



Characterization of three different IgD⁺ cell populations in channel catfish, *Ictalurus punctatus*

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Abstract

To date two different immunoglobulin (Ig) molecules have been identified in the catfish, IgM and IgD. Catfish IgM for the most part resembles mammalian IgM except that it exists as a tetramer in serum, and not a pentamer. In contrast, catfish IgD is quite different. It is expressed as a chimeric molecule consisting of a rearranged variable heavy (VH), diversity (D) and joining (JH) segment spliced to the first IgM constant C_{H1} domain, followed by seven C_δ domains and either a transmembrane or secreted tail. To study the function of IgD in catfish, monoclonal (mAb) and polyclonal (pAb) anti-IgD antibodies have been generated specific to the second IgD domain. Morevoer, RACE PCR protocols amplify the appropriate rearranged Ig messages from both cell populations indicating that these pools contain functional B cells. The third population is an IgM/IgD⁺ granular cell population of unknown origin that does not express any Ig message, but appears to be armed with exogenously produced IgD via a putative IgD-binding receptor. In contrast to the situation in mammals where IgM/IgD⁺ B cells are extremely rare, the IgM/IgD⁺ cell population can represent as much as 60% of the total PBL in some catfish. At the present, the function of the IgM/IgD⁺ granulocytes is unknown, however they degranulate in response to cross-linking of the surface bound IgD; a situation reminiscent of that of mammalian IgE Supported by a United States Department of Agriculture (2006-35204-16880) *US Veterinary Immune Reagent Network*.

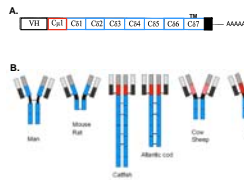


Fig 1A. Schematic representation of IgD:

Catfish IgD is expressed as a chimeric molecule consisting of a rearranged VH region spliced to a C_{μ1} domain exon, seven C_δ domains and a TM.

1B. IgD in different species

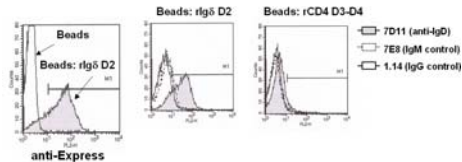
Red blocks indicate inclusion of a C_{μ1} or a C_{μ1}-like domain

Production of an anti-IgD Monoclonal Antibody

Protocol:

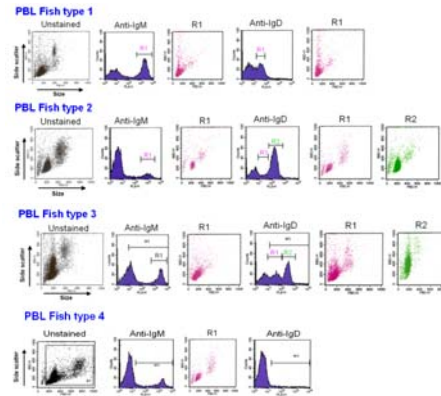
- Recombinant (r) IgD protein was generated by cloning IgD domain 2 (D2) into either a pET100 (prokaryotic) or pIB/V5 (eukaryotic) vector system.
- IgD protein was expressed in *E.coli* or S19 insect cells and purified using Magne-His nickel columns and allowed to refold via stepwise dialysis.
- Balb/c mice were immunized and boosted three times with *E.coli* produced protein; they received a final boost of S19 produced protein two days before fusion with mouse myeloma cells.
- Hybridomas were screened by Flow cytometry (FACS) using freshly isolated catfish PBL.
- One reactive mAb, 7D11 (IgM, κ) was further characterized.

mAb 7D11 reacts with domain 2 of IgD



Specificity of mAb 7D11 was verified by conjugating either rIgD-D2 or rCD4 D3-D4 produced in *E.coli* to microbeads, which were then analyzed by FACS for anti-IgD staining. Successful conjugation was determined by anti-tag mAb (anti-Express). Beads with rIgD-D2 showed positive staining when incubated with 7D11 compared to isotype controls (1.14 and 7E8). No reactivity above background was observed when mAb 7D11 was incubated with rCD4 D3-D4 conjugated beads.

Identification of 4 different IgD staining profiles



Four representative staining profiles were observed in PBL from different catfish. Panels show scatter profiles for each type, followed by histograms and scatter plots of designated regions after anti-IgM or anti-IgD staining. The FACSscanner was calibrated between each run using chicken red blood cells to eliminate instrumental variation.

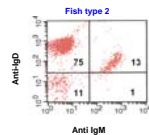
Type 1 consists almost exclusively of small, non-complex cells, with some cells staining at a high intensity for IgM and others staining at a lower intensity with anti-IgD.

Type 2 contain cells similar to those observed in Type 1 plus a population of slightly larger and more internally complex cells (R2) that stain at a medium intensity for anti-IgD.

Type 3 contain an additional population of cells, highly granular in appearance that stained with a high intensity for IgD.

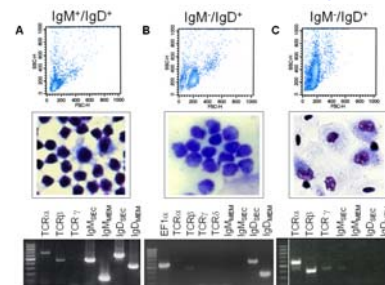
Type 4 resembles Type 2 as far as scatter profiles and anti-IgM staining. However no IgD staining was observed.

Anti-IgM and Anti-IgD double staining



Representative Type 2 PBL double stained with anti-IgM and anti-IgD show the different IgD expressing populations IgM/IgD⁺ (75%) IgM⁺/IgD⁻ (13%) IgM⁻/IgD⁺ (1%) and IgM⁻/IgD⁻ (11%).

Isolation and characterizations of IgD⁺ cells



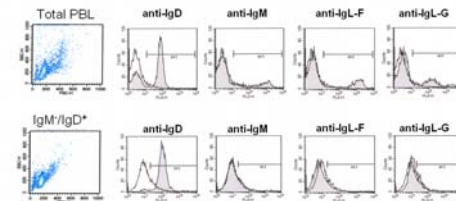
IgD⁺ cell populations. Different IgD⁺ cell populations were detected using anti-IgM and anti-IgD mAbs in magnetically activated cell sorting (MACS). Cells from a Type 2 fish were (A) first sorted using positive selection with anti-IgM. (B) The IgM⁺ depleted fraction was then positively selected with anti-IgD. Cells from (C) Type 3 fish were negatively sorted with anti-IgM. Fractions of each selected cell population were stained with Giemsa/Dip stain, analyzed on FACS and by RT-PCR.

IgM⁺/IgD⁺ cells express message for secreted (sec) and membrane (mem) IgM and IgD. Some TCR message is also detected and may be due to T cells that express IgM via a FcR.

IgM⁺/IgD⁺ lymphocyte-like cells express message for both IgDsec and IgDmem.

Type 3 negatively selected IgM⁺/IgD⁺ did not express message for IgD, although they are >80% surface positive for IgD (data not shown). Phenotypically they have a more granulocyte appearance.

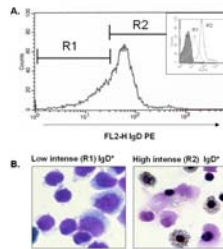
IgD surface expression



IgD is expressed on the surface without associated Ig light (L) chains:

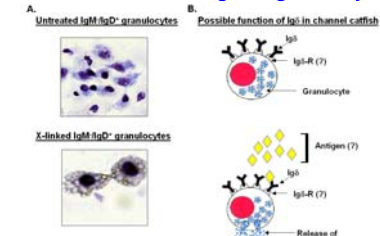
Total PBL from fish Type 2 (top panel) and MACSsorted IgM/IgD⁺ cells (bottom panel) were analyzed in FACS for light chain staining (Ig-L and Ig-L-F). Total PBL contained both IgM, IgD and IgL chain positive cells, after anti-IgM negative selection followed by anti-IgD positive selection, enriched cells were >95% positive for IgD while no staining above background was observed for anti-IgM or anti-Ig-L chain (F and G). In addition no IgL chain staining was observed on negatively selected IgM/IgD⁺ granulocytes from fish type 3 (data not shown).

Isolation of High and Low intensity IgD⁺ populations



Separation of high and low intensity staining IgD⁺ cells was accomplished using a combination of MACS and FACS. PBL from fish Type 3 were negatively selected using anti-IgM and the depleted population (>90% IgD⁺) was subjected to fluorescent activated cell sorting using anti-IgD pAb. (A) Low intensity staining cells (R1) and high intensity staining cells (R2) were collected and a fraction of the cells from each sub-population isolated were run on FACS to determine efficiency of sorting (insert); the solid histogram represents the negative control (normal mouse sera). (B) Cells from the different regions were isolated and Giemsa stained, low intense IgM/IgD⁺ cells have a lymphocyte-like appearance, while the high intense staining IgM/IgD⁺ cells display a punctuated cytoplasm reminiscent of degranulated granulocytes.

Possible function of IgD on granulocytes



A. Cross-linking of surface bound IgD on the negatively sorted IgM/IgD⁺ granulocyte population from fish Type 3 resulted in cell degranulation. PBL were negatively selected with anti-IgM using MACS. Fractions were Giemsa stained before and after treatment with anti-IgD and goat-anti-mouse Ig. Proposed mechanism for IgD expression on channel catfish granulocytes are shown in B, we postulate that IgD protein is acquired from an exogenous source and expressed on the surface of these novel small granular cells via a FcR/IgD-R type of receptor resulting in release of cell-associated granules. We also hypothesize that these granulocytes are functionally analogous to mammalian mast cells or basophils.

Conclusions

- Anti-IgD pAb and mAb (7D11) specific to IgD-D2 have been generated
- Three populations of IgD⁺ cells have been identified, two lymphocyte populations that differentially express IgM (IgD⁺/IgM⁺) and (IgD⁺/IgM⁻) and a granular cell population.
- IgD is most likely expressed on the cell surface without associated light chains
- IgD⁺ lymphocytes are low intensity staining while IgD⁺ granulocytes are high intensity staining
- No IgD message is found in the granular cell population
- X-linking surface bound IgD result in degranulation of granulocytes
- Based upon these unique and novel findings our hypothesis is that *miqD*, like *miqM*, functions as a B cell antigen receptor and that *sigD* pre-arms granular effector cells via an FcR and serves as a functional bridge between adaptive and innate immunity