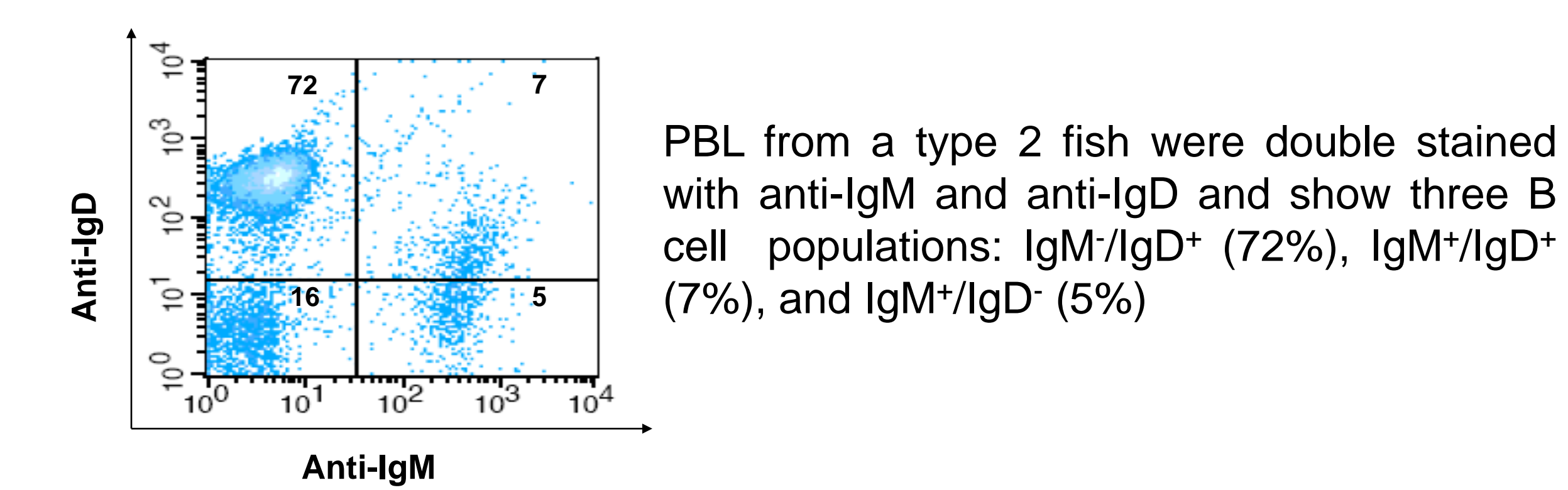
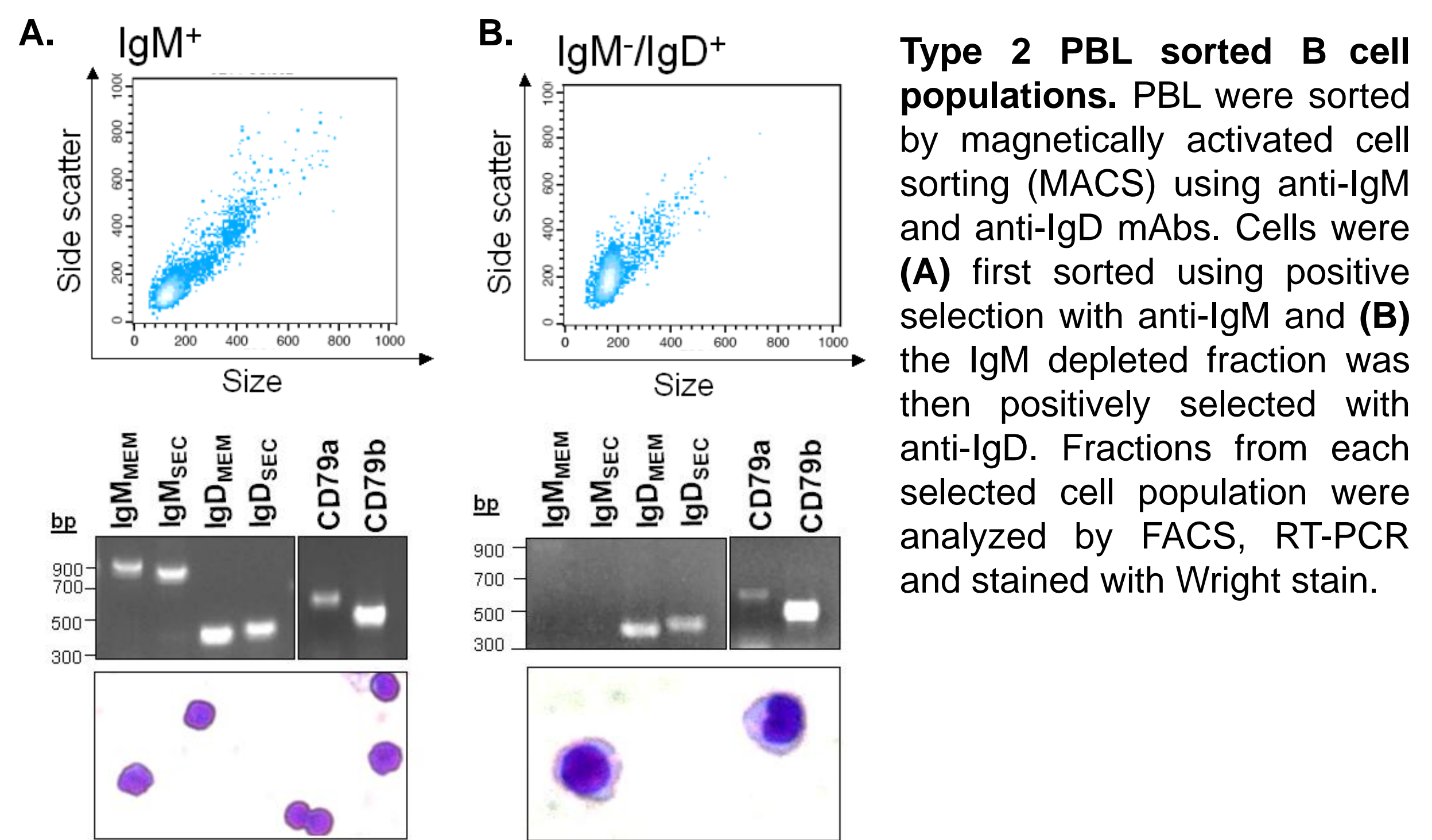


Channel catfish, *Ictalurus punctatus*, express two different IgD⁺ B cell populations. Catfish IgM⁺/IgD⁺ B cells are small and agranular, while IgM⁻/IgD⁺ B cells are larger and exhibit plasmablast morphology. The use of cell sorting, flow cytometry and RT-PCR demonstrated that IgD⁺ B cell expression varies between individual catfish. For example, some catfish have little to no IgD⁺ B cells in their peripheral blood leukocytes (PBL), while in others the IgM⁻/IgD⁺ B cell population can represent as much as 72%. This contrasts to the situation in mammals where IgM⁻/IgD⁺ B cells are rare. Also, transfection studies show IgD functions as a typical BCR since IgD chains associate with accessory molecules CD79a and CD79b. Furthermore, IgM⁻/IgD⁺ B cells preferentially express IgL σ chains, while IgM⁺/IgD⁺ B cells can express any of the four catfish IgL isotypes. As assessed by 5'-RACE, all membrane IgD transcripts from sorted IgM⁻/IgD⁺ B cells contained viable VH rearrangements with no bias in family member usage. However, all secreted IgD transcripts were V-less and began with a leader sequence spliced to the IgD CH1 domain and it is likely that these transcripts encode for the secreted form of IgD previously found in catfish serum. Importantly, the IgD leader mediated IgD protein secretion when transfected into catfish B cells. This characterization of distinct IgM⁺/IgD⁺ and IgM⁻/IgD⁺ B cells in teleosts provides an important first step in understanding the evolution of IgD function. **Supported by NIH grant R01 AI19530 and USDA (2006-35204-16880).**

Isolation and characterizations of IgD⁺ B cells



PBL from a type 2 fish were double stained with anti-IgM and anti-IgD and show three B cell populations: IgM⁺/IgD⁺ (72%), IgM⁻/IgD⁺ (7%), and IgM⁺/IgD⁻ (5%)



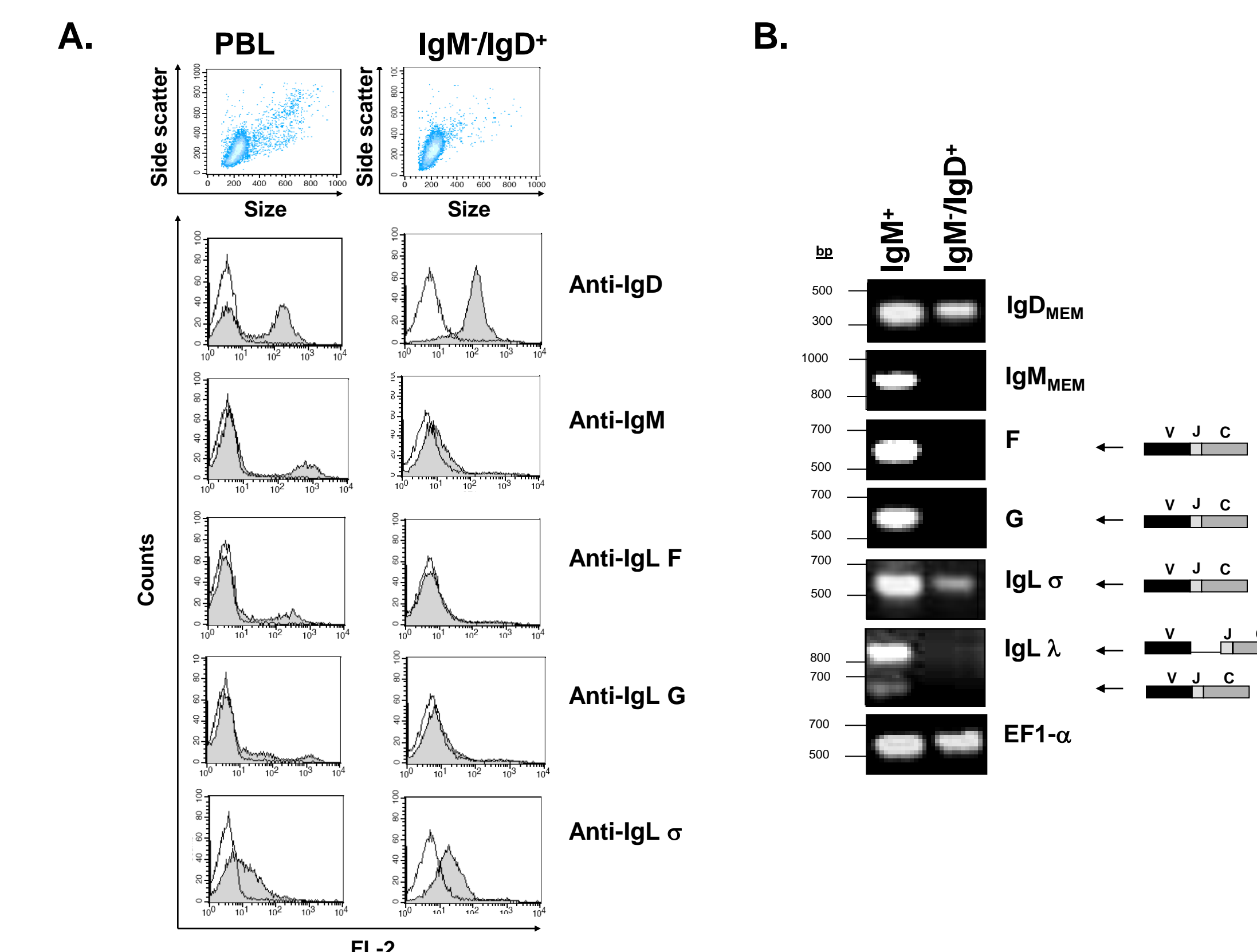
Type 2 PBL sorted B cell populations. PBL were sorted by magnetically activated cell sorting (MACS) using anti-IgM and anti-IgD mAbs. Cells were (A) first sorted using positive selection with anti-IgM and (B) the IgM depleted fraction was then positively selected with anti-IgD. Fractions from each selected cell population were analyzed by FACS, RT-PCR and stained with Wright stain.

IgM⁺/IgD⁺ cells are small agranular lymphocytes and express message for secreted (sec) and membrane (mem) IgM and IgD.

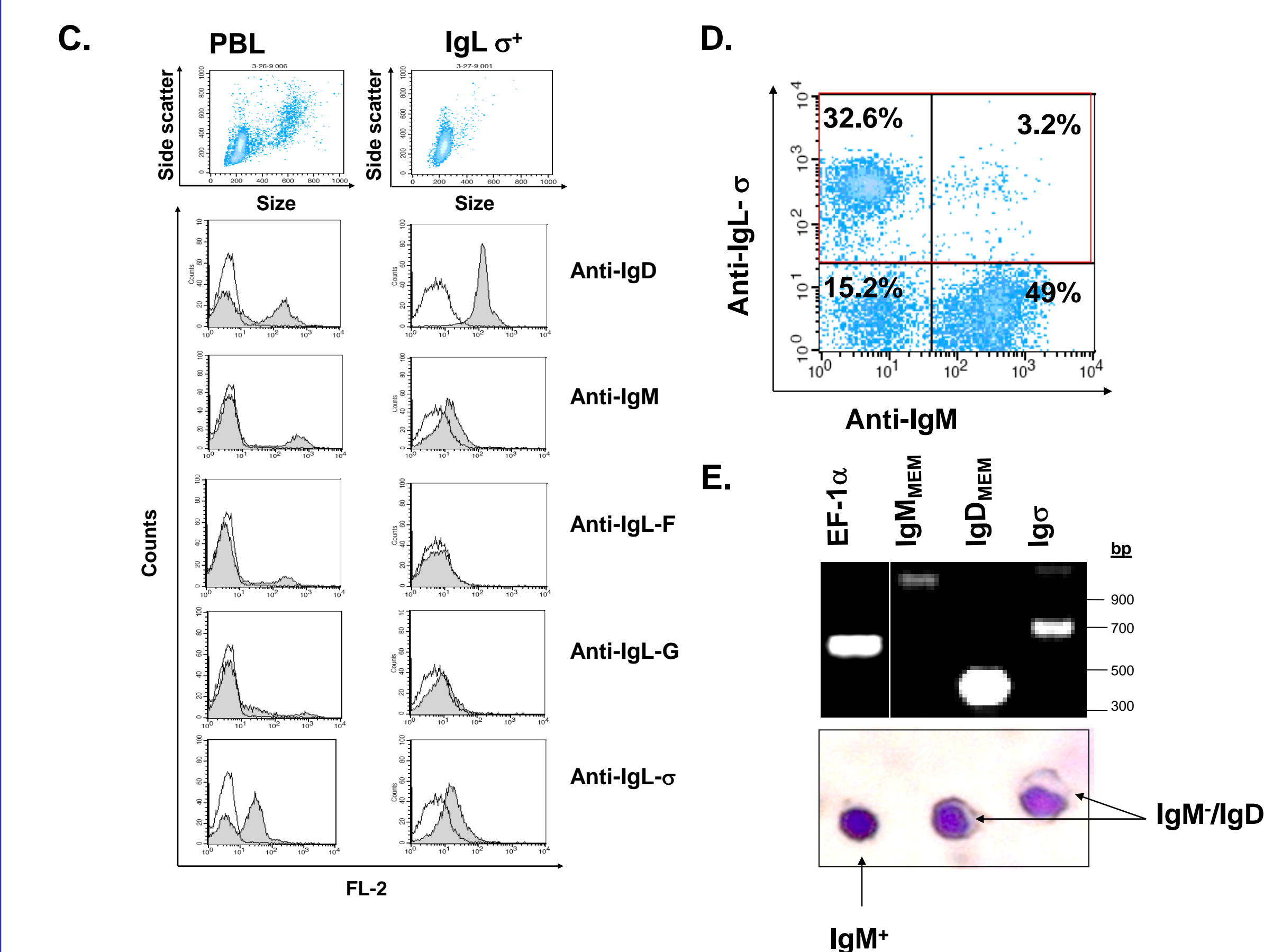
IgM⁻/IgD⁺ cells exhibit a plasmablast morphology and express message for both IgDsec and IgDmem.

Both cells express message for CD79a and CD79b molecules.

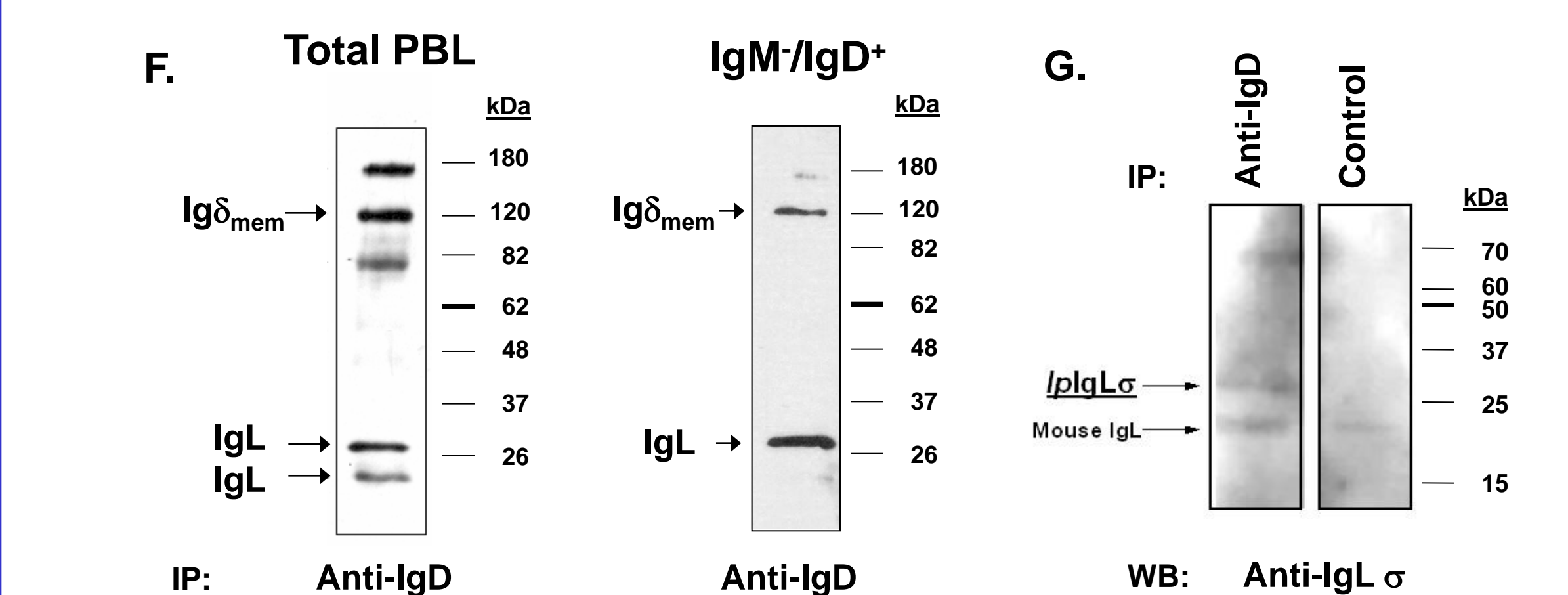
mlgD H chains preferentially associate with IgL σ



IgD, IgM, and IgL expression in PBL from a catfish with a large population of IgM⁻/IgD⁺ B cells. (A) Freshly isolated total PBL and IgM⁻/IgD⁺ MACS sorted B cells from the same fish were analyzed by flow cytometry. Aliquots of unsorted (left) and sorted cells (right) were analyzed using anti-IgD, anti-IgM, anti-IgF, anti-IgL G and anti-IgL σ mAbs (gray) and compared with their isotype controls (white). (B) Total RNA was obtained from the MACS sorted IgM⁺ and IgM⁻/IgD⁺ fractions shown in panel A. RT-PCR analysis was performed using primers specific for memIgM, memIgD, IgL F, IgL G, IgL λ , and the housekeeping gene EF1- α as a positive control. Schematics of the IgL transcripts are next to their respective panels.

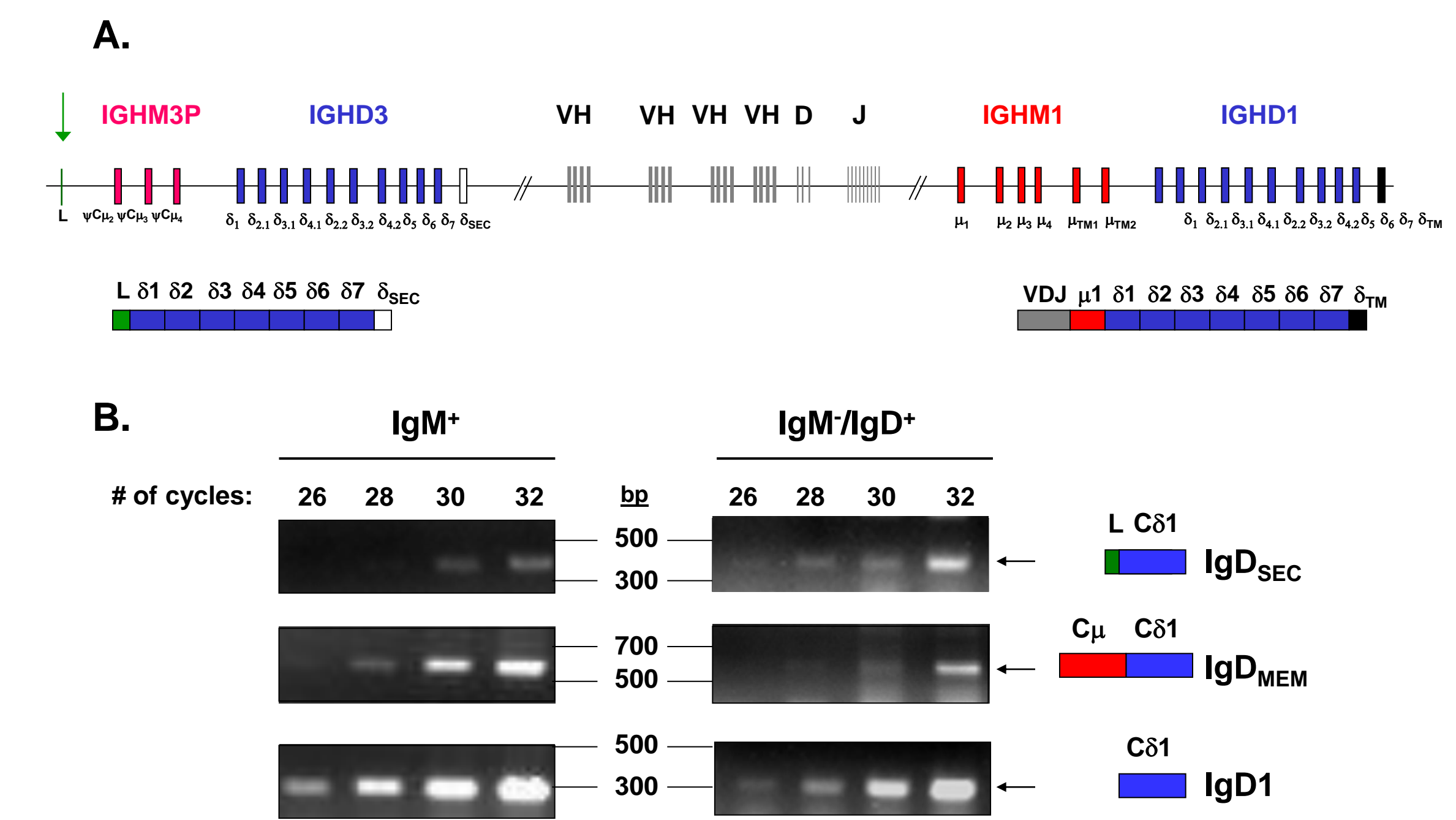


PBL from another catfish with a large population of IgM⁻/IgD⁺ B cells were sorted with anti-IgL σ mAb. (C) Freshly isolated total PBL and anti-IgL σ MACS sorted B cells from the same fish were analyzed by FACS as described above. (D) An aliquot of total PBL from the fish shown in panel C was double-stained with anti-IgM mAb and anti-IgL σ mAb followed by PE- or FITC- conjugated goat anti-mouse isotype specific secondary antibodies. The quadrants containing the IgL σ cells are boxed. (E) RT-PCR analysis and a Wright stain from the IgL σ positive MACS sort. All PCR products were verified by sequencing.



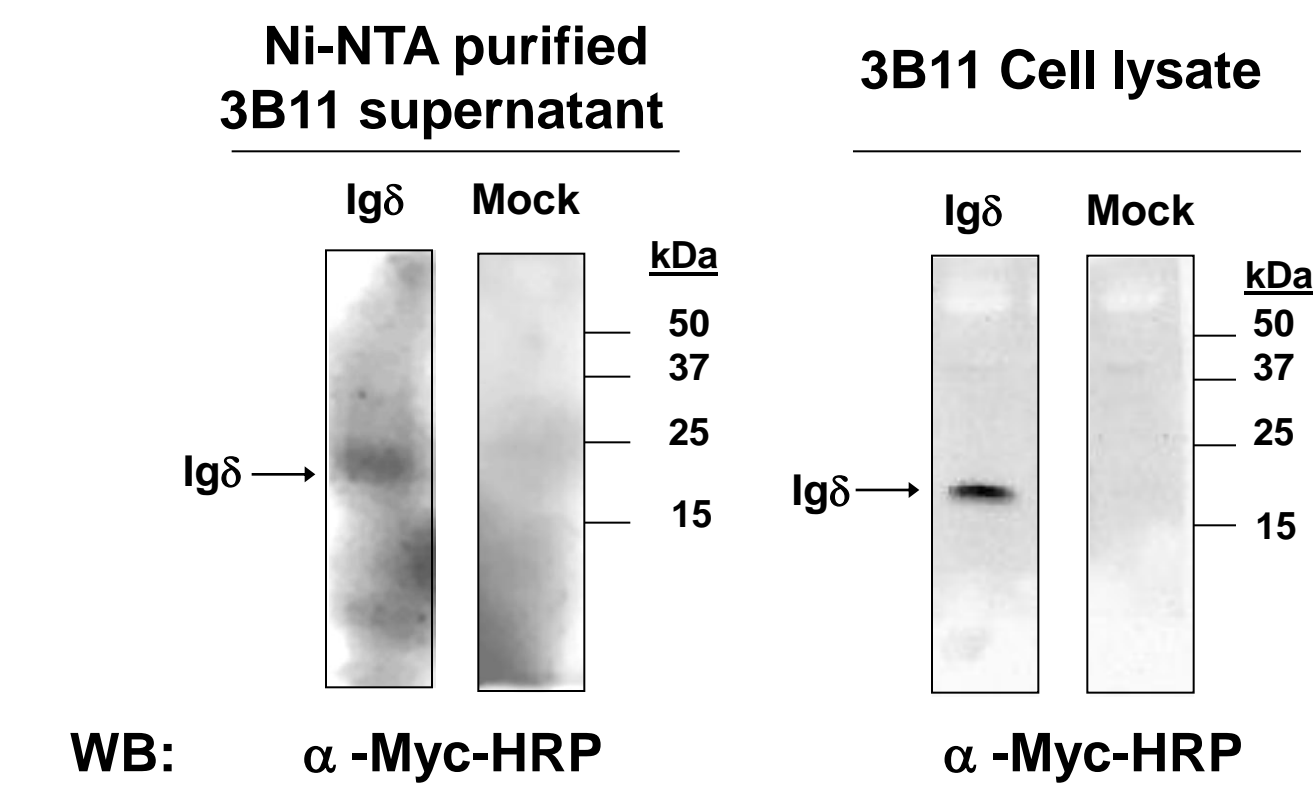
Catfish IgD preferentially associates with IgL σ chains on IgM⁻/IgD⁺ B cells. (F) Immunoprecipitations of biotinylated PBL and IgM⁻/IgD⁺ B cells using anti-IgD mAb. Labeled arrows indicate the appropriate sized IgD and IgL chains. (G) Total cell lysates were immunoselected with anti-IgD mAb, and analyzed by SDS-PAGE. Resulting proteins were visualized using anti-IgL σ as the primary mAb, followed by goat anti-mouse IgG2b-HRP as the secondary mAb. Isotype matched controls were used for all immunoprecipitations.

Expression of secreted IgD

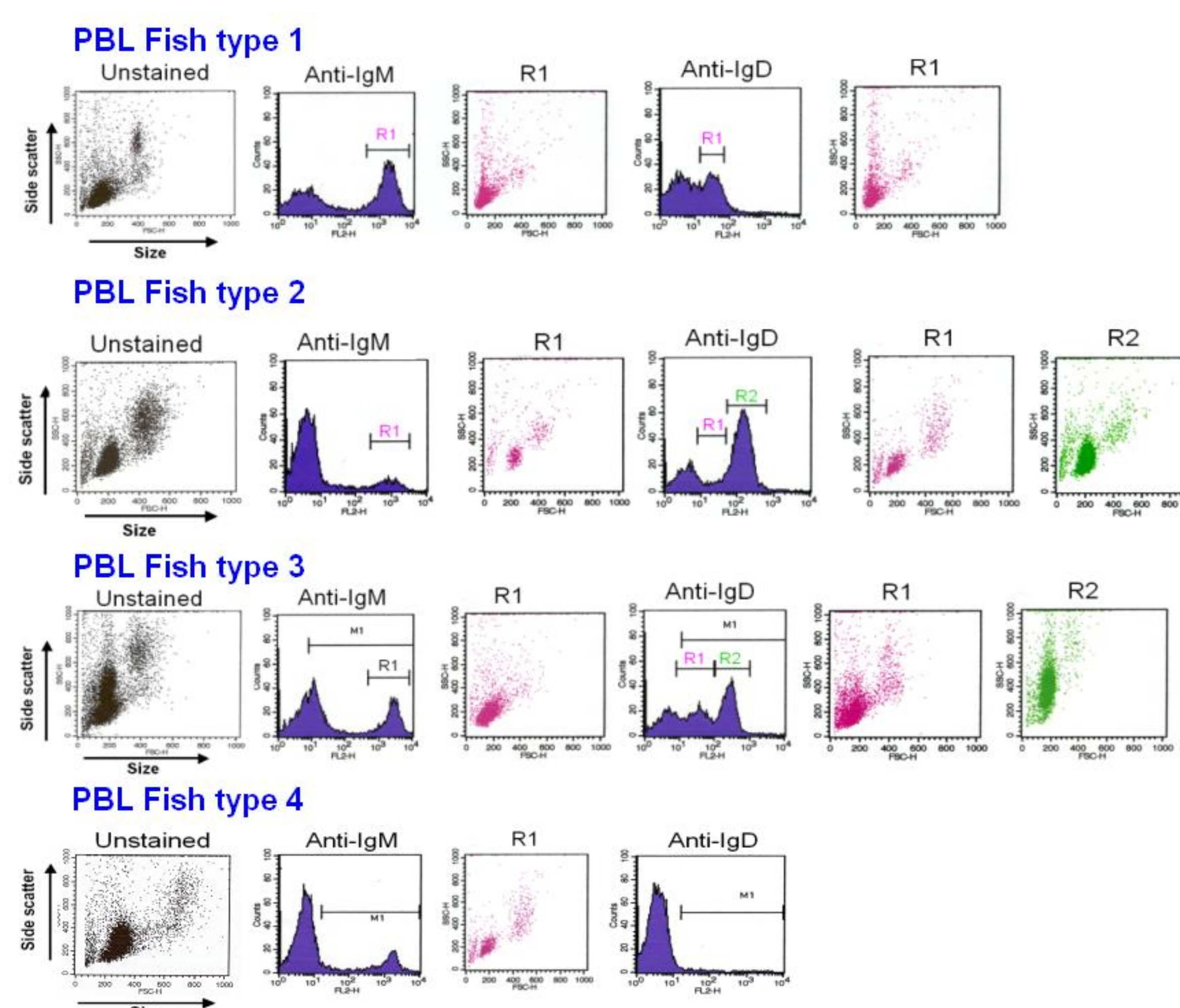


Secreted IgD expression in sorted catfish B cell populations. (A) Map showing the IGHD3 and IGHD1 region genes of the catfish IGH locus. Schematics of encoded seclgD and memlgD forms are shown below their respective genes. The green arrow indicates the location of the seclgD leader sequence. Schematic is not to scale. (B) RT-PCR analysis of seclgD and memlgD message expression in sorted IgM⁺ and IgM⁻/IgD⁺ B cells. Amplification cycle numbers and base pair markers are indicated. Schematics of the identified transcripts are next to their respective panels. PCR products were verified by sequencing and the IgD 1 exon was amplified as a template control.

Secreted IgD leader mediates IgD secretion in vivo. The seclgD leader sequence was cloned into a modified pSecTag2 vector, which did not contain its endogenous Ig κ -chain leader sequence. The modified construct was transiently transfected into catfish clonal 3B11 B-cells. Supernatants and total cell lysates were harvested 48h post-transfection. The His-tag containing proteins were purified from the supernatant and visualized using anti-Myc-HRP, which recognizes the introduced myc epitope tag.



Four types of IgD expression 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 FACSscan was calibrated between each run using chicken red blood cells to eliminate instrumental variation.

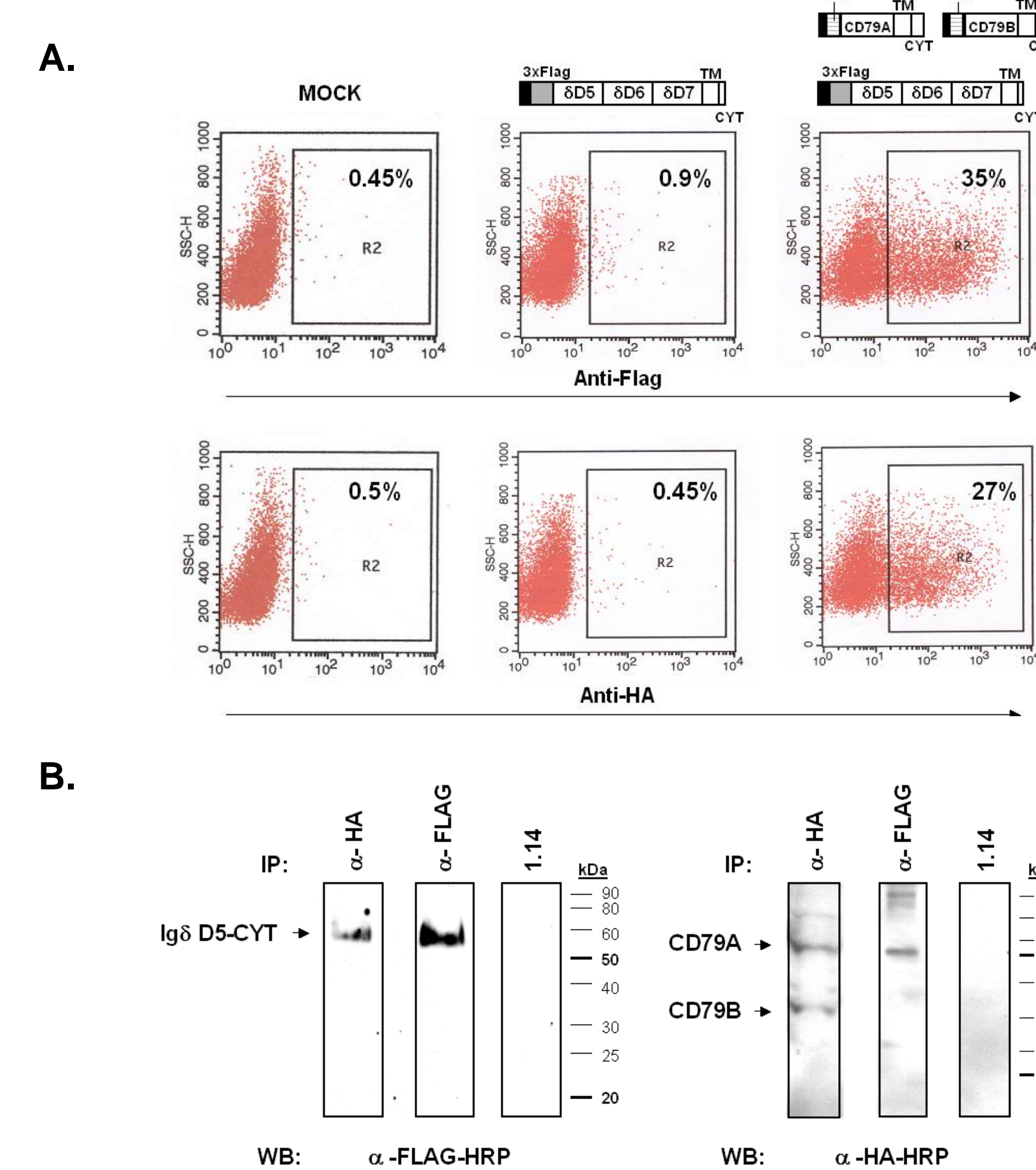
Type 1 consists almost exclusively of small, non-complex cells, with some cells staining at a high intensity for IgM and some cells staining at a lower intensity for IgD.

Type 2 consists of 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 IgD.

Type 3 consists of cells similar to Type II plus an additional population of highly granular cells that stain with a high intensity for IgD.

Type 4 PBL contain IgM⁺ cells and resemble Type 2 scatter profiles, but contain no IgD staining cells.

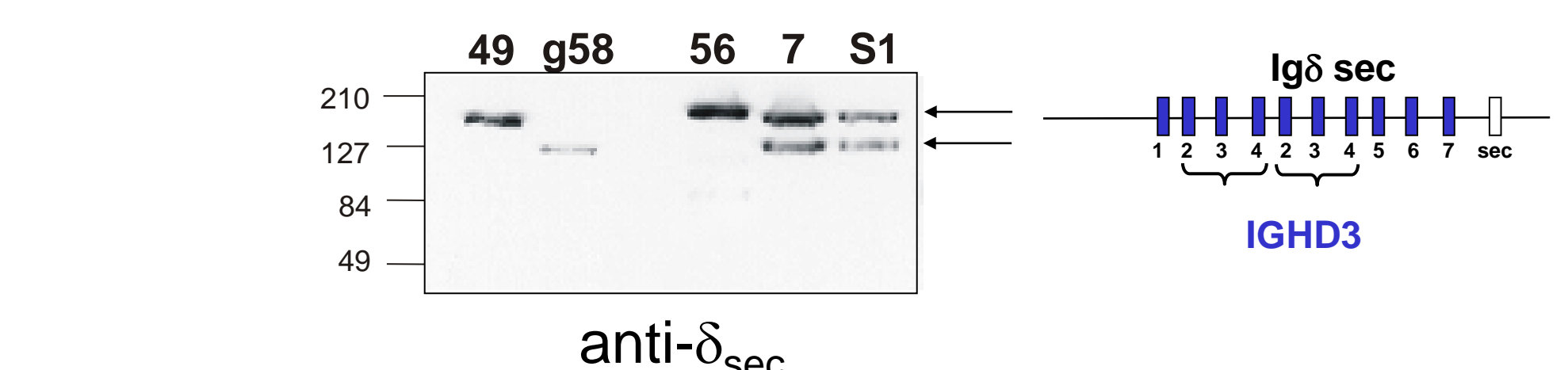
mlgD association with IpCD79 on B cells



mlgD associates with CD79 molecules on the B cell surface.

(A) Clonal catfish 3B11 B cells were nucleofected with different combinations of epitope tagged constructs; IgD (Flag), CD79A (HA) and CD79B (HA). 36 hours post nucleofection, cells were analyzed by FACS for surface expression of the tagged protein(s). Cells nucleofected with IgD alone did not express the construct on the surface while triple transfected cells (IgD, CD79A and CD79B) successfully expressed IgD on the cell surface. (B) Association of IgD with CD79 molecules was demonstrated by co-immunoprecipitations. Cells were lysed in the mild detergent octyl- β -glucoside and selected using anti-HA tag or anti-FLAG tag mAbs and compared to an isotype matched control (anti-trout Ig μ 1.14 mAb). Western blot analysis was performed using either anti-FLAG HRP or anti-HA HRP mAbs.

Evidence for secreted IgD in serum



Secreted IgD is present in catfish sera. Serum proteins from five different catfish were analyzed by 8% SDS-PAGE and visualized by Western blot using anti-IgDsec as the primary mAb, followed by goat anti-mouse IgH+L-HRP. Arrows indicate the appropriate sized IgD chains depending on inclusion/exclusion of domain duplications found in IGHD3. Schematic of IGHD3 shows the duplication of $\delta 2$ - $\delta 3$ - $\delta 4$ domains. IgD reactive bands has been verified by MS/peptide sequencing

Conclusions:

- **Three B cell populations cells that differentially express IgM and IgD have been identified.**
 - Small lymphocytes (IgM⁺/IgD⁻ and IgM⁻/IgD⁺ B cells)
 - Medium lymphocytes (IgM⁻/IgD⁺ B cells)
 - Both lymphocyte populations express message for CD79 molecules
 - Like memIgM, memIgD associates with CD79 molecules on the B cell surface
- **IgM⁻/IgD⁺ B cells preferentially express IgL σ**
 - No surface IgL F or G staining was observed in IgM⁻/IgD⁺ B cells
 - Only IgL σ message is detected in IgM⁻/IgD⁺ B cells
 - Anti-IgD co-immunoprecipitates IgL σ from IgM⁻/IgD⁺ B cells
 - Such results implies there are two B cell lineages
- **Secreted IgD is expressed without a VH region**
 - seclgD transcripts always consist of a viable leader spliced to the first seclgD exon, C δ 1
 - IgD secreted leader mediates in vitro secretion
 - IgM⁻/IgD⁺ B cells appear to be the main producers of slg δ