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Identification of Two IgD+ B Cell Populations in Channel Catfish, Ictalurus punctatus

Eva-Stina Edholm,* Eva Bengtén,* James L. Stafford,† Manoranjan Sahoo,* Erin B. Taylor,* Norman W. Miller,* and Melanie Wilson*

Channel catfish Ictalurus punctatus express two Ig isotypes: IgM and IgD. Although catfish IgM has been extensively studied at the functional and structural levels, much less is known about IgD. In this study, IgM+/IgD+ and IgM–/IgD+ catfish B cell populations were identified through the use of anti-IgM and anti-IgD Abs. Catfish IgM+/IgD+ B cells are small and agranular. In contrast, IgM–/IgD+ B cells are larger and exhibit a plasmablast morphology. The use of cell sorting, flow cytometry, and RT-PCR demonstrated that IgD+ B cell expression varies among individuals. For example, some catfish have <5% IgM–/IgD+ B cells in their PBLs, whereas in others the IgM+/IgD+ B cell population can represent as much as 72%. Furthermore, IgD expressed by IgM–/IgD+ B cells preferentially associates with IgL or. Comparatively, IgM+/IgD+ B cells can express any of the four catfish IgL isotypes. Also, transfection studies show that IgD functions as a typical BCR, because IgD–chains associate with CD79a and CD79b molecules, and all membrane IgD transcripts from sorted IgM–/IgD+ B cells contain viable VDJ rearrangements, with no bias in family member usage. Interestingly, all secreted IgD transcripts from IgM+/IgD+ and IgM–/IgD+ B cells were V-less and began with a leader spliced to CÇ1. Importantly, transfection of catfish clonal B cells demonstrated that this leader mediated IgD secretion. Together, these findings imply that catfish IgM–/IgD+ B cells likely expand in response to certain pathogens and that the catfish IgD Fc-region, as has been suggested for human IgD, may function as a pattern recognition molecule. The Journal of Immunology, 2010, 185: 000–000.

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Only two Ig classes, IgM and IgD, have been identified in channel catfish (Ictalurus punctatus). Catfish IgM and teleost IgM, in general, have been well characterized and are basically considered structural and functional homologs of mammalian IgM (1–3). In contrast, catfish (or teleost) IgD has mainly been studied at the genetic level, and very little is known about its function. In fact, it was only recently appreciated that IgD is present in rodents and primates, as well as in a wide variety of other mammals and ectothermic vertebrates. Catfish IgD was originally identified as a chimeric cDNA consisting of a rearranged VDJ region spliced to Ç1 domain, followed by seven Ç domains, a transmembrane region (TM), and a positively charged short cytoplasmic tail (4; reviewed in Refs. 2 and 5). Also, from the same cDNA screening, truncated transcripts encoding a predicted secreted form of IgD were identified. The main criteria for classifying these cDNA as IgD transcripts included sequence relatedness to mammalian IgD, coexpression with IgM in some but not all B cells, and the location of the IgD gene within the IgH locus immediately ~1.5 kb downstream of the IgM gene. In addition, the consistent inclusion of Ç1 between rearranged VDJ and the Ç1 domain implied that catfish IgD was produced by an unusual pathway of RNA processing, a process similar to the alternative pathway of RNA processing of mammalian mature IgD transcripts. This inclusion of Ç1 was hypothesized to be necessary because it permits the IgD H chain to covalently associate with catfish IgL chains. After the discovery in catfish, IgD was identified in other teleosts (6–10), artiodactyls (11–13), and later in dogs (14), horses (15), Xenopus (16), leopard gecko (17), green anole lizard (18), turtle (19), and more recently in the platypus (20, 21). The inclusion of Ç1 has been observed in all teleost IgD transcripts; interestingly, the Ç1 domain in cow, sheep, and pig exhibits >93% amino acid identity to the respective Ç1 domain in each of these species (11–13). In the pig, IgD transcripts containing the Ç1, instead of the Ç1 domain, can also be found, demonstrating that the inclusion of Ç1 or Ç1-like domains in Ig chains is not unique to the bony fish.

Another unusual feature of catfish IgD is that membrane IgD and secreted IgD forms are encoded by different genes located ~735 kb apart (5, 22). The catfish IgH locus is a large translocon-type 735 kb that has undergone multiple internal duplications and transpositions and is estimated to contain ~200 VH genes representing 14 families. It contains three IgD genes termed IGHD1, IGHD2, and IGHD3 (designated by order of discovery) that are each individually linked to the functional IgL gene (IGHM1) or an IgM pseudogene. IGHD1 is located directly 3′ of the functional Ç1 gene and encodes membrane IgD. The other two IgD genes are found linked with their respective pseudo Ç1 genes, which are located 5′ of the functional IgM gene. IGHD3 is almost identical to IGHD1, except for its terminal exon, which encodes for the secreted tail rather than a TM. Also, IGHD1 and IGHD3 have functional enhancers located directly upstream of their Ç1 exons (22).
Notably, the secreted form of IgD is readily detected in catfish serum using an anti-IgD mAb specific for the secreted tail. The third Igδ gene, IGHδ2, consists of intact Cδ1 and Cδ2 exons plus a Cδ3 exon that contains a deletion of two nucleotides creating a frame shift and an early termination. However, transcripts originating from IGHδ2 have not been found. Also, the finding that catfish have two different functional Igδ genes, with one encoding membrane IgD and the other encoding secreted IgD, is not unique because pufferfish have a similar IGH organization, with the exons encoding secreted IgD linked to a separate array of D and JH genes found 5′ of the canonical array of D and JH genes linked to the IgM and membrane Igδ genes (23). It should also be noted that the teleost third Ig isotype, IgT/IgZ (24, 25), has not been found in catfish. Our analyses of the catfish IGH 3′ end, a contig of 257,153 bp containing 55 VH, 6 D, 12 JH, and the functional IGHM1 and IGHDI genes, revealed the lack of an IgT/IgZ-like gene in this region, which is the approximate location of IgT/IgZ in the trout (Oncorhynchus mykiss) and zebrafish (Danio rerio) IGH loci. However, it is possible that this isotype exists but has been moved to a different position by transposition and duplication events within the locus. Support for this possibility comes from the identification of multiple retrotransposon elements scattered throughout the IGH locus and elsewhere in the genome (26-28). Even so, cross-hybridization and degenerate PCR strategies designed to identify putative IgT/IgZ fragments were not successful. Also, no IgT/IgZ transcripts were identified among the ~500,000 catfish-expressed sequence tags in the National Center for Biotechnology Information database. This issue of a catfish IgT/Z isotype should be resolved when sequencing and annotation of the catfish IGH locus is completed.

In humans and mice, naïve mature B cells express membrane IgM and membrane IgD and constitute the majority of B cells in the periphery. After Ag activation, these mature B cells subsequently downregulate their membrane IgD and undergo clonal expansion and differentiation (29; reviewed in Ref. 30). Although past studies using knockout mice germine deficient for IgD or IgM demonstrated that each receptor isotype is capable of compensating for the loss of the other (31-33), it remained unclear whether IgD had a specific unique function. Interestingly, in humans, a unique IgM−/IgD+ B cell population is found in tonsillar and nasal tissues. They are described as having a plasmablast morphology distinct from conventional IgM+/IgD− B cells; they are larger with more cytoplasm (34, 35). Also, 90% of human IgM−/IgD+ cells express Igλ L chains, and studies of the expressed V repertoires showed that the VH and VL chain regions of these B cells are highly mutated in the framework regions in developing 3′ end germline DNA. This has been interpreted as evidence that one or both Igδ genes encode a transmembrane IgD protein in CFA, followed by three subsequent i.p. injections of 50 μg E. coli-expressed Igδ2 protein in CFA, followed by three subsequent i.p. injections of 50 μg E. coli-expressed Igδ2 in IFA at 2-wk intervals. Three days before fusion, mice were intraperitoneally boosted with 50 μg S. typhimurium transiently expressed Igδ2. The anti-catfish IgL σ mAb S.11 was produced as part of the U.S. Veterinary Immune Reagent Network, according to the network’s cell fusion hybridoma protocol, adapted from Wagner et al. (47). This fusion also produced anti-catfish IgL G, which reacts with the denatured form of catfish IgL (48). Unless stated otherwise, hybridoma culture supernatants were used directly as the mAb source, and all supernatants contained equivalent Ig concentrations, as determined by ELISA (Southern Biotech, Birmingham, AL) and Western blot. The mAbs used were anti-catfish IgE 9E1, reacts with catfish IgH H chain (49); anti-catfish IgD 7D11 (IgM d2) mAb D11 (IgM c), reacts with catfish IgH H chain; anti-catfish secreted IgD 2E5, reacts with the catfish secreted IgD form only in Western blots of serum (22); anti-catfish IgL 3F12, reacts with catfish IgL F isoform (50, 51); and anti-catfish IgL 11A2, reacts with catfish IgL G isoform (52). Anti-trout IgM 1.14 reacts with trout Igμ H chain (53) and was used as an IgG-matched isotype control. mAb 7E8 exhibits no specificity to any catfish cells tested and was used as an IgM-matched negative control (Supplemental Table II). Hybridoma cultures were grown at 37°C in low-glucose advanced DMEM supplemented with t-glutamine, 50 μM penicillin, 50 μg/ml streptomycin, 5% hybridoma cloning factor, and 15% FBS. Serum from the Igδ2-immunized mouse was also collected as a source of polyclonal Ab (pAb).

Polylink protein coupling
To verify the specificity of anti-catfish IgM mAb 7D11, catfish Igδ2 and CD4 (as a negative control) (48), were separately coupled to latex bead microspheres (Bangs Laboratories) using a Polylink Protein Coupling Kit, according to the manufacturer’s protocol (Supplemental Fig. 1). Briefly, 12.5 μg carboxyl latex beads 5μ in size, were coupled with 100 μg IgM and CD4 proteins in 50 μl at room temperature for 2 h at room temperature, washed, and resuspended in Link Wash/Storage buffer, according to the manufacturer’s protocol (Bangs Laboratories, Fisher, IN). The reactivity of anti-catfish IgM mAb 7D11 to recombinant proteins was tested by flow cytometry; 5 μl latex beads coated

Materials and Methods

**Experimental animals and catfish 3B11 B cells**

Channel catfish (1–2 kg) were obtained from a commercial source (ConAgra, Isola, MS) and maintained in individual tanks, as previously described (45). The catfish leukocyte cell line 3B11 is a cloned autonomous B cell line generated from an outbred catfish by mitogen stimulation (4). When initially cloned in 1995, this B cell line expressed message for IgM and IgD, and both IgH chains contained the identical VHDI rearrangement. However, the subcloned 3B11 B cells used in this study express message for IgM, CD79α, and CD79b and stain positive for IgM using anti-catfish IgM mAb 9E1 (44). The 3B11 clonal cells were grown at 27°C in AL-2 medium consisting of equal parts AIM-V and Leibovitz’s-15 (Invitrogen, Carlsbad, CA) adjusted to catfish toxicity with 10% (v/v) deionized water and supplemented with 1 mg/ml NaHCO3, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-ME, and 2% heat-inactivated, pooled, normal catfish serum (45).

**Catfish recombinant proteins and mAbs**

Catfish Igδ protein was made in a prokaryotic expression system using standard protocols. The Igδ constant heavy (CH) 2 domain (Igδ2) was amplified from catfish PBL cDNA by RT-PCR (Supplemental Table I) and cloned into the pET 100 Directional TOPO expression vector (Invitrogen) that introduces an N-terminal polyhistidine tag and an X-link to a polyhistidine region at the C-terminal end of the protein. This construct was transiently transfected into Sf9 insect cells using the Cellfectin reagent (Invitrogen), according to the manufacturer’s protocol. Igδ2 was refolded using a step-wise dialysis process according to the protein refolding kit protocol (Novagen, Madison, WI). Similarly, a rIgδ2 eukaryotic expression construct was made by cloning the Igδ2 RT-PCR product into the pB/V5-His TOPO expression vector (Invitrogen) that introduces a V5 epitope and a polyhistidine region at the C-terminal end of the protein. This construct was transiently transfected into Sf9 insect cells using the Cellfectin reagent (Invitrogen), according to the manufacturer’s protocol, at 4 °C post-transfection, the Igδ2 was purified from Sf9 supernatants using MagneHis Ni-Particles.

The anti-Igδ2 mAb 7D11 was produced according to standard methods (46). Briefly, BALB/c mice were injected i.p. with 50 μg E. coli-expressed Igδ2 protein in CFA, followed by three subsequent i.p. injections of 50 μg E. coli-expressed rIgδ2 in IFA at 2-wk intervals. Three days before fusion, mice were intraperitoneally boosted with 50 μg S. typhimurium transiently expressed rIgδ2. The anti-catfish IgL σ mAb D11 was produced as part of the U.S. Veterinary Immune Reagent Network, according to the network’s cell fusion hybridoma protocol, adapted from Wagner et al. (47). This fusion also produced anti-catfish IgL G, which reacts with the denatured form of catfish IgL (48). Unless stated otherwise, hybridoma culture supernatants were used directly as the mAb source, and all supernatants contained equivalent Ig concentrations, as determined by ELISA (Southern Biotech, Birmingham, AL) and Western blot. The mAbs used were anti-catfish IgE 9E1, reacts with catfish IgH H chain (49); anti-catfish IgD 7D11 (IgM δ2), reacts with catfish IgH H chain; anti-catfish secreted IgD 2E5, reacts with the catfish secreted IgD form only in Western blots of serum (22); anti-catfish IgL 3F12, reacts with catfish IgL F isoform (50, 51); and anti-catfish IgL 11A2, reacts with catfish IgL G isoform (52). Anti-trout IgM 1.14 reacts with trout Igμ H chain (53) and was used as an IgG-matched isotype control. mAb 7E8 exhibits no specificity to any catfish cells tested and was used as an IgM-matched negative control (Supplemental Table II). Hybridoma cultures were grown at 37°C in low-glucose advanced DMEM supplemented with t-glutamine, 50 μM penicillin, 50 μg/ml streptomycin, 5% hybridoma cloning factor, and 15% FBS. Serum from the Igδ2-immunized mouse was also collected as a source of polyclonal Ab (pAb).
with rlgb2 or rCD4 were incubated with 50 μl anti-Xpress Ab (Invitrogen) diluted 1:1000 (v/v) in RPMI 1640 or 50 μl anti-catfish IgM mAb 7D11 for 30 min. Anti-trout IgM mAb 1.14 and mAb 7E8 were used as isotype controls. After incubation, beads were washed in 4 ml RPMI 1640 containing 0.5% BSA (RPMI-BSA) and incubated with 50 μl PE-conjugated goat anti-mouse Ig (H+L) diluted 1:100 (v/v) for 30 min. Microspheres were washed as above, resuspended in 500 μl RPMI-BSA, and analyzed on a BD FACScan flow cytometer (BD Biosciences, San Jose, CA).

**PBL fractionation and flow cytometry**

Catfish PBLs were isolated from heparinized blood by centrifugation using Ficoll-Hypaque (Lymphoprep Accurate Chemical, Hicksville, NY), as described previously (45). After washing and resuspending in ice cold RPMI 1640 adjusted to catfish tonicity with 10% deionized water (cRPMI), Catfish PBLs were pelleted by centrifugation at 94˚C, followed by 30 cycles of 94˚C for 30 s, 60˚C for 30 s, and 72˚C for 1 min with hybridization cycle of 25 cycles of 94˚C for 30 s, 60˚C for 30 s, and 72˚C for 1 min. One hundred thousand cells were incubated with 2 μl goat anti-mouse IgG microbeads (20 μl beads/1 × 106 cells; Milteny Biotech, Auburn, CA) and separated into fractions using MACS LS columns, according to the manufacturer’s protocol. Collected fractions were washed in cRPMI before analysis. To obtain slgM+/slgD+ cell populations, the slgM- fraction was incubated with anti-catfish IgM mAb 9E1. Briefly, 2 × 105 cells were incubated with 1 ml mAb 9E1 for 30 min on ice, washed with cRPMI, and resuspended in 300 μl degassed cRPMI-BSA containing 2 mM EDTA. The cells were then incubated with goat anti-mouse IgG microbeads (20 μl beads/1 × 106 cells; Milteny Biotech, Auburn, CA) and separated into fractions using MACS LS columns, according to the manufacturer’s protocol. Collected fractions were washed in cRPMI before analysis. For flow-cytometry analyses, aliquots of 1 × 105 PBLs or sorted populations were incubated with 50 μl mAb supernatant for 30 min at 4˚C, washed, and incubated with 50 μl goat anti-mouse secondary Abs (Southern Biotech) conjugated to PE at 1:40 (v/v) in cfRPMI for 30 min at 4˚C. After washing, cells were suspended in 0.3 ml cRPMI and analyzed for single or two-color staining by flow cytometry. The BD FACScan was calibrated using chicken RBCs (BioSure, Grass Valley, CA) and Calibrate beads (BD Biosciences).

**RT-PCR and 5’-RACE**

RNA from total and sorted PBL populations was prepared with RNA-Beetel-Test, Friendswood, TX) and treated with DNase I (Invitrogen), according to the manufacturer’s protocol. One microgram RNA was subsequently transcribed into cDNA using an oligo-dT primer and 200 U Superscript III reverse transcriptase (Invitrogen). RT-PCR was performed with specific primers for catfish IgL, IgL, IgL, IgL, F, IgL, as well as for membrane and secreted forms of catfish IgM and IgD. EF1-α was used as a template control (Supplemental Table I). Typical parameters were 3 minutes at 94˚C, followed by 30 cycles of 94˚C for 30 s, 60˚C for 30 s, and 72˚C for 1 min with hybridization cycle of 25 cycles of 94˚C for 30 s, 60˚C for 30 s, and 72˚C for 1 min. Amplification was performed in a total volume of 25 μl containing 2 μl of 1:10 diluted RT-reaction mixture. PCR products were cloned into pCR4-TOPO (Invitrogen) and verified by sequencing (Macrogen, Seoul, South Korea). Nucleotide and amino acid sequences were analyzed using DNASTAR software (Madison, WI).

**Immunoprecipitations and Western blots**

Immunoprecipitations were performed using Sepharose beads that were prepared in a stepwise fashion. First, 1 g lyophilized cyanogen bromide (CNBr)-activated Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ) was washed in 200 ml 1 mM HCl and resuspended in 5 ml coupling buffer containing 0.5 mg goat anti-mouse IgM (Southern Biotech) conjugated to PE diluted 1:80 (v/v) in RCMPI for 30 min at 4˚C. After washing, the beads were washed in 1 ml cell-fractioning buffer containing 0.5% Triton X-100 (Sigma-Aldrich) for 30 min on ice and then centrifuged at 500 × g for 5 min to separate the membrane protein from the nucleus and cytoskeletal proteins. Nine hundred and fifty microliters each of membrane and cytosolic samples were immunoprecipitated with anti-IgG 7D11/goat anti-mouse IgM-coupled CNBr beads, as described above. The resulting proteins were separated and transferred to nitrocellulose, as described, and visualized with anti-trout IgM mAb 1.14 (isotype-matched IgM) and Western blotting using Kit-Vand Amaxa protocol T-20. Transfected 3B11 cells were cultured in 0.5% BSA (RPMI-BSA) overnight at 4˚C. Membranes were then incubated with streptavidin-HRP diluted in TTBS-BSA 1:20,000 (v/v) for 1 h at room temperature. After washing, immunoreactive bands were visualized with SuperSignal West Pico chemiluminescent substrate kit (Pierce) using a Bio-Rad Molecular Imager ChemiDoc XRS system (Hercules, CA). Unbiotinylated catfish lyses were also immunoselected with anti-catfish IgD 7D11/goat anti-mouse IgM-coupled CNBr beads, as described. However, immunoselected proteins were not visualized. For Western blot using anti-catfish IgL, IgL mAb S.46 and HRP-conjugated goat anti-mouse IgD, diluted 1/5000 (v/v).

Secreted IgD protein expression was examined by immunoprecipitation and Western blot using IgM+/IgD- and IgM-/IgD+ MACS-sorted cells that were separated into membrane and cytosolic fractions, as described previously (45). Briefly, cells were lysed at 2 × 106 cells/ml in cell-fractioning buffer (300 mM sucrose, 100 mM NaCl, 10 mM PIPES, 3 mM MgCl2, 5 mM EDTA, and one tablet MINI-EDTA protease inhibitor) containing 0.1% Triton X-100 (Sigma-Aldrich) for 30 min on ice and then centrifuged at 500 × g for 5 min to separate the membrane proteins from the nucleus and cytoskeletal proteins. Nine hundred and fifty microliters each of membrane and cytosolic lyses were immunoprecipitated with anti-IgD 7D11/goat anti-mouse IgM-coupled CNBr beads, as described above. The resulting proteins were separated and transferred to nitrocellulose, as described, and visualized with anti-IgD and IgD, anti-IgM, and anti-IgG2b Abs using a goat anti-mouse IgG2b coupled to CNBr beads in TTBS-BSA containing 1% nonfat milk 1/2000 (v/v), followed by HRP-conjugated goat-anti-mouse Ig (H+L) diluted in TTBS milk 1/5000 (v/v). For comparison, 25 μl of IgM+IgD- and IgM-IgD+ cytosolic fractions were analyzed solely by Western blot, as described above.

Catfish IgL, IgL, IgL, IgL, and secreted IgG expression in serum was analyzed in Western blot, as described above, except that 1 μl serum was electrophoresed in reducing 8 or 12% SDS-PAGE, and the IgG protein bands were visualized using anti-IgG Ls 1:40, anti-IgG L 3F12, anti-IgG L 11A2, and anti-secreted IgG 2E5 mAbs.

**CD79 association**

To test whether the membrane form of IgD is expressed on the cell surface in association with catfish BCR accessory molecules CD79a and CD79b, a membrane IgD construct was produced by cloning IgD CH5–CH7, including the TM and cytoplasmic region (CTY) (with stop codon), into the p3XFLAG-CMV 9 mammalian expression vector (Sigma-Aldrich), which introduces a preprotrypsin leader and a 3XFLAG tag at the N-terminal of the protein. The IgD5-cys plasmid DNA was purified, as described previously, and electroporated into 3 × 107 B cells in cell-fractioning buffer containing 0.5% BSA, 10 μg/ml of glutation (HA)-tagged CD79 plasmid constructs (pDisplay, Invitrogen) into catfish clonal 3B11 B cells using the nucleoporation system from Amaxa Biosystems (San Francisco, CA), according to the manufacturer’s protocol, as described by Sahoo et al. (44). Five micrograms total plasmid DNA was transfected into 3B11 cells using the nucleoporation system from Amaxa Biosystems, and the IgG protein bands were visualized using anti-IgG Ls 1:40, anti-IgG L 3F12, anti-IgG L 11A2, and anti-secreted IgG 2E5 mAbs.
negative control). After washing, cells were incubated with goat anti-mouse Ig (H+L)-PE (1:100 v/v; Southern Biotech) for 30 min on ice. The cells were washed and resuspended in 0.5 ml c rPME before analysis.

Comminuum precipitation were performed 24 h posttransfection, and triple-transfected 3B11 B cells (Igδ5-CYT, CD79α, and CD79b) cells were harvested. For sorting, 4 × 10^6 cells were incubated for 20 min at 27°C with a 10-fold excess of goat anti-mouse IgG-coated Dyna beads (Invitrogen) coupled to ∼5 μg anti-HA 1.1 mAb (Sigma-Aldrich), anti-Flag M2 mAb (Sigma-Aldrich), or 1.14 mAb. The bead-bound cells were magnetically isolated, washed in media, and resuspended in 100 μl mild lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 2% octyl-β-glucoside, and one tablet complete Mini, EDTA-free protease inhibitor; Roche). Bead-associated proteins were magnetically isolated, washed four times in lysis buffer without detergent, and eluted by adding 100 μl SDS-PAGE reducing sample buffer. The dissociated beads were removed magnetically, and 25-μl protein samples were analyzed by 10% SDS-PAGE and visualized by Western blot, as described above, using anti-FLAG-HRP (1:2500 v/v; Sigma-Aldrich) or biotinylated anti-HA (Covance) diluted 1:2500 v/v in TTBS-BSA, followed by incubation with streptavidin-HRP (1:1000 v/v).

Detection of native secreted Igδ

To demonstrate that the putative secreted Igδ leader is able to mediate protein secretion in vitro, a plasmid construct was made containing the secreted Igδ leader spliced to the Igδ CHI domain. Briefly, a PCR primer pair that incorporates a BmtI site 5' of the secreted Igδ leader and a 3' HindIII site was used to amplify the secreted Igδ leader together with the first Igδ constant domain from a secreted Igδ cDNA. After digestion, the PCR product was ligated into pSecTag2 (Invitrogen), which had been cut with BmtI and HindIII to remove the Ig δ-leader sequence. This modified vector, termed pSecTag2-8L, introduces a myc epitope/polyhistidine tag at the C terminus of the protein. pSecTag-8L, pSecTag-8L, and pSecTag-8L plasmid DNA was purified, as described previously (44), and verified by sequencing prior to transfection into catfish clonal 3B11 B cells. Two micrograms of total plasmid DNA was transfected into 3.5 × 10^6 3B11 B cells, as described above, and cells and supernatant were harvested at 48 h. Secreted Igδ protein was purified from the supernatant using Ni-NTA agarose (Qiagen, Valencia, CA) by adding 300 μl Ni-NTA agarose and 2 μl 1 M PMSF protease inhibitor (Sigma-Aldrich) to 1 ml culture supernatant. Following overnight incubation at 4°C (with gentle agitation), Ni-NTA agarose slurry was transferred to a gravity chromatography polypropylene column (Qiagen). The column was washed according to the manufacturer’s protocol, and proteins were eluted in increasing amounts of imidazole (0.5–0.85 M). Ni-NTA–purified secreted proteins and total-cell lysates were analyzed by 12% SDS-PAGE and visualized by Western blot, as described above, using anti-Myc-HRP (1/2500 v/v; Invitrogen).

To examine the expression of secreted Igδ transcripts, total RNA from sorted IgM+ and IgM+/IgD+ B cell populations were isolated, as described above, and reverse transcribed using a primer specific for Igδ CH3. RT-PCR was performed using three catfish-specific forward primers: secreted IgδL, Igδ1, and Igδ1 (Supplemental Table I) in combination with an Igδ1–specific reverse primer. Parameters were 3 min at 94°C, followed by 26, 28, 30, or 32 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were cloned into pCR4-TOPO (Invitrogen) and verified by sequencing.

Results

Catfish IgD+ B cell populations

Flow-cytometry analysis of PBLs isolated from 35 individual catfish using anti-catfish IgD mAb 7D11 revealed that IgD is found on the surface of two distinct differentially expressed major B lymphocyte populations: IgM+/IgD+ and IgM+/IgD+. However, the IgD-expression pattern varies among individuals, with some catfish exhibiting as much as 72% IgM+/IgD+ PBLs, whereas PBLs isolated from other catfish have little to no surface IgD staining (Fig. 1). Moreover, catfish B cell-staining patterns can be classified into three general groups. Some catfish, termed type 1 in this study, express IgM+/IgD+ and IgM+/IgD+ B cells in their PBLs, with some individuals having very few IgM+/IgD+ B cells (data not shown). Comparatively, type 2 PBLs have a large population of IgM+/IgD+ B cells and few IgM+/IgD+ B cells. In contrast, fish with type 3 PBLs have few to no (<5%) IgD+ cells, but they do have significant numbers of IgM+/IgD+ B cells. To further characterize catfish IgM+/IgD+ B cells, cell-separation studies using stepwise MACS with anti-catfish IgM (9E1) and anti-IgD (7D11) mAbs were performed. Fig. 2 shows a representative double staining of fish type 2 PBLs

![Fish type 1 PBL](http://www.jimmunol.org/)

![Fish type 2 PBL](http://www.jimmunol.org/)

![Fish type 3 PBL](http://www.jimmunol.org/)

**FIGURE 1.** Anti-IgD staining profiles observed in PBLs from different catfish. Scatter profiles for total unstained cell populations for each fish type, followed by graphs and scatter profiles of the gated regions after anti-IgM mAb 9E1 or anti-IgD mAb 7D11 staining. Of the 35 individual catfish analyzed over a period of 2 y, the type 2 PBL staining pattern was represented in 20 of the fish (∼58%), whereas 5 fish (∼15%) displayed the type 1 profile, and 10 fish (∼27%) displayed the type 3 profile.
with a large population of IgM$^+$/IgD$^-$ B cells and some IgM$^+$/IgD$^-$ B cells. As analyzed by MACS combined with flow cytometry and Wright staining, cells displaying the IgM$^+$ phenotype (IgM$^+$/IgD$^-$ and IgM$^+$/IgD$^+$) are small, agranular, and fall within the traditionally defined catfish lymphocyte gate. In comparison, IgM$^+$/IgD$^+$ B cells are larger with more cytoplasm, and, as shown previously, they resemble the IgM$^+$/IgD$^+$ plasmablasts found in human upper respiratory mucosa (40). At the message level, membrane and secreted forms of IgM and IgD were detected in the sorted IgM$^+$ cells, but no IgM message was detected in the sorted IgM$^+$/IgD$^+$ B cells, which expressed message for secreted and membrane IgM and IgD. The resulting IgM-depleted population (the MACS sort flow-through) was then sorted using positive selection with anti-IgD and analyzed as in B. IgM$^+/IgD^+$ cells expressed message for secreted IgD and membrane IgD. All PCR products were verified by sequencing.

**FIGURE 2.** Type 2 catfish PBLs show a distinct IgM$^+$/IgD$^+$ population when double stained with anti-IgM and anti-IgD. A. Freshly isolated PBLs from a fish with a large population of IgD$^+$ only B cells were two-color stained with anti-IgM mAb 9E1 and anti-IgD mAb 7D11, followed by PE- or FITC-conjugated goat anti-mouse isotype-specific secondary Abs. Percentages of cells in each quadrant are indicated. B. PBLs from the same fish were sorted by MACS using positive selection with anti-IgM, and a fraction of the isolated cells was stained with Wright stain. Total RNA was obtained from the remaining cells and analyzed by RT-PCR using primers for secreted (SEC) and membrane (MEM) IgM and IgD, as well as for CD79a and CD79b. IgM$^+$ cells, which include IgM$^+$/IgD$^+$ cells, expressed message for secreted and membrane IgM and IgD. C. The resulting IgM-depleted population (the MACS sort flow-through) was then sorted using positive selection with anti-IgD and analyzed as in B. IgM$^+$ IgD$^+$ cells expressed message for secreted IgD and membrane IgD. All PCR products were verified by sequencing.

**Catfish IgM$^+$/IgD$^+$ B cells preferentially associate with IgL$\sigma$-chains**

Catfish express four distinct IgL isotypes. IgL F and IgL G are 30% identical at the amino acid level (56, 57) and are orthologs of κ L chains (44). Together, they are estimated to represent 90% of the IgL found associated with serum IgM (50, 51). More recently, the catfish IgL$\sigma$ and IgL$\lambda$ isotypes were identified; it was demonstrated by flow cytometry that most of the IgM$^+$ cells expressed IgL F or IgL G chains (58). To determine whether membrane IgD preferentially associates with any of the four catfish IgL chains, a combination of flow cytometry, RT-PCR, and immunoprecipitations was used (Fig. 3). First, catfish B cells from a fish with type 2 PBLs were sorted into two populations (IgM$^+$ and IgM$^+$/IgD$^+$ B cells) by consecutive MACS (i.e., IgM$^+$ B cells were first positively selected by anti-IgM and then IgD$^+$ B cells were isolated from the IgM$^+$ population using anti-IgD) (Fig. 3A). As demonstrated by flow cytometry, although total PBLs contained IgL F-, IgL G-, and IgL$\sigma$-staining cells, as well as IgM$^+$ and IgD$^+$ cells, the sorted IgM$^+$/IgD$^+$ B cells were positive only for IgD and IgL$\sigma$. There are no available anti-catfish IgL$\lambda$ Abs, and expression of this isotype cannot be measured at the protein level. Nevertheless, when the two B cell populations were examined at the message level, IgL F, IgL G, IgL$\sigma$, and IgL$\lambda$
FIGURE 3. IgD, IgM, and IgL expression in PBLs from three type 2 catfish. A, Freshly isolated total PBLs and anti-IgD MACS-sorted B cells from one individual fish were analyzed by flow cytometry. Aliquots of unsorted (left panels) and IgM<sup>+</sup>/IgD<sup>+</sup> sorted cells (right panels) were analyzed using anti-IgD, anti-IgM, anti-IgL F, anti-IgL G, and anti-IgL σ mAbs (shaded graphs) and compared with their isotype controls (white graphs). MACS sorting was performed as in Fig. 2. B, From this same fish, total RNA was obtained from the IgM<sup>+</sup> and IgM<sup>+</sup>/IgD<sup>+</sup> MACS-sorted fractions, and RT-PCR analysis was performed using primers specific for membrane IgM; membrane IgD, IgL F, IgL G, IgL σ, and IgL λ; and the housekeeping gene EF1-α as a positive control. Schematics of the IgL transcripts are shown to the right of their respective panels. The larger IgL λ product represented a 916-bp partially spliced unrearranged IgL λ transcript; the smaller 646-bp product represented the functional rearranged catfish IgL λ transcript. C, Total PBLs from a second type 2 fish were sorted by MACS using anti-IgL σ mAb and analyzed as described for A. D, An aliquot of total PBLs from a third type 2 fish, which had a larger IgM<sup>+</sup> B cell population, was double stained with anti-IgM mAb 9E1 and anti-IgL σ mAb S.11, followed by PE- or FITC-conjugated goat anti-mouse isotype-specific secondary Abs. The quadrants containing the IgL σ<sup>+</sup> cells are outlined in red. RT-PCR analysis and a Wright stain from these IgL σ<sup>+</sup> MACS-sorted cells (upper right panel). Respective anti-IgM and anti-IgD graphs of these sorted cells (bottom panels). All PCR products were verified by sequencing.
transcripts were readily detectable in the sorted IgM⁺ cells from this fish (Fig. 3B). In contrast, the sorted IgM⁺/IgD⁺ cells express message for the membrane Igδ and IgL σ and not for IgM, IgL F, IgL G, or IgL λ. Comparatively, when PBLs from another type 2 fish were analyzed after sorting with anti-IgL σ mAb S.11, the majority of the cells stained positive for IgD, supporting a preferential IgL σ usage by the IgD⁺ B cells (Fig. 3C). However, it is important to note that IgL-σ-chains can also associate with IgH chains. For example, double staining of PBLs isolated from a third type 2 fish that expressed a larger IgM population showed that ~6% of the IgM⁺ cells are IgL σ⁺ (Fig. 3D), and RT-PCR analyses of sorted IgL σ⁺ cells from this fish expressed message for rearranged IgL σ and membrane IgA and Igδ-chains. In addition, Wright staining revealed that the enriched IgL σ⁺ cell population consisted of the two morphologically distinct B cell types: the small agranular IgM⁺ B cells and the larger IgM⁺/IgD⁺ cells with more cytoplasm. Second, type 2 total PBLs and sorted IgM⁺/IgD⁺ fractions were immunoprecipitated using anti-catfish IgM mAb 9E1 or anti-catfish IgD mAb 7D11. Immunoprecipitation of total biotinylated PBLs with anti-catfish IgD yielded protein bands corresponding to the predicted size of membrane IgD (~120 kDa) and two bands of ~24 and 27 kDa indicative of IgL chains (Fig. 4A). Two unidentified bands of ~75 kDa and ~180 kDa were also observed. Anti-catfish IgM immunoprecipitated the ~70 kDa IgM H chain and IgL chains of ~23–26 kDa. In comparison, anti-catfish IgD immunoprecipitation of sorted IgM⁺/IgD⁺ B cells identified bands of ~120 kDa and ~27 kDa (Fig. 4A). The ~24 kDa IgL chain band observed in total PBLs was not present in the IgM⁺/IgD⁺ B cells. This observation is consistent with their lack of IgG F and IgL G surface staining (Fig. 3A). Duplicate samples of type 2 unbiotinylated IgM⁺/IgD⁺ cells were also immunoprecipitated with the anti-catfish IgD mAb or its isotype-matched control (mAb 7E8; Supplemental Table II). When the immunoprecipitated proteins were analyzed in Western blot using anti-IgL σ mAb S.46, followed by a type-specific goat anti-mouse IgG2b, (H + L) HRP-conjugated secondary Ab, an immunoreactive band of ~27 kDa in size was identified, confirming that the larger IgD coimmunoprecipitated IgL-sized band is IgL σ (Fig. 4B). Finally, Western blot analyses of catfish sera using the three anti-catfish IgL mAbs demonstrated that IgL σ migrates as an ~27 kDa band, whereas IgL F and IgL G migrate as an ~22/24 kDa doublet and ~26 kDa band, respectively, in SDS-PAGE (Fig. 4C). Taken together, these results strongly imply that catfish IgM⁺/IgD⁺ B cells preferentially express IgL-σ-chains.

**Catfish IgD associates with CD79 accessory molecules on B cells** To determine whether CD79α and CD79β are required for catfish IgD surface expression and whether IgD can noncovalently associate with CD79α and CD79β on B cells, a FLAG-tagged truncated Igδ construct was cotransfected in different combinations with HA-tagged CD79α and CD79β constructs into the catfish B cell line 3B11. Transfected cells were analyzed by flow cytometry using anti-tag mAbs and by immunoprecipitation combined with Western blot. To confirm successful transfection in the absence of surface expression, Western blot analyses of cellular lysates were also performed (Supplemental Fig. 2). Fig. 5A shows a representative flow-cytometry experiment using 3B11 B cells transfected with FLAG-tagged Igδ-5-CYT, HA-tagged CD79α, and HA-tagged CD79β. Mock-transfected 3B11 cells and 3B11 cells transfected with FLAG-tagged Igδ-5-CYT alone did not express the Igδ protein on their cell surface. However, when FLAG-tagged Igδ-5-CYT was cotransfected with HA-tagged CD79α or HA-CD79β, FLAG-tagged Igδ surface expression was readily detectable. At 48 h posttransfection, 26.5% of the cells transfected with Igδ-5-CYT and CD79α were positive for anti-FLAG, whereas 53.5% of the cells transfected with Igδ-5-CYT and CD79β stained positive. Igδ-5-CYT surface staining further increased to 67% when all three constructs (Igδ-5-CYT, CD79α, and CD79β) were cotransfected. Igδ-CD79 association in triple-transfected 3B11 B cells was also examined by coimmunoprecipitation. At 36 h posttransfection, cells were incubated for 20 min at 27°C with Dynabeads coated with anti-FLAG, anti-HA, or anti-trout IgM 1.14 mAb (isotype control).

**FIGURE 4.** Catfish Igδ-chains preferentially associate with IgL-σ-chains. A. Total PBL or IgM⁺/IgD⁺ MACS-sorted cells from a type 2 catfish were surface biotinylated and immunoprecipitated using anti-Igδ mAb 7D11. The immunoselected proteins were separated by reducing 10% SDS-PAGE, transferred to nitrocellulose membranes, and visualized by Western blot using streptavidin-HRP. Labeled arrows indicate appropriate-sized Igδ and IgL chains. B. Unbiotinylated IgM⁺/IgD⁺ MACS-sorted fractions from the same fish were immunoprecipitated using anti-Igδ mAb 7D11 or an isotype-matched negative control (mAb 7E8; Supplemental Table II). When the immunoprecipitated proteins were analyzed in Western blot using anti-IgL σ mAb S.46, followed by a type-specific goat anti-mouse IgG2b, (H + L) HRP-conjugated secondary Ab, an immunoreactive band of ~27 kDa in size was identified, confirming that the larger IgD coimmunoprecipitated IgL-sized band is IgL σ (Fig. 4B). Finally, Western blot analyses of catfish sera using the three anti-catfish IgL mAbs demonstrated that IgL σ migrates as an ~27 kDa band, whereas IgL F and IgL G migrate as an ~22/24 kDa doublet and ~26 kDa band, respectively, in SDS-PAGE (Fig. 4C). Taken together, these results strongly imply that catfish IgM⁺/IgD⁺ B cells preferentially express IgL-σ-chains.
The bead-bound cells were magnetically isolated and lysed in 2% octyl-β-D-glucoside. The resulting bead-bound proteins were analyzed by reducing 10% SDS-PAGE and Western blot using HRP-conjugated anti-FLAG and anti-HA mAbs. Anti-FLAG and anti-HA mAbs immunoselected the ∼60 kDa Igδ5-CYT protein, as visualized with anti-FLAG HRP (Fig. 5B). However, a duplicate blot of anti-FLAG–immunoselected proteins visualized with anti–HA-HRP identified only the ∼44 kDa HA-tagged CD79a, whereas immunoselection using anti-HA mAb yielded CD79a and CD79b (∼35 kDa; Fig. 5C). This finding that HA-tagged CD79b failed to coimmunoprecipitate with FLAG-tagged Igδ5-CYT was consistent and, at best, only a faint CD79b band could be observed. One explanation may be that the association of catfish CD79a with CD79b is weak because both molecules lack the required cysteines for forming the interchain disulfide bond commonly found in mammalian CD79a/CD79b heterodimers (44). Also, it should be emphasized that without specific anti-catfish CD79 mAbs and because 3B11 B cells express message for endogenous Igδ and CD79a and CD79b, the exact stoichiometry of the catfish IgD BCR cannot be determined. Nevertheless, our current flow-cytometry data strongly suggest that CD79a and CD79b molecules are required for surface expression of Igδ H chains on B cells.

IgM+/IgD+ B cells transcript analyses

As previously shown, IgM+/IgD+ cells express message for the membrane and secreted forms of IgD (59). Also, because the different Igδ genes encoding for the membrane and the secreted form of IgD are located ∼735 kb apart (Fig. 6A) (5, 22), 5′-RACE analysis was performed using membrane Igδ- and secreted Igδ-specific primers to examine VH usage in the IgM+/IgD+ B cells. Briefly, fish type 2 PBLs were sorted by MACS into IgM+ and IgM+IgD+ B cells; after verification of IgD+ surface staining, message profiles of the IgM+/IgD+ B cells were examined using 5′-RACE (Table I). All 13 of the membrane Igδ 5′-RACE products contained bona fide VHΔJH rearrangements and included Cμ1 as the first CH domain, which confirmed the IGHδ1 gene origin of the transcripts. Analyses of the 13 VHΔJH rearrangements revealed that 9 VH families, 8 JH segments, and at least 4 D segments were represented. No preferential usage of a specific VH family was apparent. In addition, basic local alignment search tool searches
showed that the membrane IgD VH sequences were very similar (90–97% identical) to VH sequences previously identified in association with IgM (data not shown). In contrast, each of the nine secreted IgD transcripts lacked a VH region and were spliced to a 140-nucleotide sequence encoding a 5'-untranslated region and a putative leader MQDTFWILLMFTGAAS. The exon encoding this putative leader was located ∼8.1 kb 5' of the IGHD3 δ1 exon (Fig. 6A; GenBank accession number GU799624; www.ncbi.nlm.nih.gov/genbank/). The lack of VH regions associated with the secreted form of IgD was consistent with our previous IgD cDNA analyses that indicated that VHDJH rearrangements were only found in membrane IgD transcripts (22). The expression of V-less secreted IgD transcripts was also examined using a semiquantitative RT-PCR approach. Total RNA isolated from IgM⁺ and IgM⁺/IgD⁺ B cells was reverse transcribed using a universal Cδ1 primer, which would reverse transcribe mRNA specifically for IGDH1 membrane IgD and IGDH3 secreted IgD. RT-PCR was then performed using an Cδ1 reverse primer in combination with a primer specific for the secreted IgD leader (Supplemental Table I) or with a Cµ1 primer specific for amplifying membrane IgD transcripts. A Cδ1 forward primer was used to amplify the template control. Amplifications were performed using four

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**FIGURE 6.** Secreted IgD expression in catfish sorted B cell populations. A, IGDH3 and IGDH1 region genes of the catfish IGH locus. Linkage was established by restriction mapping and sequencing of catfish bacterial artificial chromosomes (3, 5, 22). Encoded secreted (SEC) and membrane (MEM) IgD forms are shown below their respective genes. The arrow indicates the location of the secreted IgD leader sequence. Not drawn to scale. B, RT-PCR analysis of MEM and SEC IgD message expression in sorted IgM⁺ and IgM⁺/IgD⁺ B cells. Amplification cycle numbers and base pair markers are indicated. Identified transcripts are next to their respective panels. PCR products were verified by sequencing. The Cδ1 exon was amplified as a template control. C, Secreted IgD leader mediates IgD secretion in vivo. The putative secreted IgD leader sequence was cloned into a modified pSecTag2 vector, which lacked its endogenous Ig k-chain leader sequence. The construct was transiently transfected into catfish 3B11 B cells, and supernatant and total-cell lysates were harvested 48 h posttransfection. His-tagged proteins were purified from the supernatant and visualized using anti–Myc-HRP, which recognizes the introduced Myc tag. Supernatant proteins and cell lysate (∼2 × 10⁶ cells) were analyzed by 12% SDS-PAGE under reducing conditions. Molecular mass size markers are at right.
pernatant from transfected 3B11 cells using an anti–Myc-HRP 
solic fraction, but not the membrane fraction, of sorted IgM 
using a combination of immunoprecipitation and Western blot, 
below compared with membrane Ig 
seemed to be equal. In contrast, message for secreted Ig 
cycles, message levels for secreted and membrane Ig 
populations (IgM 
molecule and that the existence of two different IgD-bearing B cell 
our previous reports (4, 5, 22), demonstrate that IgD is an ancient 
Discussion

**Table I. 5’-RACE analysis of IgD transcripts in IgM+/D+ B cells**

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+, present; −, absent; CDR, complementary determining region.

different cycle numbers. As shown in Fig. 6B, message for secreted Igδ was detected in IgM−/IgD+ cells after 28 cycles; at 30 and 32 cycles, message levels for secreted and membrane Igδ forms seemed to be equal. In contrast, message for secreted Igδ seemed to be low compared with membrane Igδ in IgM+/IgD+ cells, even at 32 cycles. The lower intensity of the Cδ1 control products amplified from IgM−/IgD+ cells, compared with IgM+IgD+ cells, was observed consistently. This is likely due to a poorer RNA/cDNA quality resulting from the sorting process: IgM−/IgD+ cells are subject to extended experimental manipulation; PBLs are first sorted with anti-IgM to remove the IgM+ cells and then sorted with anti-IgD to isolate the IgD+ B cells.

Notably, it was confirmed by transfection that this putative Igδ leader sequence could mediate Igδ protein secretion in vitro. A plasmid construct containing the secreted Igδ leader sequence spliced to the Igδ CH1 domain was cloned into a modified pSecTag2 vector, where the endogenous leader had been removed by restriction-enzyme digestion. This vector (termed pSecTag2-5L) was transiently transfected into catfish clonal 3B11 B cells. Secreted Igδ protein was readily detected in Ni-NTA-purified supernatant from transfected 3B11 cells using an anti–Myc-HRP mAb, which detects the pSecTag2-Myc tag (Fig. 6C). Also, by using a combination of immunoprecipitation and Western blot, expression of secreted IgD protein could be detected in the cytosolic fraction, but not the membrane fraction, of sorted IgM−/IgD+ cells from a type 2 catfish (Supplemental Fig. 3A, 3B). The size of the cytosolic secreted IgD protein band was smaller than membrane IgD but its size was reminiscent of the secreted IgD detected in the serum of this same type 2 fish. It was also shown in a companion study that different individual catfish express size variants of secreted IgD; it may be that the observed size differences are due to the inclusion or exclusion of the duplicated block of Cδ2-Cδ3-Cδ4 exons (Supplemental Fig. 3C).

**Discussion**

The catfish IgD B cell studies reported in this paper, combined with our previous reports (4, 5, 22), demonstrate that IgD is an ancient molecule and that the existence of two different IgD-bearing B cell populations (IgM−/IgD+ and IgM+/IgD+) is evolutionarily conserved. For example, in the past, the IgM−/IgD+ B cells found in the human upper respiratory tract were considered a unique B cell subset because they are larger than conventional IgM+/IgD+ B cells, have more cytoplasm, and are characterized as exhibiting a plasmablast morphology. Similarly, catfish IgM−/IgD+ B cells are larger than catfish IgM+/IgD+ B cells and, like human IgM−/IgD+ plasmablasts, they contain more cytoplasm. Furthermore, human tonsillar IgM+/IgD+ B cells were shown to contain hypermutated VDJ regions and to preferentially use Igλ λ-chains (36, 38). In addition, it was demonstrated that human IgM+/IgD+ B cells undergo an unusual class switch involving the cryptic-switch region located between the Cμ and Cδ genes (60, 61). More recently, different VH repertoire studies showed a selected VH bias occurs in human IgM−/IgD+ B cells. First, 64 ± 22% of IgM−/IgD+ tonsillar B cells isolated from 10 donors were shown to use VH4-34, a VH gene associated with autoimmunity (38). Second, repertoire analyses of human peripheral blood B cells from four donors demonstrated that 30–47% of the IgM−/IgD+ cells used a specific VH3-30 gene in their gene rearrangements and that 40–48% preferentially used JH6 (39). In the same study, the investigators showed that a high VJ mutation rate occurred in the Igλ λ-chains expressed by tonsillar and peripheral blood IgM−/IgD+ B cells. Notably, these V-region biases were hypothesized to be the result of Ag-driven clonal expansion because there was such an observed dominance of large IgM−/IgD+ clones with related gene rearrangements (35, 36, 39). Similarly, catfish IgM−/IgD+ B cells also show preferential Igλ chain usage, albeit not with Igλ λ. In this study, by using a combination of flow cytometry, cell sorting by MACS, RT-PCR, and immunoprecipitation, we demonstrated that catfish IgM−/IgD+ B cells only express Igλ σ (58), an isotype restricted to ectothermic vertebrates (62). In contrast, any of the four catfish Igλ isotypes (IgL F, IgL G, IgL λ, and IgL σ) could be expressed by IgM+ cells. In this context, it is interesting to note that the catfish Igλ σ repertoire is very limited. Briefly, the Igλ σ locus consists of two gene clusters, each containing a single identical J and identical C, and one or two V gene segments, all belonging to the same Vσ family (57). Similarly, the Igλ λ locus also consists of only two gene clusters; however, the catfish Igλ λ isotypes IgL F and IgL G are encoded by multiple loci, which, when combined, contain 60–80 V
segments representing at least three families (56, 57). Of the 13 IgH chain rearrangements sequenced from IgM\(^{-}/\)IgD\(^{+}\) B cells isolated from a single catfish, no bias in VH or JH gene usage was observed. Notably, all rearrangements were unique; nine VH families, eight JH segments, and at least four D segments were found. VH6 was the most prevalent VH family represented, with four rearrangements; like the catfish VH1, VH2, and VH3 families, it is large and estimated to contain \(>20\)–\(30\) members (63). It should also be emphasized that determining whether the VH6, or any other VH, rearrangements are somatically mutated is not straightforward, because sequencing of the catfish IGH locus, which is estimated to contain \(~200\) VH genes and span \(~1\) Mbp, is ongoing (5). Even so, when the four rearranged VH6 segments were aligned with \(135\) catfish VH6 sequences in GenBank, they only differed by three to eight nucleotides from their corresponding matched VH6 sequence, indicating that excessive somatic mutation (20–80 nucleotide differences) of IgD VH regions in IgM\(^{-}/\)IgD\(^{+}\) B cells does not occur.

Although the relationship between IgM\(^{-}/\)IgD\(^{+}\) and IgM\(^{-}/\)IgD\(^{+}\) B cells is not clear, we believe catfish IgD functions as an Ag-binding receptor on IgM\(^{-}/\)IgD\(^{+}\) and IgM\(^{-}/\)IgD\(^{+}\) B cells, and that cross-linking of the IgD leads to activation, differentiation, and/or proliferation. For example, both B cell types express full-length membrane IgD with functionally rearranged VHDJH regions, show association of membrane IgD with IgL chain, express message for the B cell accessory molecules CD79a and CD79b, and require association of CD79 molecules for surface expression of membrane IgD. Moreover, it seems tempting to speculate that IgM\(^{-}/\)IgD\(^{+}\) B cells in catfish represent traditional naive B lymphocytes that, upon Ag activation, mature into IgM-producing plasma cells or memory cells and that IgM\(^{-}/\)IgD\(^{+}\) B cells constitute a separate B cell lineage, which constitutively expresses IgD/IgL \(\sigma\) and excludes expression of IgM. However, it may be that a small population of IgM\(^{-}/\)IgD\(^{+}\) B cells in catfish use IgL \(\sigma\)-chains, which are triggered after a specific Ag selection to differentiate into IgM\(^{-}/\)IgD\(^{+}\) B cells. In this context, it should be noted that catfish IgM\(^{-}/\)IgD\(^{+}\) B cells express high levels of IgM, and varying, but low levels of IgD, as determined by fluorescence intensity, whereas IgM\(^{-}/\)IgD\(^{+}\) B cells make up a very distinct cell population expressing high levels of IgD and no IgM (Fig. 2A). Also, the underlying molecular mechanism for the exclusion of IgM in catfish IgM\(^{-}/\)IgD\(^{+}\) B cells is not understood. However, it is clear that C\(4\)-C\(5\) class switch recombination cannot take place in the same fashion as in human IgM\(^{-}/\)IgD\(^{+}\) B cells, because the C\(\beta 1\) domain is always included in catfish membrane IgD transcripts. If switch recombination to C\(\beta\) occurs in catfish, it would have to occur between sites found within the C\(\beta 1\)–C\(\beta 2\) intron and the intron between C\(\mu 4\)-IgM TM1 exon, because a recombination event deleting the IgM TM2 exon and the intron 5\' of the C\(\delta 1\) exon would delete the IgH transcriptional enhancer (E\(\beta 3\)) (27, 64). Consequently, although catfish activation-induced cytidine deaminase was demonstrated to be capable of mediating somatic hypermutation and class switching in mouse AIDS\(^{-}\)plasmablasts, the results of these studies suggest that catfish B cells have plasmablast morphology, exhibit restricted IgL isotype usage, and produce a secreted form of IgD. We believe that such similarities between a distinct B cell population found in two phylogenetically diverged species (human and catfish) argues for their having a distinct cell population specificity. It is anticipated that our future studies will focus on determining what stimuli induce expansion of these unique IgM\(^{-}/\)IgD\(^{+}\) plasmablasts in catfish B cells and that these plasmablasts to determine how much secreted IgD is produced and whether the V-less secreted IgD made by these plasmablasts functions as a pattern-recognition molecule while bound to the surface of catfish granulocytes.


