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Microspheres-prime/rMVA-boost vaccination enhances humoral and cellular immune response in IFNAR(−/−) mice conferring protection against serotypes 1 and 4 of bluetongue virus

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ABSTRACT

Bluetongue virus (BTV) is the causative agent of bluetongue disease (BT), which affects domestic and wild ruminants. At the present, 27 different serotypes have been documented. Vaccination has been demonstrated as one of the most effective methods to avoid viral dissemination. To overcome the drawbacks associated with the use of inactivated and attenuated vaccines we engineered a new recombinant BTV vaccine candidate based on proteins VP2, VP7, and NS1 of BTV-4 that were incorporated into avian reovirus muNS-Mi microspheres (MS-VP2/VP7/NS1) and recombinant modified vaccinia virus Ankara (rMVA). The combination of these two antigen delivery systems in a heterologous prime-boost vaccination strategy generated significant levels of neutralizing antibodies in IFNAR(−/−) mice. Furthermore, this immunization strategy increased the ratio of IgG2a/IgG1 in sera, indicating an induction of a Th1 response, and elicited a CD8 T cell response. Immunized mice were protected against lethal challenges with the homologous serotype 4 and the heterologous serotype 1 of BTV. All these results support the strategy based on microspheres in combination with rMVAs as a promising multiserotype vaccine candidate against BTV.

1. Introduction

Bluetongue (BT) is a vector-borne viral disease of domestic and wild ruminants caused by Bluetongue virus (BTV), one of the most important livestock pathogens. (Saegerman et al., 2008). The disease is characterized by vascular injury that results in tissue necrosis, hemorrhage, and edema in ruminants (Maclachlan et al., 2009) and IFNAR(−/−) mice (Marin-Lopez et al., 2016). BTV belongs to the genus *Orbivirus* within the family *Reoviridae* with a non-enveloped virion and an icosahedral capsid. The genome is composed of double-stranded RNA (dsRNA) distributed in ten segments, encoding for seven structural proteins (VP1-VP7) and five/six nonstructural proteins (NS1, NS2, NS3/3A, NS4 and NS5) (Ratinier et al., 2011; Roy, 1992; Stewart et al., 2015). The virus is mainly transmitted between ruminant hosts through certain species of hematophagous *Culicoides* (*Diptera, Ceratopogonidae*) midges (Mellor et al., 2000). To date, 27 serotypes of BTV have been identified (Zientara et al., 2014) with two further putative/novel BTV serotypes identified so far (Maan et al., 2015). Since 1998 at least 8 serotypes have been detected within the European Union (Zientara and Sanchez-Vizcaino, 2013) and the introduction of new BTV serotypes is a permanent threat to the region. The constant arrival of new BTV serotypes re-emphasizes the importance of making multiserotype and more effective vaccines than those that are currently available. Although conventional vaccines have controlled or limited BTV spreading in the past, they cannot address the need for cross-protection among serotypes (Marín-López et al., 2016). Furthermore, modified live virus (MLVs) and inactivated vaccines currently used in Europe do not allow the differentiation of infected from vaccinated animals (DIVA).

In previous work in our laboratory, we demonstrated that the immunization of IFNAR(−/−) mice with an experimental subunit

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vaccine based on VP2, VP7, and NS1 proteins of BTV-4 incorporated into avian reovirus (ARV) muNS-Mi microspheres (MS-BTV) and without adjuvant protected against a homologous challenge with a lethal dose of BTV-4. Furthermore, the vaccine partially cross-protected against a heterologous challenge with a lethal dose of BTV-1 (Marin-Lopez et al., 2014). Immunized mice generated significant levels of neutralizing antibodies specific to BTV-4 and T cell responses, predominantly $CD4^{+}$. Cellular immune responses, specially cytotoxic T lymphocyte responses (CTL) have been demonstrated to be important in clearance of homologous and heterologous serotypes of BTV (Jeggo et al., 1985) and *in vitro* studies showed that BTV-specific ovine and murine CTL were cross-reactive among serotypes (Jones et al., 1996; Takamatsu and Jeggo, 1989). Furthermore, CD8 T cell epitopes have been identified in sheep and the murine model of infection from VP7 and NS1 proteins of BTV-8 (Rojas et al., 2011, 2014) and CTL epitopes have been described in sheep from VP2 and NS1 proteins of BTV-1 (Andrew et al., 1995; Janardhana et al., 1999).

In the present work, we developed a new vaccination strategy based on a heterologous prime-boost strategy with the particulate subunit vaccine MS-BTV and with the viral vaccine vector MVA expressing BTV antigens. Particulate immunogens are best for stimulating both humoral and cellular immune responses (Roy, 1996). Moreover, they are cheap, very stable, do not require the use adjuvants due to their intrinsic adjuvant effect, and are biologically safe. On the other hand, it has been described the use of rMVAs as a potent inductor of CD8 T cellular immune responses when used as a heterologous boost vaccination following a strong priming agent expressing the same antigen (Cottingham and Carroll, 2013; Whelan et al., 2009). In order to improve the serotype cross-protection of the experimental vaccine, we focused the vaccine composition on the VP2, VP7 and NS1 proteins of BTV-4. These proteins have been generally described to induce cross-serotype helper T-cell or cytotoxic T-cell responses.

2. Materials and methods

2.1. Virus and cells

Chicken embryo fibroblasts (DF-1) (ATCC, Cat. No. CRL-12203) and Vero (ATCC, Cat. No. CCL-81) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2mM glutamine, 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. Insect cells High Five (Invitrogen) were grown in TC-100 medium supplemented with 2mM glutamine, 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. BTV serotype 4 (SPA2004/02) (BTV-4) isolated from whole sheep blood and serotype 1 (ALG2006/01) (BTV-1) isolated from sheep spleen were used in the experiments. BTV and MVA virus stocks and titrations were performed as previously described (Calvo-Pinilla et al., 2009).

2.2. Mice

IFN $\alpha/\beta R^{o/o}$ IFNAR(−/−) 129/Sv mice and wild type 129/Sv mice were purchased from B&K Universal Ltd UK. Eight-week old male mice were used throughout. Upon reception, the mice were held for 7 days for acclimatization under pathogen-free conditions in the biosafety level 3 (BSL3) animal facility at the Centro de Investigación en Sanidad Animal (INIA-CISA), Madrid. All experiments with live animals were performed under the guidelines of the European Community (86/609) and were approved by the ethical review committee at the INIA-CISA and Comunidad de Madrid (Permit number: PROEX 037/15).

2.3. Generation of muNS-Mi microspheres (MS-VP2/VP7/NS1), and recombinant MVAs expressing VP2, VP7 and NS1 BTV-4 proteins (rMVA-VP2/VP7/NS1)

The production and purification of muNS-Mi-VP2/VP7/NS1 microspheres using the baculovirus expression system and the generation of rMVA-VP2/VP7/NS1 have been previously described (Brandariz-Nunez et al., 2010; Marin-Lopez and Ortego, 2016; Marin-Lopez et al., 2014).

2.4. In silico T CD8 epitope prediction

Amino acid sequences for the non-structural NS1 protein (NCBI accession number: AM778441.1) and structural VP2 protein (NCBI accession number: KP821068.1) of BTV-4 (SPA2004/02) were analized using three prediction algorithms available on the web: Immuno Epitope DataBase (IEDB Analysis Resource) (www.iedb.org), SYFPEITHI (www.syfpeithi.de), and BIMAS (www.bimas.cit.nih.gov/) for the H-2-Db MHC class I for 129/Sv mice to identify T CD8 epitopes that could be good binders to H-2-Db MHC. Theoretical T-cell epitopes were chosen by a combination of the best score in these databases (Table 1).

2.5. Peptides

The selected peptides (Table 1) were purchased from Proteogenix (Schiltigheim, France) and Sigma-Aldrich (The Woodlands, USA). Peptides were >95% pure (HPLC%). They were re-constituted in DMSO or Dimetilformamide according to the manufacturer instructions and kept frozen at −20 °C until use. All peptides were diluted in culture media: RPMI-1640 (Gibco, GreenIsland, NY) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) antibiotic-antimycotic solution (Gibco), 1% (v/v) non-essential aminoacids and 2 mM glutamine (Gibco).

2.6. Studies of cellular immune response in IFNAR(−/−) mice

Three groups of IFNAR(−/−) mice (n = 4) were immunized following a homologous prime-boost regimen with rMVA-VP2, rMVA-NS1 or MVA-wild type (non-immunized group) three weeks apart. All animals were sacrificed at 14 days post-booster and their spleens were harvested for analysis by ELISPOT and intra-cellular cytokine staining (ICCS) as previously described (de la Poza et al., 2015; Marin-Lopez et al., 2014).

2.7. Prime-boost immunization and challenge with BTV in $IFNAR^{(-/-)}$ mice

Groups of five IFNAR(−/−) mice were immunized by homologous prime-boost vaccination with MS-VP2/VP7/NS1 (50 μg of each

Table 1
Peptides selected from the epitope prediction in H-2 Db haplotype.

Protein	Position	Sequence	IEDB	SYFPEITHI	BYMAS
VP2	147	IYYDYFPL	0.3	–	–
	694	YLIQNSTGL	0.8	25	720.000
	731	LNVINFLPL	0.5	23	720.000
	743	VQDNISYW	0.8	–	–
	782	KSFYNFIRF	1.4	23	3120
	139	AKTANADTI	0.4	26	220.000
NS1	125	SALVNSERV	0.2	28	51.480
	152	GQIVNPTFI	0.2	28	720.000
	14	YANATRTFL	0.7	16	2.600
	222	IQLINFLRM	0.2	25	792.000

Using a combination of three epitope T prediction algorithms (IEDB, SYFPEITHI and BYMAS), peptides from VP2 and NS1 proteins of BTV-4 were selected and synthesized.