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Antibody responses to chimeric peptides derived from parasite antigens in mice and other animal species

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ABSTRACT

Peptide vaccines constitute an interesting alternative to classical vaccines due to the possibility of selecting specific epitopes, easy of production and safety. However, an inadequate design may render these peptides poorly immunogenic or lead to undesirable outcomes (e.g., formation of B neoepitopes). As an approach to vaccine development, we evaluated the antibody response to chimeras composed of two or three known B epitopes from *Trichinella* and *Fasciola*, and several linkers (GSGSG, GPGPG and KK) in species as different as mice, sheep and turbot. All these species could mount an effective immune response to the short chimeric peptides. Nevertheless, this response depended on several factors including a favorable orientation of B-cell epitopes, adequateness of linkers and/or probability of formation of T neoepitopes. We also observed that, at least in mice, the inclusion of a decoy epitope may have favorable consequences on the antibody response to other epitopes in the chimera.

1. Introduction

Vaccines are powerful tools for reducing the mortality and morbidity caused by infectious diseases. According to their composition, they are traditionally classified as live-attenuated vaccines, which contain laboratory-weakened versions of the original pathogen, inactivated vaccines, where the pathogen is killed, and subunit vaccines, composed of one or more molecules present in the pathogen (Kallerup and Foged, 2015). Subunit vaccines also include singular antigenic preparations, such as “toxoids” (i.e., inactivated toxins that retain all or part of the original antigenicity), bacterial capsular polysaccharides, whole proteins or fragments, and chimeric antigens, where antigenic parts from one or more pathogens are artificially combined in a single chimeric molecule (either synthetic or recombinant). Moreover, the selected antigenic parts conforming the chimeric antigens may be limited to a set of protective B- and T-cell epitopes (Blanco et al., 2013; Sette and Fikes, 2003).

Compared with classical vaccines prepared with live-attenuated or inactivated pathogens, peptide vaccines are generally less antigenic and require the use of potent adjuvants (Purcell et al., 2007), but they also offer some advantages including: i) safety of use due to the absence of infectious material, ii) no risk of integration or recombination as might occur with DNA vaccines, iii) the possibility of excluding deleterious sequences from full-length antigens, and iv) accurate delineation of the immunogen and easy scale up, transport and storage (Blanco et al., 2013; Purcell et al., 2007). For these reasons, peptide vaccines are being investigated in fields as diverse as in the treatment of cancer, immunocastration of animals, autoimmune diseases, as well as in the prevention of viral, bacterial and parasitic infectious diseases (Purcell et al., 2007). In addition, investigation of the recognition of peptide antigens is also of interest in the field of immunopeptidomics, to assess the repertoire of peptides presented at the cell surface by the major histocompatibility complex[1] (MHC)-I and MHC-II molecules in humans and animal species (Shao et al., 2018), and as effective targets for

Abbreviations: APC, antigen-presenting cells; CFA, complete Freund’s adjuvant; CL, cathepsin L; IFA, incomplete Freund’s adjuvant; MHC, major histocompatibility complex; OD, optical density; RT, room temperature; TD, T-dependent

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Short name	Extended name	Sequence	Length
D1	US9-(GSGSG)-CL1	MTFSVPISGSGSGAVPDKIDWRESGYVTEVKDQGNC	36
D2	US9-(GSGSG)-CL2	MTFSVPISGSGSGAVPESIDWRDYYYVTEVKNQGQC	36
D3	US9-(GSGSG)-CL5	MTFSVPISGSGSGAVPDRIDWRESGYVTEVKDQGGC	36
D4	CL2-(GSGSG)-MF6p2	AVPESIDWRDYYYVTEVKNQGQCGSGSGVTKAYEKARDRA	40
D5	CL2-(GSGSG)-MF6p4	AVPESIDWRDYYYVTEVKNQGQCGSGSGLNRLTDRLEKYA	40
D6	US9-(GPGPG)-CL2	MTFSVPISGPGPGAVPESIDWRDYYYVTEVKNQGQC	36
D7	US9-(KK)-CL2	MTFSVPISKKAVPESIDWRDYYYVTEVKNQGQC	33
D8	CL2-(KK)-US9	AVPESIDWRDYYYVTEVKNQGQCKKMTFSVPIS	33
T1	US9-(GSGSG)-CL2-(KK)-MF6p2	MTFSVPISGSGSGAVPESIDWRDYYYVTEVKNQGQCKKVTKAYEKARDRA	50
T2	US9-(KK)-CL2-(KK)-MF6p2	MTFSVPISKKAVPESIDWRDYYYVTEVKNQGQCKKVTKAYEKARDRA	47
T3	US9-(KK)-MF6p2-(KK)-CL2	MTFSVPISKKVTKAYEKARDRAKKAVPESIDWRDYYYVTEVKNQGQC	47
sMF6p	sMF6p/FhHDM-1	SEESREKLRESGRKMVKALRDAVTKAYEKARDRAMAYLAKDNLGEKITEVITILLNRL TDRLEKYAGN	68
sMF6a	sMF6p/FhHDM-1 (amino)	SEESREKLRESGRKMVKALRDAVTKAYEKARDR	33
TSP12	*Trichinella* (gp53)	AMKEMTFSVPIS	12
rUS9-FhpCL1	US9-(gb|CCA61803.1)	MRGSHHHHHHGSMTFSVPISSNDDLWHQWKRMYNKEYNGDDDQHRRNIWEKNVKHIQE HNLRHDLGLVTYTLGLNQFTDMTFEEFKAKYLTEMSRASDILSHGVPYEANNRAVPDK IDWRESGYVTEVKDQGNCGSCWAFSTTG.....ASLPMVARFP	331

Fig. 1. List of peptide sequences used for immunization and/or as targets in ELISA. The linker sequences between epitopes from chimeras were typed in red. The epitope sequence recognized by mAb US9 from the *T. spiralis* gp53 sequence was typed in cyan. The sequence corresponding to the CL2 epitope from the *F. hepatica* cathepsin L2 (gb| KY464953) was marked in cyan. Differences between CL1 (gb|CCA61803.1), CL2 and CL5 (gb|KY392883) epitopes were underlined. The MF6p2 and MF6p4 epitopes derived from the *Fasciola* sMF6p/FhHDM-1 protein were marked in yellow and grey, respectively. The poly-HIS region in the chimera US9-rFhpCL1 was marked in green. The number of amino acid residues corresponding to each peptide/protein is indicated on the right. A truncated, not shown, region in the chimera US9-rFhpCL1 was indicated by a succession of points. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

immunoassays (Faria et al., 2015; Mucci et al., 2017; Santos et al., 2017).

Linear chimeric peptide antigens can be constructed putting a selection of epitopes together in tandem (Golvano et al., 1990) but more conveniently using linkers, i.e., spacer sequences, between them. Natural linkers are short amino acid sequences connecting different domains into a single protein (Reddy Chichili et al., 2013). It was reported that Gly-rich regions provide flexibility and, consequently, facilitate interactions between domains, while Pro-rich regions add rigidity and unfavorable interactions (Reddy Chichili et al., 2013; Yu et al., 2015). Gly-rich linkers combined with either Ser (e.g., GSGSG) or Pro (e.g., GPGPG) were used in several studies to design chimeric vaccines (Liu et al., 2005; Nezafat et al., 2016; Wen et al., 2015). In addition, since pairs of basic residues (KK, KR, RR) were early related with peptide positions cleaved by cathepsin B during antigen presentation to T-cells (Takahashi et al., 1989), several authors used the linker KK to join T and B epitopes (Gu et al., 2017; Sarobe et al., 1993; Yano et al., 2005) to minimize the formation of neoepitopes, an issue that is more relevant as the length of the linker sequences increases (Schubert and Kohlbacher, 2016). Besides the possible formation of B neoepitopes, the combination of linker sequences with only apparent B epitopes can also lead to the formation of new T-cell epitopes (T neoepitopes) able to be recognized by the highly polymorphic MHC-II molecules on antigen-presenting cells (APC), depending on the particular set expressed by the individual being immunized. Finally, another factor to consider in the construction of chimeric peptide antigens is to define which is the best number and position to place each of the T and B cell epitopes along the peptide sequence. In this sense, it was reported that orientation (=polarization) of epitopes is of major importance to obtain optimal responses to chimeric epitope vaccines (Blanco et al., 2013; Golvano et al., 1990; Lowenadler et al., 1992).

In the field of Parasitology, chimeric epitope vaccines are being explored to confer protection against relevant protozoan and helminth diseases (Anugraha et al., 2015; Gu et al., 2017; Kaba et al., 2008; Pinheiro et al., 2014). However, the rationale of chimeric epitope vaccine constructions regarding linker selection, epitope polarization, formation of T neoepitopes and variation of the immune response between different species were poorly investigated. Consequently, and to gain insight in this area, in the present study we investigated how chimeric antigens composed of two or three B epitopes derived from the parasites *Trichinella spiralis* and *Fasciola hepatica*, and joined by the same or different linkers, induced the formation of IgG antibodies in mice. Further, the antibody response to a selection of chimeras was investigated in other species as different as sheep and turbot.

2. Material and methods

2.1. Ethics statement

All experiments with animals (mice, sheep and turbot) were done in strict accordance with Spanish and EU legislation (Law 32/2007, R.D. 53/2013 and Council Directive 2010/63/EU). For all the procedures, the animals were anaesthetized/euthanized as indicated below.

2.2. Synthetic peptides and proteins

All synthetic peptide sequences including chimeras D1-D8 and T1-T3, the complete mature sequence (sMF6p) of the *F. hepatica* MF6p/FhHDM-1 heme-binding protein (Martínez-Sernández et al., 2014, 2017a) and its truncated N-terminal region (sMF6a) were all obtained solid (95% pure, as determined by mass spectrometry) from GeneCust Europe (Dudelange, Luxembourg). The 12-mer (TSP12) peptide corresponding to the N-terminal region (A^{29}-S^{40}) of the gp53 antigen from *T. spiralis* (gb|CAD86781.1) and *T. britovi* (gb|CAD86782.1), that contains the epitope recognized by mAb US9 (US9 epitope), was synthesized (> 90% pure) on a model 433 A peptide synthesizer (Applied Biosystems, Foster City, CA). Its identity was confirmed by mass spectrometry (Reflex, Brucker Daltonics, Bremen, Germany) as reported previously (Perteguer et al., 2004). The amino acid sequences corresponding to each peptide/protein are shown in Fig. 1.

2.3. Cloning, subcloning and production of the rUS9-FhpCL1 gene

The *F. hepatica* procathepsin L1 (FhpCL1) corresponding to the L33771.1 gene was amplified by PCR and further subcloned into the pQE vector (pQE30-FhpCL1-K22) as described by Muiño et al. (2011). Then, a chimera (rUS9-FhpCL1) containing the complete FhpCL1 gene and the amino acids (MTFSVPIS) corresponding to the mAb US9

epitope (Perteguer et al., 2004) was constructed. The chimera assembly was performed by standard PCR, using standard polymerase (Biotools, Madrid, Spain) and the primers rUS9-FhCL1-F (5′_30_US9_BamH1): 5′-*GGATCC*ATGACATTTTCAGTTCCTATTTCCTCGAATGATGATTTGTG GCAT CAGTGGAAGCG-3′ and rUS9-FhCL1-R (3′_30_Sal1): 5′-*GTCGAC* CGGAAATCGTGCCACCATCGG-3′. The plasmid pQE30-pFhCL1-K22 DNA (K2.2) was used as template at a 1/1000 dilution to amplify the chimera. Directional cloning in the pQE expression vector (Qiagen; Qiagen Iberia SL, Madrid, Spain) was performed using the BamH I and Sal I restriction endonucleases (Roche Molecular Systems, Pleasanton, CA) and following standard protocols (Sambrook and Russel, 2001). Positive recombinant plasmids were selected by standard PCR with the universal primers pQE-forward and pQE-reverse. Subsequently, the plasmid rUS9-FhpCL1 DNA extracted from the gel (QIAquick Gel Extraction, Qiagen) was automatically sequenced using fluorescence-base labeling with ABI PRISM system (Perkin Elmer, Langen, Germany) to rule out any sequences errors. The universal pQE forward and reverse primers were used in the sequencing process. Recombinant pQE30-rUS9- FhpCL1 was transformed into XL1 Blue cells (Agilent, Santa Clara, CA), which were mixed with 20% of sterile glycerol and stored at −80 °C.

2.4. Obtaining and purification of rUS9-FhpCL1

The M15 [pREP4] *E. coli* strain (Qiagen) was transformed with 10 ng of recombinant pQE30-rUS9-FhpCL1 DNA. A colony of transformed cells was grown in 1 L of LB medium at 37 °C with shaking (200 rpm/min) until reaching an OD_{600} of 0.5 and the rUS9-FhpCL1 protein expression induced by the addition of 1 mM IPTG to the cell culture and further incubation for 4 h. Then, the cells were harvested by 20 min centrifugation at 5000 *g* and stored frozen during 24 h at -30 °C. Afterwards, the pellet was resuspended in 20 mL of B-PER™ Bacterial Protein Extraction Reagent (Thermo Fisher Scientific, Barcelona, Spain) supplemented with lysozyme and DNase I, as indicated by the supplier, pipetted up and down until the suspension was homogeneous and incubated with shaking (150 rpm/min) for an additional 30 min at room temperature (RT). To separate insoluble from soluble proteins, the cell lysate was centrifuged at 15,000 *g* for 15 min and the inclusion bodies containing rUS9-FhpCL1 were purified by affinity chromatography with HIS-Select Nickel Affinity Gel (Sigma–Aldrich, Madrid, Spain) under denaturing conditions (8 M urea), as indicated by the supplier. The rUS9-FhpCL1 was refolded as previously described (Muiño et al., 2011) with a few modifications. The rUS9-FhpCL1 eluted from the affinity column was pretreated with 10 mM DTT for 1 h at RT, and subsequently diluted at a ratio of 1/10 in TBS (50 mM Tris, 150 mM NaCl, pH 8) plus 10 mM cysteine, 1 mM cystine and 1 mM EDTA. Once dialyzed against TBS (pH 8), the rUS9-FhpCL1 was dialyzed against PBS and concentrated by membrane-filtration in an Amicon stirred ultrafiltration cell equipped with a Filtron Omega Series membrane (10 kDa nominal molecular weight limit; Pall Corporation, Port Washington, NY). Finally, the recombinant protein was filtered through a 0.22 µm filter and stored at −80 °C until use. The protein concentration was determined with the Micro BCA Protein Assay kit (Pierce; Thermo Fisher Scientific)

2.5. Animals and immunizations

BALB/c mice (8–12 months old, female; n = 8–12 animals per group) were immunized with a single injection of synthetic or recombinant chimeric antigens dissolved in PBS and emulsified 1:1 v/v in Complete Freund's Adjuvant (CFA; Sigma-Aldrich). Each mouse was immunized subcutaneously in the back (lumbar region) with 100 µL of the emulsion containing 50 µg of the antigen. One month after immunization, the animals were exsanguinated by heart puncture and the blood from lots of 4 animals pooled before obtaining the sera (see the end of this section). Mice were anesthetized with isoflurane before immunizations and blood withdrawals.

Galician autochthonous outbred lambs (5–6 months old, female; n = 3 animals per group) were immunized subcutaneously, in the axillary region, with 1 mL of a formulation containing 150 µg of the synthetic chimeric antigens (D2, T2 or T3), diluted in PBS plus 1 mg of Quil-A (*Quillaja saponaria*, Accurate Chemical & Scientific Corp., Westbury, NY, USA). Blood samples from each animal were collected 1 month after the immunization and processed to obtain the sera as indicated at the end of this section. The animals were reared and housed at the Centro de Investigaciones Agrarias de Mabegondo (INGACAL-CIAM), A Coruña (Spain). At the end of the experiments, the animals were sedated with xylazine hydrochloride (Rompun; Bayer, Mannheim, Germany) and then euthanized with an intravenous injection of a solution containing embutramide, mebezonium iodide and tetracaine hydrochloride (T61; MSD Animal Health, Salamanca, Spain). All procedures regarding animal handling were approved by the Animal Welfare Committee of INGACAL-CIAM.

Turbots (*Scophthalmus maximus*) of approximately 30–40 g body weight (n = 20 animals per group) were used for immunization with chimeric T2 and T3 antigens. The protocol included a first intraperitoneal (Ip) immunization with the corresponding chimera dissolved in PBS and then emulsified 1:1 v/v in CFA (day 0), and a second immunization prepared in the same conditions but emulsified in incomplete Freund's adjuvant (IFA) and injected by the same route one month later. In parallel, a group of 10 turbot were injected with the adjuvant alone and used as control. Each turbot from the immunized group received a 200 µL injection containing 50 µg of the corresponding antigen. At the end of the experiment (day 60) the animals were bled by caudal vein puncture. As for mice, blood from lots of 10 animals were pooled and sera was obtained as indicated below. For all the procedures, the fishes were anaesthetized in bath with benzocaine (50 mg/L).

For all animals (mice, sheep and turbot), the collected blood was allowed to clot for 12 h at 4 °C, centrifuged at 3000 x g for 5 min, and then the sera collected, diluted 1:1 (v/v) with glycerol, and stored at −30 °C until use.

2.6. Production and purification of mAbs US9 and UR3

Hybridoma cells secreting mAbs US9 and UR3 were obtained as previously described (Estévez et al., 1994a; Perteguer et al., 2004; Romarís et al., 2002). The secreting hybridoma cells were grown intraperitoneally in pristan-primed BALB/c mice, and the anti-*Trichinella* (US9) and anti-turbot (UR3) IgG1/κ antibodies were isolated from the ascitic fluid by affinity chromatography on a protein G column (HiTrap Protein G, GE Healthcare, Madrid, Spain) according to the manufacturer's protocol.

2.7. Indirect ELISA to measure whole IgG antibodies to chimeric antigens in mice and sheep

The wells of ELISA plates (Greiner Bio-One; Soria-Melguizo, Madrid, Spain) were coated with 100 µL of each one of the antigens described above, diluted at 10 µg/mL in PBS, incubated overnight at 4 °C, washed three times with PBS and blocked with 200 µL/well of 1.5% sodium caseinate in PBS for 1 h at RT. Aliquots of 100 µL of mice or lambs sera diluted 1/100 in PBS-T-SM (PBS containing 0.05% Tween-20 and 1% of dry skimmed milk) were added to each well of the plates, and incubated for 1 h at RT with orbital shaking at 750 rpm. The plates were then washed five times with PBS-T, and bound IgG antibodies were detected with either HRP-conjugated goat anti-mice IgG polyclonal antibodies (Bio-Rad, Madrid, Spain; dilution 1/3.000 in PBS-T-SM) or HRP-conjugated mouse anti-sheep/goat IgG monoclonal antibodies (Sigma–Aldrich; dilution 1/30.000 in PBS-T-SM). Once incubated for 30 min at RT with shaking at 750 rpm, the plates were washed and incubated with 100 µL/well of the enzyme substrate OPD (SigmaFast OPD, Sigma-Aldrich). Finally, the reaction was stopped with 3 N H_2SO_4 after 20 min at RT and the optical density (OD) was measured at

492 nm in an ELISA reader (Titertek Multiskan, Flow Laboratories, McLean, VA).

2.8. Indirect ELISA to measure IgG antibody subclasses to chimeric antigens in mice

ELISA plates were incubated with the corresponding chimeric antigen and blocked as indicated in the previous method. Then, 100 µL aliquots of each pooled sera diluted 1/100 in PBS-T-SM were added to each well of the plates and incubated for 1 h at RT with orbital shaking at 750 rpm. Afterwards, the plates were washed five times with PBS-T, and bound mouse IgG antibodies of each subclass were firstly detected using a rabbit anti-mouse isotyping kit (Bio-Rad) and, after a washing step as above, with HRP-conjugated goat anti-rabbit IgG polyclonal antibodies (Bio-Rad; dilution 1/3.000 in PBS-T-SM). Finally, the OD was read at 492 nm as indicated above.

2.9. Indirect ELISA to determine serum antibody levels to chimeric antigens in turbot

The levels of serum antibodies to the chimeric peptides were determined by indirect ELISA as previously described (Estévez et al., 1994b; Piazzon et al., 2008) with some modifications. ELISA plates were incubated with the corresponding chimeric antigen and blocked as indicated above for mice and sheep. Then, 100 µL aliquots of each pooled turbot sera diluted 1/100 in PBS-T-SM were added to each well of the plates and incubated for 30 min at RT with orbital shaking at 750 rpm. The plates were then washed five times with PBS-T, and 100 µL aliquots of the UR3 anti-turbot monoclonal antibody diluted 1/1000 in PBS-T-SM were added to each well of the ELISA plates. Afterwards, plates were washed with PBS-T and bound mouse antibodies were detected with HRP-conjugated rabbit anti-mouse Ig (Dako, Barcelona, Spain) diluted 1:2000 in PBS-T-SM and incubated for 30 min at RT with orbital shaking at 750 rpm. After five washes with PBS-T, the plates were revealed as indicated above for mice and sheep.

2.10. Bioinformatics tools

The PredBALB/c bioinformatics tool (http://cvc.dfci.harvard.edu/balbc/; Zhang et al., 2005) was used for prediction of peptide binding to class-II MHC molecules (I-A and I-E) from BALB/c mouse (H-2^d). According to the authors, this tool utilizes quantitative matrices validated using experimentally determined binders and non-binders, as well as in vivo studies with viral proteins. The BcePred bioinformatics tool (Saha and Raghava, 2004), based on physicochemical properties of the amino acid sequence (http://crdd.osdd.net/raghava/bcepred/) was used for prediction of linear B-cell epitopes in the *Fasciola* MF6p/FhHDM-1 protein. Peptide parameters as theoretical MW, aliphatic indices and grand average of hydropathicity (GRAVY) values were calculated using the ProtParam bioinformatics tool from Expasy (https://www.expasy.org/resources).

3. Results and discussion

3.1. Antibody responses induced by diepitopic B-chimeras derived from helminth sequences in mice

It has been reported that an in vivo T-dependent (TD) antibody response can be achieved by combining into a single molecule one T- and one B- contiguous epitopes (Brumeanu et al., 1997; Sakurai et al., 1993). However, when both classes of epitopes overlap, the immunogenicity of the B-cell epitope may not necessarily be impaired since peptide binding to the B-cell Ig surface receptor and to MHC molecules are two independent events (Harris et al., 1996). Consequently, certain linear peptide sequences, although initially classified as B-cell epitopes, may also meet the characteristics of T-cell epitopes if they are able to trigger a T-B cell cooperation. Moreover, when chimeric peptides are constructed by combining several B-epitopes separated by linker sequences, it may also occur that those linkers lead to the formation of junctional epitopes (neoepitopes) able to be recognized by B- and/or T-cell antigen receptors.

To gain insight into the recognition of linear chimeric B-epitopes derived from helminth antigens in different animal species, we started this study testing diepitopic chimeras in mice. These chimeras consisted of two previously reported B-epitopes: the hydrophobic sequence MTFSVPIS (GRAVY = 1.188) belonging to the *gp53* protein from *T. spiralis* and recognized by mAb US9 (US9 epitope) (Perteguer et al., 2004; Romarís et al., 2002, 2003), and a second sequence (23-mer) derived from the N-terminal region of *F. hepatica* cathepsins L (CL; Cornelissen et al., 1999), both connected by the flexible GSGSG linker (de Souza et al., 2013). Also, since *F. hepatica* adults express three clades of CL (CL1, CL2 and CL5; Robinson et al., 2008) and these molecules showed differences in antigenicity as targets in ELISA (Martínez-Sernández et al., 2018), we tested diepitopic chimeras containing the US9 epitope linked to each of the above CL-derived epitopes (see sequences D1, D2 and D3 in Fig. 1). For a better comparison, we also investigated the antibody response of mice to a recombinant chimera (rUS9-FhpCL1, 313 residues) expressed in *E. coli*, which contains the US9 epitope after the 6XHis tag and is followed by the whole *F. hepatica* procathepsin L1, excluding the signal peptide (see Fig. 1).

The data in Fig. 2 show the antibody titers obtained in mice immunized with chimeras D1, D2, D3 or rUS9-FhpCL1. It can be observed that the antibody responses to the whole chimeras D1, D2 and D3 (Fig. 2A–C) were excellent (titers > 1/25,000) in spite of the fact that these animals only received a single immunization in FCA. On the contrary, none of the chimeras tested induced an antibody response to the US9 B-epitope (contained in TSP12 peptide; Fig. 2D), which could be related to its positioning at the N-terminal region of the chimeras or with a poor immunogenicity of this epitope. The effect of the orientation of B- and T-epitopes in chimeric (dimeric) antigens on the specific antibody responses was investigated by several authors in the past with

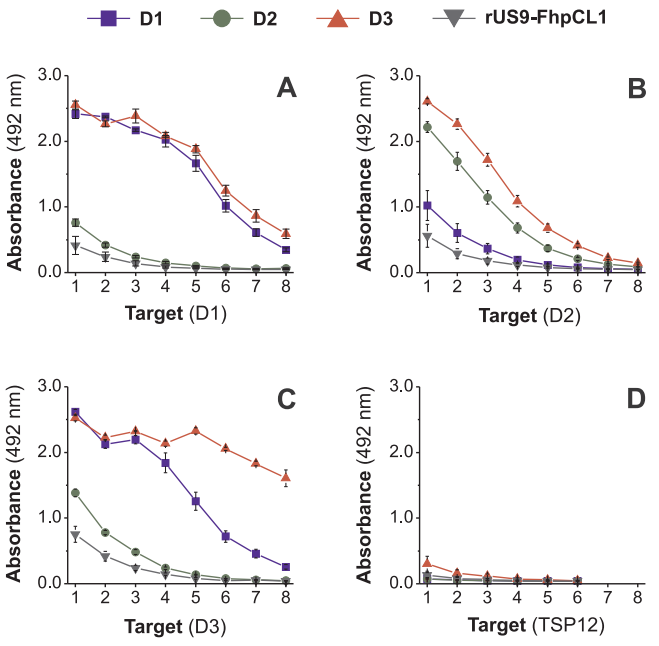

Fig. 2. Whole IgG antibody titers obtained in BALB/c mice (10 animals per group) immunized with chimeras D1, D2, D3 or rUS9-FhpCL1 in CFA. Dilutions of sera from each group were tested against the US9 sequence (TSP12) and chimeras D1, D2 and D3 in indirect ELISA. Numbers in x-axis represent the Log_2 of each dilution starting at 1/100. Vertical bars represent the SD of the mean. The mAb US9 was also used as control to confirm the availability of the US9 epitope in the TSP12 peptide bound to the plate (not shown).

contradictory results. While some authors referred a lack of antibody response to the B-epitope moiety of a B→T diepitopic chimera and a good response to the reverse T→B formulation (Sarobe et al., 1993), others reported good B-epitope antibody responses with B→T but not with T→B orientations (Blanco et al., 2013; Golvano et al., 1990). Moreover, the epitope orientation was reported to be not relevant in an experiment combining sequences containing both T and B epitopes (i.e., T/B→T/B formulations) joined by a KK linker (Yano et al., 2005).

Our data in Fig. 2 also showed that animals immunized with the rUS9-FhpCL1 chimera showed a high antibody response against this chimera (titer > 1/100,000; not shown) but low IgG response to chimera D1 (Fig. 2A), which suggests that the CL1 epitope has little contribution to the overall antigenicity of the molecule. This result is not surprising if we consider that, unlike short linear sequences, the antibody responses to correctly folded *Fasciola* cathepsins are preferentially directed to conformational epitopes (Martínez-Sernández et al., 2011; Muiño et al., 2011). In addition, our results in Fig. 2 suggested that, although similar in sequence, the CL5 epitope (derived from *Fasciola* CL5) present in chimera D3 induced higher antibody levels than the CL1 and CL2 epitopes present in chimeras D1 and D2, respectively (see Figs. 2A-C). However, this result seems not to be extrapolable to the entire molecule as it was reported that recombinant CL2 was slightly better recognized than CL1 and CL5 by antibodies induced during natural infections of cattle (Martínez-Sernández et al., 2018).

As to induce a TD antibody response it is required at least the presence of a T-cell epitope in the immunogen, the sequences of D1, D2 and D3 chimeras were analyzed with the PredBALB/c bioinformatics tool (Zhang et al., 2005) which predicts binding of peptides to mouse H-2^d class-II molecules (I-A^d and I-E^d alleles). The results in Table 1 showed that they contain 2–5 nonamers with high probability (score ≥9; range 0–10) of binding to H-2^d class-II alleles, which is in accordance with the high antibody responses obtained above. Moreover, as can be seen, most of the putative T-epitopes were located in the CL sequences, although the presence of three T neoepitopes partially overlapping the GSGSG linker was also predicted. It is important to note that, although most of the reported H-2^d class II binding peptides are 13–16 amino acids long, it has been suggested that only 9 residues are essential for binding within the class II binding pocket (Murugan and Dai, 2005), which agrees with the peptide length used by the PredBALB/c tool.

In view of the absence of an antibody response to the US9 epitope in mice immunized with chimeras D1, D2 and D3, we investigated whether positioning a CL epitope at the N-terminal position of a chimera also fails to induce antibodies in these animals. In this case, the chimeric antigen was constructed with two *F. hepatica* epitopes: the long 23-mer CL2 epitope of chimera D2 placed at the N-terminal position followed by the GSGSG linker sequence and by a second epitope derived from the sequence of the mature MF6p/FhHDM-1 heme-binding protein (Martínez-Sernández et al., 2014, 2017a; see chimeras D4 and D5 in Fig. 1). Among the latter, we tested two internal 12-mer sequences from the mature MF6p/FhHDM-1 protein: ^{23}VTKAYEKARDRA^{34} and ^{55}LNRLTDRLEKYA^{66} (Fig. 1), which are also present in the MF6p/FgHDM-1 *F. gigantica* protein (Martínez-Sernández et al., 2017b). Since there is no mapping of B-cell epitopes derived from MF6p/FhHDM-1, the above two internal protein sequences were selected based on a favorable polarity and accessibility profiles, which were calculated with the bioinformatics tool BcePred (Saha and Raghava, 2004).

The results in Fig. 3 showed that D4 and D5 chimeras induced high antibody responses to themselves in ELISA, but poor response when the sera were cross-tested, in spite of the fact that they share the same CL2-GSGSG- sequence. Moreover, while antibodies induced by immunization with the chimera D2 (which contains the CL epitope at the C-terminal position and lacks any MF6p/FhHDM-1 sequence) reacted with chimeras D4 and D5, the antibodies elicited by these latter reacted poorly with chimera D2. These results confirmed again a lack of antibody response to the B-cell epitope placed at the N-terminal region of the sequence and suggest that most of the antibodies induced by D4 and D5 chimeras are directed against the C-terminal MF6p/FhHDM-1 sequences (MF6p2 in chimera D4 or MF6p4 in chimera D5) with or without involvement of the GSGSG linker. To discard the possible implication of this linker and thus the formation of neoepitopes, we tested the same sera against two chimeras that contain the MF6p2 sequence at the central (T3, see Fig. 1) or at the C-terminal position (T2, see Fig. 1),

Table 1
Putative MHC (H-2^d) class-II binding peptide nonamers predicted for each chimeric antigen with the PredBALB/c algorithm. Numbers in parenthesis indicate the binding score obtained for each peptide (range 0–10). The higher the score the higher the probability of a given peptide to be recognized by an I-A^d or an I-E^d allele. Nonamers involving any of the spacer residues used to link the different epitopes are typed in bold.

H-2 allele	D1	D2	D3	D4	D5/D6
I-A^d	SGYVTEVKD (9.50) VTEVKDQGN (9.04)	**SGSGAVPES** (9.20) **SGAVPESID** (10.0) RDYYYVTEV (9.20) YYYVTEVKN (9.50)	**SGAVPDRID** (9.10) SGYVTEVKD (9.50)	RDYYYVTEV (9.20) YYYVTEVKN (9.50) **GSGSGVTKA** (9.50) **GSGVTKAYE** (9.10) SGVTKAYEK (9.30)	RDYYYVTEV (9.20) YYYVTEVKN (9.50)
I-E^d	KIDWRESGY (9.50)	IDWRDYYYV (9.50)		IDWRDYYYV (9.50) VTKAYEKAR (9.12)	IDWRDYYYV (9.50) **TFSVPISGP** (9.32)*

H-2 allele	D7	D8	T1	T2	T3
I-A^d	**TFSVPISKK** (9.20) **ISKKAVPES** (9.28) RDYYYVTEV (9.20) YYYVTEVKN (9.50)	RDYYYVTEV (9.20) YYYVTEVKN (9.50)	**SGSGAVPES** (9.20) **SGAVPESID** (10.0) RDYYYVTEV (9.20) **GQCKKVTKA** (9.30)	**TFSVPISKK** (9.20) **ISKKAVPES** (9.28) RDYYYVTEV (9.20) YYYVTEVKN (9.50) **GQCKKVTKA** (9.30) **CKKVTKAYE** (9.30)	**TFSVPISKK** (9.20) **SKKVTKAYE** (9.70) **DRAKKAVPE** (9.10) RDYYYVTEV (9.20) YYYVTEVKN (9.50)
I-E^d	**TFSVPISKK** (9.80) **ISKKAVPES** (10.0) IDWRDYYYV (9.50)	IDWRDYYYV (9.50) **EVKNQGQCK** (9.70)	IDWRDYYYV (9.50) **EVKNQGQCK** (9.70) **GQCKKVTKA** (9.20)	**TFSVPISKK** (9.80) **ISKKAVPES** (10.0) IDWRDYYYV (9.50) EVKNQGQCK (9.70) **GQCKKVTKA** (9.20) **KKVTKAYEK** (9.70) KVTKAYEKA (9.70) VTKAYEKAR (9.12)	**TFSVPISKK** (9.80) **PISKKVTKA** (9.20) **ISKKVTKAY** (9.40) **KKVTKAYEK** (9.70) KVTKAYEKA (9.70) VTKAYEKAR (9.12) YEKARDRAK (9.26) EKARDRAKK (9.80) IDWRDYYYV (9.50)

(*): Predicted in chimera D6 only.

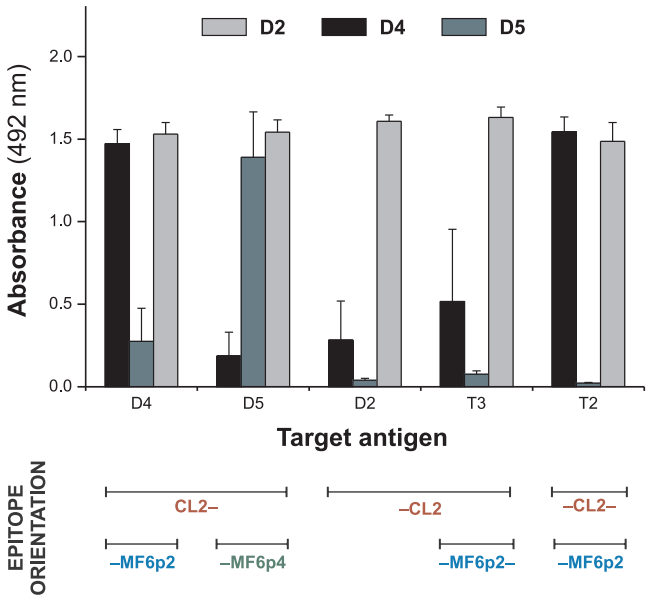

Fig. 3. Whole IgG antibody responses obtained in BALB/c mice immunized with chimeras D2 (10 animals), D4 (9 animals) and D5 (9 animals) in CFA. Each serum pool was tested at a 1/100 dilution against chimeras D2, D4, D5, T2 and T3 in indirect ELISA. Vertical bars represent the SD of the mean. The legend below the bar graph indicates the epitopes that can potentially be recognized by each group of sera as well as their orientation within the chimera (N-terminal: right dash; central: left and right dash; or C-terminal: left dash) and if they are shared or not by the different chimeras (horizontal bars).

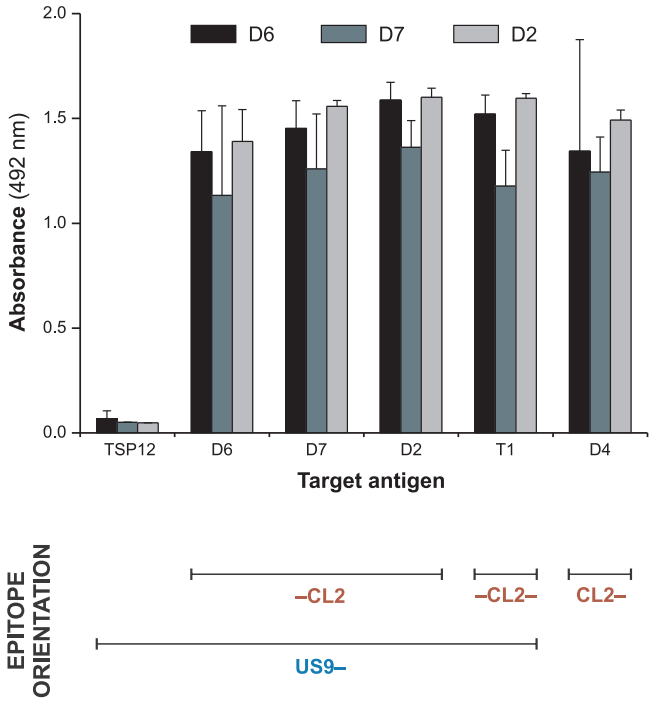

Fig. 4. Whole IgG antibody responses obtained in BALB/c mice immunized with chimeras D2 (10 animals) and D6 (8 animals) and D7 (8 animals) tested. Each serum pool was tested at a 1/100 dilution against chimeras D2, D4, D6, D7 and T1 as well as against the peptide TSP12 (US9 epitope) in indirect ELISA. Vertical bars represent the SD of the mean. The legend below the bar graph indicates the epitopes that can potentially be recognized by each group of sera as well as their orientation within the chimera (N-terminal: right dash; central: left and right dash; or C-terminal: left dash) and if they are shared or not by the different chimeras (horizontal bars). The mAb US9 was also used as control to confirm the availability of the US9 epitope in the TSP12 peptide bound to the plate (not shown).

both lacking the GSGSG linker. The results in Fig. 3 showed that mouse sera immunized with chimera D4 (black bar) were highly reactive with chimera T2 but less reactive with chimera T3. These data indicated that most of the antibodies induced by chimera D4 are directed against the MF6p2 epitope, although this epitope is better recognized when located at the C-terminal position of the target antigen.

To better explore the influence of linkers on the antigenicity of chimeric antigens, we constructed two new chimeras, which maintain the structure of chimera D2, but changing the GSGSG linker to GPGPG (chimera D6), a linker sequence used to disrupt junctional T-cell epitopes (Livingston et al., 2002; Nezafat et al., 2016), or to the sequence KK (chimera D7), recently used to isolate contiguous T-cell and B-cell epitopes (Gu et al., 2017). Again, the sera from animals immunized with chimera D2 containing the GSGSG linker were used for comparison. The results presented in Fig. 4 showed, again, no antibody responses to the US9 epitope (TSP12) present in chimeras D2, D6 and D7. However, all chimeras induced good antibody responses to the CL2 epitope, as indicated by the high OD values obtained with each one of the immunizing chimeras (self-recognition) and with the chimeras joined by other linkers (cross-recognition) in ELISA. In addition, we investigated the influence of the position of the CL2 epitope in the target sequence by testing two additional chimeras in which the CL2 epitope is in a central position (T1, see Fig. 1) or at the N-terminal region (D4, see Fig. 1). In this case, no differences were observed between self- and cross-reacting OD signals (Fig. 4), indicating that all sera recognized the CL2 epitope independently of its position in the target antigen.

In addition to B-cell epitopes, we also investigated the possible influence of linkers in altering the repertoire of T-cell epitopes along the chimeric antigen. To this end we used the PredBALB/c bioinformatics tool (Zhang et al., 2005). As can be seen in Table 1, the PredBALB/c bioinformatics tool predicted some of the diepitopic chimeras to have nonamer amino acid sequences able to bind to I-A^d and I-E^d alleles containing partial (chimeras D2, D3, D4 and D6) or complete (D7) linker sequences (all marked in bold). Interestingly, regarding chimeras D6 and D7, the addition of residues corresponding to the linker GPGPG or KK, respectively, turned the US9 sequence into a probable T-cell epitope, which could increase T-cell signaling favoring the induction of antibodies to the CL2 epitope. In the particular case of US9 sequences containing the linker KK (TFSVPISKK and ISKKAVPES) this hypothesis is in disagreement with previous reports proposing that lysosomal cathepsins B in APCs cut preferentially after pairs of basic amino acids (Takahashi et al., 1989), but agrees with more recent studies showing that such enzymes have other substrate specificities (Biniossek et al., 2011; Cotrin et al., 2004). Nevertheless, given that the endopeptidase activity of APCs is not limited to cathepsins B (Burster and Boehm, 2010) the doubt still remains about whether these neo-T-cell epitopes present in chimera D7 remain intact during antigen presentation.

3.2. Mouse antibody responses induced by triepitopic B-chimeras constructed with single or combined linkers

From the bioinformatics results presented in the previous section we concluded that the linker sequences may contribute to increase the number of available T-cell epitopes present in chimeras by inducing the formation of T neoepitopes. However, we could not elucidate which linker sequence would be more favorable to induce TD antibody responses with minimal, or no formation, of B neoepitopes, an aspect which is relevant to achieve maximum vaccine efficacy. In addition, our results did not clarify which are the most adequate positions for B-cell epitopes in multiepitope chimeras. To address these questions, we designed triepitopic chimeras using a combination of the linker GSGSG with KK, or KK alone, and permuting the position of the central and C-terminal epitopes (see chimeras T1, T2 and T3, Fig. 1). The antibody

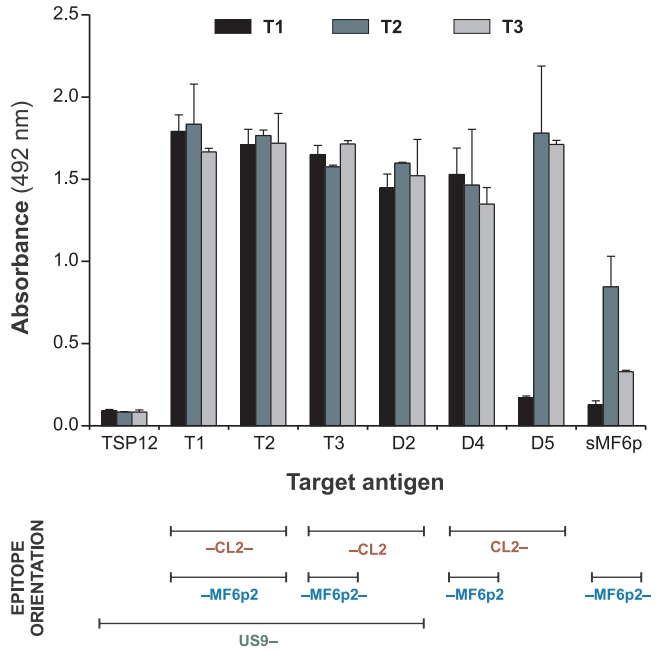

Fig. 5. Whole IgG antibody responses obtained in BALB/c mice (8 animals per group) immunized with triepitopic chimeras T1, T2 and T3. The serum pools were tested in indirect ELISA at a 1/100 dilution against peptide TSP12 (US9 epitope), the triepitopic chimeras T1, T2 and T3, the diepitopic chimeras D2, D4 and D5 and the protein sMF6p. Vertical bars represent the SD of the mean. The legend below the bar graph indicates the epitopes that can potentially be recognized by each group of sera as well as their orientation within the chimera (N-terminal: right dash; central: left and right dash; or C-terminal: left dash) and if they are shared or not by the different chimeras (horizontal bars). The mAb US9 was also used as control to confirm the availability of the US9 epitope in the TSP12 peptide bound to the plate (not shown).

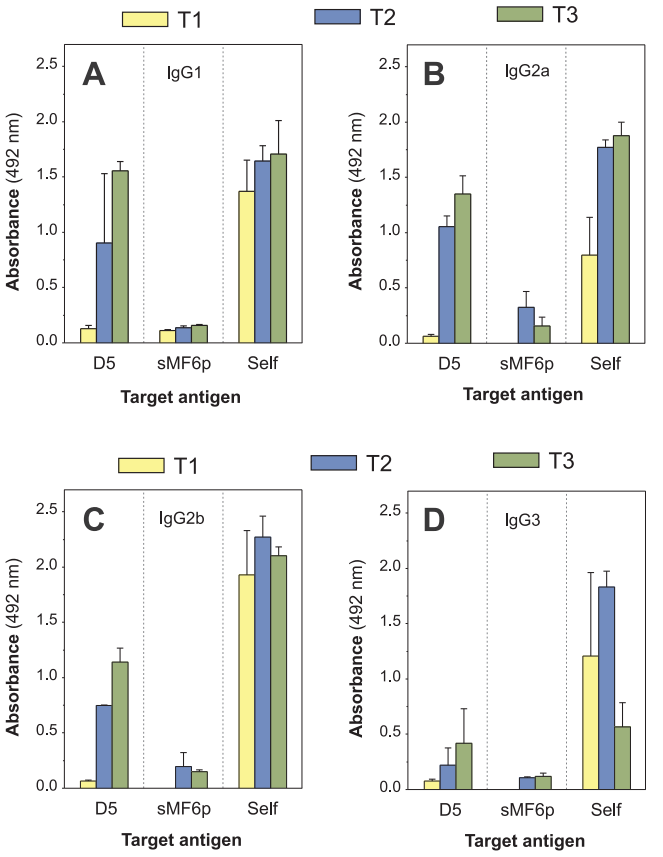

Fig. 6. Analysis of the IgG1, IgG2a, IgG2b and IgG3 antibody responses obtained in BALB/c mice after immunization with the triepitopic chimeras T1, T2 and T3 in CFA. The serum pools were tested in indirect ELISA diluted 1/100 against chimera D5, the protein sMF6p and to the corresponding chimera used for immunization (self-recognition). Vertical bars represent the SD of the mean.

responses obtained after immunization of BALB/c mice with each of these chimeras are shown in Fig. 5. Three major findings were observed: i) as for diepitopic chimeras, no antibody response was directed to the US9 epitope (TSP12 antigen) placed at the N-terminal position of the chimera; ii) the three triepitopic chimeras induced excellent antibody responses to themselves (self- and cross-recognition) and to chimeras D2 and D4, but only antibodies induced by chimeras T2 and T3 reacted strongly with chimera D5, which lacks the MF6p2 epitope; and iii) the MF6p/FhHDM-1 synthetic protein, which contains the MF6p2 (^{23}V-^{66}A) internal epitope sequence, was only recognized by antibodies induced by chimera T2 and, to a lesser extent, by those induced by chimera T3. From these results we concluded that chimeras T1, T2 and T3 were able to induce antibodies to both CL2 and MF6p2 epitopes. However, compared to T2 and T3, it seems that T1 induced a different set of antibodies as: i) these antibodies did not react with the CL2 epitope when located at the N-terminal position of the target sequence (e.g., D5 chimera, Fig. 5) and ii) the MF6p2 epitope was not recognized when located at an internal position (as in the MF6p/FhHDM-1 synthetic protein). These conclusions were also supported by several inhibition ELISAs (not shown) discarding any possible artifact due to an inadequate orientation of target peptides in the indirect ELISAs.

As for the effect of the orientation of epitopes in the antigen, our results also indicated that the linker sequences may also exert an influence on the antigenicity of triepitopic chimeras. This was deduced from the fact that, although chimeras T1 and T2 share the same epitope positioning, they generated a distinct set of antibodies, as only antibodies induced by chimera T2 were able to recognize epitopes CL2 (present in chimeras D2 and D5) and MF6p2 (present in chimera D4 and in MF6p/FhHDM-1) regardless of their position in the target antigen (Fig. 5). These results suggest that spacing epitopes by KK linkers may be a better choice for immunization experiments with chimeric antigens. This agrees with the strategy of Yano et al. (2005) of using this lysine linker to join epitopes for vaccine applications.

The comparison of the antibody response to diepitopic and triepitopic chimeras revealed that the latter were more antigenic. This finding was not surprising considering that the longer the sequence of the chimeric antigen, the more likely the probability of increasing the number of putative T epitopes, including the formation of promiscuous ones (Fonseca et al., 2016). Moreover, the PredBALB/c bioinformatics tool (see Table 1) predicted less putative T-cell epitopes in chimera T1 (n = 7) than in chimeras T2 (n = 14) and T3 (n = 14), which may be related to the differences observed in the antibody response induced by chimera T1 with respect to the latter chimeras.

Besides the determination of total IgG antibody responses induced by the triepitopic chimeras in mice, we investigated the influence of the different linkers and/or positioning of B epitopes on the repertoire of IgG subclasses induced by these chimeras. The IgG subclass antibody responses induced by chimeras T1, T2 and T3 to themselves as well as to the chimera D5 and sMF6p were determined in ELISA. The results presented in Fig. 6 show that all chimeras induced the formation of antibodies from all IgG subclasses in mice, which parallels with the antibody response obtained for anti-total IgG antibodies (see above). The high IgG2a antibody responses obtained (Fig. 6B) are probably related with the use of CFA, an adjuvant which promotes TH_1 responses in mice leading to a decrease of the IgG1/IgG2a antibody ratio (Cribbs et al., 2003).

3.3. The US9 epitope influences the response of mice to the CL2 epitope

As discussed before, the lack of a relevant antibody response in mice

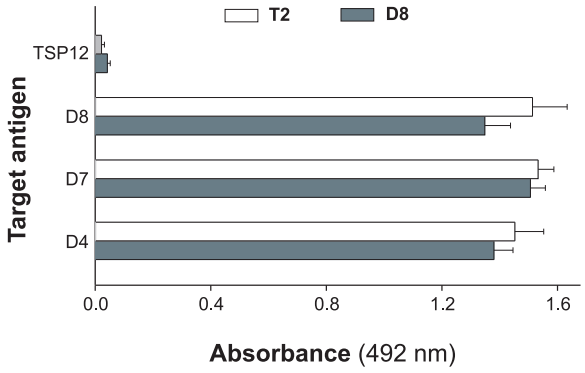

Fig. 7. Whole IgG antibody responses obtained in BALB/c mice immunized with chimeras D8 and T2. The serum pools were tested in indirect ELISA at a 1/100 dilution against peptide TSP12 (US9 epitope) and the diepitopic chimeras D4, D7 and D8. Vertical bars represent the SD of the mean. The mAb US9 was also used as control to confirm the availability of the US9 epitope in the TSP12 peptide bound to the plate (not shown).

to the epitope placed at the N-terminal region of diepitopic and triepitopic chimeras (i.e., against the US9 or CL2 epitopes) led us to assume that this may not be an adequate position to place a relevant B-cell epitope. However, considering that the CL2 sequence contains putative T-cell epitopes (Table 1), a doubt arises about whether the presence of the hydrophobic US9 epitope at any position of a chimera may modulate the responses to other boundary epitopes. To test this possibility, we immunized mice with a new diepitopic chimera (D8; see Fig. 1) which has the same US9 and CL2 epitopes as chimera D7 but inverted in their positions. After testing the sera obtained from mice immunized with chimera D8, we observed that this chimera produced an excellent antibody response to itself (Fig. 7) and that such response was exclusively directed against the CL2 epitope. This result was surprising since, as noted above, antibodies induced by chimeras D4 and D5, which bear the CL2 epitope at the same N-terminal position as D8 but lack the US9 sequence, did not react with such epitope. Although the mechanism by which the introduction of the short hydrophobic US9 sequence at the C-terminal position of the D8 chimera polarizes the antibody response to the CL2 epitope is not yet understood, we hypothesize this may be related to US9-induced changes in the affinity of the resulting processed peptides for MHC class II binding, or to changes in the immunodominance due to peptide competition for MHC class II binding (Adorini et al., 1988; Lo-Man et al., 1998). Another alternative, such as the possible induction of a higher T-cell signaling elicited by chimera D8 with respect to chimera D7 seems less probable since the PredBALB/c tool predicted less T-cell epitopes in chimera D8 than in chimera D7 (Table 1).

3.4. Analysis of the antibody responses induced by di- and triepitopic B-chimeras in other animal species

Although very convenient for conducting preliminary studies on vaccination, mice (especially syngeneic strains) are rarely representative of the immune responses generated by other animal species. Consequently, we extended our previous studies in BALB/c mice with an exploratory study on the antibody responses of sheep and turbot to a selection of the above chimeric antigens. Sheep were chosen as representative of animals for production, as this species is a natural host for *F. hepatica* and has no natural protection against this trematode (Mulcahy et al., 1999). Turbot (*Scopthalmus maximus* L), a flat fish of great commercial interest (Estévez et al., 1993), was chosen because the antibody responses in teleost fishes are very different from those in mammalian species, as they only produce antibodies of the IgM-like and IgT isotypes (Estévez et al., 1993; Salinas et al., 2011) and since the mechanism involved in TD antibody responses in these animals was

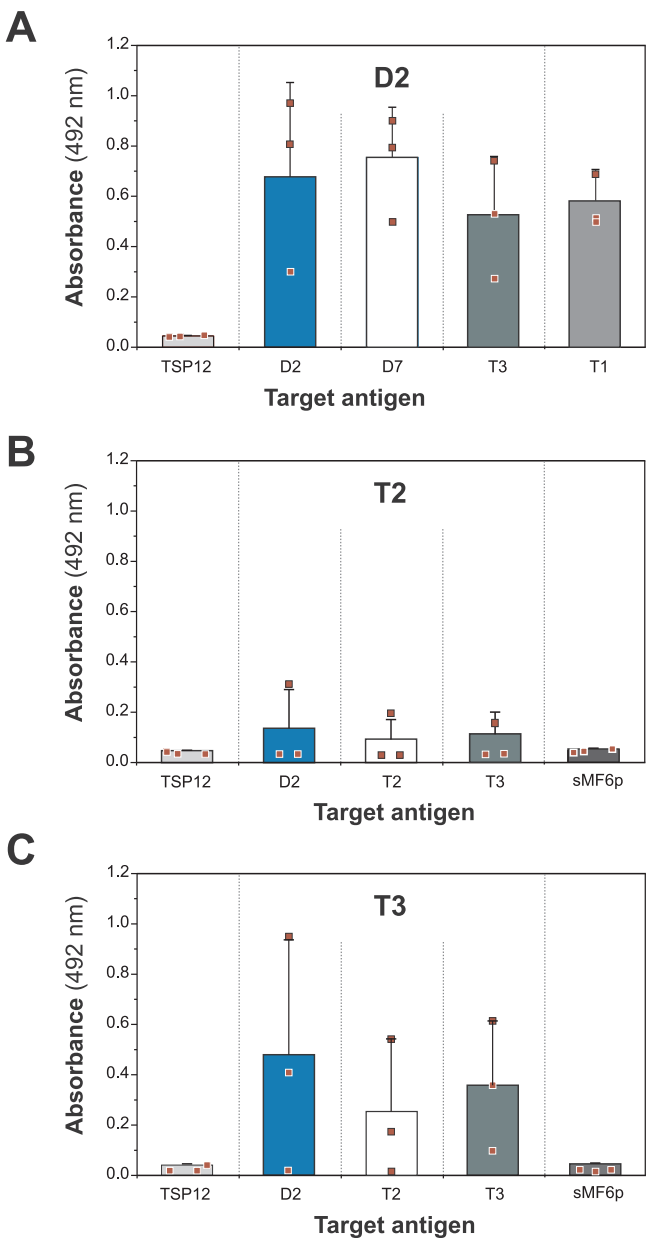

Fig. 8. Whole IgG antibody responses obtained in sheep (3 animals per group) immunized with the diepitopic chimera D2 or with the triepitopic chimeras T2 and T3 adjuvanted with Quil-A. The serum from each individual animal was tested diluted 1/100 in indirect ELISA against the peptide TSP12 (US9 epitope), the chimeras D2, D7, T1, T2 and T3, and the protein sMF6p, as appropriate. A) sheep immunized with chimera D2, B) sheep immunized with chimera T2; C) sheep immunized with chimera T3. The mean OD value obtained for each individual sheep within a group (n = 3) was represented by a small red square in the figure. Vertical bars represent the SD of the mean. The mAb US9 was also used as control to confirm the availability of the US9 epitope in the TSP12 peptide bound to the plate (not shown) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

poorly investigated.

The data on the IgG antibody responses of sheep induced by immunization with chimeras D2, T2 and T3 is shown in Fig. 8. As for mice, no antibody response was obtained to the US9 epitope with any of the three immunizing chimeras. However, the antibody responses of these animals showed some notable differences with respect to mice. In this sense, we observed that while sheep produced little, or no antibodies, to chimera T2 (Fig. 8B), these animals responded well to chimeras D2

(highest response) and T3, although a relevant variability was observed between animals within each group (represented in Fig. 8 with red dots). Also, considering the epitope composition of the chimeric antigens, it is interesting to note that most of the antibodies produced in sheep were directed against the CL2 epitope. This was deduced from the fact that there was an absence of recognition of the US9 (Figs. 8A-C) and MF6p2 (Figs. 8B-C) epitopes, and that similar OD values were obtained using as target the immunizing chimera (self-recognition) and chimeras different from the immunogen but that share the CL2 epitope (cross-recognition). Moreover, given the high antibody response observed in sheep immunized with chimera D2 to itself, as well as to chimeras D7, T1 and T3 (Fig. 8A), it seems that, like in mice, the IgG antibody responses of sheep to neoepitopes were not relevant.

Compared to mice, the poor antibody response of sheep to chimera T2 may result surprising at first glance. We hypothesize that such discrepancy may be related with the different orientation of the CL2 epitope in D2 and T3 (both at the C-terminal position) with respect to T2 (central), together with a different capability of sheep and mouse to process and present such antigens. Sheep MHC class-II (Ovine Leukocyte Antigen; OLA) DR and DQ molecules were reported to be among the highest polymorphic molecules in mammals (Koutsogiannouli et al., 2016). However, it might be that our sheep (raza gallega strain) lack an appropriate set of such MHC molecules able to stimulate resting T-cells with TCR specificity for the CL2 epitope when it is located at the central position of the chimera. However, since we currently lack adequate bioinformatics tools for prediction of peptide binding to DR and DQ molecules in sheep, this hypothesis could not be confirmed.

Once investigated the response to T2 and T3 chimeras in sheep, we evaluated the antibody response of turbot to these antigens. Among the high number of fish species of commercial interest, we selected turbot for two main reasons: i) there are many turbot farms exploited in Galicia (NW Spain) and ii) the availability in our laboratory of mAb UR3, an IgG1κ mAb previously developed by our team, which specifically recognizes the IgM of turbot (Estévez et al., 1994a). Also, since the adaptative immune response of teleosts is probably less efficient than that of mammals (Magadan et al., 2015), turbots were challenged with two sequential immunizations, the first with CFA (as with mice) and a second injection one month later in FIA, both by Ip route (see material and methods). The antibody responses of these fishes to the T2 and T3 chimeras are shown in Fig. 9. It should be underlined that, unlike sheep, turbot generated a high antibody response to the B-cell epitopes present in the chimera T2 (CL2 and MF6p2 epitopes) but, unexpectedly, little or no response to chimera T3. The good recognition of epitope MF6p2 by antibodies induced by chimera T2 was evidenced by the excellent reactivity of such antibodies to sMF6p and sMF6a (Fig. 9). Moreover, since the peptide sMF6a lacks the C-terminal residue (Ala) of the MF6p2 epitope it appears that such residue was not required for antibody binding. The data in Fig. 9 also indicate that chimera T2 did not induce a relevant antibody response to any neoepitope as the OD values obtained against chimeras D2 and D7 (which have different linker spacers) were similar.

Considering that the CL2 and MF6p2 epitopes in chimeras T2 and T3 are separated by the same linkers (KK), the differences between the antibody responses observed in mouse, sheep and turbots are again surprising. However, as antibodies induced by chimera T2 recognized the CL2 and MF6p2 epitopes either at central or C-terminal positions (see recognition of chimeras D2, D5, D6 and D7 in Fig. 9), we discarded that antibody differences in the response of turbots to T2 and T3 chimeras were due to deficiencies in the repertory of B cells. So, as we discussed before regarding sheep, it is probable that the set of class-II MHC molecules, and/or TCR repertoire, of turbots is not adequate to bind to the T-cell epitopes contained in chimera T3. In this sense, it should be noted that the turbots used in the present study were obtained from a local fish farm, where they are produced by matting a small number of breeding animals which may restrict the diversity of

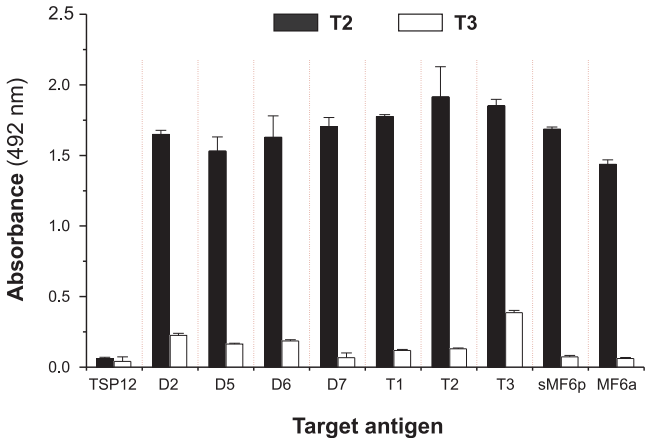

Fig. 9. Analysis of the IgM antibody response in turbots (20 animals per group) immunized with the triepitopic chimeras T2 and T3 adjuvanted in CFA (first immunization) and in IFA (second immunization). Pooled sera from each group (n = 20) were tested diluted 1/100 in indirect ELISA against the peptides TSP12 (US9 epitope), MF6a, the protein sMF6p, the diepitopic chimeras D2, D5, D6 and D7, as well as against the triepitopic chimeras T1, T2 and T3. Vertical bars represent the SD of the mean. The mAb US9 was also used as control to confirm the availability of the US9 epitope in the TSP12 peptide bound to the plate (not shown).

MHC-II molecules with respect to wild animals. Although some species of fishes as *Gadus morhua* (atlantic cod) lack MHC-II activity (Pilström et al., 2005), most of teleost fishes, including turbot (Zhang and Chen, 2006), carry classical functional MHC-II molecules and have good activation of humoral and cell-mediated immunity in response to infectious agents including bacteria, viruses and parasites (Iglesias et al., 2003; Leiro et al., 1996; Liu et al., 2017; Santos et al., 2005; Zhang et al., 2013).

4. Conclusions

Our results confirm previous findings showing that to design chimeric vaccines several factors need to be considered. These include: the polymorphisms of MHC-II molecules in the host species to be immunized, the orientation of T/B epitopes and an adequate selection of linkers between epitope sequences to avoid the formation of B-cell neoepitopes. However, our findings add important information not previously reported, namely: i) that it is possible to induce good antibody TD responses to relatively short chimeric peptides combining apparent B-cell epitopes, as long as the resulted sequence forms adequate T neoepitopes for the species being vaccinated; ii) that, at least in mice, the placement of a decoy epitope (US9 B-epitope in our study) at the N- or C-terminal regions may have favorable consequences on the antibody response to other epitopes conforming the chimeric antigen, and iii) that species as different as mice, sheep and turbot are able to produce antibodies to short chimeric peptides; nevertheless, to obtain an appropriate response, the positioning of such epitopes needs to be designed specifically for each species.

Conflict of interest

The authors declare that they have no competing interests.

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