

Molecular vaccine prepared by fusion of XCL1 to the multi-epitope protein of foot-and-mouth disease virus enhances the specific humoral immune response in cattle

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Abstract Targeting antigen to dendritic cells (DCs) is a promising way to manipulate the immune response and to design prophylactic molecular vaccines. In this study, the cattle XCL1, ligand of XCR1, was fused to the type O foot-and-mouth disease virus (FMDV) multi-epitope protein (XCL-OB7) to create a molecular vaccine antigen, and an ^ΔXCL-OB7 protein with a mutation in XCL1 was used as the control. XCL-OB7 protein specifically bound to the XCR1 receptor, as detected by flow cytometry. Cattle vaccinated with XCL-OB7 showed a significantly higher antibody response than that to the ^ΔXCL-OB7 control ($P < 0.05$). In contrast, when XCL-OB7 was incorporated with poly (I:C) to prepare the vaccine, the antibody response of the immunized cattle was significantly decreased in this group and was lower than that in the ^ΔXCL-OB7 plus poly (I:C) group. The FMDV challenge indicated that cattle immunized with the XCL-OB7 alone or the ^ΔXCL-OB7 plus poly (I:C) obtained an 80% (4/5) clinical protective rate. However, cattle vaccinated with ^ΔXCL-OB7 plus poly (I:C) showed more effective inhibition of virus replication than that in the XCL-OB7 group after viral challenge, according to the presence of antibodies against FMDV non-structural protein 3B. This is the first test of DC-targeted vaccines in veterinary medicine to use XCL1 fused to FMDV antigens. This primary result showed that an

XCL1-based molecular vaccine enhanced the antibody response in cattle. This knowledge should be valuable for the development of antibody-dependent vaccines for some infectious diseases in cattle.

Keywords Cattle dendritic cells · Vaccine · Fusion antigen · XCR1 · XCL1

Introduction

Dendritic cells (DCs) are sentinel antigen presentation cells in the immune system and play essential roles in linking the innate and acquired immune responses. The delivery of antigens to DCs is a selective strategy to manipulate the immune response and has been tested in murine models for immunotherapy and prophylactic vaccination (Boltjes and van Wijk 2014). The theoretical foundation of this manipulation is to induce tolerance to the antigen and to prevent autoimmunity or transplant rejection, to promote CTL function in tumour eradication or viral infection or to boost antibody titres in antibody-dependent vaccines (Caminschi and Shortman 2012). The exploration of DC-targeted vaccines is a major challenge for immunologists, and vaccine efficiency outcome depends on multiple factors, including the nature of selected DC receptors, the DC-subset targeted, state of DC activation, and the intermediate molecule (monoclonal antibody, ligand or modified substance) used for targeting.

Different types of immune response are obtained by targeting DC receptors, such as the C-type lectin receptors, DEC205 (CD205), Dectin-1, LOX1 and Clec9A, as well as the chemokine receptor, XCR1. DEC205 (CD205) is an endocytic receptor, and antibodies targeting DEC205 activate T cells 100-fold more efficiently than non-targeted antibodies

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(Jiang et al. 1995). However, DC capture of antigen via DEC205 induces tolerance at the steady state and enhances both CD4⁺ T and CD8⁺ T cell responses after DC maturation (Bonifaz et al. 2002; Tsuji et al. 2011). Relatedly, an improved CD4⁺ T cell response has been observed with antigens targeted to Dectin-1 (Carter et al. 2006). In addition, targeting antigens to mouse DCs via LOX1 elicits an antigen-specific CD8⁺ T cell response (Delneste et al. 2002; Xie et al. 2010) and promotes a DC-mediated class-switch B cell response (Joo et al. 2014). Both Clec9A and XCR1 are conserved molecules in mouse CD8a⁺ DC and human CD141⁺ DC, which have the special ability of antigen cross-presentation (Bachem et al. 2010; Tullett et al. 2014). Studies have shown that Clec9A specifically recognizes F-actin on necrotic cells and initiates antigen cross-presentation in the CD8⁺ T cell immune response (Ahrens et al. 2012). Antigen delivery to DC via Clec9a promotes humoral immunity, CD4⁺ T cell memory response and cytotoxic CD8⁺ T cell immune response, even in the absence of adjuvant for DC maturation (Kato et al. 2015; Li et al. 2015; Tullett et al. 2016). XCR1 is a member of the G-protein-coupled receptor family, and its ligand is XCL1, which is expressed in NK cells and activated CD8⁺ T cells. The distribution of XCR1 and XCL1 indicates that they regulate the interaction between DC and T cells; therefore, antigen delivery to XCR1⁺ DC through the XCL1-fused molecule is an advisable strategy to regulate immunity. Indeed, potent CD8⁺ T cell cytotoxicity has been induced by targeting XCR1⁺ DC via XCL1-fused OVA (Hartung et al. 2015) or haemagglutinin (HA) of the influenza virus in a mouse model (Fossum et al. 2015). These data indicate that antigen delivery to DC improves the immunogenicity of vaccines in the murine model; thus, extension of this strategy to large animals has the potential to contribute to the development of DC-targeted vaccines against infectious animal diseases.

In cattle, CD205⁺ DCs, a major subset of DCs, have been detected in skin draining lymph (Gliddon et al. 2004) and blood (Gonzalez-Cano et al. 2014). Antigens targeted to cattle CD205⁺ DCs induce significant antigen-specific antibodies and CD4⁺ T cell responses by a DNA vaccine expressing CD205-specific scFv and/or CD40L fused to MSP1a antigen from *Anaplasma marginale* with DNA-encoded Flt3L and GM-CSF as adjuvant (Njongmeta et al. 2012). Two subsets of CD205⁺ DCs have been further distinguished as CD172a⁺ CD26⁻ DC and CD172a⁻ CD26⁺ DC in skin draining lymph, and CD172a⁺ CD26⁻ DCs have a high ability to present antigen to MHC II (Gliddon and Howard 2002). Our investigation of the expression pattern of cattle blood DC subsets revealed that XCR1 and Clec9A were highly transcribed in CD26⁺ DC, similarly to sheep CD26⁺ DCs involved in antigen cross-presentation. This finding indicated that cattle CD26⁺ DC may be a subset of DCs that have cross-presentation ability and correspond to mouse CD8a⁺ DC and human CD141⁺ DC. Therefore, antigen delivery to cattle CD26⁺ DC was expected

to improve CD8⁺ T cytotoxic function, thus leading to comprehensive immune response. Moreover, cattle XCL1, the ligand of XCR1, is identified mainly in quiescent NK cells and in activated CD8⁺ T cells (Li et al. 2017). The distribution characteristics of XCR1 and XCL1 in cattle make XCR1 a good candidate receptor for exploration of a DC-targeted vaccine by using XCL1-fused antigens in cattle.

In this study, we designed a novel DC-targeted vaccine by using XCL1 fused to a multi-epitope protein (OB7) of type O foot-and-mouth disease virus (FMDV), and explored its immunological effects in cattle. The model antigen OB7 was adapted from a previous publication, and some modifications were made for use in cattle (Cao et al. 2013). The XCL1 fusion proteins (XCL-OB7) and the non-targeted ^ΔXCL-OB7 fusion protein (with a mutation in XCL1 to eliminate the ability to bind XCR1) were produced in *Escherichia coli* (*E. coli*). The fusion protein XCL-OB7 specifically bound CD26⁺ DC at an optimized concentration in vitro. Cattle vaccinated with XCL-OB7 developed higher neutralizing antibody levels than did the ^ΔXCL-OB7 vaccination group and obtained 80% protection against an FMDV challenge. These findings indicated that XCL1 is an ideal molecule to design DC-targeted vaccines and improve humoral immune response in cattle.

Materials and methods

Molecular design and expression of the XCL fusion FMDV multi-epitope proteins XCL-OB7 and ^ΔXCL-OB7

The multi-epitope protein OB7 of type O FMDV was designed on the basis of previous B4 proteins (Cao et al. 2013) with two additional FMDV-specific T cell epitopes in non-structural proteins 3A and 3D of type O FMDV (Blanco et al. 2001; Zhang et al. 2015). Cattle XCL1 (GenBank accession number KU641032) was fused to OB7 by a linker “GPGPG” to form the XCL-OB7 fusion protein (Li et al. 2017). As the control, a non-targeted variant (referred to as ^ΔXCL-OB7) of XCL-OB7 was generated by introducing two amino acid mutations in C18A and C55A of XCL1, which disrupt the function of XCL1 (Fossum et al. 2015). The detailed XCL-OB7 and ^ΔXCL-OB7 amino acid sequences are shown in Table 1.

Two coding sequences of the above two fusion proteins were synthesized by GenScript Incorporation (www.genscript.com) with optimization of the codons for expression in *E. coli*. The two codon-optimized fusion genes, XCL-OB7 (GenBank accession number MF509632) and ^ΔXCL-OB7 (GenBank accession number MF509633), were cloned into the pET28a(+) vector by using the *Nco*I and *Xho*I restriction sites. The fusion proteins were expressed as inclusion bodies in *E. coli* BL21 (DE3) and purified by using a

Table 1 The constitution of XCL fusion FMDV multi-epitopes proteins: XCL-OB7, ^ΔXCL-OB7

AA position	Sequence	Epitopes	XCL-OB7	^Δ XCL-OB7
2–83	Cattle XCL1(KU641032) AA(16–97)	Mature protein	+	+(C18/55A)
84–88	GPGPG	Linker	+	+
89–104	TAKSKKFPSYTATYQF	Invasin (universal T cell epitopes)	+	+
107–121	AAIEFFEGMVHDSIK	3A _{21–35} (O type FMDV)	+	+
124–151	SSKYGDTSTNNVRGDLQVLAKKAERALP	VP1 _{132–160} (G–H loop of O/HN/CHA/09)	+	+
154–172	AIQPSTARHKQKIVAPAKQ	VP1 _{193–211} (C-terminal of O/HN/CHA/09)	+	+
175–203	GNCKYGESPVTVNRGDLQVLAQKAARTLP	VP1 _{132–160} (G–H loop of O/Tibet/99)	+	+
206–224	AIHPNEARHKQKIVAPVKQ	VP1 _{193–211} (C-terminal of O/IRN/2010)	+	+
227–254	DCKYGESRTTVNRGDLQVLAQKAATTL	VP1 _{132–160} (G–H loop of O/IRN/2010)	+	+
257–285	GNCKYAGGSLTVNRGDLQVLDQKAARPLP	VP1 _{132–160} (G–H loop of O/Mya/98)	+	+
288–306	AVHPSAARHKQKIVAPVKQ	VP1 _{193–211} (C-terminal of O/Mya/98)	+	+
309–338	VVASYDLDFEALKPHFKSLGQTITPADKS	3D _{341–370} (A, O, Asia 1 type FMDV)	+	+
341–353	AKFVAAWTLKAAA	PADRE (universal T cell epitopes)	+	+
354–359	HHHHHH	His tag	+	+

Epitopes were connected with the Linker “GG”

+ presence of the sequence, – absence of the sequence

HisTrap™ column in the AKTA protein purification system (GE Life Sciences, Piscataway, NJ, USA) under denaturing conditions. The purified proteins were refolded in PBS buffer (pH = 7.4). The obtained fusion proteins were identified by SDS-PAGE and further confirmed by the anti-Histidine tag antibody and anti-FMDV polyclonal antibodies on a western blot. All proteins were quantified by using a BCA Protein Assay Kit (Generay Biotech, Shanghai, China).

FACS sorting of blood DC subsets and evaluation of XCL1 fusion protein binding activity

Fluorescence-activated cell sorting (FACS) was used to sort cattle blood CD26⁺ DC and CD172⁺ DC subsets from the pre-enriched DCs, which were obtained from PBMCs by depletion lineage (lin⁻) cells via indirect magnetic separation. Briefly, to deplete T cells, monocytes, B cells and NK cells, 1 × 10⁹ PBMCs were stained with a pool of mouse anti-bovine antibodies (anti-CD3/11b/CD14/21/335 antibodies, all mouse IgG1 type, 20 μg each), listed in Table 2, at 4 °C for 30 min. After cells were washed with PBS, 2 ml goat anti-mouse IgG MicroBeads (Miltenyi, Biotec, Germany) was added and incubated for 10 min at room temperature. Then, these PBMCs were applied to an LD column (Miltenyi, Biotec, Germany) for magnetic separation, and the cells in the flow-through were collected and denoted pre-enriched DCs. The pre-enriched DCs were stained with mouse anti-bovine MHC II-PE (IgG2a), anti-bovine CD11c (IgM) and anti-bovine CD172a (IgG2b) antibodies for 30 min at 4 °C, then stained with rat anti-mouse IgG1 VIVO450, anti-mouse IgM PE-Cy7 and anti-mouse IgG2b FITC. After cells were blocked with mouse

IgG (Thermo Fisher, USA), mouse anti-bovine CD26 Alexa 647 (IgG1) was added and incubated for a further 20 min in 4 °C, and this was followed by staining with 7AAD for 5 min on ice. The stained cells were immediately sorted with a Becton Dickinson FACSaria II (San Jose, CA, USA) two-way sorter via a 100-μm nozzle. The sorted CD26⁺ DC and CD172⁺ DC were incubated with the XCL1 fusion proteins for 30 min at 4 °C. After being washed two times, the cells were further incubated with mouse anti-Histidine tag-FITC or anti-Histidine tag-Alexa 647 at 4 °C for 20 min and then analysed by flow cytometry.

Real-time RT-PCR

To identify the cattle DC subset expressing XCR1, total RNA was extracted from the sorted CD26⁺ DC and CD172⁺ DC as well as PBMCs by using an RNeasy Mini Kit (Qiagen, Germany). After DNase treatment with an RNase-Free DNase Set (Qiagen, Germany), the RNAs were reverse transcribed using PrimeScript™ RT Master Mix (Takara, Dalian, China), and the obtained complementary DNA (cDNA) was quantified according to the manufacturer's protocol and then stored at – 20 °C for RT-PCR analysis. Relative qualitative RT-PCR was performed on an ABI Prism 7500 Real-Time PCR System (Applied Biosystems) using SYBR Premix Ex Taq II (TaKaRa Bio, Dalian). A total of 100 ng cDNA template and the appropriate amount of primers for the XCR1 gene (F: TGCTGTGGGTCTTGGTGAA /R: GGCAACAG GCAGGAGAACA) or GAPDH gene (F: TCGGAGTG AACGGATTTCG/R: ATCTCGCTCCTGGAAGATG) were added in the amplification system. The 2^{-ΔΔCt} method was

Table 2 Primary and secondary antibodies used for purification of cattle DC subsets by magnetic separation and FACS

Antibody	Clone	Isotype ^a	Source
CD3	MM1A	IgG1	MAC ^b , WSU
CD14	CC-G33	IgG1	AbD Serotec
CD21	CC21	IgG1	AbD Serotec
CD335	AKS1	IgG1	AbD Serotec
CD11b	MM12A	IgG1	² MAC, WSU
CD11c	BAQ153A	IgM	² MAC, WSU
MHCIPE	CC158	IgG2a	Abcam
CD26 Alexa Fluor 647	CC69	IgG1	AbD Serotec
CD172a	CC149	IgG2b	AbD Serotec
Anti-mouse IgM PE-Cy7	eB121-15F9	Rat IgG2a	eBioscience
Anti-mouse IgG1 V450	A85-1	Rat IgG1	BD Horizon™
Anti-mouse IgG2b FITC	R12-3	Rat IgG2a	BD Pharmingen™
7AAD			BD Horizon™
Anti-Histidine tag-FITC	AD1.1.10	IgG1	AbD Serotec
Anti-Histidine tag-Alexa 647	AD1.1.10	IgG1	AbD Serotec

^a Isotype: all the antibodies are derived of mouse host, exception for specific denotation

^b Source: the MAC, WSU denoted the source from the Monoclonal Antibody Center, Washington State University (MAC, WSU)

used to evaluate gene expression of the sorted cattle blood DC subsets. The GAPDH gene was selected as an internal control, and the PBMCs were selected as the calibrator sample.

Animal experiment

A total of 33 1-year-old female healthy Qinchuan cattle (*Bos taurus*) (a Chinese breed of beef cattle) with no history of FMD were raised in a local farm in Gansu, China. All of the cattle were confirmed to be seronegative for FMDV by liquid-phase blocking enzyme-linked immunosorbent assay (ELISA) before vaccination and were used for evaluation of immune efficiency of the fusion proteins. For molecular vaccine preparation, all fusion proteins or fusion proteins with poly (I:C) (Sigma-Aldrich, USA) were diluted with PBS buffer to a required concentration and added to Montanide 201 oil adjuvant (Seppic, Shanghai, China) for emulsification in a 50 : 50 volume ratio. Each animal received 2 ml molecular vaccine by intramuscular injection. A conventional inactivated FMDV vaccine prepared by chemical inactivation and Montanide 201 oil adjuvant of the O/Mya/98 strain (GenBank accession number JN998086) was used as a positive-control vaccine. The detailed grouping and vaccination doses are summarized in Table 3. Booster vaccination with identical doses was performed 28 days after primary vaccination. Serum samples were collected at days 0, 7, 14, 21 and 28 after primary and boost vaccinations. All cattle were inoculated subcutaneously at two sites on the tongue with 10,000 BID₅₀ (50% bovine

infective dose) of cattle-adapted O/Mya/98 FMDV at day 35 post boost vaccination (DPV). Clinical scoring of the cattle was recorded daily for 10 days (Table 3). At 10 days post challenge (DPC), serum samples were collected for detection of antibodies against non-structural (NS) FMDV proteins.

ELISA

The cattle IgG antibody titres after vaccination were determined by liquid-phase blocking ELISA (LPB-ELISA) kit (LVRI, Lanzhou, Gansu, China). All procedures were performed according to the manufacturer's instructions (Cao et al. 2012).

The antibodies against non-structural protein (NSP) 3B on DPC 10 were detected with a PrioCHECK® FMDV NS kit (Prionics AG, Schlieren-Zurich, Switzerland). In this test, the antibody titre was expressed as the percentage inhibition (PI) compared with the negative control. A PI of 50% is the threshold for a qualitative judgement of infection.

Micro-neutralization assays

Serum samples from cattle after vaccination at different time points were inactivated at 56 °C for 30 min and were analysed for virus neutralizing antibody (VNA) titres against three topotypes of type O FMDV (O/Mya/98, O/HN/CHA/93 [shared high homology with O/GD/China/86 (GenBank accession number AJ131468)] and O/Tibet/99 (GenBank accession number AJ539138)) by using a micro-neutralization assay,

Table 3 Cattle grouping, vaccination dose, clinical scoring and protection rate in this study

Grouping (<i>n</i> = cattle counts)	Vaccination (intramuscular injection, 2 ml/dose)	Cattle no.	Clinical scoring ^a (max)	Protection rate (percent)
A (<i>n</i> = 5)	XCL-OB7 (1 mg)	1007	0	4/5 (80%)
		1015	3	
		1016	0	
		1017	0	
		1019	1	
B (<i>n</i> = 5)	XCL-OB7 (0.1 mg)	6974	0	4/5 (80%)
		6976	0	
		6978	0	
		6981	2	
		6992	0	
C (<i>n</i> = 5)	XCL-OB7 (1 mg) plus poly (I:C) (0.5 mg)	1038	3	2/5 (40%)
		1040	0	
		1041	3	
		1044	0	
		1046	4	
D (<i>n</i> = 5)	^Δ XCL-OB7 (1 mg)	7038	0	3/5 (60%)
		2360	0	
		7041	0	
		7042	3	
		7043	3	
E (<i>n</i> = 5)	^Δ XCL-OB7 (1 mg) plus poly (I:C) (0.5 mg)	1020	0	4/5 (80%)
		1022	4	
		1023	0	
		1028	0	
		1029	0	
F (<i>n</i> = 5)	Conventional inactivated vaccine (2 ml)	7063	0	5/5 (100%)
		6971	0	
		6972	0	
		6973	0	
		2381	0	
G (<i>n</i> = 3)	PBS (2 ml)	2375	7	0/3 (0%)
		2389	6	
		2353	4	

^a Severity of FMD was scored as follows:

Fever; present (score 1)

Tongue lesion; at site of inoculation (score 1) or secondary vesiculation (score 2)

Buccal lesions; (secondary lesions on lip, dental pad or gums) when it was present (score 1)

Foot lesions; vesicular lesions on each foot (score 1), score 4 means lesion of all four feet

No clinical symptoms observed above was scored 0

Cattle with a maximum score of 8 indicated the highest level of severity. Cattle with primary lesion at site of inoculation (score 1) or secondary vesiculation limited to tongue (score 2) were considered as protected. Any secondary lesion beyond the tongue represented systemic viraemia and cattle were assigned as unprotected

as previously described (Golde et al. 2005). Briefly, serum samples were diluted 2-fold in 96-well cell culture plates in a total volume of 50 μ l, and then 100 TCID₅₀ of FMDV in 50 μ l medium was added to each well. After incubation for 1 h at 37 °C, approximately 5×10^4 BHK21 cells in 100 μ l medium were added in each well as indicators of residual

infectivity. Normal cell wells and 10, 100 and 1000 TCID₅₀ virus controls wells were used in each plate. The cell plates were incubated at 37 °C under 5% CO₂ conditions for 72 h before fixing and staining. The endpoint titres were calculated as the reciprocal of the last serum dilution to neutralize 100 TCID₅₀ FMDV in 50% of the wells.

Statistics

Prism 5.0 software (GraphPad Software, La Jolla, CA) was used to perform all statistical analyses. Differences in LPB-ELISA antibody and VNA were calculated using two-way ANOVA, followed by Bonferroni post-tests. Differences in NSP 3B antibodies after the viral challenge was calculated by using one-way ANOVA, followed by Dunnett's multiple comparison test.

Results

Design, expression and characteristics of the XCL-OB7 and its mutant Δ XCL-OB7 fusion proteins

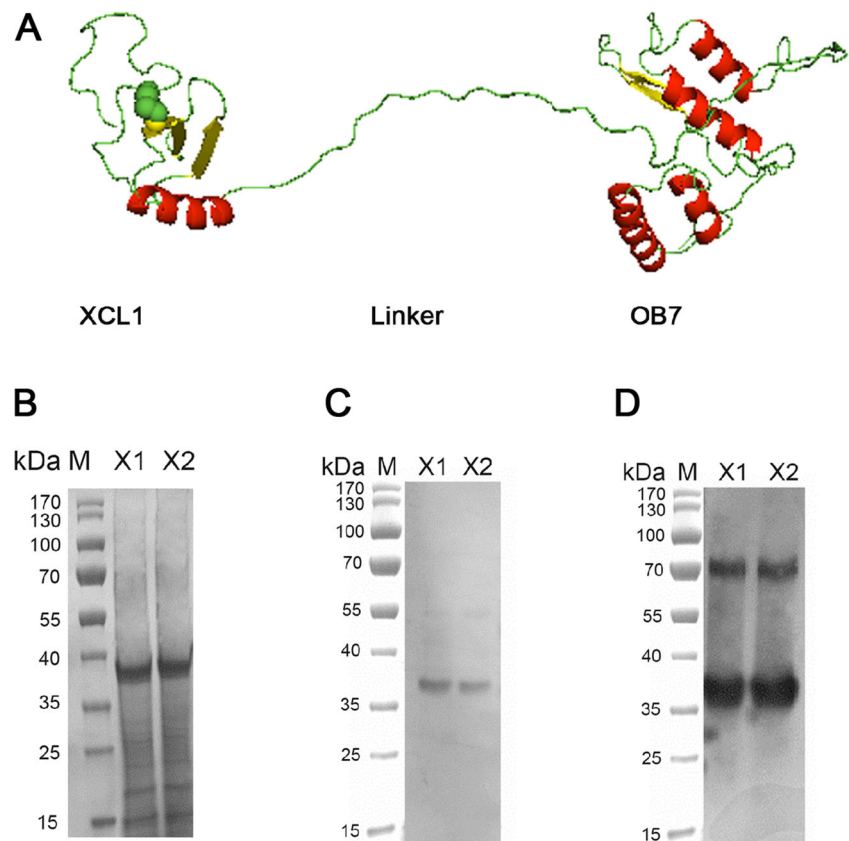
The structure of the XCL-OB7 molecule was modelled as shown in Fig. 1a. This molecule appears to comprise two separate parts linked by the “GPGPG” pentapeptide. Therefore, the function and structure of XCL1 and OB7 were expected to be unaffected by each other. XCL-OB7 and its mutant Δ XCL-OB7 fusion protein were expressed in inclusion bodies in *E. coli*. After purification with Ni-resin under denaturation conditions, the purified proteins were refolded in PBS buffer with 2 mM DTT (pH = 7.4) and then analysed by using SDS-PAGE. The purity of the XCL1 fusion proteins was

above 90% of the total protein amount (Fig. 1b). The western blot results showed that XCL-OB7 and Δ XCL-OB7 fusion proteins specifically reacted with anti-Histidine tag antibody, and the bands were both consistent with the calculated molecular masses of 37.9 kDa (Fig. 1c). Moreover, these XCL1 fusion proteins were also specifically recognized by serum from animals infected with type O FMDV (Fig. 1d).

XCL-OB7, not Δ XCL-OB7, specifically bound XCR1⁺ DC in the sorted CD26⁺ DC population

Because DCs composed less than 1% of the total PBMCs, pre-enrichment of DCs with magnetic beads (Fig. 2a–c) and FACS sorting of CD26⁺ DC and CD172a⁺ DC were performed in vitro (Fig. 2d, e). Through use of magnetic beads and FACS sorting, lin⁻ cells (Fig. 2d), cDCs (Fig. 2e), CD26⁺ DC and CD172a⁺ DC were gradually separated with increasing purity (Fig. 2f). Evaluation of the binding ability of XCL1 fusion proteins with XCR1⁺ DC was performed by staining the FACS-sorted CD26⁺ DC and CD172a⁺ DC (Fig. 2f). The results showed that XCL-OB7 selectively bound approximately 20% of CD26⁺ DC, but not the CD172a⁺ DC (Fig. 2g). Accordingly, Δ XCL-OB7 bound neither the CD26⁺ DC nor the CD172a⁺ DC (Fig. 2f). Analysis of XCR1 mRNA expression in the sorted cattle DC subsets as well as PBMCs indicated that cattle XCR1 was highly expressed on CD26⁺ DC but not

Fig. 1 Structural simulation of XCL-OB7 fusion protein and analysis of the purified production by SDS-PAGE and western blotting. The XCL-OB7 3D structure (a) was simulated by template-based protein structure modelling and analysed with the RaptorX web server. M1 represents a pre-stained molecular marker; X1 and X2 denote the purified XCL-OB7 and Δ XCL-OB7, respectively (b). Reactivity analysis of the two proteins with anti-Histidine antibody (c) and anti-FMDV polyclonal antibodies (the polyclonal antibodies were produced by purification cattle IgG from serum of cattle infected with type O FMDV using protein G chromatography in our laboratory) (d) by western blotting



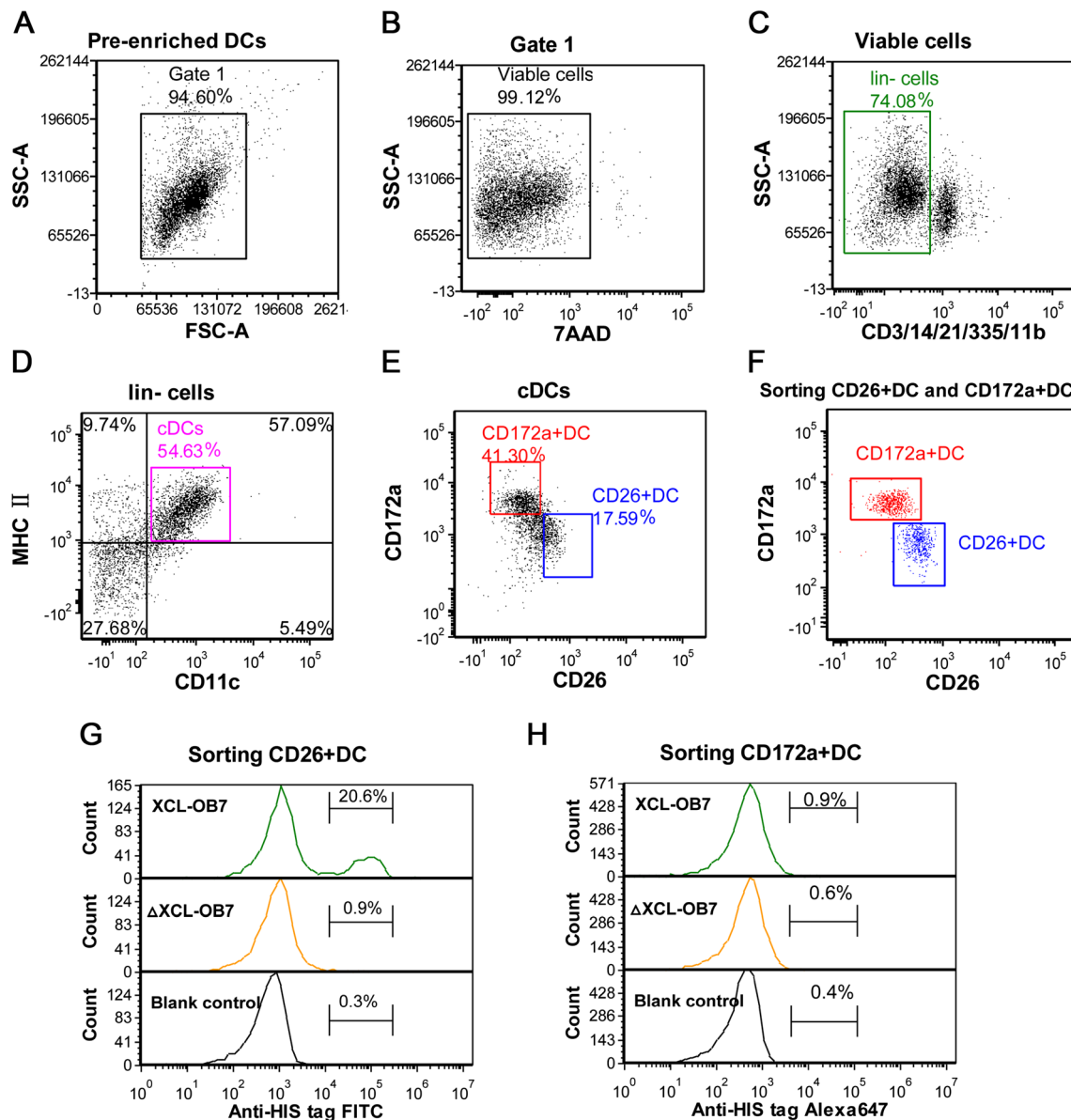


Fig. 2 Evaluation of the binding ability of XCL1 fusion protein with cattle blood CD26⁺ DC and CD172a⁺ DC subsets. After depletion of lineage cells (anti-bovine CD3/CD11b/CD14/CD21/CD335) from PBMCs, pre-enriched DCs (a) were subjected to FACS. Gate 1 (b) was selected to exclude cell debris with lower SSC-A and FSC-A values and was further analysed to gate viable cells (c) on the basis of 7AAD negativity. The remnant lineage-positive cells were excluded by staining with anti-mouse IgG1 Vivo 450, and the lin⁻ cells (d) were gated to identify the MHCII⁺CD11c⁺ cell (cDC) population (e). Then, the cDCs

were further gated to collect the CD26⁺ DC and CD172a⁺ DC. The plot (f) indicates the sorted CD26⁺ DC and CD172a⁺ DC. The stacked histogram (g) represents the results of staining of the sorted CD26⁺ DC with XCL1-OB7 (0.2 μg/ml) and Δ XCL-OB7 (0.2 μg/ml), and subsequent anti-Histidine tag-FITC. The stacked histogram (h) represents the results of staining the sorted CD172a⁺ DC with XCL1-OB7 (0.2 μg/ml) and Δ XCL-OB7 (0.2 μg/ml), and subsequent anti-Histidine tag-Alexa 647. Data shown are representative of one of two independent experiments

on CD172a⁺ DC (Fig. 3). In addition, cattle whole blood cells were surveyed with the XCL1 fusion proteins, and the results indicated that no stain lineages were stained with either XCL-OB7 or Δ XCL-OB7, including CD3⁺ T cells, CD14⁺ monocytes, CD21⁺ B cells and CD335⁺ NK cells. Therefore, these results clearly indicated that XCL-OB7 specifically binds cattle XCR1 on a subset of CD26⁺ DC.

XCL-OB7 induced enhanced IgG antibody response

Given the specific ability of XCL-OB7 to bind cattle XCR1⁺ DC in vitro, the immunological efficacy of the XCL-OB7 and Δ XCL-OB7 proteins was evaluated in cattle. The sera antibody titres of XCL-OB7 (1 mg)-immunized cattle (group A) were higher than those of Δ XCL-OB7-immunized cattle

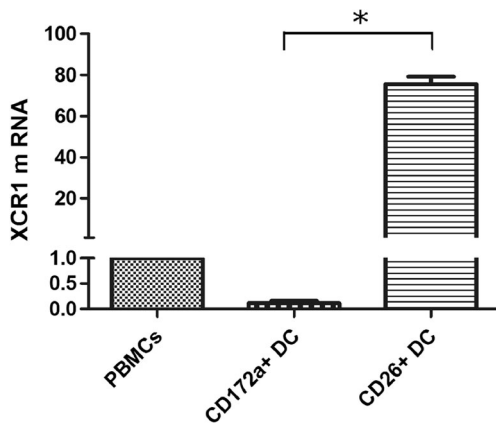


Fig. 3 Real-time RT-PCR expression analysis of cattle XCR1 from isolated blood CD26⁺ DC and CD172a⁺ DCy. Data are representative of three independent experiments. Error bars represent standard deviation. Differences in XCR1 expression between the CD26⁺ DC and CD172a⁺ DC were calculated by using a one-tailed non-parametric *t* test (Mann-Whitney test). A significant difference was defined as $P \leq 0.05$ (*)

(group D), and clear differences ($P < 0.0001$) were observed between these two groups at different time points, except at 7 DPV (Fig. 4a). The sera titres of XCL-OB7 (1 mg)-immunized cattle (group A) were comparable to those of cattle immunized with inactivated vaccine (group F), and no differences were observed between these two groups at 14 DPV as well as after boosting (Fig. 4a). For the negative control (group G), sera titres in PBS immunized cattle were below the assay sensitivity during the entire vaccination period. These results showed that XCL-fused antigen significantly improved IgG antibody titres in cattle.

Poly (I:C) inhibited IgG antibody response to XCL-OB7

It has been demonstrated that antigen targeting to immature DCs results in immunological tolerance, whereas targeting to activated DCs promotes CTLs and the humoral response in a mouse model (Tullett et al. 2014). Cattle XCR1 have previously been detected on CD26⁺ DCs that are a subset of

CD11c⁺ DCs expressing TLR3, 7, 8 and 9 (Sei et al. 2014). Therefore, we investigated the effects of poly (I:C) (the ligand of TLR3) as an adjuvant to improve the immune response to XCL-OB7. Interestingly, the antibody responses were more inhibited in the XCL-OB7 plus poly (I:C) group (group A) than the XCL-OB7 only-vaccinated group (group C) after boosting ($P < 0.0001$), as shown in Fig. 4b. Antibody titres in cattle immunized with Δ XCL-OB7 plus poly (I:C) (group E) were similar to those of cattle vaccinated with XCL-OB7 alone. In addition, Δ XCL-OB7 plus poly (I:C)-immunized cattle (group E) showed significantly higher IgG antibody titres than only Δ XCL-OB7-immunized cattle after boosting ($P < 0.0001$, Fig. 4b). These results indicated that poly (I:C) inhibits IgG antibody titres induced by XCL-OB7, not Δ XCL-OB7, in cattle.

Neutralizing antibody results indicated opposite roles of XCL1 and poly (I:C) in inducing antibody response in cattle

Neutralizing antibodies against three topotypes of type O FMDV were detectable in XCL1 fusion antigen-immunized cattle at day 28 after primary vaccination, and the antibody titres were markedly increased after boosting vaccination, especially at day 14 after the boost. Comparison of the VNA titres of all cattle groups immunized with fusion antigen indicated that the XCL-OB7-vaccinated cattle (group A) displayed higher VNA titres than other groups during the vaccination period against all three topotypes of type O FMDV, especially on day 28 after primary vaccination. Cattle in this group showed higher VNA titres against the ME-SA topotype virus than did group D cattle immunized with Δ XCL-OB7 ($P < 0.05$, Fig. 5c). Similarly to the IgG titres, VNA titres against all three topotype viruses in cattle immunized with XCL-OB7 plus poly (I:C) were clearly inhibited compared with those in group A cattle immunized with XCL-OB7 alone at day 28 after primary vaccination ($P < 0.05$, Fig. 5a, b) and days 14 and 28 after boosting ($P < 0.001$, Fig. 5a–c). These

Fig. 4 Total IgG antibodies to FMDV in sera, analysed by LPB-ELISA. Cattle were immunized at day 0 and challenged at day 35 after booster vaccination. Sera were pooled at days 0, 7, 14, 21 and 28 after primary vaccination and days 7, 14, 21 and 35 after booster vaccination. IgG titres are expressed as the reciprocal log₁₀ of the serum dilutions that yielded 50% of the absorbance value of the negative control wells in LPB-ELISA. IgG titres less than the sensitivity of the assay (0.9) were adjusted to 0.6 in the figure

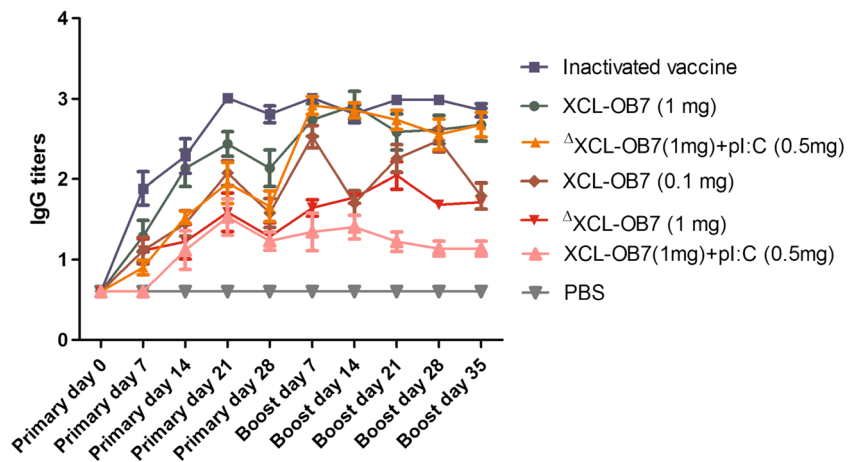
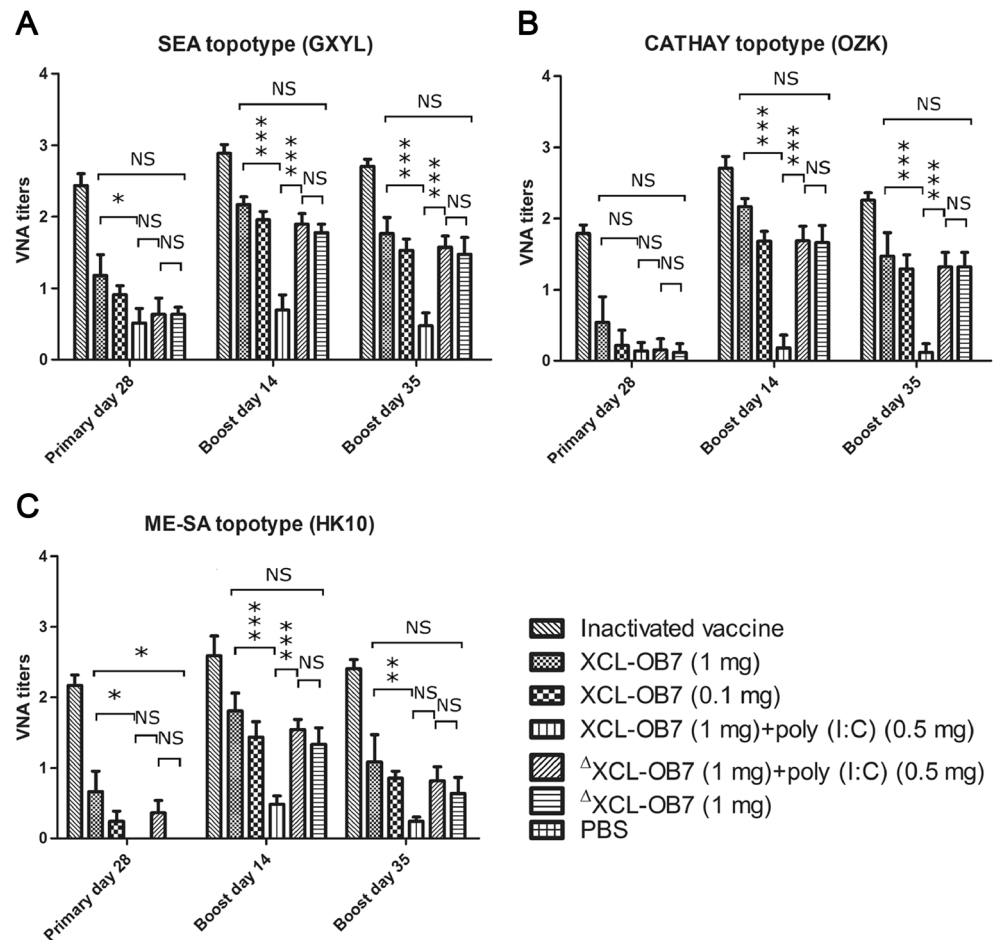


Fig. 5 Analysis of neutralizing antibody responses against three O type FMDV topotypes. Neutralizing antibody was detected for sera sampled at 28 days after primary vaccination and days 14 and 35 after booster vaccination. VNA titres are expressed as reciprocal log₁₀ of the max sera dilutions that neutralized 100 TCID₅₀ of FMDV strain O/Mya/98 (Southeast Asia topotype) (a), FMDV strain O/HN/CHA/09 (Cathay topotype) (b) and FMDV strain O/Tibet/99 (PanAsia topotype) (c). The differences between two groups are marked with asterisks (NS = $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)



results clearly indicated that poly (I:C) inhibits the VNA antibody response induced by XCL-OB7. However, VNA titres in group E cattle immunized with Δ XCL-OB7 plus poly (I:C) were slightly higher than those in group D immunized with Δ XCL-OB7, thus indicating that poly (I:C) did not inhibit VNA antibodies induced by Δ XCL-OB7. A significant difference in the VNA titres on days 14 ($P < 0.001$, Fig. 3a–c) and 28 ($P < 0.001$, Fig. 5a, b) after booster vaccination was observed between group E cattle immunized with Δ XCL-OB7 plus poly (I:C) and group C cattle immunized with XCL-OB7 plus poly (I:C). From the results of both IgG titres and VNA titres of immunized cattle, XCL1 and poly (I:C) appear to play opposite roles in inducing the antibody response in cattle vaccinated with FMDV multi-epitope protein vaccine.

Cattle vaccinated with poly (I:C) plus Δ XCL-OB7 showed a decreased FMDV NSP 3B antibody response after challenge

FMDV NSP 3B antibodies were detected at 10 DPC for cattle in all groups. The mean PIs were less than 50% in the Δ XCL-OB7 plus poly (I:C)-vaccinated cattle (group E) and inactivated vaccine-immunized cattle (group F), whereas in

the other groups, the mean PI values were all greater than 50% (Fig. 6). Compared with those in the PBS group, significantly lower NSP 3B antibodies were observed in group E and F cattle vaccinated with Δ XCL-OB7 plus poly (I:C) ($P < 0.01$) and inactivated vaccine ($P < 0.001$) (Fig. 6). These results indicated that poly (I:C) has a better antiviral immunological effect when combined with Δ XCL-OB7 than vaccination with only XCL-OB7.

Protection rate

After the FMDV challenge, the clinical score was recorded daily for 10 days. As shown in Table 3, the inactivated vaccine group cattle had no clinical signs of FMD during the entire period and obtained 100% protection. The XCL-OB7 (1 mg) (group A), XCL-OB7 (0.1 mg) (group B) and Δ XCL-OB7 plus poly (I:C)-vaccinated cattle (group E) had only one individual with slight clinical signs in each group, and the calculated protection rates were all 80%. The Δ XCL-OB7 (group D) and XCL-OB7 plus poly (I:C)-vaccinated cattle (group A) had two and three cattle with clinical signs, and the corresponding protection rates were 60 and 40%, separately. By contrast, all cattle vaccinated with PBS (group G) displayed severe clinical

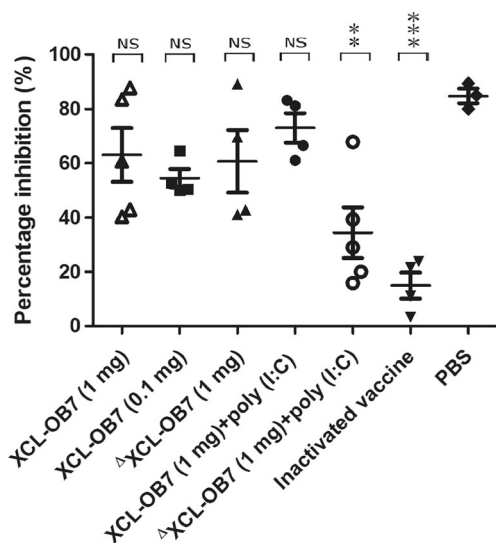


Fig. 6 Determination of the NSP 3B antibody levels at 10 days after FMDV challenge, using blocking ELISA. FMDV NSP 3B antibody levels were evaluated for sera collected at 10 DPC. $PI \geq 50\%$, NSP 3B antibody is present in the sample; $PI < 50\%$, NSP 3B antibody is absent in the samples. The NS antibodies in the PBS group were compared with those in the other immunized groups, and the significance is indicated with asterisks (NS = $P > 0.05$; ** $P < 0.01$; *** $P < 0.001$)

signs of FMD, and all were unprotected (Table 3). These results suggested that 0.1 and 1.0 mg of XCL1-fused FMDV multi-epitope protein antigens both provide sufficient protection for cattle to resist a 10,000 BID_{50} virulent viral challenge. Therefore, XCL1-fused antigen targeting to XCR1 would be a potential strategy to induce protective immunity of FMD vaccines in cattle.

Discussion

Cattle XCL1, the ligand of XCR1, is a chemokine family member of fewer than 100 aa. The XCL1 molecules produced by chemical synthesis (Le Brocq et al. 2014) or expressed in *E. coli* (Ioerger et al. 2007) have biological chemokine activity. The model antigen selected in this study was a multi-epitope protein of type O FMDV, which has been studied and has previously been found to have excellent immunogenicity in mice and pigs (Cao et al. 2013). Given the character of cattle XCL1 and the immunogenicity of the OB7 antigen, we designed the DC-targeted vaccine molecule by fusing cattle XCL1 with OB7. The XCL-OB7 fusion protein specifically bound to $CD26^+$ DC cells expressing the XCR1 receptor. Cattle immunized with 0.1 mg XCL-OB7 induced an antibody response level similar to that of 1 mg Δ XCL-OB7, thus indicating the potential to improve the immune response by targeting XCR1⁺ DC in cattle. To our knowledge, this is the first report that immunization with an XCL1 fusion protein containing the multi-epitope of FMDV significantly expands the humoral immune response in cattle.

Delivering antigens to DC via fusion with chemokines, such as CCL3 (Fredriksen and Bogen 2007), CCL5 (Fredriksen and Bogen 2007), CCL7 (Biragyn et al. 1999), CXCL10 (Biragyn et al. 1999) and MIP1 α (Ruffini et al. 2010), enhances the immune response in the absence of adjuvants. Most recent publications have also shown that targeting influenza virus haemagglutinin to human XCR1⁺ DC enhances the protective antibody response with less antigen endocytosis (Gudjonsson et al. 2017). In this work, cattle vaccinated with XCL-OB7 fusion protein alone produced a strong antibody response, thus suggesting effective antigen targeting to XCR1⁺ DC. However, the effective T cell response may have been low in the XCL-OB7 only-vaccinated cattle, because viral replication was not completely blocked in this group, as shown by detection of antibody against NSP 3B at 10 DPC, although four of five cattle were clinically protected after FMDV challenge. In contrast, the adjuvant poly (I:C) plus Δ XCL-OB7 produced good inhibition of viral replication, as revealed by a clear difference in NSP 3B antibody response after challenge. This result suggested that the cellular immune response also plays a role against FMDV infection in cattle.

Activation of DC by adjuvant was critical for ensuring an effective immune response to vaccine antigen. It has been reported that poly (I:C) strongly improves the immune response in mouse models and humans when it is incorporated with antigens targeting DC surface receptors, such as Clec9A and CD205 (Tuinstra et al. 2008; Njongmeta et al. 2012). However, in this study, when cattle were vaccinated with XCL-OB7 plus poly (I:C), the antibody response was significantly inhibited, and fewer cattle were protected after viral challenge. The detailed mechanism may be complex. We speculate that the structure of XCL-OB7 may be one of the reasons for this conflict. Human XCL1 has a unique metamorphic character, exhibiting a canonical monomeric form and an alternative dimeric form as well as an unfolding transition state under physiological solution conditions (Tuinstra et al. 2008). XCL1 in canonical form specifically binds and activates XCR1 on DC, whereas the alternative dimeric form adheres to glycosaminoglycans (GAGs) expressed on a variety of cell surfaces (Fox et al. 2016). Human XCL1 variant (V21C, V59C), which possesses an additional engineered disulphide bond, restricts the protein to the canonical monomeric form (Tuinstra et al. 2007). Similar modifications to cattle XCL1 in the XCL-OB7 molecule may promote the stability of the XCL1-bound antigen and improve the targeting specificity to XCR1⁺ DC, thus abolishing the conflicts with poly (I:C) adjuvant.

Vaccination still is key in FMD prevention. Antibody titres are related to protective immunity in cattle (Gullberg et al. 2013). Although the fusion protein XCL-OB7 and the inactivated vaccine both induced identical high antibody titres, as determined by LPB-ELISA, the protection rates between the two groups were different. This result may be

explained by the effectiveness or quality of the antibodies elicited by different antigens, as revealed by the different VNA titres in these two groups. The cellular response elicited by mature DC cells may play important roles in producing high-avidity or high-quality VNA (Cao et al. 2013; Gerdts et al. 2013; Toka and Golde 2013). Comparing the fusion protein groups with the inactivated vaccine group nonetheless indicated differences in the VNA level and protection. This observation may be attributed to differences in antigenic structure between epitope antigen and whole virus capsid antigen. Targeting a native and conformational antigen may be a better method to induce a high-affinity neutralizing antibody response. In FMD, neutralizing antibody induced by a conformational antigen may be more powerful and consequently afford effective protection against a viral challenge.

In summary, cattle XCL1-mediated antigen targeting to XCR1⁺ DC is a promising strategy to improve the pathogen-specific acquired immune response. Given the present observations, optimization of the molecular structure, including the improvement of XCL1-targeting specificity and selection of suitable antigen, should be further explored, and this strategy may be used to develop potent DC-targeted vaccines against important animal diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethics statement All cattle experiments were performed in a biosafety level 3 laboratory at Lanzhou Veterinary Research Institute (LVRI), Chinese Academy of Agricultural Sciences (CAAS). The study was approved by the Animal Ethics Committee of CAAS. The cattle were acclimated for at least 1 week before experimentation and humanely bred during the experiment. All cattle used in this study were euthanized at the end of the experiment.

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