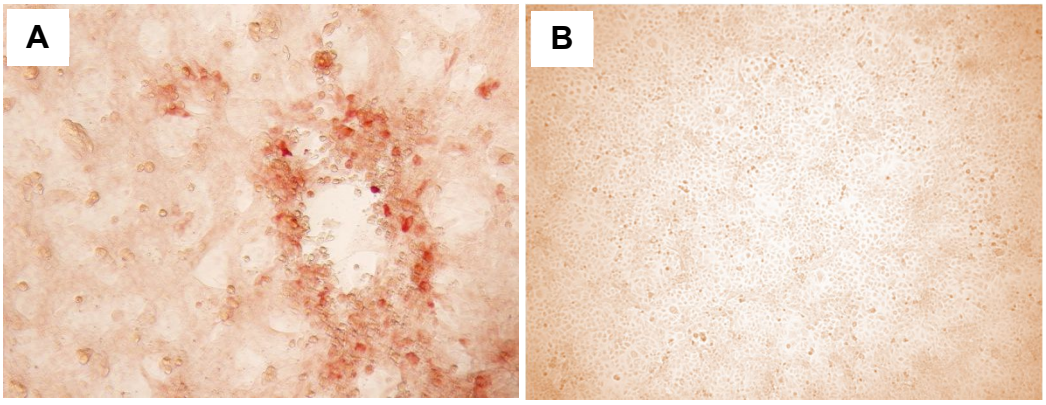

Abbildung 3.1.2.2. VP5 Nachweis von D1701-V-AH9-VP5 mit IPMA
D1701-V-AH9-VP5 infizierte Vero Zellen wurden vier Tage später mit Methanol fixiert und die Expression von VP5 (A) wurde mittels IPMA in D1701-V-AH9-VP5 infizierten Vero Zellen mit einem VP5 spezifischen monoklonalen Antikörper (Verdünnung 1:30) als primärer Antikörper sowie einem Peroxidase-markierten sekundären Antikörper nachgewiesen. Nicht-infizierte Zellen (B) dienten als Kontrolle und zeigten keine spezifische rot-braune Färbung. 10-fache mikroskopische Vergrößerung.

Rekombinanten, bei denen die Fremdgenexpression sowie die korrekte Insertion in das Genom bestätigt werden konnten, wurden zur weiteren Viruspropagierung verwendet. Immunisierungsstudien mit den beschriebenen ORFV-Rekombinanten wurden mit den in Tabelle 3.1.2. erwähnten Kollaborationspartnern geplant und teilweise durchgeführt.

3.1.3 Publizierte ORFV-Rekombinanten

Kernarbeiten der vorliegenden kumulativen Dissertation sind zwei Arbeiten, bei denen der attenuierte ORFV Stamm D1701-V zur Generierung von neuen viralen Rekombinanten diente, deren äußerst protektiven Eigenschaften als Vakzine gegen Infektionen mit RHDV, HPAIV H5N1 sowie H1N1 gezeigt werden konnte.

A new recombinant Orf virus (ORFV, Parapoxvirus) protects rabbits against lethal infection with rabbit hemorrhagic disease virus (RHDV)
Rohde J, Schirrmeier H, Granzow H, Rziha HJ; Vaccine 29 (2011) 9256-9264

Die hämorrhagische Krankheit der Kaninchen (RHD) ist eine weltweit auftretende, tödliche und hochansteckende Viruserkrankung. RHD ist verantwortlich für hohe Tierverluste in der Kaninchenzucht und ist in Europa sowie in Ostasien endemisch. Ursächlich für die Erkrankung ist das RHDV, welches ein nicht umhülltes ikosaedrisches (+)ssRNA Virus ist und zu der Gattung der Lagoviren aus der Familie der *Caliciviridae* gehört. Bestehende kommerzielle Vakzinen gegen RHDV sind inaktivierte Virusisolate, welche aus der Leber RHDV-infizierter Kaninchen gewonnen werden, da RHDV nicht *in vitro* in kultivierten Zellen propagiert werden kann. Es wird angestrebt, Vakzinen zu entwickeln, die die Notwendigkeit einer artifiziellen Infektion von Wirtstieren mit RHDV umgehen, die dem Tierschutz entsprechen und speziell generierte Totimpfstoffe aus infizierten Kaninchen ersetzen können.

Das Kapsidprotein VP1 ist das immundominante Oberflächenprotein des RHDV und wurde in den ORFV Vektor eingefügt, um die neue ORFV Rekombinante D1701-V-VP1 zu generieren. Dafür wurden zuvor für das Pockenvirus spezifische, transkriptionsunterbrechende Stoppmotive (T5NT) aus dem VP1 Gen entfernt ohne dabei die Aminosäurensequenz zu verändern (117). Verschiedene Expressionsanalysen wie IPMA, Immunfluoreszenz und Western Blot bestätigten die korrekte Expression des inserierten Gens VP1. Untersuchungen mittels Immunfluoreszenz ergaben eine ausgeprägte punktförmige Verteilung von VP1 im Zytoplasma von D1701-V-VP1 infizierten Zellen. Zudem konnten in dieser Studie durch Elektronenmikroskopie die Strukturen von akkumulierten VP1 detektiert werden und es gab deutliche Hinweise auf die Entstehung von ‚virus-like particles'. Die beobachtete Proteinakkumulation ist auch charakteristisch für die frühe Gensynthese in „Virus-Fabriken" des VV I3L Gens (213, 214).

Die neu generierte ORFV-Rekombinante wurde in Kaninchen auf ihre Fähigkeit untersucht, eine protektive Immunantwort zu induzieren. Es zeigte sich, dass bereits nach einer i.m. Immunisierung mit einer Dosis zwischen 10^5 – 10^7 pfu von D1701-V-VP1 alle Tiere die letale Belastungsinfektion mit RHDV (10.000x LD50) ohne Anzeichen einer klinischen Symptomatik wie Läsionen, Fieber oder anderen nachteiligen Nebenwirkungen überlebten. Bereits die niedrigste getestete Dosis (10^5

pfu) war bei einer einmaligen Immunisierung ausreichend, um alle Kaninchen vor der letalen RHDV Infektion zu schützen. Im Vergleich benötigen andere VP1 exprimierende Virus-Rekombinanten auf der Basis des VV und Kanarienpockenvirus Impfdosen von 10^7 – 10^9 pfu nach subkutaner, intradermaler oder oraler Applikation, um die Kaninchen erfolgreich zu schützen (215, 216). Weiterführende Untersuchungen mit der ORFV-Rekombinanten könnten aufklären, ob alternative Immunisierungsrouten höhere Vakzinierungsdosen benötigen. Eine Woche nach den Belastungsinfektionen war kein RHDV in den mit D1701-V-VP1 immunisierten Kaninchen mehr nachweisbar.

Abhängig von der Dosis und der Anzahl der Immunisierungen mit D1701-V-VP1 führte dies zur Induktion von spezifischen RHDV Antikörpern. Alle Kaninchen, die zweimal oder dreimal vakziniert wurden, erfuhren Serokonversion am Tag der Belastungsinfektion. Dies galt für alle getesteten Dosen. Einmalige Immunisierungen mit höheren Dosen (10^6 und 10^7 pfu) führten ebenfalls zur Serokonversion. Nur Kaninchen, die einmalig 10^5 pfu erhielten, zeigten dies nicht, waren aber ebenso vollständig gegen die RHDV Belastungsinfektion geschützt. Dieses Fehlen von spezifischen RHDV Antikörpern in geschützten Kaninchen ist außergewöhnlich, da ein Schutz gegen RHDV im Allgemeinen mit Serumantikörpern gegen das VP1 assoziiert ist (217-219). Jedoch beschrieb eine Studie mit einem rekombinanten Kanarienpockenvirus, welches ebenso das VP1 Gen des RHDV exprimiert, dass es auch hier keinen deutlichen Zusammenhang zwischen induziertem Schutz und der Präsenz von spezifischen RHDV Antikörpern gab (215). Ob das gemessene IL-2 im Serum von ORFV immunisierten Kaninchen im direkten Zusammenhang mit einer Beteiligung der zellulären Immunantwort steht und ob die Induktion von IL-2 abhängig von VP1 ist oder von dem Virus induziert wird, müssen weitere Analysen des Vektors zeigen. Der inaktivierte kommerzielle Impfstoff RIKA-VACC war nicht in der Lage IL-2 zu induzieren, allerdings ist für humanes Calicivirus eine TH-1 Beteiligung für den Aufbau eines Schutzes beschrieben (220). Aktivierte CD4-positive T-Zellen sind Produzenten von IL-2, welches wiederum zu Proliferation und Aktivierung von CD8-positiven CTLs führt. Es ist bekannt, dass ORFV in der Lage ist eine ausgeprägte TH-1 Antwort hervorzurufen [Übersichtsartikel (124)] (221).

Die neu generierte ORFV-Rekombinante ist eine sowohl hoch protektive als auch sichere Vakzine und geeignet die aus infizierten Kaninchenleber hergestellten inaktivierten Impfstoffe zu ersetzen.

New Orf Virus (Parapoxvirus) Recombinant Expressing H5 Hemagglutinin Protects Mice against H5N1 and H1N1 Influenza A Virus
Rohde J, Amann R, Rziha HJ; PLoS ONE 8 (12) (2013): e83802

Infektionen mit hoch-pathogenen aviären Influenzaviren (HPAIV) H5N1 weisen Mortalitätsraten um die 60% für Menschen auf [WHO (222)]. Wie alle Influenza A Viren unterliegt auch H5N1 ständiger evolutionär bedingter Veränderung. Die kurze Generationszeit und die hohe Mutationsrate während der RNA Replikation von Influenzaviren fördern die genetische Veränderung. Dieser als Gendrift beschriebene Vorgang kann durch die Vermischung des segmentierten Genoms von unterschiedlichen Influenza A Viren (Genshift) zusätzlich verstärkt werden (223). Eine Übertragung von Mensch zu Mensch wurde bisher für H5N1 nicht nachgewiesen, könnte aber durch Adaption an den Menschen und hierdurch entstehende Mutationen erfolgen. Ein potentielles Ziel solcher Veränderungen ist das HA der Influenzaviren, das an den Sialinsäurerezeptor der Wirtszelle bindet. Das AIV bindet vorzugsweise an den α-2,3-Sialinsäurerezeptor, wohingegen humane Influenzaviren an den α-2,6-Sialinsäurerezeptor binden, was das Wirtsspektrum bedingt. Nur ein oder zwei Mutationen im Genom könnten AIV an den α-2,6-Sialinsäurerezeptor binden lassen (224). Das Influenza Virus gehört zur Familie der *Orthomyxoviridae* und ist ein behülltes (-)ssRNA Virus mit segmentiertem Genom. Aufgrund der hohen Dynamik, der Influenzaviren durch Antigendrift wie auch Antigenshift unterliegen, entstehen immer neue Influenza Varianten. Daher ist das weithin angestrebte Ziel, Impfstoffe zu entwickeln, die gegen eine Vielzahl von neuartigen Varianten und Stämmen protektive Eigenschaften besitzen.

Dementsprechend wurden zwei neue ORFV-Rekombinanten entwickelt und in Infektionsstudien in der Maus auf ihre protektiven Eigenschaften gegen das Influenza A Virus untersucht. D1701-V-HAh5n exprimiert das HA und D1701-V-NPh5 das NP Gen des HPAIV Stammes H5N1 unter der Kontrolle des frühen ORFV vegf-e Promotors. Proteinexpressionsanalysen durch Western Blot und Immunfluoreszenz bestätigten die korrekte Expression des NP wie auch des HA und dessen

Vorläuferprotein HA0. Das inserierte HA Gen entstammt dem virulenten H5N1 Influenzavirus Stamm A/Vietnam/1203/2004, da dieser oftmals die Grundlage für erfolgreiche Immunität gegen H5N1 darstellte (225). Das NP unterliegt im Vergleich zu HA weniger genetischen Veränderungen und kann durch Induktion einer zellulären Immunantwort einen Schutz gegen Influenza hervorrufen (226, 227). In den vorgenommenen Studien mit letalen Infektionsdosen wies die NP exprimierende ORFV-Rekombinante jedoch keine protektiven Eigenschaften auf. Selbst die dreimalige Immunisierung mit D1701-V-NPh5 induzierte keinen Schutz gegen eine H5N1 Infektion. Hierbei wurde für die Belastungsinfektionen das Influenzavirus mit der entsprechenden Dosis (20x MLD50 für H5N1 und 20-50x MLD50 für H1N1) den Mäusen unter Betäubung in einem Volumen von 50 µl i.n. appliziert. Inwieweit eine ineffiziente Aktivierung von T- bzw. B-Zellen und somit auch das Fehlen spezifischer Antikörper für die mangelnde Protektion verantwortlich sind, bleibt zu klären. Es wurde beschrieben, dass beide Arten von Lymphozyten für eine NP bedingte Immunität gegen das Influenzavirus notwendig sind (228).

Abhängig von der Immunisierungsdosis induzierte die H5 HA exprimierende ORFV-Rekombinante in BALB/c und C57BL/6 Mäusen einen erfolgreichen Immunschutz gegen H5N1 Influenza Infektionen. Es zeigte sich, dass eine zweite i.m. Vakzinierung notwendig war, um eine Influenza induzierte Erkrankung zu vermeiden. Zweimalige Immunisierungen mit 10^6 oder 10^7 pfu von D1701-V-HAh5n führten zur kompletten Immunität gegen zwei verschiedene H5N1 Stämme (MB1, clade 2.2.1 und SN1, clade 2.2.3). Eine vergleichbare Schutzwirkung nach Kreuz-Infektionen mit den Stämmen 2.1.3, 2.2 und 2.3.4 konnte auch für eine H5 HA (Stamm 1) exprimierende MVA-Rekombinante gezeigt werden (229). Die Applikation von D1701-V-HAh5n vermittelte in BALB/c wie auch C57BL/6 Mäusen außerdem einen effektiven Schutz gegenüber dem heterologen Influenza A Stamm PR8 (H1N1). Die Übereinstimmung der Aminosäurensequenz von HA zwischen H5N1 und H1N1 liegt bei nur 66% und es bleibt zu klären, ob kreuzreaktive neutralisierende Antikörper oder spezifische CTL für den induzierten Immunschutz gegenüber diesem heterologen AIV verantwortlich sind. Generell werden neutralisierende Antikörper wie auch virusspezifische T-Zellen für einen erfolgreichen Schutz nach heterologen Influenzavirus Infektionen diskutiert (230, 231).

Für die nachweisbare Induktion von spezifischen H5 HA Antikörpern waren zwei Vakzinierungen mit D1701-V-HAh5n und einer Dosis von mindestens 10^6 pfu notwendig, wodurch die Tiere sowohl vor einer Erkrankung und somit auch vor der Mortalität nach einer AIV Infektion geschützt waren.

Eine Studie zeigte beispielsweise die Notwendigkeit von höheren (≥10^5 pfu) Immunisierungsdosen und mehrmaligen Vakzinierungen, um mit einer H5 HA (clade 1) exprimierenden MVA-Rekombinante Antikörper gegen ein genetisch unterschiedliches H5N1 Influenzavirus (clade 2.1) zu induzieren (232). Auch eine effiziente Antikörperantwort gegen ein homologes Virus benötigte höhere Dosen (≥10^5 pfu) (232). In unseren Versuchen waren Tiere, welche zweimal mit 10^5 pfu immunisiert wurden, zu 80% geschützt, obwohl diese Vakzinierungen nicht zu einer messbaren Freisetzung von Antikörpern führten. Es ist nicht auszuschließen, dass für diese protektive Immunität der Mäuse eine zusätzliche T-Zellaktivität oder die immunmodulatorischen Eigenschaften von ORFV eine unterstützende Rolle spielen [Übersichtsartikel (152)].

Um die Notwendigkeit der T-Zellen beim Aufbau eines Immunschutzes gegen H5N1 zu untersuchen, wurden CD4-positive und/oder CD8-positive T-Zellen mithilfe von Antikörpern in Mäusen depletiert. Das Entfernen der T-Zellen nach zwei Immunisierungen mit D1701-V-HAh5n und vor der AIV Infektion beeinträchtigte nicht die Immunität der Tiere. Es ist anzunehmen, dass nach zwei Immunisierungen ein ausreichender Schutz durch spezifische Antikörper besteht, um die Influenza Infektion zu kontrollieren. Das Fehlen von CD4-positiven T-Zellen oder beider T Zelltypen (CD4 und CD8) während der Immunisierungsphase führte zur Erkrankung der Tiere wie auch zu einer verringerten Überlebensrate. Vermutlich bleibt durch die Depletion von CD4-positiven T-Zellen die Reifung von B-Zellen aus und es kommt keine effektive Immunantwort durch spezifische Antikörper zustande (233). Die Versuche zeigten, dass vorrangig CD4-positive T-Zellen im Verlauf der Immunisierung notwendig waren, um einen Schutz zu induzieren, der nicht nur den Tod der Tiere, sondern auch jegliche Krankheitssymptomatik verhinderte. Über die Notwendigkeit von CD4-positiven T-Zellen und spezifischen Antikörpern zum Schutz vor H5N1 gegenüber der verminderten protektiven Rolle von CD8-positiven T-Zellen wurde berichtet (234).

Die neu generierte H5 HA exprimierende ORFV-Rekombinante zeichnete sich durch eine ausgezeichnete Schutzwirkung gegenüber Influenzavirus Infektionen mit unterschiedlicher H5N1 Stämmen wie auch gegen den heterologen H1N1 Stamm aus.

3.2 Fähigkeiten des ORFV die Immunantwort des Wirts zu modulieren

3.2.1 ORFV induzierte Immunantwort nach Expression von PRV Antigenen

Das Pseudorabiesvirus (PRV) gehört der Familie der *Herpesviridae* und der Unterfamilie *Alphaherpesvirinae* und dem Genus *Varicellovirus* an [Übersichtsartikel (235)]. Das neurotrope PRV verursacht die Aujeszkysche Krankheit, die zu substanziellen ökonomischen Verlusten in der Schweinezucht führen kann. Der natürliche Wirt sind Schweine, es können jedoch auch andere Säugetierarten wie Nagetiere mit dem Virus infiziert werden. Das Virus besitzt ein dsDNA Genom, besteht aus einem Kapsid, einem Tegument und einer Hüllmembran und zeichnet sich durch eine charakteristische Latenzphase im Lebenszyklus aus [Übersichtsartikel (236)]. Die Hauptfunktionen der 16 verschiedenen Membranproteine bestehen darin, dem Viruspartikel den Zelleintritt sowie Zellaustritt zu ermöglichen und die Virusverbreitung von Zelle zu Zelle zu gewährleisten [Übersichtsartikel (237)]. Dabei können manche virale Proteine auch modulierend auf die Immunantwort des Wirts einwirken [Übersichtsartikel (237)]. Beispielweise ist das PRV Protein UL 49.5 fähig die Funktion des Transporters der Antigenprozessierung (TAP) so zu stören, dass die Antigenpräsentation durch MHC I beeinträchtigt wird (238).

Das PRV ist in weiten Teilen Europas wie auch in Kanada, Neuseeland und in den Vereinigten Staaten von Amerika dank erfolgreicher Ausrottungsprogramme verschwunden. In Ländern, welche offiziell als PRV frei gelten, ist die Impfung gegen das Virus inzwischen verboten [Übersichtsartikel (239)]. Mitverantwortlich für die weit gehende Ausrottung von PRV ist der Impfstoff Bartha-K61. Dieser, durch Zellpassagen entstandene, attenuierte PRV Stamm wurde weltweit mehr als 30 Jahre lang für die Vakzinierung von Schweinen angewendet (240). Neue Untersuchungen in Schweinen zeigen jedoch, dass dieser PRV Impfstamm nur einen 50% Schutz gegen den neuen und virulenten PRV Stamm HeN1 induzierte (241). Somit sind neuartige Impfstoffe von weiterem Interesse, um diese bei einem

potentiellen Wiederauftreten der Seuche oder deren neuen PRV Varianten einsetzen zu können.

Zwei ORFV-Rekombinanten, welche die Glykoproteine gC (D1701-V-gC) beziehungsweise gD (D1701-V-gD) des PRV exprimieren, zeigten sehr gute protektive Eigenschaften sowohl in der Maus wie auch im natürlichen Wirt, dem Schwein (1, 164, 167). Die Oberflächenproteine gC und gD sind sehr effizient, um virusneutralisierende Antikörper zu induzieren [Übersichtsartikel (237)]. Zudem sind diese Glykoproteine auch fähig eine zelluläre Immunantwort gegen PRV auszulösen (242-245). Um die protektiven, immunologischen Eigenschaften dieser ORFV-Rekombinanten hinsichtlich der Induktion der angeborenen wie auch der humoralen Immunantwort besser zu verstehen wurden verschiedene Untersuchungen vorgenommen. Da nach der Vakzinierung von verschiedenen Knock-out Mäusen (µMT Mausstamm, CD4, CD8 und Perforin) mit gC und gD exprimierenden ORFV-Rekombinanten die Tiere weiterhin gegen eine letale PRV Infektion geschützt waren (1), war es nun interessant aufzuklären, welche zusätzlichen immunologischen Mechanismen für den Schutz verantwortlich sein könnten. Beim µMT Mausstamm ist die B-Zellentwicklung gestört, da ein defektes Exon für die Transmembrandomäne des IgM Moleküls vorliegt und sie daher auf Stufe der Prä-B-Zellen verbleibt (246).

Um den Einfluss der ORFV-Rekombinanten auf die angeborene Immunantwort zu untersuchen wurde die Aktivität von NK mit Hilfe eines Zytotoxizität-Assay (Chromfreisetzungstest) (247, 248) sowohl für BALB/c wie auch für C57BL/6 Mäuse evaluiert. Hierbei wurden die Tiere einmalig mit D1701-V-gC, D1701-V-gD oder einer 1:1 Mischung aus beiden Rekombinanten immunisiert. Es zeigte sich, dass die Kombination beider Rekombinanten mit einer Gesamtdosis von 10^7 pfu bei C57BL/6 Mäuse bereits 15 Stunden nach i.m. oder i.p. Immunisierung eine ausgeprägte NK Antwort auslöste. Verglich man dies mit der NK-Aktivität von BALB/c Tieren, so zeigte sich nach i.m. wie auch i.p. Applikation von gC und/ oder gD exprimierenden Rekombinanten eine zeitlich etwas verzögerte, aber in ihrer Zytotoxizität vergleichbare Wirkung von NK. Abbildung 3.2.1.1. zeigt die NK-Aktivität in BALB/c Tieren 24 Stunden nach i.p. Immunisierung (10^7 pfu/ Tier) mit verschiedenen ORFV-Rekombinanten. Als Kontrollen dienten BALB/c Mäuse, welche mit 10^6 pfu des LCMV i.v. infiziert wurden und Tiere, die nicht-immunisiert wurden.

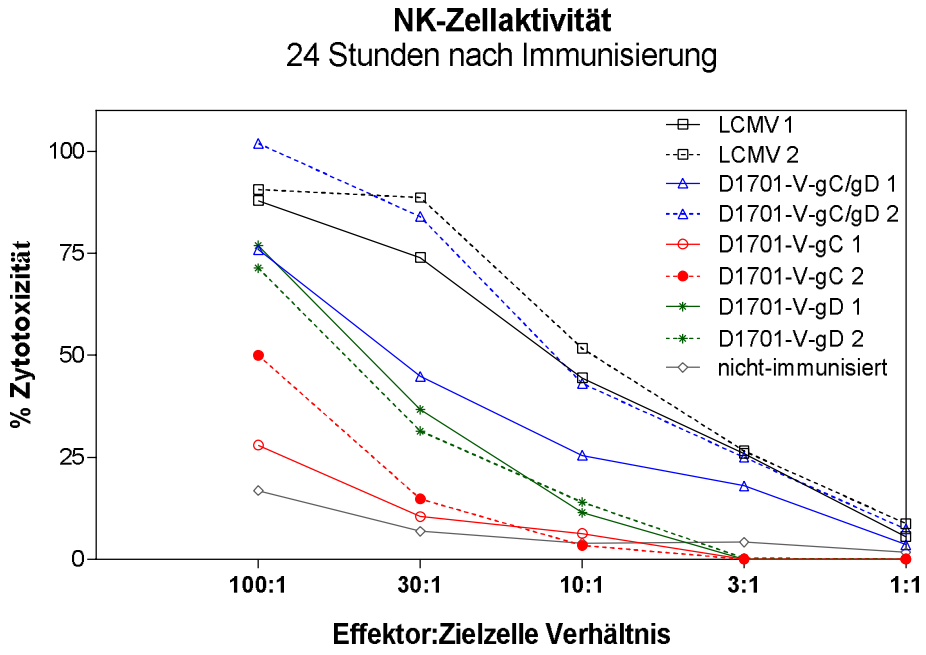

Abbildung 3.2.1.1. Aktivität von NK in BALB/c Mäusen nach Immunisierung mit ORFV-Rekombinanten
Die zytotoxische Aktivität von Splenozyten (Effektor) wurde mittels Chromfreisetzungstest von radioaktiv markierten YAC-1 Zellen (murine Lyomphoma Zellen; Zielzelle) gemessen. Jeweils zwei Tiere (1, 2) pro ORFV Gruppe wurden mit 10^7 pfu i.p. immunisiert. D1701-V-gC/gD bestand aus einer Mischung gleichen Anteils von gC und gD exprimierenden Rekombinanten. Die positiven Kontrollen wurden mit 10^6 pfu LCMV infiziert (i.v.). Spontane Chromfreisetzung 31,5%.

Die höchste Aktivität von NK wurde mit der Mischung aus D1701-V-gC und D1701-V-gD mit insgesamt 10^7 pfu erzielt. Hierbei erreicht die NK-Aktivität von Tier Nr. 2 in etwa dieselbe Intensität wie von Mäusen, welche mit LCMV infiziert waren (Abb.3.2.1.1.). Die induzierte NK-Aktivität durch die Rekombinante D1701-V-gD war etwa 2,5-fach (bei Effektor:Zielzelle Verhältnis 100:1 und 30:1) höher als durch D1701-V-gC (Abb.3.2.1.1.). Die NK-Induktion konnte auch bei BALB/c Mäusen gezeigt werden, die mit nicht-replikativen Baculovirus-Rekombinanten immunisiert wurden. Diese Rekombinanten exprimieren jeweils die Glykoproteine gB, gC und gD des PRV. Dies ist in folgender Publikation beschrieben:

New baculovirus recombinants expressing Pseudorabies virus (PRV) glycoproteins protect mice against lethal challenge infection
Grabowska AK, Lipińska AD, **Rohde J**, Szewczyk B, Bienkowska-Szewczyk K, Rziha HJ; Vaccine. 2009 Jun 2;27(27):3584-91

Für die Immunisierungsversuche wurde eine Mischung zu gleichen Teilen (1:1:1) aus allen drei Rekombinanten verwendet. Ob nun aber die ORFV- oder die Baculovirus-Vektoren eine stärkere NK Antwort vermitteln bleibt zu klären, da kein direkter

Vergleich vorgenommen werden konnte. Für einen kompletten Schutz durch die Baculovirus-Vektoren (Mischung von gB, gC und gD) waren drei Immunisierungen mit einer Dosis von 10^9 pfu notwendig. Im Gegensatz hierzu genügte bei PRV-Antigen exprimierenden ORFV-Rekombinanten (Mischung aus gC und gD, sowie gC alleine) eine einmalige Vakzinierung mit $7x10^6$ pfu um gegen eine PRV Infektion einen 100%igen Schutz zu induzieren (1). Zu späteren Zeitpunkten (48 und 72 Stunden) nach der Immunisierung mit den ORFV-Rekombinanten konnte keine erhöhte NK-Aktivität mehr nachgewiesen werden. In NK-Depletionsversuchen konnte jedoch kein direkter Einfluss der frühen NK Induktion auf die Überlebensrate nach einer PRV Belastungsinfektion gezeigt werden. Hierfür wurden C57BL/6 und B-Zell defekte µMT Mäuse vor und während der zweimaligen D1701-V-gC/-gD Immunisierung mit anti-NK [anti-asialo GM1 ;(249)] Antikörpern depletiert. Es ergaben sich für beide Mausstämme keine Veränderungen in der Überlebensrate und die Tiere waren immun gegen eine PRV Infektion unabhängig ob sie NK depletiert waren oder nicht. Die von ORFV hervorgerufene Induktion von NK konnte kürzlich ebenfalls in einem für das Virus nicht-permissiven Mausmodell gezeigt werden (157). Die hierbei beschriebene NK Aktivität ist außerdem von einer anti-tumoralen Wirkung des ORFV begleitet (157). Zudem ist beschrieben, dass inaktiviertes ORFV ebenso fähig ist eine verstärkte NK Antwort hervorzurufen (156, 250). Das ORFV hat durch die gesteigerte NK Antwort direkten Einfluss auf das angeborene Immunsystem. Jedoch hatten die daraufhin ausgelösten Mechanismen wie zum Beispiel die NK induzierte Zytokinsekretion keinen Einfluss auf die Immunität nach PRV Belastungsinfektionen, da auch NK depletierte Mäuse geschützt waren.

Um ihren jeweiligen Anteil an der induzierten Immunantwort zu evaluieren, wurden die T- und B-Lymphozyten in Blut, Milz und poplitealen Lymphknoten in Folge der Vakzinierung quantitativ analysiert. Dazu wurden BALB/c Mäuse mit der Kombination von D1701-V-gC/-gD immunisiert (10^7 pfu) und die entsprechenden Gewebe nach ein bis drei Tagen durchflusszytometrisch untersucht. Weder im Blut noch in der Milz konnten bei CD4-positiven sowie CD8-positiven T-Zellen signifikante quantitative Unterschiede zwischen ORFV immunisierten und nicht-immunisierten Tieren festgestellt werden. Bei Untersuchungen von Lymphozyten aus den poplitealen Lymphknoten konnten jedoch reproduzierbare Unterschiede festgestellt werden.

Popliteale Lymphknoten wurden untersucht, da diese dem i.m. Applikationsort am nächsten liegen. Drei Tage nach Immunisierung mit D1701-V-gC/-gD oder D1701-VrV waren CD4-positive T-Zellen (gemessen mit spezifischen anti-CD3 und anti-CD4 Antikörpern) um mehr als 10% reduziert im Vergleich zu Mäusen, die mit PBS behandelt wurden (Abb. 3.2.1.2.). Im Gegensatz hierzu war die Population von CD8-positiven T-Zellen (gemessen mit spezifischen anti-CD3 und anti-CD8 Antikörpern) in immunisierten Tieren verglichen zu den Kontrolltieren deutlich erhöht (Abb. 3.2.1.2.). Außerdem konnte gezeigt werden, dass auch 5-8% mehr B-Zellen (gemessen mit spezifischen B-Zell Antikörper B220/CD45R) vorlagen als bei PBS behandelten Tieren (Abb. 3.2.1.2.).

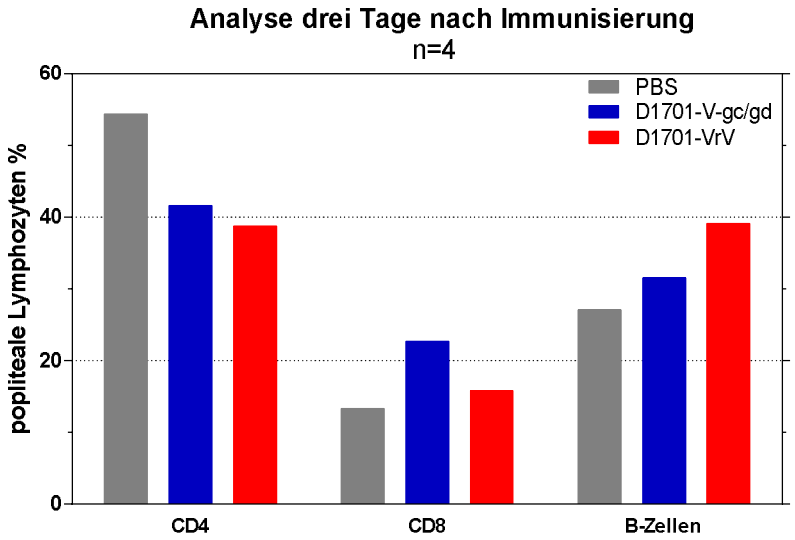

Abbildung 3.2.1.2. Durchflusszytometrische Messung von poplitealen Lymphknoten nach ORFV Immunisierung

Jeweils vier (n=4) BALB/c Mäuse wurden mit 10^7 pfu D1701-V-gC/gD oder D1701-VrV i.m. immunisiert und die Lymphozyten drei Tage später mit spezifischen T- und B-Zell Antikörpern analysiert. D1701-V-gC/gD bestand aus einer Mischung im Verhältnis 1:1 von gC und gD exprimierenden ORFV-Rekombinanten. Die Kontrolltiere wurden mit PBS behandelt. Dargestellt sind die Mittelwerte der gemessenen Proben.

Nach Immunisierungen mit dem parentalen Virus D1701-VrV konnten ähnliche Tendenzen beobachtet werden, wobei die erhöhte Menge der CD8-positiven T-Zellen geringer, die der B-Zellen stärker ausfiel als nach Vakzinierung mit den Rekombinanten D1701-V-gC/-gD (Abb. 3.2.1.2.). Die Induktion der B-Zellen lag nach Immunisierung mit parentalen und rekombinanten ORFV deutlich über der Menge mit PBS behandelten Tieren (Abb. 3.2.1.2.). Dies lässt darauf schließen, dass die ORFV bedingte Einflussnahme auf die Populationen von T- und B-Lymphozyten

unabhängig von den inserierten PRV Gene war. Die verminderte Menge an CD4-positiven T-Zellen gegenüber Tieren, die mit PBS immunisiert wurden, kann dadurch erklärt werden, dass bei ORFV vakzinierten Tieren vermutlich CD4-positiven T-Zellen aus dem Lymphknoten in dessen umliegende Bereiche einwandern um ihrer Funktion der T-Zell-Hilfe nachzukommen. Im weiteren Verlauf nach der ORFV Immunisierung könnten daraufhin CD8-positive T-Zellen rekrutiert werden, die sich aber in den vorgenommen Untersuchungen noch im Lymphknoten befanden und dann zu späteren, nicht gemessenen Zeitpunkten auswandern. Ob diese Vermutung zutreffend ist, müssten weitergehende Untersuchungen zeigen, um auch im speziellen die gemessene verringerte Menge an CD4-positiven T-Zellen nach ORFV Infektion weiter zu beleuchten. Die Spezifität und Funktionalität von CD4-positiven T-Zellen nach Kontakt mit Pockenviren ist weitgehend unklar, zumal die virusvermittelte CD4 T-Zell Antworten von sehr verwandten Pockenviren deutlich unterschiedlich sind (251). Im speziellen zeigte sich dies durch eine höhere Expression des lysosomalen Membranproteins CD107a in CD4-positiven T-Zellen nach Infektion von Mäusen mit ECTV im Vergleich zu Tieren die mit VV infiziert wurden (251). Zudem war bei diesen Versuchen VV, im Gegensatz zu ECTV, während der späten Phase der Bildung von Gedächtniszellen nicht in der Lage Virus-spezifische CD4-positive T-Zellen zu induzieren (251). Die Induktion von CD8-positiven T-Zellen passt zu den gemessenen erhöhten IFN-α Mengen (siehe unten), da Typ I Interferon dafür bekannt ist naive CD8-T-Zellen zu stimulieren (252). Die vorangegangenen Depletionsversuche zeigten jedoch, dass CD8 T-Zellen nicht essentiell für die protektive Immunität gegen PRV waren.

Die Fähigkeit von ORFV T-Zellen zu aktivieren zeigten auch Untersuchungen im Schaf, dem natürlichem Wirt von ORFV. Es wurde gezeigt, dass es nach einer Reinfektion mit ORFV zur Aktivierung von T-Zellen kommt und dies den Ursprung von Lymphokinen darstellt (253). Nicht direkt vergleichen lassen sich diese Ergebnisse mit jenen von inaktiviertem ORFV, sie zeigen aber das ORFV Lymphozyten stimulieren kann. Hierbei wurde *in vitro* die Fähigkeit von inaktiviertem ORFV beschrieben, die Proliferation speziell von CD4 T-Lymphozyten aus dem Hund zu aktivieren (154). Diese Ergebnisse zeigen das Potential von ORFV sehr früh nach Immunisierung T-Zellen wie auch B-Zellen anzuregen. Von zusätzlichem Interesse wäre zudem das intrazelluläre Zytokinexpressionsprofil der T-Lymphozyten nach

Immunisierung mit ORFV zu bestimmen, um weitere Aussagen über deren Funktionalität treffen zu können.

Weiterhin wurden im PRV Infektionsmodell Knock-out-Mäuse untersucht, welchen der TLR3 fehlte oder die den Interferon-α/β und γ Rezeptor (AG129 Mäuse) nicht aufwiesen. Die Tiere wurden wiederum zweimalig im Abstand von 14 Tagen mit der Kombination aus D1701-V-gC/-gD immunisiert (10^7 pfu/ Impfung) und 14 Tage später mit PRV (Stamm NIA-3) i.p. infiziert ($5x10^4$ pfu/Tier). Das Fehlen des TLR3 bedingte keine erhöhte Sterblichkeitsrate nach PRV Belastungsinfektion. Alle TLR3 Knock-out Tiere wie auch die Kontrolltiere überlebten die Virusinfektion. Jedoch zeigte sich, dass das Fehlen der Interferon-α/β und γ Rezeptoren massiven Einfluss auf die Überlebensrate der mit PRV infizierten Tiere hatte (Abb. 3.2.1.3.). Tiere denen die Interferon Rezeptoren fehlten und mit ORFV immunisiert waren, starben zwischen 74 bis 169 Stunden nach der Belastungsinfektion mit PRV oder wurden aufgrund von klinischen Symptomen getötet (Abb. 3.2.1.3.). Im Vergleich hierzu starben nicht-vakzinierte AG129 Mäuse etwa 12-24 Stunden früher und die Gruppe, welche die PRV Infektion nicht erhielt, überlebten zu 90% (Abb. 3.2.1.3.).

Ein Tier der nicht-infizierten Gruppe verstarb im Verlauf des Versuchs aus ungeklärten Gründen. Für die Induktion der Immunität durch D1701-V-gC/-gD gegen PRV sind die Interferon-α/β/γ Rezeptoren notwendig. Möglicherweise ist das Fehlen des Interferon-γ Rezeptors mitverantwortlich für das Sterben der Tiere nach PRV Infektion. Dies müssten weitere Versuche mit dem beschriebenen PRV Modell aufzeigen. Jedoch überlebten Interferon-α/β defekte Mäuse, welche mit MVA vakziniert und mit ECTV infiziert wurden, die Belastungsinfektion gleichermaßen wie Wildtypmäuse (254). Zudem hatte im gleichen Pockenvirus Mausmodell das Fehlen der Adaptermoleküle ($MyD88^{-/-}$ und $TRIF^{-/-}$) von allen TLR wie auch des Rezeptors von RIG-I keinen Einfluss auf die Überlebensrate der Tiere (255).

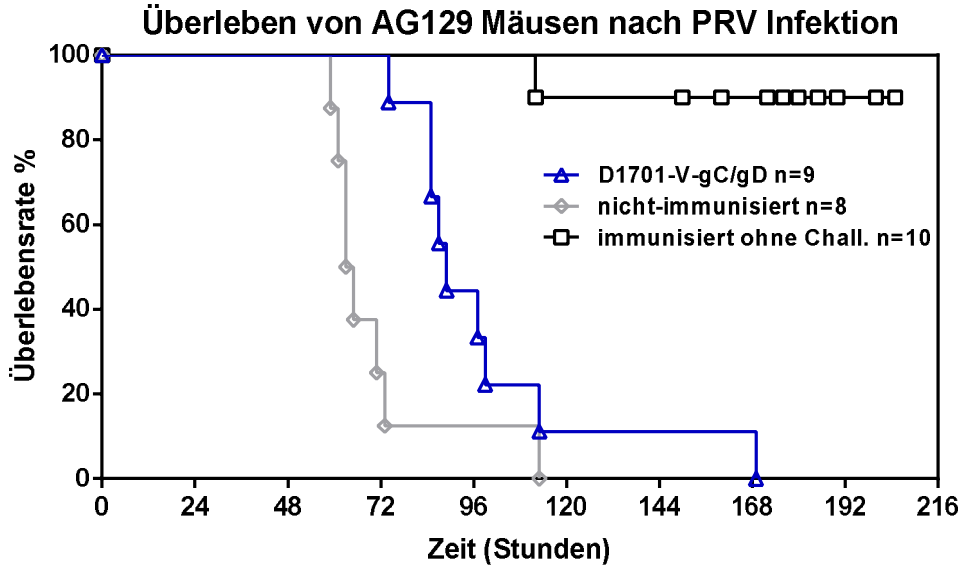

Abbildung 3.2.1.3. Überlebensrate von AG129 Mäusen nach D1701-V-gC/-gD Vakzinierung und letalen PRV Belastungsinfektion

Interferon-α/β/γ Rezeptor Knock-out Mäuse (AG129) wurden zweimal mit 10^7 pfu D1701-V-gC/-gD i.m. immunisiert und anschließend mit PRV i.p. infiziert ($5x10^4$ pfu/Tier) und danach die Überlebensrate dokumentiert. Als Kontrollen dienten AG129 Tiere, welche nicht-immunisiert oder nicht mit PRV infiziert (immunisiert ohne Chall.) wurden. n=Anzahl der Tiere.

Dagegen wurde beschrieben, dass für die MVA induzierte angeborene Immunantwort die Stoffwechselwege über TLR2, TLR6 und MyD88 eine wichtige Rolle hinsichtlich der Chemokin- sowie Zytokinproduktion von IFN-β und IL-1β übernehmen (256). Somit wäre es von Interesse, bei der Immunisierung mit ORFV die Bedeutung weiterer TLR in diesem ORFV-PRV Mausmodell zu untersuchen.

Um weitere immunrelevante Reaktionen nach D1701-V-gC/-gD und D1701-VrV Immunisierungen zu charakterisieren, wurden die Mengen verschiedener induzierter Zytokine und Interleukine (IL) im Serum von BALB/c Mäusen untersucht, die mit 10^7 pfu der ORFV-Rekombinanten vakziniert wurden. Diese Tests wurden mit Sandwich-ELISAs oder Multiplex-ELISAs durchgeführt. Exemplarisch hierfür sind in Tabelle 3.2.1. die Ergebnisse für die Zytokinexpression nach 8 und 24 Stunden der Immunisierung mit ORFV-Rekombinanten dargestellt. Es zeigte sich, dass sowohl nach 8 und 24 Stunden nach einer einmaligen Immunisierung eine 4-8-fach erhöhte Menge gegenüber PBS behandelten Mäusen von IL-1β, IL-6, TNF-α, G-CSF und eine etwa 2-fach erhöhte Menge an IFN-γ im Serum der Tiere gemessen wurde (Tabelle 3.2.1.). Der Unterschied zwischen den ORFV-Rekombinanten D1701-V-gC/-gD und D1701-VrV hinsichtlich der induzierten Zytokinmengen war hierbei gering, wie auch zwischen den beiden gemessenen Zeitwerten von 8 und 24 nach der

Immunisierung (Tabelle 3.2.1.). Diese Zytokine werden vor allem von mononukleären Phagozyten gebildet, wodurch die Ergebnisse auf eine pro-inflammatorische sowie auf eine TH-1 ausgerichtete Zytokinantwort hinweisen. Auch wurde die Expression von IFN-α (8-fach höher gegenüber PBS behandelten Tieren), IL-15 (4-8-fach höher) und IL-18 (4-8-fach höher) sowohl im Serum von D1701-V-gC/-gD wie auch D1701-VrV immunisierten Tiere nachgewiesen, was wiederrum auf eine pro-inflammatorische Reaktion hindeutet (Tabelle 3.2.1.). Die gemessene erhöhte Menge von IL-15 und IL-18 könnte auf die Stimulierung und Proliferation von NK hindeuten, die daraufhin TNF-α und IFN-γ exprimieren. Diese Ergebnisse korrelieren gut mit der zuvor beschriebenen gesteigerten NK-Aktivität in ORFV vakzinierten Tieren, da die erhöhte Menge dieser Zytokine auf die Aktivität von NK hinweisen. Hierbei ist zu erwähnen, dass in humanen Zellen des Bluts eine gesteigerte Induktion von pro-inflammatorischen sowie TH-1 relevanten Zytokinen, wie IFN-γ, TNF-α, IL-6, IL-8, IL-12 und IL-18 durch inaktiviertes ORFV beschrieben wurde (221).

Tabelle 3.2.1 Zytokinexpression im Serum von ORFV immunisierten Tieren

Immunisierung i.m 10^7 pfu / Tier	IFN-α	IL-1β	IL-6	IL-10	IL-12 p40	IL-13	IL-15	IL-18	G-CSF	IFN-γ	TNF-α
D1701-V-gC/-gD 8 hpi[a]	+++[d]	++[c]	+++	+			++	++	+++	+	++
D1701-V-gC/-gD 24 hpi		+[b]	++	+		+			+++	+	++
D1701-V-VrV 8 hpi	+++	+	+++		+		+++	+++		+	++
D1701-V-VrV 24 hpi		++	++	++		+		++	+++	+	++

Daten beruhen auf Mittelwerte aus 2 Mäusen/Gruppe
[a]Stunden nach Immunisierung
[b]+: 2-fache Erhöhung gegenüber mit PBS behandelten Tieren
[c]++: 4-fache Erhöhung gegenüber mit PBS behandelten Tieren
[d]+++: ≥ 8-fache Erhöhung gegenüber mit PBS behandelten Tieren

Zu späteren Zeitpunkten, sowohl 14 Tage nach erster als auch nach zweiter Immunisierung, blieb die pro-inflammatorische Antwort in abgeschwächter Form bestehen, da die gemessene Menge an Zytokinen im Serum von ORFV immunisierten Tieren abnahm. Jedoch konnte zudem die Induktion von TH-2 relevanten Zytokinen gemessen werden. Im Vergleich zu PBS behandelten Mäusen

lag eine deutliche Expressionssteigerung an IL-4, IL-5 und IL-13 im Serum von vakzinierten Mäusen vor.

Zudem konnte 14 Tage nach der ersten Immunisierung mit ORFV IL-10 detektiert werden. Dieses regulatorische Zytokin ist in der Lage die TH-1 Antwort zu unterdrücken und zugleich die Immunantwort hinsichtlich der Reifung von B-Zellen zu verstärken [Übersichtsartikel (257)]. Dies deutet auf eine Aktivierung von B-Zellen und hierdurch auf eine vermehrte Sekretion von Immunglobulinen im Blut von vakzinierten Tieren hin.

In vitro Untersuchungen von Überständen aus kultivierten Splenozyten, welche ebenso aus BALB/c Tieren stammten, die ebenfalls ein oder zweimal mit den ORFV-PRV-Rekombinanten oder D1701-VrV vakziniert wurden, zeigten 8 und 24 Stunden später, verglichen zu den beschriebenen Ergebnissen im Serum der Tiere ein sehr ähnliches Zytokinexpressionsmuster. Jedoch war hier der Unterschied zu nicht-immunisierten Tieren deutlich geringer im Vergleich zu Ergebnissen aus Serumproben, da die gesteigerte Induktion der Zytokine nur etwa 2-fach erhöht war.

Im Verlauf der Immunisierung mit ORFV-Rekombinanten, die PRV Antigene exprimieren, ergab sich ein Zytokinprofil mit dem Nachweis von IFN-γ, TNF-α, IL-4 und IL13, welches auf eine ausgeglichene TH1-TH2 Antwort in der für ORFV nicht-permissiven Maus hinweist. Diese Ergebnisse stützen die beschriebenen Ergebnisse einer ORFV-PRV-induzierten balancierten TH1-TH2 Immunantwort (1), welche auch für die Immunantwort einer ORFV-Rekombinanten gegen das Bornavirus vermutet wird, jedoch nicht beschrieben wurde (163). Das beschriebene Zytokinprofil galt nicht nur für die PRV Antigen exprimierenden Rekombinanten, sondern auch für den parentalen Virusstamm D1701-VrV der bei diesen Versuchen als zusätzliche Kontrollgruppe zu nicht-immunisierten Tieren diente.

Zusammengefasst deuten die Bestimmung der NK Aktivität und der inflammatorischen Zytokine darauf hin, dass ORFV sowohl Mechanismen der angeborenen wie auch der erworbenen Immunantwort induziert. Im Gegensatz hierzu war die Immunantwort nach Vakzinierung mit rekombinanten Baculoviren TH-1 von zellulären Komponenten dominiert. Die TH-2 relevanten Interleukine IL-4 und IL-10 konnten nicht nachgewiesen werden. Bei Untersuchungen mit inaktiviertem ORFV konnte eine virusinduzierte TH-1 dominierende Immunantwort festgestellt

werden (158). Aber auch das TH-2 relevante IL-4 war nach Infektion mit inaktiviertem ORFV von Relevanz für seine anti-inflammatorischen Funktionen (221).

3.2.2 Einfluss des ORFV auf den vesikulären Peptidtransport in Vero Zellen

Virale Infektionen werden unter anderem durch PRRs des infizierten Wirts registriert. Daraufhin beginnt zunächst das angeborene Immunsystem unspezifisch gegen das virale Pathogen durch die Aktivierung des Komplementsystems, Rekrutierung von Phagozyten wie Makrophagen und neutrophilen Granulozyten und die Aktivierung von NK vorzugehen und zudem die adaptive Immunabwehr einzuleiten. Pockenviren exprimieren verschiedene Gene, die es den Viren ermöglichen das Immunsystem der Wirte zu umgehen und sich somit erfolgreich zu vermehren. Unter anderem sind einige virale Proteine in der Lage Komplementfaktoren, Zytokine, Interferone und Chemokine zu neutralisieren [Übersichtsartikel (258)]. So kodiert das ORFV immunmodulatorische Gene wie vIL-10, GIF, sowie das CBP. Für die erfolgreiche Beseitigung einer viralen Infektion ist eine T-Zell vermittelte Immunantwort entscheidend. Diese wird durch die Interaktion des T-Zellrezeptors mit MHC I-Peptidkomplexen, die auf infizierten Zellen und/oder professionellen antigenpräsentierenden Zellen präsentiert werden, aktiviert. Die dafür nötige Prozessierung viraler Antigene und Mechanismen für die Oberflächenpräsentation sind weitere Angriffspunkte für immunmodulatorische Effektoren der Pockenviren [Übersichtsartikel (94, 126, 258)]. Pockenviren können diese MHC I vermittelte Antigenpräsentation verringern, indem sie das Prozessieren von viralen Antigenen oder die Antigenpräsentation durch MHC I auf der Zelloberfläche verhindern.

Orf virus interferes with MHC class I surface expression by targeting vesicular transport and Golgi
Rohde J, Emschermann F, Knittler MR, Rziha HJ; BMC Veterinary Research 2012, 8:114

Diese Studie konnte mittels fluoreszenzmikroskopischen, durchflusszytometrischen sowie Western Blot Analysen zeigen, dass eine ORFV Infektion mit dem Stamm D1701-V in Vero Zellen zur strukturellen Auflösung des Golgi Apparates führt. Darüber hinaus kommt es zu einer Anreichung von COP-I Vesikeln mit einer gesteigerten Expression von β-COP. Ein wichtiger Transport von Peptiden erfolgt

intrazellulär vom ER zur Zellmembran oder endozytotisch von der Zellmembran zu Endosomen durch das System von Vesikeln. Das hier untersuchte COP-I Vesikel ist für den anterograden wie retrograden Peptidtransport verantwortlich wobei das COP-I Coatomer aus sieben Proteinen (α, β, β‘, γ, δ, ε und ξ) und dem ADP-Ribosylierungsfaktor 1 (ARF-1) besteht [Übersichtsartikel (259)]. Es konnte außerdem eine ausgeprägte Ko-Lokalisierung zwischen MHC I und aus β-COP bestehenden Vesikeln (COP-I) gemessen werden. Des Weiteren induzierte ORFV einen veränderten Glykosylierungszustand von MHC I innerhalb des Golgi und eine reduzierte MHC I Zelloberflächenexpression sowie deren verlängerte Halbwertszeit auf der Zellmembran.

Die Veränderung der perinukleären Lage des Golgi Apparats sowie die punktförmige Auflösung der Struktur des *cis*- und *medialem*-Golgi sowie des *trans*-Golgi-Netzwerk scheinen durch frühe ORFV Genexpression bedingt zu sein. Bei Infektionen mit Varicella-Zoster Virus führte ebenfalls dessen frühe Genexpression zur Reduktion von MHC I auf der Zelloberfläche durch Einflussnahme auf den intrazellulären Transport (260). Des Weiteren konnte auch nach Infektion mit humanen Rhinovirus 1A (HRV-1A) eine Fragmentierung des Golgi zu Vesikeln gezeigt werden, was der viralen RNA Replikation dient (261). Die strukturelle Auflösung des Golgi und der Nachweis eines ORFV Hüllproteins innerhalb des Golgi Apparats während der späten Phase der Infektion wurde für ORFV bereits beschrieben (262). In diesem Zusammenhang wird vermutet, dass das Hüllprotein eingebettet zwischen zwei Golgi Membranen liegt, welche dann umhüllte und reife Virionen bilden kann. Die beschriebenen Ergebnisse zeigen jedoch, dass die Zerstörung des Golgi nicht auf der Bildung einer viralen Hüllmembran beruhen kann, da es auch in Gegenwart von Cytosin-Arabinosid, welches die Expression von späten, für die Ausbildung der Hüllmembran essentiellen ORFV Genen unterbindet, zu den strukturellen Veränderungen des Golgi Apparats kam.

Die reduzierte MHC I Expression an Zelloberflächen war in den Cytosin-Arabinosid Experimenten bedingt durch Produkte der frühen viralen Genexpression. Jedoch konnte nicht ausgeschlossen werden, dass die Verlängerung der MHC I Halbwertszeit auf der Oberfläche von Vero Zellen auch durch die späte Genexpression und Vermehrung des ORFV mit beeinflusst wird. Die beschriebene Zerstörung des TGN könnte sowohl die Endozytose wie auch die endosomale

Wiederverwertung von MHC I beeinträchtigen. Für das E5 Protein von HPV16 wurde beschrieben, dass es die Auflösung des exo-endozytotischen Zyklus bedingt und den MHC I Transfer beeinträchtigt (263).

Die Ergebnisse zeigten auch, dass ORFV eine Anhäufung von MHC I innerhalb von COP-I Vesikeln verursacht und es nach Infektion zu einer vermehrten zellulären Expression von β-COP kommt. Da COP-I sowohl für den anterograden wie retrograden sekretorischen Vesikel-Transport verantwortlich ist, ist zu vermuten, dass ORFV die weitere Prozessierung der COP-I Vesikel unterbindet. Für das Coxsackie-Virus wurde das virale Protein 3A beschrieben, welches auf den vesikulären Golgi Transport Einfluss nehmen kann, indem sie den Austausch von GDP zu GTP unterbinden und es zu einer reduzierten Menge von ARF-GTP kommt. Hierdurch bleibt die Funktion der GTPase unwirksam und es kommt nicht zur Freisetzung der Vesikel wie auch nicht zur Fusion des Vesikels mit der Zielmembran [Übersichtsartikel (264)]. Für das ORFV bleibt zu klären, welche Proteine hierfür verantwortlich sind. Für ORFV besteht kein Zusammenhang zwischen der Ausbildung der viralen Hüllmembran und der COP-I Akkumulation, da dieser Prozess unter Cytosin-Arabinosid Bedingungen zu beobachten war. Im Gegensatz hierzu ist für VV in der frühen Phase der Entstehung von Viruspartikeln der COP-I Coatamer-Komplex entscheidend wie auch der KDEL-Rezeptor (KDELR) der Zelle (265).

Um den Reifungszustand und somit Hinweise auf die Lokalisation des Glykoproteins MHC I im Golgi zu erhalten, wurde dieses auf die Sensitivität gegenüber Endo H untersucht. Dies erfolgte durch die Deglykosylierung der MHC I Moleküle mittels Endo H und anschließender Markierung mit radioaktivem Schwefel (^{35}S) sowie deren Analyse durch Immunpräzipitation und Western Blot. Die ORFV induzierte intrazelluläre Anhäufung von MHC I in COP I Vesikeln tritt zusammen mit einem verminderten Reifungszustand von N-glykosylierten MHC I Molekülen innerhalb des Golgi Apparats auf. So weist ein Großteil an MHC I eine partielle Resistenz gegenüber dem Enzym Endo H in ORFV infizierten Zellen auf. Dies deutet an, dass diese post-ER MHC I Moleküle keine oder nur eine gestörte Glykosylierung innerhalb des Golgi erfahren. Die ORFV Infektion führte nicht nur zu einer veränderten Struktur und Lage des Golgi, sondern könnte beispielsweise auch zu veränderten pH Bedingungen innerhalb des Golgi und TGN führen, wodurch es zu einer unvollständigen MHC I Reifung kommen könnte. Ähnliche Ergebnisse wurden für die

fehlerhafte Reifung von MHC I im Beisein von Concanamycin B beschrieben, welches einen spezifischen Inhibitor für die membranständige H (+)-ATPase darstellt (266).

Die Studie unterstützt die Annahme, dass auch ORFV wie andere DNA Viren die Immunantwort des Wirts umgehen kann. Das ORFV kann durch die induzierte Akkumulierung von MHC I Molekülen in COP-I Vesikeln und die dadurch erreichte verringerte Oberflächenpräsentation von MHC I vermutlich eine CTL vermittelte Immunantwort umgehen oder zumindest zeitlich verzögern. Durch die von ORFV induzierte verlängerte Halbwertszeit von bereits bestehendem MHC I auf der Zelloberfläche ist zudem davon auszugehen, dass das Virus hierdurch versucht der NK Antwort des Immunsystems entgegen zu wirken. Unterstützt wird diese Hypothese von der Tatsache, dass die ORFV-spezifische Immunantwort im natürlichen Wirt nur kurzzeitig ist und sich die Tiere mehrmals mit ORFV infizieren können [Übersichtsartikel (124)].

4 Zusammenfassung

Das Orf Virus (ORFV) gehört zur Gattung *Parapoxvirus* der *Poxviridae*. ORFV zeichnet sich durch äußerst vorteilhafte immunstimulierende und protektive Eigenschaften aus, die es für den Nutzen als vielversprechende rekombinante Vektor-Vakzine interessant machen. Die vorliegende Arbeit umfasst die Neu- und Weiterentwicklung von ORFV-Rekombinanten und die Charakterisierung deren protektiven Eigenschaften sowie die Beschreibung von potentiellen neuen Evasionsmechanismen und immunrelevanten Eigenschaften von ORFV.

Zur Herstellung und Selektion von ORFV-Rekombinanten wurden verschiedene Methoden der Transfektion optimiert, um in ORFV infizierten Zellen eine höchst mögliche Transfektionseffizienz zu erreichen. Die Etablierung der PCR für den Selektionsprozess neuer Rekombinanten löste das Problem der Abhängigkeit von Antikörpern, die gegen das inserierte Antigen gerichtet sind. Zudem ermöglichte der Einsatz von Fluoreszenz-Markergenen eine Reduktion der Arbeitsschritte, die zum Erhalt homogener Virus-Rekombinanten führen.

Des Weiteren wurden verschiedene ORFV-Rekombinanten hergestellt, in deren Genom Fremdgene verschiedener Viren (BRV, BTV, AHSV, FMDV, RHDV und Influenzavirus) eingefügt wurden. Die korrekte Insertion und Expression dieser Gene wurden durch verschiedene molekularbiologische Methoden bestimmt und die protektiven Eigenschaften einiger Rekombinanten wurden *in vivo* charakterisiert.

Die ORFV-Rekombinante D1701-V-VP1 konnte einen effektiven Schutz gegen RHDV im natürlichen Wirt, dem Kaninchen, induzieren. RHDV verursacht eine hochansteckende Erkrankung mit einer hohen Mortalitätsrate in Kaninchen. Da *in vitro* Zellkultursysteme fehlen, stammen kommerzielle Impfstoffe aus der Leber von RHDV infizierten Kaninchen. Bereits eine singuläre Immunisierung mit der Rekombinanten D1701-V-VP1 war ausreichend, um eine vollständige Immunität gegen eine letale RHDV Infektion in Kaninchen zu induzieren. Der induzierte Schutz beruhte neben der spezifischen Antikörperantwort gegen RHDV vermutlich auch auf einer Beteiligung von T-Zellen. Die generierte ORFV-Rekombinante stellt eine attraktive Alternative zu herkömmlichen Vakzinen dar.

Eine weitere neu hergestellte Rekombinante (D1701-V-HAh5n) exprimiert das Hemagglutinin (H5 HA), welches das Hauptimmunogen des hochpathogenen aviären

Influenzavirus (HPAIV) Stamm H5N1 darstellt und virus-neutralisierende Antikörper induziert. Die Immunogenität dieser ORFV-Rekombinante wurde nach Immunisierungen von Mäusen mit anschließenden Belastungsinfektionen verschiedener Stämme des HPAIV H5N1 charakterisiert. Die immunisierten Tiere zeigten nach Infektion weder eine klinische Symptomatik noch einen Verlust an Körpergewicht. Zudem waren vakzinierte Mäuse auch vollständig gegen das heterologe humane Influenzavirus A Stamm H1N1 geschützt. Depletionsversuche *in vivo* von CD4-positiven und/oder CD8-positiven T-Zellen wiesen darauf hin, dass insbesondere CD4-positve T-Zellen für die Ausbildung der protektiven Immunität notwendig waren.

Die *in vivo* Untersuchungen der protektiven immunologischen Charakteristika von ORFV-Rekombinanten, welche die Glykoproteine des Pseudorabiesvirus (PRV) exprimieren, zeigten eine ausgeglichene spezifische TH-1 und TH-2 Immunantwort. Bis zu 24 Stunden nach ORFV Immunisierung konnte eine gesteigerte Aktivität von Natürlichen Killerzellen und die Expression pro-inflammatorischer Zytokine (IL-1β, IL-6, TNFα und G-CSF) nachgewiesen werden. Zu späteren Zeitpunkten und nach einer zweiten ORFV Immunisierung war vermehrt die Sekretion von TH-2 spezifischen Zytokinen (IL-4, IL-5, IL-10 und IL-13) im Serum der Tiere nachweisbar. Des Weiteren konnte gezeigt werden, dass die Interferon-α/β und γ Rezeptoren essentiell für den Schutz vor einer PRV Infektion waren.

Pockenviren besitzen ein breites Genspektrum, das eine Umgehung der Immunantwort des Wirts ermöglicht. So wurden die immunmodulatorischen Eigenschaften von ORFV und dessen Einfluss auf den Prozess der Antigenpräsentation über MHC I in infizierten Vero Zellen untersucht. Abhängig von der Infektionsdauer war die MHC I Expression auf der Zelloberfläche bis zu 50% reduziert. Zudem konnte bei infizierten Zellen eine verlängerte Halbwertszeit von vorhandenen MHC I Molekülen an der Zelloberfläche nachgewiesen werden.

ORFV scheint die MHC I Oberflächenexpression herab zu regulieren, indem es direkt die Struktur der Golgi Kompartimente und das vesikuläre Transportsystem angreift. Dies wiederum hat Einfluss auf die Präsentation viraler Antigene und die Aktivierung von T-Zellen. Zudem zeigten Analysen eine Ko-Lokalisierung zwischen MHC I und

COP-I Vesikeln sowie einen gestörten Glykosylierungszustand von post-ER MHC I Molekülen.

Die beschriebenen Arbeiten demonstrieren eindrücklich die bemerkenswerten Eigenschaften des ORFV Stamm D1701-V und dessen großes Potential als Vektor-Impfstoff gegen virale pathogene Erreger.

5 Summary

The Orf virus (ORFV) belongs to the genus of *Parapoxvirus* of *Poxviridae*. The virus exhibits strong immune stimulating properties very beneficial for its use as a recombinant live vector vaccine. This work comprises the generation and advancements of ORFV recombinants as well as the characterization of their protective properties. Also investigations of potential new evasion mechanisms and relevant immune targeting characteristics of ORFV are described.

To improve the generation as well as the selection process of ORFV recombinants different transfection methods have been optimized, to obtain highest transfection efficiency. The inclusion of PCR allowed the selection of recombinants without the need of specific antibodies against the inserted foreign antigens. Moreover, the use of fluorescent marker genes accelerated the selection process to obtain homogenous virus recombinants.

Furthermore, several ORFV recombinants containing foreign genes of different viruses (BRV, BTV, AHSV, FMDV, RHDV and influenza virus) were generated. The correct insertion and expression of the foreign genes has been tested by different biomolecular techniques and the protective capacities of some of them have been studied *in vivo*.

In rabbits, which are the natural hosts of RHDV, the recombinant D1701-V-VP1 could induce a very protective immunity against RHDV. RHDV causes a highly contagious disease with a high mortality rate in rabbits. Due to the lack of an *in vitro* cell culture system for RHDV, the existing commercial vaccines are prepared from the liver of infected rabbits. Already a single immunization of rabbits with the recombinant D1701-V-VP1 induced complete protection against a lethal RHDV challenge infection. Besides specific antibodies against RHDV the protective immunity was presumably also based on the contribution of T-cells. The generated ORFV recombinant represents an attractive alternative to conventional vaccines.

Another generated recombinant (D1701-V-HAh5n) expresses the immunodominant hemagglutinin (H5 HA) of highly pathogenic avian influenza virus (HPAIV) strain H5N1, which is the major target antigen of neutralizing antibodies. The immunogenicity of the ORFV recombinant was characterized upon immunizations of mice followed by challenge infections with different HPAIV H5N1 strains. The

immunizations of animals led to prevention of any clinical symptoms as well as loss of body weight. Additionally, the vaccinated mice were completely protected against challenge infection with heterologous human influenza A virus strain H1N1. *In vivo* depletion of CD4-positive and/or CD8-positive T-cells demonstrated the necessity of CD4-positive T-cells for protective immunity.

Vaccination experiments of animals with ORFV recombinants expressing glycoproteins of Pseudorabies virus (PRV) exhibited the mediation of a balanced TH-1 and TH-2 protective immune response. Up to 24 hours after ORFV immunization increased activity of natural killer cells and expression of proinflammatory cytokines (IL-1β, IL-6, TNFα und GCS-F) was observed. At later time points and after a second vaccination an increased secretion of TH-2 specific cytokines (IL-4, IL-5, IL-10 and IL-13) was detectable in the sera of mice. Furthermore, challenge infection studies demonstrated that receptors for interferon-α/β and γ were essential for protection against PRV infection.

Poxviruses have a variety of genes to circumvent the host's immunity. Therefore, the immune modulating characteristics of ORFV and its influence on the intracellular transport and surface presentation by MHC I in infected Vero cells were examined. Depending on the duration of infection, the MHC I expression level on the cell surface was reduced up to 50%. The remaining cell surface MHC I on infected cells exhibited an increased half-life. Hence, ORFV seems to reduce cell surface MHC I by targeting the Golgi compartment and the vesicular export machinery. This can effect viral antigen presentation and T-cell activation. Additional analyzes showed distinct co-localisation of MHC I and COP-I vesicles as well as a disturbed carbohydrate trimming of post-ER MHC I molecules.

Conclusively, this work demonstrates impressively the remarkable properties of ORFV strain D1701-V and its excellent potential as a vector vaccine against viral pathogens.

6 Danksagung

Mein besonderer Dank gilt Herrn Dr. Hanns-Joachim Rziha für die Bereitstellung dieses äußerst spannenden Promotionsthemas, für die tolle Betreuung, seiner Bereitschaft zur kritischen und lehrreichen Diskussion und die stets gute Zusammenarbeit.

Des Weiteren möchte ich mich bei Herrn Prof. Dr. Mettenleiter bedanken, der als Präsident des Friedrich-Loeffler-Instituts, Institut für Immunologie in Tübingen, mir das Anfertigen dieser Arbeit an diesem Institut ermöglichte. Ein besonderer Dank geht außerdem an Herrn Prof. Dr. Lothar Stitz und PD Dr. Michael Knittler für viele hilfreiche Diskussionen und die gute Arbeitsatmosphäre am Institut für Immunologie.

Herrn Prof. Dr. Hans-Georg Rammensee und Herrn Prof. Dr. Friedrich Götz möchte ich für die Bereitwilligkeit zur Begutachtung der Arbeit danken.

Für die gegenseitige Hilfsbereitschaft und die freundschaftliche Zusammenarbeit möchte ich mich bei meinen Kollegen Berthilde Bauer, Karin Kegreiß und Dr. Ralf Amann bedanken, die zusammen mit vielen Studenten und Praktikanten eine freudige und lebendige Laboratmosphäre ermöglichten.

7 Literaturverzeichnis

1. **Fischer T, Planz O, Stitz L, Rziha HJ.** 2003. Novel recombinant parapoxvirus vectors induce protective humoral and cellular immunity against lethal herpesvirus challenge infection in mice. Journal of virology **77:**9312-9323.
2. **Liu MA.** 2010. Immunologic basis of vaccine vectors. Immunity **33:**504-515.
3. **Liniger M, Zuniga A, Naim HY.** 2007. Use of viral vectors for the development of vaccines. Expert review of vaccines **6:**255-266.
4. **Foged C.** 2011. Subunit vaccines of the future: the need for safe, customized and optimized particulate delivery systems. Therapeutic delivery **2:**1057-1077.
5. **Reyes-Sandoval A, Berthoud T, Alder N, Siani L, Gilbert SC, Nicosia A, Colloca S, Cortese R, Hill AV.** 2010. Prime-boost immunization with adenoviral and modified vaccinia virus Ankara vectors enhances the durability and polyfunctionality of protective malaria CD8+ T-cell responses. Infection and immunity **78:**145-153.
6. **Liu MA.** 2010. Gene-based vaccines: Recent developments. Current opinion in molecular therapeutics **12:**86-93.
7. **Rollier CS, Reyes-Sandoval A, Cottingham MG, Ewer K, Hill AV.** 2011. Viral vectors as vaccine platforms: deployment in sight. Current opinion in immunology **23:**377-382.
8. **McMichael AJ, Gotch FM, Noble GR, Beare PA.** 1983. Cytotoxic T-cell immunity to influenza. The New England journal of medicine **309:**13-17.
9. **Gianfrani C, Oseroff C, Sidney J, Chesnut RW, Sette A.** 2000. Human memory CTL response specific for influenza A virus is broad and multispecific. Human immunology **61:**438-452.
10. **Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, Gromkowski SH, Deck RR, DeWitt CM, Friedman A, et al.** 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science **259:**1745-1749.
11. **Rosenberg ES, Walker BD.** 1998. HIV type 1-specific helper T cells: a critical host defense. AIDS research and human retroviruses **14 Suppl 2:**S143-147.
12. **Einfeld DA, Schroeder R, Roelvink PW, Lizonova A, King CR, Kovesdi I, Wickham TJ.** 2001. Reducing the native tropism of adenovirus vectors requires removal of both CAR and integrin interactions. Journal of virology **75:**11284-11291.
13. **Denis-Mize KS, Dupuis M, MacKichan ML, Singh M, Doe B, O'Hagan D, Ulmer JB, Donnelly JJ, McDonald DM, Ott G.** 2000. Plasmid DNA adsorbed onto cationic microparticles mediates target gene expression and antigen presentation by dendritic cells. Gene Ther **7:**2105-2112.
14. **Small JC, Ertl HC.** 2011. Viruses - from pathogens to vaccine carriers. Curr Opin Virol **1:**241-245.
15. **Louis Jeune V, Joergensen JA, Hajjar RJ, Weber T.** 2013. Pre-existing anti-adeno-associated virus antibodies as a challenge in AAV gene therapy. Human gene therapy methods **24:**59-67.
16. **Sanchez-Sampedro L, Perdiguero B, Mejias-Perez E, Garcia-Arriaza J, Di Pilato M, Esteban M.** 2015. The evolution of poxvirus vaccines. Viruses **7:**1726-1803.
17. **Russell SJ, Peng KW.** 2009. Measles virus for cancer therapy. Current topics in microbiology and immunology **330:**213-241.

18. **Dudek T, Knipe DM.** 2006. Replication-defective viruses as vaccines and vaccine vectors. Virology **344:**230-239.
19. **Konishi E, Kurane I, Mason PW, Shope RE, Kanesa-Thasan N, Smucny JJ, Hoke CH, Jr., Ennis FA.** 1998. Induction of Japanese encephalitis virus-specific cytotoxic T lymphocytes in humans by poxvirus-based JE vaccine candidates. Vaccine **16:**842-849.
20. **Marshall JL, Hawkins MJ, Tsang KY, Richmond E, Pedicano JE, Zhu MZ, Schlom J.** 1999. Phase I study in cancer patients of a replication-defective avipox recombinant vaccine that expresses human carcinoembryonic antigen. Journal of clinical oncology : official journal of the American Society of Clinical Oncology **17:**332-337.
21. **Demento SL, Siefert AL, Bandyopadhyay A, Sharp FA, Fahmy TM.** 2011. Pathogen-associated molecular patterns on biomaterials: a paradigm for engineering new vaccines. Trends in biotechnology **29:**294-306.
22. **Lasaro MO, Ertl HC.** 2009. New insights on adenovirus as vaccine vectors. Molecular therapy : the journal of the American Society of Gene Therapy **17:**1333-1339.
23. **Liu YF, Guo QH, Chen L, Zhao J, Chang HT, Wang XW, Yang X, Wang CQ.** 2013. Induction of mucosal immunity by intranasal immunization with recombinant adenovirus expressing major epitopes of Porcine circovirus-2 capsid protein. Vet Immunol Immunopathol **154:**48-53.
24. **Kardani K, Bolhassani A, Shahbazi S.** 2016. Prime-boost vaccine strategy against viral infections: Mechanisms and benefits. Vaccine **34:**413-423.
25. **de Mare A, Lambeck AJ, Regts J, van Dam GM, Nijman HW, Snippe H, Wilschut J, Daemen T.** 2008. Viral vector-based prime-boost immunization regimens: a possible involvement of T-cell competition. Gene Ther **15:**393-403.
26. **Cawood R, Hills T, Wong SL, Alamoudi AA, Beadle S, Fisher KD, Seymour LW.** 2012. Recombinant viral vaccines for cancer. Trends Mol Med **18:**564-574.
27. **Palucka AK, Coussens LM.** 2016. The Basis of Oncoimmunology. Cell **164:**1233-1247.
28. **Smith GL, Moss B.** 1983. Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA. Gene **25:**21-28.
29. **Ciuffi A, Bushman FD.** 2006. Retroviral DNA integration: HIV and the role of LEDGF/p75. Trends in genetics : TIG **22:**388-395.
30. **Sutter G, Moss B.** 1992. Nonreplicating vaccinia vector efficiently expresses recombinant genes. Proceedings of the National Academy of Sciences of the United States of America **89:**10847-10851.
31. **Mayr A, Stickl H, Muller HK, Danner K, Singer H.** 1978. [The smallpox vaccination strain MVA: marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism (author's transl)]. Zentralbl Bakteriol B **167:**375-390.
32. **Verardi PH, Titong A, Hagen CJ.** 2012. A vaccinia virus renaissance: new vaccine and immunotherapeutic uses after smallpox eradication. Hum Vaccin Immunother **8:**961-970.
33. **Tartaglia J, Perkus ME, Taylor J, Norton EK, Audonnet JC, Cox WI, Davis SW, van der Hoeven J, Meignier B, Riviere M, et al.** 1992. NYVAC: a highly attenuated strain of vaccinia virus. Virology **188:**217-232.

34. **Tartaglia J, Cox WI, Taylor J, Perkus M, Riviere M, Meignier B, Paoletti E.** 1992. Highly attenuated poxvirus vectors. AIDS research and human retroviruses **8:**1445-1447.
35. **Gomez CE, Najera JL, Jimenez EP, Jimenez V, Wagner R, Graf M, Frachette MJ, Liljestrom P, Pantaleo G, Esteban M.** 2007. Head-to-head comparison on the immunogenicity of two HIV/AIDS vaccine candidates based on the attenuated poxvirus strains MVA and NYVAC co-expressing in a single locus the HIV-1BX08 gp120 and HIV-1(IIIB) Gag-Pol-Nef proteins of clade B. Vaccine **25:**2863-2885.
36. **Taylor J, Trimarchi C, Weinberg R, Languet B, Guillemin F, Desmettre P, Paoletti E.** 1991. Efficacy studies on a canarypox-rabies recombinant virus. Vaccine **9:**190-193.
37. **Taylor J, Weinberg R, Languet B, Desmettre P, Paoletti E.** 1988. Recombinant fowlpox virus inducing protective immunity in non-avian species. Vaccine **6:**497-503.
38. **Weli SC, Tryland M.** 2011. Avipoxviruses: infection biology and their use as vaccine vectors. Virology journal **8:**49.
39. **Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, Premsri N, Namwat C, de Souza M, Adams E, Benenson M, Gurunathan S, Tartaglia J, McNeil JG, Francis DP, Stablein D, Birx DL, Chunsuttiwat S, Khamboonruang C, Thongcharoen P, Robb ML, Michael NL, Kunasol P, Kim JH.** 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. The New England journal of medicine **361:**2209-2220.
40. **Stanley DA, Honko AN, Asiedu C, Trefry JC, Lau-Kilby AW, Johnson JC, Hensley L, Ammendola V, Abbate A, Grazioli F, Foulds KE, Cheng C, Wang L, Donaldson MM, Colloca S, Folgori A, Roederer M, Nabel GJ, Mascola J, Nicosia A, Cortese R, Koup RA, Sullivan NJ.** 2014. Chimpanzee adenovirus vaccine generates acute and durable protective immunity against ebolavirus challenge. Nat Med **20:**1126-1129.
41. **Haagmans BL, van den Brand JM, Raj VS, Volz A, Wohlsein P, Smits SL, Schipper D, Bestebroer TM, Okba N, Fux R, Bensaid A, Solanes Foz D, Kuiken T, Baumgartner W, Segales J, Sutter G, Osterhaus AD.** 2016. An orthopoxvirus-based vaccine reduces virus excretion after MERS-CoV infection in dromedary camels. Science **351:**77-81.
42. **Chavan R, Marfatia KA, An IC, Garber DA, Feinberg MB.** 2006. Expression of CCL20 and granulocyte-macrophage colony-stimulating factor, but not Flt3-L, from modified vaccinia virus ankara enhances antiviral cellular and humoral immune responses. Journal of virology **80:**7676-7687.
43. **Kibler KV, Gomez CE, Perdiguero B, Wong S, Huynh T, Holechek S, Arndt W, Jimenez V, Gonzalez-Sanz R, Denzler K, Haddad EK, Wagner R, Sekaly RP, Tartaglia J, Pantaleo G, Jacobs BL, Esteban M.** 2011. Improved NYVAC-based vaccine vectors. PLoS One **6:**e25674.
44. **Goncalves MA, de Vries AA.** 2006. Adenovirus: from foe to friend. Rev Med Virol **16:**167-186.
45. **Zhang C, Zhou D.** 2016. Adenoviral vector-based strategies against infectious disease and cancer. Hum Vaccin Immunother **12:**2064-2074.
46. **Tatsis N, Ertl HC.** 2004. Adenoviruses as vaccine vectors. Molecular therapy : the journal of the American Society of Gene Therapy **10:**616-629.

47. **Shiver JW, Fu TM, Chen L, Casimiro DR, Davies ME, Evans RK, Zhang ZQ, Simon AJ, Trigona WL, Dubey SA, Huang L, Harris VA, Long RS, Liang X, Handt L, Schleif WA, Zhu L, Freed DC, Persaud NV, Guan L, Punt KS, Tang A, Chen M, Wilson KA, Collins KB, Heidecker GJ, Fernandez VR, Perry HC, Joyce JG, Grimm KM, Cook JC, Keller PM, Kresock DS, Mach H, Troutman RD, Isopi LA, Williams DM, Xu Z, Bohannon KE, Volkin DB, Montefiori DC, Miura A, Krivulka GR, Lifton MA, Kuroda MJ, Schmitz JE, Letvin NL, Caulfield MJ, Bett AJ, Youil R, Kaslow DC, Emini EA.** 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature **415:**331-335.
48. **Vlachaki MT, Hernandez-Garcia A, Ittmann M, Chhikara M, Aguilar LK, Zhu X, Teh BS, Butler EB, Woo S, Thompson TC, Barrera-Saldana H, Aguilar-Cordova E.** 2002. Impact of preimmunization on adenoviral vector expression and toxicity in a subcutaneous mouse cancer model. Molecular therapy : the journal of the American Society of Gene Therapy **6:**342-348.
49. **Lemckert AA, Sumida SM, Holterman L, Vogels R, Truitt DM, Lynch DM, Nanda A, Ewald BA, Gorgone DA, Lifton MA, Goudsmit J, Havenga MJ, Barouch DH.** 2005. Immunogenicity of heterologous prime-boost regimens involving recombinant adenovirus serotype 11 (Ad11) and Ad35 vaccine vectors in the presence of anti-ad5 immunity. Journal of virology **79:**9694-9701.
50. **Fuchs JD, Bart PA, Frahm N, Morgan C, Gilbert PB, Kochar N, DeRosa SC, Tomaras GD, Wagner TM, Baden LR, Koblin BA, Rouphael NG, Kalams SA, Keefer MC, Goepfert PA, Sobieszczyk ME, Mayer KH, Swann E, Liao HX, Haynes BF, Graham BS, McElrath MJ, Network NHVT.** 2015. Safety and Immunogenicity of a Recombinant Adenovirus Serotype 35-Vectored HIV-1 Vaccine in Adenovirus Serotype 5 Seronegative and Seropositive Individuals. Journal of AIDS & clinical research **6**.
51. **Cheng C, Wang L, Ko SY, Kong WP, Schmidt SD, Gall JG, Colloca S, Seder RA, Mascola JR, Nabel GJ.** 2015. Combination recombinant simian or chimpanzee adenoviral vectors for vaccine development. Vaccine **33:**7344-7351.
52. **Vitelli A, Quirion MR, Lo CY, Misplon JA, Grabowska AK, Pierantoni A, Ammendola V, Price GE, Soboleski MR, Cortese R, Colloca S, Nicosia A, Epstein SL.** 2013. Vaccination to conserved influenza antigens in mice using a novel Simian adenovirus vector, PanAd3, derived from the bonobo Pan paniscus. PLoS One **8:**e55435.
53. **Manservigi R, Argnani R, Marconi P.** 2010. HSV Recombinant Vectors for Gene Therapy. The open virology journal **4:**123-156.
54. **Hastie E, Cataldi M, Marriott I, Grdzelishvili VZ.** 2013. Understanding and altering cell tropism of vesicular stomatitis virus. Virus Res **176:**16-32.
55. **Bukreyev A, Skiadopoulos MH, Murphy BR, Collins PL.** 2006. Nonsegmented negative-strand viruses as vaccine vectors. Journal of virology **80:**10293-10306.
56. **Boudreau JE, Bridle BW, Stephenson KB, Jenkins KM, Brunelliere J, Bramson JL, Lichty BD, Wan Y.** 2009. Recombinant vesicular stomatitis virus transduction of dendritic cells enhances their ability to prime innate and adaptive antitumor immunity. Molecular therapy : the journal of the American Society of Gene Therapy **17:**1465-1472.

57. **Regules JA, Beigel JH, Paolino KM, Voell J, Castellano AR, Munoz P, Moon JE, Ruck RC, Bennett JW, Twomey PS, Gutierrez RL, Remich SA, Hack HR, Wisniewski ML, Josleyn MD, Kwilas SA, Van Deusen N, Mbaya OT, Zhou Y, Stanley DA, Bliss RL, Cebrik D, Smith KS, Shi M, Ledgerwood JE, Graham BS, Sullivan NJ, Jagodzinski LL, Peel SA, Alimonti JB, Hooper JW, Silvera PM, Martin BK, Monath TP, Ramsey WJ, Link CJ, Lane HC, Michael NL, Davey RT, Jr., Thomas SJ, r V-Z-GPSG.** 2015. A Recombinant Vesicular Stomatitis Virus Ebola Vaccine - Preliminary Report. The New England journal of medicine.
58. **Matassov D, Marzi A, Latham T, Xu R, Ota-Setlik A, Feldmann F, Geisbert JB, Mire CE, Hamm S, Nowak B, Egan MA, Geisbert TW, Eldridge JH, Feldmann H, Clarke DK.** 2015. Vaccination With a Highly Attenuated Recombinant Vesicular Stomatitis Virus Vector Protects Against Challenge With a Lethal Dose of Ebola Virus. The Journal of infectious diseases **212 Suppl 2:**S443-451.
59. **Querec T, Bennouna S, Alkan S, Laouar Y, Gorden K, Flavell R, Akira S, Ahmed R, Pulendran B.** 2006. Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. J Exp Med **203:**413-424.
60. **Monath TP, Seligman SJ, Robertson JS, Guy B, Hayes EB, Condit RC, Excler JL, Mac LM, Carbery B, Chen RT, Brighton Collaboration Viral Vector Vaccines Safety Working G.** 2015. Live virus vaccines based on a yellow fever vaccine backbone: standardized template with key considerations for a risk/benefit assessment. Vaccine **33:**62-72.
61. **Lu HY, Chen YH, Liu HJ.** 2012. Baculovirus as a vaccine vector. Bioengineered **3:**271-274.
62. **van Oers MM.** 2006. Vaccines for viral and parasitic diseases produced with baculovirus vectors. Advances in virus research **68:**193-253.
63. **Yang DK, Kweon CH, Kim BH, Lim SI, Kwon JH, Kim SH, Song JY, Han HR.** 2005. Immunogenicity of baculovirus expressed recombinant proteins of Japanese encephalitis virus in mice. Journal of veterinary science **6:**125-133.
64. **Stobart CC, Moore ML.** 2014. RNA virus reverse genetics and vaccine design. Viruses **6:**2531-2550.
65. **Gomme EA, Wanjalla CN, Wirblich C, Schnell MJ.** 2011. Rabies virus as a research tool and viral vaccine vector. Advances in virus research **79:**139-164.
66. **Zhu S, Li H, Wang C, Luo F, Guo C.** 2015. Reverse genetics of rabies virus: new strategies to attenuate virus virulence for vaccine development. Journal of neurovirology **21:**335-345.
67. **Blaney JE, Marzi A, Willet M, Papaneri AB, Wirblich C, Feldmann F, Holbrook M, Jahrling P, Feldmann H, Schnell MJ.** 2013. Antibody quality and protection from lethal Ebola virus challenge in nonhuman primates immunized with rabies virus based bivalent vaccine. PLoS Pathog **9:**e1003389.
68. **Conzelmann KK, Schnell M.** 1994. Rescue of synthetic genomic RNA analogs of rabies virus by plasmid-encoded proteins. Journal of virology **68:**713-719.
69. **Mebatsion T, Schnell MJ, Cox JH, Finke S, Conzelmann KK.** 1996. Highly stable expression of a foreign gene from rabies virus vectors. Proceedings of the National Academy of Sciences of the United States of America **93:**7310-7314.

70. **Medina J, Boukhebza H, De Saint Jean A, Sodoyer R, Legastelois I, Moste C.** 2015. Optimization of influenza A vaccine virus by reverse genetic using chimeric HA and NA genes with an extended PR8 backbone. Vaccine **33:**4221-4227.
71. **Chin'ombe N, Bourn WR, Williamson AL, Shephard EG.** 2009. Oral vaccination with a recombinant Salmonella vaccine vector provokes systemic HIV-1 subtype C Gag-specific CD4+ Th1 and Th2 cell immune responses in mice. Virology journal **6:**87.
72. **Ferrari G, Kostyu DD, Cox J, Dawson DV, Flores J, Weinhold KJ, Osmanov S.** 2000. Identification of highly conserved and broadly cross-reactive HIV type 1 cytotoxic T lymphocyte epitopes as candidate immunogens for inclusion in Mycobacterium bovis BCG-vectored HIV vaccines. AIDS research and human retroviruses **16:**1433-1443.
73. **Zhang XL, Jeza VT, Pan Q.** 2008. Salmonella typhi: from a human pathogen to a vaccine vector. Cellular & molecular immunology **5:**91-97.
74. **Huang JM, Sali M, Leckenby MW, Radford DS, Huynh HA, Delogu G, Cranenburgh RM, Cutting SM.** 2010. Oral delivery of a DNA vaccine against tuberculosis using operator-repressor titration in a Salmonella enterica vector. Vaccine **28:**7523-7528.
75. **Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL.** 1990. Direct gene transfer into mouse muscle in vivo. Science **247:**1465-1468.
76. **Klinman DM, Yamshchikov G, Ishigatsubo Y.** 1997. Contribution of CpG motifs to the immunogenicity of DNA vaccines. Journal of immunology **158:**3635-3639.
77. **Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S.** 2000. A Toll-like receptor recognizes bacterial DNA. Nature **408:**740-745.
78. **McNamara MA, Nair SK, Holl EK.** 2015. RNA-Based Vaccines in Cancer Immunotherapy. Journal of immunology research **2015:**794528.
79. **Gurunathan S, Klinman DM, Seder RA.** 2000. DNA vaccines: immunology, application, and optimization*. Annual review of immunology **18:**927-974.
80. **Eisenacher K, Steinberg C, Reindl W, Krug A.** 2007. The role of viral nucleic acid recognition in dendritic cells for innate and adaptive antiviral immunity. Immunobiology **212:**701-714.
81. **Shatkin AJ.** 1976. Capping of eucaryotic mRNAs. Cell **9:**645-653.
82. **Deo RC, Bonanno JB, Sonenberg N, Burley SK.** 1999. Recognition of polyadenylate RNA by the poly(A)-binding protein. Cell **98:**835-845.
83. **Mignone F, Gissi C, Liuni S, Pesole G.** 2002. Untranslated regions of mRNAs. Genome biology **3:**reviews0004.1-reviews0004.10.
84. **Hoerr I, Obst R, Rammensee HG, Jung G.** 2000. In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies. European journal of immunology **30:**1-7.
85. **Kreiter S, Selmi A, Diken M, Koslowski M, Britten CM, Huber C, Tureci O, Sahin U.** 2010. Intranodal vaccination with naked antigen-encoding RNA elicits potent prophylactic and therapeutic antitumoral immunity. Cancer research **70:**9031-9040.
86. **Houseley J, Tollervey D.** 2009. The many pathways of RNA degradation. Cell **136:**763-776.

87. **Kallen KJ, Heidenreich R, Schnee M, Petsch B, Schlake T, Thess A, Baumhof P, Scheel B, Koch SD, Fotin-Mleczek M.** 2013. A novel, disruptive vaccination technology: self-adjuvanted RNActive((R)) vaccines. Hum Vaccin Immunother **9:**2263-2276.
88. **Scheel B, Teufel R, Probst J, Carralot JP, Geginat J, Radsak M, Jarrossay D, Wagner H, Jung G, Rammensee HG, Hoerr I, Pascolo S.** 2005. Toll-like receptor-dependent activation of several human blood cell types by protamine-condensed mRNA. European journal of immunology **35:**1557-1566.
89. **Skold AE, van Beek JJ, Sittig SP, Bakdash G, Tel J, Schreibelt G, de Vries IJ.** 2015. Protamine-stabilized RNA as an ex vivo stimulant of primary human dendritic cell subsets. Cancer immunology, immunotherapy : CII **64:**1461-1473.
90. **Petsch B, Schnee M, Vogel AB, Lange E, Hoffmann B, Voss D, Schlake T, Thess A, Kallen KJ, Stitz L, Kramps T.** 2012. Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection. Nature biotechnology **30:**1210-1216.
91. **Kubler H, Scheel B, Gnad-Vogt U, Miller K, Schultze-Seemann W, Vom Dorp F, Parmiani G, Hampel C, Wedel S, Trojan L, Jocham D, Maurer T, Rippin G, Fotin-Mleczek M, von der Mulbe F, Probst J, Hoerr I, Kallen KJ, Lander T, Stenzl A.** 2015. Self-adjuvanted mRNA vaccination in advanced prostate cancer patients: a first-in-man phase I/IIa study. Journal for immunotherapy of cancer **3:**26.
92. **Lundstrom K.** 2015. RNA-based drugs and vaccines. Expert review of vaccines **14:**253-263.
93. **Delhon G, Tulman ER, Afonso CL, Lu Z, de la Concha-Bermejillo A, Lehmkuhl HD, Piccone ME, Kutish GF, Rock DL.** 2004. Genomes of the parapoxviruses ORF virus and bovine papular stomatitis virus. Journal of virology **78:**168-177.
94. **Hosamani M, Scagliarini A, Bhanuprakash V, McInnes CJ, Singh RK.** 2009. Orf: an update on current research and future perspectives. Expert Rev Anti Infect Ther **7:**879-893.
95. **Schmidt FI, Bleck CK, Mercer J.** 2012. Poxvirus host cell entry. Curr Opin Virol **2:**20-27.
96. **Carter GC, Rodger G, Murphy BJ, Law M, Krauss O, Hollinshead M, Smith GL.** 2003. Vaccinia virus cores are transported on microtubules. The Journal of general virology **84:**2443-2458.
97. **Mallardo M, Leithe E, Schleich S, Roos N, Doglio L, Krijnse Locker J.** 2002. Relationship between vaccinia virus intracellular cores, early mRNAs, and DNA replication sites. Journal of virology **76:**5167-5183.
98. **Schramm B, Locker JK.** 2005. Cytoplasmic organization of POXvirus DNA replication. Traffic **6:**839-846.
99. **Doglio L, De Marco A, Schleich S, Roos N, Krijnse Locker J.** 2002. The Vaccinia virus E8R gene product: a viral membrane protein that is made early in infection and packaged into the virions' core. Journal of virology **76:**9773-9786.
100. **Moss B.** 2007. Poxviridae: The viruses and their replication., p. 2905-2946. *In* Knipe DM, Howley PM (ed.), In Fields virology.
101. **Moss B.** 2013. Poxvirus DNA replication. Cold Spring Harbor perspectives in biology **5**.

102. **Yang Z, Reynolds SE, Martens CA, Bruno DP, Porcella SF, Moss B.** 2011. Expression profiling of the intermediate and late stages of poxvirus replication. Journal of virology **85:**9899-9908.
103. **Broyles SS.** 2003. Vaccinia virus transcription. The Journal of general virology **84:**2293-2303.
104. **Rosel J, Moss B.** 1985. Transcriptional and translational mapping and nucleotide sequence analysis of a vaccinia virus gene encoding the precursor of the major core polypeptide 4b. Journal of virology **56:**830-838.
105. **Yang Z, Cao S, Martens CA, Porcella SF, Xie Z, Ma M, Shen B, Moss B.** 2015. Deciphering poxvirus gene expression by RNA sequencing and ribosome profiling. Journal of virology **89:**6874-6886.
106. **Marsland BJ, Tisdall DJ, Heath DD, Mercer AA.** 2003. Construction of a recombinant orf virus that expresses an Echinococcus granulosus vaccine antigen from a novel genomic insertion site. Archives of virology **148:**555-562.
107. **Davison AJ, Moss B.** 1989. Structure of vaccinia virus early promoters. Journal of molecular biology **210:**749-769.
108. **Fleming SB, Blok J, Fraser KM, Mercer AA, Robinson AJ.** 1993. Conservation of gene structure and arrangement between vaccinia virus and orf virus. Virology **195:**175-184.
109. **Sullivan JT, Mercer AA, Fleming SB, Robinson AJ.** 1994. Identification and characterization of an orf virus homologue of the vaccinia virus gene encoding the major envelope antigen p37K. Virology **202:**968-973.
110. **Baur K, Brinkmann K, Schweneker M, Patzold J, Meisinger-Henschel C, Hermann J, Steigerwald R, Chaplin P, Suter M, Hausmann J.** 2010. Immediate-early expression of a recombinant antigen by modified vaccinia virus ankara breaks the immunodominance of strong vector-specific B8R antigen in acute and memory CD8 T-cell responses. Journal of virology **84:**8743-8752.
111. **Isshiki M, Zhang X, Sato H, Ohashi T, Inoue M, Shida H.** 2014. Effects of different promoters on the virulence and immunogenicity of a HIV-1 Env-expressing recombinant vaccinia vaccine. Vaccine **32:**839-845.
112. **Wennier ST, Brinkmann K, Steinhausser C, Maylander N, Mnich C, Wielert U, Dirmeier U, Hausmann J, Chaplin P, Steigerwald R.** 2013. A novel naturally occurring tandem promoter in modified vaccinia virus ankara drives very early gene expression and potent immune responses. PLoS One **8:**e73511.
113. **Di Pilato M, Sanchez-Sampedro L, Mejias-Perez E, Sorzano CO, Esteban M.** 2015. Modification of promoter spacer length in vaccinia virus as a strategy to control the antigen expression. The Journal of general virology **96:**2360-2371.
114. **Baldick CJ, Jr., Keck JG, Moss B.** 1992. Mutational analysis of the core, spacer, and initiator regions of vaccinia virus intermediate-class promoters. Journal of virology **66:**4710-4719.
115. **Hooda-Dhingra U, Thompson CL, Condit RC.** 1989. Detailed phenotypic characterization of five temperature-sensitive mutants in the 22- and 147-kilodalton subunits of vaccinia virus DNA-dependent RNA polymerase. Journal of virology **63:**714-729.
116. **Rosel JL, Earl PL, Weir JP, Moss B.** 1986. Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes

deduced by structural and functional analysis of the HindIII H genome fragment. Journal of virology **60:**436-449.
117. **Yuen L, Moss B.** 1987. Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. Proceedings of the National Academy of Sciences of the United States of America **84:**6417-6421.
118. **Condit RC, Niles EG.** 2002. Regulation of viral transcription elongation and termination during vaccinia virus infection. Biochimica et biophysica acta **1577:**325-336.
119. **Black EP, Condit RC.** 1996. Phenotypic characterization of mutants in vaccinia virus gene G2R, a putative transcription elongation factor. Journal of virology **70:**47-54.
120. **Buttner M, Rziha HJ.** 2002. Parapoxviruses: from the lesion to the viral genome. J Vet Med B Infect Dis Vet Public Health **49:**7-16.
121. **Rogers M, Bale P, De Silva LM, Glasson MJ, Collins E.** 1989. Giant parapox infection in a two year old child. Australas J Dermatol **30:**87-91.
122. **Ara M, Zaballos P, Sanchez M, Querol I, Zubiri ML, Simal E, Horndler C.** 2008. Giant and recurrent orf virus infection in a renal transplant recipient treated with imiquimod. J Am Acad Dermatol **58:**S39-40.
123. **Haig DM, McInnes CJ.** 2002. Immunity and counter-immunity during infection with the parapoxvirus orf virus. Virus Res **88:**3-16.
124. **Haig DM.** 2006. Orf virus infection and host immunity. Current opinion in infectious diseases **19:**127-131.
125. **Haig DM, Fleming S.** 1999. Immunomodulation by virulence proteins of the parapoxvirus orf virus. Vet Immunol Immunopathol **72:**81-86.
126. **Haig DM.** 2001. Subversion and piracy: DNA viruses and immune evasion. Res Vet Sci **70:**205-219.
127. **Deane D, McInnes CJ, Percival A, Wood A, Thomson J, Lear A, Gilray J, Fleming S, Mercer A, Haig D.** 2000. Orf virus encodes a novel secreted protein inhibitor of granulocyte-macrophage colony-stimulating factor and interleukin-2. Journal of virology **74:**1313-1320.
128. **Seet BT, McCaughan CA, Handel TM, Mercer A, Brunetti C, McFadden G, Fleming SB.** 2003. Analysis of an orf virus chemokine-binding protein: Shifting ligand specificities among a family of poxvirus viroceptors. Proceedings of the National Academy of Sciences of the United States of America **100:**15137-15142.
129. **Fleming SB, McCaughan CA, Andrews AE, Nash AD, Mercer AA.** 1997. A homolog of interleukin-10 is encoded by the poxvirus orf virus. Journal of virology **71:**4857-4861.
130. **Westphal D, Ledgerwood EC, Hibma MH, Fleming SB, Whelan EM, Mercer AA.** 2007. A novel Bcl-2-like inhibitor of apoptosis is encoded by the parapoxvirus ORF virus. Journal of virology **81:**7178-7188.
131. **Diel DG, Delhon G, Luo S, Flores EF, Rock DL.** 2010. A novel inhibitor of the NF-{kappa}B signaling pathway encoded by the parapoxvirus orf virus. Journal of virology **84:**3962-3973.
132. **Diel DG, Luo S, Delhon G, Peng Y, Flores EF, Rock DL.** 2011. A nuclear inhibitor of NF-kappaB encoded by a poxvirus. Journal of virology **85:**264-275.
133. **Diel DG, Luo S, Delhon G, Peng Y, Flores EF, Rock DL.** 2011. Orf virus ORFV121 encodes a novel inhibitor of NF-kappaB that contributes to virus virulence. Journal of virology **85:**2037-2049.

134. **Perkins ND.** 2007. Integrating cell-signalling pathways with NF-kappaB and IKK function. Nature reviews. Molecular cell biology **8:**49-62.
135. **Scheidereit C.** 2006. IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. Oncogene **25:**6685-6705.
136. **Haig DM, McInnes CJ, Thomson J, Wood A, Bunyan K, Mercer A.** 1998. The orf virus OV20.0L gene product is involved in interferon resistance and inhibits an interferon-inducible, double-stranded RNA-dependent kinase. Immunology **93:**335-340.
137. **Wise L, McCaughan C, Tan CK, Mercer AA, Fleming SB.** 2007. Orf virus interleukin-10 inhibits cytokine synthesis in activated human THP-1 monocytes, but only partially impairs their proliferation. The Journal of general virology **88:**1677-1682.
138. **Lateef Z, Fleming S, Halliday G, Faulkner L, Mercer A, Baird M.** 2003. Orf virus-encoded interleukin-10 inhibits maturation, antigen presentation and migration of murine dendritic cells. The Journal of general virology **84:**1101-1109.
139. **Imlach W, McCaughan CA, Mercer AA, Haig D, Fleming SB.** 2002. Orf virus-encoded interleukin-10 stimulates the proliferation of murine mast cells and inhibits cytokine synthesis in murine peritoneal macrophages. The Journal of general virology **83:**1049-1058.
140. **Chan A, Baird M, Mercer AA, Fleming SB.** 2006. Maturation and function of human dendritic cells are inhibited by orf virus-encoded interleukin-10. The Journal of general virology **87:**3177-3181.
141. **Wise LM, Stuart GS, Real NC, Fleming SB, Mercer AA.** 2014. Orf virus IL-10 accelerates wound healing while limiting inflammation and scarring. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society **22:**356-367.
142. **Fleming SB, Haig DM, Nettleton P, Reid HW, McCaughan CA, Wise LM, Mercer A.** 2000. Sequence and functional analysis of a homolog of interleukin-10 encoded by the parapoxvirus orf virus. Virus genes **21:**85-95.
143. **Savory LJ, Stacker SA, Fleming SB, Niven BE, Mercer AA.** 2000. Viral vascular endothelial growth factor plays a critical role in orf virus infection. Journal of virology **74:**10699-10706.
144. **Lyttle DJ, Fraser KM, Fleming SB, Mercer AA, Robinson AJ.** 1994. Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus. Journal of virology **68:**84-92.
145. **Wise LM, Veikkola T, Mercer AA, Savory LJ, Fleming SB, Caesar C, Vitali A, Makinen T, Alitalo K, Stacker SA.** 1999. Vascular endothelial growth factor (VEGF)-like protein from orf virus NZ2 binds to VEGFR2 and neuropilin-1. Proceedings of the National Academy of Sciences of the United States of America **96:**3071-3076.
146. **Meyer M, Clauss M, Lepple-Wienhues A, Waltenberger J, Augustin HG, Ziche M, Lanz C, Buttner M, Rziha HJ, Dehio C.** 1999. A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. The EMBO journal **18:**363-374.
147. **Cottone R, Buttner M, McInnes CJ, Wood AR, Rziha HJ.** 2002. Orf virus encodes a functional dUTPase gene. The Journal of general virology **83:**1043-1048.

148. **Liang X, Chow B, Babiuk LA.** 1997. Study of immunogenicity and virulence of bovine herpesvirus 1 mutants deficient in the UL49 homolog, UL49.5 homolog and dUTPase genes in cattle. Vaccine **15:**1057-1064.
149. **Jons A, Gerdts V, Lange E, Kaden V, Mettenleiter TC.** 1997. Attenuation of dUTPase-deficient pseudorabies virus for the natural host. Veterinary microbiology **56:**47-54.
150. **Haig DM, McInnes C, Deane D, Reid H, Mercer A.** 1997. The immune and inflammatory response to orf virus. Comparative immunology, microbiology and infectious diseases **20:**197-204.
151. **McKeever DJ, Reid HW, Inglis NF, Herring AJ.** 1987. A qualitative and quantitative assessment of the humoral antibody response of the sheep to orf virus infection. Veterinary microbiology **15:**229-241.
152. **Weber O, Mercer AA, Friebe A, Knolle P, Volk HD.** 2013. Therapeutic immunomodulation using a virus--the potential of inactivated orf virus. Eur J Clin Microbiol Infect Dis **32:**451-460.
153. **Fachinger V, Schlapp T, Strube W, Schmeer N, Saalmuller A.** 2000. Poxvirus-induced immunostimulating effects on porcine leukocytes. Journal of virology **74:**7943-7951.
154. **Schutze N, Raue R, Buttner M, Alber G.** 2009. Inactivated parapoxvirus ovis activates canine blood phagocytes and T lymphocytes. Veterinary microbiology **137:**260-267.
155. **Weber O, Siegling A, Friebe A, Limmer A, Schlapp T, Knolle P, Mercer A, Schaller H, Volk HD.** 2003. Inactivated parapoxvirus ovis (Orf virus) has antiviral activity against hepatitis B virus and herpes simplex virus. The Journal of general virology **84:**1843-1852.
156. **Fiebig HH, Siegling A, Volk HD, Friebe A, Knolle P, Limmer A, Weber O.** 2011. Inactivated orf virus (Parapoxvirus ovis) induces antitumoral activity in transplantable tumor models. Anticancer research **31:**4185-4190.
157. **Rintoul JL, Lemay CG, Tai LH, Stanford MM, Falls TJ, de Souza CT, Bridle BW, Daneshmand M, Ohashi PS, Wan Y, Lichty BD, Mercer AA, Auer RC, Atkins HL, Bell JC.** 2012. ORFV: a novel oncolytic and immune stimulating parapoxvirus therapeutic. Molecular therapy : the journal of the American Society of Gene Therapy **20:**1148-1157.
158. **Friebe A, Friederichs S, Scholz K, Janssen U, Scholz C, Schlapp T, Mercer A, Siegling A, Volk HD, Weber O.** 2011. Characterization of immunostimulatory components of orf virus (parapoxvirus ovis). The Journal of general virology **92:**1571-1584.
159. **Mayr A, Herlyn M, Mahnel H, Danco A, Zach A, Bostedt H.** 1981. [Control of ecthyma contagiosum (pustular dermatitis) of sheep with a new parenteral cell culture live vaccine]. Zentralblatt fur Veterinarmedizin. Reihe B. Journal of veterinary medicine. Series B **28:**535-552.
160. **Cottone R, Buttner M, Bauer B, Henkel M, Hettich E, Rziha HJ.** 1998. Analysis of genomic rearrangement and subsequent gene deletion of the attenuated Orf virus strain D1701. Virus Res **56:**53-67.
161. **Rziha HJ, Henkel M, Cottone R, Meyer M, Dehio C, Buttner M.** 1999. Parapoxviruses: potential alternative vectors for directing the immune response in permissive and non-permissive hosts. Journal of biotechnology **73:**235-242.
162. **Rziha H, Henkel M, Cottone R, Bauer B, Auge U, Gotz F, Pfaff E, Rottgen M, Dehio C, Buttner M.** 2000. Generation of recombinant parapoxviruses:

non-essential genes suitable for insertion and expression of foreign genes. J Biotechnol **83:**137-145.

163. **Henkel M, Planz O, Fischer T, Stitz L, Rziha HJ.** 2005. Prevention of virus persistence and protection against immunopathology after Borna disease virus infection of the brain by a novel Orf virus recombinant. Journal of virology **79:**314-325.
164. **Dory D, Fischer T, Beven V, Cariolet R, Rziha HJ, Jestin A.** 2006. Prime-boost immunization using DNA vaccine and recombinant Orf virus protects pigs against Pseudorabies virus (Herpes suid 1). Vaccine **24:**6256-6263.
165. **Voigt H, Merant C, Wienhold D, Braun A, Hutet E, Le Potier MF, Saalmuller A, Pfaff E, Buttner M.** 2007. Efficient priming against classical swine fever with a safe glycoprotein E2 expressing Orf virus recombinant (ORFV VrV-E2). Vaccine **25:**5915-5926.
166. **Amann R, Rohde J, Wulle U, Conlee D, Raue R, Martinon O, Rziha HJ.** 2013. A new rabies vaccine based on a recombinant ORF virus (parapoxvirus) expressing the rabies virus glycoprotein. Journal of virology **87:**1618-1630.
167. **van Rooij EM, Rijsewijk FA, Moonen-Leusen HW, Bianchi AT, Rziha HJ.** 2010. Comparison of different prime-boost regimes with DNA and recombinant Orf virus based vaccines expressing glycoprotein D of pseudorabies virus in pigs. Vaccine **28:**1808-1813.
168. **Rohde J, Schirrmeier H, Granzow H, Rziha HJ.** 2011. A new recombinant Orf virus (ORFV, Parapoxvirus) protects rabbits against lethal infection with rabbit hemorrhagic disease virus (RHDV). Vaccine **29:**9256-9264.
169. **Rohde J, Amann R, Rziha HJ.** 2013. New Orf virus (Parapoxvirus) recombinant expressing H5 hemagglutinin protects mice against H5N1 and H1N1 influenza A virus. PLoS One **8:**e83802.
170. **Sun Y, Luo Y, Wang CH, Yuan J, Li N, Song K, Qiu HJ.** 2016. Control of swine pseudorabies in China: Opportunities and limitations. Veterinary microbiology **183:**119-124.
171. **Tan JL, Ueda N, Heath D, Mercer AA, Fleming SB.** 2012. Development of orf virus as a bifunctional recombinant vaccine: surface display of Echinococcus granulosus antigen EG95 by fusion to membrane structural proteins. Vaccine **30:**398-406.
172. **Hain KS, Joshi LR, Okda F, Nelson J, Singrey A, Lawson S, Martins M, Pillatzki A, Kutish G, Nelson EA, Flores EF, Diel DG.** 2016. Immunogenicity of a Recombinant Parapoxvirus Expressing the Spike Protein of Porcine Epidemic Diarrhea Virus. The Journal of general virology.
173. **Rziha HJ, Rohde J, Amann R.** 2016. Generation and Selection of Orf Virus (ORFV) Recombinants. Methods in molecular biology **1349:**177-200.
174. **Zeitelhofer M, Vessey JP, Xie Y, Tubing F, Thomas S, Kiebler M, Dahm R.** 2007. High-efficiency transfection of mammalian neurons via nucleofection. Nature protocols **2:**1692-1704.
175. **Gurskaya NG, Fradkov AF, Pounkova NI, Staroverov DB, Bulina ME, Yanushevich YG, Labas YA, Lukyanov S, Lukyanov KA.** 2003. A colourless green fluorescent protein homologue from the non-fluorescent hydromedusa Aequorea coerulescens and its fluorescent mutants. The Biochemical journal **373:**403-408.
176. **Ning Z, Peng Y, Hao W, Duan C, Rock DL, Luo S.** 2011. Generation of recombinant Orf virus using an enhanced green fluorescent protein reporter gene as a selectable marker. BMC veterinary research **7:**80.

177. **Kaplon J, Fremy C, Bernard S, Rehby L, Aho S, Pothier P, Ambert-Balay K.** 2013. Impact of rotavirus vaccine on rotavirus genotypes and caliciviruses circulating in French cattle. Vaccine **31:**2433-2440.
178. **Greenberg HB, Estes MK.** 2009. Rotaviruses: from pathogenesis to vaccination. Gastroenterology **136:**1939-1951.
179. **Zade JK, Kulkarni PS, Desai SA, Sabale RN, Naik SP, Dhere RM.** 2014. Bovine rotavirus pentavalent vaccine development in India. Vaccine **32 Suppl 1:**A124-128.
180. **Papp H, Laszlo B, Jakab F, Ganesh B, De Grazia S, Matthijnssens J, Ciarlet M, Martella V, Banyai K.** 2013. Review of group A rotavirus strains reported in swine and cattle. Veterinary microbiology **165:**190-199.
181. **Estes MK, Graham DY, Mason BB.** 1981. Proteolytic enhancement of rotavirus infectivity: molecular mechanisms. Journal of virology **39:**879-888.
182. **Denisova E, Dowling W, LaMonica R, Shaw R, Scarlata S, Ruggeri F, Mackow ER.** 1999. Rotavirus capsid protein VP5* permeabilizes membranes. Journal of virology **73:**3147-3153.
183. **Crouch CF, Oliver S, Francis MJ.** 2001. Serological, colostral and milk responses of cows vaccinated with a single dose of a combined vaccine against rotavirus, coronavirus and Escherichia coli F5 (K99). The Veterinary record **149:**105-108.
184. **Saif LJ, Fernandez FM.** 1996. Group A rotavirus veterinary vaccines. The Journal of infectious diseases **174 Suppl 1:**S98-106.
185. **Tang B, Gilbert JM, Matsui SM, Greenberg HB.** 1997. Comparison of the rotavirus gene 6 from different species by sequence analysis and localization of subgroup-specific epitopes using site-directed mutagenesis. Virology **237:**89-96.
186. **Maclachlan NJ.** 2011. Bluetongue: history, global epidemiology, and pathogenesis. Preventive veterinary medicine **102:**107-111.
187. **Maan S, Maan NS, van Rijn PA, van Gennip RG, Sanders A, Wright IM, Batten C, Hoffmann B, Eschbaumer M, Oura CA, Potgieter AC, Nomikou K, Mertens PP.** 2010. Full genome characterisation of bluetongue virus serotype 6 from the Netherlands 2008 and comparison to other field and vaccine strains. PLoS One **5:**e10323.
188. **Maan NS, Maan S, Belaganahalli MN, Ostlund EN, Johnson DJ, Nomikou K, Mertens PP.** 2012. Identification and differentiation of the twenty six bluetongue virus serotypes by RT-PCR amplification of the serotype-specific genome segment 2. PLoS One **7:**e32601.
189. **Roy P.** 1992. Bluetongue virus proteins. The Journal of general virology **73 (Pt 12):**3051-3064.
190. **Calvo-Pinilla E, Castillo-Olivares J, Jabbar T, Ortego J, de la Poza F, Marin-Lopez A.** 2014. Recombinant vaccines against bluetongue virus. Virus Res **182:**78-86.
191. **Rojas JM, Rodriguez-Calvo T, Pena L, Sevilla N.** 2011. T cell responses to bluetongue virus are directed against multiple and identical CD4+ and CD8+ T cell epitopes from the VP7 core protein in mouse and sheep. Vaccine **29:**6848-6857.
192. **Andrew M, Whiteley P, Janardhana V, Lobato Z, Gould A, Coupar B.** 1995. Antigen specificity of the ovine cytotoxic T lymphocyte response to bluetongue virus. Vet Immunol Immunopathol **47:**311-322.

193. **Jeggo MH, Wardley RC, Brownlie J.** 1984. A study of the role of cell-mediated immunity in bluetongue virus infection in sheep, using cellular adoptive transfer techniques. Immunology **52:**403-410.
194. **Jeggo MH, Wardley RC, Taylor WP.** 1984. Role of neutralising antibody in passive immunity to bluetongue infection. Res Vet Sci **36:**81-86.
195. **Guthrie AJ.** 2007. African horse sickness, p. 164-171. *In* Sellon D.C. LMT (ed.), Equine infectious diseases. Saunders Elsevier, St, Louis.
196. **Maclachlan NJ, Guthrie AJ.** 2010. Re-emergence of bluetongue, African horse sickness, and other orbivirus diseases. Veterinary research **41:**35.
197. **Kanai Y, van Rijn PA, Maris-Veldhuis M, Kaname Y, Athmaram TN, Roy P.** 2014. Immunogenicity of recombinant VP2 proteins of all nine serotypes of African horse sickness virus. Vaccine **32:**4932-4937.
198. **Mellor PS, Hamblin C.** 2004. African horse sickness. Veterinary research **35:**445-466.
199. **Maree S, Maree FF, Putterill JF, de Beer TA, Huismans H, Theron J.** 2016. Synthesis of empty african horse sickness virus particles. Virus Res **213:**184-194.
200. **van de Water SG, van Gennip RG, Potgieter CA, Wright IM, van Rijn PA.** 2015. VP2 Exchange and NS3/NS3a Deletion in African Horse Sickness Virus (AHSV) in Development of Disabled Infectious Single Animal Vaccine Candidates for AHSV. Journal of virology **89:**8764-8772.
201. **Jamal SM, Belsham GJ.** 2013. Foot-and-mouth disease: past, present and future. Veterinary research **44:**116.
202. **Rijks JM, Cito F, Cunningham AA, Rantsios AT, Giovannini A.** 2015. Disease Risk Assessments Involving Companion Animals: an Overview for 15 Selected Pathogens Taking a European Perspective. Journal of comparative pathology.
203. **Smith MT, Bennett AM, Grubman MJ, Bundy BC.** 2014. Foot-and-mouth disease: technical and political challenges to eradication. Vaccine **32:**3902-3908.
204. **Valarcher JF, Leforban Y, Rweyemamu M, Roeder PL, Gerbier G, Mackay DK, Sumption KJ, Paton DJ, Knowles NJ.** 2008. Incursions of foot-and-mouth disease virus into Europe between 1985 and 2006. Transboundary and emerging diseases **55:**14-34.
205. **Arzt J, Juleff N, Zhang Z, Rodriguez LL.** 2011. The pathogenesis of foot-and-mouth disease I: viral pathways in cattle. Transboundary and emerging diseases **58:**291-304.
206. **Grubman MJ, Baxt B.** 2004. Foot-and-mouth disease. Clinical microbiology reviews **17:**465-493.
207. **Paton DJ, Sumption KJ, Charleston B.** 2009. Options for control of foot-and-mouth disease: knowledge, capability and policy. Philosophical transactions of the Royal Society of London. Series B, Biological sciences **364:**2657-2667.
208. **Reid SM, Ferris NP, Hutchings GH, Zhang Z, Belsham GJ, Alexandersen S.** 2002. Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay. Journal of virological methods **105:**67-80.
209. **Rodriguez LL, Grubman MJ.** 2009. Foot and mouth disease virus vaccines. Vaccine **27 Suppl 4:**D90-94.
210. **Cao Y, Lu Z, Liu Z.** 2016. Foot-and-mouth disease vaccines: progress and problems. Expert review of vaccines **15:**783-789.

211. **Pacheco JM, Brum MC, Moraes MP, Golde WT, Grubman MJ.** 2005. Rapid protection of cattle from direct challenge with foot-and-mouth disease virus (FMDV) by a single inoculation with an adenovirus-vectored FMDV subunit vaccine. Virology **337:**205-209.
212. **Cubillos C, de la Torre BG, Jakab A, Clementi G, Borras E, Barcena J, Andreu D, Sobrino F, Blanco E.** 2008. Enhanced mucosal immunoglobulin A response and solid protection against foot-and-mouth disease virus challenge induced by a novel dendrimeric peptide. Journal of virology **82:**7223-7230.
213. **Nerenberg BT, Taylor J, Bartee E, Gouveia K, Barry M, Fruh K.** 2005. The poxviral RING protein p28 is a ubiquitin ligase that targets ubiquitin to viral replication factories. Journal of virology **79:**597-601.
214. **Resch W, Weisberg AS, Moss B.** 2005. Vaccinia virus nonstructural protein encoded by the A11R gene is required for formation of the virion membrane. Journal of virology **79:**6598-6609.
215. **Fischer L, Le Gros FX, Mason PW, Paoletti E.** 1997. A recombinant canarypox virus protects rabbits against a lethal rabbit hemorrhagic disease virus (RHDV) challenge. Vaccine **15:**90-96.
216. **Bertagnoli S, Gelfi J, Petit F, Vautherot JF, Rasschaert D, Laurent S, Le Gall G, Boilletot E, Chantal J, Boucraut-Baralon C.** 1996. Protection of rabbits against rabbit viral haemorrhagic disease with a vaccinia-RHDV recombinant virus. Vaccine **14:**506-510.
217. **Parra F, Prieto M.** 1990. Purification and characterization of a calicivirus as the causative agent of a lethal hemorrhagic disease in rabbits. Journal of virology **64:**4013-4015.
218. **Barcena J, Morales M, Vazquez B, Boga JA, Parra F, Lucientes J, Pages-Mante A, Sanchez-Vizcaino JM, Blasco R, Torres JM.** 2000. Horizontal transmissible protection against myxomatosis and rabbit hemorrhagic disease by using a recombinant myxoma virus. Journal of virology **74:**1114-1123.
219. **Laurent S, Vautherot JF, Madelaine MF, Le Gall G, Rasschaert D.** 1994. Recombinant rabbit hemorrhagic disease virus capsid protein expressed in baculovirus self-assembles into viruslike particles and induces protection. Journal of virology **68:**6794-6798.
220. **Lindesmith L, Moe C, Lependu J, Frelinger JA, Treanor J, Baric RS.** 2005. Cellular and humoral immunity following Snow Mountain virus challenge. Journal of virology **79:**2900-2909.
221. **Friebe A, Siegling A, Friederichs S, Volk HD, Weber O.** 2004. Immunomodulatory effects of inactivated parapoxvirus ovis (ORF virus) on human peripheral immune cells: induction of cytokine secretion in monocytes and Th1-like cells. Journal of virology **78:**9400-9411.
222. **WHO** 2016, posting date. http://www.who.int/influenza/human_animal_interface/avian_influenza/h5n1_research/faqs/en/. [Online.]
223. **Tao H, Steel J, Lowen AC.** 2014. Intrahost dynamics of influenza virus reassortment. Journal of virology **88:**7485-7492.
224. **Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, Uiprasertkul M, Boonnak K, Pittayawonganon C, Cox NJ, Zaki SR, Thawatsupha P, Chittaganpitch M, Khontong R, Simmerman JM, Chunsutthiwat S.** 2005. Probable person-to-person transmission of avian influenza A (H5N1). The New England journal of medicine **352:**333-340.

225. **Mayrhofer J, Coulibaly S, Hessel A, Holzer GW, Schwendinger M, Bruhl P, Gerencer M, Crowe BA, Shuo S, Hong W, Tan YJ, Dietrich B, Sabarth N, Savidis-Dacho H, Kistner O, Barrett PN, Falkner FG.** 2009. Nonreplicating vaccinia virus vectors expressing the H5 influenza virus hemagglutinin produced in modified Vero cells induce robust protection. Journal of virology **83:**5192-5203.
226. **Ulmer JB, Fu TM, Deck RR, Friedman A, Guan L, DeWitt C, Liu X, Wang S, Liu MA, Donnelly JJ, Caulfield MJ.** 1998. Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. Journal of virology **72:**5648-5653.
227. **Rimmelzwaan GF, Kreijtz JH, Bodewes R, Fouchier RA, Osterhaus AD.** 2009. Influenza virus CTL epitopes, remarkably conserved and remarkably variable. Vaccine **27:**6363-6365.
228. **LaMere MW, Lam HT, Moquin A, Haynes L, Lund FE, Randall TD, Kaminski DA.** 2011. Contributions of antinucleoprotein IgG to heterosubtypic immunity against influenza virus. Journal of immunology **186:**4331-4339.
229. **Hessel A, Schwendinger M, Holzer GW, Orlinger KK, Coulibaly S, Savidis-Dacho H, Zips ML, Crowe BA, Kreil TR, Ehrlich HJ, Barrett PN, Falkner FG.** 2011. Vectors based on modified vaccinia Ankara expressing influenza H5N1 hemagglutinin induce substantial cross-clade protective immunity. PLoS One **6:**e16247.
230. **Guo H, Santiago F, Lambert K, Takimoto T, Topham DJ.** 2011. T cell-mediated protection against lethal 2009 pandemic H1N1 influenza virus infection in a mouse model. Journal of virology **85:**448-455.
231. **Laidlaw BJ, Decman V, Ali MA, Abt MC, Wolf AI, Monticelli LA, Mozdzanowska K, Angelosanto JM, Artis D, Erikson J, Wherry EJ.** 2013. Cooperativity between CD8+ T cells, non-neutralizing antibodies, and alveolar macrophages is important for heterosubtypic influenza virus immunity. PLoS Pathog **9:**e1003207.
232. **Kreijtz JH, Suezer Y, de Mutsert G, van Amerongen G, Schwantes A, van den Brand JM, Fouchier RA, Lower J, Osterhaus AD, Sutter G, Rimmelzwaan GF.** 2009. MVA-based H5N1 vaccine affords cross-clade protection in mice against influenza A/H5N1 viruses at low doses and after single immunization. PLoS One **4:**e7790.
233. **Swain SL, McKinstry KK, Strutt TM.** 2012. Expanding roles for CD4(+) T cells in immunity to viruses. Nat Rev Immunol **12:**136-148.
234. **Droebner K, Haasbach E, Fuchs C, Weinzierl AO, Stevanovic S, Buttner M, Planz O.** 2008. Antibodies and CD4(+) T-cells mediate cross-protection against H5N1 influenza virus infection in mice after vaccination with a low pathogenic H5N2 strain. Vaccine **26:**6965-6974.
235. **Mettenleiter TC.** 2000. Aujeszky's disease (pseudorabies) virus: the virus and molecular pathogenesis--state of the art, June 1999. Veterinary research **31:**99-115.
236. **Roizman B. PPE.** 2001. The family herpesviridae: a brief introduction, p. 2381-2397, Fields Virology, 4th ed.
237. **Pomeranz LE, Reynolds AE, Hengartner CJ.** 2005. Molecular biology of pseudorabies virus: impact on neurovirology and veterinary medicine. Microbiology and molecular biology reviews : MMBR **69:**462-500.
238. **Koppers-Lalic D, Verweij MC, Lipinska AD, Wang Y, Quinten E, Reits EA, Koch J, Loch S, Marcondes Rezende M, Daus F, Bienkowska-Szewczyk**

K, Osterrieder N, Mettenleiter TC, Heemskerk MH, Tampe R, Neefjes JJ, Chowdhury SI, Ressing ME, Rijsewijk FA, Wiertz EJ. 2008. Varicellovirus UL 49.5 proteins differentially affect the function of the transporter associated with antigen processing, TAP. PLoS Pathog **4:**e1000080.

239. **Muller T, Hahn EC, Tottewitz F, Kramer M, Klupp BG, Mettenleiter TC, Freuling C.** 2011. Pseudorabies virus in wild swine: a global perspective. Archives of virology **156:**1691-1705.
240. **Lomniczi B, Blankenship ML, Ben-Porat T.** 1984. Deletions in the genomes of pseudorabies virus vaccine strains and existence of four isomers of the genomes. Journal of virology **49:**970-979.
241. **An TQ, Peng JM, Tian ZJ, Zhao HY, Li N, Liu YM, Chen JZ, Leng CL, Sun Y, Chang D, Tong GZ.** 2013. Pseudorabies virus variant in Bartha-K61-vaccinated pigs, China, 2012. Emerg Infect Dis **19:**1749-1755.
242. **Andries K, Pensaert MB, Vandeputte J.** 1978. Effect of experimental infection with pseudorabies (Aujeszky's disease) virus on pigs with maternal immunity from vaccinated sows. American journal of veterinary research **39:**1282-1285.
243. **Mettenleiter TC.** 1996. Immunobiology of pseudorabies (Aujeszky's disease). Vet Immunol Immunopathol **54:**221-229.
244. **Ober BT, Teufel B, Wiesmuller KH, Jung G, Pfaff E, Saalmuller A, Rziha HJ.** 2000. The porcine humoral immune response against pseudorabies virus specifically targets attachment sites on glycoprotein gC. Journal of virology **74:**1752-1760.
245. **Ober BT, Summerfield A, Mattlinger C, Wiesmuller KH, Jung G, Pfaff E, Saalmuller A, Rziha HJ.** 1998. Vaccine-induced, pseudorabies virus-specific, extrathymic CD4+CD8+ memory T-helper cells in swine. Journal of virology **72:**4866-4873.
246. **Kitamura D, Roes J, Kuhn R, Rajewsky K.** 1991. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. Nature **350:**423-426.
247. **Stitz L, Althage A, Hengartner H, Zinkernagel R.** 1985. Natural killer cells vs cytotoxic T cells in the peripheral blood of virus-infected mice. Journal of immunology **134:**598-602.
248. **Grabowska AK, Lipinska AD, Rohde J, Szewczyk B, Bienkowska-Szewczyk K, Rziha HJ.** 2009. New baculovirus recombinants expressing Pseudorabies virus (PRV) glycoproteins protect mice against lethal challenge infection. Vaccine **27:**3584-3591.
249. **Stitz L, Baenziger J, Pircher H, Hengartner H, Zinkernagel RM.** 1986. Effect of rabbit anti-asialo GM1 treatment in vivo or with anti-asialo GM1 plus complement in vitro on cytotoxic T cell activities. Journal of immunology **136:**4674-4680.
250. **Mayr A, Buttner M, Wolf G, Meyer H, Czerny C.** 1989. [Experimental detection of the paraspecific effects of purified and inactivated poxviruses]. Zentralblatt fur Veterinarmedizin. Reihe B. Journal of veterinary medicine. Series B **36:**81-99.
251. **Siciliano NA, Hersperger AR, Lacuanan AM, Xu RH, Sidney J, Sette A, Sigal LJ, Eisenlohr LC.** 2014. Impact of distinct poxvirus infections on the specificities and functionalities of CD4+ T cell responses. Journal of virology **88:**10078-10091.

252. **Agarwal P, Raghavan A, Nandiwada SL, Curtsinger JM, Bohjanen PR, Mueller DL, Mescher MF.** 2009. Gene regulation and chromatin remodeling by IL-12 and type I IFN in programming for CD8 T cell effector function and memory. Journal of immunology **183:**1695-1704.
253. **Haig DM, Deane DL, Myatt N, Thomson J, Entrican G, Rothel J, Reid HW.** 1996. The activation status of ovine CD45R+ and CD45R- efferent lymph T cells after orf virus reinfection. Journal of comparative pathology **115:**163-174.
254. **Volz A, Langenmayer M, Jany S, Kalinke U, Sutter G.** 2014. Rapid expansion of CD8+ T cells in wild-type and type I interferon receptor-deficient mice correlates with protection after low-dose emergency immunization with modified vaccinia virus Ankara. Journal of virology **88:**10946-10957.
255. **Kremer M, Suezer Y, Volz A, Frenz T, Majzoub M, Hanschmann KM, Lehmann MH, Kalinke U, Sutter G.** 2012. Critical role of perforin-dependent CD8+ T cell immunity for rapid protective vaccination in a murine model for human smallpox. PLoS Pathog **8:**e1002557.
256. **Delaloye J, Roger T, Steiner-Tardivel QG, Le Roy D, Knaup Reymond M, Akira S, Petrilli V, Gomez CE, Perdiguero B, Tschopp J, Pantaleo G, Esteban M, Calandra T.** 2009. Innate immune sensing of modified vaccinia virus Ankara (MVA) is mediated by TLR2-TLR6, MDA-5 and the NALP3 inflammasome. PLoS Pathog **5:**e1000480.
257. **Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A.** 2001. Interleukin-10 and the interleukin-10 receptor. Annual review of immunology **19:**683-765.
258. **Smith GL, Benfield CT, Maluquer de Motes C, Mazzon M, Ember SW, Ferguson BJ, Sumner RP.** 2013. Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity. The Journal of general virology **94:**2367-2392.
259. **Kirchhausen T.** 2000. Three ways to make a vesicle. Nature reviews. Molecular cell biology **1:**187-198.
260. **Abendroth A, Lin I, Slobedman B, Ploegh H, Arvin AM.** 2001. Varicella-zoster virus retains major histocompatibility complex class I proteins in the Golgi compartment of infected cells. Journal of virology **75:**4878-4888.
261. **Quiner CA, Jackson WT.** 2010. Fragmentation of the Golgi apparatus provides replication membranes for human rhinovirus 1A. Virology **407:**185-195.
262. **Tan JL, Ueda N, Mercer AA, Fleming SB.** 2009. Investigation of orf virus structure and morphogenesis using recombinants expressing FLAG-tagged envelope structural proteins: evidence for wrapped virus particles and egress from infected cells. The Journal of general virology **90:**614-625.
263. **Ashrafi GH, Haghshenas MR, Marchetti B, O'Brien PM, Campo MS.** 2005. E5 protein of human papillomavirus type 16 selectively downregulates surface HLA class I. Int J Cancer **113:**276-283.
264. **Pierini R, Cottam E, Roberts R, Wileman T.** 2009. Modulation of membrane traffic between endoplasmic reticulum, ERGIC and Golgi to generate compartments for the replication of bacteria and viruses. Semin Cell Dev Biol **20:**828-833.
265. **Zhang L, Lee SY, Beznoussenko GV, Peters PJ, Yang JS, Gilbert HY, Brass AL, Elledge SJ, Isaacs SN, Moss B, Mironov A, Hsu VW.** 2009. A role for the host coatomer and KDEL receptor in early vaccinia biogenesis. Proceedings of the National Academy of Sciences of the United States of America **106:**163-168.

266. **Yilla M, Tan A, Ito K, Miwa K, Ploegh HL.** 1993. Involvement of the vacuolar H(+)-ATPases in the secretory pathway of HepG2 cells. J Biol Chem **268:**19092-19100.

8 Anhang

Publikationen

Chapter 12

Generation and Selection of Orf Virus (ORFV) Recombinants

Hanns-Joachim Rziha, Jörg Rohde, and Ralf Amann

Abstract

Orf virus (ORFV) is an epitheliotropic poxvirus, which belongs to the genus *Parapoxvirus*. Among them the highly attenuated, apathogenic strain D1701-V is regarded as a promising candidate for novel virus vector vaccines. Our recent work demonstrated that those ORFV-based recombinants were able to induce protective, long-lasting immunity in various hosts that are non-permissive for ORFV. In this chapter we describe procedures for the generation, selection, propagation, and titration of ORFV recombinants as well as transgene detection by PCR or immunohistochemical staining.

Key words Orf virus (ORFV), Parapoxvirus, Recombinant vector vaccine

1 Introduction

Poxviruses are used manifold as viral vectors. Vaccinia virus (VACV) was among the earliest eukaryotic viruses to be engineered for expression of foreign genes already more than 30 years ago [1, 2], and also paved the way for the general development of recombinant viral vectors (reviewed in ref. [3]). Increasing knowledge of poxviral gene regulation and developments in molecular biological techniques commonly facilitated the generation of poxvirus recombinants including VACV, fowlpox virus, and canarypox virus (reviewed in ref. [4]). Subsequently, recombinant poxviruses also became attractive live vaccine vectors against various infectious diseases, for human gene therapy, and for anticancer immunotherapy [5–7].

The use of recombinant poxviruses as excellent candidate vaccine vectors is reasoned mainly by (1) their stability, (2) their large genomic size allowing flexible integration of multiple foreign genes, (3) their exclusive cytoplasmic gene expression, which is independent from the host cell machinery and therefore, (4) essentially no risk of integration into the host genome and subsequent insertional cellular gene inactivation, (5) the very low mutation

Alejandro Brun (ed.), *Vaccine Technologies for Veterinary Viral Diseases: Methods and Protocols*, Methods in Molecular Biology, vol. 1349, DOI 10.1007/978-1-4939-3008-1_12,

rates of the recombinants' genome, and (6) most importantly their ability to stimulate long-lasting transgene-specific B- and T-cell immunity. However, replication-competent attenuated VACV vectors caused some inadvertent, serious complications after immunization [6, 8]. Due to their inability of replication in mammalian cells, the highly attenuated VACV strains modified vaccinia virus Ankara (MVA) and NYVAC turned out to promising poxvirus vectors for human use [4, 8, 9]. Concern exists that the profound attenuation of MVA or NYVAC might be responsible for reduced immunogenicity observed in some clinical trials, and several strategies have used to enhance their immunogenic capacity [10–14].

Recently the *Parapoxvirus* Orf virus (ORFV) has been recognized as a valuable new virus vector system combining several important demands for a safe recombinant vector virus: The very restricted host range, the absence of systemic virus spread, the short-term vector-specific immunity and the lack of serum antibodies efficiently neutralizing ORFV enabling repeated immunizations, still unraveled immune-modulating properties, and the induction of strong and long-lasting immune responses against vector-encoded foreign antigens [15, 16]. During recent years, we demonstrated the successful use of this novel virus vector system based on the apathogenic, Vero cell culture-adapted highly attenuated ORFV strain D1701-V, which is used to generate recombinants by substituting the viral vegf-e gene with a foreign gene, and thereby removing an ORFV virulence gene [15, 17]. We have chosen the original vegf-e early ORFV promoter for the transgene control, which allows its expression before ORFV DNA replication and consequently does not need production of infectious progeny of ORFV recombinant. Therefore, transgene expression is also achieved in cells non-permissive for ORFV [18]. Adaption for growth in the non-ruminant cell line Vero led to additional genomic deletions, which are most probably responsible for the strongly reduced pathogenicity of D1701-V [17]. Various D1701-V recombinants were reported to mediate excellent protective immunity against a number of different viral infections [18–25].

Here we describe up-dated procedures for the generation and selection of recombinant ORFV derived from strain D1701-V or D1701-VrV. The protocols include two different selection techniques and optimized procedures for production, titration, and identification of recombinant ORFV.

1.1 General Considerations

Since poxvirus DNA is not infectious and the large genome is not efficiently taken up by cells, poxvirus recombinants must be generated by homologous recombination taking place between a transfer plasmid, which contains the transgene(s) of interest under the control of defined poxviral promoter, and flanking poxviral genomic DNA. To this end, cells infected with the parental ORFV

are transfected with the transfer plasmid at the approximate time point of ORFV DNA replication. Because infection of too many cells ultimately leads to high background of parental ORFV impeding efficient selection of new recombinants, low multiplicities of infection (moi) like for example 0.01–0.2 must be pretested. Consequently, the low moi demands for highest transfection rates allowing to target a sufficient number of infected cells with transfer plasmid DNA.

After assaying various transfection reagents and techniques including Lipofectamine, Fugene, or magnetofection, the best recombination rates were reproducibly obtained using nucleofection [26]. The electroporation-based principle of nucleofection allows most efficient transfection of different cell types either as cell monolayer or single cell suspension with high cell viability. To our knowledge two versions of nucleofector devices are available, either the original Amaxa nucleofector or the CLB-Transfection system, which both are used with excellent results.

To achieve highest possible titers of ORFV progeny, the so-called simultaneous ORFV infection is preferred to standard infection of monolayer cells. To this end, the necessary amount of ORFV is mixed together with the needed amount of trypsinized cells in growth medium and seeded directly into the culture dishes or flasks. Cells in best condition and regularly tested to be free of mycoplasma must be used not only for transfection.

2 Materials

2.1 Virus and Cell Culture

1. *Virus*: The attenuated ORFV strain D1701-V and the β-galactosidase expressing derivative D1701-VrV has been described earlier [18, 27].
2. *Vero cells*: The African green monkey kidney cell line was originally obtained from the ATCC (CCL-81).
3. *Growth medium*: Minimum Essential Medium (MEM) Eagle, supplemented with Earle's salt, nonessential amino acids, and l-glutamine, including 5 % fetal calf serum (FCS), 10^5 units penicillin per liter, and 100 mg streptomycin per liter.
4. *Versene-trypsin (VT)*: 0.125 % (w/v) trypsin, 0.025 % (w/v) EDTA, 0.4 % (w/v) NaCl, 0.01 % (w/v) KCl, 0.01 % (w/v) KH_2PO_4, 0.057 % (w/v) Na_2HPO_4 (*see* **Notes 1** and **2**).
5. *Fetal calf serum (FCS)*: Endotoxin-free, sterile filtrated, before use heat-inactivated (30 min, 56 °C) and stored at –20 °C.
6. *TB, Trypan-blue*: 0.25 % w/v TB in PBS.
7. *PBS*: Dulbecco's PBS, phosphate-buffered saline without Mg^{2+} and Ca^{2+}.
8. *384-well plate*: Perkin-Elmer, viewplate (OptiPlate), suited for fluorescence.

9. *Multi(12-)channel reagent reservoir*: Best with V-shaped troughs, which facilitates the serial dilution and mixing of virus lysate with cells etc.
10. *Nucleofection*: The nucleofection solutions and cuvettes are delivered with the kit.

2.2 Agarose Overlay

1. 2× MEM: or 2× medium T199, without phenol red.
2. *Low melting temperature (LMT) agarose*: Agarose is solved in aqua bidest. by boiling in a microwave to obtain 2 % (w/v), and after cooling down to approximately 37 °C distributed into 6 mL portions into tubes and subsequently sterilized by autoclaving. These portions can be stored tightly closed at RT (*see* **Note 3**).
3. *BluoGal*: Stock solution contains 30 mg/mL DMSO or DMF (Dimethyl-formamide), store at –20 °C in the dark, i.e., wrapped in aluminum foil. For use dilute 1:100 to obtain 0.3 mg/mL final concentration (*see* **Note 4**).

2.3 DNA

1. *The transfer plasmid* used for nucleofection should be of high quality, endotoxin-free.
2. *Phenol*: Tris-equilibrated phenol for molecular biological use.
3. *CIA*: Mixture of 24 parts of chloroform and 1 part isoamyl alcohol, nucleic acid grade.
4. *Glycogen-blue*: Life Technologies.
5. *7.5 M Ammonium acetate*: Autoclaved.
6. *Eppendorf tabletop centrifuge*, refrigerated.
7. *DNA isolation kit*: For instance Master Pure DNA isolation kit, Epicentre, including lysis buffer, RNase and proteinase K enzyme solution.
8. *Isopropanol*.
9. *Ethanol*: absolute and 70 % (v/v) in sterile H_2O; can be stored at –20 °C.
10. *Filter-tips* (aerosol-tight) recommended for PCR.

2.4 Immune Staining

1. *FALDH*: 16 % methanol-free formaldehyde, tightly sealed in glass ampoules.
2. *TBST (Tris-buffer saline plus Tween 20)*: Can be prepared from a 10× concentrated TBS stock solution (0.5 M Tris, 1.5 M NaCl solved in H_2O dest., pH adjusted with HCl to 7.4–7.6) by 1:10 dilution and adding Tween 20 to a final concentration of 0.05 % (v/v).
3. *Block solution*: TBST plus 10 % FCS or 10 % BSA (bovine serum albumin).

4. *Peroxidase staining substrate*: 1 mg DAB or DAB-black per mL 0.1 M Tris–HCl, pH 7.4, shortly before use add 0.01 % (v/v) H_2O_2.
5. Vector VIP stain kit is commercially available from Vector Laboratories.
6. *Beta Blue staining kit*: Available from Novagen-Merck.

3 Methods

3.1 Nucleofection of ORFV-Infected Cells

To obtain best possible transfection efficiency, Vero cells are seeded the day before and grown to an approximately 80 % confluent monolayer. After trypsinization (VT) the number of viable cells is determined by Trypan Blue (TB) exclusion, and cells are diluted in MEM plus 5 % (v/v) FCS to obtain 1.5×10^6 cells per mL. This is achieved as follows:

1. Pre-incubate the VT solution in a 37 °C water bath.
2. Pour off the medium of the fresh overnight cell culture (T75 flask).
3. Wash the cell layer once with 5 mL PBS or medium without FCS (tempered at 37 °C).
4. Rinse the monolayer with 2 mL VT, remove the VT completely with a pipette.
5. Add 1 mL fresh VT, slightly move the flask to distribute the VT solution evenly over the cell layer.
6. Place in a 37 °C incubator until cells start to detach (*see* also **Note 2**).
7. When monolayer cells are beginning to detach, some taps against the side of the flask help to dislodge the cells.
8. Add 0.5 mL FCS to stop trypsinization, vigorously suspend the cells by strongly flushing the solution on that side of the flask where the cells were grown.
9. Add 3.5 mL medium plus FCS, again vigorously suspend the cells.
10. Remove 50 µL of the cell suspension and mix with 50 µL of TB solution.
11. Pipette the mixture at the edge of a counting chamber covered with a coverslip to allow suspension to spread evenly by capillary action.
12. Place the chamber under a light microscope and count the viable, not stained cells in four large quadrants (*see* **Note 5**).
13. For each transfection, 1 mL containing 1.5×10^6 suspended Vero cells are infected with 3.0×10^5 plaque-forming units (PFU) of the ORFV strain D1701-VrV (moi = 0.2) and incu-

bated at 37 °C in a 2 mL Eppendorf cup under continuous slow rotation for 2 h (*see* **Note 6**).

14. Prepare a 6-well culture plate by filling 2.5 mL of MEM plus 5 % FCS per well for each nucleofection.
15. Let the nucleofection solution equilibrate to room temperature.
16. Two hours after infection (**step 13**) centrifuge the cell-virus suspension at 90×*g* for 10 min at room temperature and remove the supernatant completely (*see* **Note 7**).
17. Resuspend the cell pellet carefully with 0.1 mL nucleofection solution.
18. Add immediately 2 μg plasmid DNA, gently mix and transfer into a cuvette included in the nucleofection kit avoiding air bubbles (*see* **Note 8**).
19. Close the cuvette and insert it into the cuvette holder of the apparatus.
20. Select the correct pulse program for Vero cells and start.
21. After the pulse is finished, remove the cuvette from the holder, and add 0.2–0.5 mL of the pre-warmed medium-FCS under a laminar flow hood by using sterile Pasteur pipettes.
22. Gently transfer the solution back to the well of the 6-well plate and incubate at 37 °C, 5 % CO_2.
23. After 2–5 days, up to 80 % cpe (cytopathogenic effect) should be recognized.
24. Medium and detached cells from each well are transferred to sterile tubes and placed on ice.
25. Cells remaining on the bottom of the well are harvested by treatment with 0.5 mL VT and combined with the medium from **step 24**.
26. The obtained nucleofection-cell lysates (NL) are broken by three times freezing at −70 °C and alternating briefly thawing at 37 °C.
27. Preferably the NL is sonicated (100 W) 5–7 times 20 s on ice (with a 10 s break between) to release infectious virus, and stored at −70 °C until use.

3.2 Selection of lacZ-Negative Recombinant Virus

The following procedure describes our original protocol for selecting new recombinants starting with the parental β-galactosidase expressing, blue ORFV D1701-VrV [18, 23, 24].

1. The nucleofection lysate (NL; as described in Subheading 3.1) is thawed on ice.
2. Five dilutions of NL (1:4–1:2500) are prepared on ice in PBS or medium (*see* **Note 9**).

3. For each NL dilution 3×10^5 Vero cells in 2 mL MEM plus 5 % FCS are freshly prepared as described in Subheading 3.1.
4. Added per well of a 6-well plate and mixed with 0.1 mL of each NL dilution. One well receiving non-infected cells is used as negative control.
5. After approx. 3 days incubation at 5 % CO_2, 37 °C plaques should become visible.
6. Pre-warm 2× MEM in 37 °C water bath.
7. For each well of a 6-well plate 1.5–2.0 mL agarose overlay is needed, i.e., 12 mL agarose-medium-BluoGal overlay must be prepared per plate.
8. To this end boil one 6 mL portion of LMT agarose followed by equilibrating to approximately 37 °C in a water bath (*see* also **Note 2**).
9. Thoroughly mix with 6 mL of 2× MEM equilibrated at 37 °C, but be careful to avoid creating air bubbles.
10. Finally mix 0.12 mL of the BluoGal stock to obtain 0.3 mg/mL final concentration.
11. Carefully remove the medium, take care not to let dry out the cells, and slowly pour 2 mL of the overlay from the edge of each well followed by carefully moving the plate to prepare an evenly distributed overlay covering the complete cell monolayer.
12. Let the overlay harden for a short time at room temperature (*see* also **Note 4**), before incubating at 5 % CO_2, 37 °C.
13. Blue parental D1701-VrV plaques should become visible after 4–48 h.
14. Isolation of potentially new recombinant plaques: Identify single, colorless plaques under the light microscope, and label them with a lab pen outside on the bottom of the well.
15. For each virus plaque fill 0.2 mL PBS per well of a 48-well plate.
16. Under the sterile work bench pick the plaques for example with a sterile Pasteur pipette.
17. Transfer each plaque agarose block into the PBS of the individual wells.
18. Incubate the 48-well plate overnight at 4 °C to elute virus from the agarose blocks.
19. Next day, freshly prepare Vero cells (*see* Subheading 3.1), and add 0.5 mL MEM plus 5 % FCS containing 1×10^5 cells to each well containing the picked plaques.
20. Incubate the plate at 37 °C, 5 % CO_2 for 3–5 days until cpe or plaque formation becomes visible (*see* **Note 10**).

21. The plate is frozen and thawed three times (−70 °C, 37 °C), in order to break cells and to release virus.
22. Medium and cells are harvested from each well and stored at −70 °C until used for: PCR screening (Subheading 3.6), virus plaque titration (Subheading 3.8), X-gal staining, and additional plaque-purification by re-isolation of white plaques (*see* **Note 11**).

    For DNA preparation 0.1 mL of the lysates can be used, too (Subheadings 3.5 and 3.6).

3.3 Fluorescence-Based Positive Selection

As compared to the blue-white screening described in Subheading 3.2, the positive selection based for instance on fluorescence has several advantages:

- Less expensive and laborious, no agarose overlay containing special substrate (like X-Gal) is necessary.
- The fluorescent signal can be detected earlier and faster.
- The initial screening for new fluorescence-positive recombinants can be performed by limiting dilution procedure, which is clearly easier and faster. The strategy of the "endpoint-dilution" method is to detect in the best case a dilution of the NL, which contains per well one single pfu, here derived from one fluorescent recombinant infectious particle.
- Several fluorescent colors can be used, which might facilitate the generation of polyvalent vectors. Various fluorescent marker genes are commercially available, which can be used not only as marker genes expressed alone or in addition to another gene of interest, but of course also for tagging or fusing with the foreign gene.

The nucleofection of Vero cells infected with D1701-VrV (moi = 0.2) is performed exactly as described in Subheading 3.1. In the following procedure the selection of an ORFV recombinant is described, where the LacZ gene of D1701-VrV is replaced for the AcGFP gene. The used transfer plasmid pdV-AcGFP contains the AcGFP gene under the control of the early vegf-e promoter of ORFV (*see* **Note 12**).

After freeze-thaw and sonication the obtained NL is diluted and screened for AcGFP-positive recombinants as follows:

1. Freshly prepared Vero cells (*see* Subheading 3.1) are diluted to (a) 1×10^5 cells per mL and (b) 1.5×10^5 cells per mL.
2. In trough one of a multichannel reservoir prepare a 1:3 dilution of NL achieved by mixing 1.0 mL of NL thoroughly with 2.0 mL MEM containing 5 % FCS and 3×10^5 Vero cells.
3. Troughs 2–12 of a multichannel reservoir are filled each with 2.0 mL medium plus 5 % FCS and 2×10^5 Vero cells.

4. Next, transfer 1.0 mL from trough 1 to trough 2 containing the 2.0 mL cells-medium, mix thoroughly to obtain the next 1:3 dilution.
5. This 1:3 dilution steps are identically repeated ending up in trough 12 with a dilution of 1:531,441.
6. Using a multichannel pipette (12 channels) 50 µL of the different dilutions from troughs 1–12 can be easily transferred to the 384-well plate as follows:

   Wells A to P of rows 1 and 2, i.e., 32 wells of the plate receive the first 1:3 dilution from trough 1.
7. Wells A to P of rows 3 and 4 receive the next dilution from trough 2 (1:9 dilution), and so on.
8. Ending with wells A to P, rows 23 and 24 with the highest dilution from trough 12 (*see* **Note 13**).
9. After 24 h of incubation at 37 °C And 5 % CO_2 the plate can be monitored under a fluorescence microscope (*see* **Note 14**).
10. Wells exhibiting green fluorescent cells in the highest NL dilutions are recorded.
11. Continue incubation until virus plaque formation has proceeded (usually 72 h after seeding).
12. Determine the ratio of the number of green plaques to white plaques (fluorescence versus bright field).
13. Harvest those wells showing the highest ratio of green plaques by transferring medium and cells (detached with 30 µL VT per well) into single wells of a 48-well plate (*see* **Note 15**).
14. Freeze-thaw the harvested lysates three times (as described before).
15. Add 0.5 mL of MEM containing 5 % FCS and 1×10^5 Vero cells to each well.
16. Incubate at 37 °C, 5 % CO_2 until clear cpe and plaque formation can be visualized (usually after 72 h).
17. Harvest the medium and cells (treating with 0.1 mL VT per well), freeze-thaw three times.
18. Use 0.1 mL for DNA preparation (Subheading 3.6), store the lysates at –70 °C.

Negative selection:

It is self-evident that the above described procedure of selection can be identically used for a negative selection. Here a parental D1701-V is applied, which for instance is expressing a fluorescent marker gene and new nonfluorescent recombinant ORFV are screened. The latter might require some expertise, because starting ORFV-specific cpe or ORFV plaques are not always unambiguously identifiable.

3.4 DNA Isolation from Single ORFV Plaques for PCR

The following method modified from Pasamontes et al. [28] we found reliably working with good success. However, other reported methods or commercially available DNA purification kits might be suitable, too.

Following important points should be strictly considered:

- Work sterile at a place reserved for PCR and use filter-tips recommended for PCR.
- Use clean pipettes reserved for PCR, which are never used for pipetting of DNA templates.
- Extreme care must be taken to prevent contamination or carry-over with virus or DNA (plasmid or viral DNA).
- All solutions used must be reserved for PCR and bottled in single use portions.
- Working with phenol must be performed under a fume hood.

1. Mix 0.1 mL of the plaque virus lysates, as obtained in Subheading 3.2 or 3.3, with 0.1 mL PCR grade H_2O.
2. Add successively 0.1 mL phenol and 0.1 mL CIA, vortex, and centrifuge 3–5 min at 12,000 × *g* in an Eppendorf centrifuge.
3. Save the DNA containing supernatant, add 0.2 mL CIA, vortex and centrifuge as above (*see* **Note 16**).
4. Repeat **step 3**, take the supernatant and add 1–3 μL Glycogen-Blue (*see* **Note 16**).
5. Ethanol precipitate the DNA (0.2 mL) by mixing successively with 0.1 mL 7.5 M ammonium-acetate, and adding 0.6 mL absolute ethanol, mix and chill 10–30 min on ice.
6. Centrifuge at 10,000 rpm in an Eppendorf centrifuge, 4 °C for 20–30 min, pour off ethanol and wash pellet twice with 0.2 mL 70 % (v/v) Ethanol (*see* **Note 17**).
7. Remove the ethanol completely and dry DNA in the opened cup at room temperature or 37 °C (*see* **Note 17**).
8. Thoroughly dissolve DNA in 12 μL PCR-H_2O.
9. Use 3 μL containing approximately 100–500 ng DNA for PCR as described in Subheading 3.7.

3.5 Quick Preparation of Viral DNA

The protocol is adapted from the Master Pure DNA isolation kit from Epicentre Biotechnol (Biozym Scientific). In our hands, this method results in reproducibly good quantity and quality of ORFV DNA, and can be also used for other DNA analyses like Southern blotting.

The given volumes refer to infected cells taken from one well of a 6-well plate (1.0–1.5 mL medium per well for infection is sufficient); however, for the use of smaller cell numbers as derived from 48- or 96-well plates the volumes can be adapted proportionally.

1. Harvest cells when cpe has proceeded to approximately 80 % (*see* **Note 18**).
2. Transfer cells and medium in a 2 mL Eppendorf cup, keep on ice.
3. Trypsinize remaining cell monolayer with 0.3 mL VT at 37 °C, thoroughly suspend cells and combine with the corresponding medium from **step 2**.
4. Spin down cells by brief centrifugation and discard supernatant.
5. Add 1.0 mL PBS, vortex to resuspend cells completely, and centrifuge again.
6. Remove supernatant but leave one drop (approximately 50 μL) to suspend cell pellet thoroughly by vortexing.
7. Add 0.3 mL lysis buffer premixed with Proteinase K (kit) and completely suspend the cell pellet (*see* **Note 19**).
8. Heat for 15 min at 65 °C and mix by inversion every 5 min.
9. Equilibrate the lysate to 37 °C before adding 1 μL RNase A (kit) and incubate additional 30 min at 37 °C.
10. Place on ice, add 0.15 mL MPC reagent (kit), and mix thoroughly by inversion.
11. Centrifuge, for 10 min at 4 °C and 12,000 × *g* in an Eppendorf centrifuge.
12. Transfer supernatant without any precipitate into a fresh Eppendorf cup (1.5 mL) and add 0.5 mL isopropanol.
13. Precipitate DNA by inverting the cup 30-times (can be stored overnight at 4 °C).
14. Centrifuge for 10 min at 4 °C and 12,000 x g in an Eppendorf centrifuge, and pour off ethanol (*see* also **Note 17**).
15. Wash DNA pellet twice with 0.2 mL 70 % ethanol, centrifuge for 5 min, and drain off ethanol as above (**step 14**).
16. Dry DNA pellet until ethanol is completely evaporated (*see* also **Note 17**).
17. Solve the DNA pellet with 10–50 μL TE (kit) and leave overnight at 4 °C for completely resolving DNA. Thoroughly suspend with a cut yellow tip (*see* **Note 20**), and determine DNA concentration.

3.6 Screening by PCR

The successful isolation and purification of new recombinants is monitored by PCR assays, which are (1) specific for the new inserted foreign gene of interest, (2) specific for the marker gene of the parental virus (the lacZ gene in the case of D1701-VrV), and (3) ORFV-specific. The result of such a PCR analysis is representatively shown in Fig. 1.

Specific PCR primers are selected, which amplify internal gene fragments of 300–700 bp in size, and which allow most sensitive and specific detection of the corresponding gene. We recommend establishing PCRs that are able to detect less than 50 fg of the inserted gene. Using such PCR sensitivity, we never detected growth of parental blue or fluorescent virus from plaque-purified ORFV recombinants after several cell culture passages, which have been PCR-negative for the marker gene of the parental virus. In that respect, we recommend to test recombinant virus passages also routinely by PCR for the absence of the parental virus and to verify stable insertion of transgene.

In the very most cases, we found that ready-to-use double-concentrated Taq polymerase-based PCR mixes, which already contain gel loading dye, are most suitable for screening of larger numbers of DNA isolated from potential recombinant virus plaques or virus lysates. The only exception represents detection of the lacZ gene of D1701-VrV, which needs for highest sensitivity a Pfx or Pfu polymerase (*see* protocol in Subheading 3.6.1).

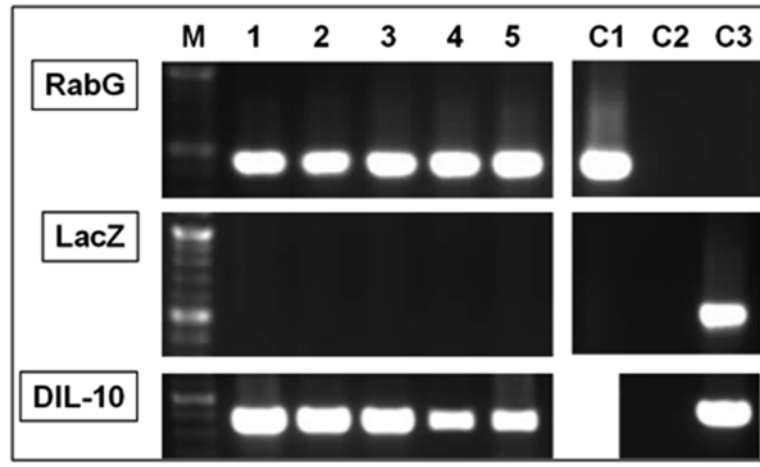

Fig. 1 PCR screening of recombinant ORFV plaques. *Lanes 1–5* represent PCR products of DNA directly isolated from single recombinant ORFV plaques, which are positive for the inserted Rabies virus G gene (RabG, 433 bp), negative for the parental LacZ gene (508 bp), and positive for the ORFV-specific IL-10 gene (DIL-10, 363 bp). As controls PCR was performed with RabG plasmid DNA (*C1*), with DNA from non-infected cells (*C2*), and with lacZ plasmid DNA (*C3*-LacZ) or with IL-10 plasmid DNA (*C3*-DIL-10). *M* represents 1 kb size marker (BioLabs)

The chosen times for denaturation, annealing, and extension of the amplification protocol is depending on the apparatus used. Due to the relatively high G+C content of the ORFV DNA (on the average 64 %), we recommend to start with a first denaturation step at *98 °C for 2 min*, which helps to obtain complete single strand denaturation of ORFV DNA. The following 35 amplification cycles consist of denaturation at 96 °C, the appropriate annealing temperature and extension at 72 °C using Taq polymerase.

Hot-start PCR is used to improve the performance of PCR and to increase specificity as well as target yield. Several hot-start PCR systems are commercially available. These approaches avoid DNA polymerase extension at lower temperatures and minimize nonspecific amplification and formation of primer dimers. Similar hot-start effect can be achieved by starting the thermocycler with the chosen PCR program, but stop the program after reaching 80 °C during the first step. At this temperature all samples just lacking the template but already including primers and polymerase mix are preheated in the thermoblock at 80 °C for a couple of minutes. Next, the template is added to each vial and placed back to the thermoblock. After adding DNA to the last vial the program is continued for cycling.

3.6.1 LacZ Gene-Specific PCR

The following PCR has been established for the sensitive detection of the parental D1701-VrV (as used in Subheadings 3.1–3.3).

1. The primer mix LacZ-FR is prepared by mixing together 3.95 pmol of the forward primer lacZ-F (5′-CGA TAC TGT CGT CGT CCC CTC AA-3′) and 4.13 pmol of the reverse primer lacZ-R (5′-CAA CTC GCC GCA CAT CTG AAC T-3′).
2. Per PCR reaction use 1 µL LacZ-FR, 1 µL PCR-H_2O, 3 µL (ca. 100 ng) template DNA isolated from the NL or virus lysates, and 5 µL twofold concentrated AccuPrime II (Life Technologies).
3. The PCR program consists of 98 °C and 2 min, 35 cycles consisting of 96 °C and 60 s, 62 °C and 30 s, 68 °C and 90 s, ending with a final step at 68 °C for 2 min.
4. The expected amplicon size amounts to 508 bp, as seen in Fig. 1, LacZ.

3.6.2 ORFV-Specific IL-10 PCR

Negative PCR results cannot be excluded to result from poor quality of the DNA isolated from the different virus isolates (Subheadings 3.1–3.3). This can be tested by the use of an ORFV-specific PCR to verify the presence of ORFV DNA. ORFV encodes a functional IL-10 homologue (PP42) not present in other poxviruses. We found that ovIL10-specific PCR is very sensitive for D1701-V, which of course does not exclude the suitability of other ORFV genes.

1. The primer mix DIL10-FR is prepared by mixing 4 pmol forward primer DIL10-F (5′-CAC ATG CTC AGA GAA CTC AGG G-3′) and 4 pmol reverse primer DIL10-R (5′-CGC TCA TGG CCT TGT AAA CAC C-3′).
2. Per PCR reaction 3 µL DIL10-FR are mixed with 100 ng template DNA (2 µL) and 5 µL 2× DreamTaq Green PCR Master Mix available from Thermo Scientific-Fermentas (*see* **Note 21**).
3. The PCR program consists of 98 °C and 2 min, 35 cycles consisting of 96 °C and 30 s, 65 °C and 30 s, 72 °C and 30 s, ending with the final step at 72 °C for 2 min.
4. The expected amplicon size amounts to 363 bp, as seen in Fig. 1, DIL-10.

3.7 Production of Concentrated ORFV Preparation

ORFV titers obtained by in vitro cell culture propagation, not only with ORFV D1701-V, are generally hardly exceeding 10^6–10^7 pfu/mL. In order to achieve virus stocks with higher titers the following procedure can be recommended (*see* **Note 22**).

1. Vero cells are seeded in a T175 culture flask to obtain an almost confluent cell monolayer of approximately 2×10^7 cells after overnight incubation at 37 °C and 5 % CO_2.
2. After pouring off the medium, briefly wash the cell monolayer with VT, add 2.0 mL VT and incubate 3–5 min at 37 °C until cells are detached completely.
3. Add 0.9 mL FCS and resuspend cells by vigorous pipetting.
4. Add 1×10^7 pfu virus, finally corresponding to 0.5 moi, fill up with medium to 9.0 mL, and swirl the flask for mixing.
5. Divide the virus–cell suspension into three T150 flasks (3 mL for each).
6. Fill up to 40–50 mL with medium plus 5 % FCS.
7. Incubate 3–4 days at 37 °C, 5 % CO_2 until cpe has developed to approximately 80 % (*see* **Note 23**).
8. Tap the flasks vigorously to dislodge infected cells, pour medium plus cells into a rotor cup (Beckman, JA-14, 250 mL),
9. In case cells are still retained in the flask, add 2.0 mL VT and incubate briefly at 37 °C.
10. Vigorously suspend the trypsinized cells with the medium transferred in **step 8** to the rotor cup.
11. Centrifuge 2 h at 26–30,000 × *g* and 4 °C, remove supernatant carefully without disturbing pellet.
12. Add 1 mL PBS and leave the rotor cup at 4 °C overnight in a sloped position to cover and to resolve the pellet completely.
13. Transfer the crude virus preparation into appropriate vials on ice.

14. Freeze-thaw three times (−70 °C, 37 °C).
15. Immerse the sterile bar of an ultrasonic device into the upper part of the virus suspension on ice, sonicate with 8–10 pulses (100 W, 20 s each) with a 5–10 s break between each pulse.
16. Now immerse the bar into the lower part of the virus suspension and again pulse four times.
17. Centrifuge 5 min at 4 °C and 500–700 × *g* to remove cell debris.
18. Save supernatant on ice.
19. Suspend the pellet in 1.0 mL PBS and transfer it to an Eppendorf cup.
20. Sonicate (100 W) again in an ultrasonic bowl, on ice 20 s twice (10 s break) and once for 30 s.
21. Centrifuge at 2000 × *g* for 10 min at 4 °C and combine this supernatant with supernatant from **step 17**.
22. Determine the virus titer, which should be higher than 10^8 pfu/mL. Store in aliquots at −70 °C.

3.8 ORFV Plaque Titration

The classical virus plaque titration assay is applied to determine the titer of D1701-V derived recombinant virus preparations. Vero cells are seeded the day before use and prepared as described in Subheading 3.1 (*see* **Note 24**).

1. Prepare on ice 1.0 mL tenfold virus dilutions ranging from 1:10^2 to 1: 10^8 in medium,
2. Pipette 0.1 mL of each dilution into wells A–F of rows 1–7 (sextuple) of a 48-well plate.
3. Wells 8A–8F represent the non-infected controls and are filled with cells only.
4. Pipette 0.5 mL cell suspension containing 5×10^4 cells in MEM plus 5 % FCS into each well.
5. Mix with the virus dilution by careful manual agitation, and incubate at 37 °C, 5 % CO_2.
6. After 3–4 days plaques can be counted and the mean plaque-forming unit (pfu) titers are calculated.
7. For additional virus plaque staining remove the medium, carefully wash once with PBS without destroying the cell monolayer, and proceed as described in Subheading 3.9.

3.9 Immunostaining of Infected Cells and Virus Plaques

The availability of appropriate specific antibodies allows the proof of successful transgene expression by performing immunohistochemical staining of infected cells (shown in Fig. 2). In the following, immune peroxidase monolayer assays (IPMA) are described for antigen detection in fixed or non-fixed, live cells.

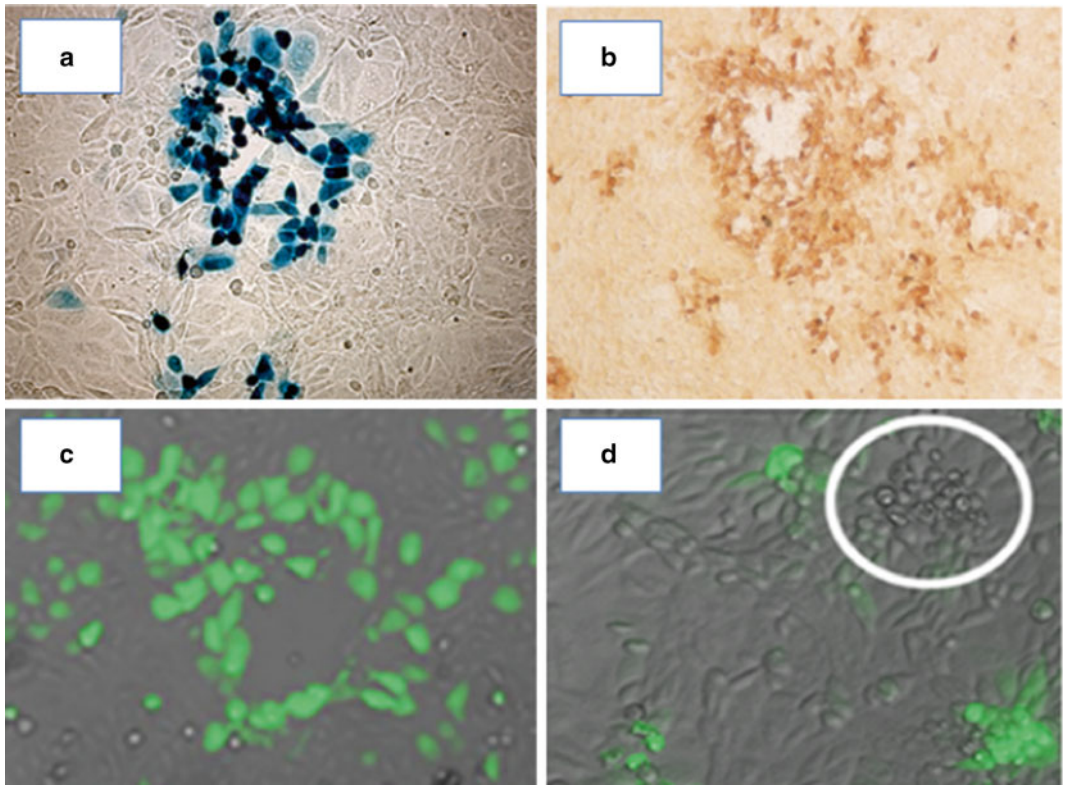

Fig. 2 Identification of recombinant ORFV plaques. (**a**) LacZ gene expression from D1701-VrV is detected by X-Gal staining using agarose-overlay. (**b**) RabG gene expressing recombinant virus plaque detected by specific IPMA, as described in Amann 2013 [24]. (**c**) Exchange of the lacZ gene for the AcGFP gene leading to green fluorescent recombinant virus plaques of D1701-V-AcGFP as described in Subheading 3.3. (**d**) Nonfluorescent, white recombinant virus (*circled*) was obtained by substitution of the AcGFP gene (Microscopic magnification: 40-fold)

3.9.1 IPMA Plaque Assay

This assay allows the discrimination of new foreign gene expressing recombinant ORFV plaques or foci from plaques of parental, transgene-negative ORFV (Fig. 2b).

1. Vero cells are prepared as described in Subheadings 3.1–3.3.
2. Virus lysate dilutions are prepared on ice.
3. For each well of a 24-well plate 0.1 mL virus dilution is mixed with 0.5 mL MEM plus 5 % FCS containing 1×10^5 cells (*see* **Note 25**).
4. One well contains non-infected and parental virus-infected cells as negative controls.
5. The plate is incubated for 2–4 days at 37 °C and 5 % CO_2 until distinct cpe or virus plaques have been formed.
6. Aspirate medium carefully not to damage the intact cell monolayer.

7. Let dry cells by placing the uncovered plate in the laminar flow hood for ca. 10 min, which helps to maintain intact monolayer.
8. Add slowly 0.5 mL ice-cold 100 % methanol (prechilled at –20 °C) to each well.
9. Fix the cells for 15 min at –20 °C.
10. Aspirate methanol, wash twice with ice-cold PBS containing either 1 % (w/v) BSA or 1 % (v/v) FCS.
11. Blocking unspecific antibody binding sites is achieved with 0.5 mL PBS plus 10 % FCS per well for 60–90 min at room temperature or overnight in the refrigerator.
12. Remove block solution and without washing add 0.2 mL per well of first, antigen-specific antibody diluted in TBST-BSA/FCS (*see* **Note 26**).
13. Incubate for 1 h at room temperature under constant slow shaking.
14. Wash three times for 5 min with 0.5 mL TBST-BSA/FCS at room temperature for instance on a rocking platform.
15. Incubate for another 30–60 min at room temperature with 0.2 mL appropriate peroxidase-labeled second antibody, diluted for example 1:2000 in TBST (*see* also **Note 26**).
16. Wash the wells three times 5 min with 0.5 mL TBST and once with PBS at room temperature.
17. Add substrate (e.g., DAB or DAB-black) prepared as recommended by the manufacturer until distinct brown or black color had developed.
18. Stop reaction by rinsing with tap water (which helps to intensify brownish staining) before counting stained plaques or foci.
19. The tightly closed plate can be stored at 4 °C and also used for further histological staining.

3.9.2 X-Gal Staining

Instead of using a X-gal-agarose-overlay (Subheading 3.2), β-galactosidase can be also monitored in fixed, infected cells as follows. Instead of the BetaBlue Staining kit (Novagen-Merck) other comparable kits should be also suitable for similar quick, direct visualization of β-galactosidase expression.

1. Vero cells are infected in 24-well plates and incubated until cpe and/or plaque formation is achieved as described above (Subheading 3.9.1).
2. Aspirate culture medium from cells.
3. Wash the cells twice with PBS plus NP-40 (*see* **Note 27**).
4. The cells are fixed by the addition of 0.5 mL methanol-free FALD for 15 min at room temperature.

5. Remove the fixative and wash cells four times with PBS.
6. Gently add 0.5 mL of the BetaBlue staining solution and incubate at 37 °C (*see* **Note 28**).
7. Staining is finished after 1–3 h and stopped by washing with PBS.
8. For storage, the stained cell layer can be covered with 15 % (v/v) glycerol in PBS.

3.9.3 Combined Detection of β-Galactosidase and Antigen

ORFV D1701-VrV expressing β-galactosidase can be discriminated from new transgene-expressing recombinants by the simultaneous staining for β-galactosidase and the foreign antigen.

1. Remove supernatant from cells infected in a 24-well plate as described above (Subheading 3.9.1).
2. Wash at room temperature three times 5 min with PBS (0.5 mL).
3. Dry the cell monolayer by leaving the open plate for ca. 10 min in laminar flow hood.
4. Cells are fixed at 4 °C (in the refrigerator) after adding 0.5 mL cold methanol-free FALD.
5. Gently add 0.5 mL of the BetaBlue staining solution and incubate for 1 h at 37 °C (*see* **Note 28**).
6. Wash three times 5 min at room temperature with 0.5 mL TBST containing 1 % FCS.
7. Incubation with first and peroxidase-labeled secondary antibody as well as substrate reaction is performed exactly as described from **steps 12–19** in Subheading 3.9.1.

3.9.4 Plaque Staining of Unfixed Infected Cells

The following procedure can be used to detect foreign gene expression in non-fixed, viable cells forming virus plaques, which can be subsequently isolated. This method works very well for recombinant proteins expressed on the infected cell surface; staining of internally expressed proteins has not yet been tested.

1. Vero cells, $3–5 \times 10^5$ cells in 3 mL MEM plus 5 % FCS are mixed with 0.1 mL virus lysates (dilution series from 10^{-2} to 10^{-6}).
2. Seeded simultaneously in wells of a 6-well plate and incubated until plaque formation is clearly visible.
3. After removal of medium, cells are washed carefully twice with sterile PBS.
4. Incubated with 1 mL first antibody (specific for the foreign gene product), diluted in MEM plus 2 % FCS for 2 h by gentle rocking.

5. After removal of antibody solution, the cells are washed once with PBS (2 mL).
6. Incubated for 1 h with 1 mL secondary peroxidase-labeled antibody diluted in MEM.
7. Cells are washed once with PBS (2 mL).
8. Incubation can be performed with for example the peroxidase substrate "VECTOR VIP" substrate kit (Vector) until purple plaque staining becomes visible during the next 1–3 h.
9. Wash twice with 2 mL PBS.
10. Cover the wells with LMT-Agarose overlay, which is prepared as described in Subheading 3.2, **steps 6–9**.
11. After cooling down the overlay to 37 °C, slowly pour 1.5 mL per well.
12. Allow the agarose-overlay to harden in the refrigerator.
13. Now the antigen-positive, purple virus plaques can be picked with a sterile Pasteur pipette and used for virus isolation exactly as described (Subheading 3.2, **steps 15–22**).

4 Notes

1. Vero cells should not be overgrown or completely confluent for splitting, because that might cause sticking together and clumping of cells.
2. Incubate cells not too long in VT (versene-trypsin solution) at 37 °C. Check after approximately 3 min; too long trypsin treatment or too high trypsin concentration can lead to damaged, clumped or stuck cells. VT pre-warmed at 37 °C leads to reproducible times for cell displacing. Forcing cells to detach by harsh, too extensive tapping or pipetting can cause permanent cell damage. Before counting or seeding, carefully check that the cells are evenly suspended.
3. LMT, low melting point agarose has the advantage to become completely molten at 65 °C and to remain fluid even between 37 and 30 °C. Weigh the needed amount of agarose (e.g., 2 g) into water (100 mL), and after boiling take care to refill the evaporated amount of water.
4. Agarose-BluoGal-overlay: We found BluoGal to result in fast and strong blue staining; however, other X-Gal substrates work equally.

   The pH of the agarose overlay should be between 7.0 and 7.4.

   Cool the agarose overlay solution down to ca. 30 °C, pour it very slowly from the edge of the wells, in order to prevent cell damage that can be erroneously noted as plaques or

cpe; be aware that the cell layer does not dry out after removal of the culture medium.

After pouring the overlay, allow the agarose to harden briefly at room temperature before incubating at 37 °C.

5. For cell counting preparation of more dilutions might improve calculation of cell density. The calculation depends on the type of counting chamber used.

   Neubauer improved chamber:

   Number of cells/4 quadrants × 2× dilution × 10^4 = cells/mL.

   Fuchs-Rosenthal chamber:

   Number of cells/4 quadrants × 2× dilution × 5 × 10^3 = cells/mL.

6. The optimal multiplicity of infection (moi) might be pretested, in our hands moi of 0.2–0.01 works well. If it is not possible to place a rotator in the incubator, the virus–cell suspension can be slowly shaken by hands several times during incubation. Two hours of incubation are chosen to become ORFV DNA replication started and increasing the possibility of recombination with transfer plasmid DNA in the infected cells.
7. Caution, the cell pellet is very unstable.
8. We found 2 μg plasmid DNA optimal. Pilot tests can be performed by monitoring the number of fluorescent or X-gal stained cells 24 and 48 h after nucleofection.

   We recommend performing not more than two nucleofections simultaneously, because incubation of cells plus DNA in transfection buffer should not exceed 15 min to avoid cell death. Prepare in advance the needed amount of cuvette, Eppendorf cups, pipettes, and cut filter tips.
9. Nucleofection lysates dilutions: That dilution must be found resulting in a reasonable number of separated virus plaques to allow single virus plaque picking. Because the virus titers in the different NL can vary, additional dilutions might be needed.
10. In case that no cpe or clear plaque formation becomes visible, nevertheless harvest the cells by trypsinization, freeze-thaw and sonicate, and use a 1:5 or 1:10 dilution for simultaneous infection of Vero cells in a 24- or 48-well plate. Due to very low amount of virus in the plaque eluate, such a "blind passage" can increase the virus titer.
11. After this point, at least additional three up to five more rounds of virus plaque purification are necessary to obtain genetically homogeneous recombinant viruses.
12. Genetic variants of the green fluorescent protein (GFP) are available with improved photostability, fluorescence strength, or spectral characteristics. The AcGFP gene encodes a GFP

consisting of human codons to enhance translation and expression in mammalian cells (Clontech, BD).

13. The described dilution series can be changed or adapted. The reason for preparing 32 wells of identical dilutions is to enhance the chance of identifying a sufficient number of wells with single green plaques. Similar endpoint dilution series can be of course also performed for example in 96-well plates.
14. Twenty-fold microscopic magnification is recommended to allow clear visualization of beginning plaque formation in the bright field and identification of green fluorescent cells and/or plaques by UV fluorescence.
15. For reasonable handling, maximally 24 wells are recommended to harvest.
16. Instead of transferring each supernatant into fresh cups after each extraction, the removal of the lower phenol-CIA and CIA phase by withdrawing it with a pipette from the bottom of the cup and using it for the next extraction step, represents a good alternative. Finally, the supernatant of last CIA extraction is transferred to a fresh cup.

    Glycogen-blue can be added to facilitate recovery of low amounts of DNA. Alternatively, yeast tRNA (10 μg) could be also used.
17. To prevent loss of the tiny DNA precipitate, for instance by swapping out during ethanol removal, we recommend the following procedure:

    Immediately after centrifugation slowly turn the closed cup upside down, which separates the DNA pellet from the ethanol solution. After placing it in that position onto a clean filter (e.g., Kleenex) paper, slowly open the lid, let the ethanol drain off by putting the cup with the opened lid upside down on the filter paper.

    Depending on the amount of remaining traces of ethanol, drying can take from several to 10 min. Take care that the ethanol is completely evaporated, which can be recognized by the DNA pellet becoming translucent.
18. Harvesting the infected cells at the appearance of approximately 80 % cpe should result in the best yield of ORFV DNA.
19. Complete suspension of the cell pellets is important, because unsolved cell clumps will drastically reduce the DNA recovery.
20. To cut the filter tips for DNA solving avoids mechanical shearing of high mol. wt. poxviral DNA.
21. Other available ready-to-use double-concentrated Taq polymerase-based PCR mixes could work equally.

22. Only a relatively crude, partially purified virus preparation is obtained. However, we found no detectable differences in the strength and quality of the protective immune response as compared to sucrose-gradient purified preparation (unpublished data). Nevertheless, for more routine use as vaccine more purified virus preparations are advised.
23. We found that that simultaneous infection with moi 0.5 and harvesting at the appearance of ca. 80 % cpe resulted in highest final virus titers.
24. One T75 flask containing 80–90 % confluent Vero cell should be sufficient for three 48-well plates.
25. Virus dilutions should be used that lead to maximally 10–20 plaques per each well. Of course this procedure can be adapted to 12- or 6-well plate.
26. Optimal dilution of first and second antibodies has to be determined; the peroxidase-labeled secondary antibody must be directed against that species used for production of the first antibody.
27. For higher sensitivity, we use PBS containing 0.02 % (v/v) NP-40; however, PBS without detergent can work properly, too.
28. Be careful by using a tissue culture incubator as the CO_2 may alter the pH and can lead to unacceptable background staining.

Acknowledgements

The excellent contributions of all members of the Rziha laboratory since 1999 are greatly acknowledged without the possibility to name each individually. However, we want to express special thanks to Mathias Büttner for initiating the ORFV project and helping with his expertise, and to Timo Fischer and Marco Henkel, whose pioneering work finally enabled the development of the novel ORFV vector system. Berthilde Bauer and Karin Kegreiß are gratefully acknowledged for their permanent excellent technical assistance. Finally, we also like to thank Lothar Stitz for his consistent support and his assistance with animal experiments. Financial support was obtained during all the years by different grants obtained from the EU, Bayer AG Animal Health, Riemser AG, Pfizer Animal Health—Zoetis.

References

1. Mackett M, Smith GL, Moss B (1982) Vaccinia virus: a selectable eukaryotic cloning and expression vector. Proc Natl Acad Sci U S A 79:7415–7419
2. Panicali D, Paoletti E (1982) Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc Natl Acad Sci U S A 79:4927–4931
3. Moss B (2013) Reflections on the early development of poxvirus vectors. Vaccine 31:4220–4222
4. Draper SJ, Cottingham MG, Gilbert SC (2013) Utilizing poxviral vectored vaccines for antibody induction-progress and prospects. Vaccine 31:4223–4230
5. Gomez CE, Najera JL, Krupa M et al (2011) MVA and NYVAC as vaccines against emergent infectious diseases and cancer. Curr Gene Ther 11:189–217
6. Walsh SR, Dolin R (2011) Vaccinia viruses: vaccines against smallpox and vectors against infectious diseases and tumors. Expert Rev Vaccines 10:1221–1240
7. Kim JW, Gulley JL (2012) Poxviral vectors for cancer immunotherapy. Expert Opin Biol Ther 12:463–478
8. Gomez CE, Najera JL, Krupa M et al (2008) The poxvirus vectors MVA and NYVAC as gene delivery systems for vaccination against infectious diseases and cancer. Curr Gene Ther 8:97–120
9. Drexler I, Staib C, Sutter G (2004) Modified vaccinia virus Ankara as antigen delivery system: how can we best use its potential? Curr Opin Biotechnol 15:506–512
10. Chavan R, Marfatia KA, An IC et al (2006) Expression of CCL20 and granulocyte-macrophage colony-stimulating factor, but not Flt3-L, from modified vaccinia virus ankara enhances antiviral cellular and humoral immune responses. J Virol 80:7676–7687
11. Russell TA, Tscharke DC (2014) Strikingly poor CD8(+) T-cell immunogenicity of vaccinia virus strain MVA in BALB/c mice. Immunol Cell Biol 92:466–469
12. Gomez CE, Perdiguero B, Najera JL et al (2012) Removal of vaccinia virus genes that block interferon type I and II pathways improves adaptive and memory responses of the HIV/AIDS vaccine candidate NYVAC-C in mice. J Virol 86:5026–5038
13. Quakkelaar ED, Redeker A, Haddad EK et al (2011) Improved innate and adaptive immunostimulation by genetically modified HIV-1 protein expressing NYVAC vectors. PLoS One 6, e16819
14. Kibler KV, Gomez CE, Perdiguero B et al (2013) Improved NYVAC-based vaccine vectors. PLoS One 6, e25674
15. Büttner M, Rziha H-J (2002) Parapoxviruses: from the lesion to the viral genome. J Vet Med B Infect Dis Vet Public Health 49:7–16
16. Haig D, Mercer AA (2008) Parapoxvirus. Encyclopedia Virol 5:57–63, Mahy BWJ, Van Regenmortel MHV (Eds) Elsevier, Oxford
17. Rziha H-J, Henkel M, Cottone R et al (2000) Generation of recombinant parapoxviruses: non-essential genes suitable for insertion and expression of foreign genes. J Biotechnol 83:137–145
18. Fischer T, Planz O, Stitz L et al (2003) Novel recombinant parapoxvirus vectors induce protective humoral and cellular immunity against lethal herpesvirus challenge infection in mice. J Virol 77:9312–9323
19. Henkel M, Planz O, Fischer T et al (2005) Prevention of virus persistence and protection against immunopathology after Borna disease virus infection of the brain by a novel Orf virus recombinant. J Virol 79:314–325
20. Dory D, Fischer T, Beven V et al (2006) Prime-boost immunization using DNA vaccine and recombinant Orf virus protects pigs against Pseudorabies virus (Herpes suid 1). Vaccine 24:37–39
21. van Rooij EM, Rijsewijk FA, Moonen-Leusen HW et al (2010) Comparison of different prime-boost regimes with DNA and recombinant Orf virus based vaccines expressing glycoprotein D of pseudorabies virus in pigs. Vaccine 28:1808–1813
22. Voigt H, Merant C, Wienhold D et al (2007) Efficient priming against classical swine fever with a safe glycoprotein E2 expressing Orf virus recombinant (ORFV VrV-E2). Vaccine 25:5915–5926
23. Rohde J, Schirrmeier H, Granzow H et al (2011) A new recombinant Orf virus (ORFV, Parapoxvirus) protects rabbits against lethal infection with rabbit hemorrhagic disease virus (RHDV). Vaccine 29:9256–9264
24. Amann R, Rohde J, Wulle U et al (2013) A new rabies vaccine based on a recombinant ORF virus (parapoxvirus) expressing the rabies virus glycoprotein. J Virol 87:1618–1630
25. Rohde J, Amann R, Rziha HJ (2013) New Orf virus (Parapoxvirus) recombinant expressing H5 hemagglutinin protects mice against H5N1

and H1N1 influenza A virus. PLoS One 8, e83802

26. Zeitelhofer M, Vessey JP, Xie Y et al (2007) High-efficiency transfection of mammalian neurons via nucleofection. Nat Protoc 2:1692–1704
27. Rziha HJ, Henkel M, Cottone R et al (1999) J Biotechnol 73:235–242
28. Pasamontes L, Gubser J, Wittek R et al (1991) Direct identification of recombinant vaccinia virus plaques by PCR. J Virol Methods 35:137–141

Vaccine 29 (2011) 9256–9264

Contents lists available at SciVerse ScienceDirect

Vaccine

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

A new recombinant Orf virus (ORFV, Parapoxvirus) protects rabbits against lethal infection with rabbit hemorrhagic disease virus (RHDV)

Joerg Rohde[a], Horst Schirrmeier[b], Harald Granzow[b], Hanns-Joachim Rziha[a,*]

[a] Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Immunology, Paul-Ehrlich-Str. 28, D-72074 Tuebingen, FRG
[b] Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Diagnostic Virology, Suedufer 10, D-17493 Greifswald-Insel Riems, FRG

ARTICLE INFO

Article history:
Received 29 July 2011
Received in revised form
20 September 2011
Accepted 22 September 2011
Available online 11 October 2011

Keywords:
Parapoxvirus
Orf virus
ORFV recombinant
Rabbit hemorrhagic disease virus (RHDV)
Protection

ABSTRACT

This report describes the generation of a new recombinant Orf virus (ORFV; *Parapoxvirus*) expressing the major capsid protein VP1 (VP60) of the *calicivirus*, rabbit hemorrhagic disease virus (RHDV). Authentic expression of VP1 could be demonstrated in cells infected with the recombinant D1701-V-VP1 without the need for production of infectious ORFV progeny. Notably, infected cells also released empty calicivirus-like particles (VLPs). Challenge experiments showed that even a single immunization with $\geq 10^5$ PFU of D1701-V-VP1 protected rabbits against lethal RHDV infection. ELISA tests indicated that the protective immunity mediated by D1701-V-VP1 did not strictly depend on the presence of detectable RHDV-specific serum antibodies. The induction of interleukin-2 found only in the sera of rabbits immunized with the D1701-V-VP1, but not in sera of rabbits immunized with the inactivated commercial vaccine RIKA-VACC, might indicate also some involvement of T-cells in protection. Collectively, this work adds another example of the successful use of the ORFV vector system for the generation of a recombinant vaccine, and demonstrates its potential as an alternative vaccine to protect rabbits against RHDV infection.

1. Introduction

Rabbit hemorrhagic disease (RHD) was first observed in China 1984 [1] and represents an acute, fatal, and highly contagious disease of wild and domestic rabbits particularly affecting the European rabbit (*Oryctolagus cuniculus*). RHD is observed worldwide, and is endemic in Europe and East Asia [2]. The disease causes important economic losses in commercial rabbit production and leads to high mortality in wild rabbit populations [3]. The etiological agent is the rabbit hemorrhagic disease virus (RHDV), a non-enveloped RNA virus belonging to the Genus *Lagovirus* within the *Caliciviridae* [4,5]. Animals usually die within 2–3 days after infection from necrotizing hepatitis and haemorrhages in the liver, spleen, kidney, and lungs [6]. Experimental RHDV infection of rabbits is also used as a clinical model of fulminant liver failure [7]. The positive-sense single-stranded RNA genome of RHDV is 7.5 kb in length and encodes a polyprotein precursor from the open reading frame ORF1, which is finally cleaved into the 60 kDa major capsid protein (VP1, formerly VP60) and several non-structural proteins [8,9].

Various efforts have been made to create effective vaccines for the prevention of the RHDV infection of rabbits, most of them relying on the use of the VP1 capsid protein. VP1 expression in several heterologous systems, including bacteria, baculovirus, poxvirus, yeast or plants, clearly showed its importance for the induction of a protective immunity [10–20]. Expression of the VP1 gene can result in the formation of calicivirus-like particles (VLPs), which were found to be very immunogenic [21–28]. Nevertheless, presently used commercial vaccines are still prepared from the liver of experimentally infected rabbits, because RHDV cannot be propagated *in vitro* in cell culture systems. Therefore, replacing the use of infectious animal material for vaccine production with new recombinant vector systems would be very desirable.

Recently we reported the development of the *Parapoxvirus* Orf virus (ORFV) as a new virus vector system for the expression of foreign antigens. The very restricted host range, the skin tropism and the lack of systemic spread even in immune-compromised animals make ORFV a promising viral vector candidate [29–31]. Additional benefits of the ORFV vector are the reported adjuvant properties and the short-term vector specific immunity without the formation of ORFV neutralizing antibodies, which allows repeated inoculations [32–34]. For generating recombinant ORFV the highly attenuated and apathogenic virus strain D1701-V is used [31,32]. This strain offers several advantages for its use as a viral vector including its ability to be propagated in a cell line (Vero), and its property to induce a strong immune-stimulating response even in hosts non-permissive for ORFV [29,32,35]. Recombinant ORFV are generated by inserting foreign genes in place of the viral vegf-e gene

* Corresponding author. Tel.: +49 7071 967 253.
E-mail address: achim.rziha@fli.bund.de (H.-J. Rziha).

0264-410X/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2011.09.121

[31,36], thereby removing an ORFV virulence gene [33]. Recently, ORFV D1701-V recombinants proved useful for delivering foreign viral antigens and generating protective immunity to control acute and persistent virus infections, which require different immune mechanisms [36–38].

In this report we describe the construction of the ORFV recombinant D1701-V-VP1 expressing the VP1 protein of RHDV, and evaluated its potential to protect rabbits against RHDV infection. Rabbit experiments demonstrated that already a single intramuscular (i.m.) immunization with the recombinant D1701-V-VP1 mediated excellent protective immunity against lethal RHDV challenge infection. Interestingly, protection did not strictly depend on the magnitude of specific serum antibody titres. Cytokine-ELISA revealed the induction of interleukin-2 in the sera from D1701-V-VP1 immunized animals, but not in sera from animals immunized with the inactivated commercial RHDV vaccine, which might indicate some role of T-cells in protection of rabbits against RHDV.

2. Materials and methods

Cells and viruses. The ORFV recombinants were propagated in Vero cells and titrated as described [36]. To arrest ORFV infection in the early stage, cells were infected in the presence of Cytosine arabinoside (AraC, 40 μg/ml, Sigma). RHDV strain Eisenhuettenstadt (Acc. No.: Y15440) was obtained from the liver of infected rabbits [39].

Antibodies. The polyclonal rabbit antiserum specific for RHDV VP1 (R-aVP60) was generously provided by B. Szewczyk (University Gdansk, Poland), the monoclonal antibody (mAb) 1G8 is directed against VP1 [39], the rabbit anti-VP1 antiserum C1 [40] was obtained from G. Meyers (FLI, Inst. Immunol., Tuebingen, FRG). Rabbit antibody against cellular β-actin was purchased from Sigma–Aldrich (FRG).

Construction and selection of ORFV recombinant D1701-V-VP1. The VP1 coding sequence (GenBank accession no. M67473.1) was synthesized by Mr. Gene (Regensburg, FRG). To prevent premature stop of VP1 expression, at two positions the poxviral early transcript stop motif TTTTTNT (where N represents any nucleotide) was removed by a silent mutation from codons TTT to TTC. In addition, at the 3′-end a second translational TGA stop was added followed by the sequence T5CT to provide correct early RNA stop. Finally, a new Hind*III* and *EcoRI* restriction site was introduced at the 5′ and the 3′ end, respectively, and used for insertion into the transfer plasmid pdV-Rec1 as described earlier [36] to obtain plasmid pdV-VP60n. Correct insertion of the VP1 gene was verified by restriction enzyme analysis and DNA sequencing.

Trypsinized Vero cells were infected in suspension for 2–3 h with moi 0.2 of strain D1701-VrV containing the LacZ gene, before nucleofection with 2 μg pdV-VP60n DNA using the nucleofector device as recommended by the manufacturer (Lonza, FRG). Thereafter the transfected and infected cells were seeded into 6-well plates and grown until the appearance of cytopathogenic effects. The cells were lysed, titrated on Vero cells and white virus plaques were picked, propagated, and VP1 expressing virus plaques were detected with the VP1-specific rabbit antiserum by immunostaining as described [36]. Plaque purification was performed four times until viral progeny was found by plaque PCR positive for VP1 gene and negative for parental LacZ-gene expressing virus.

Single plaque PCR. Viral DNA was prepared from each single virus plaque essentially as described [41]. Oligonucleotides used as PCR primers were purchased from Metabion (FRG). VP1-specific amplification (449 bp) was achieved with 3 pmol primer vp60-N1, 5′-CGA CTG ATG GCA TGG ATC CTG GCG TTG TG-3′, and 3 pmol primer Vp60-N2, 5′-GGG ACG CAA GTC TGG CAT GGT GAT GGT AAC-3′ in a 10 μl reaction containing 2× Reddy (ABgene, Fisher Scientific). The samples were heated at 98 °C for 2 min followed by 35-times cycling at 96 °C (1 min), 70 °C (30 s), and 72 °C (30 s) in a TRIO-Thermoblock (Biometra, FRG). Amplification of the LacZ gene fragment (508 bp) was achieved using 4,3 pmol primer lacZ-F, 5′-CGA TAC TGT CGT CGT CCC CTC AA-3′, 4,1 pmol primer lacZ-R, 5′-CAA CTC GCC GCA CAT CTG AAC T-3′, and AccuPrime SuperMix II (Invitrogen, Fisher Scientific, FRG). After denaturation for 2 min at 98 °C, cycling was performed 35-times at 96 °C (1 min), 62 °C (30 s), and 68 °C (90 s). PCR products were detected by electrophoresis in horizontal 0.8% (w/v) agarose gels.

RNA isolation and Northern blot hybridization was performed as described [36], using UltraHyb as annealing solution according to the recommendation of the manufacturer (Ambion, FRG). The VP1-specific PCR product was gel isolated (Qiaex II, Qiagen, FRG) and used as a probe after radioactive labelling with ^{32}P-dCTP (MP Biomedicals, FRG) and RediPrime (GE Healthcare, Amersham-Biosciences, FRG).

Western blot analysis was performed as described [37]. All antibodies were diluted in 1× RotiBlock (Roth, FRG) and for enhanced chemiluminescence (ECL) the substrate Immobilon Western HRP (Millipore, FRG) was used. X-ray films for ECL were purchased from Pierce (Fisher Scientific, FRG).

Immunofluorescence. Vero cells were grown in 4-chamber cell culture slides (Becton Dickinson, FRG), infected with the ORFV recombinants, fixed at different times after infection in 2% methanol-free formaldehyde (Polysciences, FRG) at room temperature for 15 min, and permeabilized in 0.2% (v/v) TritonX-100 in Phosphate-buffered saline (PBS) for 5 min. Antibodies were diluted in PBS containing 1% (v/v) foetal calf serum and incubated for 45 min at 37 °C followed by extensive washing in PBS. Secondary dye-coupled antibodies (Fisher Scientific, Invitrogen, FRG) were diluted 1:1000 and after 30 min at 37 °C the slides were washed as above, and cellular F-actin staining was achieved by incubation with Phalloidin CF647 conjugate (Biotium, FRG) during 20 min at room temperature followed by staining of nuclei with DAPI (4′,6-diamidino-2-phenylindole-dihydrochloride, 1 μg per ml, Sigma, FRG). Thereafter, the slides were thoroughly washed and embedded in Mowiol-DABCO. Fluorescence images were recorded with Axiovision using the ApoTome (Zeiss, FRG).

2.1. Electron microscopy

Cell culture supernatant was adsorbed directly at formvar-coated 300 mesh nickel grids for 7 min. After removing the supernatant fluid, grids were stained with phosphotungstic acid (pH 6.0) for another 7 min. For intracellular labelling of viral proteins non-infected and infected Vero cells were fixed with 0.5% (v/v) glutaraldehyde in PBS, pH 7.2, for 30 min, embedded in LMP agarose (Biozym, Germany), and post fixed in the above fixative for 30 min. Thereafter, samples were blocked with 0.5 M ammonium-chloride in PBS for 60 min, washed in PBS, stained overnight in 0.5% aqueous uranyl-acetate, dehydrated in ethanol under progressive lowering of temperature, infiltrated with the acrylic resin Lowicryl K4M (Lowi, Waldkraiburg, Germany) at −35 °C, and polymerized by 360 nm UV light [42].

The post-embedding labelling of ultrathin sections was performed after blocking of surfaces with 1% cold water fish gelatin, 0.02 M glycine, 1% bovine serum albumin (BSA fract. V; Sigma, Germany) in PBS, by 2 h incubation at room temperature with anti-VP1 mAb or anti-RHDV hyperimmune serum diluted in PBS–BSA. Diluted gold-conjugated goat-anti-species antibodies (GAM_{10}, GAR_{10} for single labelling; British BioCell Int. Cambridge, U.K.) were added for 60 min at room temperature. Specificity of the reaction was controlled on uninfected and infected Vero cells by using gold-conjugate without primary antibody and by using a RHDV VP1 non-related, anti-Newcastle disease virus antibody.

Table 1
Protection against RHDV challenge of rabbits intramuscularly immunized with different doses (PFU) of D1701-V-VP1.

PFU		D1701-V-VP1			RIKA-VACC	Non immunized
		Number of immunizations				
		1×	2×	3×	1×	
10^7	Exp-A			4/4[a]		
	Exp-B	4/4	4/4	4/4		
10^6	Exp-A			4/4		
	Exp-B	4/4	3/3[b]	2/2[b]		
10^5	Exp-A			4/4	4/4	0/4
	Exp-B	3/3[b]	3/3[b]	3/3[b]	3/3[b]	0/5

[a] Survivors/total number of animals.
[b] Reduced number of animals due to intercurrent, not RHDV-related mortality prior to challenge infection.

All preparations were analyzed with a transmission electron microscope (Tecnai 12, Eindhoven, The Netherlands).

2.2. Animal experiments

RHDV seronegative "Zika-bred" rabbits aged 8 weeks of mixed sex were distributed into groups as outlined in Table 1. The animals were immunized once, twice or 3-times by the intramuscular (i.m.) route with 1 ml D1701-V-VP1 containing 10^7 PFU (plaque-forming units), 10^6 PFU, or 10^5 PFU, respectively. For controls, groups of rabbits were vaccinated with the commercial RHDV vaccine "RIKA-VACC" (Riemser Arzneimittel AG, Germany) or remained non-vaccinated. Sera were collected prior to the vaccination and in weekly intervals. Twenty two days after the last vaccination, the animals were inoculated i.m. with 1 ml containing 10^4 LD50 (lethal dose 50) of the virulent RHDV strain "Eisenhüttenstadt". Rabbits were monitored clinically and livers of rabbits which died after challenge infection were collected and tested by haemagglutination test and antigen ELISA for RHDV as described [39].

ELISA. The presence of RHDV-specific serum antibodies was monitored by indirect ELISA as reported [39]. The results are presented as ELISA indices and the cut-off value was set to 0.2 as described [43]. An antigen ELISA for detection of RHDV in liver samples after challenge was performed as previously published [39]. Induction of serum cytokines was investigated by ELISA according to the instruction of the manufacturer (USCN Life Sci., Hoelzel, Germany).

3. Results

3.1. Selection of VP1-expressing ORFV recombinant D1701-V-VP1

After three rounds of plaque-purification several recombinant virus isolates were obtained containing the VP1 gene instead of the LacZ gene cassette of the parental D1701-VrV as proven by PCR (data not shown). Three single plaque virus isolates (2.2.6, 3.4.4, and 4.1.9) were tested for expression of VP1 protein by IPMA and were chosen for further analysis. Fig. 1 shows that the rabbit

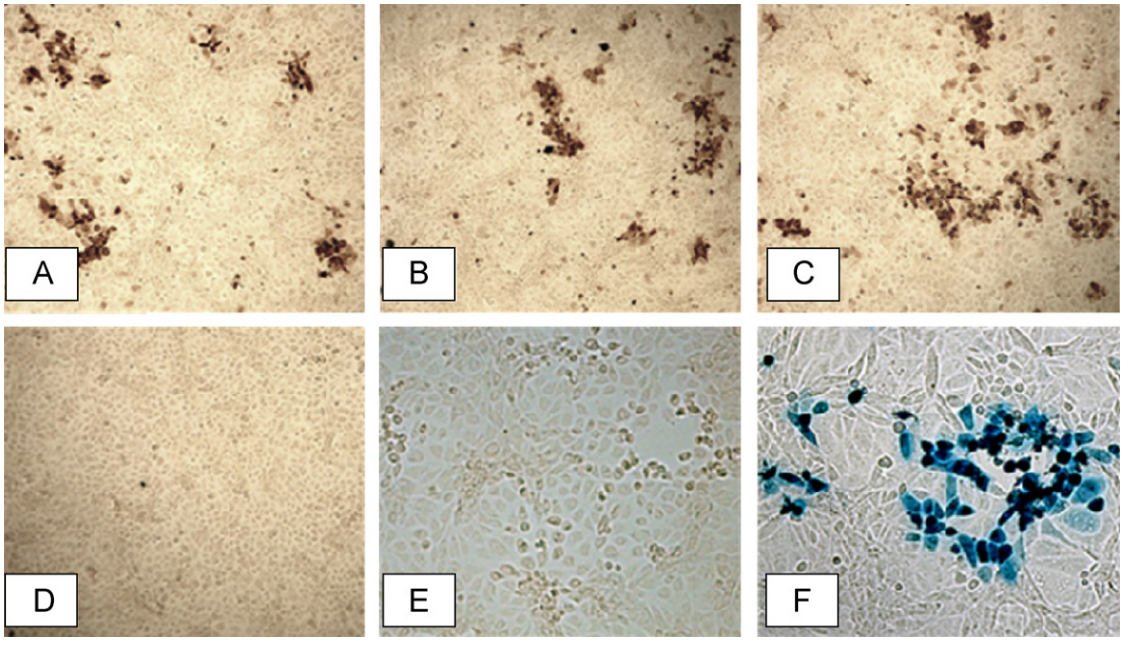

Fig. 1. VP1 expression from single plaque virus isolates of recombinant ORFV. Vero cells were infected with the 3 different single plaque virus isolates 2.2.6 (A), 3.4.4 (B), and 4.1.9 (C), non-infected (D), or infected with the parental virus D1701-VrV (E and F), fixed 2 days pi, and VP1 expression was detected by IPMA staining with the R anti-VP60 antiserum (1:1000 diluted). Virus-infected cells starting to form plaques are specifically stained brown (A–C), whereas non-infected or D1701-VrV-infected cells remained negative (D and E). Blue plaques of D1701-VrV-infected cells are demonstrated by X-gal staining (F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

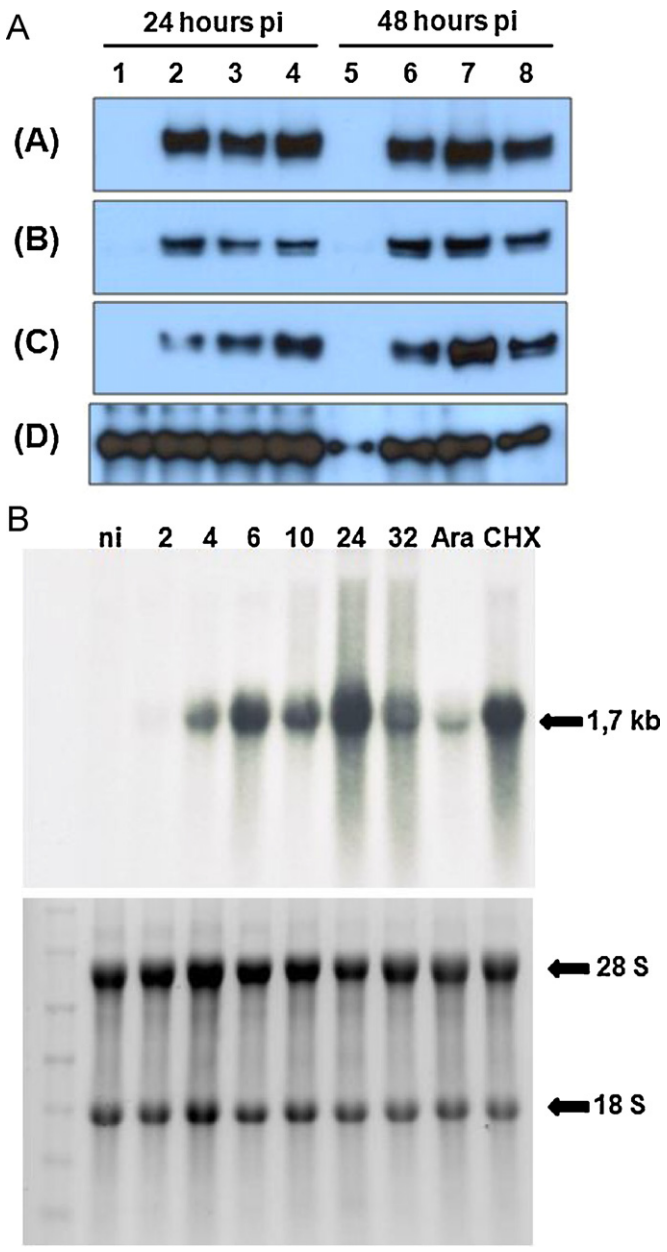

Fig. 2. (A) VP1 expression shown by Western blot analysis. Protein lysates were prepared from non-infected cells (lanes 1) and from cells infected with virus plaque isolate 2.2.6 (lanes 2 and 6), 3.4.4 (lanes 3 and 7), 4.1.9 (lanes 4 and 8) or parental D1701-V (lane 5) 24 h and 48 hpi as indicated. Detection of VP1 (60 kDa) was achieved with (A) specific antisera C1 diluted 1:15,000, (B) R-antiVP60 diluted 1:20,000, and (C) mAb 1G8 diluted 1:2000. (D) Detection of cellular β-actin as a gel loading control showing a slightly lower amount of protein of the parental D1701-V lysate in lane 5. All 3 plaque virus isolates reacted equally well with the 3 different anti-VP1 antibodies. (B) Northern blot hybridization to detect early expression of the VP1 gene. Total RNA was isolated from D1701-V-VP1 infected cells at the indicated hours pi. Lanes Ara and CHX indicate RNA isolated from cells treated with AraC or Cycloheximide during 24 h of infection, respectively. RNA from non-infected cells was separated in lane ni, lower part, first lane shows RNA size markers (Sigma–Aldrich, FRG). The upper part displays the result of the hybridization with the VP1-specific probe recognizing the approx. 1.7 kb mRNA of VP-1, showing early expression of the VP1 gene. The lower part shows the ethidium bromide-stained agarose gel to demonstrate comparable quality and amount of RNA loaded onto each lane. The ribosomal 28S and 18S cell RNAs are indicated.

anti-VP1 antiserum specifically stained cells infected with the 3 different recombinant plaque-isolates, but not non-infected cells (Fig. 1D) or cells infected with the parental virus D1701-VrV (Fig. 1E) expressing the *Escherichia. coli* lacZ gene (Fig. 2F). The correct insertion of the VP1 gene into the vegf-e gene locus was finally proven by Southern blot hybridization with restriction enzyme digested viral DNA isolated from each plaque virus isolate and radioactively labelled probes specific for the VP1 gene and the flanking ORFV genomic regions, respectively (data not shown).

3.2. Expression of RHDV VP1 in ORFV recombinant infected cells

Protein lysates were prepared 24 and 48 h after infection (hpi) of Vero cells with each plaque virus isolate (moi = 3.0) and tested by Western blotting for the expression of VP1. As depicted in Fig. 2, the three plaque virus isolates expressed equally well the correct sized VP1 of RHDV, which was recognized by the 3 different anti-VP1 antibodies. No specific reaction was found with non-infected cells or with cells infected with parental D1701-V (Fig. 2A, lanes 1 and 5). The plaque virus isolate 3.4.4 was finally chosen for preparing high titre virus stocks, designated as D1701-V-VP1, and used for all further experiments. In time course experiments the VP1 protein was detectable by Western blotting from 4 to 6 hpi on with increasing amounts translated at later times pi (data not shown).

Northern blot hybridization corroborated the expected early expression of the inserted VP1 gene (Fig. 2B), due to its control from the early ORFV vegf-e promoter. Using the VP1 gene-specific PCR product as a radioactively labelled probe, the specific RNA of approximately 1.7 kb in size was detected already at 2 hpi with increasing amounts until later times pi. The VP1-specific RNA was also found after arresting the D1701-V-VP1-infected cells in the very early phase of gene expression by treatment with Cycloheximide (Fig. 2B, CHX) or preventing viral DNA replication by treatment with AraC (Fig. 2B, AraC).

Comparably, immunofluorescence of cells infected with D1701-V-VP1 revealed VP1 expression at 6 hpi (Fig. 3) or in the presence of AraC (not shown). Notably, the VP1-specific fluorescence appeared as sharply contoured, globular staining in the cytoplasm of the infected cells. This distinctive staining pattern was also found at later times pi in an increasing number of cells (Fig. 3), indicating a compact formation of the expressed VP1.

Single step growth curve experiments showed that the insertion of the VP1 gene did not alter the *in vitro* growth characteristics in the ORFV permissive Vero cells compared to the parental virus D1701-V (Fig. 4).

3.3. Electron microscopy

The ultrathin sections of the infected cells showed all known stages of *Parapoxvirus* morphogenesis including different immature and mature ORFV particles as well as typical viral factories or viromatrix (Fig. 5A and B, vm). In addition, the infected cells contained “electron lucent” fibrogranular cytoplasmic inclusions of unknown composition and function (Fig. 5A and B, ib). These often perinuclear localized structures of slightly fibrogranular appearance showed a distinct labelling pattern with the different RHDV VP1-specific antibodies in electron immune cytochemistry (Fig. 5A and B, insets). The distribution of labelling at ultrathin sections can explain the spot-like VP1-specific fluorescence pattern shown in Fig. 3. These inclusion bodies containing synthesized VP1 were seen in all examined ultrathin sections of only D-1701-V-VP1 infected cells, but were never observed in non-infected cells or in cells infected with wild-type ORFV. Also no specific gold-labelling was obtained with the VP1 unrelated antibody (data not shown).

Remarkably, the presence of scattered calicivirus-like particles (VLPs) was found only in the supernatant of infected cells, in addition to typical ORFV particles (Fig. 5C). This indicates excretion of VLP formed by the ORFV expressed VP1 of RHDV. However, examination of the ultrathin sections of infected cells failed so far to

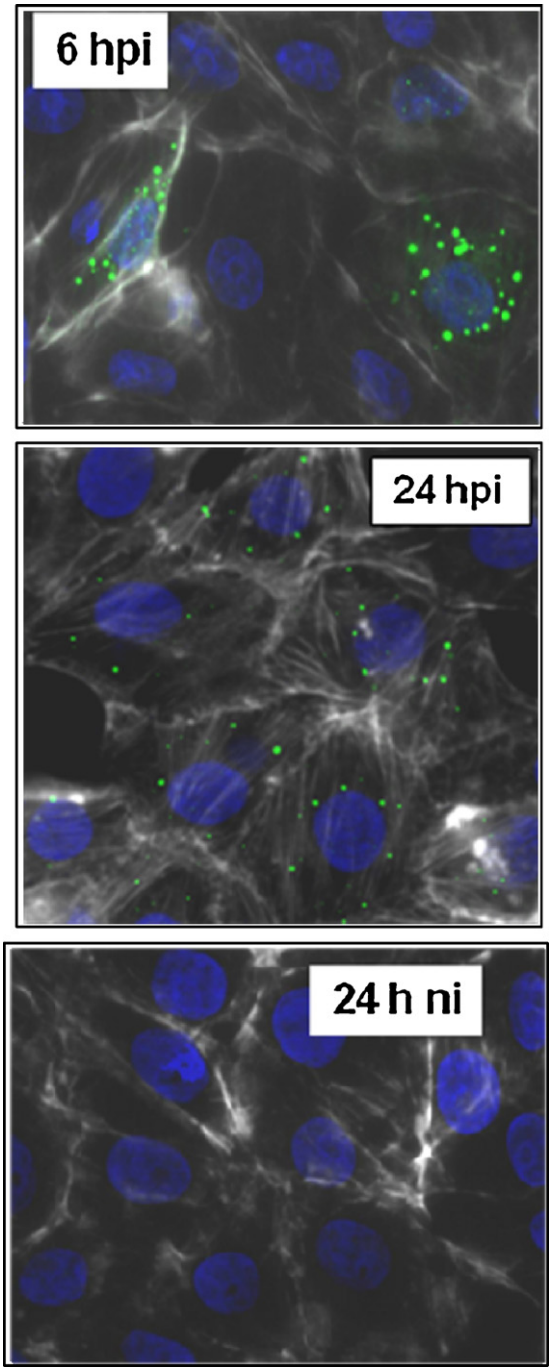

Fig. 3. Immunofluorescence detection of RHDV VP1 in D1701-V-VP1 infected Vero cells. Cells were infected with D1701-V-VP1 (moi = 1.0), fixed at the indicated hours pi (hpi), and incubated with the VP1-specific mAb 1G8 (diluted 1:500) and Alexa-488 coupled secondary antibody followed by F-actin staining with PhalloidinCF-647 (white) and blue staining of nuclei (DAPI) as described in Section 2. As negative controls, non-infected (ni) cells did not show VP1-specific green fluorescence. VP1 expressed by the recombinant appeared in the cytoplasm as a distinct globular green staining early pi (6 hpi). One picture taken at 24 hpi (2 hpi) representatively demonstrates VP1-specific fluorescence unchanged in intensity, but found in an increasing number of cells as compared to 6 h pi.

illustrate formation of intracellular VLP, and therefore, needs more detailed studies.

3.4. Protection of rabbits against lethal challenge infection

Earlier studies indicated that i.m. application of ORFV recombinants induced a better protective immune response compared to

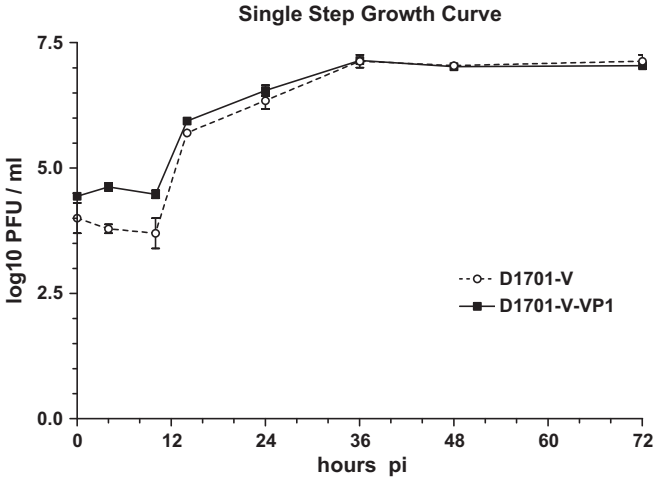

Fig. 4. Single step growth curve. Infected cells (moi = 5.0) were harvested approx. 1 h after adsorption (0 h pi) and at the indicated hours pi, and total cell lysates were titrated on Vero cells. The virus growth curve of the recombinant D1701-V-VP1 resembled that of the parental D1701-V.

intravenous immunization [37]. Therefore, we tested in 2 independent animal experiments the protective capacity of i.m. injection of different quantities of D1701-V-VP1 against lethal RHDV challenge infection. The first experiment A (Table 1, Exp-A) demonstrated that 3 immunizations given in 2 weeks intervals protected all animals using 10^7, 10^6 or 10^5 PFU of the ORFV recombinant. The second experiment (Exp-B, Table 1) confirmed the results, and moreover, showed that even a single immunization with each dose tested mediated protection of all animals. All animals immunized with the ORFV-vectored vaccine survived without any clinical signs, similar to animals control immunized with the commercially available inactivated RHDV vaccine RIKA-VACC. As expected, all non-immunized rabbits died within 48 h after challenge with RHD-specific clinical symptoms, and their livers contained high virus titres as measured by haemagglutination test and antigen ELISA (data not shown). All survivors tested 7 days post challenge were negative for RHDV in both assays (data not shown).

3.5. Immune response induced by D1701-V-VP1

The induced serum antibody response was monitored with serum samples taken from the animals listed in Table 1 at the days of immunization (V1, V2, V3), at the day of challenge infection, and from the protected animals one week after challenge infection. The results of the RHDV-specific ELISA are depicted in Fig. 6. Three i.m. immunizations led to a seroconversion of all animals independent from the immunization dose applied, and at the day of challenge the ELISA serum titres were similar to those induced by the commercial, inactivated vaccine RIKA-VACC (Fig. 6A). Two weeks after prime immunization weakly positive serum antibody titres were induced by 10^7 or 10^6 PFU of the recombinant, although close to the cut-off limit of the ELISA specificity, whereas a single application of 10^5 PFU did not induce detectable serum antibodies (V2, Fig. 6A and B). Interestingly, one week later, 3 weeks after the prime immunization (at the day of challenge) increasingly higher positive serum antibody titres were found using 10^7 or 10^6 PFU, but using 10^5 PFU still did not exhibit detectable serum antibodies (Fig. 6, compare V2 in A or B with "Challenge" in C).

The magnitude of the serum antibody titres increased 2 weeks after the second application with all applied doses of the ORFV recombinant, including 10^5 PFU, indicating successful booster immunization (Fig. 6A, V3), and after the third immunization only a slight rise in serum antibody titre was seen in all animals at the

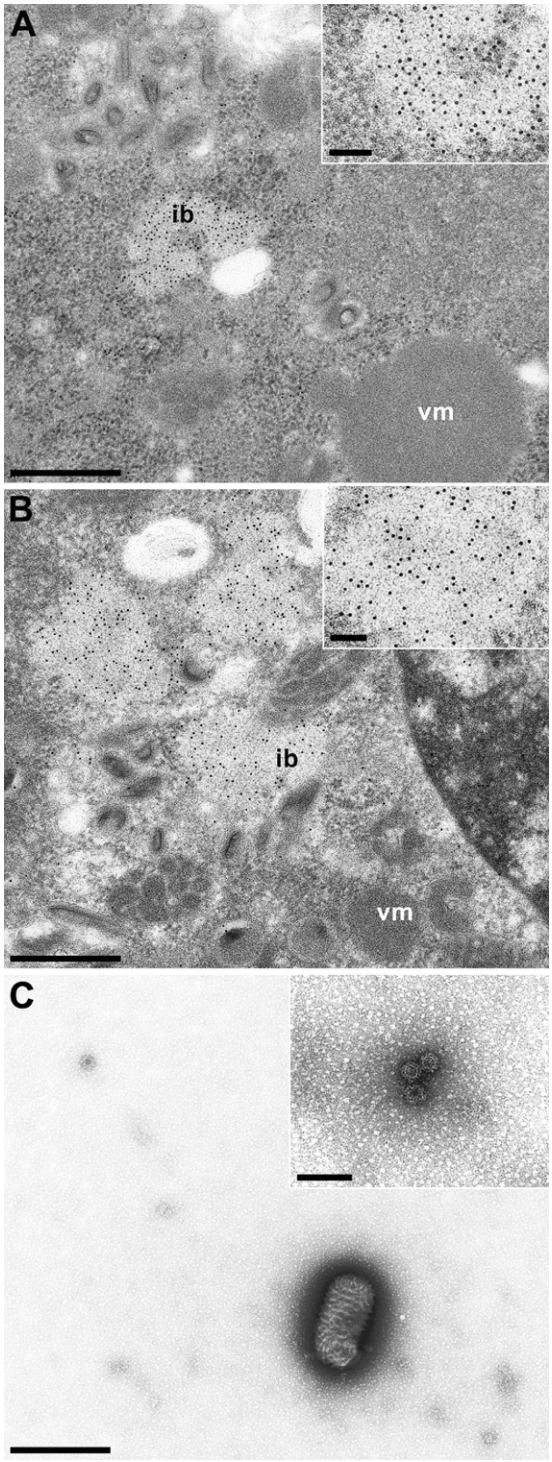

Fig. 5. Electron microscopy. (A and B) Ultrathin section of Vero cells 72 hpi with D1701-V-VP1. In addition to various maturation stages of ORFV, areas resembling inclusion bodies (ib) were only found in recombinant ORFV infected cells that were specifically labelled with RHDV hyperimmune serum (A, bar represents: A 3.0 μm) or with antibody 1G8 recognizing VP1 of RHDV (B, bar delineates 1.0 μm). Larger magnifications are shown in the insets (bars representing 100 nm) to better visualize recognition of expressed VP1 by immune EM. Electron dense viromatrix (vm) was regularly visible. The results indicate that VP1 accumulated in condensed areas (ib) in the recombinant ORFV-infected cells. (C) Detection of small VLPs with typical calicivirus morphology (see also inset for higher magnification, bar represents 100 nm) together with mature ORFV particles by negative staining electron microscopy of the supernatant of D1701-V-VP1 infected Vero cells; bar represents 300 nm.

day of challenge. Similarly all animals immunized twice with the different doses had sero-converted at the day of challenge, with the lowest serum antibody titre induced by 10^5 PFU of the recombinant (Fig. 6B). One week after challenge infection, the serum antibody titres increased only marginally, except of the rabbits immunized with 10^5 PFU showing a more pronounced titre increase (Fig. 6B). After challenge infection, all animals immunized only once showed a relatively strong serum antibody titre increase (Fig. 6C), which also indicates successful boosting of the induced immune response.

Notably, all rabbits primed with a single dose of 10^5 PFU, which were sero-negative in ELISA, were nevertheless protected against the lethal RHDV challenge infection. Therefore, protection did not strictly correlate with the serum antibody titres. These data prompted us to test the available sera for the presence of induced cytokines, which might be involved in cellular immune mechanisms additionally to the humoral antibody response. By cytokine ELISA, interferon-gamma and interferon-alpha was not detectable in sera from all D-1701-V-VP1 or RIKA-VACC immunized rabbits before challenge infection, as also not in sera from non-immunized rabbits. However, one week after challenge infection sera from all immunized rabbits were positive for both interferon-gamma and -alpha (data not shown). By contrast, interleukin-2 (IL-2) was only found in sera of the rabbits immunized with the ORFV recombinant, but not in sera from the RIKA-VACC or from non-immunized animals. Fig. 6D shows that a single application of increasing amounts of D-1701-V-VP1 induced increasing amounts of serum IL-2. A single dose of 10^5 PFU induced only in 3 animals increased serum IL-2 (150–5000 pg per ml serum), all animals immunized with 10^6 PFU exhibited elevated amounts of serum IL-2 (150–3900 pg per ml), and after the application of 10^7 PFU the sera of all animals contained between 200 and 1000 up to 15,900 pg IL-2 per ml (Fig. 6D). Two or three immunizations seemed not to induce significantly more elevated levels of serum IL-2, but due to the availability of only a small number of those serum samples, it needs further confirmation.

4. Discussion

Novel efficacious vaccines against RHDV are still desirable, also to substitute the traditional vaccine preparations from organs of infected rabbits. Since the expressed capsid protein VP1 (VP60) of RHDV has been protective in most cases, we decided to use this capsid protein of RHDV for expression in the novel ORFV vector system. The present study now demonstrates the generation of a recombinant ORFV expressing the VP1 gene of RHDV, designated D1701-V-VP1, that protects rabbits against high dose lethal RHDV infection. The vast majority of the coding sequences of the VP1 gene published in GeneBank exhibits at two positions the sequence T5NT known to act as a poxviral early transcription stop motif [44]. Our pilot experiments indeed demonstrated that insertion of the original VP1 gene into the ORFV genome led to truncated VP1 transcripts (data not shown). Therefore, the VP1 gene was newly synthesized to contain silent sequence changes for removal of the early stop motifs, but remaining the authentic amino acid sequence of VP1. As demonstrated by immunostaining (IPMA) and Western blot analyses, cells infected with D1701-V-VP1 correctly expressed the RHDV VP1 protein in good quantity. As expected, the VP1 gene was expressed early in infection due to its control by the early vegf-e promoter [36,37].

Notably, the expressed VP1 exhibited a very distinct fluorescent staining of globular or spot-like shape (Fig. 3), which gives the impression of VP1 expression mainly restricted to distinct areas in the cytoplasm of the infected cells, and which reminds on the synthesis of early VACV proteins like I3L in viral factories

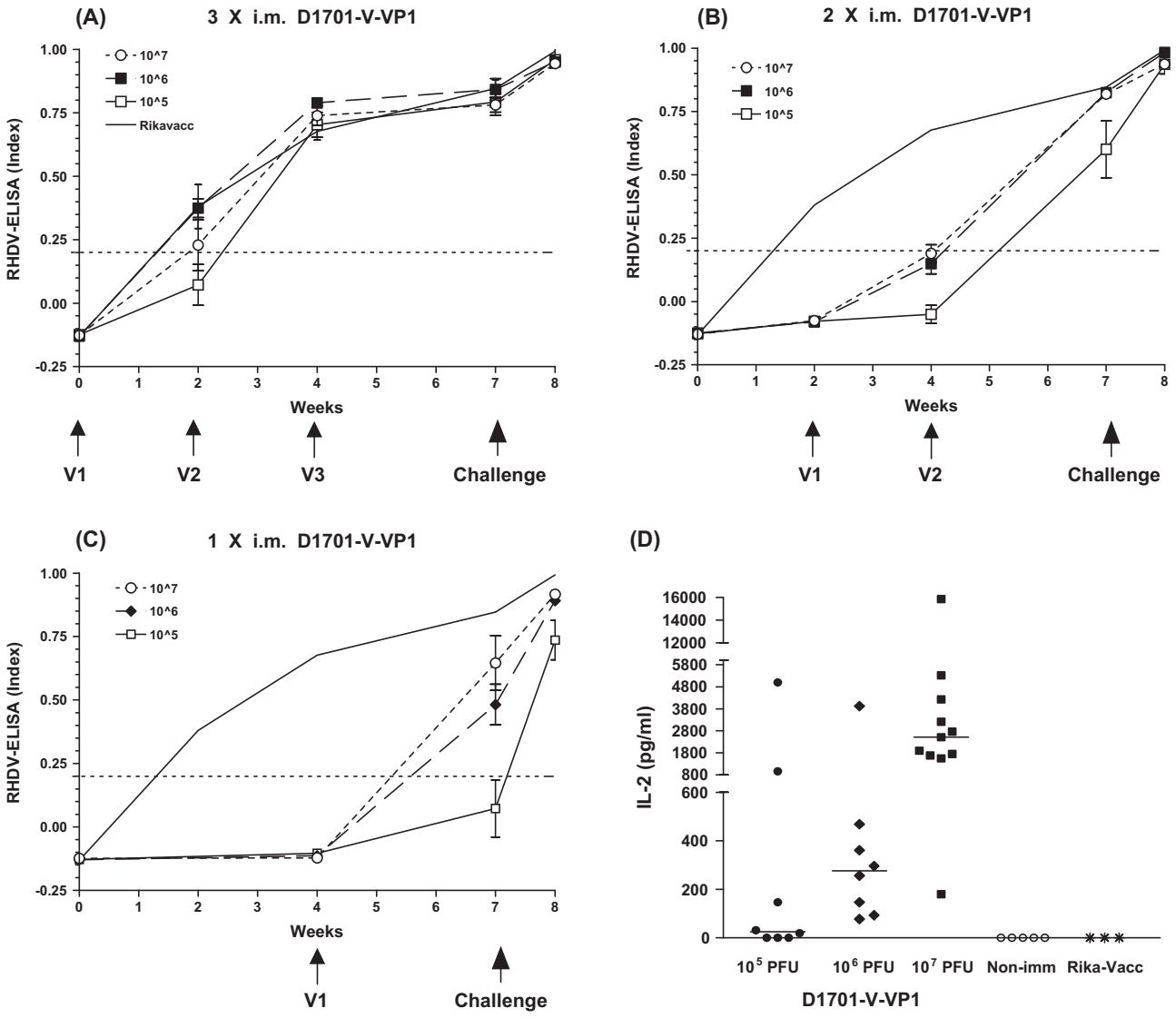

Fig. 6. Serum antibody response induced by different dose of D1701-V-VP1. RHDV-specific serum antibodies were determined by ELISA after 3 (A), 2 (B), or 1 (C) immunizations with 10^7, 10^6, or 10^5 PFU of D1701-V-VP1. V1 indicates prime immunization, V2 and V3 the booster immunizations in a 2 weeks interval, respectively. A single immunization with the commercial, inactivated vaccine RIKA-VACC was performed at day 0, and the sera were tested 2, 4, 7 and 8 weeks later (black line). The dashed line marks the cut-off limit of the ELISA index (0.2). (D) Amounts of IL-2 detected in individual sera of rabbits after a single immunization with 10^5 PFU (circles), 10^6 PFU (diamonds) or 10^7 PFU (squares) of D-1701-V-VP1. No IL-2 was detectable in the sera of Rika-Vacc immunized or not immunized animals.

[45,46]. Examination by immune electron microscopy revealed inclusion body-like structures of delimited shapes in the cytoplasm, which represent sites of VP1 synthesis as seen by the specific labelling with the gold-conjugated VP1-specific antibodies (Fig. 5). Due to their lower electron density, these 'factories' of VP1 can be clearly distinguished from the adjacent electron-dense viral factories or viroplasm (virus matrix) characteristic for poxviruses [46,47]. These areas reminding on inclusion bodies represent the major detectable sites of VP1 accumulation in the infected cell, at least at the investigated times after infection. The particular immunofluorescence staining pattern and the intracellular protein synthesis in inclusion bodies seems to be a property of the RHDV VP1 protein and not of the ORFV vector, because it was never observed with recombinants expressing other foreign viral genes. A somewhat comparable granular or punctate fluorescence staining of the capsid protein was reported earlier for porcine or human calicivirus [48,49]. The sites of RHDV VP1 accumulation seen by immune electron microscopy (Fig. 5A and B) are organized as granulofibrillar structures in the cells, which might favor the formation of calicivirus-like particles and indeed VLPs could be demonstrated in the culture supernatant of D-1701-V-VP1 infected cells together with released mature ORFV particles (Fig. 5C). However, intracellular calicivirus VLP formation was not detectable in the examined ultrathin sections. Therefore, the site of assembly of the excreted VLP remains unclear and must be now subject of more detailed electron microscopy studies. The results of immune electron microscopy also corroborate that the early expressed VP1 does not become part of the ORFV replication machinery and of the maturation cycle of ORFV. VP1 expression in AraC-treated infected cells also proves early foreign gene regulation from recombinant ORFV, which does not require ORFV replication or production of infectious progeny as reported for other ORFV recombinants [36–38,50].

The protective potential of the new ORFV recombinant was investigated in rabbits, and the results clearly demonstrated solid protection of all animals after i.m. immunization with doses of D-1701-V-VP1 ranging between 10^5 and 10^7 PFU. All animals survived the high lethal dose of challenge virus (10,000 × LD50) without any clinical signs, a challenge virus dose that can be assumed to exceed

the amount of RHDV occurring in the field. One week after infection no challenge virus was detectable in the immunized rabbits. Even animals immunized by a single administration of the lowest dose of 10^5 PFU of the ORFV recombinant resisted the challenge. This is remarkable, because much higher amounts (10^7–10^9 PFU) of RHDV VP1 expressing VACV or Canarypoxvirus recombinants were needed for protection after subcutaneous, intra-dermal or oral application [15,51]. Therefore, additional studies must now demonstrate the induction of a protective immune response after various routes of administration of the ORFV recombinant as well as the ultimate minimal protective dose.

As anticipated the immunization of rabbits with D-1701-V-VP1 resulted in RHDV-specific serum antibody responses depending somewhat on the amount of recombinant ORFV injected and on the number of applications. After two or three i.m. injections with all tested doses of D-1701-V-VP1 (10^5, 10^6, 10^7 PFU) all animals had clearly seroconverted at the day of challenge and were all protected. This was also the case for rabbits immunized only once with 10^6 or 10^7 PFU and most interestingly even a single dose of only 10^5 PFU not inducing detectable specific serum antibodies mediated protection against challenge infection. After challenge infection all immunized animals displayed an additional increase in serum antibody titres, independent from the used dose of the recombinant. This indicates perfect priming of the animals by the ORFV recombinant or by the challenge virus, which enables a fast specific immune response to prevent lethal RHDV infection.

The finding of protection of sero-negative, immunized rabbits was remarkable, because several reports indicate correlation between protection against RHDV infection and detectability of anti-RHDV or anti-VP1 serum antibodies [5,10,22,52,53]. Additionally, the protective role of humoral RHDV-specific antibodies was supported by adoptive serum transfer experiments [54] and by passive immunization with monoclonal antibodies against VP1 [55]. Supporting our data, Fischer et al. [15] could not found a clear correlation of protection with the presence and magnitude of serum antibodies after immunization with canarypoxvirus vectored RHDV VP1 vaccine. The duration of RHDV-specific serum antibodies is suggested to indicate the presence of a protective immunity. Barcena et al. [10] demonstrated the persistence of anti-RHDV serum antibodies for at least 8 months, however, whether those immunized animals could resist challenge infection is unknown. Studies now have to investigate, whether also the application of this new ORFV recombinant can achieve long-term protection as shown earlier against Borna disease virus [37], which perhaps could exceed the 14 weeks duration of immunity reported for the inactivated vaccine RIKA-VACC [43].

The immunity against RHDV is poorly understood due to the lack of cell culture systems, and any involvement of cell-mediated immunity for protection against RHDV is completely unknown. The fact that the inactivated vaccine RIKA-VACC is successfully used to protect against RHDV might contradict an important role of cellular immunity in protection. On the other hand, for human calicivirus it was reported that also cell-mediated, T-helper cell type 1 immunity is involved in protection [56]. Whether the induction of serum IL-2 only after immunization with the live ORFV recombinant, but not with the inactivated vaccine might indicate activation of cellular immune responses remains to be proven. A major source of IL-2 production are activated CD4-positive T-helper cells, which supports proliferation and activation of CD8-positive cytotoxic T-cells as well as the differentiation into memory effector cells (for review see [57]). Recently it was shown that ORFV can modulate antigen presenting cells and generate a strong T-helper cell type 1 response [33,58], which might explain induction of IL-2 in the serum of the immunized rabbits. Most probably the ORFV vector is responsible for the IL-2 induction, which however, must be shown by immunization with the parental vector virus D1701-V. Future studies have now to determine the origin and contribution of serum IL-2 to a protective immunity apart from the induced humoral immune response. In addition, the formation of calicivirus-like VLPs needs further clarification, because of their beneficial vaccination effect and known stimulation of cellular-mediated immunity [25,55]. Using ORFV with its immune-stimulating properties [30] and the expression of foreign antigens as chimeric VLPs could open another door for additional highly effective vector vaccines.

The application of D1701-V-VP1 did not lead to lesions, febrile response or other harmful side effects even after using higher doses of the recombinant. The overall health of rabbits remains also unaffected after application of 10^9 PFU of the parental virus D1701-V (Buettner & Rziha, unpublished data) without replication of infectious ORFV, again demonstrating the innocuousness of the ORFV-derived recombinants. Collectively, the present study adds another example of the successful use of this new vector virus. Demonstrating the efficacy of ORFV – (strain D1701-V) vectored vaccines against herpesvirus, against Borna disease virus, against pestivirus, and now against calicivirus further supports its usefulness as an universal vector virus platform with excellent immunizing properties.

Acknowledgements

The excellent technical assistance of Mrs. B. Bauer, K. Kegreiβ, P. Meyer, M. Jörn and B. Hillmann is greatly appreciated. Mrs. Edith Schneider' contributions to the recombinant construction during her practical training are greatly acknowledged.

For financial support of the animal experiments we thank the Riemser Arzneimittel AG, Island of Riems, Germany.

Many thanks to Lothar Stitz and Ralf Amann for fruitful discussions and critically reading the manuscript.

References

[1] Liu SJ, Xue HP, Quian NH. A new viral disease in rabbits. Anim Husb Vet Med 1984;16:253–5.
[2] Mitro S, Krauss H. Rabbit hemorrhagic disease: a review with special reference to its epizootiology. Eur J Epidemiol 1993;9(1):70–8.
[3] McIntosh MT, Behan SC, Mohamed FM, Lu Z, Moran KE, Burrage TG, et al. A pandemic strain of calicivirus threatens rabbit industries in the Americas. Virol J 2007;4:96.
[4] Green KY, Ando T, Balayan MS, Berke T, Clarke IN, Estes MK, et al. Taxonomy of the caliciviruses. J Infect Dis 2000;181(Suppl. 2):S322–30.
[5] Parra F, Prieto M. Purification and characterization of a calicivirus as the causative agent of a lethal hemorrhagic disease in rabbits. J Virol 1990;64(8):4013–5.
[6] Alonso C, Oviedo JM, Martin-Alonso JM, Diaz E, Boga JA, Parra F. Programmed cell death in the pathogenesis of rabbit hemorrhagic disease. Arch Virol 1998;143(2):321–32.
[7] Tunon MJ, Sanchez-Campos S, Garcia-Ferreras J, Alvarez M, Jorquera F, Gonzalez-Gallego J. Rabbit hemorrhagic viral disease: characterization of a new animal model of fulminant liver failure. J Lab Clin Med 2003;141(4):272–8.
[8] Meyers G, Wirblich C, Thiel HJ. Rabbit hemorrhagic disease virus—molecular cloning and nucleotide sequencing of a calicivirus genome. Virology 1991;184(2):664–76.
[9] Meyers G, Wirblich C, Thiel HJ, Thumfart JO. Rabbit hemorrhagic disease virus: genome organization and polyprotein processing of a calicivirus studied after transient expression of cDNA constructs. Virology 2000;276(2):349–63.
[10] Barcena J, Morales M, Vazquez B, Boga JA, Parra F, Lucientes J, et al. Horizontal transmissible protection against myxomatosis and rabbit hemorrhagic disease by using a recombinant myxoma virus. J Virol 2000;74(3):1114–23.
[11] Bertagnoli S, Gelfi J, Le GG, Boilletot E, Vautherot JF, Rasschaert D, et al. Protection against myxomatosis and rabbit viral hemorrhagic disease with recombinant myxoma viruses expressing rabbit hemorrhagic disease virus capsid protein. J Virol 1996;70(8):5061–6.
[12] Boga JA, Martin Alonso JM, Casais R, Parra F. A single dose immunization with rabbit haemorrhagic disease virus major capsid protein produced in *Saccharomyces cerevisiae* induces protection. J Gen Virol 1997;78(Pt. 9):2315–8.
[13] Castanon S, Marin MS, Martin-Alonso JM, Boga JA, Casais R, Humara JM, et al. Immunization with potato plants expressing VP60 protein protects against rabbit hemorrhagic disease virus. J Virol 1999;73(5):4452–5.
[14] Fernandez-Fernandez MR, Mourino M, Rivera J, Rodriguez F, Plana-Duran J, Garcia JA. Protection of rabbits against rabbit hemorrhagic disease virus by

immunization with the VP60 protein expressed in plants with a potyvirus-based vector. Virology 2001;280(2):283–91.
[15] Fischer L, Le Gros FX, Mason PW, Paoletti E. A recombinant canarypox virus protects rabbits against a lethal rabbit hemorrhagic disease virus (RHDV) challenge. Vaccine 1997;15(1):90–6.
[16] Gil F, Titarenko E, Terrada E, Arcalis E, Escribano JM. Successful oral prime-immunization with VP60 from rabbit haemorrhagic disease virus produced in transgenic plants using different fusion strategies. Plant Biotechnol J 2006;4(1):135–43.
[17] Marin MS, Martin Alonso JM, Perez Ordoyo Garcia LI, Boga JA, rguello-Villares JL, Casais R, et al. Immunogenic properties of rabbit haemorrhagic disease virus structural protein VP60 expressed by a recombinant baculovirus: an efficient vaccine. Virus Res 1995;39(2–3):119–28.
[18] Mikschofsky H, Schirrmeier H, Keil GM, Lange B, Polowick PL, Keller W, et al. Pea-derived vaccines demonstrate high immunogenicity and protection in rabbits against rabbit haemorrhagic disease virus. Plant Biotechnol J 2009;7(6):537–49.
[19] Perez-Filgueira DM, Resino-Talavan P, Cubillos C, Angulo I, Barderas MG, Barcena J, et al. Development of a low-cost, insect larvae-derived recombinant subunit vaccine against RHDV. Virology 2007;364(2):422–30.
[20] Torres JM, Sanchez C, Ramirez MA, Morales M, Barcena J, Ferrer J, et al. First field trial of a transmissible recombinant vaccine against myxomatosis and rabbit hemorrhagic disease. Vaccine 2001;19(31):4536–43.
[21] Gromadzka B, Szewczyk B, Konopa G, Fitzner A, Kesy A. Recombinant VP60 in the form of virion-like particles as a potential vaccine against rabbit hemorrhagic disease virus. Acta Biochim Pol 2006;53(2):371–6.
[22] Laurent S, Vautherot JF, Madelaine MF, Le GG, Rasschaert D. Recombinant rabbit hemorrhagic disease virus capsid protein expressed in baculovirus self-assembles into viruslike particles and induces protection. J Virol 1994;68(10):6794–8.
[23] Nagesha HS, Wang LF, Hyatt AD. Virus-like particles of calicivirus as epitope carriers. Arch Virol 1999;144(12):2429–39.
[24] Nagesha HS, Wang LF, Hyatt AD, Morrissy CJ, Lenghaus C, Westbury HA. Self-assembly, antigenicity, and immunogenicity of the rabbit haemorrhagic disease virus (Czechoslovakian strain V-351) capsid protein expressed in baculovirus. Arch Virol 1995;140(6):1095–108.
[25] Peacey M, Wilson S, Baird MA, Ward VK. Versatile RHDV virus-like particles: incorporation of antigens by genetic modification and chemical conjugation. Biotechnol Bioeng 2007;98(5):968–77.
[26] Peacey M, Wilson S, Perret R, Ronchese F, Ward VK, Young V, et al. Virus-like particles from rabbit hemorrhagic disease virus can induce an anti-tumor response. Vaccine 2008;26(42):5334–7.
[27] Plana-Duran J, Bastons M, Rodriguez MJ, Climent I, Cortes E, Vela C, et al. Oral immunization of rabbits with VP60 particles confers protection against rabbit hemorrhagic disease. Arch Virol 1996;141(8):1423–36.
[28] Young SL, Wilson M, Wilson S, Beagley KW, Ward V, Baird MA. Transcutaneous vaccination with virus-like particles. Vaccine 2006;24(26):5406–12.
[29] Brun A, Albina E, Barret T, Chapman DA, Czub M, Dixon LK, et al. Antigen delivery systems for veterinary vaccine development. Viral-vector based delivery systems. Vaccine 2008;26(51):6508–28.
[30] Friebe A, Friederichs S, Scholz K, Janssen U, Scholz C, Schlapp T, et al. Characterization of immunostimulatory components of orf virus (parapoxvirus ovis). J Gen Virol 2011;92(Pt. 7 (July)):1571–84.
[31] Rziha HJ, Henkel M, Cottone R, Bauer B, Auge U, Goetz F, et al. Generation of recombinant parapoxviruses: non-essential genes suitable for insertion and expression of foreign genes. J Biotechnol 2000;83:137–45.
[32] Buettner M, Rziha HJ. Parapoxviruses: from the lesion to the viral genome. J Vet Med B: Infect Dis Vet Public Health 2002;49(1):7–16.
[33] Haig DM. Orf virus infection and host immunity. Curr Opin Infect Dis 2006;19(2):127–31.
[34] Siegemund S, Hartl A, von BH, Dautel F, Raue R, Freudenberg MA, et al. Conventional bone marrow-derived dendritic cells contribute to toll-like receptor-independent production of alpha/beta interferon in response to inactivated parapoxvirus ovis. J Virol 2009;83(18):9411–22.
[35] Hosamani M, Scagliarini A, Bhanuprakash V, McInnes CJ, Singh RK. Orf: an update on current research and future perspectives. Expert Rev Anti Infect Ther 2009;7(7):879–93.
[36] Fischer T, Planz O, Stitz L, Rziha HJ. Novel recombinant parapoxvirus vectors induce protective humoral and cellular immunity against lethal herpesvirus challenge infection in mice. J Virol 2003;77(17):9312–23.
[37] Henkel M, Planz O, Fischer T, Stitz L, Rziha HJ. Prevention of virus persistence and protection against immunopathology after Borna disease virus infection of the brain by a novel Orf virus recombinant. J Virol 2005;79(1):314–25.
[38] Voigt H, Merant C, Wienhold D, Braun A, Hutet E, Le Potier MF, et al. Efficient priming against classical swine fever with a safe glycoprotein E2 expressing Orf virus recombinant (ORFV VrV-E2). Vaccine 2007;25(31):5915–26.
[39] Schirrmeier H, Reimann I, Kollner B, Granzow H. Pathogenic, antigenic and molecular properties of rabbit haemorrhagic disease virus (RHDV) isolated from vaccinated rabbits: detection and characterization of antigenic variants. Arch Virol 1999;144(4):719–35.
[40] Wirblich C, Meyers G, Ohlinger VF, Capucci L, Eskens U, Haas B, et al. European brown hare syndrome virus: relationship to rabbit hemorrhagic disease virus and other caliciviruses. J Virol 1994;68(8):5164–73.
[41] Pasamontes L, Gubser J, Wittek R, Viljoen GJ. Direct identification of recombinant vaccinia virus plaques by PCR. J Virol Methods 1991;35(2):137–41.
[42] Carlemalm E, Garavito RM, Villiger W. Resin development for electron microscopy and an analysis of embedding at low temperature. J Microstruct 1982;126:123–43.
[43] Gall A, Hoffmann B, Teifke JP, Lange B, Schirrmeier H. Persistence of viral RNA in rabbits which overcome an experimental RHDV infection detected by a highly sensitive multiplex real-time RT-PCR. Vet Microbiol 2007;120(1–2):17–32.
[44] Yuen L, Moss B. Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. Proc Natl Acad Sci USA 1987;84(18):6417–21.
[45] Nerenberg BT, Taylor J, Bartee E, Gouveia K, Barry M, Fruh K. The poxviral RING protein p28 is a ubiquitin ligase that targets ubiquitin to viral replication factories. J Virol 2005;79(1):597–601.
[46] Resch W, Weisberg AS, Moss B. Vaccinia virus nonstructural protein encoded by the A11R gene is required for formation of the virion membrane. J Virol 2005;79(11):6598–609.
[47] Roberts KL, Smith GL. Vaccinia virus morphogenesis and dissemination. Trends Microbiol 2008;16(10):472–9.
[48] Cheetham S, Souza M, Meulia T, Grimes S, Han MG, Saif LJ. Pathogenesis of a genogroup II human norovirus in gnotobiotic pigs. J Virol 2006;80(21):10372–81.
[49] Flynn WT, Saif LJ. Serial propagation of porcine enteric calicivirus-like virus in primary porcine kidney cell cultures. J Clin Microbiol 1988;26(2):206–12.
[50] Dory D, Fischer T, Beven V, Cariolet R, Rziha HJ, Jestin A. Prime-boost immunization using DNA vaccine and recombinant Orf virus protects pigs against Pseudorabies virus (Herpes suid 1). Vaccine 2006.
[51] Bertagnoli S, Gelfi J, Petit F, Vautherot JF, Rasschaert D, Laurent S, et al. Protection of rabbits against rabbit viral haemorrhagic disease with a vaccinia-RHDV recombinant virus. Vaccine 1996;14(6):506–10.
[52] Boga JA, Casais R, Marin MS, Martin-Alonso JM, Carmenes RS, Prieto M, et al. Molecular cloning, sequencing and expression in *Escherichia coli* of the capsid protein gene from rabbit haemorrhagic disease virus (Spanish isolate AST/89). J Gen Virol 1994;75(Pt. 9):2409–13.
[53] Chasey D, Lucas MH, Westcott DG, Sharp G, Kitching A, Hughes SK. Development of a diagnostic approach to the identification of rabbit haemorrhagic disease. Vet Rec 1995;137(7):158–60.
[54] Ferreira PG, Dinis M, Costa ES, Aguas AP. Adult rabbits acquire resistance to lethal calicivirus infection by adoptive transfer of sera from infected young rabbits. Vet Immunol Immunopathol 2008;121(3–4):364–9.
[55] Capucci L, Frigoli G, Ronshold L, Lavazza A, Brocchi E, Rossi C. Antigenicity of the rabbit hemorrhagic disease virus studied by its reactivity with monoclonal antibodies. Virus Res 1995;37(3):221–38.
[56] Lindesmith L, Moe C, Lependu J, Frelinger JA, Treanor J, Baric RS. Cellular and humoral immunity following Snow Mountain virus challenge. J Virol 2005;79(March (5)):2900–9.
[57] Bachmann MF, Oxenius A. Interleukin 2: from immunostimulation to immunoregulation and back again. EMBO Rep 2007;8(December (12)):1142–8.
[58] Friebe A, Siegling A, Friederichs S, Volk HD, Weber O. Immunomodulatory effects of inactivated parapoxvirus ovis (ORF virus) on human peripheral immune cells: induction of cytokine secretion in monocytes and Th1-like cells. J Virol 2004;78(Sepetmber (17)):9400–11.

OPEN ACCESS Freely available online

PLOS ONE

New Orf Virus (Parapoxvirus) Recombinant Expressing H5 Hemagglutinin Protects Mice against H5N1 and H1N1 Influenza A Virus

Jörg Rohde[¤], Ralf Amann[¤], Hanns-Joachim Rziha*[¤]

Institute of Immunology, Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Island of Riems, Greifswald, Germany

Abstract

Previously we demonstrated the versatile utility of the *Parapoxvirus* Orf virus (ORFV) as a vector platform for the development of potent recombinant vaccines. In this study we present the generation of new ORFV recombinants expressing the hemagglutinin (HA) or nucleoprotein (NP) of the highly pathogenic avian influenza virus (HPAIV) H5N1. Correct foreign gene expression was examined *in vitro* by immunofluorescence, Western blotting and flow cytometry. The protective potential of both recombinants was evaluated in the mouse challenge model. Despite adequate expression of NP, the recombinant D1701-V-NPh5 completely failed to protect mice from lethal challenge. However, the H5 HA-expressing recombinant D1701-V-HAh5n mediated solid protection in a dose-dependent manner. Two intramuscular (i.m.) injections of the HA-expressing recombinant protected all animals from lethal HPAIV infection without loss of body weight. Notably, the immunized mice resisted cross-clade H5N1 and heterologous H1N1 (strain PR8) influenza virus challenge. *In vivo* antibody-mediated depletion of CD4-positive and/or CD8-posititve T-cell subpopulations during immunization and/or challenge infection implicated the relevance of CD4-positive T-cells for induction of protective immunity by D1701-V-HAh5n, whereas the absence of CD8-positive T-cells did not significantly influence protection. In summary, this study validates the potential of the ORFV vectored vaccines also to combat HPAIV.

Citation: Rohde J, Amann R, Rziha H-J (2013) New Orf Virus (Parapoxvirus) Recombinant Expressing H5 Hemagglutinin Protects Mice against H5N1 and H1N1 Influenza A Virus. PLoS ONE 8(12): e83802. doi:10.1371/journal.pone.0083802

Editor: Matthias Johannes Schnell, Thomas Jefferson University, United States of America

Received September 30, 2013; **Accepted** November 16, 2013; **Published** December 20, 2013

Funding: Part of the work was financially supported by Pfizer Animal Health. No additional external funding was received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The indicated funding from Pfizer-Animal Health did not influence at any time the planning, performance, analysis, interpretation and discussion of the presented results. It also does not alter the authors' adherence to all PLOS ONE policies on sharing data and materials.

* E-mail: achim.rziha@fli.bund.de

¤ Current address: Department of Immunology, Interfaculty Institute for Cell Biology, University Tuebingen, Tuebingen, Germany.

Introduction

Influenza A virus is a member of the *Orthomyxoviridae* and can infect numerous hosts, including aquatic birds, poultry, swine and humans (for review [1]). Its negative-sense, single-stranded RNA genome is composed of eight gene segments encoding the viral proteins. The genetic variation of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) is the basis for further subtyping influenza A viruses in H1 to H16 and N1–N9, respectively [2], and a novel subtype H17N10 has recently been detected in bats [3,4]. Cross-species transmission of influenza viruses to humans has been documented frequently, and in 2009 the new H1N1 influenza A virus (pH1N1) resulted from recombination of gene segments from human, swine and avian influenza A virus causing a new pandemic human flu [5]. The highly pathogenic avian influenza virus (HPAIV) H5N1 has caused outbreaks in wild birds and poultry leading to severe, fatal disease [6], and transmission from birds to humans was reported [1,7]. The World Health Organization registers approximately 600 confirmed human H5N1 virus infections, approximately 60% resulting in death (WHO, August 2013; http://www.who.int/influenza/human_animal_interface/EN_GIP_20130829CumulativeNumberH5N1cases.pdf). Thus, serious concerns exist about the emergence of a pandemic H5N1 strain transmissible between humans. The trimeric HA is an important viral factor determining virulence, host tropism and transmission of influenza A virus [8,9,10,11]. For entering the host cell the HA0 precursor form of the trimeric HA must be proteolytically cleaved into HA1, which binds to sialic acid-containing host cell receptors, and into HA2, which mediates membrane fusion. This cleavage site differs amongst HA subtypes, which in part, can determine the degree of virulence (for review [12]). Influenza virus infections can be effectively controlled and prevented by vaccination. Currently, inactivated vaccines are produced according to the HA and NA subtypes of circulating virus strains. Virus-neutralizing and receptor-blocking antibodies directed against HA1, the globular head of HA, can mediate sterilizing immunity provided that they have the proper strain-specificity. However, the rapid mutation rate of NA and of HA1 can impede the production of effective vaccines matching currently circulating virus types. Therefore, several attempts are reported for the generation of effective, more universal influenza virus vaccines (reviewed in [13]). Plasmid DNA vaccines expressing consensus sequences of HA and NA mounted cross-reactive cellular and humoral immune responses [14,15] and were able to protect mice against divergent H5N1 strains [16]. Other approaches comprise the development of headless constructs, also to limit the suggested immunodominance of the

globular head of HA [17]. Recent reports on the construction of various chimeric head and stalk HA proteins or functional influenza viruses expressing those chimeras offer another strategy for cross-protecting vaccines [18,19].

Besides the humoral immune response against Influenza virus, T-cells that either eliminate infected cells or help B-cells to mount a more rapid and efficient neutralizing antibody response are also important to relieve the disease [20]. Especially cytolytic and cytokine-secreting T-cells directed to conserved influenza virus proteins, like the nucleoprotein (NP) or matrix protein (M1), can represent effectors in protective immunity [21,22,23] and are considered another promising approach for the development of more universal influenza vaccines [24,25,26]. HA epitopes, which are recognized by virus-specific human and mouse CD8-positive cytolytic T-cells, have also been identified (reviewed in [27]). The role of additional viral targets in adaptive, protective immunity against influenza A virus has recently been reviewed comprehensively [28,29].

Various strategies are pursued to develop improved, safe, effective and cross-protecting vaccines not only against H5N1 strains but also against different influenza A virus subtypes. Those approaches comprise the generation of baculovirus-based multivalent vaccines [30] or self-assembling viral-like particles [31,32], or DNA vaccines preferentially now in prime boost combinations with e.g. adenovirus recombinants [13]. The efficient and fast technology of reverse genetics allows the safe and effective creation of recombinant or attenuated influenza viruses with almost every desired gene alteration and constellation (reviewed in [33]). Moreover, attenuated influenza virus designed by a synthetic engineering approach to recode and synthesize the viral genome induced protective immunity in mice [34]. Finally, very recently the successful vaccination with optimized mRNA of HA, NA, and NP was reported, which stimulated T- and B-cell dependent protection against influenza A H1N1, H3N2 and H5N1 viruses [35]. Poxvirus-vectored vaccines are attractive due to the possibility for inserting multiple antigens by established methods, and their potential of rapid stimulation of good humoral and cell-mediated immune responses also mediating protection against e.g. HPAIV challenge infection [36,37,38,39]. For safety reasons attenuated or replication-deficient poxviral vectors have been developed and used to mount protective immune responses against different influenza A virus subtypes [40,41].

The *Orf Virus* (ORFV) from the genus *Parapoxviridae* (*PPV*) represents a promising candidate for novel vectored vaccines [42,43,44,45,46,47,48,49]. ORFV has a very restricted host range *in vivo and in vitro*, a restricted skin tropism and an absence of systemic infection [50]. Ideal vector vaccine properties are the short-lived ORFV vector-specific immunity allowing repeated immunizations, and still not entirely understood immunomodulating properties, which lead to the induction of strong innate and adaptive Th1-Th2 balanced immune responses [44,45,50,51]. The inserted foreign genes are regulated by an early ORFV promoter, which results in the induction of foreign antigen-specific immunity without the need of replication and multiplication of mature, infectious ORFV.

The present study describes the generation of new ORFV recombinants expressing the HPAIV genes H5 HA (D1701-V-HAh5n) or H5 NP (D1701-V-NPh5). After demonstrating proper expression of the inserted HPAIV genes, the protective potential of both recombinants was investigated by challenge infection of mice. Whereas the HA-expressing recombinant was able to protect all mice against lethal H5N1 virus challenge, the NP-expressing recombinant failed to mount protective immunity. Intramuscular (i.m.) immunization with D1701-V-HAh5n mediated cross-clade (H5N1 clades 1, 2.2.2, and 2.2.3) and heterosubtypic (H1N1) protection in different mouse strains. *In vivo* T-cell depletion experiments and a dose dependent increase of H5 HA-specific antibodies indicated that both arms of the immune response seem to be essential for protection after immunization with the HA-expressing ORFV recombinant.

Materials and Methods

Ethics statement

All animal studies were reviewed and approved by the local authorities (Regional council of Tuebingen) and were carried out in strict accordance with the regulations of the German animal welfare law set forth by this authority (permit number FLI 250/10).

Cells and viruses

Propagation and titration of ORFV in Vero cells has been described earlier [44]. The highly pathogenic H5N1 avian influenza A viruses (HPAIV) A/Mallard/Bavaria/1/2006 (MB1, clade 2.2.1), A/mute swan/Germany/R1349/07 (SN1, clade 2.2.3), and the H1N1 human influenza A virus A/Puerto Rico/8/34 (PR8) were kindly provided by O. Planz (Univ. Tübingen, Dep. Immunology) and L. Stitz (Friedrich-Loeffler-Institut, Germany). The HPAIV were propagated and titrated as described [20]. For inactivation, the MB1 virus was incubated with 0.02% formalin at 4°C for three days and then stored at −20°C.

Generation and selection of new ORFV recombinants

The HA coding sequence of H5N1 influenza A strain Vietnam/1203/2004 (Acc. no. AY818135) and the NP coding sequence of strain MB1 (Acc. no. DQ792924) were chemically synthesized by GeneArt (Regensburg, Germany) changing poxviral early transcript stop motifs (TTTTTCT) by silent mutations from codon TTT to TTC. In addition, new restriction sites were added to the 5′ and 3′ ends of both genes allowing to clone the HA gene as a HindIII – BamHI fragment and the NP gene as a KpnI – EcoRI fragment into plasmid pdV-Rec1 [44]. Correct insertion of the AIV genes into the obtained transfer plasmids pdV-HAh5n3 and pdV-NPh5n were tested by DNA-sequencing and restriction enzyme analysis (data not shown). Electroporation of *LacZ* positive ORFV D1701-VrV-infected Vero cells (moi 0.1–0.2) with 2 µg pdV-HAh5n3 DNA or pdV-NPh5n DNA, respectively, and selection of the new ORFV recombinants was described recently [46]. Single plaque PCR was used to screen virus progeny positive for the HA or the NP gene and negative for the *LacZ* gene of the parental virus D1701-VrV. Oligonucleotides used as PCR primers were purchased from Metabion (Martinsried, Germany). H5 HA-specific amplification (459 bp) was achieved with 3.8 pmol primer HA5Fn 5′-GTG AGC AGC GCA TGT CCT TAC CAG-3′ and 3.8 pmol primer HA5-Rnn 5′-CTC CCA TAG GGG TCT GGC ACT TTG-3′, NP-specific amplification (452 bp) with 4 pmol primer NP5-F 5′-GGA GGA TTT GGC GTC AAG CGA AC-3′ and 3.8 pmol primer NP5-R 5′-CTC TCA GGA TGA GTG CAG ACC TTG-3′. The PCR reactions contained 2X Reddy mix (ABgene, Fisher Scientific, Germany) and were denatured at 98°C for 2 minutes followed by 35 cycles at 96°C (1 min), annealing (30 sec) at 66°C for HA or 70°C for NP, and extension at 72°C (30 sec) in a T3-Thermocycler (Biometra, Germany). The amplification of the *LacZ* gene fragment was performed as described [46]. PCR amplicons were detected by electrophoresis using 0.8 % (w/v) agarose-ethidium bromide gels.

Antibodies

Specific detection of H5 HA was accomplished with the mouse monoclonal antibody (mAb) 15A3 (Rockland, USA) and the polyclonal rabbit LGL antiserum (kindly provided by M. Büttner, Bavarian Health and Food Safety Authority, Oberschleissheim, Germany). The mouse mAb 2442 (Abnova, Germany) was used for specific recognition of the NP protein. The mAb 4D9 [52] allowed detection of the ORFV major envelope protein (F1L), the β-actin specific antibody was purchased from Sigma-Aldrich (Germany). Goat anti-mouse Alexa Fluor 488-conjugated (Fisher Scientific, Invitrogen, Germany), horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG (Dianova, Germany) and goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (Dianova, Germany) were used as second antibodies.

Western blot analysis

Non-infected or infected Vero cells were suspended in 1% (v/v) Triton-X100 (Sigma-Aldrich, Germany) in PBS and incubated for 30 minutes at 4°C. Western blot analysis was performed as described [46]. Protein concentration of the lysates was determined using Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Germany) according to the recommendation of the manufacturer. Afterwards, the lysates were adjusted to equal protein concentrations. The antibodies were diluted in 1X RotiBlock (Roth, Germany) and the substrate Immobilion Western HRP (Millipore, Germany) was used for enhanced chemiluminescence (ECL). X-ray films for ECL were purchased from Pierce (Thermo Fisher Scientific, Germany).

Immune peroxidase monolayer assay (IPMA)

Expression of inserted HA and NP genes in recombinant-infected cells was demonstrated by IPMA exactly as described [42] using HRP substrate (Vector NovaRED, USA).

Immunofluorescence

Vero cells infected with the ORFV recombinants were grown in chamber slides (BD Biosciences, Germany), fixed with 2% (v/v) methanol-free formaldehyde (Pierce, Thermo Fisher Scientific, Germany) in PBS and permeabilized with 0.2% Triton-X100 (Sigma, Germany) as reported [46]. Microscopy was performed with ApoTome confocal fluorescence microscope (Axiovert 200 M; Zeiss, Germany) and AxioVision Rel. 4.8 software (Zeiss).

Flow cytometry

Vero cells were harvested by trypsinization and washed once with FACS buffer (10% v/v foetal bovine serum, 0.1% v/v sodium azide in PBS). Approximately 10^6 cells were stained with H5 HA-specific primary antibody mAb15A3 for 30 minutes at 4°C. After three times washing the cells were stained in the dark with FITC-conjugated secondary antibody for another 30 minutes at 4°C. To exclude nonviable cells staining with 7-AAD (7-Amino-Actinomycin D; BD Bioscience, Germany) was performed 10 minutes prior to flow cytometry with FACSCalibur (BD Bioscience, Germany) and CellQuest Pro (BD Bioscience, Germany). Gates were set for viable cells negative for 7-AAD.

Vaccination of mice and Influenza A virus challenge

BALB/c and C57BL/6 mice at the age of 8-12 weeks were obtained from the animal breeding facility of the Friedrich-Loeffler-Institut (Germany). Mice were instilled intranasally (i.n.) under anaesthesia [20] using 50 µl of the indicated mouse 50% lethal dose (MLD50) of HPAIV. For BALB/c mice 1× MLD50 corresponded to 7×10^1 plaque-forming units (pfu) of strain MB1, 2×10^1 pfu of strain SN1 and 1×10^4 pfu of strain PR8. For C57BL/6 mice 2×10^3 pfu of strain MB1, 1.4×10^3 pfu of strain PR8 matched to 1× MLD50. Weight loss and survival of infected mice was daily monitored during 14 days after challenge infection. According to the German animal-protection law, animals that lost approximately 25% of their body weight were sacrificed, documented as dead, and thereafter excluded from calculation of the body weight graph. The challenge experiments were performed under BSL3 conditions at the Friedrich-Loeffler-Institut, Tübingen (Germany).

Table 1. Monitoring the success of T-cell depletion.

Days [a]	CD4-positive T-cells (%)	CD8-positive T-cells (%)
2	0.20 (n = 4: 0.00–0.80) [b]	0.03 (n = 5: 0.00–0.07)
6	0.15 (n = 4: 0.00–0.39)	0.03 (n = 5: 0.00–0.08)
9	0.06 (n = 4: 0.01–0.06)	0.01 (n = 5: 0.00–0.04)
14	1.55 (n = 4: 0.34–3.10)	0.22 (n = 5: 0.06–0.45)

[a]Days after second antibody treatment.
[b]Mean percentage; number of animals (n) and range of percentage is given in parenthesis.
doi:10.1371/journal.pone.0083802.t001

In vivo depletion of T-cell subpopulations

Monoclonal antibodies directed against murine CD4 (mAB YTS 191.1) or CD8 (mAB YTS 169.4) [53] were kindly provided by L. Stitz, (Friedrich-Loeffler-Institut, Germany) and used for depletion of T-cell subsets as described recently [42]. The 1:25 diluted mAbs were administered intraperitoneally, and 0.2 ml of each mAb was applied per mouse or 0.4 ml of an equal mixture of

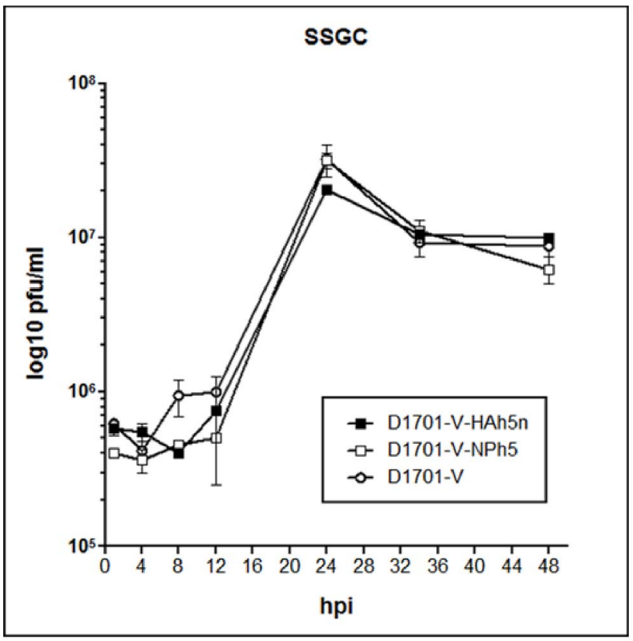

Figure 1. Single step growth curve. Comparison of the *in vitro* growth characteristics of D1701-V-HAh5n, D1701-V-NPh5 and parental D1701-V. Vero cells were infected with moi 5.0 and total cell lysates were taken for virus titration at the indicated hours post infection (hpi). The results demonstrate very similar growth kinetics of both ORFV recombinants and the parental D1701-V.
doi:10.1371/journal.pone.0083802.g001

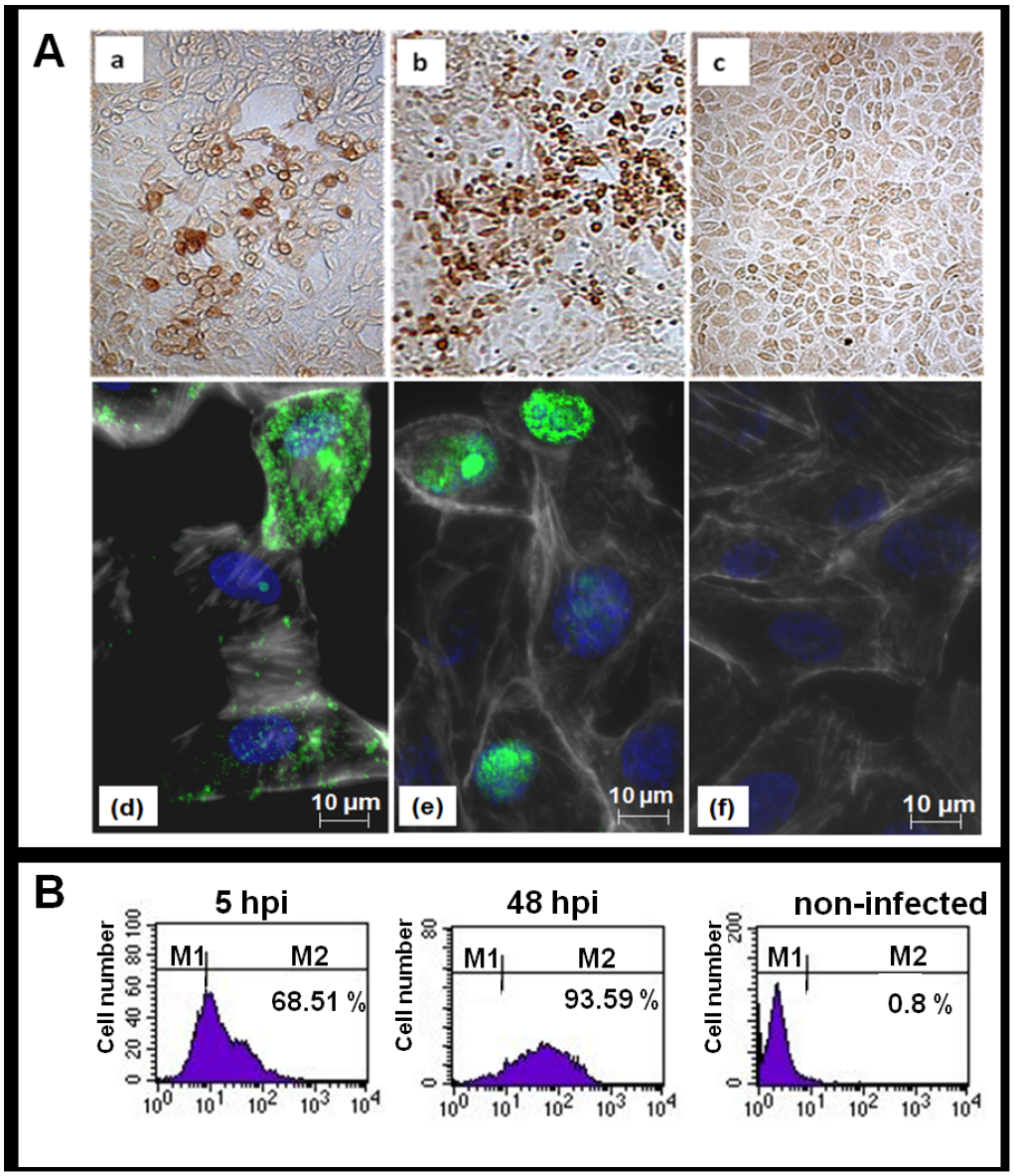

Figure 2. AIV gene expression of D1701-V-HAh5n and D1701-V-NPh5. (A) Expression of HA and NP in ORFV recombinant-infected cells demonstrated by IPMA (panel a–c) and by immunofluorescence (panel d–f). Vero cells were infected with D1701-V-HAh5n (panel a and d), D1701-V-NPh5 (panel b and e) or non-infected (panel c and f). Three days post infection transgene expression (brown) is detected with the HA-specific, 1:250 diluted LGL antiserum (panel a, magnification ×40) and with the NP-specific, 1: 500 diluted mAb 2442 (panel b, magnification ×20), whereas non-infected cells (panel c, magnification ×40) remained unstained. HA-specific immunofluorescence (green) is shown 24 hpi with the 1:250 diluted mAb 15A3 (panel d), and nuclear NP expression with the 1:1,000 diluted mAb 2442 (panel e). Non-infected cells as negative control are depicted in panel f. The cell nuclei are DAPI-stained (blue) and the actin cell skeleton is stained by Phalloidin-CF647 (white). **(B)** Cell surface expression of H5 HA was quantified by flow cytometry. Vero cells were harvested 5 hours (5 hpi) and 48 hours (48 hpi) after D1701-V-HAh5n infection (moi 1.0) and stained with mAb 15A3. The histograms show the cell number (ordinate) plotted against the fluorescence intensity (abscissa) gated for 7-AAD negative, viable cells. HA-positive cells are gated in M2, negative cells in M1. Distinct H5 HA cell surface expression was demonstrable already 5 hpi increasing with later times after infection.
doi:10.1371/journal.pone.0083802.g002

both mAb for the simultaneous depletion of CD4- and CD8-positive T-cells. The timeline of the injections of mAb is detailed in the Results part. Efficacy of depletion and kinetic of T-cell repopulation was monitored by flow cytometry in a preliminary experiment. Blood was taken from the retro-orbital plexus 2, 6, 9, and 14 days after antibody-treatment at days −2 and 0. The gated lymphocytes were used for double-staining with PE- or FITC-labelled CD3- and CD4- or CD3- and CD8-specific antibodies (BD Biosciences, Germany). Non-depleted mice contained on the average approximately 30 % CD-4 positive T-cells (n = 4: 20.6%–33.5%) and 5 % CD-8 positive T-cells (n = 4: 3.8%–5.9%), respectively. After antibody treatment more than 99% of each T-cell subpopulation remained absent for at least 9 days, before T-cell repopulation started (Table 1), similarly as reported earlier [53,54].

The mice (BALB/c; n = 8) were i.m. immunized twice (14 days interval) with 10^7 pfu of the recombinant D1701-V-HAh5n.

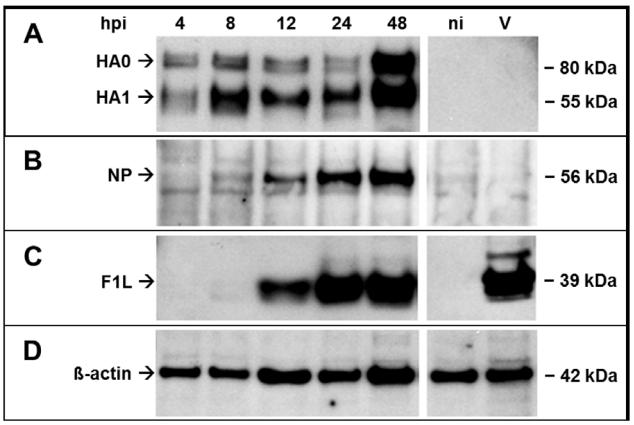

Figure 3. Western Blot analysis for the detection of HA and NP. Cell lysates were harvested at indicated hours post infection (hpi) with **(A, C)** D1701-V-HAh5n (moi 3.0) or **(B, D)** D1701-V-NPh5 (moi 1.0). As controls non-infected cells (lanes ni) or parental D1701-V- (moi 1.0) infected cells (lanes V) were tested at 24 hpi. **(A)** H5 HA was detected with the specific mAb 15A3 (diluted 1:5,000), **(B)** NP was detected with specific mAb 2442 (diluted 1:2,000). **(C)** The mAb 4D9 (diluted 1:800) was used to detect the ORFV major envelope protein F1L expressed at late times pi. **(D)** Beta-actin was demonstrated as loading control. The apparent mol. wt. of the specific proteins is indicated in kilodalton (kDa).
doi:10.1371/journal.pone.0083802.g003

Fourteen days after last vaccination the i.n. challenge infection was performed with 20× MLD_{50} HPAIV MB1.

Hemagglutination inhibition (HI) test

HI test was performed according to the OIE (World Organization for Animal health) instruction manual (Manual of diagnostic tests and Vaccines for terrestrial animals, 5th edition, 2004) in 96-well microtiter plates (Greiner bio-one, Germany) using 25 µl 1% (v/v) suspension of chicken red blood cells in PBS. Twenty-five µl of two-fold dilutions of heat-inactivated (30 min, 56°C) sera were incubated for 40 min at RT with 4 hemagglutination units (HAU) of formalin-inactivated H5N1 MB1 virus. The HI titre was defined as the reciprocal of the highest serum dilution inhibiting hemagglutination.

Results

Generation of the new ORFV recombinants

The transfer plasmids containing the H5 HA gene (pdV-HAh5n3) or the NP gene (pdV-NPh5n) were used for electroporation of Vero cells infected with D1701-VrV, which expresses the *LacZ* gene and enables the blue-white selection as described [44,46]. New white ORFV recombinants were selected by PCR as described in Material and Methods. Single plaque virus isolates of D1701-V-HAh5n and D1701-V-NPh5 were subject to five and four additional rounds of plaque purification, respectively, to obtain genetically homogeneous new ORFV recombinants. Single step growth curve experiments demonstrated that the insertion of the H5 HA or NP gene had no influence on the *in vitro* growth characteristics of both ORFV recombinants compared to the parental ORFV D1701-V (Fig. 1). Transgene expression of the virus plaque isolates was tested by IPMA. As shown in Figure 2A (panel a–c) expression of the H5 HA and NP transgene in ORFV recombinant-infected cells was demonstrable by specific brown immune staining (Fig. 2A, panel a, b), but not in non-infected cells (Fig. 2A, panel c) or in cells infected with the parental D1701-VrV (data not shown). Correct insertion of the HA or NP gene into the *vegf-e* gene locus of D1701-V was verified by PCR and Southern blot hybridization of recombinant virus DNA (data not shown).

Expression of the H5 HA and NP gene

Indirect immunofluorescence assays demonstrated expression of H5 HA and NP gene in recombinant virus-infected cells (Fig. 2A). As expected, the NP gene was expressed in the nuclei of D1701-V-NPh5n-infected cells (Fig. 2A, panel e), whereas non-infected (Fig. 2A, panel f) or parental virus-infected Vero cells (data not shown) remained negative. Cell surface expression of H5 HA was further demonstrated by flow cytometry of D1701-V-HAh5n-infected Vero cells. Already five hours after infection (hpi) 68.5% of the infected cells expressed the H5 HA on the cell surface increasing to 93.6% at 48 hpi (Fig. 2B).

Expression of the inserted influenza A virus HA and NP gene was also inspected by Western blot analysis at different times after infection of Vero cells with D1701-V-HAh5n or D1701-V-NPh5. Using mAb 15A3 the expression of the H5 HA was detectable from 4 hpi onwards with increasing amounts at later times after infection (Fig. 3A). At all tested time points after infection the precursor protein HA0 was recognized as a double band migrating with a mol. wt. of approximately 80 kDa as well as the subunit HA1 (55 kDa). The subunit HA2 is not recognized by the used antibody. Non-infected (ni) cells and cells infected with parental D1701-V (V) remained negative. The NP protein (56 kDa) was demonstrable with mAb 2242 by Western blot analysis (Fig. 3B). In the presented experiment, protein lysates were obtained from cells infected with a lower moi (1.0) of D1701-V-NPh5 in contrast to the D1701-V-HAh5n lysates (moi = 3.0), which explains the weaker NP expression compared to HA. In addition, early NP expression could be unequivocally proven by Northern blot analysis (data not shown). The late ORFV major envelope protein F1L (39 kDa) was recognized beyond 12 hpi with the mAb 4D9, which reflected multiplication of the ORFV recombinants in Vero cells (Fig. 3C). Nearly comparable protein loading was verified by detection of cellular β-actin (Fig. 3D).

Protection of mice from challenge with divergent H5N1 strains

The protective potential of the new ORFV recombinants was evaluated first in C57BL/6 mice after i.m. immunization with different doses followed by i.n. challenge infection with strain MB1 (20× MLD50). A single immunization with 1×10^5 pfu of D1701-V-HAh5n was not able to mediate proper protection against the MB1 challenge infection. Except of 2 mice, all animals suffered from severe disease and 6 out of the 12 mice had to be euthanized at days 6 to 8 after challenge (Fig. 4A). The surviving mice gradually lost body weight 2 to 4 days after challenge (Fig. 4B), individually ranging from 8% to 15%, and one animal lost 24% of weight. Thereafter all survivors recovered and regained their body weight (Fig. 4B). A booster immunization with 10^5 pfu improved protection rate to 89% survival, 8 out of 9 mice survived the challenge (Fig. 4A). One survivor suffered from severe illness associated with 24% weight loss before recovering, whereas the body weight loss of the other 7 mice ranged only from 8% to 17% around 3 days after challenge (Fig. 4B, mean 12%). Increasing the immunization dosage of D1701-V-HAh5n to 1×10^6 pfu, again as a single application mediated only partial protection from challenge (Fig. 4C) and from body weight loss (Fig. 4D). The 4 surviving animals experienced weight loss of 7%, 11%, 21%, and 24%, respectively, before recovering beyond day 4 (Fig. 4D). Two immunizations with 1×10^6 pfu, however, conferred complete protection from lethal challenge (Fig. 4C and 4D). Only 3 mice

lost 11% body weight, whereas the other mice sustained their body weight and remained healthy.

The administration of 10^7 pfu of D1701-V-HAh5n clearly improved the generation of protective immunity against 20× MLD50 MB1 challenge. Single vaccination with that immunization dosage was sufficient to mediate 100% survival (Fig. 4E). At day 3 after challenge a mean body weight loss of 18% was observed (individually ranging from 14% to 23%), but thereafter all mice fully recovered and regained their body weight (Fig. 4F). All C57BL/6 mice receiving 2 doses of 1×10^7 pfu of that

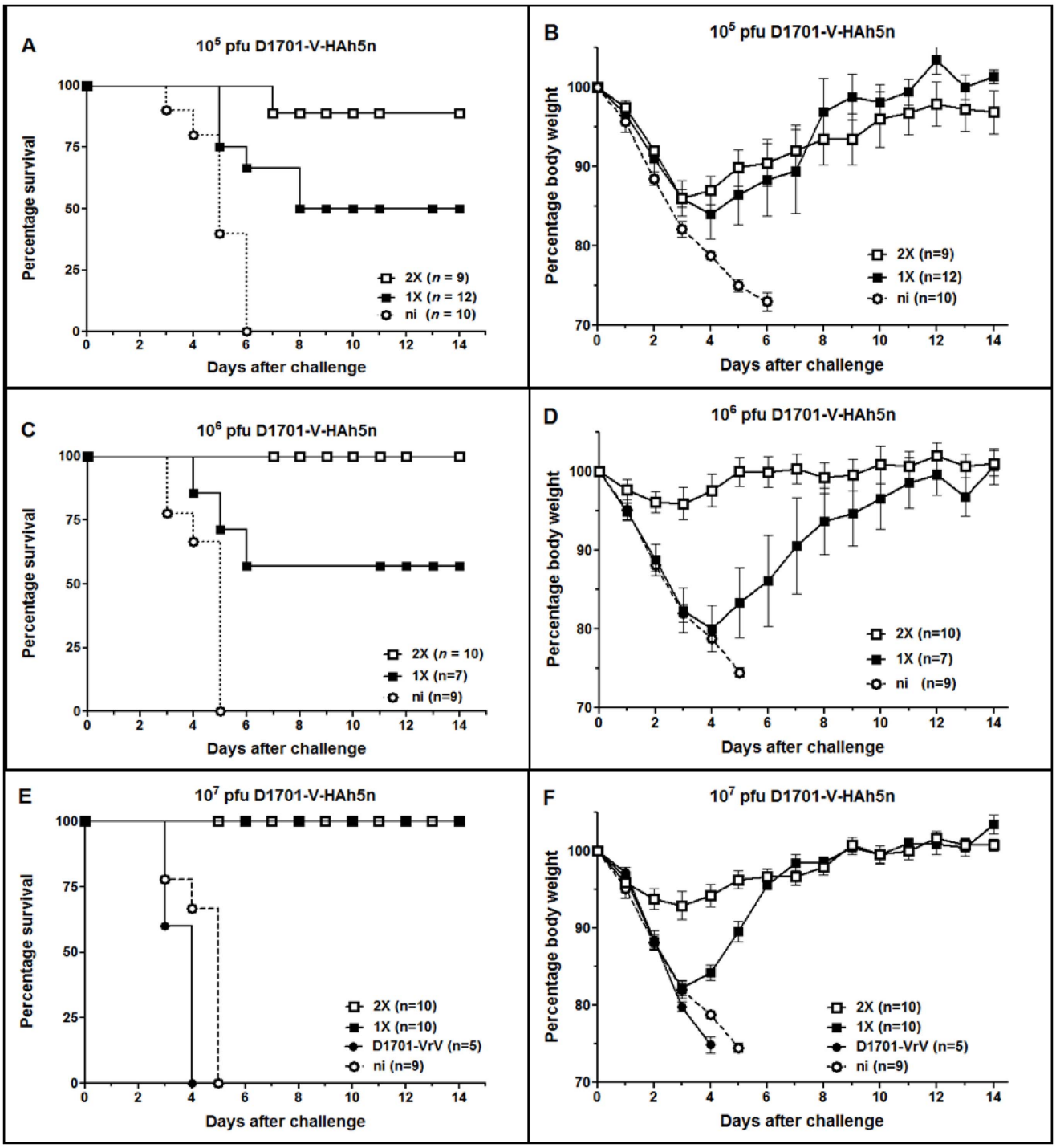

Figure 4. Protective efficacy of D1701-V-HAh5n in C57BL/6 mice. Survival rates (A, C, E) and mean body weight changes (B, D, F) of C57BL/6 mice i.m. immunized once (1X) or twice (2X) with the indicated pfu of D1701-V-HAh5n. The mice were monitored during 14 days after i.n. challenge infection with 20× MLD50 of H5N1 strain MB1. As controls mice were immunized two times with 10^7 pfu of parental ORFV D1701-VrV (E, F) or were non-immunized (ni). SEM is shown by bars, *n* indicates the total number of mice in each group. Mice exhibiting more than 25% loss of body weight were sacrificed according to the German animal protection law.
doi:10.1371/journal.pone.0083802.g004

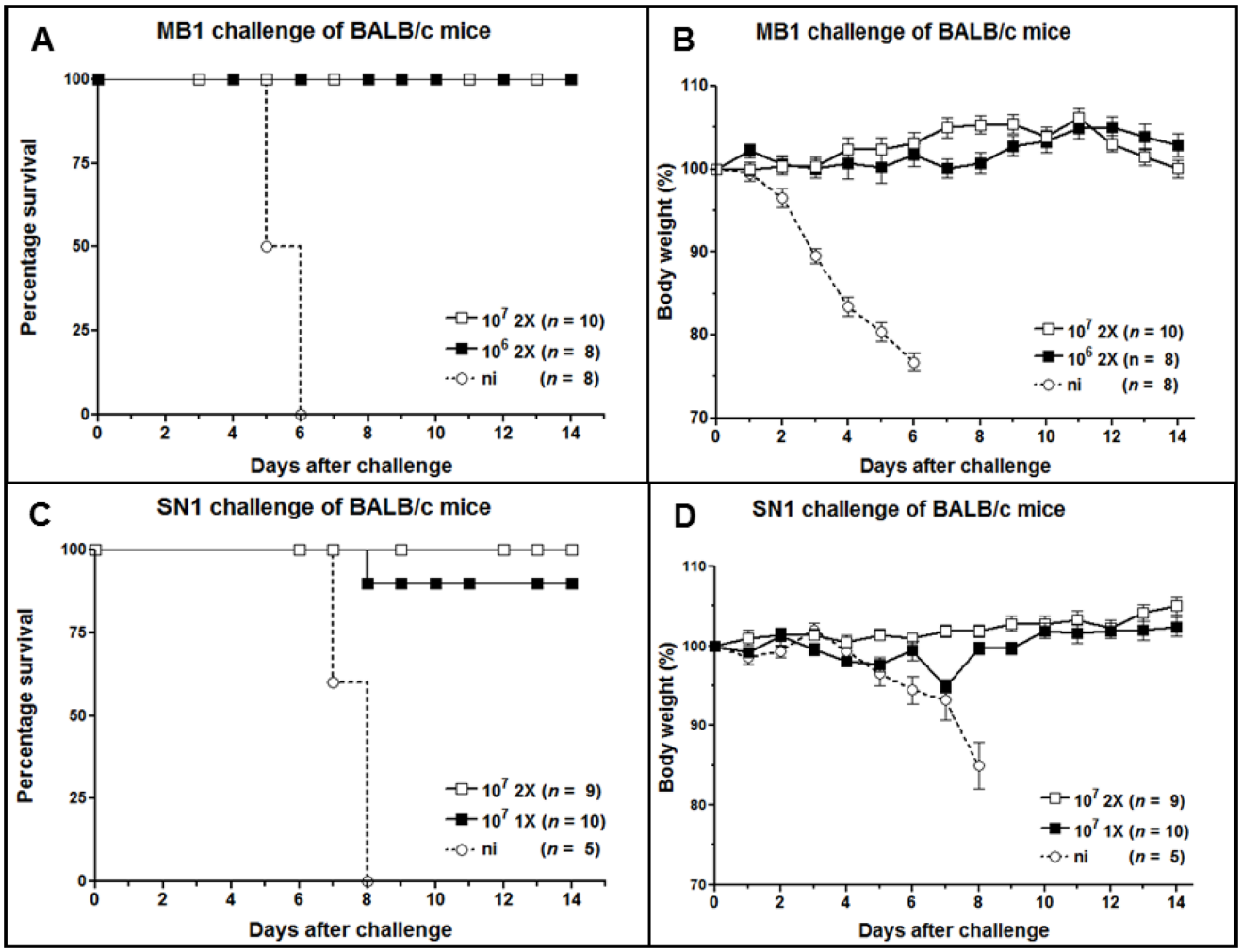

Figure 5. Protection of BALB/c mice from cross-clade HPAIV challenge infection. Survival rates (A, C) and body weight changes (B, D) of BALB/c mice after i.n. challenge with 20× MLD50 of H5N1 strain MB1 (A, B) or SN1 (C, D). Single (1X) or booster (2X) i.m. immunization was performed with 10^6 pfu or 10^7 pfu D1701-V-HAh5n and mice were monitored during 14 days after challenge infection. Mean percentage of body weight change is shown (bars indicate SEM), and *n* indicates the number of mice per group. After approximately 25% loss of body weight mice were sacrificed according to the German animal protection law.
doi:10.1371/journal.pone.0083802.g005

recombinant survived the challenge (Fig. 4E) and stayed healthy (Fig. 4F). Only 2 animals exhibited a transient weight loss of 17% until day 3 before also retrieving their original body weight. Control mice receiving 2 injections of 10^7 pfu of the parental ORFV D1701-VrV were not protected against the MB1 challenge infection. Onset of disease and loss of body weight were similar to non-immunized animals, and the mice had to be euthanized 4 days after challenge (Fig. 4E and F).

The NP gene expressing recombinant D1701-V-NPh5 did not confer protection from lethal challenge or prevent morbidity after infection with 20× MLD50 MB1. Groups of mice (C57BL/6, n = 10) were i.m. immunized once or twice and neither 10^6 nor 10^7 pfu of D1701-V-NPh5n were able to protect. All animals became diseased and had to be sacrificed during days 4 to 6 after challenge, likewise the non-immunized or parental D1701-VrV vaccinated control animals. Also three doses of 5×10^6 pfu did not confer protection against challenge infection (data not shown).

The protective potential of D1701-V-HAh5n was also tested in BALB/c mice, which are approximately 30-fold more susceptible to MB1 challenge as compared to C57BL/6 mice (see Material and Methods). Two i.m. applications of either 1×10^6 or 1×10^7 pfu also elicited complete protection against 20× MLD50 of strain MB1. All immunized animals survived the lethal challenge without body weight loss or any sign of disease, in contrast to all non-immunized mice (Fig. 5A and B). As for C57BL/6 mice again vaccination with D1701-V-HAh5n, expressing H5 HA from a clade 1 HPAIV (A/Vietnam/1203/2004), could protect from a cross-clade challenge infection with HPAIV strain MB1 (clade 2.2.1). Finally we found protection of mice from challenge with 20× MLD50 HPAIV strain SN1, which belongs to influenza A virus clade 2.2.3. As depicted in Figure 5C, 9 out of 10 BALB/c mice survived the challenge infection after a single administration of 10^7 pfu D1701-V-HAh5n, and all animals immunized twice survived the challenge. Both groups of mice did not show loss of body weight (Fig. 5D) and the non-immunized mice had to be sacrificed within 8 days after challenge infection.

Protection from challenge with H1N1 strain PR8

After demonstrating the induction of protective immunity against HPAIV H5N1 strains of clade 2, we tested the potential

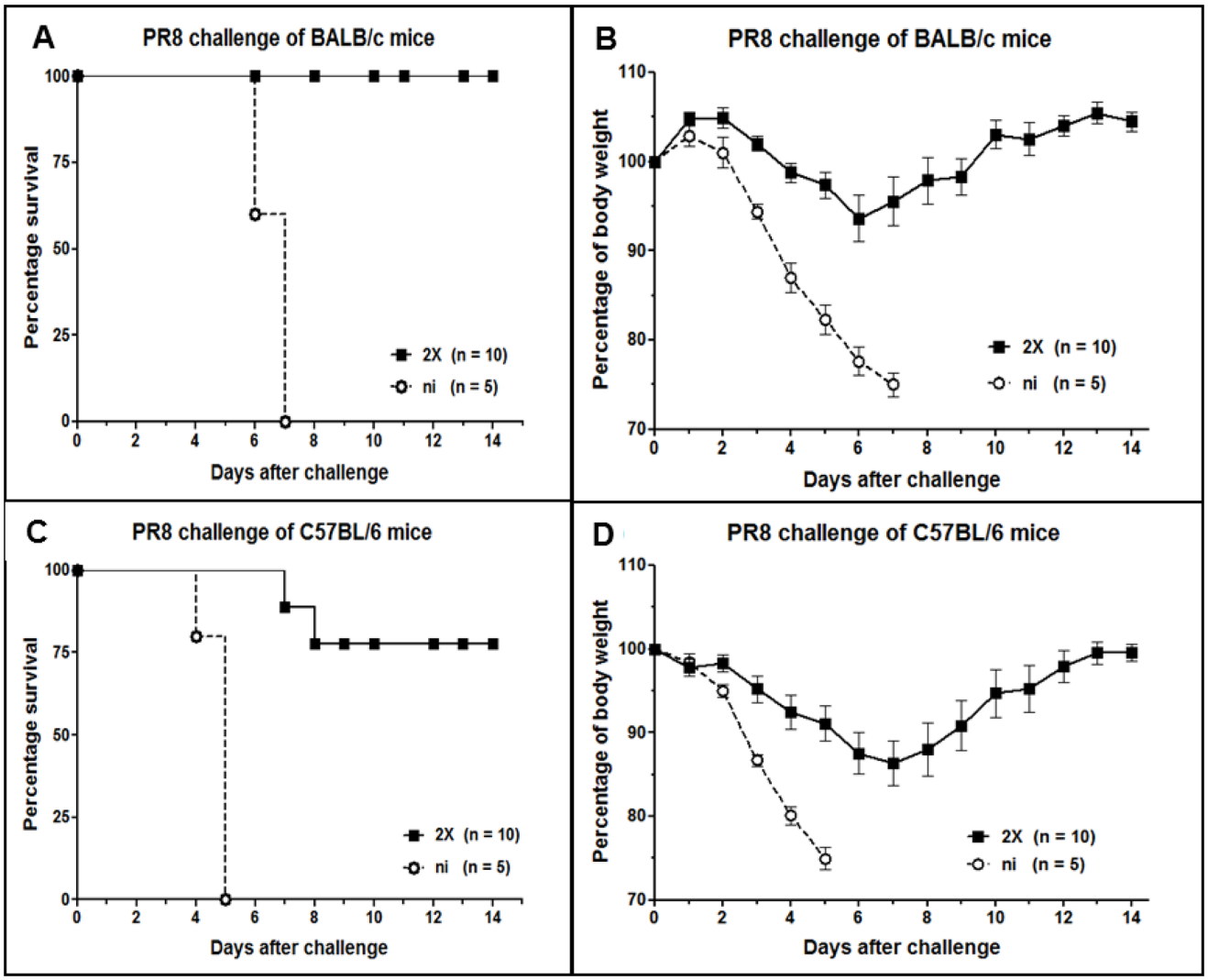

Figure 6. Protection against heterologous influenza A virus H1N1. Survival rates (A, C) and average body weight loss (B, D) of BALB/c (A, B) or C57BL/6 (C, D) mice i.m. immunized twice (2X) with 10^7 pfu D1701-V-HAh5n. The percentage of body weight (bars indicate SEM) was monitored during 14 days after i.n. challenge infection with 50× MLD50 (A, B) or 20× MLD50 (C, D) of H1N1 strain PR8. Mice suffering from more than 25% body weight loss were sacrificed. Control non-immunized mice (ni) are shown, *n* indicates the number of mice per group.
doi:10.1371/journal.pone.0083802.g006

of the recombinant D1701-V-HAh5n to protect mice against heterologous H1N1 influenza A virus. BALB/c and C57BL/6 mice were i.m. immunized once or twice with 10^7 pfu of D1701-V-HAh5n followed by i.n. challenge infection with lethal doses of the H1N1 strain PR8. Whereas a single immunization was not sufficient to protect BALB/c mice (data not shown), after two vaccinations all BALB/c mice survived the challenge using 50× MLD50 (Fig. 6A). Transient, slight loss of body weight ranging from 5% to 17% was observed with 6 out of the 10 mice followed by complete recovery during days 7 to 10 (Fig. 6B). On the contrary, 2 out of the 10 double-immunized C57BL/6 mice were not protected from the 20× MLD50 PR8 challenge (Fig. 6C). Six out of the 8 survivors exhibited weight losses at days 6 to 9 (individually ranging from 8% to 22%) before completely recovering (Fig. 6D). In contrast to H5N1 strain MB1 (Fig. 4), the peak of disease was delayed by 4–5 days after challenge infection with H1N1 strain PR8 (Fig. 6). The most pronounced body weight loss was found around day 6–7 (BALB/c mice) or day 7–8 (C57BL/6 mice) after PR8 challenge, but at day 3 after MB1 challenge.

Taken together, two immunizations with the H5 HA-expressing ORFV recombinant mediated potent protection of BALB/c or C57BL/6 mice against cross-clade strains MB1 or SN1, and against the human H1N1 strain PR8.

HA-specific serum antibody response

The immune response stimulated by D1701-V-HAh5n immunization of C57BL/6 mice was determined by HI tests as described in *Material and Methods*. Figure 7 exemplary demonstrates the HI titres obtained by the i.m. application of 10^7 pfu of D1701-V-HAh5n. One week after prime immunization relevant HA-specific antibodies were detectable in the serum of only one out of 10 mice, but one week after booster immunization all animals except of one had seroconverted (Fig. 7, week 3). The titres of the individual sera ranged between 1:32 and 1:512 resulting in a mean titre of 1:144. Thereafter, only a slight decline to a mean titre of 1:74 was found just before challenge infection (Fig. 7, Ch). Very

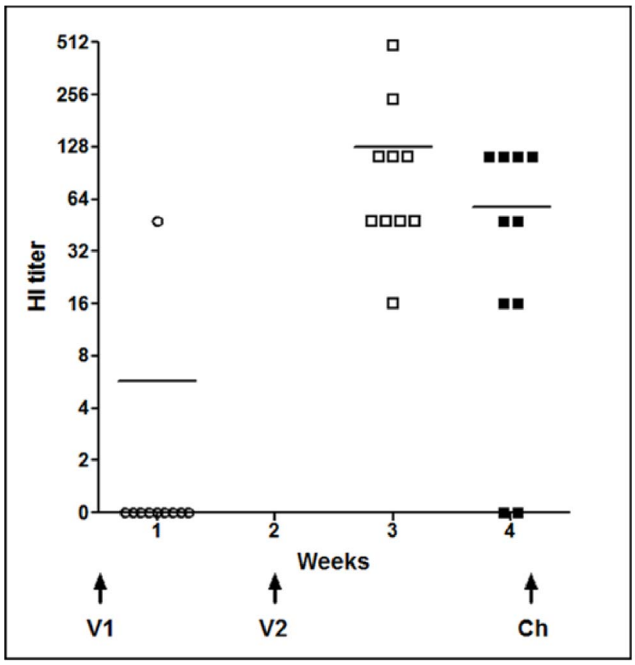

Figure 7. HA-specific serum antibody response. H5 HA-specific serum antibody response of mice elicited after i.m. immunization with 10^7 pfu of D1701-V-HAh5n. The hemagglutination inhibition (HI) titers (reciprocal log2) of individual mice were determined 1 week after prime immunization (V1), after booster immunization (V2), and at the day of challenge infection (Ch). Sera from control mice immunized twice with 10^7 pfu of D1701-VrV (VrV) displayed unspecific HI titers of 1:16. The lines denote the mean titers calculated from the individual sera.
doi:10.1371/journal.pone.0083802.g007

similar HI titres were also found after applying 10^6 pfu of D1701-V-HAh5n (data not shown), whereas after immunization with 10^5 pfu no specific immune response could be detected. Sera from non-immunized mice (data not shown) or control mice immunized twice with 10^7 pfu of the parental virus D1701-VrV exhibited unspecific HI titres of 1:16 (Fig. 7, VrV). In summary, the stimulation of a distinct cross-clade HA serum antibody response needed two immunizations with 10^6 or 10^7 pfu of D1701-V-HAh5n.

Importance of T-cell subsets in D1701-V-HAh5n-immunized mice

To examine the relevance of T-cells for the D1701-V-HAh5n mediated immunity the CD4-positive and/or CD8-positive T-cell subsets of BALB/c mice were depleted *in vivo*. Flow cytometry ensured successful depletion of the T-cells (data not shown). The T-cell populations were removed as described in *Materials and Methods* and depicted in Figure 8, panels c. At first we examined the relevance of T-cells present during H5N1 challenge infection (Fig. 8A, Depletion-A, panel c). In this situation the lack of CD4- and/or of CD8-positive T-cells had no influence on the protective immunity generated by D1701-V-HAh5n. All animals of the 3 groups survived without body weight loss (Fig. 8A, panel a and b).

Next, we analysed the importance of the T-cell subsets for priming the protective anti-HA response (Fig. 8B, depletion-B). After eliminating CD4- or CD8-positive T-cells all immunized mice survived the challenge infection, and after simultaneous removal of both T-cell subsets still 7 out of 8 mice resisted the challenge (Fig. 8B, panel a). All challenged animals depleted for CD8-positive T-cells during immunization retained their body weight (Fig. 8B, panel b). The lack of CD4-positive T-cells during prime immunization resulted in slight decrease of body weight, ranging from 8% to 18%, during days 3–5 after challenge (Fig. 8B, panel b). Thereafter all animals recovered and regained their original body weight (Fig. 8B, panel b). A similar effect was observed after removing both CD4- and CD8-positive T-cells. Five out of the 7 surviving mice showed weight losses ranging from 9% to 21% around days 4–5 after challenge (Fig. 8B, panel b).

Finally, we tested the effect of the absence of CD4- and/or CD8-positive cells during immunization and challenge infection (Fig. 8C, Depletion-C, panel c). Again, all mice missing only CD8-positive T-cells survived the lethal challenge without loss of body weight (Fig. 8C, panel a, b). After depletion of CD4-positive cells 7 out of 8 immunized mice survived the challenge, one animal exhibited 23% weight loss, the three other mice lost weight between 11% and 14% at days 4 and 5 after challenge (Fig. 8C). Two out of 8 mice did not survive challenge infection after combined removal of CD4- and CD8-positive T-cells (Fig. 8C, panel a). Four mice lost 17% to 22% of body weight at day 6 after challenge before completely recovering (Fig. 8C, panel b). Collectively, the presented results implicate the importance of CD4-positive T-cells for eliciting a robust, protective immunity in mice by the use of the new H5 HA-expressing ORFV recombinant.

Discussion

In previous studies we demonstrated the utility of recombinant ORFV vectored vaccines [42,43,44,45,46]. Here we describe the generation and evaluation of two new ORFV recombinants, which express the H5 HA gene or the NP gene of H5N1 HPAIV. Both AIV genes were expressed under the control of the early promoter of the ORFV vegf-e gene, which allows expression of the inserted genes without the need of recombinant virus multiplication as also reported for other ORFV recombinants [42,44]. In the ORFV-permissive Vero cell line both recombinants demonstrate comparable virus growth kinetics to each other and also to the parental virus D1701-V used for recombinant virus generation. Western blot and immunofluorescence analyses demonstrated correct expression of both genes including the cleavage of the HA precursor protein HA0. The H5 HA was also demonstrable on the surface of the ORFV recombinant infected cells, and the NP protein was expressed in the cell nucleus. HA and NP genes were chosen because both are of importance for the induction of a protective immune response. The crucial role of HA-specific virus-neutralizing antibodies for protection has been manifold documented. We used the H5 HA from influenza strain A/Vietnam/1203/ 2004, because most H5N1 vaccines based on the HA from the highly virulent human isolates of this Vietnam strain confer solid protection against H5N1 strains [38]. The contribution of the conserved NP antigen to protection is mainly attributed to the activation of cellular immune responses including the induction of specific cytolytic T-cells [23,55,56]. Additionally, it was reported that NP-specific antibodies can exert potent antiviral activity [55].

The protective capacity of the new ORFV recombinants was assessed in the mouse challenge model. The results showed that the ORFV recombinant expressing the conserved NP was not protective in mice. None of the animals survived the lethal challenge infection, also not after three immunizations with the recombinant. It remains to be determined whether the lack of protection can be explained by insufficient activation of T-cells or dendritic cells and/or missing induction of specific antibodies. Both B- and T-cells were reported to be of importance for NP-mediated protection [55]. Another explanation might be that the

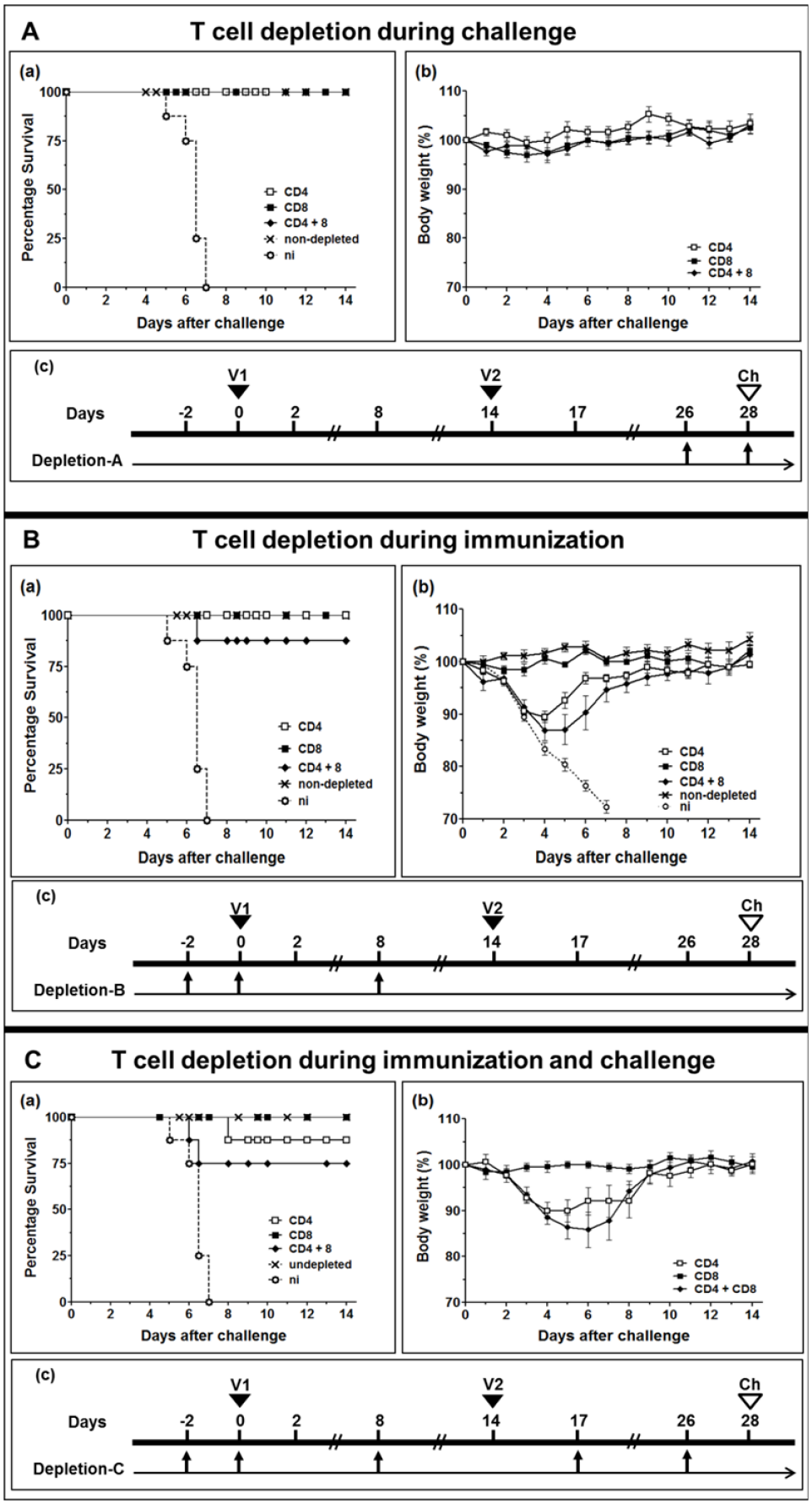
A
T cell depletion during challenge
(a)
Percentage Survival
Days after challenge
CD4
CD8
CD4 + 8
non-depleted
ni
(b)
Body weight (%)
Days after challenge
CD4
CD8
CD4 + 8
(c)
V1
V2
Ch
Days
-2
0
2
8
14
17
26
28
Depletion-A
B
T cell depletion during immunization
(a)
Percentage Survival
Days after challenge
CD4
CD8
CD4 + 8
non-depleted
ni
(b)
Body weight (%)
Days after challenge
CD4
CD8
CD4 + 8
non-depleted
ni
(c)
V1
V2
Ch
Days
-2
0
2
8
14
17
26
28
Depletion-B
C
T cell depletion during immunization and challenge
(a)
Percentage Survival
Days after challenge
CD4
CD8
CD4 + 8
undepleted
ni
(b)
Body weight (%)
Days after challenge
CD4
CD8
CD4 + CD8
(c)
V1
V2
Ch
Days
-2
0
2
8
14
17
26
28
Depletion-C

Figure 8. Role of T-cells for D1701-V-HAh5n-induced protection. BALB/c mice were immunized twice (V1 and V2) with 10^7 pfu of D1701-V-HAh5n and depletion was performed during immunization **(A)**, during challenge **(B)** or during immunization and challenge **(C)**. Challenge (Ch) was performed with 20× MLD50 strain MB1 and mice were monitored during 14 days after challenge. Survival of mice ($n = 8$) is shown in panels a, the mean percentage of body weight (bars indicate SEM) is demonstrated in panels b. Mice were sacrificed after having dropped more than 25% of their original body weight. Panels c schematically depict the days of *in vivo* depletion of CD4-positive, CD8-positive or both CD4- and CD8-positive T-cell subset as described in *Material and Methods*. For control, challenge infection of immunized non-depleted animals or non-immunized mice (ni) was also performed.
doi:10.1371/journal.pone.0083802.g008

immunity mediated by D1701-V-NPh5 was not sufficient to protect from the dose of lethal challenge virus used in our experiments. Reduced protective efficacy of NP against increasing challenge virus dose was found in mice and ferrets [57]. Although non-protective the described experiments do not exclude the utility of D1701-V-NPh5 in prime-boost vaccination regimens, similarly as reported for DNA prime-recombinant adenoviral boost immunization with NP [58].

The H5 HA-expressing recombinant D1701-V-HAh5n was found to elicit very good protection in a dose-dependent manner. The results showed that booster i.m. immunization was superior to single application of the recombinant. The animals of both mouse strains used were protected not only from lethal challenge but also from disease according to weight loss determination. All mice immunized twice with 10^6 or 10^7 pfu of the recombinant survived and remained healthy after challenge infection with two different cross-clade H5N1 strains, namely MB1 (clade 2.2.1) and SN1 (clade 2.2.3). Similar findings were reported for MVA recombinants expressing the H5 HA of clade 1, which were able to protect mice against infection with H5N1 AIV of clades 2.1.3, 2.2 and 2.3.4 [39]. Despite only 66% HA amino acid homology, the presented recombinant D1701-V-HAh5n was able to protect BALB/c and C57BL/6 mice against the heterologous AIV strain PR8 (H1N1). The results indicate slightly better protection of BALB/c mice, which resisted a higher challenge virus dose (50× MLD50) compared to C57BL/6 mice (20× MLD50). Whether the different genetic background of the two mouse strains might influence the anti-AIV immune response is not known. The major determinants of the D1701-V-HAh5n induced cross-protective immunity, cross-neutralizing HA antibodies and/or specific cytotoxic T-cells, must be clarified. Both are suspected to work in protection from heterologous AIV infection [59], as also cooperation of virus-specific CD8-positive T-cells and non-neutralizing antibodies was described [60].

The presence of HI antibodies with titres of 1:40 or higher are considered to predict protection (for review [27]), but animals with low or without detectable HI antibodies were also protected, as for instance by poxvirus based vaccination [61]. The presented data indicate that only the twofold application of higher doses of D1701-V-HAh5n elicited moderate HI antibody responses and protected all animals from disease and death. Similarly it was reported that 2 injections of HA-based vaccines can be necessary to elicit higher HI antibody titres (for review [62]). Also higher doses of MVA-based H5 HA recombinant have been necessary to induce detectable antibodies [41]. Two immunizations with 10^5 pfu of D1701-V-HAh5n did not induce detectable HI antibodies also suggesting that the magnitude of the antibody responses can depend on the vaccine dose. But nevertheless more than 80% of these animals survived the lethal challenge infection, which implies a certain immune control. In addition, the still not completely unravelled immunomodulating properties of ORFV strain D1701 [63] can be considered to improve not only cross-protective immunity. Collectively, these results might indicate the additional involvement of T-cells for the formation of a protective immunity.

The mouse immunization experiments demonstrated that two injections of D1701-V-HAh5 were beneficial to mediate robust protective immunity from lethal AIV challenge. To investigate the involvement of T-cells in protection, immune mice were *in vivo* depleted of CD4-positive and/or CD8-positive T-cells as described. Elimination of the T-cell subsets after the two vaccinations before challenge infection did not affect protective immunity. That can be explained by the development of a complete robust protective immunity before depleting the T-cell subsets. Most probably specific antibodies present at the time of challenge infection control the virus. The body weight loss indicated that the presence of CD4-positive T-cells at prime vaccination contributed to disease control, although all challenge infected animals recovered (Fig. 8B). The lack of B-cell help by CD4-positive T-cells during prime immunization can be expected to impair the production of antibodies that effectively neutralize the virus [21]. Depletion of CD4-positive T-cells alone or in combination with CD8-positive T-cells during immunization and challenge resulted in loss of body weight and slightly reduced survival rate (Fig. 8C). Most probably the missing CD4-T-cell help until the time of challenge impeded maturation of B-cells and consequently an effective specific antibody response necessary for early control of challenge virus (for review [64]). That can be also suggested from a prolonged course of disease for 2 days. The question on a possible contribution of CD8-positive T-cells for protective immunity mediated by the HA-expressing ORFV recombinant could be answered by the use of B-cell knock-out mice for *in vivo* deletion of T-cell subsets. Whether CD8-positive T-cells add some effector functions and/or cytokine production remains to be investigated in more detail. Conclusively, the presented findings show that CD4-positive T-cells are needed to prime protective immunity, but deleting these T-cell subset later, e.g. before challenge infection, does not substantially reduce protection. This supports recent reports on the importance of CD4-positive T-cells and of specific antibodies for protection from H5N1 and on the minor protective role of CD8-positive T-cells [20]. CD4-positive T-cells are also important for the development of memory B- and T-cells and thus, additionally aid to increase the protective immune response against AIV [22,28]. Moreover, they help to clear infected cells early after infection also in the absence of CD8-positive T-cells by antibody-independent, cytotoxic mechanisms or interferon-gamma secretion [65] (for review [64]).

In conclusion, the presented study adds another example of the utility of the *Parapoxvirus* ORFV strain D1701-V as a versatile vector virus platform for the development of live non-adjuvanted recombinant vaccines, which can be used for repeated immunizations. The ORFV based vaccines can be easily propagated in the non-tumorigenic, permanent Vero cell line, also accepted for influenza virus vaccine production [66]. The application of the H5 HA-expressing ORFV recombinant to protect against cross-clade HPAIV or heterologous AIV can be of great interest for vaccination of pets that have the potential to transmit H5N1 from domestic animals to humans. HPAIV H5N1 or H7N7 strains have the capacity to cross the species barrier by infecting dogs and domestic cats, respectively (for review [67]), [68,69]. The excellent applicability and safety of ORFV-vectored vaccines in pets was

demonstrated recently [42]. Due to the very good experience of using ORFV recombinants for immunization of pigs [43,48,49] the application of AIV gene expressing ORFV recombinants could also represent alternative vaccines for this AIV relevant host. Based on the presented findings more detailed studies must now scrutinize the induced immune response. In addition, improved cross-protective immunity against AIV can be attempted by using additional recombinants expressing other immune-relevant proteins of AIV.

Acknowledgments

The generous help of Annette Vogel, Emanuel Haasbach, Oliver Planz and Benjamin Petsch during the work of influenza virus, the brilliant technical assistance of K. Kegreiss and B. Bauer is greatly acknowledged. We thank Lothar Stitz (FLI, Inst. Immunology) for his assistance with mice experiments as well as his fruitful discussions. The authors also want to thank Mathias Büttner (Bavarian Health and Food Safety Authority, Oberschleissheim, Germany) for providing valuable information on the HPAIV strain MB1, and Oliver Planz (Univ. Tübingen, Dep. Immunology) for critically reading the manuscript.

Author Contributions

Conceived and designed the experiments: HJR JR RA. Performed the experiments: JR. Analyzed the data: JR RA HJR. Contributed reagents/materials/analysis tools: JR HJR RA. Wrote the paper: HJR JR.

References

1. Tumpey TM, Belser JA (2009) Resurrected pandemic influenza viruses. Annual review of microbiology 63: 79–98.
2. Bouvier NM, Palese P (2008) The biology of influenza viruses. Vaccine 26 Suppl 4: D49–53.
3. Li Q, Sun X, Li Z, Liu Y, Vavricka CJ, et al. (2012) Structural and functional characterization of neuraminidase-like molecule N10 derived from bat influenza A virus. Proceedings of the National Academy of Sciences of the United States of America 109: 18897–18902.
4. Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, et al. (2012) A distinct lineage of influenza A virus from bats. Proceedings of the National Academy of Sciences of the United States of America 109: 4269–4274.
5. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, et al. (2009) Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325: 197–201.
6. Peiris JS, de Jong MD, Guan Y (2007) Avian influenza virus (H5N1): a threat to human health. Clinical microbiology reviews 20: 243–267.
7. Gillim-Ross L, Subbarao K (2006) Emerging respiratory viruses: challenges and vaccine strategies. Clinical microbiology reviews 19: 614–636.
8. Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, et al. (2012) Airborne transmission of influenza A/H5N1 virus between ferrets. Science 336: 1534–1541.
9. Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, et al. (2012) Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature 486: 420–428.
10. Russell CA, Fonville JM, Brown AE, Burke DF, Smith DL, et al. (2012) The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. Science 336: 1541–1547.
11. Srinivasan A, Viswanathan K, Raman R, Chandrasekaran A, Raguram S, et al. (2008) Quantitative biochemical rationale for differences in transmissibility of 1918 pandemic influenza A viruses. Proceedings of the National Academy of Sciences of the United States of America 105: 2800–2805.
12. Skehel JJ, Wiley DC (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annual review of biochemistry 69: 531–569.
13. Chen JR, Ma C, Wong CH (2011) Vaccine design of hemagglutinin glycoprotein against influenza. Trends in biotechnology 29: 426–434.
14. Laddy DJ, Yan J, Kutzler M, Kobasa D, Kobinger GP, et al. (2008) Heterosubtypic protection against pathogenic human and avian influenza viruses via in vivo electroporation of synthetic consensus DNA antigens. PloS one 3: e2517.
15. Xu K, Ling ZY, Sun L, Xu Y, Bian C, et al. (2011) Broad humoral and cellular immunity elicited by a bivalent DNA vaccine encoding HA and NP genes from an H5N1 virus. Viral immunology 24: 45–56.
16. Chen MW, Cheng TJ, Huang Y, Jan JT, Ma SH, et al. (2008) A consensus-hemagglutinin-based DNA vaccine that protects mice against divergent H5N1 influenza viruses. Proceedings of the National Academy of Sciences of the United States of America 105: 13538–13543.
17. Bommakanti G, Citron MP, Hepler RW, Callahan C, Heidecker GJ, et al. (2010) Design of an HA2-based Escherichia coli expressed influenza immunogen that protects mice from pathogenic challenge. Proceedings of the National Academy of Sciences of the United States of America 107: 13701–13706.
18. Hai R, Krammer F, Tan GS, Pica N, Eggink D, et al. (2012) Influenza viruses expressing chimeric hemagglutinins: globular head and stalk domains derived from different subtypes. Journal of virology 86: 5774–5781.
19. Pica N, Palese P (2013) Toward a universal influenza virus vaccine: prospects and challenges. Annual review of medicine 64: 189–202.
20. Droebner K, Haasbach E, Fuchs C, Weinzierl AO, Stevanovic S, et al. (2008) Antibodies and CD4(+) T-cells mediate cross-protection against H5N1 influenza virus infection in mice after vaccination with a low pathogenic H5N2 strain. Vaccine 26: 6965–6974.
21. Swain SL, Agrewala JN, Brown DM, Jelley-Gibbs DM, Golech S, et al. (2006) CD4+ T-cell memory: generation and multi-faceted roles for CD4+ T cells in protective immunity to influenza. Immunological reviews 211: 8–22.
22. Teijaro JR, Verhoeven D, Page CA, Turner D, Farber DL (2010) Memory CD4 T cells direct protective responses to influenza virus in the lungs through helper-independent mechanisms. Journal of virology 84: 9217–9226.
23. Ulmer JB, Fu TM, Deck RR, Friedman A, Guan L, et al. (1998) Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. Journal of virology 72: 5648–5653.
24. Doherty PC, Turner SJ, Webby RG, Thomas PG (2006) Influenza and the challenge for immunology. Nature immunology 7: 449–455.
25. Heiny AT, Miotto O, Srinivasan KN, Khan AM, Zhang GL, et al. (2007) Evolutionarily conserved protein sequences of influenza a viruses, avian and human, as vaccine targets. PloS one 2: e1190.
26. Hillaire ML, Osterhaus AD, Rimmelzwaan GF (2011) Induction of virus-specific cytotoxic T lymphocytes as a basis for the development of broadly protective influenza vaccines. Journal of biomedicine & biotechnology 2011: 939860.
27. Bodewes R, Osterhaus AD, Rimmelzwaan GF (2010) Targets for the induction of protective immunity against influenza a viruses. Viruses 2: 166–188.
28. Dormitzer PR, Galli G, Castellino F, Golding H, Khurana S, et al. (2011) Influenza vaccine immunology. Immunological reviews 239: 167–177.
29. Osterhaus A, Fouchier R, Rimmelzwaan G (2011) Towards universal influenza vaccines? Philosophical transactions of the Royal Society of London Series B, Biological sciences 366: 2766–2773.
30. Tang XC, Lu HR, Ross TM (2010) Hemagglutinin displayed baculovirus protects against highly pathogenic influenza. Vaccine 28: 6821–6831.
31. Khurana S, Wu J, Verma N, Verma S, Raghunandan R, et al. (2011) H5N1 virus-like particle vaccine elicits cross-reactive neutralizing antibodies that preferentially bind to the oligomeric form of influenza virus hemagglutinin in humans. Journal of virology 85: 10945–10954.
32. Pushko P, Pearce MB, Ahmad A, Tretyakova I, Smith G, et al. (2011) Influenza virus-like particle can accommodate multiple subtypes of hemagglutinin and protect from multiple influenza types and subtypes. Vaccine 29: 5911-5918.
33. Ozawa M, Kawaoka Y (2011) Taming influenza viruses. Virus research 162: 8–11.
34. Mueller S, Coleman JR, Papamichail D, Ward CB, Nimnual A, et al. (2010) Live attenuated influenza virus vaccines by computer-aided rational design. Nature biotechnology 28: 723–726.
35. Petsch B, Schnee M, Vogel AB, Lange E, Hoffmann B, et al. (2012) Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection. Nature biotechnology 30: 1210–1216.
36. Kyriakis CS, De Vleeschauwer A, Barbe F, Bublot M, Van Reeth K (2009) Safety, immunogenicity and efficacy of poxvirus-based vector vaccines expressing the haemagglutinin gene of a highly pathogenic H5N1 avian influenza virus in pigs. Vaccine 27: 2258–2264.
37. Poon LL, Leung YH, Nicholls JM, Perera PY, Lichy JH, et al. (2009) Vaccinia virus-based multivalent H5N1 avian influenza vaccines adjuvanted with IL-15 confer sterile cross-clade protection in mice. Journal of immunology 182: 3063–3071.
38. Mayrhofer J, Coulibaly S, Hessel A, Holzer GW, Schwendinger M, et al. (2009) Nonreplicating vaccinia virus vectors expressing the H5 influenza virus hemagglutinin produced in modified Vero cells induce robust protection. Journal of virology 83: 5192–5203.
39. Hessel A, Schwendinger M, Holzer GW, Orlinger KK, Coulibaly S, et al. (2011) Vectors based on modified vaccinia Ankara expressing influenza H5N1 hemagglutinin induce substantial cross-clade protective immunity. PloS one 6: e16247.
40. Karaca K, Swayne DE, Grosenbaugh D, Bublot M, Robles A, et al. (2005) Immunogenicity of fowlpox virus expressing the avian influenza virus H5 gene (TROVAC AIV-H5) in cats. Clinical and diagnostic laboratory immunology 12: 1340–1342.
41. Kreijtz JH, Suezer Y, de Mutsert G, van Amerongen G, Schwantes A, et al. (2009) MVA-based H5N1 vaccine affords cross-clade protection in mice against influenza A/H5N1 viruses at low doses and after single immunization. PloS one 4: e7790.

42. Amann R, Rohde J, Wulle U, Conlee D, Raue R, et al. (2013) A new rabies vaccine based on a recombinant ORF virus (parapoxvirus) expressing the rabies virus glycoprotein. Journal of virology 87: 1618–1630.
43. Dory D, Fischer T, Beven V, Cariolet R, Rziha HJ, et al. (2006) Prime-boost immunization using DNA vaccine and recombinant Orf virus protects pigs against Pseudorabies virus (Herpes suid 1). Vaccine 24: 6256–6263.
44. Fischer T, Planz O, Stitz L, Rziha HJ (2003) Novel recombinant parapoxvirus vectors induce protective humoral and cellular immunity against lethal herpesvirus challenge infection in mice. Journal of virology 77: 9312–9323.
45. Henkel M, Planz O, Fischer T, Stitz L, Rziha HJ (2005) Prevention of virus persistence and protection against immunopathology after Borna disease virus infection of the brain by a novel Orf virus recombinant. Journal of virology 79: 314–325.
46. Rohde J, Schirrmeier H, Granzow H, Rziha HJ (2011) A new recombinant Orf virus (ORFV, Parapoxvirus) protects rabbits against lethal infection with rabbit hemorrhagic disease virus (RHDV). Vaccine 29: 9256–9264.
47. Rziha H, Henkel M, Cottone R, Bauer B, Auge U, et al. (2000) Generation of recombinant parapoxviruses: non-essential genes suitable for insertion and expression of foreign genes. Journal of biotechnology 83: 137–145.
48. van Rooij EM, Rijsewijk FA, Moonen-Leusen HW, Bianchi AT, Rziha HJ (2010) Comparison of different prime-boost regimes with DNA and recombinant Orf virus based vaccines expressing glycoprotein D of pseudorabies virus in pigs. Vaccine 28: 1808–1813.
49. Voigt H, Merant C, Wienhold D, Braun A, Hutet E, et al. (2007) Efficient priming against classical swine fever with a safe glycoprotein E2 expressing Orf virus recombinant (ORFV VrV-E2). Vaccine 25: 5915–5926.
50. Buttner M, Rziha HJ (2002) Parapoxviruses: from the lesion to the viral genome. Journal of veterinary medicine B, Infectious diseases and veterinary public health 49: 7-16.
51. Haig DM (2006) Orf virus infection and host immunity. Current opinion in infectious diseases 19: 127–131.
52. Czerny CP, Waldmann R, Scheubeck T (1997) Identification of three distinct antigenic sites in parapoxviruses. Archives of virology 142: 807–821.
53. Cobbold SP, Jayasuriya A, Nash A, Prospero TD, Waldmann H (1984) Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. Nature 312: 548–551.
54. Le Gros GS, Herbert AG, Watson JD (1984) In vivo modulation of thymus-derived lymphocytes with monoclonal antibodies in mice. II. Separation of natural killer cells and cytotoxic T cells. Immunology 51: 103–113.
55. LaMere MW, Lam HT, Moquin A, Haynes L, Lund FE, et al. (2011) Contributions of antinucleoprotein IgG to heterosubtypic immunity against influenza virus. Journal of immunology 186: 4331–4339.
56. Rimmelzwaan GF, Kreijtz JH, Bodewes R, Fouchier RA, Osterhaus AD (2009) Influenza virus CTL epitopes, remarkably conserved and remarkably variable. Vaccine 27: 6363–6365.
57. Rao SS, Kong WP, Wei CJ, Van Hoeven N, Gorres JP, et al. (2010) Comparative efficacy of hemagglutinin, nucleoprotein, and matrix 2 protein gene-based vaccination against H5N1 influenza in mouse and ferret. PloS one 5: e9812.
58. Epstein SL, Kong WP, Misplon JA, Lo CY, Tumpey TM, et al. (2005) Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. Vaccine 23: 5404–5410.
59. Guo H, Santiago F, Lambert K, Takimoto T, Topham DJ (2011) T cell-mediated protection against lethal 2009 pandemic H1N1 influenza virus infection in a mouse model. Journal of virology 85: 448–455.
60. Laidlaw BJ, Decman V, Ali MA, Abt MC, Wolf AI, et al. (2013) Cooperativity between CD8+ T cells, non-neutralizing antibodies, and alveolar macrophages is important for heterosubtypic influenza virus immunity. PLoS pathogens 9: e1003207.
61. Stittelaar KJ, Lacombe V, van Lavieren R, van Amerongen G, Simon J, et al. (2010) Cross-clade immunity in cats vaccinated with a canarypox-vectored avian influenza vaccine. Vaccine 28: 4970–4976.
62. El Sahly HM, Keitel WA (2008) Pandemic H5N1 influenza vaccine development: an update. Expert review of vaccines 7: 241–247.
63. Weber O, Mercer AA, Friebe A, Knolle P, Volk HD (2013) Therapeutic immunomodulation using a virus–the potential of inactivated orf virus. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology 32: 451–460.
64. Swain SL, McKinstry KK, Strutt TM (2012) Expanding roles for CD4(+) T cells in immunity to viruses. Nature reviews Immunology 12: 136–148.
65. Brown DM, Lee S, Garcia-Hernandez Mde L, Swain SL (2012) Multifunctional CD4 cells expressing gamma interferon and perforin mediate protection against lethal influenza virus infection. Journal of virology 86: 6792–6803.
66. Barrett PN, Portsmouth D, Ehrlich HJ (2010) Developing cell culture-derived pandemic vaccines. Current opinion in molecular therapeutics 12: 21–30.
67. Harder TC, Vahlenkamp TW (2010) Influenza virus infections in dogs and cats. Veterinary immunology and immunopathology 134: 54–60.
68. Chen Y, Zhong G, Wang G, Deng G, Li Y, et al. (2010) Dogs are highly susceptible to H5N1 avian influenza virus. Virology 405: 15–19.
69. Kuiken T, Rimmelzwaan G, van Riel D, van Amerongen G, Baars M, et al. (2004) Avian H5N1 influenza in cats. Science 306: 241.

Vaccine 27 (2009) 3584–3591

Contents lists available at ScienceDirect

Vaccine

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

New baculovirus recombinants expressing Pseudorabies virus (PRV) glycoproteins protect mice against lethal challenge infection

Agnieszka K. Grabowska[a,b], Andrea D. Lipińska[a], Jörg Rohde[b], Boguslaw Szewczyk[a], Krystyna Bienkowska-Szewczyk[a], Hanns-Joachim Rziha[b,*]

[a] Dept. of Mol. Virology, Faculty of Biotechnology, University of Gdansk, Gdansk, Poland
[b] Friedrich-Loeffler Institute, Federal Research Institute of Animal Health, Institute of Immunology, Tuebingen 72076, Germany

ARTICLE INFO

Article history:
Received 21 November 2008
Received in revised form 14 March 2009
Accepted 18 March 2009
Available online 9 April 2009

Keywords:
Baculovirus recombinant
VSV-G protein
Pseudorabies virus
Viral vector vaccine
Protective immunization

ABSTRACT

The present study demonstrates the protective potential of novel baculovirus recombinants, which express the glycoproteins gB, gC, or gD of Pseudorabies virus (PRV; Alphaherpesvirus of swine) and additionally contain the glycoprotein G of Vesicular Stomatitis Virus (VSV-G) in the virion (Bac-G-PRV). To evaluate the protective capacity, mixtures of equal amounts of the PRV gB-, gC-, and gD-expressing baculoviruses were used for immunization. Three intramuscular immunizations with that Bac-G-PRV mixture could protect mice against a lethal PRV challenge infection. To achieve complete protection high titers of Bac-G-PRV and three immunizations were necessary. This immunization with Bac-G-PRV resulted in the induction of high titers of PRV-specific serum antibodies of the IgG2a subclass and of interferon (IFN)-gamma, indicating a Th1-type immune response. Moreover, splenocytes of immunized mice exhibited natural killer cell activity accompanied by the production of IFN-alpha and IFN-gamma. Collectively, the presented data demonstrate for the first time that co-expression of VSV-G in baculovirus recombinant vaccines can improve the induction of a protective immune response against foreign antigens.

1. Introduction

Live viral vectors based on, e.g. poxvirus, adenovirus, or herpesvirus are favoured as vaccine vectors due to their ability to elicit humoral as well as cell-mediated immune responses against the inserted antigen [1,2,4,5,9]. However, potential disadvantages of some of these vectors consist in their cytotoxicity, in vaccine virus multiplication in the immunized host, and in pre-existing or induced immunity against the vector virus diminishing or preventing efficient immunity against foreign antigens [3,10]. An alternative platform for vector vaccines offers the use of recombinant baculoviruses. The baculovirus expression system is widely used for the production of recombinant proteins, the development of subunit vaccines in insect cells [6,7], and the generation of virus-like particles (VLPs) for the use as vaccines [11,12]. In recent years, baculovirus was adopted for gene delivery into mammalian cells *in vitro* and *in vivo* [8,13–16,18]. Even with a high multiplicity of infection (MOI), transduction of mammalian cells leads to strong expression of foreign proteins without baculovirus reproduction. Moreover, baculovirus-specific neutralizing serum antibodies are not induced in animals [15,17,21]. Abe et al. [20] demonstrated that immunization with a baculovirus recombinant expressing the hemagglutinin (HA) gene of influenza virus elicited an innate immune response and protected mice against challenge infection with influenza A and B viruses.

A new generation of baculovirus vectors (Bac-G) has been recently established, which express the surface glycoprotein G of Vesicular Stomatitis Virus (VSV) for increased foreign gene transfer [15,17,21]. VSV-G enhances the escape of the baculovirus vector from endosomes by its membrane fusion activity, thereby improving the transduction efficiency [17]. Those VSV-G-pseudotyped baculovirus recombinants were utilized in vaccination trials by Facciabene et al. [21]. Intramuscular application of a baculovirus expressing the E2 glycoprotein of Hepatitis C virus (HCV) or the carcinoembryonic antigen (CEA) induced both humoral and cell-mediated immune responses against the inserted antigens, and additionally stimulated innate immune mechanisms. Antigens expressed by baculovirus displaying VSV-G appear to be more efficiently immunogenic as opposed to their expression by baculoviruses without VSV-G [21]. However, it has not yet been tested whether Bac-G recombinants have the potential to elicit protective immunity.

The objective of the present study was to investigate the utility of recombinant baculoviruses as a non-replicative vector vaccine against infection with Pseudorabies virus (PRV; Suid herpesvirus 1) as a clinically relevant pathogen. Infection of swine with PRV

* Corresponding author. Tel.: +49 7071 967 253; fax: +49 7071 967 303.
E-mail address: achim.rziha@fli.bund.de (H.-J. Rziha).

0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.03.067

causes the epidemic Aujeszky's disease (AD) [23], which can lead to economically important losses in pig industry. Therefore, active immunization of swine is performed with modified live or inactivated vaccines [22,24]. Those vaccines provide varying protection against clinical signs of the disease and can reduce PRV shedding, but they do not prevent infection and establishment of latent infection of PRV [25,26].

Commonly, mice are used to investigate the immunoprotective capacity of potential vaccines against lethal PRV challenge infection as well as to analyze the induced PRV-specific immune response. Among the 12 different PRV glycoproteins, gB, gC, and gD are regarded as being most important for the development of the antiviral humoral and cellular immune response [27,32]. PRV neutralizing serum antibodies are reported to be mainly directed against the glycoproteins gB, gC and gD [29,30], and PRV gC was found to represent a target for cytotoxic T lymphocytes [28]. The gB and gC glycoproteins were also shown to be involved in the induction of the lymphoproliferative response [27,31]. Recently, ORFV (Parapoxvirus) recombinants expressing only PRV gC or gD were found to protect mice and swine from a lethal PRV challenge infection [33,34].

The present study describes the generation of new VSV-G-pseudotyped baculovirus recombinants encoding glycoproteins gB, gC or gD of PRV (Bac-G-gB, Bac-G-gC, Bac-G-gD), which were efficiently expressed in different mammalian cell lines. The protective capacity of combinations of these recombinants (Bac-G-PRV) was tested in a PRV mouse challenge model, and first analyses of the induced immune response are presented. Collectively, the results demonstrate that a threefold intramuscular (i.m.) vaccination of mice with Bac-G-PRV mediated complete protection against lethal PRV challenge infection accompanied by the induction of high titers of PRV-specific Th1-type serum antibodies.

2. Materials and methods

2.1. Cell lines and viruses

Baculoviruses were propagated in Spodoptera frugiperda (Sf9) insect cells using HyQ SFX-Insect MP (HyClone) or Grace's Insect medium mixed 1:1 (v/v) with TC-100 Insect Medium (Invitrogen, USA) supplemented with 6% fetal bovine serum (FBS), 100 U of penicillin G, and 100 U of streptomycin/ml (Invitrogen, USA). SK6 (swine kidney) cells were maintained in minimum essential medium Eagle (MEM; Sigma, USA) supplemented with 10% FBS, 2 mM L-glutamine and antibiotics. The PRV strain NIA-3 was produced in VERO cells (African green monkey kidney cell line) with Dulbecco's modified Eagle medium (DMEM; Sigma, USA) containing 5% FBS and antibiotics.

2.2. Generation of recombinant baculovirus vectors

For the generation of baculovirus recombinants the pFastBacDUAL plasmid and Bac-To-Bac baculovirus expression system was used (Invitrogen, USA). To construct pFD-G-CMV transfer plasmid, the CMV-MCS-BGH polyA cassette was isolated from pcDNA3 plasmid by digestion with NruI and SmaI and ligated into the BamHI and HindIII digested pFastBacDUAL after end filling with Klenow polymerase. The VSV-G gene was amplified from plasmid pVSV-G (Invitrogen, USA) using primers containing an XhoI restriction site (underlined): 5′-CTCGAGATGAAGTGCCTTTTGTACTTAGCC-3′ and 5′-CTCGAGTTACTTTCCAAGTCGGTTCATCTC-3′. The purified PCR product was inserted into the XhoI restriction site downstream of the p10 baculovirus promoter of pFD-CMV (Fig. 1A). Plasmid pFD-G-CMV-gB was generated by inserting the complete open reading frame of PRV gB into the EcoRI site of pFD-G-CMV downstream of the hCMV early promoter (Fig. 1A). The 1.8-kb EcoRI DNA-fragment of PRV gB was isolated from plasmid pcDNA-gB (PRV) (Department of Molecular Virology, University of Gdansk, Poland). The complete PRV gC gene was amplified by PCR from PRV genomic DNA with primer Fw: 5′-GAATTCATGGCCTCGCTCGCGCGTGCGATG-3′ and primer Rv: 5′-GAATTCTCACGGCCCCGCCCGGCGGTAGTA-3′ (the introduced EcoRI sites are underlined) and inserted into the EcoRI site of pFD-G-CMV to result in plasmid pFD-G-CMV-gC. The PRV gD gene was also amplified by PCR from the PRV genome with the primers 5′-GAATTCATGCTGCTCGCAGCGCTATTG-3′ and 5′-GAATTCCTACGGACCGGGCTGCGCT-3′ (introduced EcoRI sites are underlined) and ligated into EcoRI cleaved pFD-G-CMV to obtain plasmid pFD-G-CMV-gD (Fig. 1). For proving correct insertions all plasmids have been molecularly characterized. The different recombinant baculoviruses were purified from supernatants of infected insect cells on a 27% (w/v) sucrose cushion in a SW28 rotor (Beckman, USA) at 24,000 rpm for 75 min at 4 °C. The viral pellet was resuspended in phosphate-buffered saline (PBS) and titrated by plaque assays on Sf9 cells.

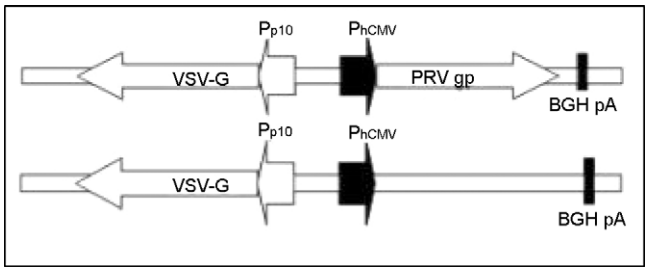

Fig. 1. Schematic representation of recombinant baculoviruses. Schematic depiction of the construction of the VSV-G pseudotyped recombinants expressing the single PRV glycoprotein (gp) genes (Bac-G-PRV) under the control of the human CMV promoter (Phcmv) and the VSV-G protein controlled by the baculovirus p10 promoter (Pp10), as described in Section 2.

2.3. Detection of PRV glycoproteins in baculovirus-infected cells

Vero or SK6 cells were transduced with the different baculoviruses (MOI of 10) in the presence of 5 mM sodium butyrate as previously described [35]. After 24 h, the harvested cells were lysed in a mixture of 125 mM Tris–HCl pH 6.8, 4% SDS, 10% glycerol, 0.02% bromophenol blue, 4% 2-mercaptoethanol, resolved by electrophoresis in SDS-10% polyacrylamide gel, and transferred onto a polyvinylidene difluoride membrane (Roti-PVDF, Carl Roth, Germany). After blocking overnight at 4 °C in 5% non-fat dry milk in TBST (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.05% Tween 20), the membrane was incubated with primary antibodies diluted in 1.5% (w/v) non-fat dry milk in TBST for 2 h at room temperature. The polyclonal PRV gB-specific rabbit antiserum was used in a dilution of 1:2000, the PRV gC-specific mouse antiserum (B16-d6) was diluted 1:10 and the polyclonal PRV gD-specific rabbit antiserum 1:1000. All PRV-specific antisera were kindly provided by T.C. Mettenleiter (FLI, Island of Riems, Germany). The VSV-G-specific mouse monoclonal antibody P5D4 (Sigma, USA) was used in a dilution of 1:500. The membrane was washed three times with TBST and incubated for 1 h at room temperature with alkaline phosphatase-conjugated species-specific secondary antibodies (Santa Cruz Biotechnologies, USA). After final washing, the membranes were incubated with the BCIP/NBT (Fermentas, Lithuania) substrate solution.

2.4. Animal experiments

Groups of 8-week-old BALB/c mice were immunized intramuscularly (i.m.) at 2-weeks intervals with the indicated doses of

mixtures of the three PRV glycoproteins expressing Bac-G recombinants. These immunization cocktails were prepared by combining equal amounts of plaque forming units (PFU) of each of recombinant to obtain the indicated final dose of PFU. For control, BALB/c mice were vaccinated with non-recombinant baculovirus Bac-G-WT or with PBS. Two weeks after the last immunization, all mice were intraperitoneally (i.p.) challenge-infected with the virulent PRV strain NIA-3 (10^4 PFU/animal). Serum antibody and cell-mediated immune responses from the immunized mice were recorded until day 14 after challenge. All animal experiments were performed at the FLI, Institute of Immunology in Tuebingen, Germany as approved by the animal protection authority.

2.5. Serum antibody analyses

Sera from immunized and control mice were analyzed for PRV-specific IgG1 and IgG2a serum antibody titers by ELISA as previously described [33]. The mean titers are calculated from $\log_{10}$ of the highest serum dilution giving a twofold higher OD value compared to that of sera from Bac-G-WT vaccinated mice. Virus-neutralizing antibodies were evaluated by a complement-independent neutralization assay. Twofold serial dilutions (at least in duplicates, starting with a serum dilution of 1:4) of heat-inactivated (30 min at 56 °C) sera were incubated with 50 PFU of PRV strain NIA-3 for 24 h at 37 °C and plaque reduction was determined as described [33].

2.6. Determination of cytokine release from murine splenocytes

Spleens of individual immunized mice and control mice were homogenized in PBS (pH 7.4), and erythrocytes were lysed with Red Blood Cell Lysing Buffer (Sigma, Germany) for 2–5 min at room temperature. After three times washing with PBS, the splenocytes were seeded in 96-well flat-bottom plates at a density of 2.5×10^6 cells/ml in MEM medium containing antibiotics, 10% bacto tryptose-phosphate broth, and 2% FBS. Splenocytes were stimulated *in vitro* with PRV strain NIA-3 (MOI = 1) or with 1 μg/ml Concanavalin A (Sigma, Germany). After 4 days incubation at 37 °C in 5% CO_2 atmosphere, the supernatants from cell cultures were collected. Secretion of interferon (IFN)-alpha, gamma and interleukin (IL)-4 into the supernatant was determined by specific ELISA (IL-4, IL-10-ELISA Ready-SET-Go!; eBioscience, NatuTec, Germany, and IFN-α ELISA Kit, PBL Biomedical Laboratories, USA) according to the manufacturer's instructions.

2.7. Cytotoxicity assay

The cytolytic activity of NK cells was analyzed by chromium release assay as described [36]. BALB/c mice were injected i.p. with the combination of 10^9 PFU of the three baculovirus recombinants (Bac-G-PRV) or for control with wild-type baculovirus (Bac-G-WT). Splenocytes were isolated 24 h after vaccination and suspended in MEM medium containing 2% FBS at a density of 10^7 cells/ml. Different numbers of splenocytes (effector cells) were plated in 96-well round-bottom plates. Target Yac-1 murine lymphoma cells were labeled with 100 μCi ^{51}Cr (Amersham Biosciences, Germany) by incubation for 1 h at 37 °C, and seeded at a density of 10^4 cells/well. Effector to target (E:T) ratios of 100:1, 30:1, 10:1, 3:1 and 1:1 were incubated for 5 h at 37 °C in 5% CO_2 atmosphere. After incubation, 50 μl of supernatants were transferred to LumaPlate-96 (Perkin-Elmer, Germany), allowed to dry overnight, and the amount of ^{51}Cr released was determined per well at least in duplicates. Spontaneous release of ^{51}Cr was determined for cultures containing labeled Yac-1 cells alone, and maximum release was performed by adding 1N HCl to the labeled Yac-1 cells. Percentage of cytotoxicity was calculated by the formula: 100 × (experimental cpm − spontaneous cpm)/(maximal cpm − spontaneous cpm).

3. Results

3.1. Characterization of the recombinant baculoviruses

Baculoviruses were generated expressing the PRV gB, gC, or gD coding sequences under the control of the hCMV immediate-early promoter. The VSV-G coding sequence was additionally inserted downstream of the p10 baculovirus promoter as depicted in Fig. 1 (see also Section 2). Expression of VSV-G was confirmed by Western blotting of purified Bac-G-PRV using the VSV-G-specific monoclonal antibody P5D4. As shown in Fig. 2A, the VSV-G protein of approximately 64 kDa in size was detected in all Bac-G-PRV virus particles, but not in Bac-G-WT preparation. For examin-

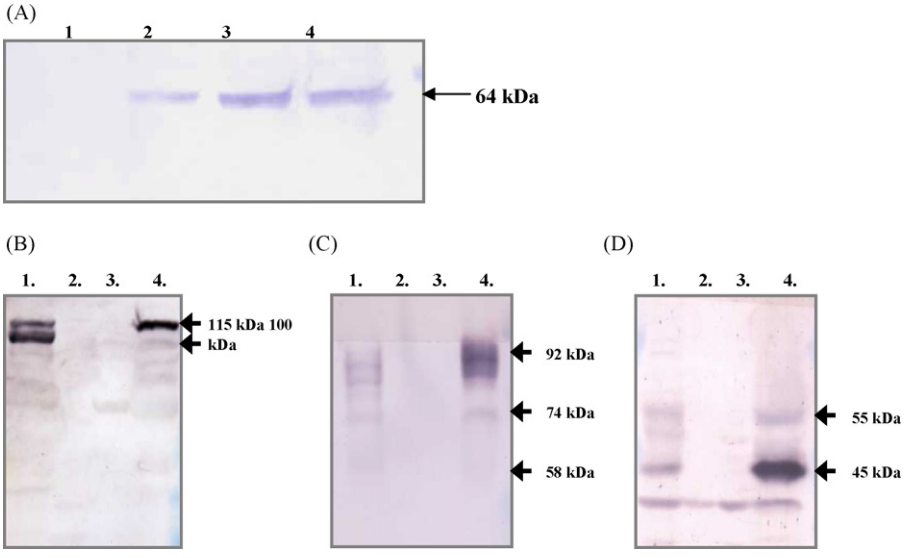

Fig. 2. Western blot analysis of the different Bac-G-PRV. (A) Purified non-recombinant Bac (lane 1), Bac-G-gB (lane 2), Bac-G-gC (lane 3), and Bac-G-gD (lane 4) were separated on SDS polyacrylamide gels and Western blotting was performed with antibodies specific for VSV-G protein. Lysates from baculovirus-transduced SK6 cells were analyzed by Western blotting with PRV monospecific antibodies for (B) gB, (C) gC, and (D) gD glycoproteins of PRV. Lane 1 represent lysates from PRV-infected SK cells, lane 2 from non-infected SK cells, lane 3 from Bac-G-WT and lane 4 in (B) from Bac-G-gB, in (C) from Bac-G-gC, and in (D) from Bac-G-gD transduced cells. The arrows mark the different mature or precursor protein forms as described in the text.

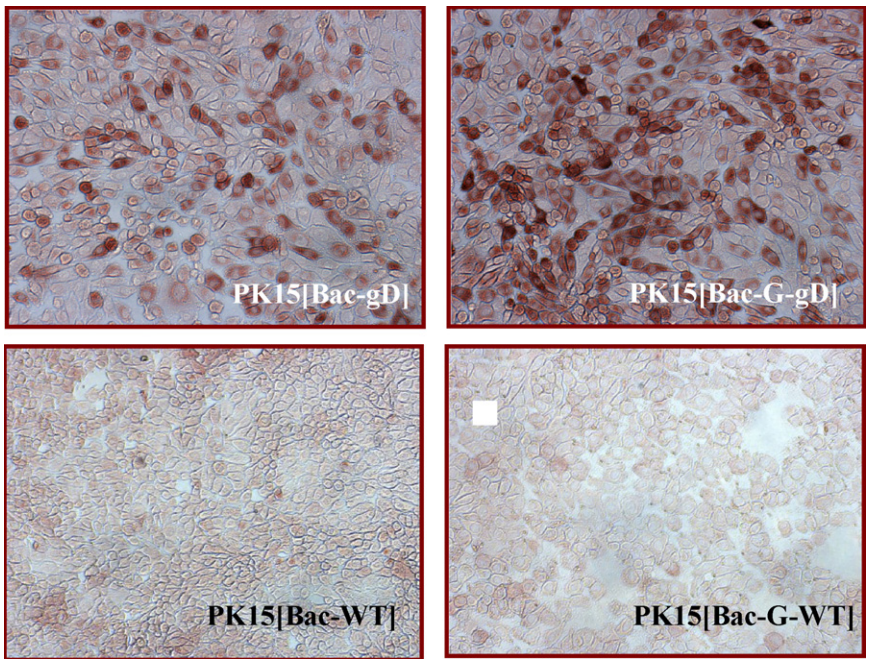

Fig. 3. Bac-G-gD shows higher transduction efficiency as compared to Bac-gD. Detection of PRV glycoproteins *in situ* in recombinant baculovirus-transduced PK15 cells by IPMA assay as described in Section 2.

ing the expression of PRV glycoproteins, swine kidney (SK6) cells were infected with a MOI of 10 of Bac-G-PRV and 24 h later cell lysates were analyzed by Western blotting with PRV monospecific antibodies. Cells infected with PRV (strain NIA-3), with non-recombinant baculovirus (Bac-G-WT) or non-infected SK6 cells were used as controls. Cells transduced with Bac-G-gB exhibited the expression of the 115 kDa mature form and the 100 kDa precursor form of gB similar to PRV-infected cells (Fig. 2B). The mature form (94 kDa), immature form (74 kDa) and precursor form (58 kDa) of PRV gC were detectable in Bac-G-gC-transduced cells as in PRV-infected cells (Fig. 2C). Finally, the 55 kDa mature form and the 45 kDa precursor form of PRV gD was found in Bac-G-gD-transduced cells (Fig. 2D). Monitoring the foreign gene expression *in situ* it appears that co-expression of the VSV-G gene clearly improved the transduction efficiency and/or expression of the PRV genes. This is representatively demonstrated in Fig. 3 for the PRV gD gene by comparing Bac-G-gD with Bac-gD that lacks the VSV-G gene. It can be seen that the number of cells strongly expressing the PRV gD after Bac-G-gD transduction outnumbers that after Bac-gD transduction (Fig. 3). Similar results were also found by the use of Bac-G-gB or Bac-G-gC (data not shown).

Finally, results of confocal fluorescence microscopy demonstrated intracellular and surface expression of each PRV glycoprotein after transduction of SK6 cells or murine L929 cells (data not shown). These data confirmed the expression and modification of the PRV glycoproteins with all baculovirus recombinants, but co-expression of VSV-G protein improved transduction efficiency and strength of expression of the foreign antigens.

3.2. Protection against PRV challenge infection

In order to evaluate the protective potential of the baculovirus recombinants, mice were challenged with a lethal dose of PRV after i.m. application of Bac-G-PRV, each representing a cocktail of equal amounts of the gB-, gC- and gD-expressing recombinants as described in Section 2. First, we intended to specify the dose of the baculovirus recombinants needed to protect mice against lethal PRV challenge infection after three i.m. immunizations. As shown in Fig. 4A, all animals that were not immunized (PBS) or were control-immunized with the different doses of Bac-G-WT died between 66 and 99 h after challenge infection. After three immunizations with 10^6 PFU of Bac-G-PRV no protection was found though some delay of death was observed. All animals showed severe neurological symptoms and died between 66

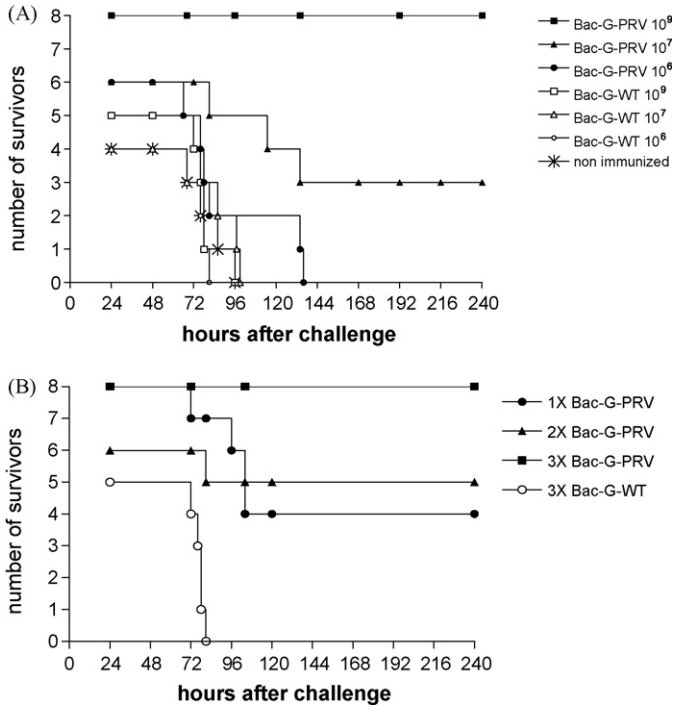

Fig. 4. PRV challenge infection of immunized mice. Mice were challenge infected with a lethal dose of the PRV strain NIA-3 (A) after three immunizations with the indicated doses of Bac-G-PRV recombinants or the parental Bac-G-WT. (B) Protection of mice after one (1×), two (2×) or three (3×) immunizations with 10^9 PFU of Bac-G-PRV.

and 136 h after PRV infection. Partial protection was found after the application of 10^7 PFU of Bac-G-PRV, where 50% of mice survived and 50% of mice showed a delayed death (Fig. 4A). Increasing the immunization dose to 10^9 PFU, three i.m. applications of Bac-G-PRV protected all mice against lethal PRV challenge infection (Fig. 4A), and none of these animals exhibited clinical symptoms. Therefore, the protection rate was dependent on the amount of Bac-G-PRV applied. Finally, we determined the number of i.m. immunizations of 10^9 PFU Bac-G-PRV necessary to protect the mice. As shown in Fig. 4B, again three applications of Bac-G-PRV completely protected the mice against PRV challenge infection. After two vaccinations, the protection rate decreased to 83% (five out of six mice), and after one single vaccination with Bac-G-PRV only four out of eight mice (50%) survived the challenge infection.

3.3. Serum antibody response

For determining the humoral immune response elicited by the immunization with Bac-G-PRV, PRV-specific serum antibodies of IgG1 and IgG2a subclasses were evaluated by ELISA. Serum samples were taken 1 day before challenge infection (pre-challenge) as well as from animals surviving the PRV challenge at day 5 and day 14, respectively. The results demonstrate that already the first immunization before challenge infection induced high IgG2a serum antibody titers (1:8000 to 1:12,800) and lower specific IgG1 (1:600 to 1:800) serum antibody titers, which were not substantially increased by the booster immunization (Fig. 5, pre-challenge). Challenge infection of mice vaccinated one-, two- or three-times slightly increased the PRV-specific IgG1 serum antibody titers (Fig. 5). At day 5 and day 14 after challenge infection (p.i.), comparable serum antibody levels as well as similar IgG2a:IgG1 ratios were found.

Interestingly, PRV-neutralizing antibodies were hardly detectable in the sera of all immunized animals pre- and post-challenge infection. Independent of whether the animals were immunized with one, two or three doses of 10^9 PFU Bac-G-PRV, the neutralizing antibody titers did not exceed serum dilutions of 1:10 (data not shown). In sum, the data indicate that Bac-G-PRV immunization induced predominantly specific IgG2a serum antibodies favouring the stimulation of a T-helper class 1 (Th-1) immune response against PRV.

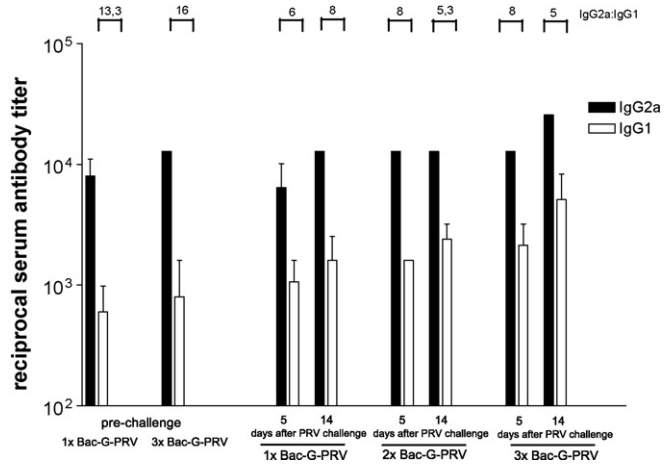

Fig. 5. PRV-specific serum antibody response. Sera were taken from one-time (1×), two-times (2×) or three-times (3×) immunized BALB/c mice 1 day before (pre-challenge, $n = 4$) or 5 ($n = 3$) and 14 days ($n = 5$) after PRV challenge infection. PRV-specific IgG1 (white columns) or IgG2a (black columns) antibodies were determined by ELISA. The ratios of IgG2a to IgG1 subclasses are given above the columns. Bars indicate standard deviations.

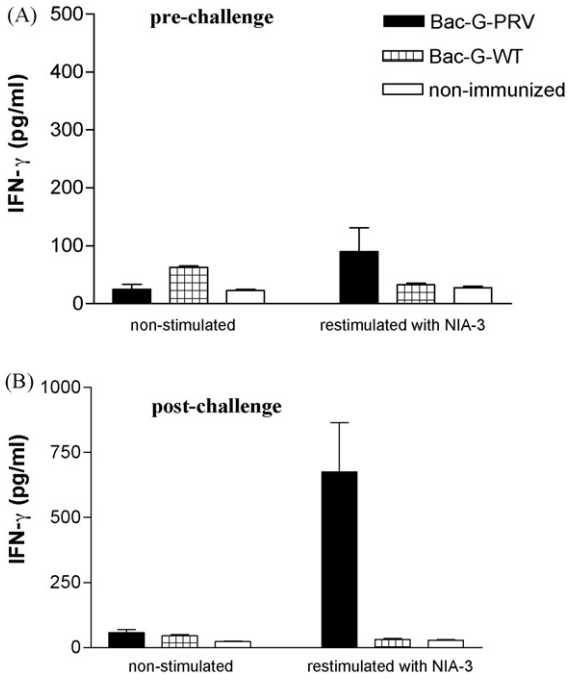

Fig. 6. Production of IFN-gamma from splenocytes. Spleens were obtained from mice three times immunized as indicated (A) 1 day before or (B) 3–5 days after challenge infection. The splenocytes were *in vitro* re-stimulated for 4 days with MOI 1.0 of NIA-3 or culture medium as negative control. The supernatants were tested for the production of IFN-gamma by ELISA. Mean values are given in pg IFN-g per ml, the bars indicate standard deviations.

3.4. Induction of interferon gamma

Whereas IFN-gamma is known to mediate the induction of an antiviral Th-1 immune response, the induction of IL-4 is regarded to promote a B-cell response during viral infection. The production of IFN-gamma and IL-4 from splenocytes of immunized, protected mice and control-immunized animals was tested by ELISA after *in vitro* re-stimulation with PRV. As shown in Fig. 6A, before challenge infection splenocytes from all mice secreted only low amounts of IFN-gamma, whereas 5 days after challenge infection an approximately 10-fold increased production of IFN-gamma was found in the spleen from the immunized animals but not from control-immunized mice (Fig. 6B). In contrast, none of the splenocytes secreted detectable IL-4 or IL-10 before or after challenge infection (data not shown).

3.5. Induction of an early immune response by Bac-G-PRV

Twenty-four hours after Bac-G-PRV prime vaccination, splenocytes were monitored for the production of IFN-alpha, IFN-gamma and NK-mediated cytotoxic activity in order to assess the induction of an early immune response. As representatively shown in Fig. 7, only splenocytes derived from vaccinated mice (animals #1 and #2) exhibited high cytolytic activity against Yac-1 target cells (Fig. 7A). Effector splenocytes prepared from not or control-immunized mice exhibited background NK cell activity. Sera taken from the same mice 24 h after a single vaccination with Bac-G-PRV displayed clearly elevated amounts of IFN-alpha (mean concentration: 750 pg/ml) and IFN-gamma (mean concentration: 140 pg/ml) were detectable by ELISA (Fig. 7B). Comparable results have been obtained by determining NK cell activity of splenocytes and serum cytokine response in two other independent experiments (data not shown). These results indicate that prime immunization with the Bac-G-PRV induced an efficient early NK cell response accompanied

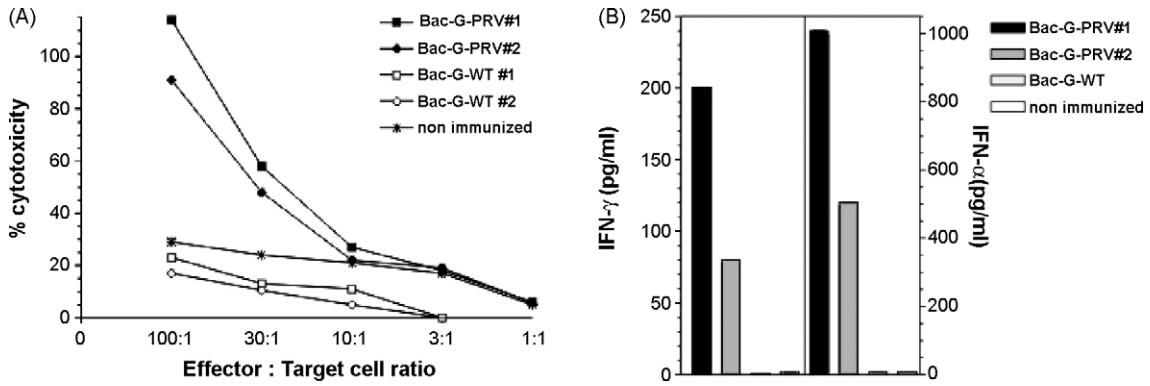

Fig. 7. Analysis of the immune response 24 h after first immunization. (A) Cytolytic activity was evaluated by chromium release assays of labeled target Yac-1 cells in the presence of different ratios of effector splenocytes (spontaneous release 30%). Effector–target cell ratios are indicated on the abscissa and the percent of cytotoxicity on the vertical axis. (B) The production of interferon-alpha and -gamma was determined by specific ELISA from individual serum samples taken from immunized and control mice. Two Bac-G-PRV immunized animals (#1 and #2) were tested.

by the production IFN-alpha and IFN-gamma known to activate NK cells.

4. Discussion

Numerous reports demonstrate the versatility of the baculovirus vector system for transduction and expression of foreign genes, including viral proteins, into mammalian cells. However, investigations of the potential of recombinant baculoviruses as immunizing reagents to mediate protective immunity against viral infections are very limited (for review [38,39,42]). The present study was designed to evaluate the suitability of the VSV-G protein pseudotyped baculovirus (Bac-G) expression system [17] as potential vector vaccine and to analyze the induced immune response. To this end, we have used the well-established murine model of Pseudorabies virus (PRV) challenge infection, a member of the alphaherpesvirinae (Suid herpes 1). As immunogens the PRV glycoproteins gB, gC, and gD were chosen, which represent major targets for anti-PRV humoral and cellular immune responses [27]. Also the induction of protective immunity in mice and pigs against PRV challenge infection has been reported by the use of vaccines containing these PRV glycoproteins [33,34,37].

Bac-G recombinants were generated to express the PRV genes under the control of the hCMV promoter that is active in mammalian cells, and the VSV-G protein incorporated into the virion of the recombinants by expression from the p10 promoter of baculovirus that is silent in mammalian cells. As reported earlier for the expression of other foreign genes in Bac-G [15,17,21] the presented results also indicate that VSV-G protein pseudotyping enhanced the transduction efficiency and led to increased expression of the PRV glycoproteins in different cell species.

For immunization equal amounts of each recombinant were mixed together to obtain the Bac-G-PRV cocktail and mice were vaccinated intramuscularly (i.m.), because this route of baculovirus vector inoculation has previously been shown to achieve superior antigen-specific B- and T-cell responses compared to intraperitoneal, subcutaneous or intranasal injection [19–21]. Moreover, prolonged antigen expression in the muscle of i.m. inoculated mice has been reported [17]. The results show for the first time complete protection of mice against lethal PRV challenge infection after immunization with a combination of baculovirus expressed glycoproteins gB, gC, and gD. Reliable protection, however, was only achieved by the use of the Bac-G-PRV cocktail, but not after application of identical mixtures of 10^9 PFU or even 10^{10} PFU of Bac-PRV that lack the VSV-G protein (data not shown). This is in line with data reported by Facciabene et al. [21] demonstrating that Bac-G-E2 (expressing the E2 protein of Hepatitis C virus) was more immunogenic than the same dose of applied Bac-E2. The rate of protection clearly depends on the amount of the injected Bac-G-PRV. We found that 10^9 PFU as the minimal dose must be inoculated three times (Fig. 4). Decreasing the amounts of Bac-G-PRV and the use of only one or two immunizations led at best to partial protection. The observed protection of the mice was mediated by the expression of the PRV glycoproteins, since none of the animals survived the challenge infection after application of non-recombinant Bac-G, and the mean time to death of the control-immunized mice was comparable to non-immunized, challenge-infected animals. This is interesting, because earlier results showed that non-recombinant baculovirus can protect mice non-specifically against EMCV challenge infection [43] or against influenza virus infection [20]. It was supposed that baculovirus per se can act as an adjuvant by stimulating innate immunity. The injection of live baculovirus was described to induce type I interferon (IFN) *in vitro* and *in vivo* in mice [43], and non-recombinant Bac-G elicited a strong IFN-gamma induction in mouse splenocytes [21].

To examine the stimulation of innate immune responses, splenocytes from mice were analyzed 24 h after vaccination with non-recombinant Bac-G or Bac-G-PRV for natural killer (NK) cell activity and induction of serum IFN. Application of non-recombinant Bac-G did neither induce cytolytic activity in the spleen nor detectable stimulation of IFN-alpha or IFN-gamma secretion into serum. In contrast, splenocytes of the Bac-G-PRV inoculated mice exhibited pronounced NK cell activity accompanied by increased amounts of serum type I and type II interferon. According to the presented results, we suppose that the applied non-recombinant Bac-G does not mount a considerable innate immune response as reported by Facciabene et al. [21] and Abe et al. [20], although we used the same mouse strain (BALB/c) and comparable purity of the applied Bac-G recombinants. However, at present the reported and presented findings cannot be fully compared due to the use of different methods of measuring the induction of innate immunity.

Therefore, in our experiments the early antiviral immune response seems primarily induced by the PRV glycoproteins expressed from the Bac-G-PRV. The importance of type I IFN for protection against Alphaherpesvirus infection is well described in murine models. Early after herpesvirus infection IFN-alpha is produced from antigen-presenting cells, which leads to activation of NK cells (for review [44,45]). The importance of distinct herpesviral gene products for stimulation of innate immunity is unknown. From other reports one might suggest that gC and gB of PRV could function in stimulating innate immunity. DNA immunization studies demonstrated the role of PRV gC in triggering IFN-alpha secretion, which subsequently supports the production of specific serum

antibodies of IgG2a class [46]. The use of each individual Bac-G recombinant can now help to decipher the role of these PRV proteins for stimulation of NK activity and associated innate immune mechanisms, at least in interrelation with the baculovirus virion proteins.

There is growing evidence for the implication of innate immunity including NK cells in regulating and modulating the adaptive immune response as well as the immunological memory. The quality and quantity of the adaptive immunity seem to depend on the innate response sensed after infection or vaccination (for review [47–50]). We have not determined the type of immune cells producing IFN-alpha and IFN-gamma early after Bac-G-PRV prime immunization, however, the results would fit into the concept that IFN-gamma secreted by NK cells enhances the decision for a Th1 response [51]. This is also supported by our finding of a strong anti-PRV antibody response dominated by the production of IgG2a serum antibodies, which was induced already after prime immunization. Notably, the following booster immunizations did no more significantly increase the anti-PRV serum antibody titers and also did not substantially change the ratio of IgG2a to IgG1 antibody subclasses. This indicates a rapid induction of a Th class 1 immune response after immunization with Bac-G-PRV. Nevertheless, two more booster immunizations are needed to protect all mice against lethal PRV challenge infection. This implies that three injections of high dose of Bac-G-PRV are necessary to mount an anti-PRV T-cell response sufficient to protect against PRV challenge infection. The importance of Th1-type CD4+ T-cells and IFN-gamma for protecting mice and pigs against PRV infection is well documented [40,41,52–55]. The PRV gB was reported to play an important role in generating CD4 positive Th1 cells [52,56]. PRV gC is suspected to function in stimulating the specific T-cell and B-cell response [57,58] and to promote a CD-8 positive T-cell response against PRV [52]. In conclusion, the protective potential of the Bac-G-PRV cocktail is supposed to be mediated predominantly by the induction of PRV-specific T-cell immunity, although some role of complement-dependent antibodies cannot be ruled out. Further studies to elucidate the T-cell subsets induced after immunization are now needed to prove that assumption.

In addition to gC, the gD of PRV must be regarded as a main antigen for the anti-PRV immune response, which essentially contributes to virus neutralization and protection [33,52,59,60]. Therefore, Bac-G expressing gD was included into the immunizing cocktail. However, even two booster injections did not lead to the generation of significant titers of PRV neutralizing serum antibodies. It is remarkable that all mice were protected against challenge infection despite of the absence of higher virus-neutralizing serum antibodies. However, several reports show that *in vivo* protection must not necessarily correlate with the level of virus-neutralizing serum antibodies [61,62]. The *in vitro* determined serum neutralizing activity does not obviously reflect the neutralizing capacity of those antibodies *in vivo* [63]. Interestingly, it has been reported that protection against PRV infection positively correlates with the induced amount of IFN-gamma rather than with the level of generated virus-neutralizing serum antibodies [61,62]. Our data indicate that the application of Bac-G-PRV rapidly shifted the antiviral response to a Th1, cell-mediated immunity, at the expense of a Th2, anti-PRV humoral immunity. This assumption is supported by the stimulation of IFN-gamma but not of interleukin-4 or interleukin-10 in the spleen of three times immunized mice before and after challenge infection. Therefore, the baculovirus vector system seems to favour the stimulation of T-cells, both CD4+ and CD8+ T lymphocytes, independent of the inserted antigen as reported for the Malaria CS antigen [64] or the E2 protein of HCV [21]. A kind of adjuvant effect of Bac-G to drive primarily a Th1-type immunity can be concluded, because expression of the gC and gD genes of PRV as ORFV (Orf virus, Parapoxvirus) recombinants stimulated a balanced Th1 and Th2 immune response [33]. Collectively, the protective potential of Bac-G-PRV can be explained by the generation of an efficient T-cell response, combined with the induction of an early strong PRV-specific humoral immune response.

The present study demonstrates for the first time the successful use of Bac-G recombinants for the generation of protective immunity against a herpesvirus infection. The described Bac-G recombinants represent valuable tools for analyzing the contribution of the individual PRV glycoproteins in protection and antiviral innate and adaptive immune mechanisms, which will be subject of an ongoing study. The described baculovirus recombinants will now allow the establishment of assays to investigate the contribution of the individual glycoproteins in the PRV-specific CTL immune response. Finally, the protective potential of Bac-G-PRV can now be investigated in the natural host of PRV, swine. In the future, the use of the Bac-G vector system for the expression of other antigens will show its benefit for other antimicrobial vaccines.

Acknowledgments

The monoclonal and polyclonal PRV-specific antibodies were kindly provided by T.C. Mettenleiter, Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Island of Riems, Germany. The generous help of Simone Nebrich and Lothar Stitz during the animal experiments is gratefully acknowledged. This study was supported by EU grant QLK2-1999-459/QLRT-2001-2896 and by the Polish Ministry of Science grant 0969/PO1/2006/30.

References

[1] Marshall E. Gene therapy death prompts review of adenovirus vector. Science 1999;286:2244–5.
[2] Brave A, Ljungberg K, Wahren B, Liu MA. Vaccine delivery methods using viral vectors. Mol Pharm 2007;4:18–32.
[3] Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. Nature Rev Genet 2003;4:346–58.
[4] Shams H. Recent developments in veterinary vaccinology. Vet J 2005;170:289–99.
[5] Paoletti E. Applications of pox virus vectors to vaccination: an update. Proc Natl Acad Sci USA 1996;93:11349–53.
[6] Patterson RM, Selkirk JK, Merrick BA. Baculovirus and insect cell gene expression—review of baculovirus biotechnology. Environ Health Perspect 1995;103:756–9.
[7] Beljelarskaya SN. A baculovirus expression system for insect cells. Mol Biol 2002;36:281–92.
[8] Boyce FM, Buchner NLR. Baculovirus-mediated gene transfer into mammalian cells. Proc Natl Acad Sci USA 1996;93:2348–52.
[9] Dudek T, Knipe DM. Replication-defective viruses as vaccines and vaccine vectors. Virology 2006;344:230–9.
[10] Smith CL, Mirza F, Pasquetto V, Tscharke DC, Palmowski MJ, Dunbar PR, et al. Immunodominance of poxviral-specific CTL in a human trial of recombinant-modified vaccinia Ankara. J Immunol 2005;175:8431–7.
[11] Gromadzka B, Szewczyk B, Konopa G, Mitzner A, Kesy A. Recombinant VP60 in the form of virion-like particles as a potential vaccine against rabbit hemorrhagic disease virus. Acta Biochim Pol 2006;53:371–6.
[12] Matassov P, Cupo A, Galarza JM. A novel intranasal virus-like particle (VLP) vaccine designed to protect against the pandemic 1918 influenza A virus (H1N1). Viral Immunol 2007;20(3):441–52.
[13] Hofmann C, Sanding V, Jennings G, Rudolph M, Schlag P, Strauss M. Efficient gene-transfer into human hepatocytes by baculovirus vectors. Proc Natl Acad Sci USA 1995;92:10099–103.
[14] Sarkis C, Serguera C, Peters S, Buichet D, Rider JL, Edelman L, et al. Efficient transduction of neural cells in vitro and in vivo by a baculovirus-derived vector. Proc Natl Acad Sci USA 2000;97:14638–43.
[15] Pieroni L, Maione D, La Monica N. *In vivo* gene transfer in mouse skeletal muscle mediated by baculovirus vectors. Hum Gene Ther 2001;12:871–81.
[16] Ho YC, Chen HC, Wang KC, Hu YC. Highly efficient baculovirus-mediated gene transfer into rat chondrocytes. Biotechnol Bioeng 2004;88:643–51.
[17] Barsoum J, Brown R, McKee M, Boyce FM. Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. Hum Gene Ther 1997;8:2011–8.
[18] Shoji I, Aizaki H, Tani H, Ishii K, Chiba T, Saito I, et al. Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. J Gen Virol 1997;78:2657–64.
[19] Aoki H, Sakoda Y, Jukuroki K, Takada A, Kida H, Fukusho A. Induction of antibodies in mice by a recombinant baculovirus expressing pseudorabies virus glycoprotein B in mammalian cells. Vet Microbiol 1999;68:197–207.

[20] Abe T, Takahashi H, Hamazaki H, Miyano-Kurosaki N, Matsuura Y, Takaku H. Baculovirus induces an innate immune response and confers protection from lethal influenza virus infection in mice. J Immunol 2003;171:1133–9.
[21] Facciabene A, Aurisicchio L, La Monica N. Baculovirus vectors elicit antigen-specific immune responses in mice. J Virol 2004;78:8663–72.
[22] Kimman TG, Gielkens AJL, Glazenburg K, Jacobs L, De Jong MCM, Mulder WAM, et al. Characterization of live pesudorabies virus vaccines. Dev Biol Stand 1995;84:89–96.
[23] Wittmann G, Rziha HJ. Aujeszky's disease (pseudorabies) in pigs. In: Herpesvirus diseases of cattle, horses and pigs. Boston, MA: Kulwer Academic; 1989. p. 230–325.
[24] Van Oirschot JT. Intranasal vaccination of pigs against Aujeszky's disease: comparison with one or two doses of attenuated vaccines in pigs with high maternal antibody titers. Res Vet Sci 1987;42:12–6.
[25] Visser N, Rziha HJ. Is recombination of PRV vaccine strains a real problem? Acta Vet Hung 1994;42:183–93.
[26] Stegeman A, de Jong MC, van Nes A, Bouma A. Dynamics of pseudorabies virus infections in vaccinated pig populations: a review. Vet Q 1997;19:117–22.
[27] Mettenleiter TC. Immunobiology of pseudorabies (Aujeszky's disease). Vet Immunol Immunopathol 1996;54:221–9.
[28] Zuckermann FA, Zsak L, Mettenleiter TC, Ben-Porat T. Pseudorabies virus glycoprotein gIII is a major target antigen for murine and swine virus-specific cytotoxic T lymphocytes. J Virol 1990;64:802–12.
[29] Eloit M, Fargeaund D, L'Haridon R, Toma B. Identification of the pseudorabies glycoprotein gp50 as a major target of neutralizing antibodies. Arch Virol 1988;99:45–56.
[30] Ben-Porat T, DeMarchi J, Lomniczi B, Kaplan AS. Role of glycoproteins of pseudorabies virus in eliciting neutralizing antibodies. Virology 1986;154:325–34.
[31] Kimman TG, De Bruin TGM, Voermans JJM, Peeters BPH, Bianchi ATJ. Development and antigen specificity of the lymphoproliferation response of pigs to pseudorabies virus: dichotomy between secondary B- and T-cell responses. Immunology 1996;86:372–8.
[32] Mukamoto M, Watanabe I, Kobayashi Y, Icatlo Jr FC, Ishii H, Kodama Y. Immunogenicity in Aujeszky's disease virus structural glycoprotein gVI (gp50) in swine. Vet Microbiol 1991;29:109–21.
[33] Fischer T, Planz O, Stitz L, Rziha HJ. Novel recombinant parapoxvirus vectors induce protective humoral and cellular immunity against lethal herpesvirus challenge infection in mice. J Virol 2003;77:9312–23.
[34] Dory D, Fischer T, Beven V, Cariolet R, Rziha HJ, Jestin A. Prime-boost immunization using DNA vaccine and recombinant Orf virus protects pigs against Pseudorabies virus (herpes suid 1). Vaccine 2006;24:6256–63.
[35] Hsu CS, Ho YC, Wang KC, Hu YC. Investigation of optimal transduction conditions for baculovirus-mediated gene delivery into mammalian cells. Biotechnol Bioeng 2004;88:42–51.
[36] Planz O, Stitz L. Borna disease virus nucleoprotein (p40) is a major target for CD8(+)-T-cell-mediated immune response. J Virol 1999;73:1715–8.
[37] Gerdts V, Joens A, Mettenleiter TC. Potency of an experimental DNA vaccine against Aujeszky's disease in pigs. Vet Microbiol 1999;66:1–13.
[38] Kost TA, Condreay JP, Jarvis DL. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat Biotechnol 2005;23:567–75.
[39] Hu YC. Baculovirus as a highly efficient expression vector in insect and mammalian cells. Acta Pharmacol Sin 2005;26:405–16.
[40] van Rooij EMA, de Bruin MGM, de Visser YE, Middel WGJ, Boersma WJA, Bianchi ATJ. Vaccine-induced T cell-mediated immunity plays a critical role in early protection against pseudorabies virus (suid herpes virus type 1) infection in pigs. Vet Immunol Immunopathol 2004;99:113–25.
[41] Bianchi ATJ, Moonen-Leusen HWM, van Millingen FJ, Savelkoul HFJ, Zwart RJ, Kimman. A mouse model to study immunity against pseudorabies virus infection: significance of $CD4^+$ and $CD8^+$ cells in protective immunity. Vaccine 1998;16:1550–8.
[42] Hu YC, Yao K, Wu TY. Baculovirus as an expression and/or delivery vehicle for vaccine antigens. Expert Rev Vaccines 2008;7:363–71.
[43] Gronowski AM, Hilbert DM, Sheehan KCF, Garotta G, Schreiber RD. Baculovirus stimulates antiviral effects in mammalian cells. J Virol 1999;73:9944–51.
[44] Cunningham AL, Diefenbach RJ, Miranda-Saksena M, Bosnjak L, Kim M, Jones C, et al. The cycle of human herpes simplex virus infection: virus transport and immune control. J Infect Dis 2006;15(194 Suppl. 1):S11–8.
[45] Onodera T, Yoshihara K, Suzuki T, Tsuda T, Ikeda T, Awaya A, et al. Resistance to pseudorabies virus with enhanced interferon production and natural killer cell activity in mice treated with serum thymic factor. Microbiol Immunol 1994;38:47–53.
[46] Tudor D, Riffault S, Carrat C, Lefèvre F, Bernoin M, Charley B. Type I IFN modulates the immune response induced by DNA vaccination to pseudorabies virus glycoprotein C. Virology 2001;20:197–205.
[47] Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol 2008;9:503–10.
[48] Pulendran B, Ahmed R. Translating innate immunity into immunological memory: implications for vaccine development. Cell 2006;24(124):849–63.
[49] Guy B. The perfect mix: recent progress in adjuvant research. Nat Rev Microbiol 2007;5:505–17.
[50] Kawai T, Akira S. Innate immune recognition of viral infection. Nat Immunol 2006;7:131–7.
[51] Martín-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat Immunol 2004;5:1260–5.
[52] Yoon HA, Aleyas AG, George JA, Park SO, Han YW, Kang SH, et al. Differential segregation of protective immunity by encoded antigen in DNA vaccine against pseudorabies virus. Immunol Cell Biol 2006;84:502–11.
[53] Schijns VE, Haagmans BL, Rijke EO, Huang S, Aguet M, Horzinek MC. IFN-gamma receptor-deficient mice generate antiviral Th1-characteristic cytokine profiles but altered antibody responses. J Immunol 1994;153:2029–37.
[54] Fischer T, Büttner M, Rziha HJ. T helper 1-type cytokine transcription in peripheral blood mononuclear cells of pseudorabies virus (Suid herpesvirus 1)-primed swine indicates efficient immunization. Immunology 2000;101:378–87.
[55] Hoegen B, Saalmüller A, Röttgen M, Rziha HJ, Geldermann H, Reiner G, et al. Interferon-gamma response of PBMC indicates productive pseudorabies virus (PRV) infection in swine. Vet Immunol Immunopathol 2004;102:389–97.
[56] van Rooij EM, Haagmans BL, de Visser YE, de Bruin MG, Boersma W, Bianchi AT. Effect of vaccination route and composition of DNA vaccine on the induction of protective immunity against pseudorabies infection in pigs. Vet Immunol Immunopathol 1998;66:113–26.
[57] Ober BT, Summerfield A, Mattlinger C, Wiesmüller KH, Jung G, Pfaff E, et al. Vaccine-induced, pseudorabies virus-specific, extrathymic CD4+ CD8+ memory T-helper cells in swine. J Virol 1998;72:4866–73.
[58] Ober BT, Teufel B, Wiesmüller KH, Jung G, Pfaff E, Saalmüller A, et al. The porcine humoral immune response against pseudorabies virus specifically targets attachment sites on glycoprotein gC. J Virol 2000;74:1752–60.
[59] Mengeling WL, Brockmeier SL, Lager KM. Evaluation of a recombinant vaccinia virus containing pseudorabies (PR) virus glycoprotein genes gp50, gII, and gIII as a PR vaccine for pigs. Arch Virol 1994;134:259–69.
[60] Riviere M, Tartaglia J, Perkus ME, Norton EK, Bongermino CM, Lacoste F, et al. Protection of mice and swine from pseudorabies virus conferred by vaccinia virus-based recombinants. J Virol 1992;66:3424–34.
[61] Xiao S, Chen H, Fang L, Liu C, Zhang H, Jiang Y, et al. Comparison of immune responses and protective efficacy of suicidal DNA vaccine and conventional DNA vaccine encoding glycoprotein C of Pseudorabies virus in mice. Vaccine 2004;22:345–51.
[62] Gerdts V, Jöns A, Makoschey B, visser N, Mettenleiter TC. Protection of pigs against Aujeszky's disease by DNA vaccination. J Gen Virol 1997;78:2139–46.
[63] Bachmann MF, Kalinke U, Althage A, Freer G, Burkhart C, Roost H-P, et al. Science 1997;276:2024–7.
[64] Strauss R, Hüser A, Ni S, Tuve S, Kiviat N, Sow PS, et al. Baculovirus-based vaccination vectors allow for efficient induction of immune responses against *Plasmodium falciparum* circumsporozoite protein. Mol Ther 2007;15:193–202.

BMC Veterinary Research

RESEARCH ARTICLE Open Access

Orf virus interferes with MHC class I surface expression by targeting vesicular transport and Golgi

Jörg Rohde[1,2], Frederic Emschermann[2], Michael R Knittler[2†] and Hanns-Joachim Rziha[1,2*†]

Abstract

Background: The Orf virus (ORFV), a zoonotic Parapoxvirus, causes pustular skin lesions in small ruminants (goat and sheep). Intriguingly, ORFV can repeatedly infect its host, despite the induction of a specific immunity. These immune modulating and immune evading properties are still unexplained.

Results: Here, we describe that ORFV infection of permissive cells impairs the intracellular transport of MHC class I molecules (MHC I) as a result of structural disruption and fragmentation of the Golgi apparatus. Depending on the duration of infection, we observed a pronounced co-localization of MHC I and COP-I vesicular structures as well as a reduction of MHC I surface expression of up to 50%. These subversion processes are associated with early ORFV gene expression and are accompanied by disturbed carbohydrate trimming of post-ER MHC I. The MHC I population remaining on the cell surface shows an extended half-life, an effect that might be partially controlled also by late ORFV genes.

Conclusions: The presented data demonstrate that ORFV down-regulates MHC I surface expression in infected cells by targeting the late vesicular export machinery and the structure and function of the Golgi apparatus, which might aid to escape cellular immune recognition.

Keywords: Orf virus, Parapoxvirus, MHC class I, Subversion, Immunomodulation, Golgi apparatus

Background

The Orf virus (ORFV; Parapoxvirus ovis) is the type species of the *Genus Parapoxvirus* belonging to the family *Poxviridae*. It is a skin epitheliotropic double-stranded DNA virus that causes pustular skin lesions in sheep and goats, known as contagious ecthyma [1]. Most interestingly, animals are not protected against ORFV re-infections, which might also be due to the short-lived ORFV-specific adaptive immunity. Orf is a zoonotic disease [2] that can be transmitted to humans by contact with infected animals. While Orf is usually a benign self-limiting illness, it can be very progressive in immune-compromised hosts [2].

Poxviruses provide considerable inventories of gene products that allow them to evade the host immune response [3]. It has been previously shown that ORFV encodes immunomodulators like ORFV IL-10, the GM-CSF- and IL-2-inhibitory factor (GIF) or the ORFV chemokine binding protein CBP, which have the ability to inhibit cytokine synthesis of monocytes [4-8]. These evasion strategies seem to play an important role in supporting ORFV replication and enabling repeated re-infections.

Cell-mediated immunity is critical for the clearance of virus-containing cells. Infected hosts normally react by activating their MHC I - mediated cellular immune response [9]. MHC I transmembrane glycoproteins function by binding intracellularly processed peptide antigens and presenting them on the cell surface to cytotoxic T cells [10]. During viral infection, a spectrum of antigenic peptides is displayed by MHC I molecules, resulting in the specific recognition of the infected cells by cytotoxic T cells (CTL). However, many viruses, including poxviruses [3,11], evade the T cell-mediated immune response, primarily by

* Correspondence: achim.rziha@fli.bund.de
†Equal contributors
[1]Present address: Department of Immunology, Interfaculty Institute for Cell Biology, University of Tuebingen, Auf der Morgenstelle 15, 72076, Tuebingen, Germany
[2]Friedrich-Loeffler-Institute, Federal Research Institute of Animal Health, Institute of Immunology, Greifswald-Insel Riems, Germany

decreasing the levels of surface MHC I, thus reducing the presentation of pathogen-derived antigens [12] to escape cellular immunosurveillance mechanisms [13]. MHC I down-regulation of infected cells increases susceptibility to natural killer (NK) cells, and many viruses have also evolved strategies to escape this immune detection [14].

The ability to inhibit proinflammatory cytokines (TNF and IFN) that regulate MHC expression is a mechanism of poxviruses to prevent the up-regulation of MHC I [3]. The gene product M153R of myxoma virus interferes directly with the antigen presentation pathway and induces the loss of β2-microglobulin associated MHC I, both at the cell surface and in an intracellular post-Golgi compartment [15]. Genes of cowpox virus modulate the MHC I antigen processing and expression. The CPXV203 protein is responsible for decreased surface expression of mouse and human MHC I molecules by using the physiologic KDEL-pathway to retain MHC I in the ER [16,17], whereas the CPVX12 protein prevents TAP-dependent peptide loading [18,19].

We are interested to identify possible immune evasion mechanisms of ORFV, the type species of Parapoxvirus. Also *in vitro* propagation of wild-type ORFV is very restricted and mostly primary ovine or bovine cells are used, which limits the availability of MHC I or cell compartment specific reagents. Therefore, we took advantage from the Vero cell-adapted ORFV strain D1701-V to analyse virus induced alterations of MHC I surface expression in infected permissive Vero cells. We show that this Parapoxvirus impairs MHC I surface expression by structurally disrupting the Golgi apparatus. Most interestingly, Golgi fragmentation is accompanied by a defective intracellular MHC I transport, pronounced co-localization of MHC I and COP-I-vesicles, disturbed carbohydrate trimming of Golgi-localized MHC I molecules and a reduction of MHC I surface expression of up to 50%. In addition to these effects, we also noticed that the half-life of the remaining MHC I surface population is remarkably increased. All observed evasion phenotypes except for the MHC I half-life effect are linked to the expression of early ORFV genes. On the basis of our findings we postulate that ORFV modulates MHC I surface expression in infected cells by targeting the vesicular transport machinery and the structure and function of the Golgi apparatus. Thus, it is tempting to speculate that the discovered ORFV-mediated effects on MHC I act in concert to facilitate infection and allow the acute virus to replicate and shed prior to clearance by the host immune response.

Results

ORFV induces down-regulation of surface MHC I molecules

Surface expression of MHC I was investigated in ORFV-infected and non-infected Vero cells by flow cytometry using the MHC I specific monoclonal antibody (mAb) W6/32 as described in *Methods*. As shown in Figure 1a, ORFV infection resulted in a significant decrease of the MHC I surface expression. Twelve hours post infection (hpi) about 80% of MHC I was detectable on the cell surface compared to non-infected cells, which was further reduced to 70% at 24 hpi, and to almost 50% at 36 hpi. These decreases were statistically highly significant as determined by One-way ANOVA analysis ($P < 0.001$). Reduction of MHC I surface expression was dependent on live, replicating ORFV. Thus, infection of the cells with β-propiolactone-inactivated virus did not change the amount of MHC I expressed on the surface of Vero cells (Figure 1a, inact. ORFV).

To analyze whether expression of early or late ORFV genes might be responsible for the MHC I down-regulation, AraC was used to inhibit viral DNA synthesis and thereby preventing intermediate and late gene expression of ORFV [6]. Figure 1b demonstrates that blocking of ORFV intermediate and late gene transcription (+ AraC) did not abolish MHC I down-regulation or affect MHC I surface presentation in non-infected cells. Infection of Vero cells and the effect of AraC were controlled by immunofluorescence studies using the mAb 13 C10, which is directed against the late major envelope protein of ORFV (Figure 1e).

ORFV infection increases the half-life of remaining surface MHC I molecules

Virus-infected cells were treated with Brefeldin A (BFA) to examine the biological stability of cell surface expressed MHC I molecules. BFA prevents the anterograde MHC I transport from the endoplasmic reticulum (ER) to the Golgi apparatus, and thereby inhibits the supply of newly synthesized MHC I to the cell surface. This experimental design allows the analysis of the half-life of surface expressed pre-existing MHC I by using flow cytometry. BFA-treated, non-infected Vero cells showed a 40 and 60% reduction of surface MHC I after 8 and 20 h incubation, respectively (Figure 1c, ni). In contrast, virus-infected Vero cells showed at the same BFA-incubation time points only a marginal MHC I decrease of 10% and 30% (Figure 1c, ORFV). These results suggest that ORFV infection increases the half-life of the remaining MHC I surface population by affecting surface stability and/or recycling of MHC I molecules. To examine whether early and/or late ORFV gene expression might be responsible for the increase in MHC I surface survival, cells were additionally treated with AraC during ORFV infection and BFA treatment. Figures 1c, d show that the MHC I half-life on the surface of non-infected cells was not altered by AraC. In infected cells the presence of AraC has some neutralizing influence on the ORFV mediated half-life effect on surface MHC I (compare Figure 1c, d). Thus, the ORFV-

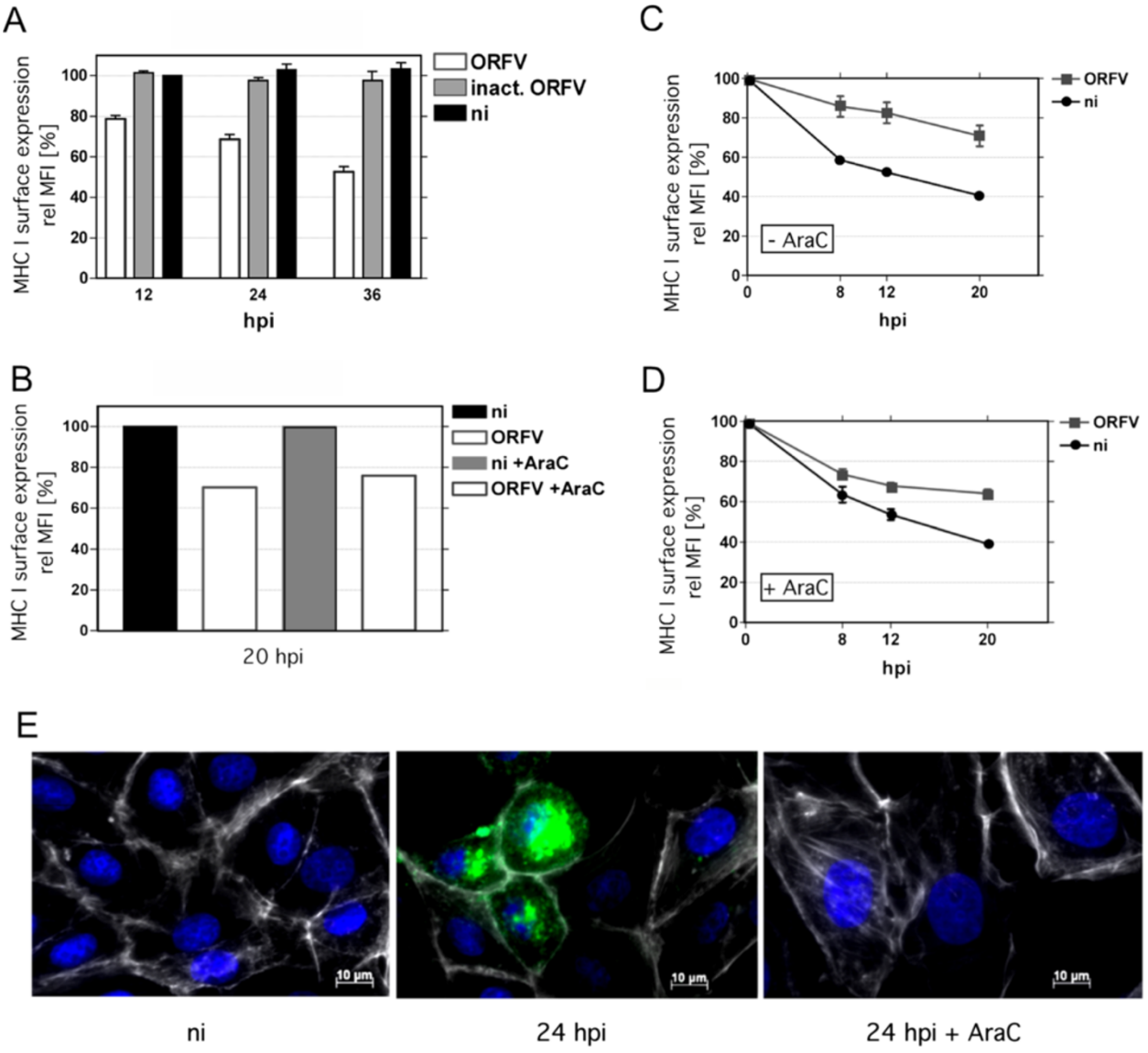

Figure 1 Modulation of MHC I surface expression in ORFV-infected cells. (A) Vero cells were harvested at 12, 24, and 36 hpi (m.o.i. 1.0) and stained with the anti-MHC I mAb W6/32 as described in Methods. The effect of non-replicating ORFV was tested by the use of ß-propiolactone inactivated ORFV (inact. ORFV; m.o.i. 1.0 before inactivation), non-infected (ni) cells were used as negative controls. The average of three separate virus culturing experiments is shown. ORFV infection decreased cell surface expressed MHC I. **(B)** Twenty hours post infection (m.o.i. 1.0), MHC I cell surface expression (W6/32) was determined by FACS in the presence and absence of AraC. No effect of AraC treatment on MHC I surface expression was observed. One representative experiment is shown. **(C)** ORFV infected (m.o.i. 1.0) or non-infected Vero cells were treated with BFA or **(D)** with BFA plus AraC. Virus infection increased the half-life of MHC I on the cell surface, determined at 8, 12 and 20 hpi using W6/32 anti-MHC I antibody by flow cytometry. The average of three independent experiments is shown in C, D. The relative mean fluorescence intensity (rel MFI) is given in percentages. **(E)** Infection (m.o.i. 1.0) of Vero cells (green staining) and the effect of AraC was controlled (24 hpi) by immunofluorescence studies using the mAb 13 C10 (diluted 1:1000) recognizing the late major envelope protein of ORFV. Nuclei and F-actin are stained blue by DAPI and white by phalloidin-TRITC, respectively.

dependent increase of MHC I surface stability might be partially controlled also by late gene products.

ORFV infection does not influence MHC I-transcription

A semi-quantitative RT-PCR was used to determine whether ORFV infection might influence the MHC I mRNA synthesis and thereby reduces MHC I surface expression. The amount of mRNA specific for the housekeeping gene GAPDH was related to the amount of MHC I mRNA at different times after infection. Each PCR product taken at the linear phase of PCR amplification was analyzed by gel densitometry. As shown in Figure 2a, the ratio of MHC I to GAPDH mRNA in non-infected cells ranged between 0.63 and 0.65 (Lanes

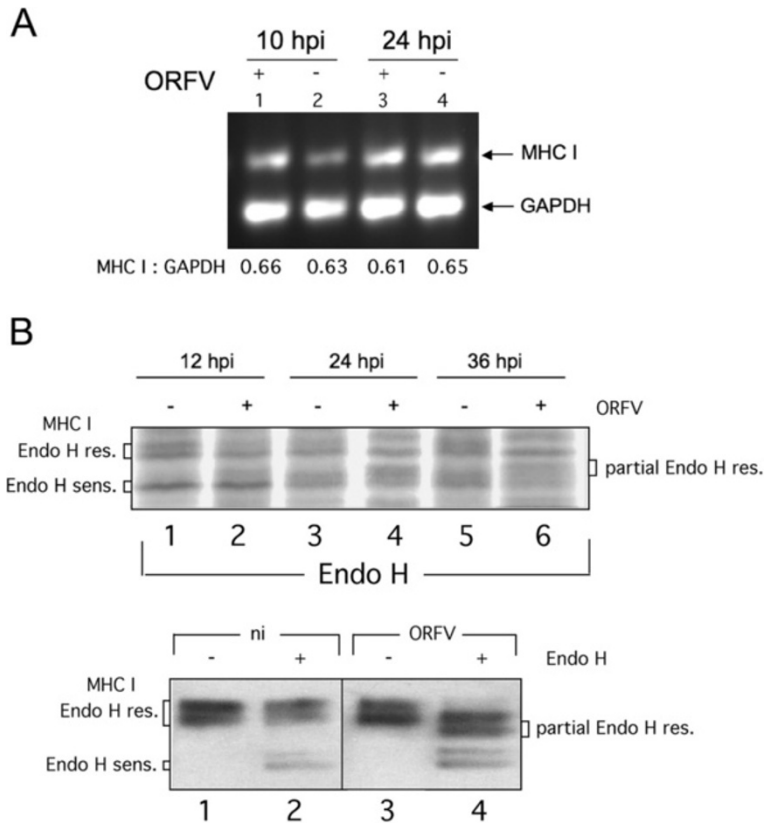

Figure 2 Effects of ORFV-infection on expression and intracellular transport of MHC I . (A) MHC I- and GAPDH-specific RT-PCR was performed as described in Methods. After gel electrophoresis the amplicon band intensities were quantified by densitometry and their calculated ratios are indicated below each gel lane. The transcription rate of MHC I was not affected significantly by ORFV infection. **(B, upper panel)** ORFV infection affects carbohydrate trimming of MHC I. Infected (+; m.o.i. 2.0) or not infected (–) cells were labelled with Trans-^{35} S-Label, lysed at 12, 24 and 36 hpi, and MHC I was immunoprecipitated with W6/32 antibody. The immunoprecipitates were digested with Endo H before separation by SDS-PAGE. Fluorographs were analyzed using GelEval 1.32 software (FrogDance Software). Endo H-resistant, -sensitive and partially Endo H-resistant MHC I forms are indicated. (**B, lower panel)** Infected (+; m.o.i. 1.0) or not infected (–) cells were lysed at 12 hpi, digested with Endo H and analyzed by Western blots probed with anti-MHC I mAb LY5.1.

2 and 4), which remained almost unaltered 10 or 24 h after ORFV infection (lanes 1 and 3). Thus, the observed decrease of MHC I surface expression cannot be attributed to a prevention or inhibition of MHC I mRNA transcription by ORFV.

ORFV infection disturbs carbohydrate trimming and maturation of MHC I

Next we analyzed whether and to what extent intracellular maturation of MHC I along the secretory route might be affected by ORFV infection. Endoglycosidase H (Endo H) – cleavage experiments were performed with anti-MHC I immunoprecipitates from detergent extracts of biosynthetically labelled, infected or non-infected Vero cells. Endo H is used to monitor posttranslational modification of glycosylated proteins within the Golgi. The MHC I-attached high mannose oligosaccharides are modified by a series of different ER and Golgi enzymes. Endo H is able to cleave oligosaccharides until the medial Golgi enzyme α-mannosidase II removes two mannose subunits. Since all later carbohydrate structures are Endo H-resistant, the enzyme monitors MHC I maturation within the late secretory route.

As can be seen from the SDS-PAGE analysis in Figure 2b upper panel, 12 h after ORFV infection intracellular MHC I-maturation is comparable in infected and non-infected Vero cells. In both situations we observed an approximately 1:1 signal ratio between Endo H-sensitive and -resistant MHC I molecules (Figure 2b upper panel, compare lanes 1 and 2). An additional minor species (approximately 10% of total MHC I signal) of partially resistant MHC I was also visible in infected cells (Figure 2b, upper panel, lane 2). After 24 and 36 h of infection, the population of Endo H-resistant MHC I was almost unaffected whereas the amount of Endo H-

sensitive MHC I decreased by more than half (Figure 2b upper panel, lanes 4 and 6) as determined by densitometric scanning. Most importantly, the latter phenomenon was linked to a simultaneous increase of partially Endo H-resistant MHC I molecules by 45 and 55%, respectively. No such formation of unusual MHC I forms could be observed for non-infected control cells after 24 or 36 h of incubation (Figure 2b, upper panel, compare lanes 1, 3 and 5). The distinct behaviour of MHC I maturation in ORFV-infected cells was also seen in Western blot experiments, in which lysates of infected and non-infected Vero cells were assayed by using a different anti-MHC I antibody (mouse mAb, clone LY5.1, see Figure 2b, lower panel) with apparently higher specificity for the mature forms of MHC I. The two Endo H-resistant and -sensitive protein bands found after immunoprecipitation (Figure 2b, upper panel) or in Western blotting (Figure 2b, lower panel) by the two different anti-MHC I antibodies (W6/32 and LY5.1) most likely represent different allelic MHC I products expressed in Vero cells. Taken together, these findings suggest that ORFV-infection interferes with the functional requirements for proper carbohydrate trimming of MHC I within the *cis*- and/or *medial*-Golgi or the transport between the exocytic compartments.

ORFV infection results in morphological changes of the Golgi apparatus

Next, we investigated whether ORFV infection might affect the secretory pathway and Golgi transport of MHC I and thereby prevents intracellular trafficking of newly synthesized MHC I to the cell surface. Therefore, we analyzed infected cells by confocal immunofluorescence after co-staining of intracellular MHC I and Giantin, a main component of the *cis*- and *medial*-Golgi. The results in Figure 3a demonstrate that virus infection caused substantial changes in the localization patterns of MHC I and Giantin. Already 10 hpi, MHC I dispersed into the cytoplasm with a punctuated vesicular structure (Figure 3a - panel A) continuing to 24 hpi (Figure 3a - panel G), whereas MHC I in non-infected Vero cells showed a dense and ring-shaped perinuclear staining (Figure. 3a - panels D and J).

In non-infected cells, Giantin-staining was characterized by a compact perinuclear pattern (Figure 3a - panels E and K) that disappeared during ORFV infection and scattered throughout the cytoplasm (Figure 3a - panels B and H). Simultaneously, co-localization between Giantin and MHC I, which was clearly seen in non-infected cells (Figure 3a - panels F and L), was reduced during virus infection (Figure 3a – panels C and I) as verified by calculating the coefficient of co-localization (Pearson value; data not shown). The ORFV-induced Golgi spreading was also found in AraC-treated infected cells (Figure 3a, panels M to R) indicating the involvement of early ORFV gene(s). The ORFV-induced dislodgment of Golgi from its original nucleus-associated location into the cytoplasm could be confirmed by quantitative analysis of the distances between Golgi and nucleus in infected and non-infected cells (Figure 3b). The distance from the centre of the nucleus of each cell to the peripheral fringe of the Golgi was almost duplicated in infected cells, in the presence as well as in the absence of AraC, when compared to non-infected cells, and was highly significant according to *T* test ($P < 0.0001$).

The *trans*-Golgi network (TGN) represents another important constituent of the late secretory route involved in exo- as well as endocytic processes [20]. The possible influence of ORFV on the TGN structure was examined with a TGN46-specific antibody. Partial co-localization between TGN46 and MHC I was visible in infected and non-infected cells. Similar to Giantin and MHC I, TGN46 lost its prominent perinuclear distribution after virus infection in favour of a punctuated vesicular pattern within the cytoplasm (Figure 4a, compare panels A and D, B and E), which was also seen in infected cells arrested for early gene expression by AraC (data not shown). Quantitative analysis of the images (Figure 4b) revealed a significantly increased distance ($P < 0.0001$) between TGN and nucleus (17 to 23 μm) in comparison to non-infected cells (9 to 12 μm). In summary, in virus infected cells Golgi and TGN are structurally dispersed into the cytoplasm and these processes are linked to early gene expression.

Influence of ORFV on the intracellular transport of MHC I molecules

Since ORFV-infection leads to a fragmentation of Golgi, we explored the viral influence on Golgi-transport of MHC I. COP-I is a protein complex that coats vesicles transporting polypeptides between different Golgi compartments and from the *cis*-Golgi back to the ER [21]. Therefore, we analyzed intracellular staining of MHC I and COP-I-component β-COP in infected and non-infected cells by fluorescence microscopy. Non-infected Vero cells displayed a characteristic juxtanuclear staining pattern of MHC I (Figure 5a - panels D and J) but only partial intracellular co-labelling of MHC I and β-COP (Figure 5a - panels F and L). In infected Vero cells a prominent perinuclear and vesicular MHC I-staining was observed 10 hpi that, however, dispersed into the cytoplasm after 24 hpi (Figure 5a - panels A and G). In contrast to non-infected cells, MHC I/β-COP co-localization could be seen for both infection time points (Figure 5a - panels C and I) confirmed by Pearson value calculation (data not shown). It must be noted that the non-infected cells were photographed with longer exposure time for

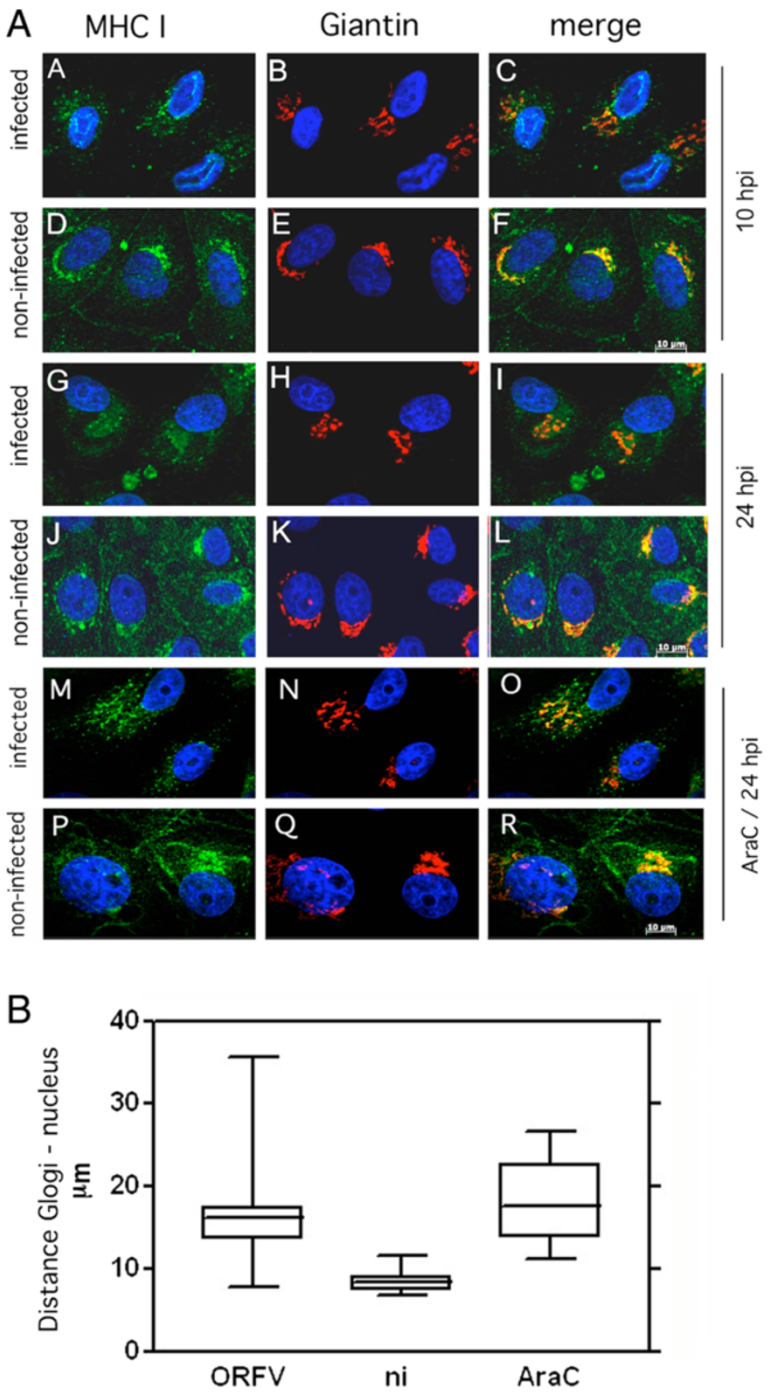

Figure 3 ORFV-induced dispersion of the Golgi apparatus. (A) Vero cells were infected (m.o.i. 0.5) and stained with the MHC I-specific mAb W6/32 (green) and the Giantin antibody specific for Golgi (red) after 10 hpi **(panels A-F)** and 24 hpi **(panels G-L)** or 24 hpi in the presence of AraC **(panels M-R)**. In infected cells MHC I dispersed into the cytoplasm and Golgi structures dispersed into the cytoplasm. Nuclei are stained blue by DAPI. Representative results of five independent experiments are shown. **(B)** The distances of the Golgi from the nucleus in non-infected and infected cells in the presence or absence of AraC were quantified using with AxioVision Rel. 4.8 software. The evaluation of 46 infected cells (ORFV), 20 infected and AraC-treated cells (ORFV/AraC), and 42 non-infected cells (ni) demonstrates significantly (*T* test: $P < 0.0001$) increased distance between Golgi and cell nucleus in infected cells. Box plots with median percentile were accomplished with GraphPad Prism 5 software.

the sake of better visualization. Additional AraC experiments showed that this effect is also controlled by early ORFV gene expression (data not shown).

Given expression levels of β-COP were analyzed by Western blot experiments in infected and non-infected cells. Figure 5b demonstrates that the 95 kDa β-COP

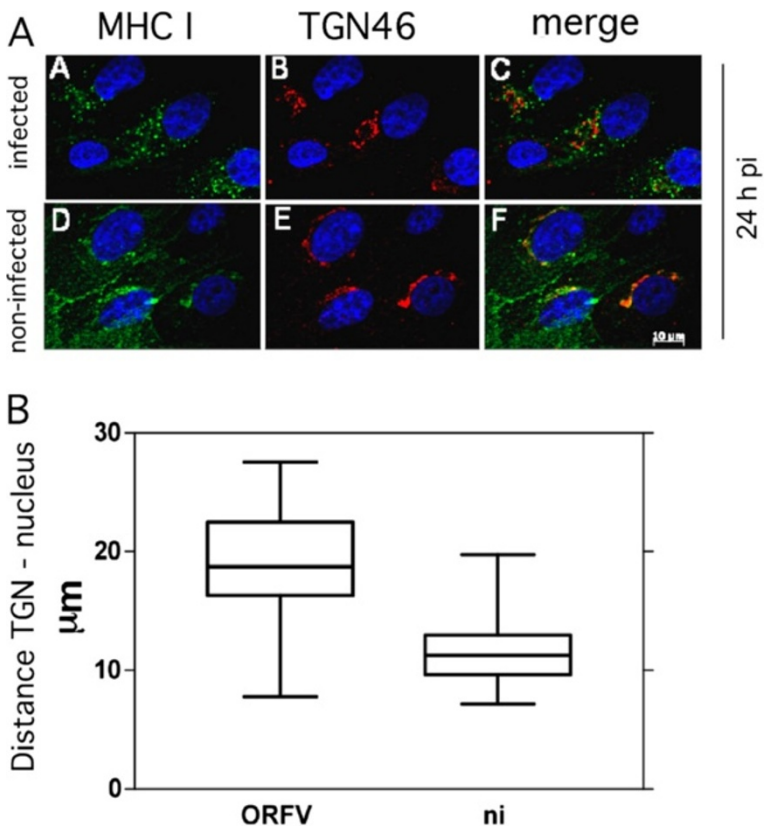

Figure 4 Structural changes of the trans-Golgi network (TGN) after ORFV infection. (A) Infected cells (m.o.i. 0.5) or non-infected cells were fixed 24 hpi and stained with W6/32 antibody (green) and anti-TGN46 antibody (red) **(panels A-F)**. Partial co-localization of MHC I and TGN can be seen in infected cells by merging the fluorescent images (merge). Nuclei are stained blue by DAPI. After infection TGN lost its perinuclear location and moved into the cytoplasm. A representative result of confocal fluorescence microscopy of three experiments is shown. **(B)** TGN-dislocation in ORFV infected cells. The distances of the TGN and the nucleus in infected and non-infected cells were quantified using with AxioVision Rel. 4.8 software (Zeiss). The evaluation of 27 infected and 27 non-infected cells is summarized as box plots and demonstrates an increased distance between TGN and cell nucleus in infected cells.

protein was hardly detectable in cell extracts of non-infected Vero cells, most likely due to the fact that β-COP, like other COP-I components, does not stably exist out of the coatomer complex [22]. Nevertheless, 24 h after ORFV infection β-COP was clearly visible with reduced amounts expressed after 36 hpi suggesting that the population of stably assembled COP-I structures is drastically enlarged in infected cells. Comparable protein loading was controlled by β-actin staining (Figure 5b, lower panel). Taken together, our findings provide evidence that the amount of MHC I-containing stable COP-I vesicles increased significantly during the first 24 hours after ORFV infection.

Discussion

The presented study shows that cellular ORFV infection leads to structural dispersion of the Golgi/TGN compartments and enrichment of COP-I vesicular structures. These processes are accompanied by an increase in the steady state expression of β-COP (Figure 5b), defective carbohydrate trimming of MHC I within the Golgi (Figure 2b), reduction of surface expressed MHC I molecules and a prolonged half-life of pre-existing MHC I on the plasma membrane (Figure 1). Upcoming studies have to prove whether the described interferences of ORFV with the MHC I expression also occur in natural host cells.

Our findings demonstrate that in ORFV-infected cells the intra-Golgi- and endosome/TGN-transport of MHC I was severely disturbed. ORFV seems to utilize early gene expression to block MHC I export within the late secretory route and thereby reduces MHC I surface expression. As shown by our experiments ORFV alters the perinuclear localization as well as the overall structure of the Golgi and TGN in infected Vero cells. Similar effects on the Golgi have also been described for a variety of different viruses. Early gene expression of Varicella zoster virus leads to MHC I down-regulation by

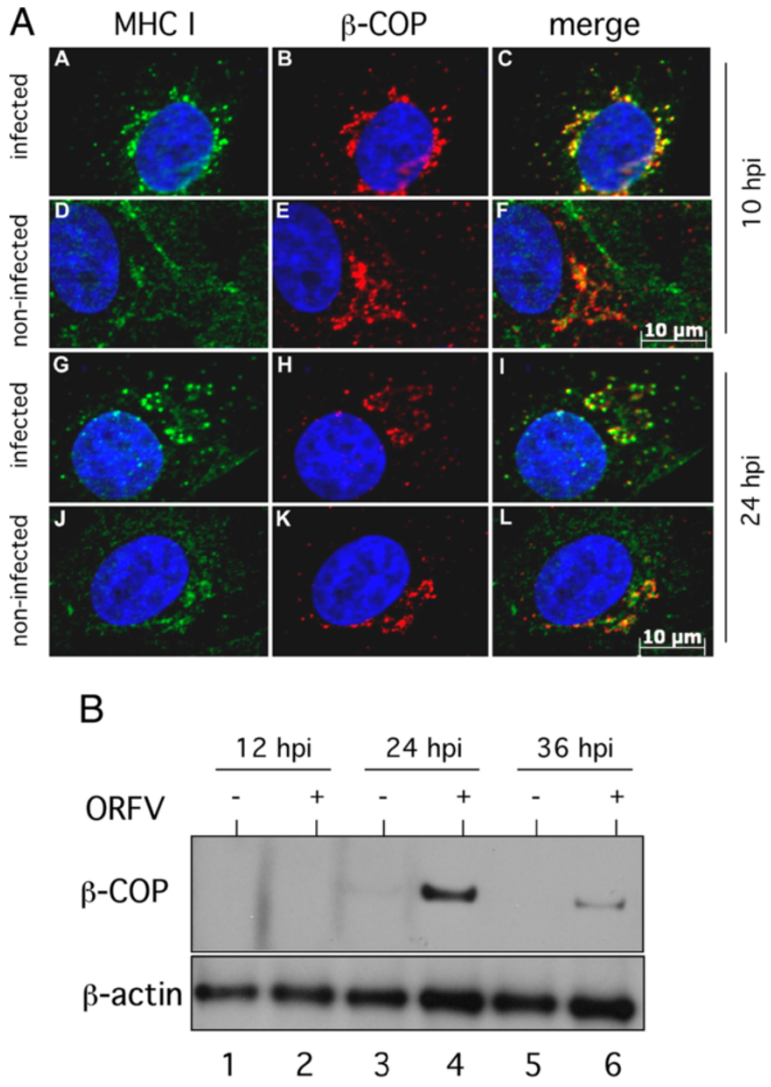

Figure 5 ORFV-infection interferes with COP-I mediated vesicular transport. (A) MHC I co-localizes with COP-I vesicles after ORFV infection. In infected cells (m.o.i. 0.5) MHC I was stained with mAb W6/32 (green) and anti-β-COP antibody (red). A representative result of three experiments at 10 hpi **(panels A-F)** and 24 hpi **(panels G-L)** is shown. Cell nuclei are stained blue with DAPI. A distinct co-localization (merge, yellow) of MHC I and β-COP was found in infected cells. Note that non-infected cells had to be photographed with longer exposure times as infected cells for the sake of better MHC-I/ß-COP visualization. **(B)** ORFV induced expression levels of β-COP. ß-COP (95 kDa) was detectable by Western blot analysis in infected cells (m.o.i. 1.0; lanes +) during 24 to 36 hpi. Detection of cellular ß-actin demonstrates comparable protein loading.

impairing its transport to the cell surface [23]. A late event in the reproductive cycle of Herpes simplex virus type 1 causes fragmentation and dispersal of the Golgi in infected Vero cells, which coincides with virion assembly [24]. The infection with human rhinovirus 1A (HRV-1A) induces Golgi-fragmentation into vesicles that appear to be used as a substrate for viral RNA replication [25]. Another positive-strand RNA virus, the poliovirus, induces dramatic disruption of the Golgi with consequences for the secretory complex [26,27]. Furthermore, it is known that vaccinia virus becomes enwrapped by cisternae derived from the intermediate compartment between ER and Golgi stacks as well as the TGN [28]. Recently Tan et al. also observed fragmentation of the Golgi during ORFV infection, and reported the Golgi localization of an ORFV envelope protein during late stage of infection [29]. The authors suggested that it is concealed between two Golgi membranes, which are forming wrapped mature virions. In the present study, the destruction of the Golgi structure is clearly not linked to virus envelope formation since the observed structural modifications are also visible in the presence of AraC, which prevents the expression of late ORFV genes essentially required for the virus envelope.

ORFV-infected cells are characterized by a reduced amount of newly synthesized MHC I on the plasma membrane as well as a prolonged half-life of the remaining pre-existing surface MHC I molecules (Figure 1). Down-regulation of MHC I is clearly AraC-insensitive and thus apparently linked to the expression of early ORFV genes whereas it cannot be excluded that the observed MHC I half-life effect might be also controlled by late ORFV gene expression. It is tempting to speculate that the respective viral gene products target compartments within the late secretory route. Since structural and functional integrity of the TGN are essentially required for endosomal/TGN-trafficking, the observed disruption of the TGN in infected cells (Figure 4) might be suspected to interfere with endocytosis as well as endosomal recycling of MHC I. A similar phenotype has been described for the HPV16 protein E5 [30], which mediates disruption of the exo- and endocytic trafficking, including transport of the MHC I [30], which causes reduced MHC I surface presentation and extends the half-life of the remaining MHC I molecules on the plasma membrane (M. R. Knittler, manuscript in preparation).

The ORFV infection leads to an accumulation of MHC I in COP-I vesicles (Figure 5a). COP-I is the cytoplasmic membrane-coat complex (coatomer) of seven distinct proteins and is required for both anterograde and retrograde transport in the secretory pathway [31,32]. The observation that ORFV infection increases the cellular expression levels of β-COP (Figure 5b) and the amount of COP-I vesicular structures suggests inhibition of uncoating of COP-I vesicles by ORFV. The identification of responsible ORFV protein(s), as found in Coxsackievirus [33], requires further detailed studies. In contrast to vaccinia virus, which hijacks the COP-I coatomer for viral particle formation [34], no correlation between accumulation of COP-I vesicles and viral biogenesis was observed, since the ORFV-mediated effect was also detectable in the presence of AraC.

The Endo H-experiments suggest that destruction of Golgi and TGN structures as well as intracellular accumulation of MHC I in COP-I vesicles is accompanied by impaired post-ER maturation of the N-linked carbohydrates of MHC I. In contrast to non-infected cells, a substantial amount of the MHC I molecules exhibits partial Endo H-resistance in ORFV-infected cells indicating that these molecules are not correctly processed by carbohydrate-trimming within Golgi. This reminds of the defective maturation of MHC I in the presence Concanamycin B, a specific inhibitor of the vacuolar type H (+)-ATPase [35], suggesting that ORFV infection not only affects the intracellular location and structure of Golgi and TGN, but also the functional pH conditions within these two compartments.

In addition to MHC I, ORFV infection also interferes with the surface expression of the transferrin receptor (TfR, CD71) (data not shown), which suggests that the ORFV-induced reduction of MHC I-antigen presentation is mediated by subversion of the host cell export machinery and not via specific targeting of MHC I molecules. Thus, one could assume that the ORFV-mediated modulation of vesicular transport has a more pleiotropic effect that also includes the reduction of antigen presentation and thereby provides an immune subversion strategy in advantage of the viral pathogen.

ORFV does clearly not interfere with the expression of MHC I molecules (Figure 2a) but uses an evasion strategy that accumulates newly synthesized MHC I molecules within the late secretory pathway (COP-I vesicles) possibly to down-modulate MHC I presenting viral antigens (for evasion of a cytotoxic T cell -mediated response), while simultaneously increasing the half-lives of pre-existing self peptide MHC I complexes at the plasma membrane (for evasion of an NK cell-mediated response). This suggests that ORFV like other large DNA viruses (e.g. Herpesviruses) uses different evasion strategies to interfere with antigen presentation at different levels of MHC I processing.

Conclusion

We assume that the reduction of surface expressed MHC I and the impaired structure and function of the Golgi apparatus, which are possibly controlled by different ORFV gene products, independently affect intracellular transport and surface stability of MHC I and cooperatively undermine immune recognition of ORFV-infected cells by CTLs as well as NK cells. In view of the fact that the immunity elicited by ORFV is short-lived, and animals can be repeatedly infected [2], MHC I subversion may contribute to rescuing ORFV from host immunity and supporting viral replication in epidermal cells.

Methods

Cells and virus

The attenuated ORFV strain D1701-V was propagated and titrated in Vero cells as described [36]. Virus inactivation was achieved with 0.05% (v/v) β-Propiolactone (Serva) by incubation at 37 °C for 4 h and maintaining the pH-value of 7.6. After overnight incubation at 4 °C the supernatant was collected by centrifugation and plaque assays proved the successful virus inactivation.

Antibodies

The mouse mAb W6/32 specific for HLA-ABC also recognizing simian MHC I [37] was used for flow cytometry, confocal fluorescence microscopy and immunoprecipitation. LY5.1 is a mAb recognizing MHC class I

heavy chains of HLA-ABC (Acris). Antibodies specific for Giantin, TGN46 and β-COP were purchased from Abcam, the antibody against ß-actin from Sigma-Aldrich . The mAb 13 C10 is directed against the 39 K major envelope protein of ORFV [38] and was a generous gift of C. McInnes and P. Nettleton (MRI, Pentlands Science Park, Penicuik, Scotland). As second antibodies we used anti-mouse FITC-conjugated antibody (Dianova), anti-mouse Alexa Fluor 488- and Alexa Fluor 555-conjugated antibodies and anti-rabbit Alexa Fluor 488- and Alexa Fluor 555-conjugated antibodies (Fisher Scientific, Invitrogen) and HRP-conjugated anti-rabbit IgG (Dianova).

Flow cytometry

Vero cells were infected with a m.o.i. of 1.0, harvested and stained successively with primary antibody and FITC-conjugated secondary antibody for 30 minutes at 4 °C. Brefeldin A (BFA, Sigma-Aldrich) was used in a concentration of 10 μg ml^{-1}, cytosine arabinoside (AraC, Sigma-Aldrich) was added (40 μg ml^{-1}) during virus infection. For viable cell determination dead cells were stained with 7-AAD (BD Bioscience) prior to FACS analyses using a FACSCalibur (BD Bioscience) and CellQuest Pro (BD Bioscience).

RNA isolation and semi-quantitative reverse transcription PCR

RNA kit (SurePrep True Total RNA Purification Kit, Fisher Scientific) was used to isolate total RNA from infected (m.o.i. 1.0) and non-infected Vero cells according to the manufacturer's instructions. RNA was treated with DNase (DNA-free, Ambion) and 300 ng were used for RT-PCR. Specific RNA of MHC I and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene was amplified by RT-PCR according to the manufacturer's recommendation (OneStep RT-PCR Kit, Qiagen) in a total volume of 10 μl, using GAPDH-specific primers at an annealing temperature of 64 °C [39] or using MHC I generic primers at an annealing temperature of 62 °C [40]. PCR products were taken during the linear phase of amplification, separated by gel electrophoresis and the amplicon DNA band intensities were quantified using GelEval 1.32 software (FrogDance Software).

Immunofluorescence

Vero cells were grown and infected (m.o.i. 0.5) in chamber slides (BD Biosciences) and fixed with 2% (v/v) methanol-free formaldehyde (Pierce, Fisher Scientific) in PBS and permeabilized with 0.2% (v/v) Triton-X100 (Sigma-Aldrich) in PBS. After 30 minutes blocking at room temperature in 5% (v/v) FCS in PBS, all antibody incubations were performed in PBS containing 1% (v/v) FCS for 30 minutes at 37 °C. F-actin was stained with Phalloidin-TRITC (Sigma-Aldrich), nuclei were stained with DAPI (1 μg ml^{-1}, Sigma-Aldrich) before embedding of slides in Mowiol-DABCO. Confocal microscopy was performed with ApoTome confocal fluorescence microscope (Axiovert 200 M; Zeiss) and arranged with AxioVision Rel. 4.8 (Zeiss). The Pearson coefficient showing degree of colocalization was determined using the program CoLocalizer Express (CoLocalizer).

Biosynthetic labelling and immunoprecipitation of proteins

Cells were starved for one hour in methionine-cysteine free Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 4 mM L-glutamine and 1 mM Na-Pyruvate, followed by incubation for additional 12 h in the presence of 10.5 mCi ml^{-1} Trans-^{35}S-Label (MP Biomedicals). Washed labelled cells were solubilised in PBS containing 1% Triton- X100 (Sigma-Aldrich) on ice for 45 minutes. After centrifugation at 14.000 rpm for 5 minutes the supernatants were used for immunoprecipitations at 4 °C overnight with anti MHC I mAb W6/32, which has been coupled directly to cyanogen bromide-activated sepharose (Amersham Life Sci.). Precipitates were digested with 10 mU of Endo H (Sigma-Aldrich) for 12 h at 37 °C and MHC I was eluted with 2.4 M urea, 2% SDS, 20% Glycerine, 125 mM Tris (pH 6.8) for 5 minutes at 95 °C prior to SDS-PAGE. Following electrophoresis fixed and dried gels were exposed to X-ray films (Kodak).

SDS-PAGE and western blot analysis

Non-infected and infected (m.o.i. 1.0) cells were dissolved with 1% (v/v) Triton- X100 (Sigma-Aldrich) in PBS for 30 minutes at 4 °C. SDS-PAGE and Western Blot were performed as reported [41]. All antibodies were diluted in 1 x RotiBlock (Roth) and for enhanced chemiluminescence (ECL) the substrate Immobilon Western HRP (Millipore) was used. X-ray films for ECL were purchased from Pierce (Fisher Scientific).

Statistical analysis

Statistical significances were evaluated by One-way ANOVA analysis (Figure 1) or by the *T* test (Figures 2 and 3) using GraphPad Prism 5 software (La Jolla).

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

JR carried out the studies, participated in the design of the studies and drafted the manuscript. FE participated in flow cytometry analysis and in the design of the studies. MRK and H-JR designed and coordinated the studies, aided in the interpretation of the data and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Lothar Stitz, Gregor Meyers, and Ralf Amann for helpful discussions and for critically reading the manuscript.

Received: 7 March 2012 Accepted: 29 May 2012
Published: 18 July 2012

References

1. Büttner M, Rziha HJ: **Parapoxviruses: from the lesion to the viral genome.** *J Vet Med B Infect Dis Vet Public Health* 2002, **49**(1):7–16.
2. Haig DM: **Orf virus infection and host immunity.** *Curr Opin Infect Dis* 2006, **19**(2):127–131.
3. Seet BT, Johnston JB, Brunetti CR, Barrett JW, Everett H, Cameron C, Sypula J, Nazarian SH, Lucas A, McFadden G: **Poxviruses and immune evasion.** *Annu Rev Immunol* 2003, **21**:377–423.
4. Fleming SB, McCaughan CA, Andrews AE, Nash AD, Mercer AA: **A homolog of interleukin-10 is encoded by the poxvirus orf virus.** *J Virol* 1997, **71**(6):4857–4861.
5. McInnes CJ, Wood AR, Mercer AA: **Orf virus encodes a homolog of the vaccinia virus interferon-resistance gene E3L.** *Virus Genes* 1998, **17**(2):107–115.
6. Deane D, McInnes CJ, Percival A, Wood A, Thomson J, Lear A, Gilray J, Fleming S, Mercer A, Haig D: **Orf virus encodes a novel secreted protein inhibitor of granulocyte-macrophage colony-stimulating factor and interleukin-2.** *J Virol* 2000, **74**(3):1313–1320.
7. Imlach W, McCaughan CA, Mercer AA, Haig D, Fleming SB: **Orf virus-encoded interleukin-10 stimulates the proliferation of murine mast cells and inhibits cytokine synthesis in murine peritoneal macrophages.** *J Gen Virol* 2002, **83**(Pt 5):1049–1058.
8. Lateef Z, Baird MA, Wise LM, Young S, Mercer AA, Fleming SB: **The chemokine-binding protein encoded by the poxvirus orf virus inhibits recruitment of dendritic cells to sites of skin inflammation and migration to peripheral lymph nodes.** *Cell Microbiol* 2010, **12**(5):665–676.
9. Abendroth A, Arvin A: **Immune evasion mechanisms of varicella-zoster virus.** *Arch Virol Suppl* 2001, **17**:99–107.
10. Williams DB, Vassilakos A, Suh WK: **Peptide presentation by MHC class I molecules.** *Trends Cell Biol* 1996, **6**(7):267–273.
11. Ambagala AP, Solheim JC, Srikumaran S: **Viral interference with MHC class I antigen presentation pathway: the battle continues.** *Vet Immunol Immunopathol* 2005, **107**(1–2):1–15.
12. Hewitt EW: **The MHC class I antigen presentation pathway: strategies for viral immune evasion.** *Immunology* 2003, **110**(2):163–169.
13. Heemels MT, Ploegh H: **Generation, translocation, and presentation of MHC class I-restricted peptides.** *Annu Rev Biochem* 1995, **64**:463–491.
14. Jonjic S, Babic M, Polic B, Krmpotic A: **Immune evasion of natural killer cells by viruses.** *Curr Opin Immunol* 2008, **20**(1):30–38.
15. Zuniga MC, Wang H, Barry M, McFadden G: **Endosomal/lysosomal retention and degradation of major histocompatibility complex class I molecules is induced by myxoma virus.** *Virology* 1999, **261**(2):180–192.
16. Byun M, Wang X, Pak M, Hansen TH, Yokoyama WM: **Cowpox virus exploits the endoplasmic reticulum retention pathway to inhibit MHC class I transport to the cell surface.** *Cell Host Microbe* 2007, **2**(5):306–315.
17. Dasgupta A, Hammarlund E, Slifka MK, Fruh K: **Cowpox virus evades CTL recognition and inhibits the intracellular transport of MHC class I molecules.** *J Immunol* 2007, **178**(3):1654–1661.
18. Byun M, Verweij MC, Pickup DJ, Wiertz EJ, Hansen TH, Yokoyama WM: **Two mechanistically distinct immune evasion proteins of cowpox virus combine to avoid antiviral CD8 T cells.** *Cell Host Microbe* 2009, **6**(5):422–432.
19. Alzhanova D, Edwards DM, Hammarlund E, Scholz IG, Horst D, Wagner MJ, Upton C, Wiertz EJ, Slifka MK, Fruh K: **Cowpox virus inhibits the transporter associated with antigen processing to evade T cell recognition.** *Cell Host Microbe* 2009, **6**(5):433–445.
20. Traub LM, Bannykh SI, Rodel JE, Aridor M, Balch WE, Kornfeld S: **AP-2-containing clathrin coats assemble on mature lysosomes.** *J Cell Biol* 1996, **135**(6 Pt 2):1801–1814.
21. Rabouille C, Klumperman J: **Opinion: The maturing role of COPI vesicles in intra-Golgi transport.** *Nat Rev Mol Cell Biol* 2005, **6**(10):812–817.
22. Lowe M, Kreis TE: **In vivo assembly of coatomer, the COP-I coat precursor.** *J Biol Chem* 1996, **271**(48):30725–30730.
23. Abendroth A, Lin I, Slobedman B, Ploegh H, Arvin AM: **Varicella-zoster virus retains major histocompatibility complex class I proteins in the Golgi compartment of infected cells.** *J Virol* 2001, **75**(10):4878–4888.
24. Campadelli G, Brandimarti R, Di Lazzaro C, Ward PL, Roizman B, Torrisi MR: **Fragmentation and dispersal of Golgi proteins and redistribution of glycoproteins and glycolipids processed through the Golgi apparatus after infection with herpes simplex virus 1.** *Proc Natl Acad Sci U S A* 1993, **90**(7):2798–2802.
25. Quiner CA, Jackson WT: **Fragmentation of the Golgi apparatus provides replication membranes for human rhinovirus 1A.** *Virology* 2010, **407**(2):185–195.
26. Sandoval IV, Carrasco L: **Poliovirus infection and expression of the poliovirus protein 2B provoke the disassembly of the Golgi complex, the organelle target for the antipoliovirus drug Ro-090179.** *J Virol* 1997, **71**(6):4679–4693.
27. Beske O, Reichelt M, Taylor MP, Kirkegaard K, Andino R: **Poliovirus infection blocks ERGIC-to-Golgi trafficking and induces microtubule-dependent disruption of the Golgi complex.** *J Cell Sci* 2007, **120**(Pt 18):3207–3218.
28. Schmelz M, Sodeik B, Ericsson M, Wolffe EJ, Shida H, Hiller G, Griffiths G: **Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network.** *J Virol* 1994, **68**(1):130–147.
29. Tan JL, Ueda N, Mercer AA, Fleming SB: **Investigation of orf virus structure and morphogenesis using recombinants expressing FLAG-tagged envelope structural proteins: evidence for wrapped virus particles and egress from infected cells.** *J Gen Virol* 2009, **90**(Pt 3):614–625.
30. Ashrafi GH, Haghshenas MR, Marchetti B, O'Brien PM, Campo MS: **E5 protein of human papillomavirus type 16 selectively downregulates surface HLA class I.** *Int J Cancer* 2005, **113**(2):276–283.
31. Girod A, Storrie B, Simpson JC, Johannes L, Goud B, Roberts LM, Lord JM, Nilsson T, Pepperkok R: **Evidence for a COP-I-independent transport route from the Golgi complex to the endoplasmic reticulum.** *Nat Cell Biol* 1999, **1**(7):423–430.
32. Kirchhausen T: **Three ways to make a vesicle.** *Nat Rev Mol Cell Biol* 2000, **1**(3):187–198.
33. Pierini R, Cottam E, Roberts R, Wileman T: **Modulation of membrane traffic between endoplasmic reticulum, ERGIC and Golgi to generate compartments for the replication of bacteria and viruses.** *Semin Cell Dev Biol* 2009, **20**(7):828–833.
34. Zhang L, Lee SY, Beznoussenko GV, Peters PJ, Yang JS, Gilbert HY, Brass AL, Elledge SJ, Isaacs SN, Moss B, *et al*: **A role for the host coatomer and KDEL receptor in early vaccinia biogenesis.** *Proc Natl Acad Sci U S A* 2009, **106**(1):163–168.
35. Yilla M, Tan A, Ito K, Miwa K, Ploegh HL: **Involvement of the vacuolar H(+)-ATPases in the secretory pathway of HepG2 cells.** *J Biol Chem* 1993, **268**(25):19092–19100.
36. Fischer T, Planz O, Stitz L, Rziha HJ: **Novel recombinant parapoxvirus vectors induce protective humoral and cellular immunity against lethal herpesvirus challenge infection in mice.** *J Virol* 2003, **77**(17):9312–9323.
37. Brodsky FM, Parham P: **Evolution of HLA antigenic determinants: species cross-reactions of monoclonal antibodies.** *Immunogenetics* 1982, **15**(2):151–166.
38. Housawi FM, Roberts GM, Gilray JA, Pow I, Reid HW, Nettleton PF, Sumption KJ, Hibma MH, Mercer AA: **The reactivity of monoclonal antibodies against orf virus with other parapoxviruses and the identification of a 39 kDa immunodominant protein.** *Arch Virol* 1998, **143**(12):2289–2303.
39. Fischer T, Büttner M, Rziha HJ: **T helper 1-type cytokine transcription in peripheral blood mononuclear cells of pseudorabies virus (Suid herpesvirus 1)-primed swine indicates efficient immunization.** *Immunology* 2000, **101**(3):378–387.
40. Ashrafi GH, Tsirimonaki E, Marchetti B, O'Brien PM, Sibbet GJ, Andrew L, Campo MS: **Down-regulation of MHC class I by bovine papillomavirus E5 oncoproteins.** *Oncogene* 2002, **21**(2):248–259.
41. Henkel M, Planz O, Fischer T, Stitz L, Rziha HJ: **Prevention of virus persistence and protection against immunopathology after Borna disease virus infection of the brain by a novel Orf virus recombinant.** *J Virol* 2005, **79**(1):314–325.

doi:10.1186/1746-6148-8-114
Cite this article as: Rohde *et al.*: Orf virus interferes with MHC class I surface expression by targeting vesicular transport and Golgi. *BMC Veterinary Research* 2012 **8**:114.