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Short communication

Affinity maturation occurs in channel catfish (*Ictalurus punctaus*) following immunization with a T-cell dependent antigen

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ABSTRACT

Affinity maturation of the antibody response, a process of antibody affinity increasing over response, is one of the key features of the mammalian immune system. However, the process is incompletely understood in teleost, including channel catfish (*Ictalurus punctaus*). In this study, IgM affinity maturation in channel catfish was investigated by estimating the kinetics of antibody affinity using ELISA and ELISPOT assays. Fish were immunized with a T-cell dependent antigen (TNP-KLH), and individual serum IgM antibody titers and affinities, and IgM^+ antibody-secreting cells (ASCs) in peripheral blood were analyzed over a period of 14 weeks. A detectable serum anti-TNP response developed by 2-weeks post-immunization, and the maximal antibody production was observed by 6-weeks post-immunization. The average affinity of anti-TNP serum antibody increased consistently and reached the maximum by 10-weeks post-immunization. The increase of antibody affinity beyond the point of optimal antibody titer revealed that the affinity maturation of IgM antibody response occurred in channel catfish. Dissection of dynamics of individual affinity subpopulations indicated that a significant proportion of low affinity subpopulations appeared at early response, and high affinity subpopulations appeared predominantly at later, resulting in a 100-fold increase in affinity over response. Additional, TNP^+ IgM^+ ASCs was detected by 2-weeks post-immunization and achieved the maximal number by 6-weeks post-immunization. Using an inhibition ELISPOT assay, the findings of a consistent increase in the average affinity of secreted IgM antibody by peripheral blood ASCs, as the immune response progressed, confirmed the occurrence of the affinity maturation. Taken together, the results of this study indicated that affinity maturation occurred in channel catfish following immunization with a TD antigen TNP-KLH.

1. Introduction

Antibody affinity maturation is an important, unique feature of humoral immunity, referring to the strength with which the epitope binds to an individual antigen-binding site on the antibody increased during an immune response [1–3]. The mechanisms of antibody affinity maturation in mammals include somatic hypermutation and antigen-driven clonal selection [4–9]. Different from the mammals, in the early years, the fish were thought to lack antibody affinity maturation because studies found that affinity of serum antibody only increased very little following immunization in teleost species, including the coho salmon, channel catfish, and Atlantic salmon [10–12]. However, recently, the molecular analyses in zebrafish had demonstrated that teleost V genes do undergo somatic mutation upon response to antigen [13], which was also demonstrated in vaccinated channel catfish with detection of the mismatchment of the VH or JH segments of μ-chain [14]. The discovery of somatic hypermutation of a new antigen receptor gene (NAR) in the nurse shark revealed the possible role in antigen-driven reactions, which is similar to that of mammalian Ig genes and to spontaneous mutations in evolution [15]. Thus, these studies indicated affinity maturation could be exist in fish. Until 2002, employing surface plasmon resonance [16] and a partition-based immunoassay [17,18], IgM antibody affinity maturation was proved to be occurred in rainbow trout over a T cell-dependent antigen response. T cell-dependent antigen refers to the antigens that activate B cells with the help of T-cell [19]. Relatively, antigens that activate B cells without T cell help are known as T cell-independent antigens [20]. B cell activated by T cell-dependent antigens takes multiple days, and antibodies generated have a higher affinity and are more functionally versatile than those generated from T cell-independent activation [21]. Although the affinity maturation was demonstrated that existed in rainbow trout, the process remains uncompleted understood in teleost since there are

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so many fish species. In channel catfish, the early study found only very little increase in affinity; however, the recent findings of the somatic mutation in VH genes [14] with the identification of B cell mutator enzyme activation-induced cytidine deaminase (AID) [14,22,23] suggest that antibody affinity maturation may be existing in channel catfish.

In mammals, it is widely accepted that antibody affinity maturation is the result of somatic hypermutation of V region genes combined with the positive selection of B cells whose mutated B cell antigen receptors (BCR) acquire higher affinity [5,7,24,25]. The differentiation of B cells, switching from expression of membrane-bound antibody to secretion of antibody, into plasmablasts and then, ultimately, into plasma cells (including short-lived plasma cells and long-lived plasma cells) causes B cells with the ability to secrete antibody [26,27]. At the early antigen response, antibody-secreting cells (ASCs) are dominated by plasmablasts. With co-stimulation of activated T cells and differentiation factors, these plasmablasts can develop into terminally differentiated, short-lived plasma cells in the periphery [27] and may become long-lived plasma cells [28] upon migration to survival niches within the bone marrow in mammal. Particularly, due to the mechanism of affinity-driven selection, the long-lived plasma cells express antibodies that have been affinity matured by somatic hypermutation [29]. Similar to the findings in mammals, the recent discovery in rainbow trout indicate that B cell subset diversity is existing in teleost, including resting B cells, plasmablasts and plasma cells [30–36]. In rainbow trout, the B cell differentiation from activated B cells into plasmablasts then plasma cells were demonstrated in both *in vitro* and *in vivo* stimulation over the antigen response [33,36,37]. Among the process, long-lived plasma cells are found existing predominantly at the late stage of immunization [30], which is coincident with the appearance of high affinity antibody dominant at the late response. In channel catfish, a comparable discovery of B cell subpopulations and differentiation [32] and B cell memory [22,23] may reply that antibody affinity maturation exists in channel catfish as the rainbow trout.

In this study, the affinity partitioning ELISA was employed to dissect affinity dynamics of serum IgM antibody following immunization with TNP-KLH, and an inhibition ELISPOT method was established to examine the affinity of IgM antibodies secreted by ASCs *in vitro* culture. We believe that this study will enrich the knowledge of affinity maturation in teleost and provide a new perspective for humoral immunity study in channel catfish.

2. Materials and methods

2.1. Fish

Healthy channel catfish (*Ictalurus punctatus*) (600 ± 100 g) were obtained from Guangdong Catfish Breeding Farm in Guangzhou (Guangdong, China) and maintained in semi-automatic circulating water system using biologically filtered, dechlorinated, chemically balanced, and UV-treated city water. Fresh water exchange was 4%/day, with 75% of the volume recirculated through the biofilter per hour. Water temperature was maintained at 28 ± 2 °C, and photoperiod was adjusted to match seasonal change. Fish were fed for 3 weeks prior to any experiment and no clinical sign of diseases was observed. All animal protocols were reviewed and approved by the University Animal Care and Use Committee of the South China Normal University.

2.2. Antigen preparation and fish immunization

The antigen, trinitrophenylated-keyhole limpet hemocyanin (TNP-KLH) was prepared as previously described [18] with some modifications. In brief, 10 mL of 5 mg/mL KLH (Sigma, USA) was dialyzed against 1 L exchanges of phosphate buffered saline (PBS, 1.85 mM NaH_2PO_4, 8.41 mM Na_2HPO_4 and 150 mM NaCl, pH 7.3) for three times, 2 h every time at room temperature (RT). After dialyzed, the KLH solution was stored in a 15 mL foil-covered sterility tube. The TNP-KLH was accomplished by the drop-wise addition of 65 μL 2,4,6-trinitrobenzenesulfonic acid solutions (TNBS, 5% w/v, Sigma, USA) to the KLH, and this solution was mixed on a rotator for 1 h at RT. The solution was then dialyzed against three 1-L exchanges of PBS to remove the unreacted TNBS. After dialyzed, the solution was filter-sterilized using a 0.22 μm filter (Millipore, USA) and stored in a sterile stoppered amber bottle at 4 °C. Protein concentrations were determined by Nanodrop 2000 (Thermo, USA). TNP-BSA was employed as the antigen for antibody affinity and titer determinations.

Channel catfish were anesthetized with a 0.02% solution of 3-aminobenzoic acid ethyl ester (MS-222, Aladdin, USA) prior to immunization. After anesthetizing them, 1 mL bleeding blood was collected by venipuncture from the caudal vessel, then placed them in a sterile 1.5 mL tube as week 0 sample. The blood was centrifuged at 4 °C, 500 × *g* for 10 min and the serum was collected and stored at −80 °C. Fish were immunized by intraperitoneal injection with 100 μg TNP-KLH per fish in a final volume of 100 μL (TNP-KLH in PBS emulsified 1:1 with Freund's complete adjuvant (FCA, Sigma, USA), n = 5), and five fish were used for the control group immunized with the same volume of PBS emulsified 1:1 in FCA.

2.3. Antibody titers and affinities determinations

The anti-TNP sera titers of channel catfish were determined by the standard antibody titration ELISA as described in trout [17]. Since the substrate was 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), the optical density (O.D.) rate was measured using a microplate reader (Thermo, USA) at 405 nm. Titers were expressed as the number of antibody units per milliliter of serum. A unit of antibody activity is defined as the volume of sample required to produce O.D. rate of half the maximum rate.

Anti-TNP sera antibody affinity subpopulations were assessed by a partitioning ELISA-based analysis as previous described [38]. All of anti-TNP antibodies competed with inhibition TNP-Lysine to combined with antigen TNP_8-BSA, the affinity of different antibody subpopulations (aK) is determined by the equation as published [18]. The discrete individual aK values were distinguished as half -log aK increments (3.00–3.49, 3.50–3.99, 4.00–4.49, 4.50–4.99, 5.00–5.49, 5.50–5.99, 6.00–6.49, 6.50–6.99, and 7.00–7.49).

2.4. Isolation of leukocytes from peripheral blood (PBL)

The procedure of leukocytes isolation was referred to previous description with some modification, and the brief steps are as followed [30,34]. Channel catfish were anesthetized in water containing approximately 0.04% MS-222 at week 0, 2, 4, 6, 8, 10, and 14 post-immunization. PBL was immediately collected from the caudal vein with 2.5 mL syringe. After centrifuged at 4 °C, 500 × *g* for 10 min, the serum was collected. The cells were diluted to the total volume about 10 mL with RPMI-1640 (Gibco, USA), containing 100 U/mL penicillin G and 100 mg/mL streptomycin (Sigma, USA), suspend the cells gently then put it on the ice.

The 10 mL cell suspension was layered upon an equal volume of Histopaque 1077 (Sigma, USA) in 50 mL conical centrifuge tubes slowly, 500 × *g* centrifuged for 40 min at 4 °C [30,34]. Leukocytes were collected from the interface layer were washed three times by centrifugation in RPMI-1640. Viability was determined by 0.4% trypan blue, and cells were resuspended to a concentration of 1×10^7 cells/mL in RPMI-1640.

2.5. ELISPOT to dissect ASCs of PBL and secreted antibody affinity

ASCs were enumerated by ELISPOT analyses as previously described [30,34]. Briefly, after activated PVDF membrane (Millipore, USA) with methanol, washed by PBS three times. Then 20 mL PBS containing

50 μg/mL TNP_8-BSA was added to incubate the membrane at RT for 2 h on shaker. Then membrane was rinsed with PBS three times and blocked with blocking solution (0.5% BSA in PBS) for 2 h. Washed 3 times as previous, the membrane backed with parafilm was inserted into a dot-blot apparatus (BioRad, USA) and 10^4-10^6 cells per well was added in a total volume of 200 μL. As for the inhibition ELISPOT, diluted TNP-Lysine to a range of concentrations (1×10^{-4} M, 2×10^{-5} M, 4×10^{-6} M, 8×10^{-7} M, 1.6×10^{-7} M, 3.2×10^{-8} M, 6.4×10^{-9} M and 0 M) and joined 100 μL to every well, each sample set three repetitions. Mixed 100 μL leukocytes in a count of 1×10^6 cells with the corresponding inhibitor (TNP-Lysine), cultured at 25 °C overnight. Then the membrane was removed, wiped with a Kimwipes (Kimwipes, USA), and washed with PBS three times, incubated with gentle shaking in blocking solution for half an hour. The membrane was then washed with PBS and incubated with gentle shaking at for 2 h in PBS containing mouse monoclonal antibody anti-channel catfish IgM heavy chain (0.5 μg/mL, 1:1000 dilution) [32,39]. Washed with PBS 3 times incubated with gentle shaking at RT for 2 h in PBS containing goat anti-mouse IgG conjugated with HRP (0.5 μg/mL, 1:4000 dilution) (Southern Biotech, USA). Finally, after washed the membrane 3 times, AEC (3-amino-9-ethylcarbazole, Sigma, USA) development solution (8 mg AEC in 0.05 M acetate buffer with 0.04% H_2O_2) was applied to the membrane. Flushing with tap water terminated the reaction, then dry the membrane naturally.

The ASCs were enumerated using a stereoscope (Olympus, Japan) and spots counted on the enlarged photographs. One spot represented an antibody-secreting cell. The total ASCs of PBL were determined by multiplying the ASCs/10^6 leukocytes by the total leukocytes. Total blood volumes were calculated as previously described [34,40]. The spots number of each well was calculated in different inhibitor concentration and draw a curve as Fig. 4A. The standard is 50% of the maximum value, indicating the hapten concentration that inhibits antibody to produce 50% of the largest number spots.

3. Results and discussion

Channel catfish produced a distinct specific IgM immune response after immunization with TNP-KLH. A group of five fish was immunized with TNP-KLH, and IgM titers to TNP were monitored at weeks 0, 2, 4, 6, 8, 10 and 14 post-immunization (Fig. 1A). It can be seen that the anti-TNP titers increased early first, and achieve the peak at week 6, then slowly declined. In the 14-week period, the detection of TNP-specific sera IgM titers of channel catfish indicated that there was about a 55-fold increase at 2 weeks post-immunization (p.i.) compared to that at week 0, and the IgM antibody titer reached the peak at 6 weeks p.i. with an average titer of approximately 112,724 units/mL, then it decreased slowly and maintained at a level about 14,923 units/mL in the late immunization (at 14 weeks p.i.) (Fig. 1A). The dynamics of IgM antibody exhibited was similar to the findings in rainbow trout that the antibody titer increased first and attained the maximal, then began to slowly decline [17,18]. It was also consistent with the reported kinetics of anti-DNP IgM antibody after immunization with DNP-HoSA in an early study of channel catfish [10].

The measurement of IgM antibody affinity showed that the anti-TNP affinities increased at every time point through week 10 (Fig. 1B), 4 weeks past the point at which the highest titers were observed (Fig. 1A), indicating affinity maturation undergoing in channel catfish. The affinity achieved a peak about -log aK = 6.5 at 10 weeks p.i. with a 100-fold increase in affinity, and the high affinity antibodies maintained at the late of immunization (14 weeks p.i.) (Fig. 1B). It was similar to the kinetics of IgM antibody affinity in rainbow trout after immunization with TNP-KLH [17,18]. In addition to the average affinities revealing affinity maturation (Fig. 1B), examination of the behavior of the individual affinity subpopulations is considerably more informative (Fig. 2). In order to investigate the dynamics of antibody affinity subpopulations, a solid-phase affinity-partitioning ELISA assay was adopted, which had been widely employed in estimate of antibody affinity such as in rainbow trout [17,18], duck [41], and mouse [42]. Different to the classical techniques such as equilibrium dialysis or fluorescence quenching only providing single average affinity estimation, the affinity-ELISA can provide estimates of individual affinity for antibody subpopulations within a single serum sample [38]. The analysis of affinity subpopulations behavior provided more information, revealing there existed different subpopulations during the immunization (Fig. 2A). The low affinities appeared early in the response, while the high affinity subpopulations, including -log aK = 4.50–4.99, 5.00–5.45 and 5.50–5.99, appeared after 2 weeks post-immunization. The higher affinity antibodies (6.00–6.49, 6.50–6.99, and 7.00–7.49) appeared later (such as 4–8 weeks p.i.), persisted longer and maintained dominantly through week 14. At the peak of antibody affinity reached at 10 weeks p.i., the high affinity subpopulation such as -log aK = 7.00–7.49 occupied about 30%. The titers of antibody affinity subpopulations were calculated, shown in Fig. 2B, revealed that the high affinity subpopulations were dominant in the late phase. Thus, not only the average antibody affinity but also the affinity subpopulations in the process of immunization indicated that the affinity maturation occurred in channel catfish. These findings were consistent with the reported differential dynamics of antibody subpopulation expression during affinity maturation in rainbow trout [17,18].

In contrast to the only little increase in affinity in the previous study [9], in this study, a significant 100-fold increase in IgM antibody affinity was observed, mainly with the contribution of the late and de novo appearance of the high affinity subpopulations (such as -log aK = 7.00–7.49). The late appearance of the high affinity subpopulations was possible due to antigen-driven selection of somatic mutations that had only appeared late, which had been previously observed in

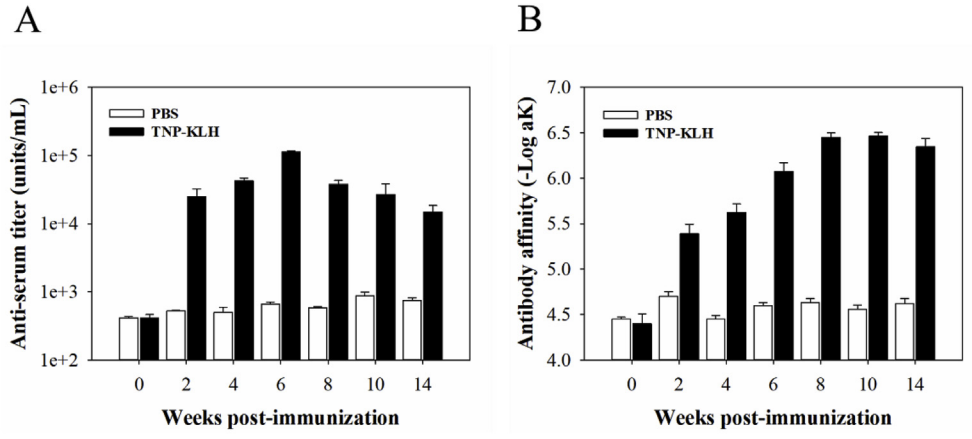

Fig. 1. Change in serum IgM antibody titer (A) and average affinity (B) induced to TNP-KLH. Each of five TNP-KLH immunized channel catfish was bled prior to immunization (week 0) and periodically over the ensuing 14 weeks (week 2, 4, 6, 8, 10 and 14). PBS immunized as control. Error bars represent standard error about the mean.

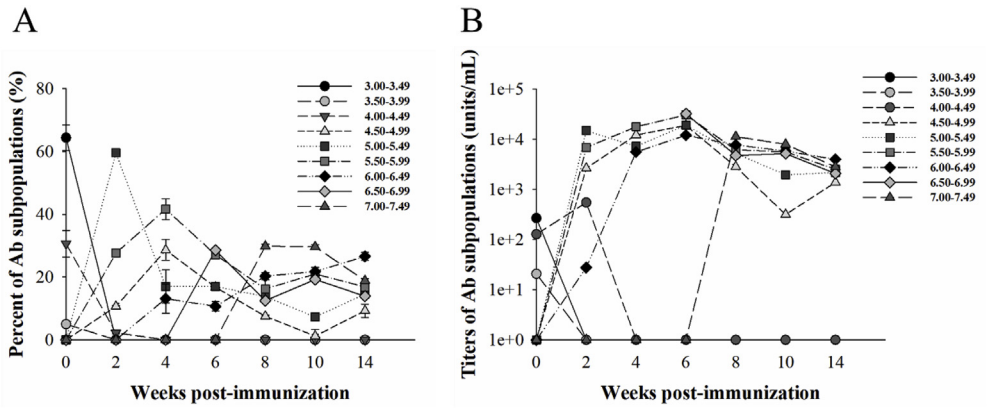

Fig. 2. Change in affinity profiles of serum IgM antibodies over time. The responses were summarized for each time point (week 0, 2, 4, 6, 8, 10 and 14). The titers (A: the average titer) of each affinity distribution was converted into the percent of the total antibody titer for each individual sample. The average percent (B) of each affinity was then calculated using all individuals in the group and depicted in the figure. Error bars represent standard error about the mean.

trout [43]. More specifically, the somatic mutations had been reported in the catfish [14] as well. Further, another possibility is because of high-dose suppression [44–46] that the high concentration of antigen suppressed the highest affinity B cells, which would not be relieved until the concentration diminished late in the response. It may be the reason to explain the only little increase in affinity observed in the previous catfish study [10], since the immunization dose was much higher (500 μg). The similar findings were also found in trout, when exposing the fish to high concentration of the antigen and more than one antigen injection, the greater persistence of the low affinity subpopulations was observed [17]. It corroborates the classical mechanism of antigen-driven selection that only low concentration of antigen is able to selectively elicit high affinity response [17,24].

To further explore the affinity maturation undergoing in channel catfish, the affinities of TNP-specific antibodies secreted by ASCs were examined *in vitro* with ELISPOT analysis. Leukocytes were separated from PBL over a period of 14 weeks after immunization by Histopaque-1077 gradients. Here, one million leukocytes were performed in the plate each well and the TNP-specific ASCs occupied as showed in Fig. 3A by ELISPOT. One spot represents a specific ASC. It was found that the TNP-specific ASCs reached the peak at week 6 p.i. by both per 10^6 leukocytes and the whole leukocytes in PBL (Fig. 3B and C). Then, an inhibition ELISPOT was employed to explore the affinities of TNP-specific antibodies secreted by ASCs. After added different concentration of TNP-lysine to compete the binding of TNP_8-BSA, the spots number decreased with the increased concentration of TNP-Lysine (Fig. 4B). Anti-TNP specific ASCs at week 14 p.i. were too few to be measured by inhibition ELISPOT, so here the affinities until week 10 were displayed (Fig. 4C). It's clear that the affinities of anti-TNP antibodies from ASCs presented the same trend with serum antibody affinities, which increased gradually then stayed at a high level in the late phase (Fig. 4C). The dynamic of anti-TNP specific ASCs responses reflected a TD antibody response in channel catfish, similar to that in rainbow trout [30,34], demonstrating that the ASCs were plasmablasts and plasma cells. In mammals, when stimulated by TD antigens, somatic hypermutation takes place through B cell stages and B cells with mutated B cell receptor are continuously selected on the basis of antigen specificity as well as affinity [5,8]. The somatic mutation and B cell heterogeneity (plasmablasts and plasma cells) happened [14,30,31,34] in teleost as well. Plasmablasts appeared in the early immunization [30] corresponding to low affinity antibodies appeared at the primary immunization [17,18]. At the late immunization stage,

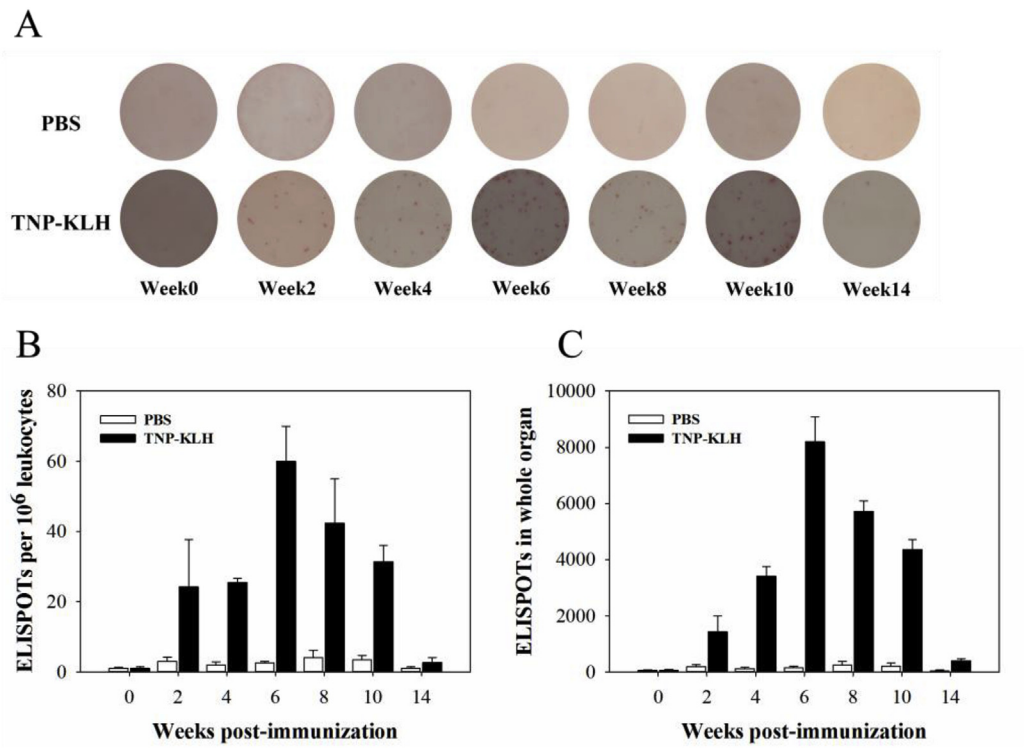

Fig. 3. Kinetics of TNP-specific ASCs response in peripheral blood over time. The ELISPOT dynamics of PBL anti-TNP special ASCs per 10^6 leukocytes (A), the calculation of specific ASCs in 10^6 leukocytes (B) and in the whole organ (C). Each channel catfish was bled prior to immunization (week 0) and periodically over the ensuing 14 weeks (week 2, 4, 6, 8, 10 and 14). Error bars represent standard error of the mean (n = 5).

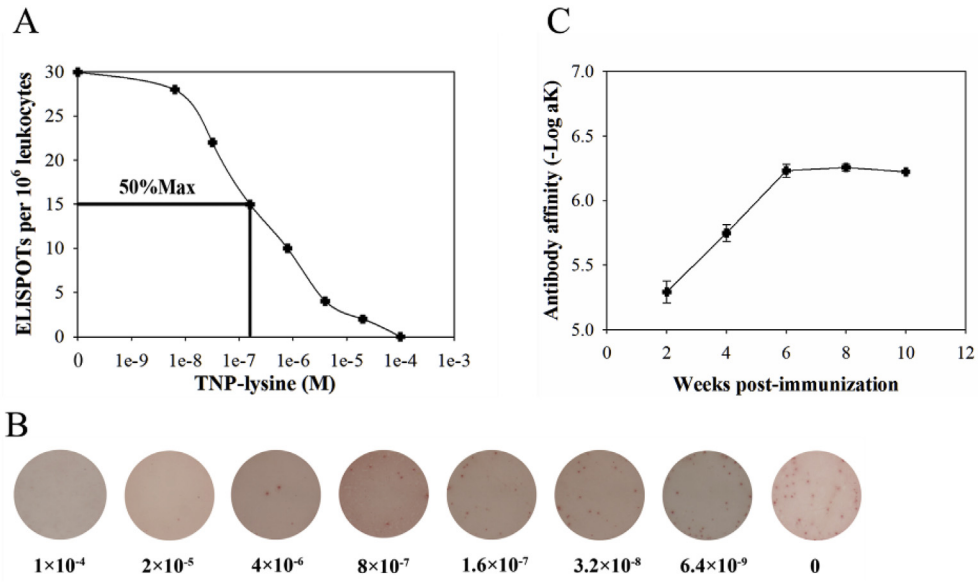

Fig. 4. Dynamics of average affinity of secreted antibodies by peripheral blood TNP-specific ASCs over time. Diagram of a model to calculate the average affinity of antibodies secreted by ASCs by examination of ASCs (ELISPOTs) per 10^6 leukocytes related with the concentration of inhibitor TNP-lysine added (A and B), and (C) kinetic of average affinity of secreted antibody by PBL TNP-specific ASCs in catfish. Error bars represent standard error of the mean (n = 5).

however, plasmablasts differentiate into the plasma cells secreted high affinity antibody and gradually occupied the main position in anterior kidney [30]. Though the normal PBL is regarded possessing mature naive B cells that cannot generate and maintain a population of plasma cells, the migration of B cell subsets from anterior kidney or spleen makes the plasmablasts and plasma cells exist in PBL possible [30,31]. The phenomenon of long-lived plasma cells existed in anterior kidney and continued to secrete antibodies [30] seems to demonstrate that the antibodies in serum at the late phase were sustained by these cells.

4. Conclusion

After immunized with TNP-KLH, the maximal anti-TNP IgM antibody affinity reached at week 10 beyond the IgM antibody titer peak at week 6. Low affinity antibodies greatly appeared early and the high affinity antibodies dominantly expressed later, indicating that affinity maturation occurred in channel catfish. In addition, the occurrence of affinity maturation was confirmed by an inhibition ELISPOT assay, appearing a consistent increase in the affinity of antibodies secreted by ASCs at the response. The analysis of affinity maturation occurring in channel catfish may provide a new perspective to understand the affinity maturation in teleost and teleost B cell development and differentiation.

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