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(Title): Promiscuous T cell epitopes boosts specific IgM immune response against a P0 peptide antigen from sea lice in different teleost species.

(Authors): Yeny Leal[1], Janet Velazquez[1], Liz Hernandez[1], Jaya Kumari Swain[2,3], Rebeca Martínez[1], Claudia Garcia[1], Yassel Ramos[4], Mario Pablo Estrada[1]*, Yamila Carpio[1]*

(Affiliations): [1]Animal Biotechnology Division, Center for Genetic Engineering and Biotechnology, Havana, Cuba; [2]NOFIMA, Tromso, Norway; [3]Fish Immunology and Vaccinology Research Group, Norwegian College of Fishery Science, UiT The Arctic University of Norway, Tromso, Norway; [4]Physico-Chemistry Department, CIGB, Havana, Cuba

*Corresponding authors:

yamila.carpio@cigb.edu.cu

mario.pablo@cigb.edu.cu

Abstract

The development of vaccines employing conserved protein antigens, for instance ribosomal protein P0, has as disadvantage the high degree of identity between pathogen and host proteins due to possible induction of tolerance or auto antibodies in the host organism. To overcome this drawback, peptide-based vaccines have been designed with a proved high efficacy. The use of defined peptides as antigens has the problem that they are generally poor immunogenic unless coupled to a carrier protein. Several studies have established the potential for promiscuous T cell epitopes incorporated into chimeric peptides to enhance the immunogenicity in mammals. On the contrary, studies about the role of these epitopes on teleost immune system are scarce. Therefore, the main objective of our present study was to evaluate the potential of promiscuous T cell epitopes to boost specific IgM immune response in teleost fish against a peptide antigen. With this aim, we used a peptide of 35 amino acids from the ribosomal P0 protein of *Lepeophtheirus salmonis*, an important parasite in salmon aquaculture. We fused this peptide to the C-terminal of T cell epitopes from tetanus toxin and measles virus and produced the chimeric protein in *Escherichia coli*. Following vaccination, antibody production was monitored in different immunization schemes in Tilapia, African catfish and Atlantic salmon. The results demonstrated for first time that the addition of T cell epitopes at the N-terminal of a target peptide increased IgM specific response in different teleost species, revealing the potential of this approach to develop peptide-based vaccines for aquaculture. The results are also of great importance in the context of vaccine development against sea lice using ribosomal protein P0 as antigen taking into account the key role of P0 in protein synthesis and other essential physiological processes.

Key Words: Immunoglobulin M, *Lepeophtheirus salmonis*, epitopes, teleost, peptide, vaccine

1. Introduction

The development of a vaccine candidate based on conserved proteins between the pathogen and the host has as a drawback that the high degree of identity can result in the induction of tolerance or the generation of auto antibodies in the host organism. To overcome this disadvantage, peptide-based vaccines have been designed with a proved high efficacy; for example, a vaccine developed against ticks based on a 20 amino acid peptide of the acidic ribosomal protein P0 from *Rhipicephalus* sp. ticks [1, 2]. The use of peptides as antigens has the problem that they are generally not very immunogenic unless coupled to a carrier protein. There are a number of contributing reasons, among which is that the peptide sequences used to present epitopes are generally short. Therefore, they contain insufficient information to fold into the correct shape necessary to mimic conformation-dependent epitopes and even if the peptide is recognized by a B cell, these cells must still receive help from a helper T cell recognizing a sequence within the same immunogen. Traditionally the site of T cell recognition has been provided by a carrier protein to which peptides are covalently coupled [3]. Several studies have established the potential for promiscuous T cell epitopes (TCE's) incorporated into chimeric peptides to enhance the immunogenicity of other epitopes within the chimeric peptide in mammalian immune systems [4-7].

Teleost fish has an adaptive immune system, as they have immunoglobulins, T cell antigen receptors, major histocompatibility complex class I and II molecules, spleen and thymus and many other features, which are similar to and in some cases differ from those of the mammalian immune system [8]. In order to overcome the problem associated with the poor immunogenicity of peptides, multiple antigen peptide system (MAPS) was used to bypass the need of a carrier protein for antibody production in rainbow trout *Oncorhynchus mykiss* [9]. To test the effectiveness of MAPS, rainbow trout were immunized with two MAPS containing the decapeptide

72 Gonadotropin Releasing Hormone (GnRH). One of these MAPS was heterologous and contained
73 alternating sequences of GnRH and a measles virus T cell epitope. The results show that MAPS are
74 a suitable delivery system in fish for the generation of anti-peptide antibodies but not a real
75 improvement was seen with the addition of TCE's. In another previous work, promiscuous TCE's
76 from measles virus fusion protein (MVF) (288–302) [10] and *Clostridium tetani* tetanus toxin (tt) P2
77 epitope (830–844) [11] were used to construct chimeric fusion proteins with OspA to determine if
78 these mammalian TCE's could enhance the immunogenicity of recombinant OspA within the
79 salmonid immune system. OspA is a 17 kDa putative outer surface protein from *Piscirickettsia*
80 *salmonis*, the etiological agent of salmonid rickettsial septicaemia (SRS) and a devastating disease
81 of farmed salmonid fish. The authors demonstrated that addition of these TCE's dramatically
82 improved the efficacy of the OspA vaccine, reflected by a three-fold increase in vaccine efficacy.
83 Nevertheless, the mechanisms of the increased efficacy were not measured at that time [12] and
84 no further studies about the role of mammalian TCE's on teleost immune system or the potential
85 of this strategy to develop peptide-based vaccines has been published.
86 The aim of the present study was to evaluate the potential of TCE's to boost specific IgM immune
87 response in teleost fish against a peptide antigen. In this connection and based on the results
88 obtained in ticks, we were interested also in the development of a vaccine against sea lice based
89 on ribosomal P0 protein. Sea lice are crustacean ectoparasites affecting Atlantic salmon (*Salmo*
90 *salar*) production worldwide, causing huge economic losses [13]. Thus, we used a peptide of 35
91 amino acids from the ribosomal P0 protein of *Lepeophtheirus salmonis* (the most important sea
92 lice species affecting salmon aquaculture in northern hemisphere) as antigen. This peptide was
93 chosen from a highly immunogenic region within the P0 protein, which also showed low sequence
94 identity (45%) with *S. salar* P0 protein. We fused this peptide to the C-terminal of TCE's from
95 tetanus toxin and measles virus positioned in tandem, similar to the strategy used by Kuzyk et al

2001 [12] and produced the protein in *Escherichia coli*. Later, antibody production was monitored after vaccination in Tilapia (*Oreochromis niloticus*), African catfish (*Clarias gariepinus*) and Atlantic salmon. The results demonstrated for first time that addition of TCE's at the N-terminal of small peptide antigen increased humoral specific response in different teleost species, revealing the potential of this approach to develop peptide-based vaccines for aquaculture.

2. Materials and Methods

2.1 Cloning and expression in *Escherichia coli* of TT-P0 antigen

The tt P2 (830–844 QYIKANSKFIGITEL; GenBank X04436) and MVF protein (288–302 LSEIKGVIVHRLEGV; GenBank M81903) TCE's were obtained by PCR from the plasmid 0807716_ttantigen-MVF_prote_pGA14 generated by GENEART containing the synthetic gene. The PCR primers used to amplify the TCE's were A and B (Table 1) and they contain *Nco* I-*Hind* III restriction sites. A 35 aa peptide between the amino acids 267 to 301 from *L. salmonis* P0 sequence was amplified from pMOS-Blue-P0 [14] using the primers C and D containing the restriction sites *Xho* I- *Hind* IIII (Table 1) to allow the fusion to the C-terminal of promiscuous T cell epitopes. The PCR fragments were sub-cloned into pGEM-T-easy (Promega), extracted by the corresponding endonuclease digestion and the fragments were inserted into the corresponding cloning sites of pET28a. The final vector was titled as pET28a-TT-P0.

For expression of the recombinant polypeptide, the pET28a-TT-P0 expression plasmid was transformed into *E. coli* BL21(DE3) strain. Single clones of BL21 (DE3) transformed with pET28a-TT-P0 were grown overnight at 37°C in Luria Bertani (LB) medium containing 50 µg/mL of kanamycin. Cultures were then diluted (1:20) in fresh LB medium and grown at 37°C until the OD_{600} reached approximately 0.5. The expression of recombinant proteins was initiated by the addition of isopropyl-β-D-thiogalactoside (IPTG) (Sigma) to a final concentration of 1 mM and incubation continued another 5 h for induction of recombinant protein expression.

120 **2.2 TT-P0 protein purification**

121 After induction, the bacterial cells were harvested by centrifugation at 10,000 x g for 10 min at
122 4°C. The cell pellet was resuspended in 300 mM NaCl, 10 mM Tris, pH 6 and the cells were
123 disrupted in French Press (Ohtake, Japan) at 1 200 kgf/cm2. After bacterial cells disruption, the
124 cells were harvested by centrifugation at 10,000 x g for 10 min at 4°C. The cell pellet containing
125 the protein was resuspended in 150 mL of Solubilization buffer (300 mM NaCl, 10 mM Tris, 10 mM
126 Imidazol, 6M urea, pH 8) and it was incubated for 2 h at room temperature with gentle agitation.
127 Afterwards, the sample was centrifuged at 10,000 x g for 20 min at 4°C and the supernatant was
128 used for further purification steps. Affinity chromatography was performed under denaturing
129 conditions employing IMAC SepharoseTM Fast Flow (GE Healthcare) according to the
130 manufacturer's instructions. The clarified lysate with 10 mM Imidazole was loaded onto the
131 previously equilibrated column with equilibration buffer (NaCl 300 mM, Tris-HCl 10 mM, Imidazol
132 10 mM, urea 1.5M, pH 8) at a flow rate of 1 mL/min. Then, wash was performed with the same
133 buffer but 40 mM Imidazole. Protein elution was done with 200 mM Imidazole. For refolding, the
134 fraction purified by affinity chromatography was dialyzed against NaCl 150 mM, Tris-HCl 10 mM,
135 pH 8 buffer. Each fraction was checked by 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel
136 electrophoresis under reducing conditions. Protein concentration was determined with a BCA
137 protein assay kit (Pierce) according to the manufacturer's instructions. The purity of recombinant
138 protein was assayed by densitometry scanning of protein gels taking into account total protein
139 concentration.

140 **2.3 Protein gel electrophoresis and *western blotting***

141 Protein samples were loaded on 15% polyacrylamide gels that were stained with Coomassie
142 Brilliant Blue or transferred to nitrocellulose membranes. Membranes were blocked with 5% skim

143 milk for 60 min at room temperature. *Western blotting* was performed using anti-His monoclonal
144 antibody peroxidase conjugate (Sigma) at a dilution 1:2000, or a rabbit serum against P0.
145 The polyclonal sera against P0 was prepared in New Zealand White rabbits (6 weeks old) that were
146 immunized subcutaneously with three doses (weeks 0, 3 and 7) containing 500 µg of synthetic
147 35 aa P0 peptide (CIGB peptide synthesis department) conjugated to KLH (Sigma) per dose in
148 Freund's complete adjuvant (Sigma) at week 0 and Freund's incomplete adjuvant at weeks 3 and
149 7. Blood extraction was performed one week after the last immunization. After washing with PBS-
150 Tween 0.01% once and with PBS twice, the membrane was incubated with a 1:100 dilution of the
151 polyclonal serum against P0 for 2 h. After the washing steps, the membrane was incubated with
152 gentle shaking for 1 h at room temperature with 1:5000 dilution of anti-rabbit polyclonal antibody-
153 horseradish peroxidase (HRP) conjugate (Amersham Biosciences) as secondary antibody.
154 Chromogenic detection was carried out using 3,3'-diaminobenzidine as an HRP detection
155 substrate.

156 **2.4 In-gel protein digestion and Mass Spectrometry analysis**

157 The identity of the purified protein was confirmed by mass spectrometry analysis. The Coomassie
158 blue-stained band was excised from SDS-PAGE gels and incubated at 37 °C with 50% acetonitrile in
159 1% ammonium bicarbonate until they became colorless. The gel slice was dried and rehydrated in
160 25mM ammonium bicarbonate buffer containing sequencing grade trypsin at 12.5 ng/µL. The in-
161 gel digestion was for 16 h at 37 °C. The resulting proteolytic peptides were passively eluted in 0.2%
162 of formic acid solution, desalted by using a ZipTips reverse phase microcolumn and loaded into
163 gold-coated borosilicate nanotips for mass spectrometry analysis.
164 Low-energy ESI-MS and MS/MS spectra were acquired using a QTOF-2™ mass spectrometer from
165 Waters (Manchester, UK). The capillary and cone voltages were set to 1200 and 35 V, respectively.
166 The multiply-charged signals of highest intensity corresponding to tryptic peptides were further

analyzed by ESI-MS/MS using appropriate collision energies to obtain either partial or complete amino acid sequences.

2.5 Fish immunization experiments

2.5.1 Animals

Tilapias (*Oreochromis niloticus*) were obtained from the Aquaculture Research Station at the Center for the Genetic Engineering and Biotechnology (CIGB), Havana, Cuba. African catfish (*Clarias gariepinus*) were provided by the Center for Aquaculture of Mampostón (CPAM). Atlantic salmon (*Salmo salar*) were from the Aquaculture Research Station (Tromsø, Norway).

All animal experiments were previously approved by the Ethics Committee of the CIGB, Havana, Cuba (Tilapia and African catfish) or by 'FDU' (http://www.mattilsynet.no/fdu/) to be in accordance with the animal welfare act as required by Norwegian law (Atlantic salmon). Prior to vaccination and sampling, fish were anaesthetized with benzocaine at recommended doses.

2.5.2 Adjuvants

All antigens, unless specified, were formulated in Montanide ISA 50 V2 adjuvant (Seppic, France) at a ratio 50/50. Formulations were made in a Politron (Ultra-Turrax T25, IKA WERKE, Germany).

2.5.3 Experiments in tilapia

Experiment 1

Animals were maintained in a circular 500 L tanks supplied with recirculating fresh water at 26±2°C and 12:12 light-dark cycle. Fish were fed with pelleted feed (CENPALAB, Cuba). Ten tilapias per group (65±5 g) were immunized by intraperitoneal (i.p.) injection on days 1 and 15. Three experimental groups were settled. One group of Tilapia was injected with the TT-P0 antigen at the dose of 1 μg per g of body weight (1 μg/gbw). The second group received the same quantity of 35 aa synthetic P0 (CIGB synthesis Department, Cuba) and the third group (Control) received equal

190 volume of buffer (NaCl 150 mM, Tris-HCl 10 mM, pH 8). Blood was collected from the caudal vein
191 of all fish on days 0 and 21 and serum was prepared for antibody detection.
192 *Experiment 2*
193 A second experiment was developed to evaluate the need of the oil adjuvant to obtain the
194 antibody response. A similar immunization scheme than in previous experiment was developed
195 but the experimental groups were: buffer in Montanide ISA 50 V2, TT-P0 purified protein without
196 adjuvant and TT-P0 adjuvated in Montanide ISA 50 V2. Blood was collected from the caudal vein of
197 all fish on days 0 and 21, 28 and 35 from the beginning of the experiment (7, 15 and 21 days after
198 re-immunization).
199 *Experiment 3*
200 A third similar immunization scheme was performed to compare the antibody titers against P0
201 induced by TT-P0 protein and P0 fused to a carrier protein. One group of tilapias was i.p. injected
202 with the TT-P0 antigen at 1 µg/gbw. The second group received the same quantity of P0-my32 [13]
203 and the third group (Control) received equal volume of buffer. Blood was collected from the
204 caudal vein of all fish on days 0 and 21, 28 and 35 from the beginning of the experiment (7, 15 and
205 21 days after re-immunization).
206 **2.5.4 Experiment in African catfish**
207 Animals were maintained in a circular 500 L tanks supplied with recirculating fresh water at 26±2°C
208 and 12:12 light-dark cycle. Fish were fed with pelleted feed (CENPALAB, Cuba). Twelve African
209 catfish (65±5 g) per group were immunized by i.p. injection on days 1 and 15. One group of catfish
210 was injected with the antigen TT-P0 (1 µg/gbw). Control fish were immunized with buffer. Blood
211 was collected from the caudal vein of all fish at days 0 and 35.
212
213

214 **2.5.5 Experiment in Atlantic salmon**

215 Atlantic salmon (40±5 g) were kept in a circular 500 L tanks supplied with recirculating fresh water
216 for 2 weeks at an ambient temperature of approximately 10 °C with 24 h illumination (summer
217 stimuli) for acclimation. Fish were fed with a commercial pellet diet (Nutra Olympic, Skretting).
218 One hundred and twenty fish were placed in each tank per group and two experimental groups
219 were settled. Control group received PBS and the other group received 1 µg/gbw of TT-P0, both by
220 i.p. injection. Fifteen days after first vaccination, the fish were transferred to the sea water. The
221 fish were kept under the following conditions during the experiment: Temperature: 10 °C; Light:
222 24 h; Oxygen level: ~80-90 %; Salinity: 34-35 ppt. After 20 days in sea water (35 days from first
223 vaccination), a booster was given. Blood samples were taken at day 0 and 69 from 30 fish per
224 group.

225 **2.6 Serum IgM levels**

226 P0-specific IgM antibodies in the serum of vaccinated fish were determined by indirect ELISA. High
227 binding microtiter plates (Nunc, Denmark) were coated for 16 h at 4 °C with 10 µg/mL of 35 aa
228 synthetic P0. After three washes with PBS-Tween 0.05 %, blocking was performed with 5 %
229 skimmed milk in PBS (16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 120 mM NaCl, pH 7.4) for 2 h at room
230 temperature (RT). Afterwards, two-fold serial dilutions or required dilution of sera were applied
231 and incubated for 16 h at 4 °C. Bound antigen-specific antibodies were incubated for 2 h at 25 °C
232 with anti-tilapia IgM or anti-catfish IgM or anti-rainbow trout/Atlantic salmon IgM monoclonal
233 antibody (ADL Aquatic Diagnostics, UK), depending on the specie evaluated, and they were
234 detected by sequential incubation for 1 h at 25 °C with anti-mouse IgG conjugated with peroxidase
235 (Sigma) according with the instructions of the manufacturer.

236 After washing, the chromogen TMB in substrate buffer was added and incubated for 10 min or
237 until color development. After stop the reaction, color intensity was measured at 450 nm with a

238 Varioskan Flash microplate reader. Antibody titers were defined as the dilution of serum giving
239 twice the OD value of the pre-immune serum for each animal. Cut off value was defined for each
240 experiment and it was set at twice the lowest dilution of the negative control serum (pre-immune
241 serum). An internal positive control was included in each assay.

242 **2.7 *Western blotting* for P0 recognition by immunized fish sera**

243 Recombinant TT-P0 purified protein was loaded on 15% polyacrylamide gels that were stained
244 with Coomassie Brilliant Blue or transferred to nitrocellulose membranes. Membranes were
245 blocked with 5% skim milk for 60 min at room temperature. *Western blotting* was performed as
246 described above but using as primary antibody serum from immunized tilapia at a dilution 1:100
247 and anti-tilapia IgM as secondary antibody.

248 **2.8 Statistical analysis**

249 All the statistical analysis was done in Graphpad Prism version 6.0. In the case of comparison of
250 two groups, Unpaired t-test or Mann-Whitney test was performed were done depending on the
251 normal distribution and equal variance of the data. In case of three experimental groups, One-way
252 ANOVA or Kruskall-Wallis test was done followed by Tukey or Dunn's Multiple Comparison post-
253 test in each case. The normal distribution of data was analyzed with D'Agostino Pearson's test and
254 the variance homogeneity with Bartlett's test.

255 **3. Results**

256 **3.1 Production of recombinant TT-P0 protein**

257 Analysis by SDS-PAGE and *western blotting* with the anti-His showed two bands between 6 and 15
258 kDa in the lanes corresponding to the cell extracts of the strains of *E. coli* BL21 (DE3) transformed
259 with the genetic constructions pET28-TT-P0 clones 1 and 4 (Fig. 1 A, B, C). This agrees with the
260 expected molecular weight for the TT-P0 protein of 8.3 kDa, according to the prediction based on
261 the amino acid sequence deduced from the nucleotide sequence. The result of the *western*

blotting also confirmed the presence of the tail of six His (Fig. 1B). Recombinant protein was also recognized by the polyclonal serum anti pP0. This polyclonal antibody has immunoreaction with higher molecular weight bands (Fig. 1C). These bands identified between 27 and 41 kDa are recognized in *E. coli* cells transformed with pET28a, thus they are not related to P0 immunization. After cell disruption most of the protein was obtained forming inclusion bodies (data not shown).

The purification of the TT-P0 protein, after its solubilization in urea, was carried out by affinity chromatography to metal chelates. For the optimization of this process, different imidazole molarities were tested (data not shown). The wash step of the TT-P0 protein was carried out at a concentration of 40 mM of imidazole where important contaminants are eliminated without significant losses of the proteins of interest. Elution at 200 mM concentration of Imidazole resulted in TT-P0 protein with purity higher than 90% (Fig. 1D, E). The mass spectrum of tryptic digestion of the most intense band showed few signals (Fig. 2A), typical of a low molecular weight protein and multiple tryptic sites. The most intense signals were fragmented and the MS/MS spectra were analyzed manually. The sequences obtained (60% coverage from full sequence) confirmed the identity of TT-P0 chimeric protein (Figure 2B, C).

3.2 Promiscuous T cell epitopes boosts the humoral immune response to P0 peptide in tilapia

In order to evaluate the immune-potentiating action of the TCE's on the pP0 peptide, an immunization scheme was performed on tilapia. Sera from tilapia were evaluated by indirect ELISA at 21 days after re-immunization. Chimeric TT-P0 protein adjuvanted in Montanide ISA 50 V2 induced a specific antibody response against pP0 after intraperitoneal immunization ($p<0.001$) whereas the synthetic peptide in the same adjuvant or placebo (buffer) does not produce any IgM response (Fig. 3A). In this group, the 67% of the fish had specific IgM titers. The specificity of the response was confirmed by *western blotting.* The results showed that sera from TT-P0 immunized

285 fish are able to recognize the antigen. On the contrary, sera from synthetic pP0 immunized fish
286 didn't give any signal (Fig. 3B).
287 In order to compare the response induced by immunization of TT-P0 with or without the oil
288 adjuvant, we conducted another experiment in tilapia. In this experiment, only the group
289 immunized with TT-P0 adjuvanted in Montanide ISA 50 V2 had significant higher IgM titers
290 ($p<0.01$) as compared to buffer-adjuvanted injected group and 80% of immunized fish developed
291 an antibody response. In TT-P0 injected group without adjuvant, only 43% of fish had titers against
292 pP0 (Fig. 4).
293 To compare with another carrier, a third experiment in tilapia was performed where IgM response
294 to TT-P0 immunization was compared to IgM response to P0-my32. As result, the specific IgM
295 titers against P0 were significantly higher in sera from TT-P0 immunized fish as compared to buffer
296 injected ($p<0.01$) and 70% of immunized fish developed the response after 7, 15 and 21 days after
297 booster whereas only 20% had IgM titers in P0-my32 vaccinated fish and significant differences
298 with control group were only found at 7 days post re-immunization ($p<0.05$) (Fig. 5).
299 **3.3 Promiscuous T cell epitopes enhances IgM response in African catfish and Atlantic salmon**
300 We also test the potential of TT-P0 antigen to induce antigen-specific IgM response in other
301 teleost species such as African catfish and Atlantic salmon. Two groups of catfish were immunized
302 with TT-P0 and buffer (negative control). At day 35 from the beginning of the experiment (20 days
303 after booster), the IgM antibody response against P0 was measured by ELISA. Results show that
304 P0-specific total IgM response was developed in 100% of TT-P0 injected animals compared to no
305 response in buffer injected group ($p<0.001$) (Fig. 6A) and in all cases antibody titers were equal or
306 higher than 4000. In Atlantic salmon, TT-P0 was also able to induce specific IgM response at day 69
307 from the beginning of the experiment (Fig. 6B), being the antibody titers significantly higher as

compared to negative control group ($p<0.001$) but with less titers when compared to tilapia and catfish.

4. Discussion

The prevention of diseases is essential for the development of sustainable aquaculture worldwide. In this context, vaccination is the most effective method for combating diseases and currently there are some commercially available vaccines for use in fish. Modern advances in vaccines and vaccinology offer valuable opportunities to discover new vaccine candidates to combat fish pathogens such as parasitic agents, for which vaccines are still lacking [15, 16].

The present results clearly show that the fusion of TCE's to the N-terminal of P0 peptide and production of chimeric protein in *E. coli* constitute a suitable delivery system in fish for the generation of anti-P0 antibodies, suggesting the use of this cost-effective strategy to develop vaccines against conserved antigens in fish. Increased antibody titers had been found in human and murine studies using tt P2 and MVF epitopes [4, 6]. The tt P2 and MVF epitopes have been established as strong T helper cell epitopes that exhibit universal antigenicity in mammals. This universality of the TCE's was confirmed in the present study by the induction of specific IgM titers against pP0 after intraperitoneal injection in three different teleost species: Tilapia, African catfish and Atlantic salmon. Previously, its action in terms of increased vaccine efficacy was also demonstrated in salmon coho [12]. These TCE's are MHC class II restricted and are capable to bind MHC class II molecules from a wide variety of haplotypes [10, 11]. Farmed fish are outbred and presumably exhibit heterologous MHC haplotypes. Therefore, the expansion of protection across MHC haplotypes is highly beneficial to fish vaccinology.

A significant response was obtained only when the recombinant TT-P0 was injected in "water in oil" formulation with Montanide ISA 50 V2. The need for an oil adjuvant had been demonstrated

before in fish [9, 17, 18]. For example, MAPS administered in saline solution elicited no response in rainbow trout [9].

In this work, a 35 aa peptide of *L. salmonis* ribosomal protein P0 was chosen due to its less amino acid identity with respect to its hosts *S. salar*. This sequence is part of a linear B cell epitope and presents a high degree of accessibility that suggests it is exposed on the surface of the protein, forming part of a natural epitope thereof [14]. This agrees with that reported by Rodríguez-Mallon and colleagues, who used a 20-amino acid peptide as a vaccine candidate against another ectoparasite, the *R. sanguineus* tick with 90% efficacy [1]. The chimeric protein TT-P0 was designed focused on locating the designed epitopes T, measles virus and tetanus toxin, fused in tandem towards the N-terminal of the peptide of 35 amino acids pP0, because it has been reported that tandem TCE's improves the immunogenicity of chimeric proteins, with respect to the fusion of a single T cell epitope [4, 12, 19].

For the expression of TT-P0 protein *in E. coli*, the pET28a vector was selected due to bacteriophage T7 promoter robustness that ensures high levels of expression of the genes under its control. In addition, it contains a coding sequence for a tail of histidine that can be fused to both the N-terminus and the C-terminus of the target protein and it is essential for further purification by affinity chromatography to metal chelates. The immune-identification by *western blotting* showed two bands at the expected size. Similar results were obtained by us [20], using pET28a as expression vector for the recombinant tilapia IFN-γ production. In that case we confirmed by mass spectrometry that the two bands corresponded to IFN-γ and they resulted from a shift in the open reading frame in the C-terminus of the protein. Further deep characterization of the recombinant protein will allow us corroborating this hypothesis also for TT-P0.

IgM constitutes the most abundant Ig class in fish serum and this isotype plays most important role in the adaptive immune response at the systemic level but also has a role in mucosal immune

responses [21]. The first immunological evaluation carried out in this work was the demonstration of the immunopotentiating effect of the T epitopes in the IgM type humoral immune response specific against the pP0 peptide in tilapia. The titers of antibodies obtained against the TT-P0 protein adjuvanted in Montanide ISA 50 V2 were statistically superior to those obtained for animals immunized only with the synthetic pP0 peptide formulated in the same adjuvant. The synthetic pP0 was not able to induce a specific IgM humoral immune response against pP0 in tilapia. This is in consistent with the main disadvantage of peptide vaccines, which is the low immunogenicity. In the case of pP0, it contains only a linear B epitope, so it should not be recognized by T lymphocytes that cooperate in the induction of a specific humoral response and high affinity. Additionally, the group immunized with TT-P0 chimera showed the largest number of fish responders with higher titers. Concurrently, these results suggest that the incorporation of the TCE's in the chimeric construction provide an immunostimulatory effect in other T cells and in humoral epitopes within the peptide. The immunoidentification of the chimeric protein TT-P0 by *western blotting* using the sera of tilapia immunized with the formulations of the protein TT-P0 and synthetic pP0 in Montanide ISA 50 V2 confirmed the specific response against this antigen.

Previously, a chimeric protein based on pP0 fused to the N-terminal of the my32 protein of *L. salmonis* (P0-my32) was obtained by us [14]. The chimeric protein P0-my32, adjuvanted in Montanide was able to induce a specific IgM type response against pP0 in tilapia [18]. This prototype provided 28-35 relative percent protection in *S. salar* vaccinated groups at 44 days post infection with the parasite under different vaccination-boost strategies in an immunization-challenge experiment in controlled laboratory conditions [14]. Based on these results, we decided to compare the humoral immune response of this protein and the TT-P0 chimera adjuvanted in Montanide ISA 50 V2. In this experiment, higher titers against pP0 were obtained in animals immunized with TT-P0 and Montanide ISA 50 V2 compared to animals immunized with P0-my32,

379 in addition to which a greater number of fish with titers higher than 1:1000 responded. This result
380 suggests that T epitopes confer greater antigenicity to the pP0 peptide in terms of IgM response
381 and it could be a potential candidate for sea lice vaccine development.
382 As observed in the experiments, the humoral response induced in the fish, especially tilapia and
383 catfish, is very heterogeneous. This may be due to the fact that these species constitutes open
384 genetic lines, since the animals are obtained by the uncontrolled crossing between unrelated
385 individuals; unlike syngenic and pure lines, which constitute closed populations composed of
386 genetically identical individuals [22].
387 In conclusion, we showed for first time that non-responsiveness to P0 peptide in teleost fish can
388 be overcome by adding foreign T cell epitopes. The response is characterized by increased IgM
389 titers and more fish responding to vaccination as compared to synthetic peptide or another
390 chimeric protein P0-my32. TCE's, MVF and tt P2, which are highly immunogenic in human and
391 murine models, were shown to retain their immunostimulatory properties not only in the context
392 of the salmonid model but also in perciformes and siluriformes teleost species. The results are also
393 important in the context of peptide-vaccine development. In other studies in teleost, chemical
394 conjugation to carrier proteins such as hemocyanin from *Megathura cranulata* (KLH) had been
395 used to increase the immunogenicity of peptides-based vaccines. This approach could have some
396 disadvantages such as: the need of carrier purification from its natural source, reproducibility of
397 chemical conjugation procedures and the fact that some fish could have natural antibodies to KLH,
398 probably due to its presence in the marine environment. The potential lytic activity to KLH of
399 natural antibodies might have decreased the serum half-life of the peptide, reducing its
400 interaction with the fish immune system although this potential lytic action was probably
401 negligible according to results obtained in sea bass [23]. The chimeric peptide herein can be
402 obtained in controlled laboratory conditions by *E. coli* fermentation methodology adding another

403 value to the approach used. Next steps are the validation of this vaccine candidate in a
404 vaccination-challenge experiment in *S. salar*. Further characterizations of the same approach using
405 other candidate peptides will broad the application range of this strategy for peptide-vaccine
406 development to fight against important diseases in farmed species.

407 **Acknowledgements**

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Captions

Figure 1. Recombinant expression in *E. coli* of TT-P0 protein (A-C) and affinity purification (D-E). (A and D) SDS-PAGE 15 % (B) *Western blotting* using anti-His monoclonal antibody (C and E) *Western blotting* using a polyclonal serum against P0 generated in rabbits. (A-C) Lane 1: Molecular weight marker Pre-stained SDS-PAGE Standards Broad Range (BioRad, EE.UU) (MWM), Lane 2: BL21(DE3)-TT-P0 cell extract clone 1, Lane 3: BL21(DE3)-TT-P0 cell extract clone 4, Lane 4: BL21(DE3)-pET28a. (D-E) Lane 1: Not bound fraction, Lane 2: Wash, Lane 3: Elution, Lane 4: MWM. Arrows indicate TT-P0 protein.

Figure 2. Mass spectrometry identification of TT-P0 protein. (A) ESI-MS/MS spectrum of the TT-P0 tryptic digestion (B) Peptides sequences obtained (C) Underlined the sequence region obtained.

Figure 3. Antibody response in tilapia (*Oreochromis niloticus*) immunized with TT-P0 antigen in comparison with synthetic P0. Tilapia (n = 10) were injected twice intraperitoneally (days 1 and 15) with the antigens formulated in Montanide ISA 50 V2. Three experimental groups were settled: Buffer, injected with synthetic peptide (P0) and injected with recombinant TT-P0 protein. Data represents the mean + SD. The statistical analysis of data was performed using a Kruskall-Wallis

494 followed by Dunn's multiple comparison test. *** indicates p < 0.001 (B) Specific recognition of TT-
495 P0 by sera from immunized tilapia: SDS-PAGE 15% (lanes 1,2) and *western blotting* using as
496 primary antibody the sera from injected fish as primary antibody and anti-tilapia IgM as secondary
497 antibody (lanes 3-8). Lanes 3-5 immunized with TT-P0. Lanes 6-8 immunized with synthetic pP0.
498 **Figure 4.** Comparison of antibody response in tilapia (*Oreochromis niloticus*) immunized with TT-P0
499 antigen in Montanide or without oil adjuvant. Tilapias (n = 10) were injected twice
500 intraperitoneally (days 1 and 15). Three experimental groups were settled: Buffer, injected with
501 recombinant TT-P0 protein adjuvanted in Montanide ISA50 V2, injected with recombinant TT-P0
502 protein without adjuvant. Data represent the mean + SD. The statistical analysis of data was
503 performed using a Kruskall-Wallis followed by Dunn's multiple comparison test. ** indicates p <
504 0.01 with buffer injected fish.
505 **Figure 5.** IgM antibody response in tilapia (*Oreochromis niloticus*) immunized with TT-P0 antigen in
506 comparison with P0-my32. Tilapia (n = 10) were injected twice intraperitoneally (days 1 and 15)
507 with the antigens formulated in Montanide ISA 50 V2. Three experimental groups were settled:
508 injected with buffer, injected with recombinant TT-P0 protein and a third group injected with
509 recombinant P0-my32. Data represent the mean + SD. The statistical analysis of data was
510 performed using a Kruskall-Wallis followed by Dunn's multiple comparison test. * indicates p<0.05;
511 ** indicates p < 0.01
512 **Figure 6**. (A) IgM antibody response in African catfish (*Clarias gariepinus*) immunized with TT-P0
513 antigen or buffer. Catfish (n = 12) were injected twice intraperitoneally (days 1 and 15). Data
514 shows P0-specific IgM antibody response at day 35. The statistical analysis of data was performed
515 using a Mann-Whitney test. *** indicates p < 0.001. (B) IgM Antibody response in Atlantic salmon
516 (*Salmo salar*) immunized with TT-P0 antigen. Salmon (n = 120) were injected twice
517 intraperitoneally (days 1 and 35). The antigen was formulated in Montanide ISA 50 V2. Data shows

P0-specific antibody response at day 69 of 30 animals per group represented as mean + SD. The statistical analysis of data was performed using a Mann-Whitney test. *** indicates $p < 0.001$.

Figure 1

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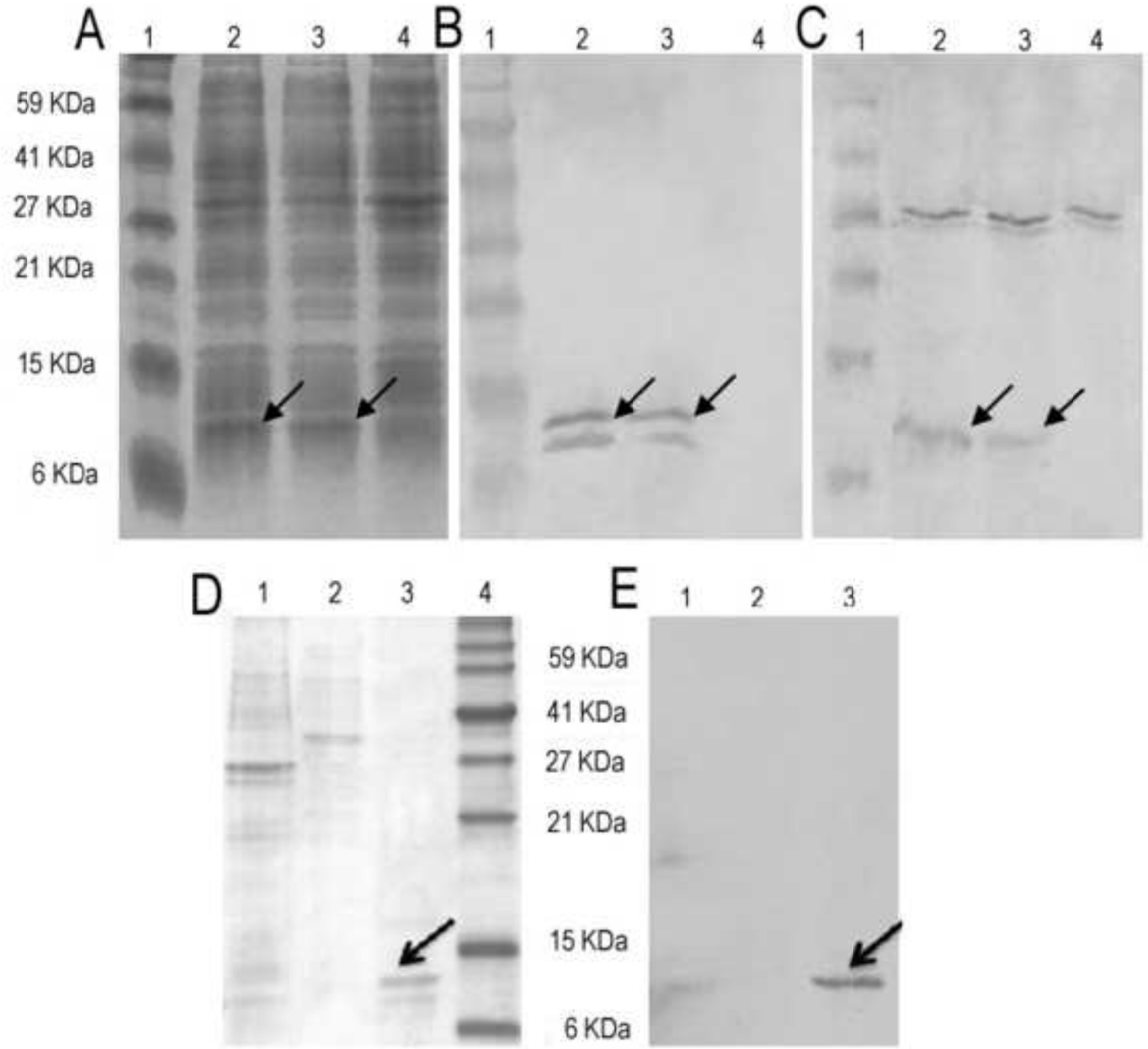

Figure 2

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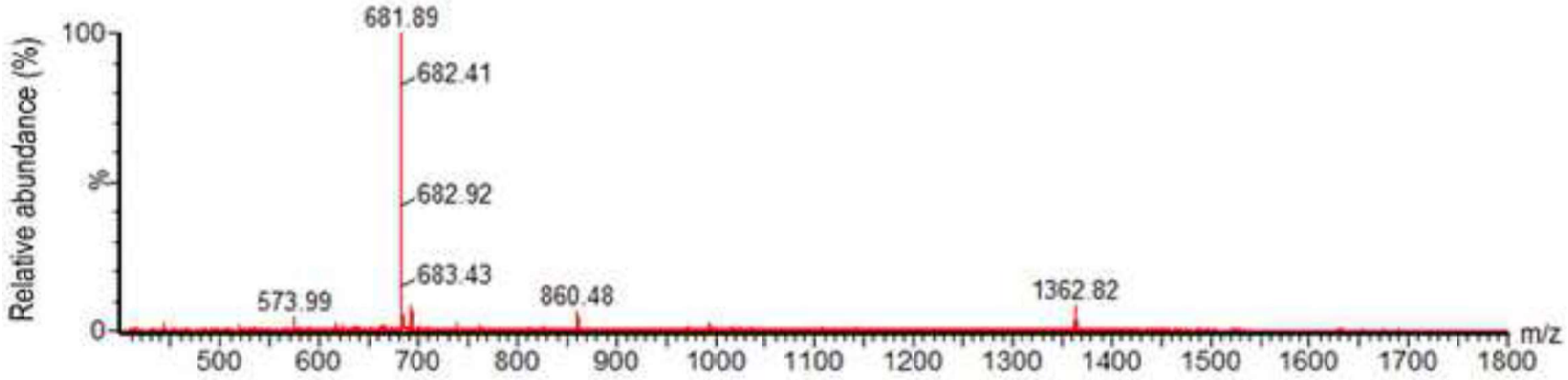

B

Protein	Sequence	Calculated mass (Da)	Experimental mass (Da)
TT-P0	LEYLADPSK	1034.53	1034.56
	FASVAAA...	1231.66	1231.70
	FIGITELLSEIK	1361.78	1361.78
	LEGVGTKLEYLADPSK	1718.91	1718.96
	FIGITELLSEIKGV...	2023.18	2023.23

C

MGQYIKANSKFIGITELLSEIKGVIVHRLEGVGTKLEYLADPSKFASVAAAPAAGATKAAAAAPAKADE

PELEHHHHHH

Figure 3

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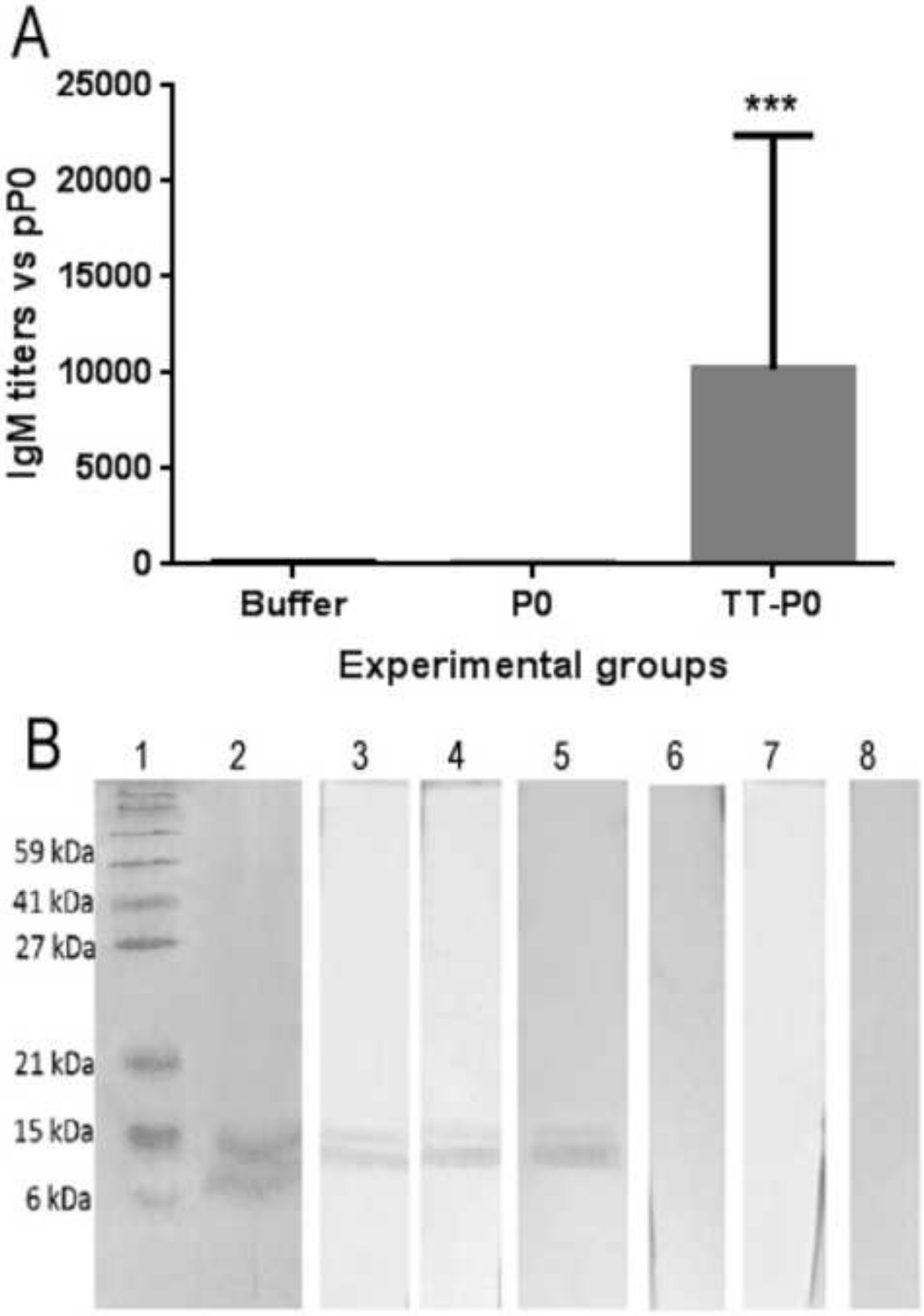

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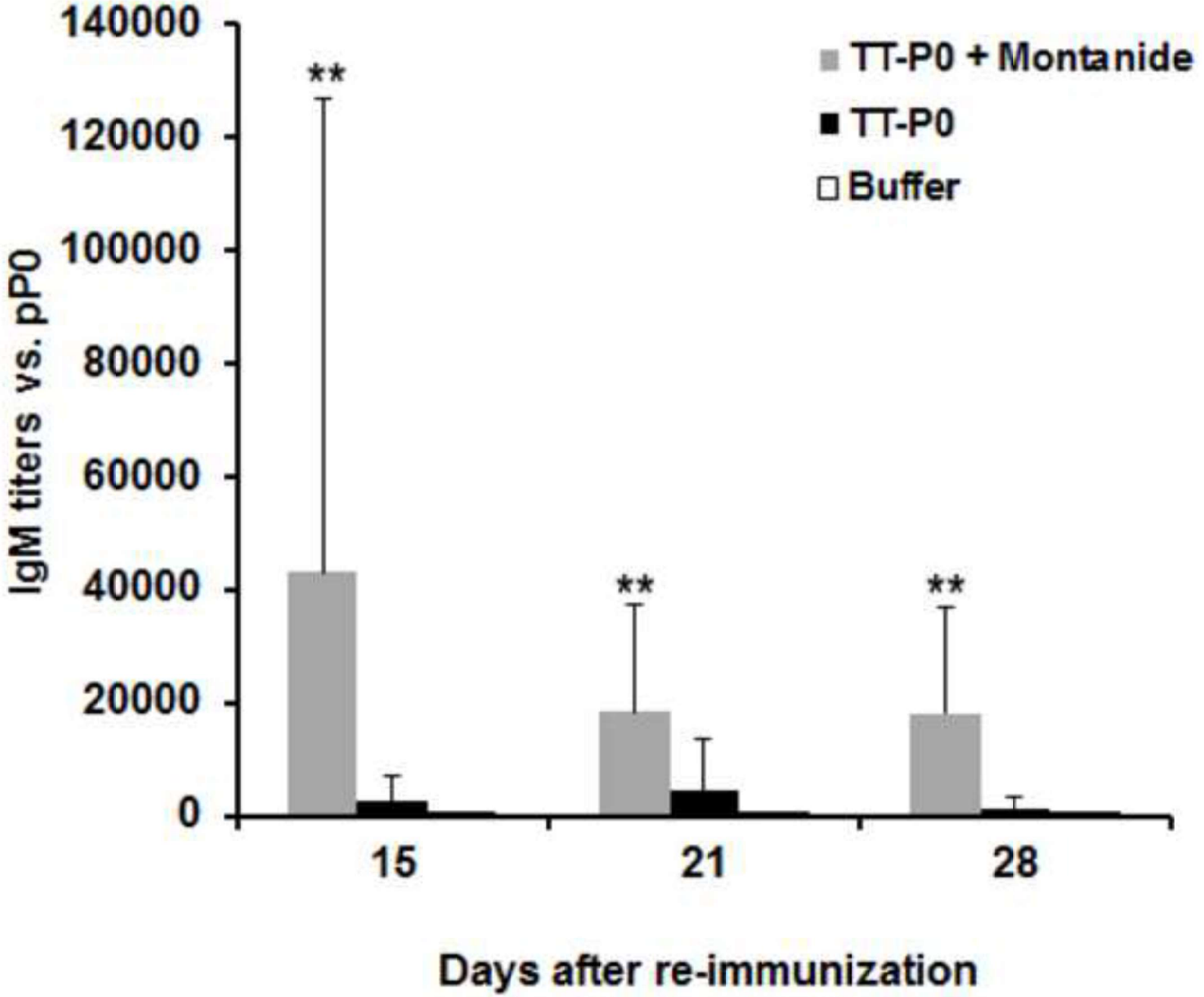

Figure 5

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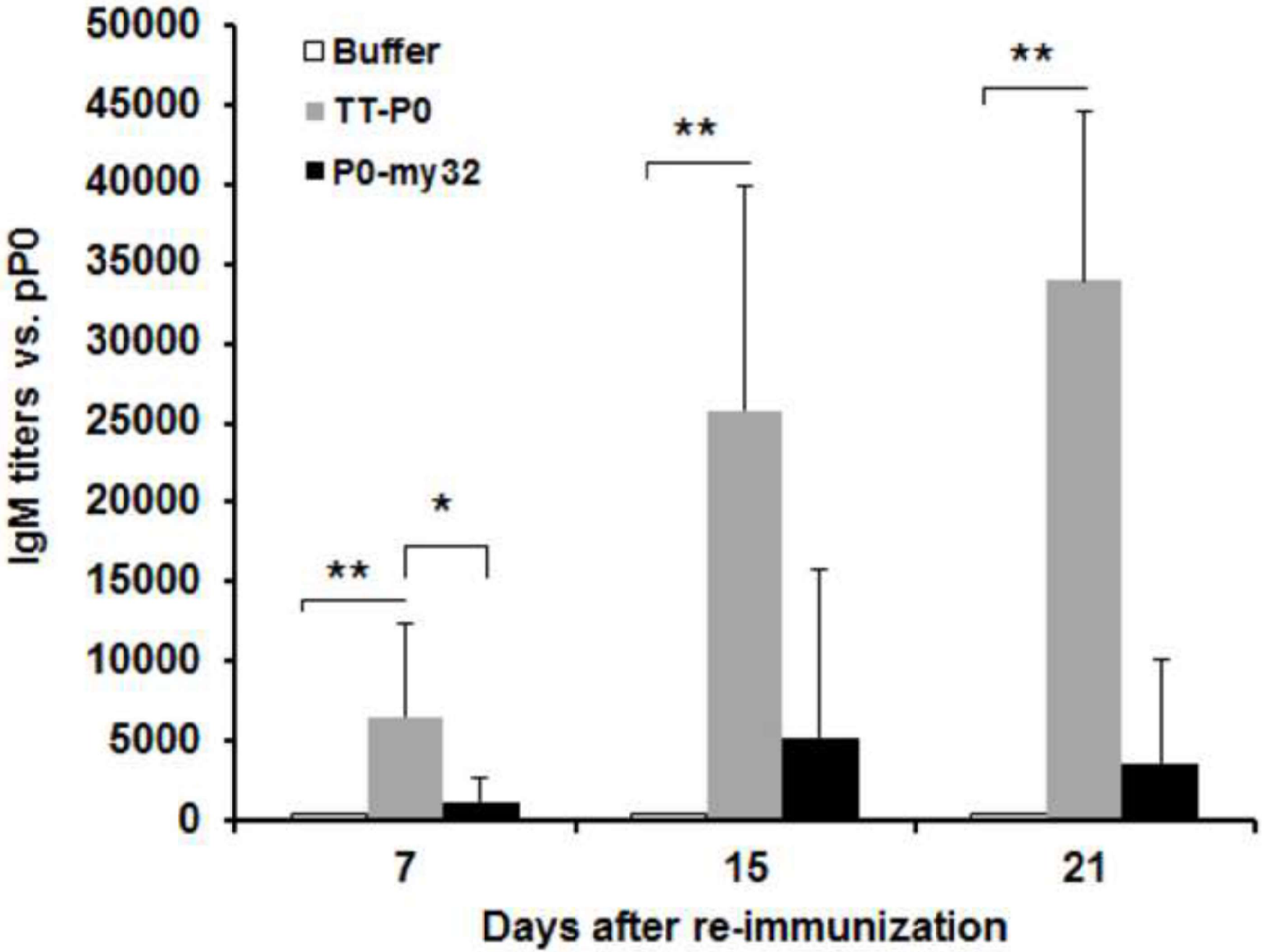

Figure 6
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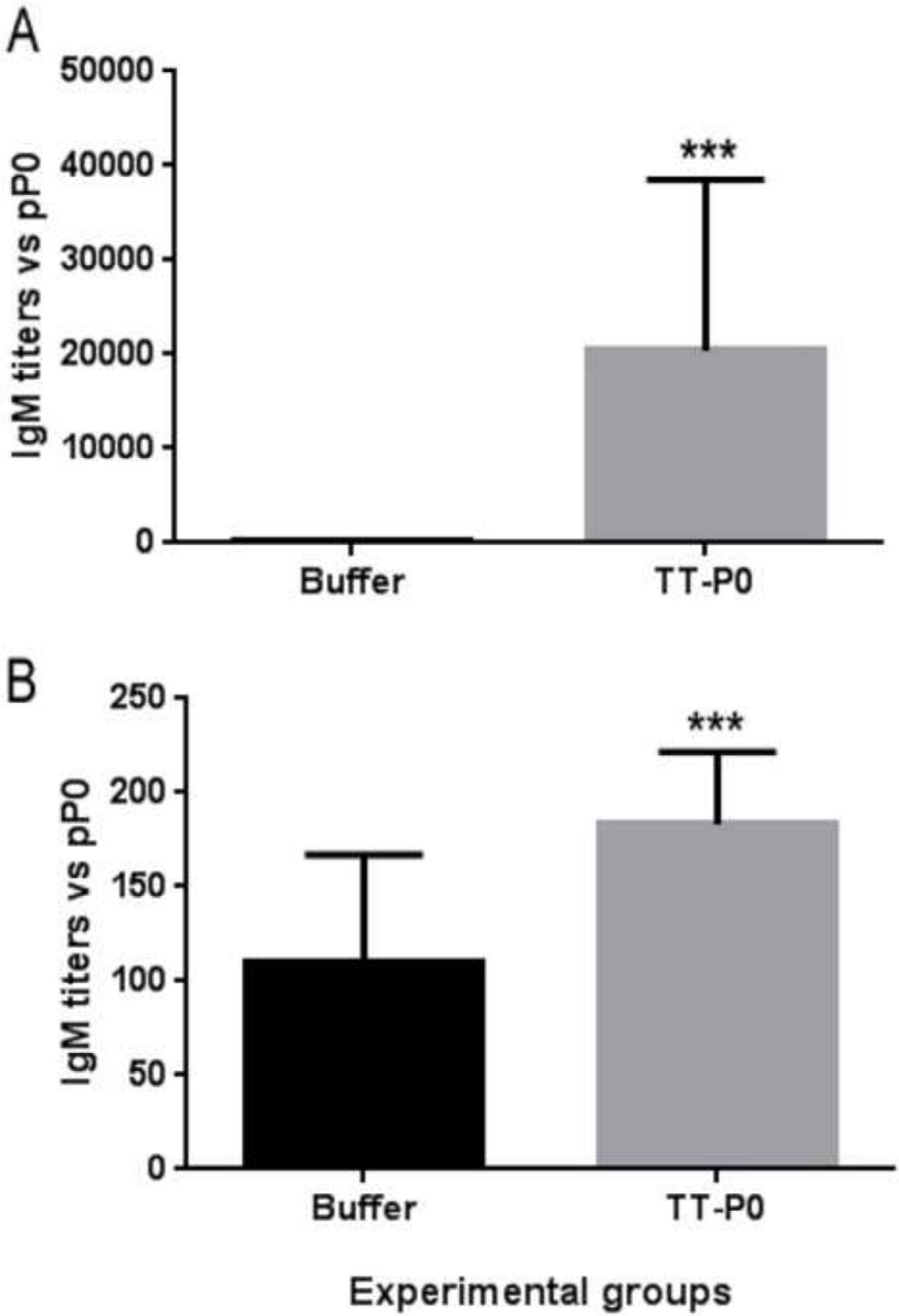

Table 1

Table 1. Primers used for cloning of promiscuous T cell epitopes and P0 into pET28a *Escherichia coli* expression vector. Endonuclease restriction sites are underlined.

Primers	Sequence 5'-3'	Direction	Application
A	CCATGGGACAATACATCAAGGCTAACTCC	Forward	Amplification of TCE's with *Nco* I site for insertion into *E. coli* expression vector
B	AAGCTTGGTACCAACACCCTCTAATCTG	Reverse	Amplification of TCE's with *Hin*d III site for insertion into *E. coli* expression vector
C	AAGCTTGAATATCTGGCTGATCCCA	Forward	Amplification of P0 with *Hin*d III site for insertion into *E. coli* expression vector
D	CTCGAGCTCAGGTTCATCCGCCTTAG	Reverse	Amplification of P0 with *Xho* I site for insertion into *E. coli* expression vector