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Technical report

Characterization of anti-channel catfish IgL σ monoclonal antibodies

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ABSTRACT

This study characterizes three monoclonal antibodies (mAbs) developed against the constant (C) region of the immunoglobulin light (IgL) σ chain isotype of the channel catfish, *Ictalurus punctatus*. Microsphere bead assays and Western blot analyses utilizing different recombinant (r) proteins show that these anti-catfish IgL σ chain mAbs each specifically recognize the denatured form of IgL σ. Importantly, Western blotting of catfish sera using the anti-IgL σ mAbs also identified an IgL chain-sized immunoreactive band(s) of ~27 kDa. It is anticipated that these mAbs in combination with the already existing anti-catfish Ig heavy (H) and IgL chain mAbs will be useful in future studies examining the functional roles of the different catfish IgL isotypes.

As for most fish species, the current lack of immunologically relevant antibody reagents for the channel catfish, *Ictalurus punctatus*, severely limits functional studies in this species. Thus, development of polyclonal (pAb) and monoclonal antibodies (mAbs) is vital to the unambiguous identification and functional characterization of various leukocyte populations within the catfish. Currently there are only a limited number of anti-catfish mAb reagents available, these include anti-IgM (Miller et al., 1987), anti-IgD δsec (Bengten et al., 2002) anti-IgD δ2 (Chen et al., 2009), anti-IgL F (Lobb and Clem, 1982; Lobb et al., 1984), anti-IgL G (Lobb and Clem, 1982; Lobb et al., 1984; Shen et al., 2003), anti-CD45 (Kountikov et al., 2004) and anti-MHC II α and β mAbs (Dijkstra et al., 2003; Moulana et al., 2008; Thankappan et al., 2006). Also, there are a number of catfish antibody reagents that recognize specific cell populations but the epitopes are less well defined. These include anti-neutrophil mAb 51A (Ainsworth et al., 1990), anti-thrombocyte mAb 4-20 (Passer et al., 1997), and anti-cytotoxic cell mAb CC41 (Shen et al., 2003). With the recent genetic identification of two new catfish immunoglobulin light (IgL) chain isotypes IgL σ and IgL λ, we undertook the development of anti-IgL σ specific mAbs as part of the US Veterinary Reagent Network (http://www.umass.edu/vetimm/).

Channel catfish express four IgL chain isotypes: IgL λ, IgL σ (Edholm et al., 2009), and two subclasses of IgL κ (F and G; Ghaffari and Lobb, 1993, 1997). IgL F and G were the first IgL chains to be described in catfish and each represents a distinct isotype since the C domains share only ~30% amino acid identity with each other. However, phylogenetic analysis clearly demonstrates them to be IgL κ orthologs (Criscitiello and Flajnik, 2007). Previously, the use of anti-catfish IgL F and IgL G mAbs established that F and G light chains were expressed on different B-cell subsets and together constitute the majority of catfish IgM associated IgL chains. Consistent with this, >90% of purified catfish anti-dinitrophenyl (DNP) IgM in serum was immunoprecipitated when anti-catfish F and G mAbs are used in combination (Lobb, 1986; Lobb and Clem, 1982; Lobb et al., 1984). As compared to the catfish Igκ isotypes, the genes encoding the recently described catfish

Abbreviations: IgL, immunoglobulin light chain; IgH, Ig heavy chain; mAb, monoclonal antibody; PBL, peripheral blood leukocytes.

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IgL λ and IgL σ chains are considerably less diverse (Edholm et al., 2009). For example, while the genes encoding IgL σ and IgL λ are found in a cluster organization of one or two variable (V) gene segments, followed by single joining (J) and constant (C) region genes like other teleost IgL chain genes, they are few in number. Both catfish IgL σ and IgL λ are each encoded by only two clusters. In contrast, Southern blot analyses showed there were at least 30 V segments and 15 C genes for the catfish G isotype and at least 50 different fragments hybridized with V and C probes for the F isotype (Ghaffari and Lobb, 1993, 1997).

Anti-catfish IgL σ mAbs were produced by immunizing Balb/c mice with recombinant (r) IgL σ made in a prokaryotic expression system using standard protocols. Briefly, the complete constant region of IgL σ as well as the two membrane proximal domains of catfish CD4 (used as a negative control) (Edholm et al., 2007) were amplified from catfish PBL cDNA by RT-PCR (see Table 1 and Fig. 1A) and cloned into the Champion pET 100 Directional TOPO expression vector (Invitrogen), which introduces a N-terminal polyhistidine region and a Xpress epitope tag. After sequence verification the constructs were transformed into BL21 competent *E. coli* (Invitrogen) induced with 1 mM IPTG and harvested according to the manufacturers recommendations. Approximately 1 mg each of rIgL σ and rCD4 was purified from 1 L lysed bacterial cultures using MagneHis Ni-particles (Promega) according to the manufacturer's protocol. The resulting r proteins were refolded using 1.0 kDa cutoff mini dialysis chambers (Amersham Pharmacia Bioscience) in a step-wise dialysis process in the presence of a redox-pair according to the Novagen protein refolding kit. The refolding steps in neutral buffer were included in the purification since *E. coli* generated r proteins are purified from bacterial lysates under harsh conditions, i.e. 4–8 M urea. We believe that refolding in neutral buffer is an essential part of the purification process and by using a single domain protein which has been dialyzed/refolded in a neutral buffer for immunization, the chances of obtaining a mAb that reacts with native protein is increased. In the past we have used this refolding protocol to produce the soluble catfish rFcRI protein (Stafford et al., 2006; Nayak et al., 2009) and catfish rIgD (domain 2), which was used successfully for immunization and production of anti-catfish IgD mAb 7D11 (Chen et al., 2009).

The rIgL σ was ~15.4 kDa in size including an ~4.1 kDa tag while the rCD4 including the tag was ~30.0 kDa in size, both proteins were readily detectable using the anti-Xpress mAb. Anti-catfish IgL σ mAbs were produced at the University of Massachusetts Amherst as part of the U.S.

Table 1
Primers used for making pET 100 constructs.

Primer	Sequence 5′–3′	Accession number[a]
IgLμpET F	CACCGACGCTGCTCTCCCGGATCCT	EU872021
IgLμpET R	CTTTAACATCTATAAGCCTTTGCTG	EU872021
CD4L1pET F	CACCGAACCAAAGGGGATTTTTG	DQ435301
CD4L1pET R	CTAGAGGAGGCGAGTTCTACAATGA	DQ435301

[a] GenBank accession number.

Veterinary Immune Reagent Network according to the Networks Cell Fusion Hybridoma Production Protocol http://www.umass.edu/vetimm/docs/Wagner Hybridoma.pdf adapted from (Wagner et al., 2003). Supernatants from the resulting hybridomas were used directly as an antibody source and screened using a combination of ELISA with the rIgL σ as an antigen, Western blotting, and flow cytometry (FACS).

ELISA screening identified three anti-catfish IgL σ mAbs, S.4 (IgG_3 kappa), S.28 (IgG_3 kappa) and S.46 (IgG_{2b} kappa) and their specificity for the denatured form of Igσ was confirmed by microsphere bead protein assays and Western blot analysis. For the microsphere bead assay 12.5 μg of 6 μm carboxyl latex bead microspheres (Bangs Laboratories) were covalently coupled to ~100 μg of either catfish rIgL σ or catfish rCD4 using a polylink protein coupling kit (Bangs Laboratories) according to the manufacturer's protocol. This protocol conjugates proteins via their amino-terminus (NH_2–) using 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide hydrochloride as a linker. Coated microspheres were stored in PolyLink wash/storage buffer (Bangs Laboratories) at 4 °C until use. Briefly, 5 μl of microspheres coated with either catfish rIgL σ or catfish rCD4 were incubated with 100 μl of anti-IgL σ S.4, S.28, S.46 mAb, or 50 μl of anti-Xpress antibody (Invitrogen) diluted 1:100 (v/v) in RPMI to detect the protein tag, or anti-trout IgM 1.14 as a negative control for 30 min. The microsphere beads were then washed in 4 ml RPMI containing 0.5% BSA (RPMI-BSA) and incubated with 50 μl phycoerythrin (PE)-conjugated goat-anti-mouse Ig (H + L; Southern Biotechnology) diluted 1:80 in RPMI-BSA (v/v) for 30 min. After a final wash the beads were re-suspended in 0.5 ml RPMI-BSA and analyzed on a BD FACScan flow cytometer (Becton Dickinson). Each of the three mAbs specifically reacted with rIg σ microspheres, and no staining above background levels was observed on microspheres coated with the rCD4 protein, which served as an irrelevant Xpress tagged protein control (Fig. 1B). For the microsphere bead assay, anti-trout IgM 1.14 mAb, which reacts with rainbow trout, *Oncorhynchus mykiss*, Igμ H chain (DeLuca et al., 1983) was used as an isotype control. As expected, it did not react with either of the catfish r proteins. In addition, when rIgL σ and rCD4 were electrophoresed under reducing conditions by 10% SDS-PAGE and visualized by Western blot, the mAbs specifically reacted with the ~15.4 kDa sized rIgL σ protein (~11.3 kDa Igσ C region + 4.1 kDa tag). That reactivity was not observed against rCD4 demonstrates that S.4, S.28 and S.46 are not "anti-tag" specific mAbs (Fig. 1C). Here it should also be noted that none of the anti-IgL σ mAbs tested here showed any reactivity in flow cytometry when screened against catfish PBL that were depleted of IgL F and IgL G expressing cells even though message for IgL σ could be amplified from cDNA isolated from these negatively sorted PBL (data not shown). In addition, we recently demonstrated that catfish PBL routinely express message for all four IgL isotypes, IgL F, IgL G, IgL σ and IgL λ and that PBL depleted of IgL F and IgL G expressing cells, expressed message for both IgL σ and IgL λ (Edholm et al., 2009).

Moreover, Western blot analysis of catfish sera collected from three different outbred fish (SV14, SV13

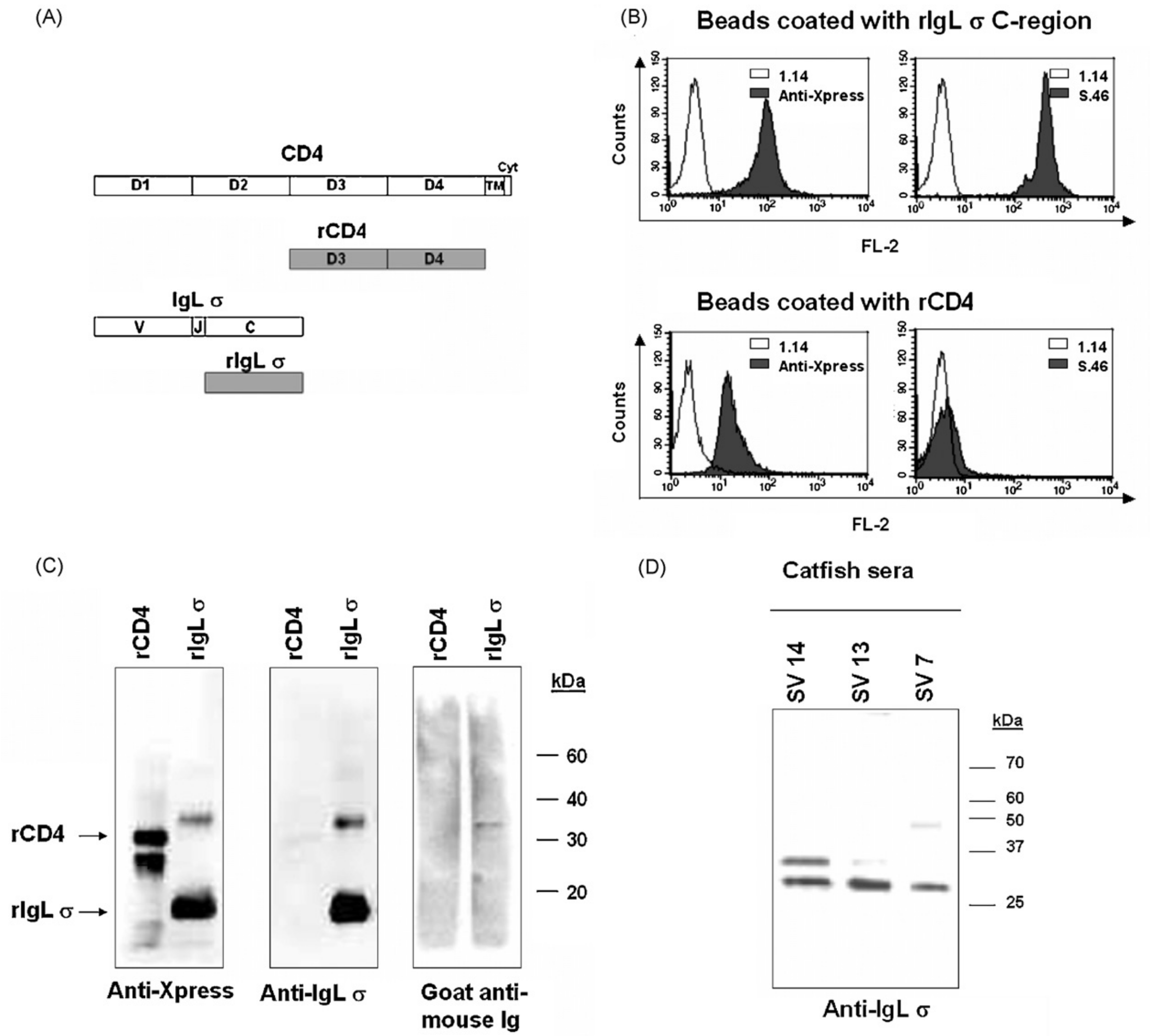

Fig. 1. Anti-catfish mAb S.46 is specific to denatured catfish Igσ. (A) Schematics of the constructs used to generate catfish CD4 and IgL σ recombinant (r) proteins. (B). Microbeads coated with rIgL σ protein stain positive with anti-IgL σ mAb and the anti-Xpress tag mAb. However microbeads coated with rIpCD4 protein, as a negative control, only stain positive with the anti-Xpress tag mAb. (C) 5 μg of catfish rIgL σ and rCD4 proteins were analyzed by 10% SDS-PAGE under reducing conditions and visualized by Western blot using anti-IgL σ mAb. The anti-Xpress mAb served as a positive control and goat-anti-mouse Ig (H + L) HRP was used as the secondary antibody. Arrows mark the identified proteins; the larger band observed with the rIgL σ protein (second lane) was due to cross-reactivity of the secondary goat-anti-mouse Ig (H + L) mAb with an unidentified *E. coli* protein as shown in the third panel labeled Goat-anti-mouse Ig. It should be noted that the presence of this band is not consistent between r protein preparations. (D) One microliter of catfish sera from three individual outbred catfish, SV14, SV13, and SV7 was analyzed by 10% SDS-PAGE under reducing conditions and IgL σ proteins were visualized by Western blot using anti-IgL σ mAb. As above, goat-anti-mouse Ig (H + L) HRP was used as the secondary antibody. Molecular weight size markers are at the right of panels (C) and (D). Since comparable results were observed with S.4 and S.28 anti-catfish IgL σ mAbs, only results with S.46 are shown.

and SV7) revealed that each of the three anti-catfish IgL σ mAbs recognizes a band of ∼27 kDa, which is in the predicted size range of an IgL σ chain (Fig. 1D). However, depending on the individual fish examined, two immunoreactive bands of ∼27 kDa and 30 kDa could also be observed consistently in some fish. This finding of two anti-Igσ reactive bands may be similar to what has been previously observed with catfish IgL F where individual catfish were shown to have different IgL chain variants. For example, using affinity chromatography and/or immunoprecipitations, anti-catfish IgL F mAb 3F12 reacted with ∼24 kDa and ∼22 kDa IgL F chain variants. Comparatively anti-IgL G mAb 1G7 reacted with only ∼26 kDa IgL G chains (Lobb et al., 1984). Also, both IgL F and G isotypes were always found in catfish serum.

In summary, three anti-IgL σ mAbs that specifically react with the denatured form IgL σ (S.4, S.28 and S.46) have been developed and characterized. We anticipate that these anti-catfish IgL σ mAbs will be useful in future studies examining the role of catfish IgL σ chain expression in immune responses. The anti-catfish IgL σ mAbs are listed on the U.S. Veterinary Immune Reagent Network website (http://www.umass.edu/vetimm/) and can be obtained by contacting M. Wilson or E. Bengtén at the University of Mississippi Medical Center.

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