Virology 468-470 (2014) 283–292

Contents lists available at ScienceDirect

Virology

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

Poly ICLC increases the potency of a replication-defective human adenovirus vectored foot-and-mouth disease vaccine

Fayna Diaz-San Segundo [a,1], Camila C. Dias [a,b,1], Mauro P. Moraes [a,c,2], Marcelo Weiss [a,b], Eva Perez-Martin [a,b], Andres M. Salazar [d], Marvin J. Grubman [a,*], Teresa de los Santos [a,*]

[a] Plum Island Animal Disease Center, North Atlantic Area, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 848, Greenport, NY 11944, United States

[b] Oak Ridge Institute for Science and Education, PIADC Research Participation Program, Oak Ridge, TN 37831, United States

[c] Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT 06269, United States

[d] Oncovir, Inc., Washington, DC 20008, United States

ARTICLE INFO

Article history:
Received 27 June 2014
Returned to author for revisions
15 July 2014
Accepted 14 August 2014
Available online 12 September 2014

Keywords:
Foot-and-mouth disease
Foot-and-mouth disease virus
Replication-defective adenovirus
Vaccines
Adjuvants
Neutralizing antibodies
Cell-mediated immunity
Poly ICLC

ABSTRACT

Foot-and-mouth disease virus (FMDV) causes a highly contagious disease of cloven-hoofed animals. We have previously demonstrated that a replication-defective human adenovirus 5 vector carrying the FMDV capsid coding region of serotype A24 Cruzeiro (Ad5-CI-A24-2B) protects swine and cattle against FMDV challenge by 7 days post-vaccination. However, since relatively large amounts of Ad5-CI-A24-2B are required to induce protection this strategy could be costly for livestock production. Poly ICLC is a synthetic double stranded RNA that activates multiple innate and adaptive immune pathways. In this study, we have tested for the first time, the adjuvant effect of poly ICLC in combination with Ad5-CI-A24-2B in swine. We found that the combination resulted in a reduction of the vaccine protective dose by 80-fold. Interestingly, the lowest dose of Ad5-CI-A24-2B plus 1 mg of poly ICLC protected animals against challenge even in the absence of detectable FMDV-specific neutralizing antibodies at the time of challenge.

Published by Elsevier Inc.

Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals that can have a significant economic impact on both FMD-free countries and countries in which the disease is enzootic. While FMD does not result in high mortality it can cause significant morbidity effecting animal growth, milk production, etc. The disease results in well characterized clinical signs consisting of fever, lameness and vesicular lesions in the mouth, tongue, nose, feet and teats (Grubman and Baxt, 2004). Since FMD is a "listed disease" by the World Organization of Animal Health (OIE) (OIE, 2004), FMD-free OIE member countries impose trade restrictions on susceptible animals and their products from countries in which the disease is present, resulting in considerable economic and social consequences.

The etiologic agent, FMD virus (FMDV), that belongs to the *Aphthovirus* genus, family *Picornaviridae*, is antigenically variable and consists of 7 serotypes and multiple subtypes and strains within each serotype (Grubman and Baxt, 2004). The virus contains a single-stranded positive-sense RNA genome surrounded by an icosahedral capsid composed of 60 copies each of 4 structural proteins. The viral genome codes for the 4 structural proteins (VP1–VP4) as well as 10 nonstructural (NS) proteins required at different stages of viral replication (Grubman and Baxt, 2004; Mason et al., 2003).

FMD outbreaks are controlled by restriction of animal movement, decontamination of infected premises, and slaughter of infected and in-contact animals. Vaccination with an inactivated whole virus vaccine is also used in enzootic countries. However, vaccination is often not used in previously FMD-free countries since current OIE regulations favor slaughter or vaccination followed by slaughter, rather than only vaccination as the approach to most rapidly regain FMD-free status and thus resume international trade (Grubman and Baxt, 2004).

While the current inactivated vaccine has been successfully used to eliminate FMD from Western Europe and parts of South America, there are a number of limitations in its use including difficulty in distinguishing infected from vaccinated animals

* Corresponding authors. Fax: +1 631 323 3006.
E-mail addresses: marvin.grubman@ars.usda.gov (M.J. Grubman), teresa.delossantos@ars.usda.gov (T. de los Santos).

[1] These authors contributed equally to this publication.

[2] Current address: Ceva Biomune, 8906 Rosehill Road, Shawnee Mission, KS, United States.

http://dx.doi.org/10.1016/j.virol.2014.08.012
0042-6822/Published by Elsevier Inc.

(DIVA), the requirement of an expensive high-containment facility for vaccine production, and the possibility of escape of live or inadequately inactivated virus from production facilities. As a result investigators have attempted to develop alternative vaccine approaches (Mason and Grubman, 2008). The use of replication-defective human adenovirus (Ad5-) to deliver FMD vaccines has been reported by different groups. Du et al. (2007, 2008a, 2008b) have tested Ad5 vectors expressing multiple VP1 epitopes combined with immunomodulatory molecules. However, several boosts were required to achieve complete protection, even in mice. On the other hand, we have successfully used a similar approach to deliver FMDV empty capsids (Ad5-FMD) and effectively addressed most of the concerns of the inactivated vaccine in the FMDV natural host. Our Ad5-FMD vaccine contains the FMDV capsid-coding region, P1-2A, as well as the coding region for the viral protease $3C^{pro}$, that is required for processing P1-2A into the individual capsid proteins (Mayr et al., 1999, 2001). One inoculation of this first generation Ad5-FMD vaccine resulted in complete protection from challenge of swine and cattle as early as 7 days post-vaccination (Moraes et al., 2002; Pacheco et al., 2005). More recently in collaboration with the Department of Homeland Security, and a partner company, GenVec, Inc., this platform has been further developed for production purposes (Grubman et al., 2010) and in June of 2012 the Center for Veterinary Biologics, Animal Plant and Health Inspection Service, USDA, granted a conditional license for use of the Ad5-FMDV serotype A24 Cruzeiro vaccine in cattle in an emergency situation (Grubman et al., 2012).

One of the limitations of the Ad5-FMD approach is that it requires a relatively high protective dose which is problematic for utilization as a veterinary vaccine. In an effort to address this concern, we have evaluated several approaches. We constructed a second-generation Ad5-FMD vaccine (Ad5-CI-A24-2B) which contained the P1-2A coding region of FMDV A24 and incorporated the full length 2B coding region of the same serotype, in addition to the partial 3B and full length $3C^{pro}$ coding regions of FMDV A12 present in our first-generation vaccine (Pena et al., 2008). The 2B protein is involved in the rearrangement of cellular membranes that act as sites for picornavirus replication and capsid assembly (Aldabe and Carrasco, 1995; Cho et al., 1994; Suhy et al., 2000; de Jong et al., 2002; Monaghan et al., 2004; Buenz and Howe, 2006; Moffat et al., 2005; García-Briones et al., 2006). Our hypothesis was that creation of an intracellular milieu that resembled natural infection would enhance capsid formation and stability. Inoculation of swine or cattle with this vector containing the full length 2B region enhanced vaccine efficacy and induced a more robust cell-mediated immune response (Pena et al., 2008; Moraes et al., 2011). More recently we showed that subcutaneous (sc) inoculation at 2 sites on either side of the neck compared to intramuscular inoculation (im) at one site resulted in at least an additional 25-fold dose-sparing (protective at 2×10^8 pfu/animal as compared to 5×10^9 pfu/animal) (Grubman et al., 2012). These results indicated that there is a strict correlation between the vaccine dose, route of administration and induced protection, as previously reported for other Ad vector-based vaccines (Tatsis and Ertl, 2004).

An additional approach to enhance the immune response induced by a vaccine is the inclusion of an adjuvant in the vaccine formulation. Adjuvants can significantly enhance the maturation of antigen-presenting dendritic cells (DCs) and the antigen specific cellular response (Pulendran and Ahmed, 2006; Kool et al., 2008). Poly ICLC is a synthetic, double-stranded polyriboinosinic-polyribocytidylic acid molecule stabilized with poly-L-lysine and carboxymethyl cellulose which has enhanced biostability in animals, as compared to poly IC (Nordlund et al., 1970) and is a known toll-like receptor 3 (TLR3) and MDA-5 agonist that can activate multiple innate immune pathways (Meylan and Tschopp, 2006; Stahl-Hennig et al., 2009). Moreover, poly IC enhances DC maturation and B cell activation, leading to induction of a potent adaptive immune response when combined with protein antigens (Stahl-Hennig et al., 2009; Flynn et al., 2011) and also potentiates differentiation of effector $CD8^+$ T cells (Wiltrout et al., 1985; Ngoi et al., 2008). In rodents and primates, poly ICLC is a strong interferon alpha (IFN-α) inducer and provides antiviral and adjuvant activity (Levy et al., 1975; Harrington et al., 1979; Caskey et al., 2011; Tenbusch et al., 2012). In addition, treatment with poly IC has been effective in providing protection in animal models against a number of viral infections (Houston et al., 1976; Stephen et al., 1977, 1979; Baer et al., 1979; Kende, 1985; Kende et al., 1987; Stahl-Hennig et al., 2009; Li et al., 2011; Levy et al., 1976), as well as inducing rapid innate protection against FMDV in swine (Dias et al., 2012). Furthermore, it has been recently shown that poly IC has an adjuvant effect when combined with an FMD multiepitope protein or inactivated FMD vaccines (Cao et al., 2012, 2013, 2014; Zhou et al., 2014).

In this study we examined the effectiveness of poly ICLC when combined with our second-generation Ad5-CI-A24-2B vaccine delivered sc at 2 sites. We demonstrate that vaccination with the combination reduces the vaccine protective dose approximately 80-fold as compared to administration of vaccine alone. Interestingly, the enhanced efficacy of the combination approach correlated with a stronger antigen specific cell-mediated immune response.

Results

Poly ICLC has been used as an adjuvant in some animal models, but its use in vaccine formulations to immunize large animals has never been described. We performed a preliminary study to estimate the dose of poly ICLC required to induce an adjuvant effect when combined with an Ad5-FMD vaccine (experiment #1). We compared the effect of combining 1 or 4 mg poly ICLC with a suboptimal dose of Ad5-CI-A24-2B vaccine (4×10^7 pfu/animal). Twenty-one days postvaccination (dpv) animals were challenged. All the control animals (Ad5-Blue vaccinated) developed severe disease by 2 days postchallenge (dpc) with clinical scores of 16–17 (maximum score possible is 17). As expected, the three animals treated with a dose of 2×10^8 pfu/animal of Ad5-CI-A24-2B were fully protected with no detectable viremia, virus shedding, or viral RNA in serum (Table 1). In contrast, a reduction in the vaccine dose to 4×10^7 pfu/animal, only induced partial protection and all three animals developed clinical signs between 3–6 dpc, but with reduced severity (clinical score 1–3). Only viral RNA was detected in 2 of the 3 animals by RT-PCR, a more sensitive method than conventional virus detection. Interestingly, a combination of 4×10^7 pfu/animal vaccine with 1 or 4 mg of poly ICLC resulted in enhanced protection. One animal in each group was fully protected and the other 2 developed milder disease as compared to the group inoculated with vaccine alone. Only one out of the six animals had detectable viral RNA. No significant differences were observed in the clinical or antibody responses. Based on these results we decided to use 1 mg of poly ICLC to further examine its effectiveness in enhancing the efficacy of the Ad5-CI-A24-2B vaccine.

Poly ICLC improves humoral immune response induced by Ad5-CI-A24-2B vaccine

Following the preliminary study, we performed another experiment (experiment #2) and compared the efficacy of a 20-fold lower vaccine dose in the presence or absence of 1 mg poly ICLC (Table 2). Four groups of 3 animals each were vaccinated with either 2×10^8 pfu Ad5-Blue vector control, 2×10^8 pfu Ad5-CI-A24-2B, or 1×10^7 pfu

Table 1
Clinical performance of swine immunized with varying doses of Ad5-CI-A24-2B in the absence or presence of poly ICLC (experiment #1).

Vaccine	Dose[a] (pfu)	Adjuvant[b]	Pig #	Clinical score[c]	Viremia[d]	Shedding virus[e]	SN[f]	RT-PCR[g]
Ad5-Blue	2×10^8	–	29	2/16	$2/1.0 \times 10^5/2$	$2/8.2 \times 10^1/2$	$< 0.3/3.0$	SP
			30	2/17	$2/4.0 \times 10^5/3$	$2/5.6 \times 10^2/2$	$< 0.3/3.0$	SP
			31	2/17	$2/2.7 \times 10^5/2$	$2/8.2 \times 10^2/2$	$< 0.3/3.3$	SP
Ad5-CI-A24-2B	2×10^8	–	12	0	0	0	1.2/2.4	N
			13	0	0	0	1.2/2.1	N
			14	0	0	0	1.2/2.1	N
Ad5-CI-A24-2B	4×10^7	–	26	3/3	0	0	1.2/3.6	WP
			27	3/2	0	0	1.2/4.2	N
			28	6/1	0	0	0.9/3.6	WP
Ad5-CI-A24-2B	4×10^7	pICLC 1 mg	23	0	0	0	1.5/3.0	N
			24	6/1	0	0	1.2/3.9	N
			25	6/1	0	0	1.2/3.3	N
Ad5-CI-A24-2B	4×10^7	pICLC 4 mg	19	6/3	0	0	$< 0.3/3.3$	N
			20	5/2	0	0	1.2/3.3	WP
			21	0	0	0		

[a] Dose of inoculum per animal expressed as number of pfu in a total volume of 2 ml.
[b] Dose of adjuvant per animal in a total volume of 2 ml.
[c] dpc first signs of lesions are detected/highest lesion score achieved throughout the entire experiment.
[d] First day post-challenge (dpc) that viremia was detected; maximum amount of viremia in pfu/ml detected in sera samples; and the duration (days) of viremia.
[e] First dpc that shedding virus was detected; maximum amount of shedding virus in pfu/ml detected in nasal swab samples; and the duration (days) of shedding.
[f] SN=serum neutralizing antibody response reported as Log $TCID_{50}$ at 0 and 21 dpc, respectively.
[g] RT-PCR, 1–7 dpc sera tested, N=negative, Ct=40; WP=weak positive, $35 \leq Ct < 40$; SP=strong positive, $Ct < 35$.

Table 2
Clinical performance of swine immunized with varying doses of Ad5-CI-A24-2B in the absence or presence of poly ICLC (experiment #2).

Vaccine	Dose[a] (pfu)	Poly ICLC[b]	Pig #	Clinical score[c]	Viremia[d]	Shedding virus[e]	SN[f]	3ABC ELISA[g]	RT-PCR[h]	3D (RIP)[i]
Ad5-Blue	2×10^8	–	16	2/15	$1/6.0 \times 10^6/4$	$2/1.6 \times 10^4/4$	$< 0.3/2.7$	SP	SP	SP
			17	2/13	$1/6.3 \times 10^6/3$	$2/1.4 \times 10^3/2$	$< 03/D$[j]	D	SP	D
			18	2/17	$1/6.7 \times 10^6/3$	$2/7.8 \times 10^4/2$	$< 0.3/D$	D	SP	D
Ad5-CI-A24-2B	2×10^8	–	1	0	0	0	1.8/2.1	N	N	N
			2	0	0	0	2.7/3.3	SP	N	N
			3	0	0	0	2.1/2.4	N	N	N
Ad5-CI-A24-2B	1×10^7	–	13	4/2	0	$3/1.1 \times 10^3/4$	1.8/2.7	WP	SP	SP
			14	3/17	$4/5.5 \times 10^2/1$	$2/6.5 \times 10^2/4$	$< 0.3/2.7$	SP	SP	WP
			15	3/15	$4/3.0 \times 10^2/1$	$3/1.3 \times 10^3/2$	1.2/3.0	SP	SP	SP
Ad5-CI-A24-2B	1×10^7	1 mg	10	0	0	0	1.5/2.4	N	N	N
			11	0	0	0	1.5/2.4	N	N	N
			12	0	0	0				

[a] Dose of inoculum per animal expressed as number of pfu in a total volume of 2 ml.
[b] Dose of adjuvant per animal in a total volume of 2 ml.
[c] dpc first signs of lesions are detected /highest lesion score achieved throughout the entire experiment.
[d] First day post-challenge (dpc) that viremia was detected; maximum amount of viremia in pfu/ml detected in sera samples; and the duration (days) of viremia.
[e] First dpc that shedding virus was detected; maximum amount of shedding virus in pfu/ml detected in nasal swab samples; and the duration (days) of shedding.
[f] SN=serum neutralizing antibody response reported as Log $TCID_{50}$ at 0 and 21 dpc, respectively.
[g] 3ABC ELISA, 21 dpc sera tested, N=negative, 0–45; WP=weak positive, 45–55; SP=strong positive, > 55.
[h] RT-PCR, 1–7 dpc sera tested, N=negative, Ct=40; WP=weak positive, $35 \leq Ct < 40$; SP=strong positive, $Ct < 35$.
[i] Detection of serum antibodies against FMDV 3D protein tested by radioimmunoprecipitation at 21 dpc sera tested, N=negative; WP=weak positive; P=positive; SP=strong positive.
[j] D=animal #s 17 and 18 died on 4 dpc from FMD related causes.

Ad5-CI-A24-2B in the presence or absence of 1 mg poly ICLC, followed by challenge with FMDV at 21 dpv.

In the group inoculated with 2×10^8 pfu of the Ad5-CI-A24-2B vaccine, all animals developed serum neutralizing (SN) antibodies starting at 7 dpv with a peak at 14 dpv (Fig. 1; Table 2). Reducing the vaccine dose to 1×10^7 pfu resulted in significantly lower levels of SN antibodies that were only detected by 21 dpv, and one animal, #14, did not show any response before challenge (Table 2). In contrast, animals vaccinated with the combination of the low dose vaccine and poly ICLC developed SN antibodies that were detected starting at 7 dpv with a maximum at 14 dpv that was statistically significant ($P < 0.05$), suggesting that poly ICLC had an adjuvant effect on the humoral immune response (Fig. 1). Nevertheless, the level of SN antibodies prior to challenge in these animals was lower than in the pigs inoculated with the highest dose (2×10^8 pfu) of vaccine alone.

All animals in the control group developed viremia by 1 dpc lasting for 3–4 days, while clinical disease was evident by 2 dpc (Fig. 2A; Table 2). None of the animals in the high dose group (2×10^8 pfu) developed clinical disease or had virus in serum or nasal swabs, as assayed by either virus isolation or viral RNA by rRT-PCR (Fig. 2B; Table 2). With the exception of one animal (#2), which had antibodies against NS proteins by the 3ABC ELISA, no challenge-virus replication was detected in this group. A slight increase in the levels of SN antibodies was detected in the three animals in this group after challenge (Table 2; Fig. 1). All animals that received only the low vaccine dose (1×10^7 pfu) developed clinical disease (Fig. 2C), but one animal (#13) had a low clinical score (Table 2). Furthermore, all animals in this group were positive in all the assays that evaluated challenge-virus replication including detection in serum of antibodies against FMDV NS proteins (3ABC and radioimmunoprecipitation [RIP]) and increase in the SN antibody titer. In contrast, all the animals treated with the low vaccine dose (1×10^7 pfu) in combination with poly ICLC were clinically protected (Fig. 2D) and did not show signs of

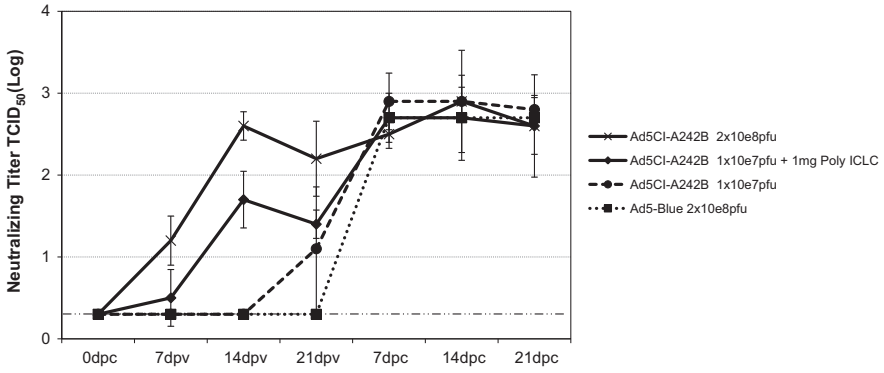

Fig. 1. Kinetics of neutralizing antibodies against FMDV A24 in pigs vaccinated with different doses of Ad5 vectors with or without poly ICLC and challenged at 21 dpv (experiment #2). Serum samples were collected weekly following vaccination and challenge. Titers are expressed as the inverse dilution of serum yielding a 50% reduction of virus titer (Log_{10} $TCID_{50}$/ml). Dashed line represents limit of detection (values ≤ 0.3). Each data point represents the mean ($\pm$SD) of each group.

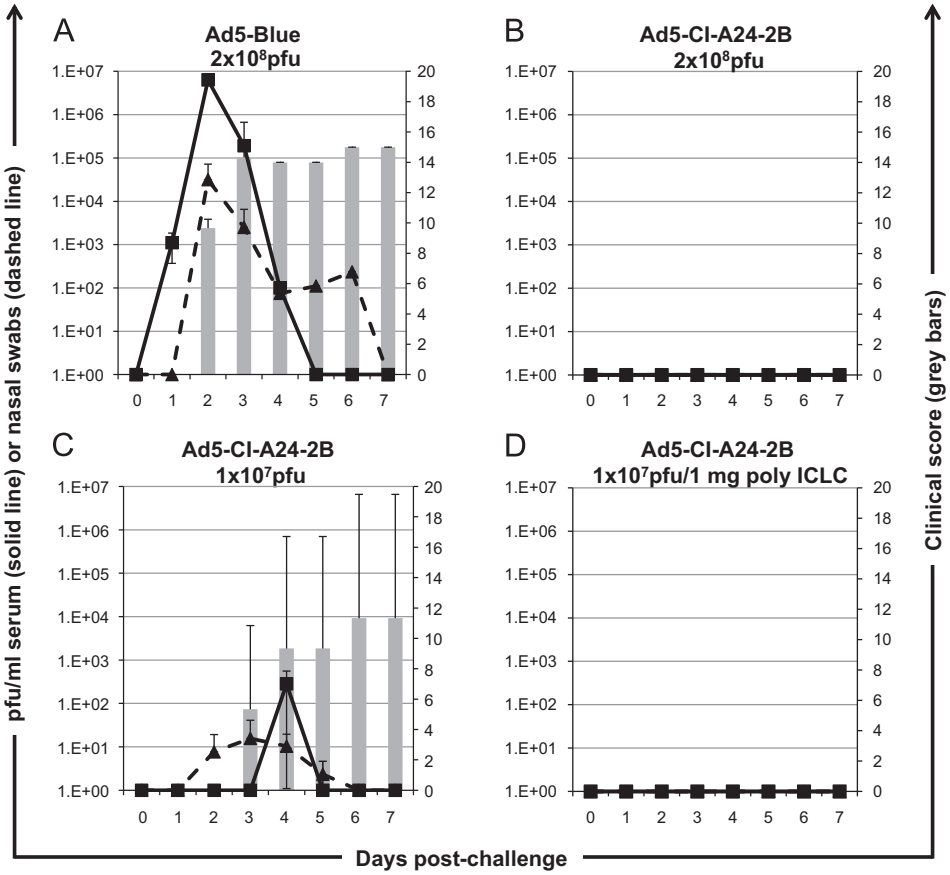

Fig. 2. Clinical outcome of pigs vaccinated with different doses of Ad5 vectors with or without poly ICLC and challenged at 21 dpv (experiment #2). Clinical signs (bars), the presence of virus in serum (solid lines) and nasal swabs (dashed lines) were monitored daily for 7 dpc. Clinical score is expressed as number of toes showing lesions plus one more point scored when lesions were present in either the mouth or snout or both (maximum score is 17). Virus levels are expressed as the number of plaque forming units per ml (pfu/ml) of serum or media (in which the swabs were collected). Each data point represents the mean ($\pm$SD) of each group.

challenge-virus replication (Table 2). Interestingly, an increase in the level of SN antibodies was detected after challenge of this group, to levels similar to the protected animals treated with the high dose of vaccine (Fig. 1).

Use of poly ICLC allows for an 80-fold dose sparing in the protective Ad5-CI-A24-2B vaccine dose

Since the addition of poly ICLC allowed us to lower the protective dose of vaccine by 20-fold (from 2×10^8 to 1×10^7 pfu), we next used a 4-fold lower vaccine dose to test the limits of efficacy of the combination of vaccine and poly ICLC. In experiment #3, 4 groups of 3 animals/group were vaccinated with 1×10^7 pfu Ad5-Blue, 1×10^7 pfu Ad5-CI-A24-2B plus 1 mg poly ICLC, 1×10^7 pfu Ad5-CI-A24-2B plus 0.5 mg poly ICLC, 2.5×10^6 pfu Ad5-CI-A24-2B plus 1 mg poly ICLC and challenged 21 dpv. Two additional groups were vaccinated with 1×10^7 pfu Ad5-CI-A24-2B plus 1 mg poly ICLC or 2.5×10^6 pfu Ad5-CI-A24-2B plus 1 mg poly ICLC and challenged at 7 dpv (Table 3).

All animals vaccinated with 1×10^7 pfu of Ad5-CI-A24-2B in combination with different amounts of poly ICLC and challenged at 21 dpv developed SN antibodies prior to challenge with a peak at 14 dpv and maintained an SN response at the day of challenge (Fig. 3). In contrast animals vaccinated with a lower dose (2.5×10^6 pfu) and

challenged at 21 dpv did not have a SN antibody response at the time of challenge, even when the vaccine was combined with poly ICLC (Fig. 3; Table 3). Likewise, none of the animals challenged at 7 dpv developed detectable SN antibody response before challenge.

All animals in the control group developed viremia and clinical disease by 2 dpc, and had detectable virus shedding by 3 dpc (Fig. 4A; Table 3). In the group given vaccine at a dose of 1×10^7 pfu with 1 mg poly ICLC and challenged at 21 dpv one animal was clinically and serologically protected, while the other 2 animals developed a delayed and significantly reduced severity of disease in comparison to the control group. In these 2 animals viremia was not detected by virus isolation, but viral RNA and FMDV-specific antibodies were positive (Fig. 4B; Table 3). Animals given the same dose of vaccine and 0.5 mg poly ICLC and also challenged at 21 dpv were completely protected from clinical disease (Fig. 4C. Table 3) and did not show signs of challenge-virus replication (Table 3). Interestingly, when the vaccine dose was further reduced by 4-fold to 2.5×10^6 pfu and was used in combination with 1 mg of poly ICLC, all animals challenged at 21 dpv were completely protected from clinical disease (Fig. 4D), even in the absence of a SN antibody response at the time of challenge (Fig. 3, Table 3). A weak positive viral RNA level was detectable in these animals.

All animals in both groups challenged at 7 dpv showed clinical signs, although there was a delay and reduced severity of disease in the group given 1×10^7 and poly ICLC as compared to the control group (Fig. 4E). Although the group given 2.5×10^6 pfu of vaccine and poly ICLC, had similar, but slightly less severe disease than the control, this group had approximately 10-fold less virus shedding (Fig. 4F).

Poly ICLC enhances the antigen specific T cell response induced by Ad5-CI-A24-2B vaccine

In experiment #3, animals given an 80-fold reduced dose of vaccine (2.5×10^6 pfu) plus poly ICLC and challenged at 21 dpv did

Table 3
Clinical performance of swine immunized with varying doses of Ad5-CI-A24-2B combined with different amounts of poly ICLC and challenged at different days post-vaccination (experiment #3).

Vaccine	Dose[a] (pfu)	pICLC[b]	Pig#	Clinical score[c]	Viremia[d]	Shedding virus[e]	SN[f]	3ABC ELISA[g]	RT-PCR[h]	3D (RIP)[i]
Ad5-Blue challenge 21 dpi	1×10^7	–	31478	2/16	$2/8.5 \times 10^4/2$	$3/4.3 \times 10^2/1$	$< 0.3/2.1$	SP	SP	SP
			31479	2/16	$2/4.7 \times 10^5/2$	$3/4.6 \times 10^2/1$	$< 0.3/1.8$	P	SP	SP
			31480	2/16	$2/9.3 \times 10^4/2$	$3/3.0 \times 10^2/1$	$< 0.3/2.4$	P	SP	SP
Ad5-CI-A24-2B challenge 21 dpi	1×10^7	1 mg	31463	4/3	0	$3/5.0 \times 10^0/1$	$< 0.3/1.8$	SP	WP	SP
			31464	0	0	0	0.9/1.5	N	N	N
			31465	4/3	0	$3/3.3 \times 10^1/1$	0.9/2.1	WP	WP	WP
Ad5-CI-A24-2B challenge 21 dpi	1×10^7	0.5 mg	31466	0	0	0	0.9/1.2	N	N	N
			31467	0	0	0	0.9/1.2	N	N	N
			31468	0	0	0	0.9/1.2	N	N	N
Ad5-CI-A24-2B challenge 7 dpi	1×10^7	1 mg	31469	7/2	0	0	$< 0.3/1.8$	N	WP	P
			31470	4/9	0	0	$< 0.3/1.8$	P	WP	P
			31471	5/6	0	0	$< 0.3/1.8$	WP	WP	SP
Ad5-CI-A24-2B challenge 21 dpi	2.5×10^6	1 mg	31472	0	0	0	$< 0.3/D$[j]	D	D	D
			31473	0	0	0	$< 0.3/1.2$	N	WP	N
			31474	0	0	0	$< 0.3/1.2$	N	WP	N
Ad5-CI-A24-2B challenge 7 dpi	2.5×10^6	1 mg	31475	3/13	$3/8.8 \times 10^3/2$	$3/2.0 \times 10^1/1$	$< 0.3/1.8$	WP	SP	SP
			31476	4/10	0	$3/2.0 \times 10^1/1$	$< 0.3/2.4$	SP	SP	SP
			31477	2/14	$3/1.42 \times 10^5/1$	$3/1.0 \times 10^1/1$	$< 0.3/1.8$	WP	SP	SP

[a] Dose of inoculum per animal expressed as number of pfu in a total volume of 2 ml.
[b] Dose of adjuvant per animal in a total volume of 2 ml.
[c] dpc first signs of lesions are detected /highest lesion score achieved throughout the entire experiment.
[d] First day post-challenge (dpc) that viremia was detected; maximum amount of viremia in pfu/ml detected in sera samples; and the duration (days) of viremia.
[e] First dpc that shedding virus was detected; maximum amount of shedding virus in pfu/ml detected in nasal swab samples; and the duration (days) of shedding.
[f] SN=serum neutralizing antibody response reported as Log $TCID_{50}$ at 0 and 21 dpc, respectively. D=died.
[g] 21 dpc sera tested, N=negative; WP=weak positive; P=positive; SP=strong positive.
[h] RT-PCR, 1–7 dpc sera tested, N=negative Ct=40; WP=weak positive, $35 \leq Ct < 40$; SP=strong positive, $Ct < 35$.
[i] Detection of serum antibodies against FMDV 3D protein tested by radioimmunoprecipitation at 21 dpc sera tested, N=negative; WP=weak positive; P=positive; SP=strong positive.
[j] Pig #31472 died at 3 dpc from other than FMD related causes.

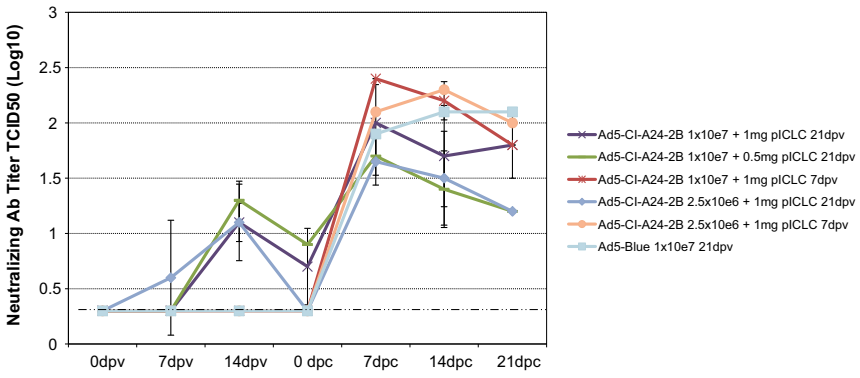

Fig. 3. Kinetics of neutralizing antibodies against FMDV A24 in pigs vaccinated with different doses of Ad5 vectors in combination with poly ICLC and challenged at 7 or 21 dpv (experiment #3). Serum samples were collected weekly following vaccination and challenge. Titers are expressed as the inverse dilution of serum yielding a 50% reduction of virus titer (Log_{10} $TCID_{50}$/ml). Dashed line represents limit of detection (values ≤ 0.3). Each data point represents the mean ($\pm$SD) of each group.

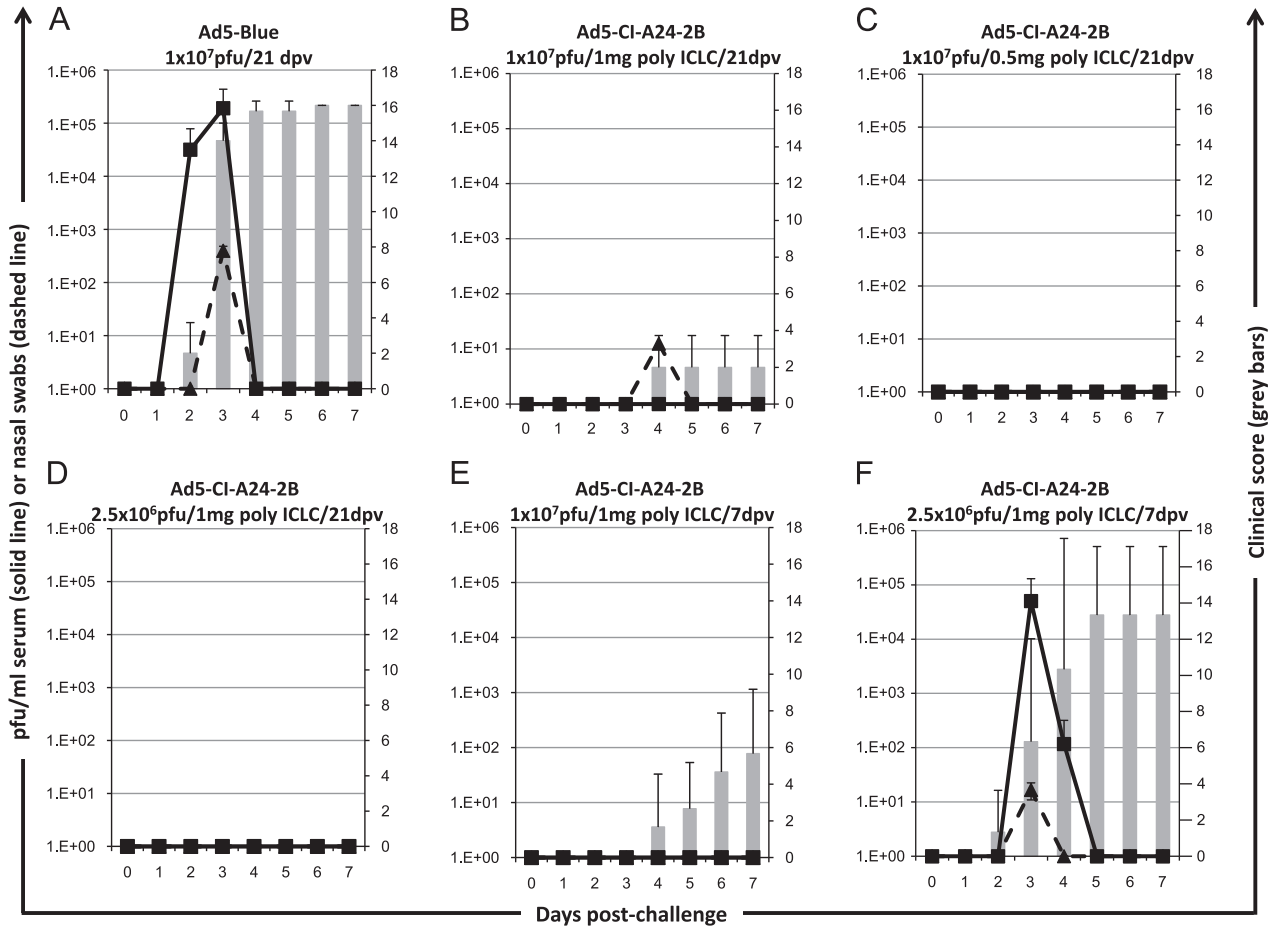

Fig. 4. Clinical outcome of pigs vaccinated with different doses of Ad5 vectors in combination with poly ICLC and challenged at 7 or 21 dpv (experiment #3). Clinical signs (bars), the presence of virus in serum (solid lines) and nasal swabs (dashed lines) were monitored daily for 7 dpc. Clinical score and virus levels are expressed as described in Fig. 2 legend. The error bars represent the variation within the three animals from each group.

not develop a stable SN antibody response, albeit they were protected from clinical disease after challenge. We had previously shown that the Ad5-FMD vaccine, at a dose of 5×10^9 pfu, is able to induce a T cell response in cattle and this response correlates with protection in animals challenged 21 days after vaccination (Moraes et al., 2011). We therefore examined if a T cell response is induced and correlates with protection after vaccination of swine with the Ad5-CI-A24-2B vaccine alone or in combination with poly ICLC. Peripheral blood mononuclear cells (PBMCs) were purified from most of the animals from experiments #2 and #3 at different times post-vaccination and the percentage of specific IFN-γ producing $CD4^+$ and $CD8^+$ cells was determined by FACS analysis (Fig. 5)

In experiment #2 we observed that animals vaccinated with the highest dose of vaccine alone (Ad5-CI-A24-2B; 2×10^8 pfu) showed a specific IFN-γ $CD4^+$ and $CD8^+$ T cell response prior to challenge, with an immediate boost after challenge, and reaching a maximum at 1–3 dpc as compared to the control group (Ad5-Blue; 2×10^8 pfu) (Fig. 5, B vs A). When the dose of vaccine was reduced by 20-fold (Ad5-CI-A24-2B; 1×10^7 pfu), the T cell response was considerably lower than in the group with high dose vaccine, prior to and after challenge (Fig. 5, D vs B), with statistically significant differences for $CD4^+$ cells at days 3 and 5 dpc and for $CD8^+$ cells at days 0 and 3 dpc ($P \leq 0.05$). However, when the same lower dose was used in combination with 1 mg of poly ICLC, the IFN- γ producing $CD8^+$ T cell response increased and was statistically significantly higher as compared with the animals inoculated with the same dose of vaccine without poly ICLC ($P < 0.01$) (Fig. 5, panel C vs D) or with the group inoculated with the highest dose of vaccine ($P < 0.05$, after challenge) (Fig. 5, C vs B). Although the same trend was observed for IFN-γ producing $CD4^+$ T cells, the differences were not statistically significant. Furthermore, analyses of the percentage of the IFN-γ producing $CD4^+$ and $CD8^+$ cells in experiment #3 (Fig. 5, E–H), showed that all animals treated with the Ad5-CI-A24-2B vaccine in combination with poly ICLC had a specific response at 1 dpc which was statistically significant as compared with the Ad5-Blue control group (compare Fig. 5, F–H vs E). However, when these groups were compared with the animals inoculated with 2×10^8 pfu of vaccine alone from experiment #2, the differences were not statistically significant (Fig. 5, F–H vs B), indicating that giving less vaccine in combination with poly ICLC results in a similar T cell response as a higher dose of vaccine alone. In addition, animals inoculated with 2.5×10^6 pfu Ad5-CI-A24-2B plus 1 mg of poly ICLC developed a similar IFN-γ $CD4^+/CD8^+$ specific response even in the absence of a detectable SN antibody response at the time of challenge.

Discussion

To be considered as a practical alternative, any novel FMD vaccine candidate must address the shortcomings of the current inactivated FMD vaccine and in addition, it has to be cost effective. Our Ad5-vectored FMD vaccine only contains a portion of the FMDV genome, and thereby it does not cause disease and can be produced in non-containment BSL2 facilities. Animals vaccinated

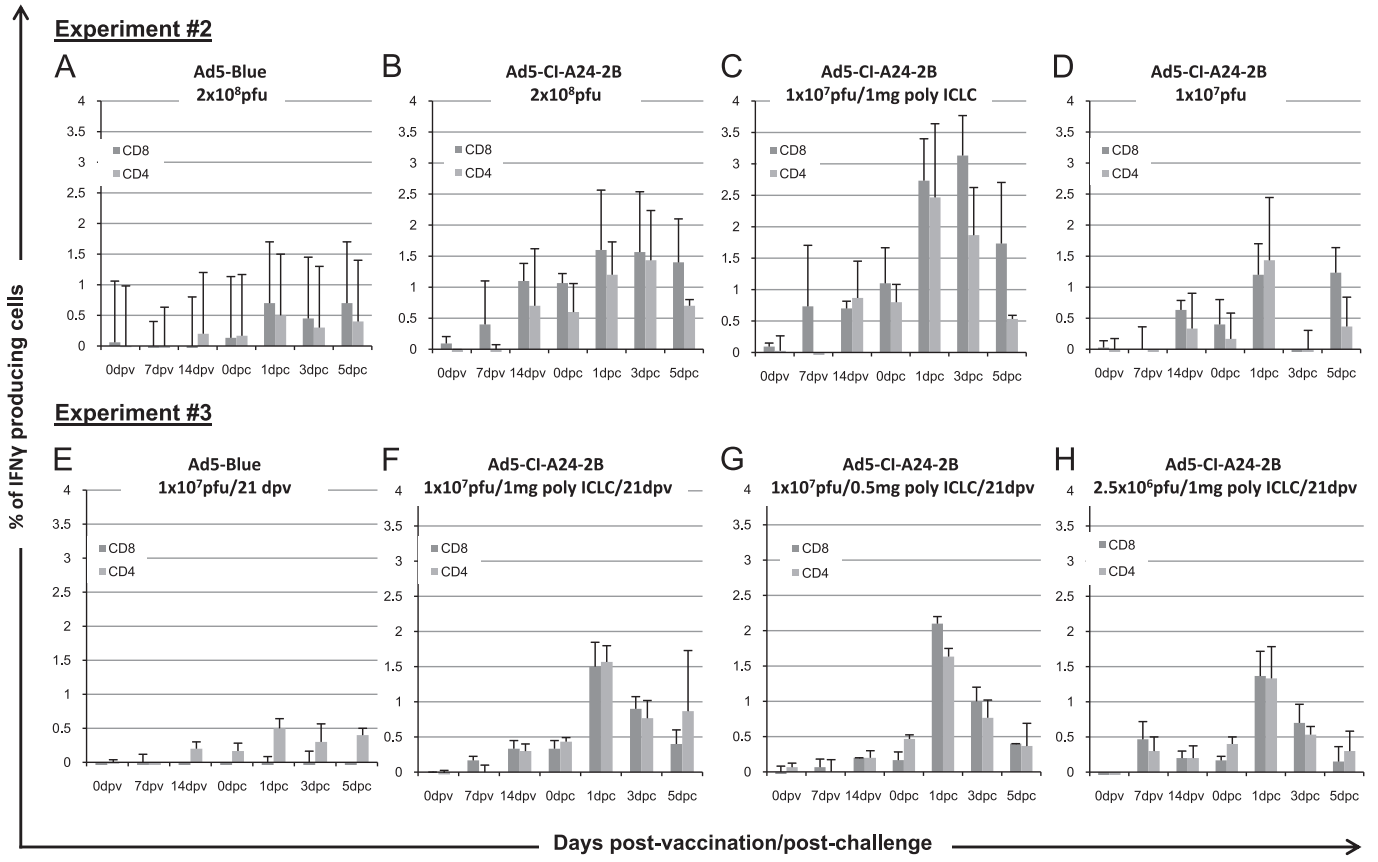

Fig. 5. Cell-mediated immunity induced in pigs vaccinated with different doses of Ad5 vectors with or without poly ICLC and challenged at 21 dpv (experiment #2 [panels A–D] and #3 [panels E–H]). Specific cellular response was measured by intracellular cytokine staining (ICCS). PBMCs from vaccinated and control animals were extracted at different times during vaccination (dpv) and after challenge (dpc) with FMDV A24, stimulated with homologous FMDV A24 ex vivo and the capacity of $CD4^+$ or $CD8^+$ T cells to produce IFN-γ was evaluated by ICCS. The percentage of $CD4^+$ or $CD8^+$ T cells that produce IFN-γ is shown. The error bars represent the variation within the three animals from each group.

with this agent can be unequivocally differentiated from infected animals (DIVA). Furthermore, since the Ad5-FMD vaccine is produced as a cDNA platform there is little concern about antigenic variation arising during the manufacturing process as compared to the current inactivated FMDV vaccine that relies on the viral error-prone RNA polymerase (3D) for replication (Domingo and Holland, 1988).

To address the economics of vaccine production we have evaluated a number of approaches aiming to reduce the vaccine dose required for complete protection. We previously demonstrated that a single dose of our second generation Ad5-CI-A24-2B vaccine can protect swine given 2×10^8 pfu when delivered sc at 2 sites as compared to 5×10^9 pfu when delivered im at 1 site (Grubman et al., 2012), resulting in a 25-fold dose-sparing.

In this study we demonstrated, for the first time, the potential of using poly ICLC to enhance the protective response of a live viral-vectored FMD vaccine. Our results show that addition of poly ICLC to the Ad5-FMD vaccine allows for an additional 80-fold reduction in the vaccine dose required for protection when swine are challenged at 21 dpv.

Despite the animal to animal variation we demonstrated that the addition of poly ICLC to groups given vaccine doses of 1×10^7 or 2.5×10^6 pfu and challenged at 21 dpv resulted in full protection in 9 out of 11 animals showing no clinical disease, no detectable viremia or virus shedding (Tables 2 and 3). The remaining two animals had at least a 10-fold reduction in virus shedding with delayed and significantly reduced clinical signs as compared to the control group. In one experiment (experiment #2) we had observed that a combination of 1×10^7 pfu and 1 mg of poly ICLC was sufficient to protect 3 animals when challenged at 21 dpv. However although the same dose was not fully protective in the next experiment (experiment #3), reduction of the amount of poly ICLC from 1 to 0.5 mg, or the vaccine dose from 1×10^7 to 2.5×10^6 pfu provided the desired protection, but only when used in combination. These results clearly indicated that poly ICLC works as an effective adjuvant of the live Ad5 vectored FMD vaccine. Of particular interest, animals given the lowest dose of vaccine, 2.5×10^6 pfu (experiment #3), did not have a detectable FMDV-specific SN antibody response at the day of challenge albeit they were clinically protected. Noticeably, these animals displayed a $CD4^+/CD8^+$ IFN-γ producing T cell response at 1 dpc as compared to the control group. These results are consistent with previous animal studies demonstrating that poly ICLC can work as an adjuvant enhancing the antigen specific T cell response. Studies comparing different TLR agonists in non-human primates have shown that poly IC was the most efficient in inducing SIV Gag-specific $CD4^+$ Th1 responses in blood and bronchoalveolar lymphocytes with a significant induction of a $CD8^+$ T cell response after boosting; further, animals were completely protected against SIV challenge (Park et al., 2013). Recently, Martins et al. (2014) have reported that a strong antigen specific polyfunctional $CD4^+/CD8^+$ T cell response is induced when a virus-like particle vaccine is used in combination with poly ICLC significantly enhancing protection against Ebola virus infection. Most importantly, and similar to our previous studies in cattle, (Moraes et al., 2011), the results presented here suggest that a $CD4^+/CD8^+$ T cell response

may play an important role in improving the Ad5-FMD elicited protection against FMDV challenge in swine, and the addition of poly ICLC can further boost this response.

Animals challenged at 7 dpv also did not develop a detectable SN antibody response, but had delayed and/or less severe clinical disease as compared to the control group (Table 3). The group inoculated with 1×10^7 pfu vaccine plus poly ICLC had no detectable viremia or virus in nasal swabs and clinical signs were delayed 2–5 days. The group inoculated with a 4-fold lower vaccine dose had more severe disease, but clinical signs and virus shedding were less severe than the control group indicating that even at such a low vaccine dose the presence of poly ICLC ameliorated disease severity as early as 7 dpv.

Two other groups have also recently tested the effectiveness of poly IC as an adjuvant for FMD vaccines. Cao et al. (2013) combined poly IC with a multiple epitope FMD protein vaccine resulting in 100% protection against clinical disease of swine challenged at 28 dpv as compared to 60% protection of swine vaccinated with the vaccine alone. In parallel experiments, the same group evaluated cross-protection in pigs against different topotypes of FMDV serotype O using a multi-epitope subunit vaccine emulsified in oil in combination with poly IC. The animals were clinically protected even when the dose of vaccine was reduced 9-fold (Cao et al., 2014). Furthemore, Zhou et al. (2014) inoculated mice with aluminum hydroxide adjuvanted inactivated FMD vaccine and examined the effect of the addition of either poly IC, or resiquimod – a TLR7 agonist, or both, on the immune response. They found a small immune enhancement with the addition of only poly IC compared to vaccine and aluminum hydroxide, but a significant enhancement when both, poly IC and resiquimod, were added.

Our data clearly indicates that poly ICLC can also work as an adjuvant of a live vectored Ad5-FMD vaccine. Addition of poly ICLC allowed for an Ad5-CI-A24-2B vaccine dose sparing of approximately 2000-fold compared to the dose used in our initial studies with our first generation Ad5-A24 vaccine (Grubman et al., 2010). Poly ICLC is more stable than naked poly IC and resists nucleases that are present in the serum of mammals (Nordlund et al., 1970, Levy et al., 1975). Our demonstration that this compound has an adjuvant effect when administered in combination with the Ad5-FMD vectored vaccine makes this approach more practical, although the cost of poly ICLC needs to be considered. In this regard we found that one swine group given 0.5 mg poly ICLC was as well protected as the group administered with 1 mg poly ICLC (Table 3). Future studies to determine the minimum amount of poly ICLC required to induce protection with a relative low vaccine dose (i.e. 2.5×10^6 pfu), as well as the identification of other adjuvants that could provide the effectiveness of poly ICLC at an economically reasonable cost are within our immediate scope in evaluating countermeasures against FMD.

Materials and methods

Cells and viruses

Human 293 cells (ATTC CRL-1573) from the American Type Culture Collection (ATCC; Rockville, MD) were used to generate, grow and titer all recombinant human Ad5 viruses used in this study (Graham and Prevec, 1991; de Avila Botton et al., 2006). The recombinant Ad5 viruses included: Ad5-Blue, a negative control containing the α gene fragment of β-galactosidase (Moraes et al., 2001) and Ad5-CI-A24-2B, which contains the FMDV A24 P1-2A and full-length 2B coding regions and the 3B (lacking the first 6 amino acids of $3B_1$) and $3C^{pro}$ coding regions of FMDV A12 under the control of the cytomegalovirus promoter/enhancer, intron, and T7 RNA polymerase region from the vector pCI (Promega, Madison, WI) (Pena et al., 2008). All Ad5 vectors were purified by CsCl gradient centrifugation and viral titer determined by standard protocols (de Avila Botton et al., 2006). To measure FMDV transgene expression swine kidney cells, IB-RS-2 cells, were infected with Ad5-CI-A24-2B and cell lysates were examined by Western blot analysis with a polyclonal antibody to detect FMDV structural proteins. Plaque reduction neutralization assays and FMDV titration from blood and nasal swabs samples were performed on BHK-21 cells (ATCC CCL-10). FMDV A24 Cruzeiro used as a challenge virus in this study was collected from the vesicular fluid of an infected swine and titered in IB-RS-2 cells and in pigs and stored in aliquots at −70 °C. Poly ICLC was provided by Oncovir, Inc.

Vaccine trials in swine

Three vaccine experiments were performed in the high-containment facilities of the Plum Island Animal Disease Center (PIADC) under animal protocol number 151-10-R that was approved by the Institutional Animal Care and Use Committee of PIADC (USDA/APHIS/AC Certificate number: 21-F-0001). Animals were acclimated for 5–7 days before the start of the experiments. In all experiments, female Yorkshire swine (five weeks old and weighing approximately 40–50 lbs each) were divided into groups according to the vaccination regimen and each group was housed in separate rooms. All animals were vaccinated sc at two sites on either side of the neck with a total volume of 2 ml of a formulation containing various amounts of Ad5-CI-A24-2B and poly ICLC diluted in PBS, when applicable (Grubman et al., 2012).

In all the experiments the immunogenicity and efficacy of the vector were tested, at varying doses, in the absence or presence of varying amounts of poly ICLC. In experiment #1, 15 pigs were divided into 5 groups. The first 2 groups were inoculated with 2×10^8 pfu/animal Ad5-Blue or Ad5-CI-A24-2B, respectively, while the other three groups were inoculated with 4×10^7 pfu/animal Ad5-CI-A24-2B in the absence or presence of 1 or 4 mg poly ICLC. Twenty-one dpv all pigs were challenged intradermally (id) in the heel bulb in the left rear foot (4 inoculation sites of 100 μl each) with a total of 1×10^5 pfu/animal of FMDV A24 Cruzeiro.

In experiment #2, 12 pigs were divided into 4 groups. The first 2 groups were inoculated with 2×10^8 pfu/animal Ad5-Blue or Ad5-CI-A24-2B, respectively, while the third and fourth groups were inoculated with 1×10^7 pfu/animal Ad5-CI-A24-2B in the absence or presence of 1 mg poly ICLC, respectively. Twenty-one dpv all pigs were challenged as above.

In experiment #3, 18 pigs were divided into 6 groups. The first group was treated with 1×10^7 pfu/animal Ad5-Blue, the second and third groups were treated with 1×10^7 pfu/animal Ad5-CI-A24-2B plus 1 or 0.5 mg poly ICLC, respectively, the fourth group was treated with 1×10^7 pfu/animal Ad5-CI-A24-2B plus 1 mg poly ICLC, and the fifth and sixth groups were treated with 2.5×10^6 pfu/animal Ad5-CI-A24-2B plus 1 mg poly ICLC. Groups 1, 2, 3 and 5 were challenged at 21 dpv and groups 4 and 6 challenged at 7 dpv as above.

Serum samples were collected from all animals weekly until the termination of the experiment (21 dpc), when the animals were humanely euthanized. Serum samples were inactivated at 56 °C for 30 min.

After challenge, animals were examined daily for 7 days and a clinical score was recorded. A final clinical examination was performed at 14 dpc. Rectal temperature data was monitored on a daily basis throughout the experiment. Clinical scores were determined by the number of toes presenting FMD lesions plus the presence of lesions in the snout and/or mouth. The maximum score was 17, and lesions restricted to the site of challenge were not counted.

Nasal swabs and serum samples were taken daily for seven days after challenge to assess virus shedding and viremia, respectively. Heparinized blood was obtained weekly after vaccination and on 1, 3, 5, 7, 14 and 21 dpc to extract PBMCs to study the cellular immune response.

Evaluation of humoral and cellular immune responses

Determination of serum neutralizing (SN) antibody titer

Sera samples were tested for the presence of FMDV-specific SN antibodies by a plaque reduction neutralization assay (PRN) as previously described (Mason et al., 1997). Serum neutralizing antibody titers were reported as the serum dilution yielding a 50% reduction in the number of plaques ($TCID_{50}$) induced by FMDV A24 Cruzeiro in BHK-21 cells.

Flow cytometry analysis

$CD8^+$ and $CD4^+$ IFN-γ-producing cells in total PBMCs were prepared and analyzed by flow cytometry as previously described (Díaz-San Segundo et al., 2012). Expression of cell surface molecules was analyzed using mouse anti-swine CD4-FITC and mouse anti-swine CD8-AF647 (AbD Serotec, Raleigh, NC).

3ABC ELISA assay

Zero and 21 dpc sera were examined for antibodies against FMDV NS protein 3ABC using a PrioCHECK™ FMDV-NS ELISA kit (Prionics AG, Lelystad, Netherlands) (Sorensen et al., 1998).

Radioimmunoprecipitation (RIP) of [^{35}S]methionine/cysteine labeled FMDV A24 infected cell lysates

Sera obtained at 0 and 21 dpc were tested for antibodies against FMDV NS proteins by a RIP assay as previously described (Wu et al., 2003).

Virus titration in blood and nasal secretions

Serum samples and nasal swabs were assayed for the presence of virus by a standard plaque assay on BHK-21 cells (passage levels 60–70) (Moraes et al., 2007). Virus titers were expressed as $\log_{10}$ pfu per ml of blood or nasal swab.

Detection of FMDV RNA by real-time RT-PCR (rRT-PCR)

Serum samples from animals that had no detectable clinical disease were processed for RNA extraction and rRT-PCR as described (Arzt et al., 2010). Samples were considered positive when Ct values were < 40.

Statistical analysis

Statistical significance of differences was evaluated by Student's *t* test. Differences were considered significant for $P < 0.05$.

Acknowledgments

This research was supported in part by the Plum Island Animal Disease Research Participation Program administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Department of Agriculture (USDA) (appointments of Camila C.A. Dias, Marcelo Weiss, and Eva Perez-Martin), by USDA CRIS project 1940-32000-057-00D, (M.J. Grubman and T. de los Santos), and by an interagency agreement between Agricultural Research Service, USDA and the Science and Technology Directorate of the U.S. Department of Homeland Security under awards HSHQPD-07-X-00003 and HSHQDC-09-X-00373 (M. J. Grubman and T. de los Santos). The authors thank Fawzi Mohamed, FADDL, for performing a histopathological analysis on the animals that died in the various trials; and the animal care staff at PIADC for their professional support and assistance.

References

Aldabe, R., Carrasco, L., 1995. Induction of membrane proliferation by poliovirus proteins 2C and 2BC. Biochem. Biophys. Res. Commun. 206, 64–76.
Arzt, J., Pacheco, J.M., Rodriguez, L.L., 2010. The early pathogenesis of foot-and-mouth disease in cattle after aerosol inoculation. Identification of the nasopharynx as the primary site of infection. Vet. Pathol. 47, 1048–1063.
Baer, G.M., Moore, S.A., Shaddock, J.H., Levy, H.B., 1979. An effective rabies treatment in exposed monkeys: a single dose of interferon inducer and vaccine. Bull. World Health Organ. 57, 807–813.
Buenz, E.J., Howe, C.L., 2006. Picornaviruses and cell death. Trends Microbiol. 14, 28–36.
Cao, Y., Lu, Z., Li, D., Fan, P., Sun, P., Bao, H., Fu, Y., Li, P., Bai, X., Chen, Y., Xie, B., Liu, Z., 2014. Evaluation of cross-protection against three topotypes of serotype O foot-and-mouth disease virus in pigs vaccinated with multi-epitope protein vaccine incorporated with poly(I:C). Vet. Microbiol. 168, 294–301.
Cao, Y., Lu, Z., Li, P., Sun, P., Fu, Y., Bai, X., Bao, H., Chen, Y., Li, D., Liu, Z., 2012. Improved neutralising antibody response against foot-and-mouth-disease virus in mice inoculated with a multi-epitope peptide vaccine using polyinosinic and polycytidylic acid as an adjuvant. J. Virol. Methods 185, 124–128.
Cao, Y., Lu, Z., Li, Y., Sun, P., Li, D., Li, P., Bai, X., Fu, Y., Bao, J., Zhou, C., Xie, B., Chen, Y., Liu, Z., 2013. Poly(I:C) combined with multi-epitope protein vaccine completely protects against virulent foot-and-mouth disease virus challenge in pigs. Antiviral Res. 97, 145–153.
Caskey, M., Lefebvre, F., Filali-Mouhim, A., Cameron, M.J., Goulet, J.P., Haddad, E.K., Breton, G., Trumpfheller, C., Pollak, S., Shimeliovich, I., Duque-Alarcon, A., Pan, L., Nelkenbaum, A., Salazar, A.M., Schlesinger, S.J., Steinman, R.M., Se'kaly, R.P., 2011. Synthetic double-stranded RNA induces innate immune responses similar to a live viral vaccine in humans. J. Exp. Med. 208, 2357–2366.
Cho, M.W., Teterina, N., Egger, D., Bienz, K., Ehrenfeld, E., 1994. Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells. Virology 202, 129–145.
de Avila Botton, S., Brum, M.C.S., Bautista, E., Koster, M., Weiblen, R., Golde, W.T., Grubman, M.J., 2006. Immunopotentiation of a foot-and-mouth disease virus subunit vaccine by interferon alpha. Vaccine 24, 3446–3456.
de Jong, A.S., Schrama, I.W., Willems, P.H., Galama, J.M., Melchers, W.J., van Kuppeveld, F.J., 2002. Multimerization reactions of coxsackievirus proteins 2B, 2C and 2BC: a mammalian two-hybrid analysis. J. Gen. Virol. 83, 783–793.
Dias, C.C., Moraes, M.P., Weiss, M., Diaz-San Segundo, F., Perez-Martin, E., Salazar, A.M., de los Santos, T., Grubman, M.J., 2012. Novel antiviral therapeutics to control foot-and-mouth disease. J. Int. Cyt. Res. 32, 1–12.
Díaz-San Segundo, F., Weiss, M., Koster, M.J., Dias, C.C., Grubman, M.J., de los Santos, T., 2012. Inoculation of swine with foot-and-mouth disease SAP-mutant virus induces early protection against disease. J. Virol. 86, 1316–1327.
Domingo, E., Holland, J.J., 1988. High error rates, population equilibrium and evolution of RNA replication systems. In: Domingo, E., Holland, J.J., Ahlquist, P. (Eds.), RNA Genetics, Vol. III. Variability of RNA Genomes. CRC Press, Boca Raton, Fl, USA, pp. 3–36.
Du, Y., Jiang, P., Li, Y., He, H., Jiang, W., Wang, X., Hong, W., 2007. Immune responses of two recombinant adenoviruses expressing VP1 antigens of FMDV fused with porcine granulocyte macrophage colony-stimulating factor. Vaccine 25, 8209–8219.
Du, Y., Li, Y., He, H., Qi, J., Jiang, W., Wang, X., Tang, B., Cao, J., Wang, X., Jiang, P., 2008a. Enhanced immunogenicity of multiple-epitopes of foot-and-mouth disease virus fused with porcine interferon alpha in mice and protective efficacy in guinea pigs and swine. J. Virol. Methods 149, 144–152.
Du, Y., Dai, J., Li, Y., Li, C., Qi, J., Duan, S., Jiang, P., 2008b. Immune responses of recombinant adenovirus co-expressing VP1 of foot-and-mouth disease virus and porcine interferon alpha in mice and guinea pigs. Vet. Immunol. Immunopathol. 124, 274–283.
Flynn, B.J., Kastenmüller, K., Wille-Reece, U., Tomaras, G.D., Alam, M., Lindsay, R.W., Salazar, A.M., Perdiguero, B., Gomez, C.E., Wagner, R., Esteban, M., Park, C.G., Trumpfheller, C., Keler, T., Pantaleo, G., Steinman, R.M., Seder, R., 2011. Immunization with HIV Gag targeted to dendritic cells followed by recombinant New York vaccinia virus induces robust T-cell immunity in nonhuman primates. Proc. Natl. Acad. Sci. USA 26, 7131–7136.
García-Briones, M., Rosas, M.F., González-Magaldi, M., Martín-Acebes, M.A., Sobrino, F., Armas-Portela, R., 2006. Differential distribution of non-structural proteins of foot-and-mouth disease virus in BHK-21 cells. Virology 349, 409–421.
Graham, F.L., Prevec, L., 1991. Manipulation of adenovirus vectors. In: Murray, E.J. (Ed.), Methods In Molecular Biology. The Humana Press, Clinton, NJ, pp. 109–128.
Grubman, M.J., Baxt, B., 2004. Foot-and-mouth disease. Clin. Microbiol. Rev. 17, 227–234.
Grubman, M.J., Diaz-San Segundo, F., Dias, C.C.A., Moraes, M.P., Perez-Martin, E., de los Santos, T., 2012. Use of replication-defective adenoviruses to develop vaccines and biotherapeutics against foot-and-mouth disease. Future Virol. 7 (8), 767–778.

Grubman, M.J., Moraes, M.P., Schutta, C., Barrera, J., Nielan, J., Ettyreddy, D., Butman, B.T., Brough, D.E., Brake, D.A., 2010. Replication-defective human adenovirus serotype 5 vectored foot-and-mouth disease subunit vaccines: the first decade. Future Virol. 5, 51–64.

Harrington, D.G., Crabbs, C.L., Hilmas, D.E., Brown, J.R., Higbee, G.A., Cole Jr, F.E., Levy, H.B., 1979. Adjuvant effects of low doses of nuclease-resistant derivative of polyinosinic acid polycytidylic acid on antibody responses of monkeys to inactivated Venezuelan equine encephalomyelitis virus vaccine. Infect. Immun. 24, 160–166.

Houston, W.E., Crabbs, C.L., Stephen, E.L., Levy, H.B., 1976. Modified polyriboinosinic-polyribocytidylic acid, an immunological adjuvant. Infect. Immun. 14, 318–319.

Kende, M., 1985. Prophylactic and therapeutic efficacy of poly(I,C)-LC against Rift Valley fever virus infection in mice. J. Biol. Response Modif. 4, 503–511.

Kende, M., Lupton, H.W., Rill, W.L., Levy, H.B., Canonico, P.G., 1987. Enhanced therapeutic efficacy of poly(ICLC) and ribavirin combinations against Rift Valley fever virus infection in mice. Antimicrob. Agents Chemother. 31, 986–990.

Kool, M., Soullie, T., Van Nimwegen, M., Willart, M.A., Muskens, F., Jung, S., Hoogsteden, H.C., Hammad, H., Lambrecht, B.N., 2008. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J. Exp. Med. 205, 869–882.

Levy, H.B., Baer, G., Baron, S., Buckler, C.E., Gibbs, C.J., Iadarola, M.J., London, W.T., Rice, J.A., 1975. A modified polyriboinosinic-polyribocytidylic acid complex that induces interferon in primates. J. Infect. Dis. 132, 434–439.

Levy, H.B., London, W., Fuccillo, D.A., Baron, S., Rice, J., 1976. Prophylactic control of simian hemorrhagic fever in monkeys by an interferon inducer polyriboinosinic-polyribocytidylic acid-poly-L-lysine. J. Infect. Dis. (133 Suppl.), A256–A259.

Li, Y., Hu, Y., Jin, Y., Zhang, G., Wong, J., Sun, L.Q., Wang, M., 2011. Prophylactic, therapeutic and immune enhanced effect of liposome-encapsulated poly ICLC on highly pathogenic H5N1 influenza infection. J. Gene Med. 13, 60–72.

Martins, K.A.O., Steffens, J.T., van Tongeren, S.A., Wells, J.B., Bergeron, A.A., Dickson, S.P., Dye, J.M., Salazar, A.M., Bavari, S., 2014. Toll-like receptor agonist augments virus-like particle-mediated protection from Ebola virus with transient immune activation. PLoS One 9, e89735.

Mason, P.W., Grubman, M.J., 2008. Foot-and-mouth disease (Chapter 22). In: Barrett, A., Stanberry, L. (Eds.), Vaccines for Biodefense and Emerging and Neglected Diseases. Elsevier Press, London, U.K., pp. 359–376.

Mason, P.W., Grubman, M.J., Baxt, B., 2003. Molecular basis of pathogenesis of FMDV. In: Rowlands, D. (Ed.), Foot-And-Mouth Disease (Virus Res. 91, 9–32).

Mason, P.W., Piccone, M.E., McKenna, TSt.-C., Chinsangaram, J., Grubman, M.J., 1997. Evaluation of a live-attenuated foot-and-mouth disease virus as a vaccine candidate. Virology 227, 96–102.

Mayr, G.A., Chinsangaram, J., Grubman, M.J., 1999. Development of replication-defective adenovirus serotype 5 containing the capsid and 3C protease coding regions of foot-and-mouth disease virus as a vaccine candidate. Virology 263, 496–506.

Mayr, G.A., O'Donnell, V., Chinsangaram, J., Mason, P.W., Grubman, M.J., 2001. Immune responses and protection against foot-and-mouth disease virus (FMDV) challenge in swine vaccinated with adenovirus-FMDV constructs. Vaccine 19, 2152–2162.

Meylan, E., Tschopp, J., 2006. Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. Mol. Cell 22, 561–569.

Moffat, K., Howell, G., Knox, C., Belsham, G.J., Monaghan, P., Ryan, M.D., Wileman, T., 2005. Effects of foot-and-mouth disease virus nonstructural proteins on the structure and function of the early secretory pathway: 2BC but not 3 A blocks endoplasmic reticulum-to-golgi transport. J. Virol. 79, 4382–4395.

Monaghan, P., Cook, H., Jackson, T., Ryan, M., Wileman, T., 2004. The ultrastructure of the developing replication site in foot-and-mouth disease virus-infected BHK-38 cells. J. Gen. Virol. 85, 933–946.

Moraes, M.P., de los Santos, T., Koster, M., Turecek, T., Wang, H., Andreyev, V.G., Grubman, M.J., 2007. Enhanced antiviral activity against foot-and-mouth disease virus by a combination of type I and II porcine interferons. J. Virol. 81, 7124–7135.

Moraes, M.P., Mayr, G.A., Grubman, M.J., 2001. pAd5-Blue: an easy to use, efficient, direct ligation system for engineering recombinant adenovirus constructs. BioTechniques 31, 1050–1056.

Moraes, M.P., Díaz-San Segundo, F., Dias, C.C.A., Pena, L., Grubman, M.J., 2011. Increased efficacy of an adenovirus-vectored foot-and-mouth disease capsid subunit vaccine expressing nonstructural protein 2B is associated with a specific T cell response. Vaccine 29, 9431–9440.

Moraes, M.P., Mayr, G.A., Mason, P.W., Grubman, M.J., 2002. Early protection against homologous challenge after a single dose of replication-defective human adenovirus type 5 expressing capsid proteins of foot-and-mouth disease virus (FMDV) strain A24. Vaccine 20, 1631–1639.

Ngoi, S.M., Tovey, M.G., Vella, A.T., 2008. Targeting poly(I:C) to the TLR3-independent pathway boosts effector CD8 T cell differentiation through IFN-alpha/beta. J. Immunol. 181 (11), 7670–7680.

Nordlund, J.J., Wolff, S.M., Levy, H.B., 1970. Inhibition of biologic activity of poly I: poly C by human plasma. Proc. Soc. Exp. Biol. Med. 133, 439–444.

OIE, 2004. (Modified May, 2006. Section 2.1 List A diseases. Chapter 2.1.1 Foot and mouth disease). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Fifth edition, pp. 111–128.

Pacheco, J.M., Brum, M.C.S., Moraes, M.P., Golde, W.T., Grubman, M.J., 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.

Park, H., Adamson, L., Ha, T., Mullen, K., Hagen, S.I., Nogueron, A., Sylwester, A.W., Axthelm, M.K., Legasse, A., Piatak Jr, M., Lifson, J.D., McElrath, J.M., Picker, L.J., Seder, R.A., 2013. Polyinosinic-polycytidylic acid is the most effective tlr adjuvant for siv gag protein-induced T cell responses in nonhuman primates. J. Immunol. 190, 4103–4115.

Pena, L., Moraes, M.P., Koster, M., Burrage, T., Pacheco, J.M., Diaz-San Segundo, F., Grubman, M.J., 2008. Delivery of a foot-and-mouth disease virus empty capsid subunit antigen with nonstructrual protein 2B improves protection of swine. Vaccine 26, 5689–5699.

Pulendran, B., Ahmed, R., 2006. Translating innate immunity into immunological memory: implications for vaccine development. Cell 124, 849–863.

Sorensen, K.J., Madsen, K.G., Madsen, E.S., Salt, J.S., Nqindi, J., Mackay, D.K., 1998. Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. Arch. Virol. 143, 1461–1476.

Stahl-Hennig, C., Eisenblatter, M., Jasny, E., Rzehak, T., Tenner-Racz, K., Trumpfheller, C., Salazar, A.M., Uberla, K., Nieto, K., Kleinschmidt, J., Schulte, R., Gissmann, L., Muller, M., Sacher, A., Racz, P., Steinman, R.M., Uguccioni, M., Ignatius, R., 2009. Synthetic double-stranded RNAs are adjuvants for the induction of T helper 1 and humoral immune responses to human papillomavirus in rhesus macaques. PLoS Pathog. 5, 1–15.

Stephen, E.L., Hilmas, D.E., Levy, H.B., Spertzel, R.O., 1979. Protective and toxic effects of a nuclease-resistant derivative of polyriboinosinic-polyribocytidylic acid on Venezuelan equine encephalomyelitis virus in rhesus monkeys. J. Infect. Dis. 139, 267–272.

Stephen, E.L., Sammons, M.L., Pannier, W.L., Baron, S., Spertzel, R.O., Levy, H.B., 1977. Effect of a nuclease-resistant derivative of polyriboinosinic-polyribocytidylic acid complex on yellow fever in rhesus monkeys (Macaca mulatta). J. Infect. Dis. 136, 122–126.

Suhy, D.A., Giddings Jr, T.H., Kirkegaard, K., 2000. Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: an autophagy-like origin for virus-induced vesicles. J. Virol. 74, 8953–8965.

Tatsis, N., Ertl, H.C.J., 2004. Adenoviruses as vaccine vectors. Mol. Ther. 10, 616–629.

Tenbusch, M., Ignatius, R., Nchinda, G., Trumpfheller, C., Salazar, A.M., Töpfer, K., Sauermann, U., Wagner, R., Hannaman, D., Tenner-Racz, K., Racz, P., Stahl-Hennig, C., Überla, K., 2012. Immunogenicity of DNA vaccines encoding simian immunodeficiency virus antigen targeted to dendritic cells in rhesus macaques. PLoS One 7, e39038.

Wiltrout, R.H., Salup, R.R., Twilley, T.A., Talmadge, J.E., 1985. Immunomodulation of natural killer activity by polyribonucleotides. J. Biol. Response Modif. 4, 512–517.

Wu, Q., Brum, M.C.S., Caron, L., Koster, M., Grubman, M.J., 2003. Adenovirus-mediated type I interferon expression delays and reduces disease signs in cattle challenged with foot-and-mouth disease virus. J. Int. Cyt. Res. 23, 371–380.

Zhou, C.-X., Li, D., Chen, Y.-L., Lu, Z.-J., Sun, P., Cao, Y.-M., Hui-Fang, B., Fu, Y.-F., Li, P.-H., Bai, X.-W., Xie, B.-X., Liu, Z.-X., 2014. Resiquimod and polyinosinic-polycytidylic acid formulation with aluminum hydroxide as an adjuvant for foot-and-mouth disease vaccine. BMC Vet. Res. 10, 2. http://dx.doi.org/10.1186/1746-6148-10-2.