Supplementary Information

IgT, a primitive immunoglobulin class specialized in mucosal immunity

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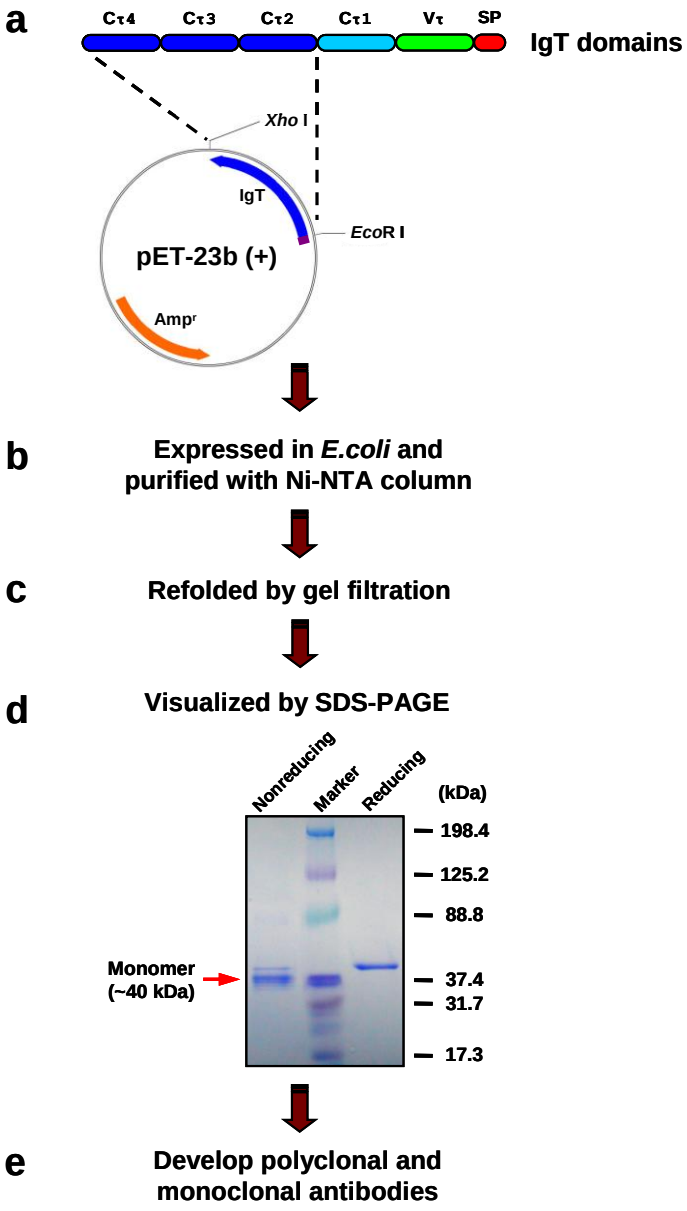
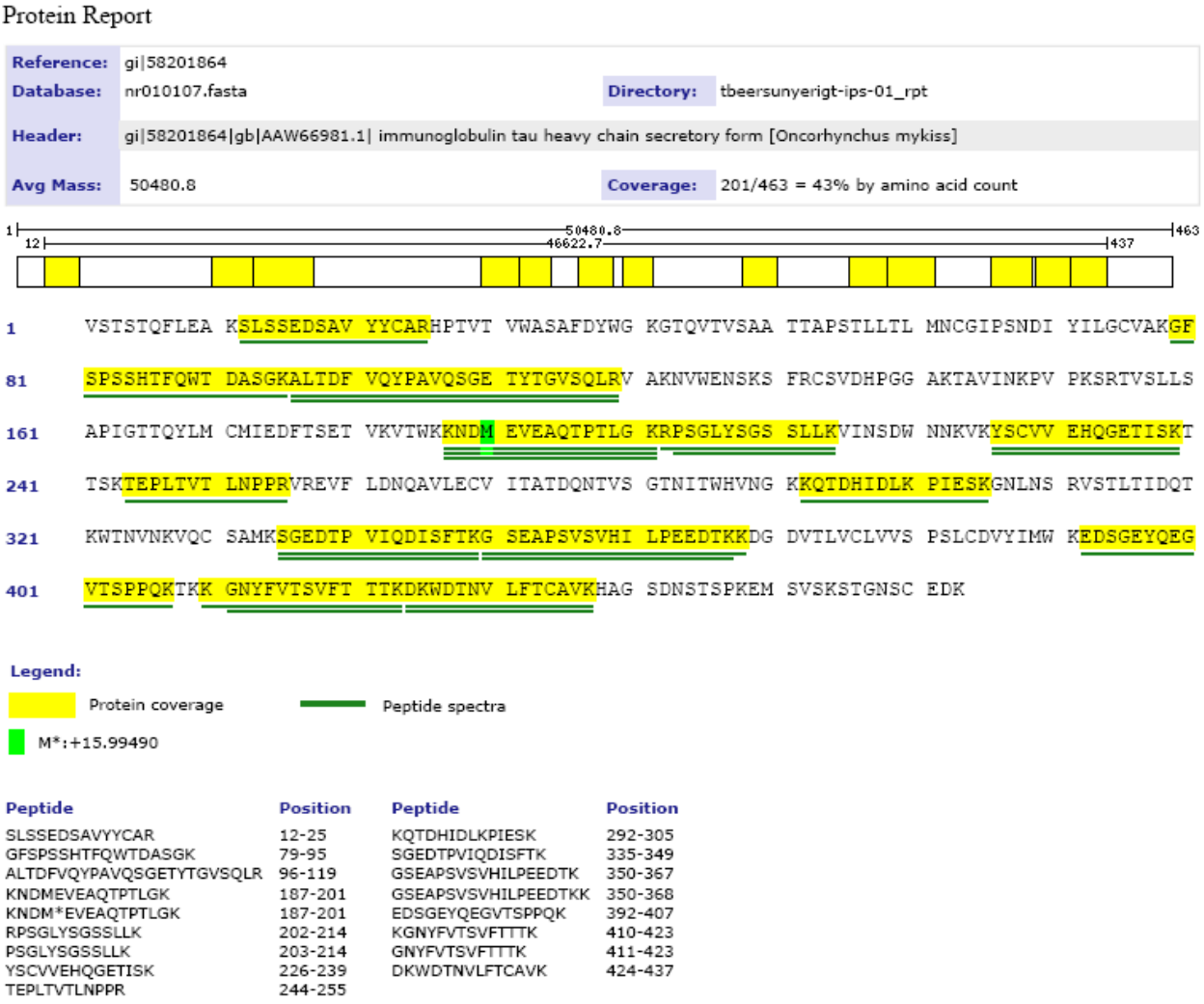
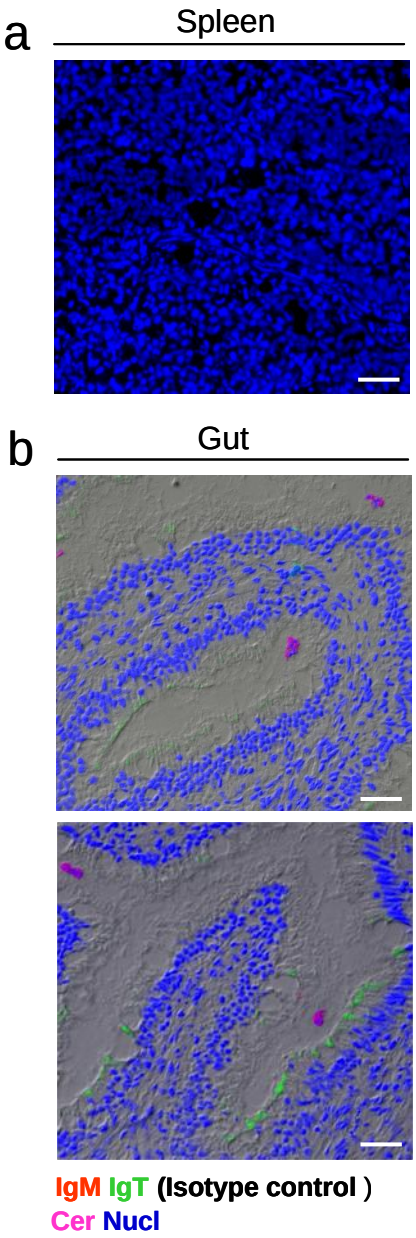


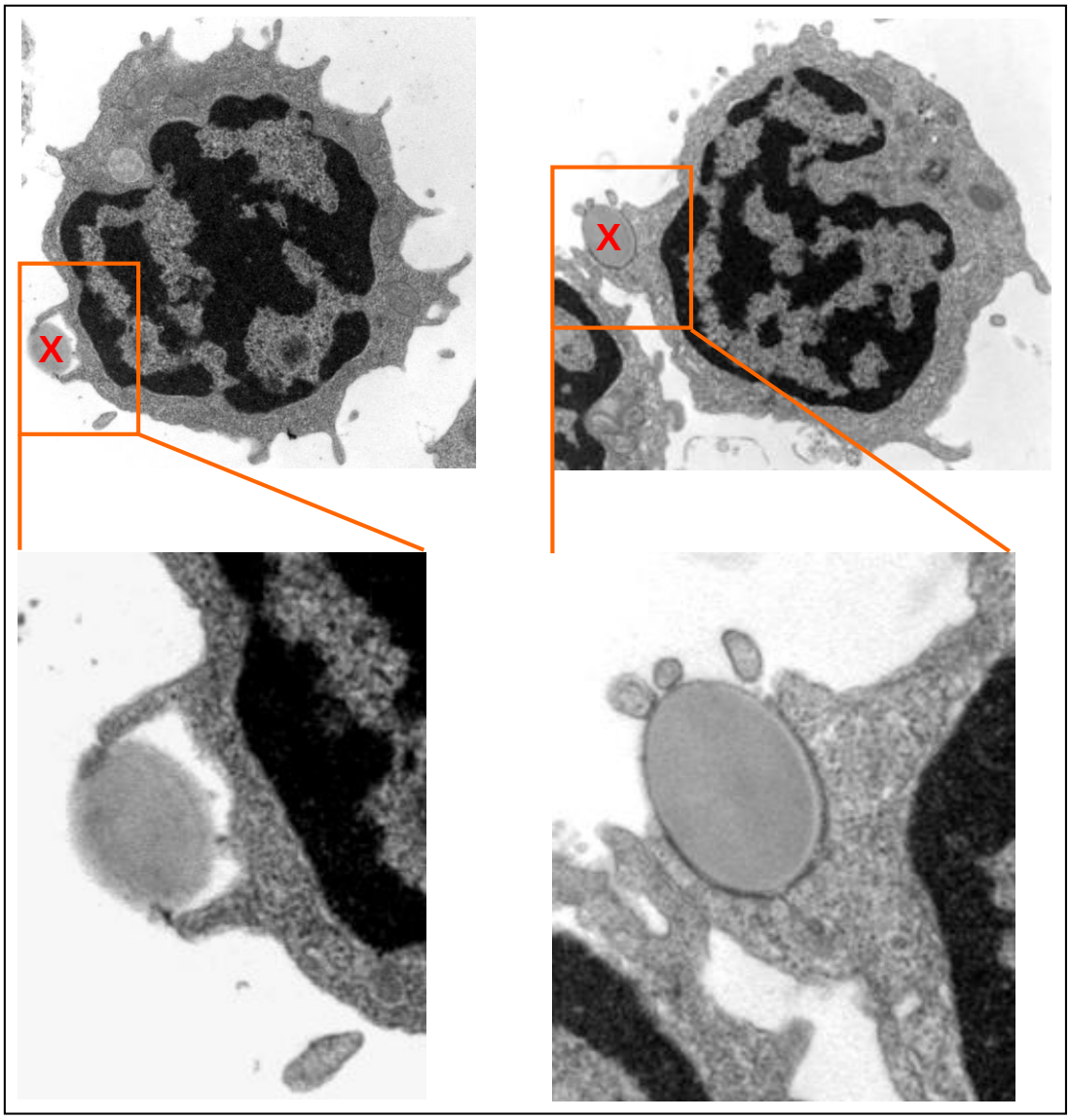
Supplementary Figure 1. Development of polyclonal and monoclonal antibodies to trout IgT. (**a**) The expression plasmid to produce a recombinant fragment of trout IgT was constructed by inserting the cDNA encoding for the CH2-4 domains of trout IgT heavy chain into a modified pET23b(+) vector with an N-terminal 6×His-Tag. (**b-e**) The recombinant trout IgT fragment was produced in *E.coli*, purified with Ni-NTA column, and refolded in a glutathione redox buffer (pH 7.5) by gel filtration using a Superdex 200 FPLC column. The gel filtration fractions containing the refolded recombinant trout IgT fragment (~2 μg) were resolved on a 4-15% SDS-PAGE under nonreducing and reducing conditions, and stained with Coomassie blue (**d**). The resulting recombinant trout IgT fragment (~40 kDa, red arrow) was used to raise pAbs in rabbits and mAbs in mice (**e**). SP, signal peptide; Vτ, variable domain of trout IgT heavy chain; Cτ1-4, constant domains 1-4 of trout IgT heavy chain.



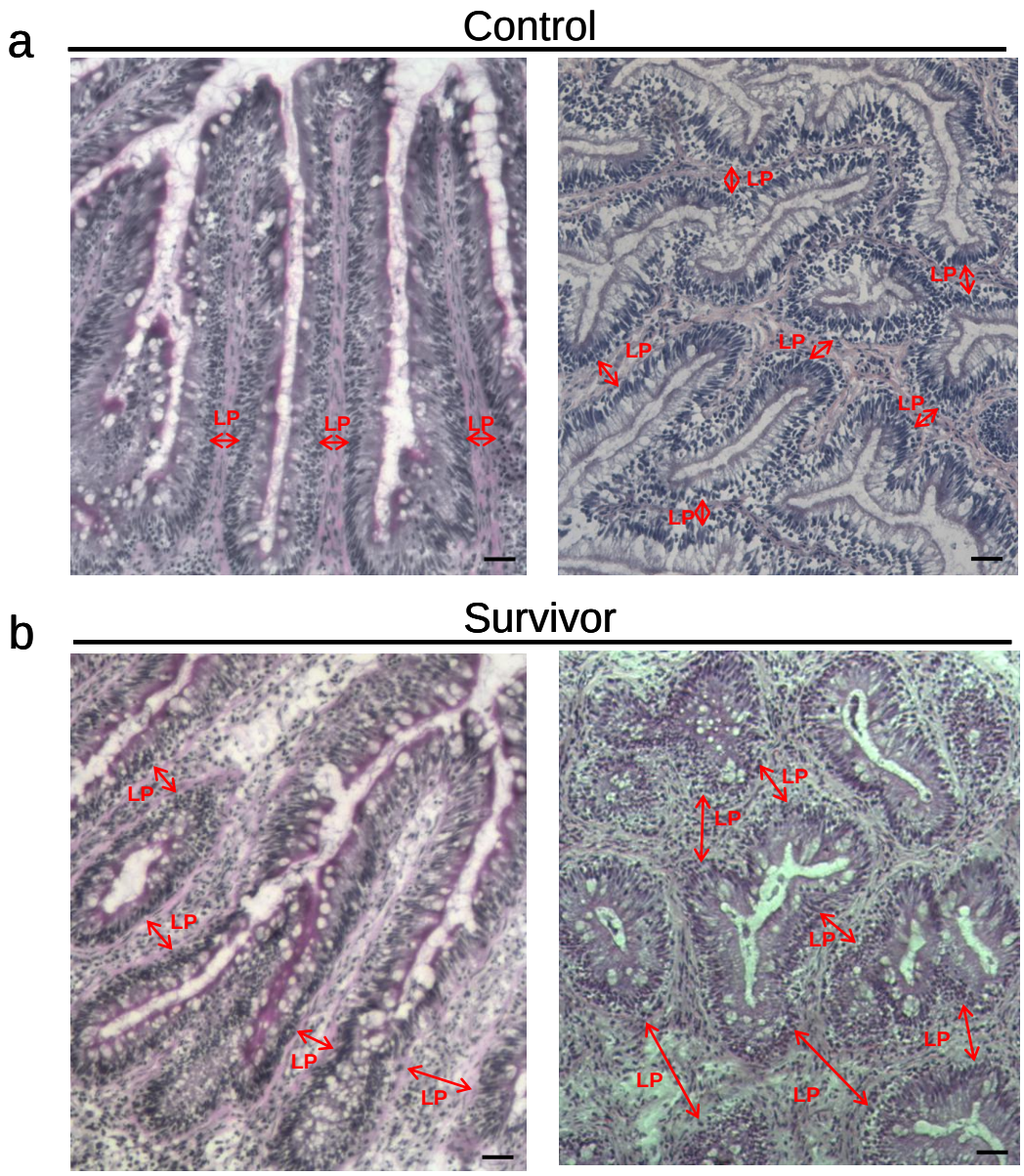
Supplementary Figure 2. LC-MS/MS analysis of trout IgT heavy chain. Approximately 2 μg of affinity purified trout IgT was resolved on a 4-15% SDS-PAGE under reducing conditions and stained with Coomassie blue. The band corresponding to IgT heavy chain was subjected to in-gel digestion and LC-MS/MS analysis. The resulting masses and MS/MS spectra were searched against the non-redundant NCBI database. The database searching showed that 17 peptides matched with the secretory form of trout IgT heavy chain (accession no. AAW66981), comprising 43% of the IgT heavy chain sequence. The amino acid sequence of the parent protein (IgT) is shown numbered, and the protein sequence coverage is shown in highlight yellow. The peptide spectra are shown underlined in green. An 'M' highlighted in green indicates oxidation of methionine with a mass increase of about 16 Da. The sequences and positions of each peptide hit are shown in the bottom panel.



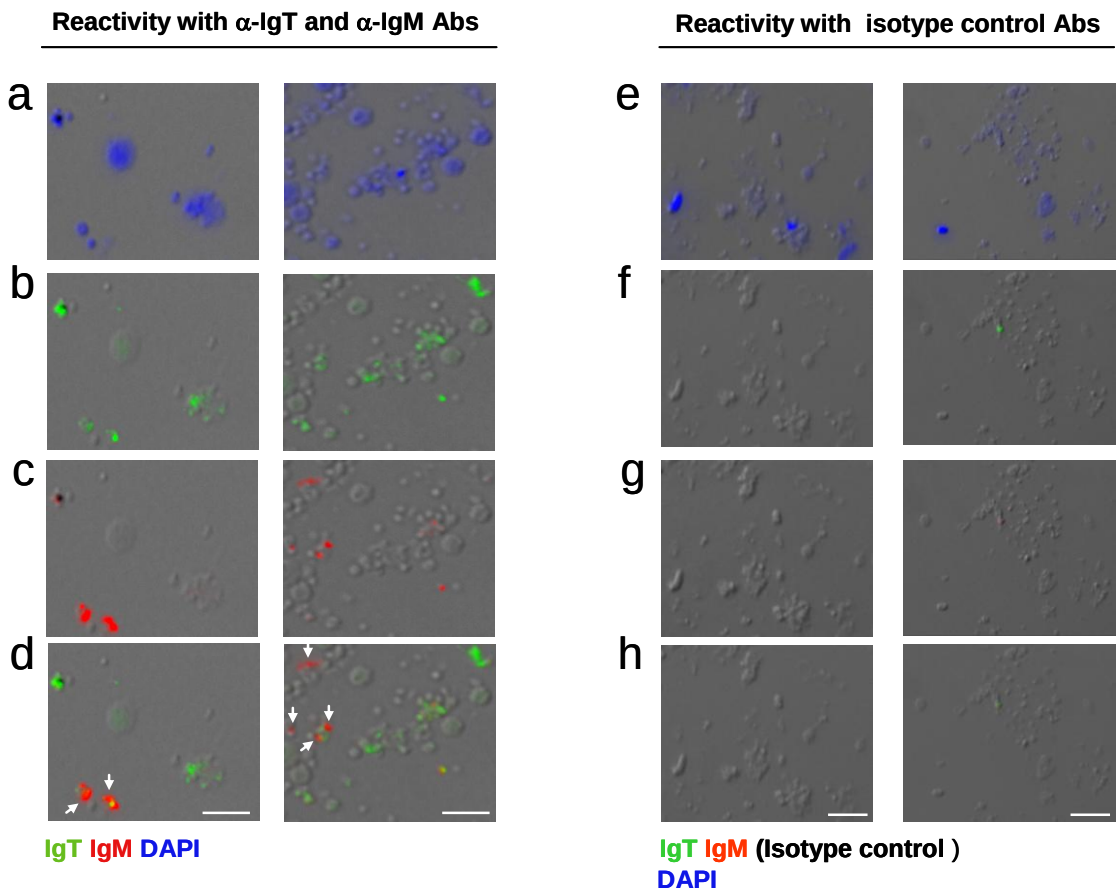
Supplementary Figure 3. Isotype control staining of trout spleen and gut cryosections. Isotype control Abs for anti-IgT (green) and anti-IgM (red) were used to stain trout spleen cryosections from control fish (**a**) and gut cryosections from survivor fish (**b**). Nuclei (Nucl) were stained with DAPI (blue). For the gut cryosections (**b**), images were taken with DIC, and *C. Shasta* (Cer) was stained with anti-*C.shasta* for better visualization of the parasite (magenta). Isotype control Abs for anti-*C. shasta* did not produce any staining (data not shown). Scale bar, 10 µm. Data are representative of three independent experiments.



Supplementary Figure 4. Formation of small and large pseudopodia by IgT$^+$ B cells. Magnified TEM images from **Fig. 3d** show the ultrastructural features of bead uptake by trout phagocytic IgT$^+$ B cells.



Supplementary Figure 5. Leukocytic infiltration in the lamina propria of survivor fish. Hematoxylin-eosin staining of gut cryosections from control (**a**) and survivor (**b**) fish. Lamina propria (LP) from survivor fish showed a notable leukocytic infiltration as evidenced by its increased thickness (i.e., as indicated by the increased arrow length when compared to that of LP from control fish). Scale bar, 30 μm. Data are representative of three independent experiments.



Supplementary Figure 6. Staining of gut luminal bacteria with anti-IgM, anti-IgT and their respective isotype control Abs. (**a-d**) DIC images of gut luminal bacteria stained with a DAPI-Hoeschst solution (blue, **a**), anti-IgT (green, **b**) or anti-IgM (red, **c**). Merged IgT and IgM staining is shown in (**d**). White arrows in (**d**) point to examples of bacteria stained prevalently (left image) or uniquely (right image) for IgM. (**e-h**) DIC images of luminal bacteria stained with a DAPI-Hoeschst solution (blue, **e**), isotype control Abs for anti-IgT (green, **f**) or for anti-IgM (red, **g**). Merged isotype control Abs for anti-IgT and anti-IgM staining is shown in (**h**). Scale bar, 5 μm. Data are representative of at least five independent experiments.

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CACATAGTCACAGTCTGGAACATGATGACTCCTCTACTCTTCTTCGCCTTCCTCCTCCTC   60
                      M  M  T  P  L  L  F  F  A  F  L  L  L    13
TCTCGTCTGCCAGACTCTCTCTGTAGGGTGACCACCGTGGGTGATCTGGCGGTTCTGGAA  120
 S  R  L  P  D  S  L  C  R  V  T  T  V  G  D  L  A  V  L  E    33
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 G  R  S  V  M  I  P  C  H  Y  G  P  Q  Y  A  S  Y  V  K  Y    53
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 W  C  R  G  S  V  K  D  L  C  T  S  L  V  R  S  D  A  P  R    73
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 G  P  A  A  A  G  E  D  K  V  V  M  F  D  D  P  V  Q  Q  V    93
TTCACAGTAACAATGACAGAGCTGCAGAAGGAGGACTCTGGATGGTACTGGTGTGGTGTG  360
 F  T  V  T  M  T  E  L  Q  K  E  D  S  G  W  Y  W  C  G  V   113
GAGGTGGGAGGAGTCTGGAGTGCTGATGTCACAGCCTCCCTTCACATCAATGTTATCCAA  420
 E  V  G  G  V  W  S  A  D  V  T  A  S  L  H  I  N  V  I  Q   133
GGTCTGTCTGTGGTGAACAGCATGGTGAGTGGAGAGGAAGGGACCAGCGTCACTGTCCAG  480
 G  L  S  V  V  N  S  M  V  S  G  E  E  G  T  S  V  T  V  Q   153
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TCCACAACAGCGCCTGCTCCTACAGTTACGTCCCCACCAGAAGAAAGCCCTCAGTCTGTT  780
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CCTGTGTCTCCTCTTCCCAGACACATAGCTAAAGGAGCAGACCACCAAAGGCCACTATGG  840
 P  V  S  P  L  P  R  H  I  A  K  G  A  D  H  Q  R  P  L  W   273
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 A  S  Q  K  V  Y  G  F  *                                    341
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ACAGACCTGCTAAGTACCACAAATGGTAATAATGTTAGTTATTGTGATCATTACCTGACA  1260
TGTTAAATAATGTTACAATGTTTACTGAAATCAGTGTGTATATAATCTACTGTAACTGTT  1320
TGTTTTGTAT                                                    1330
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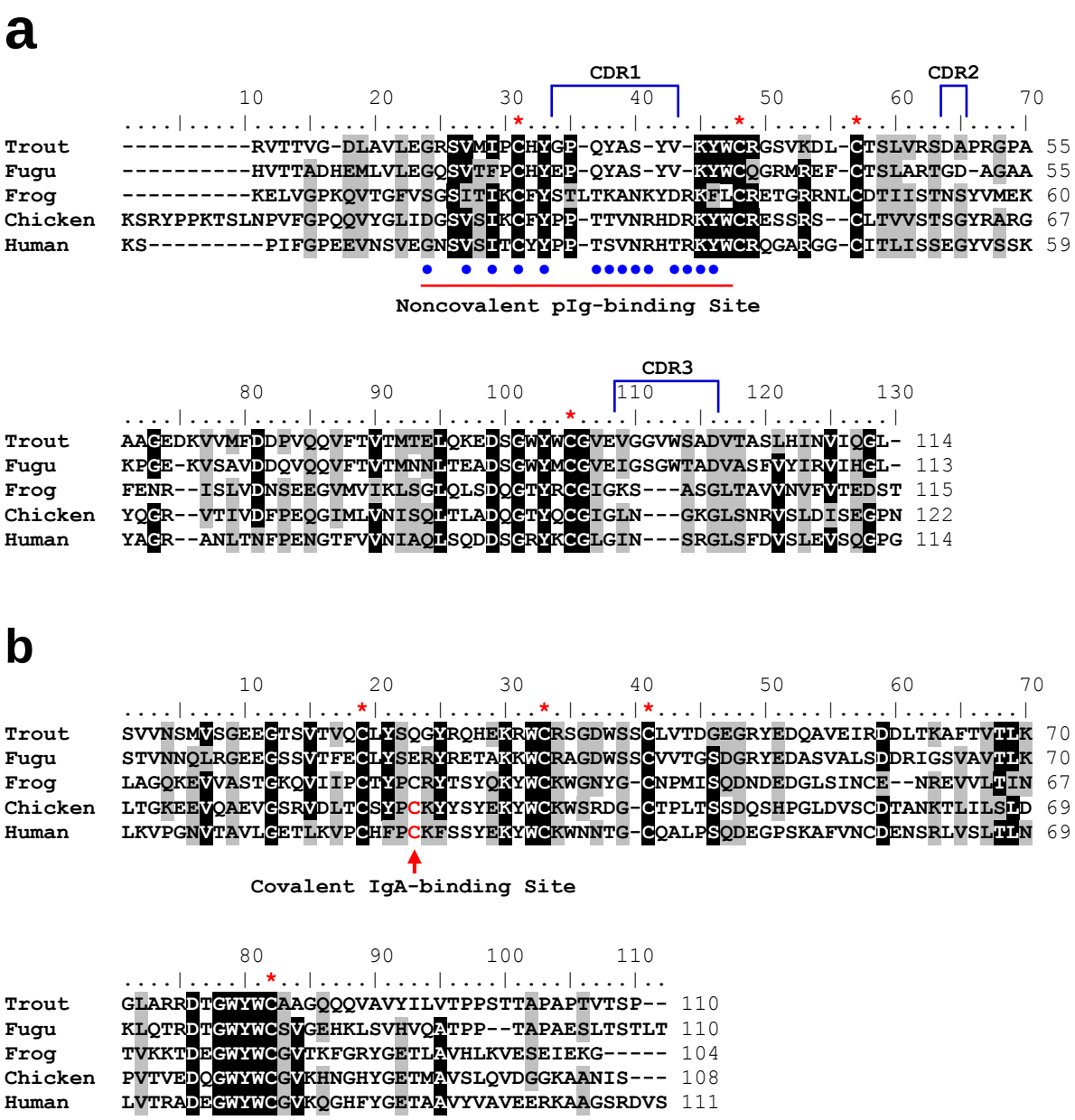
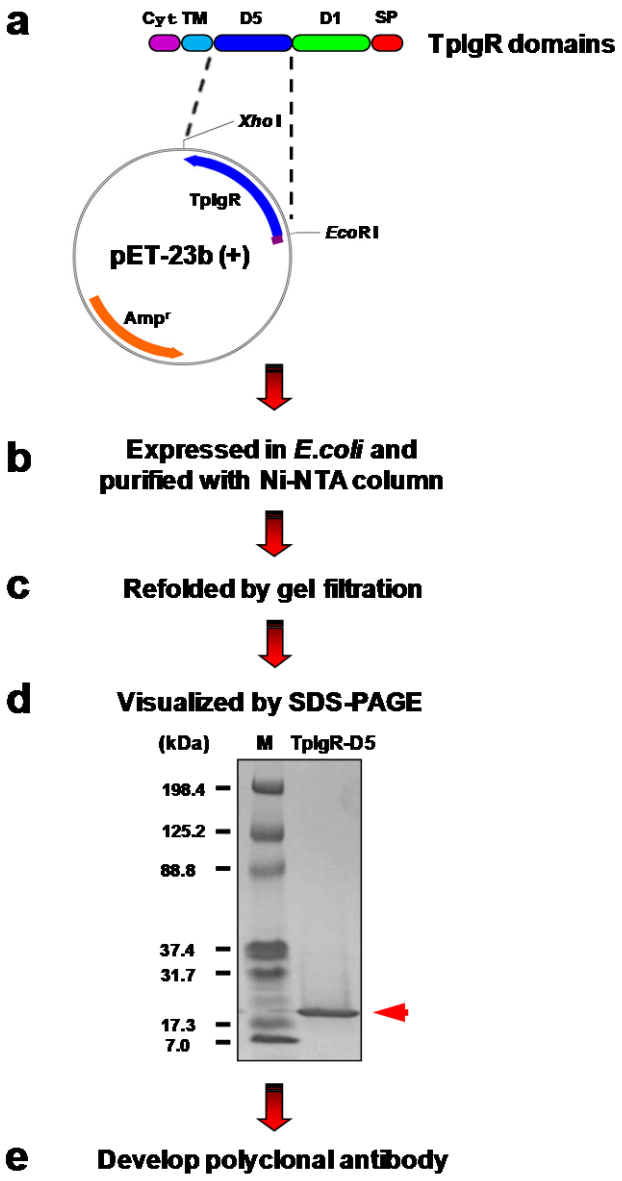
Supplementary Figure 7. Nucleotide and deduced amino acid sequences of trout pIgR (accession no. FJ940682). The start and stop codons are bold red. The predicted signal peptide and transmembrane region are bold underlined. The potential *O*-glycosylation sites are bold circled.



Supplementary Figure 8. Multiple alignments of the domains 1 (a) and 5 (b) of trout pIgR with the corresponding domains from other vertebrates. The identical (black) and similar (grey) residues from the aligned sequences are shaded. Dashes indicate gaps introduced to maintain maximal sequence alignment. Conserved cysteine residues within domains 1 and 5 are indicated by "*". The amino acids from domain 1 of human pIgR participating in the noncovalent binding with polymeric Ig are indicated by "●" below the alignment, whereas the cysteine residue in domain 5 of human pIgR involved in the covalent IgA-binding is indicated by "↑" below the alignment. The complementarity-determining regions (CDR1-3) of human pIgR are indicated. GenBank accession numbers of the sequences used in the alignments are the same as **Fig. 8a**.



Supplementary Figure 9. Development of polyclonal antibody against trout pIgR. (**a**) The expression plasmid to produce a recombinant fragment of trout pIgR was constructed by inserting the cDNA encoding for the domain 5 of trout pIgR into a modified pET23b(+) vector with an N-terminal 6×His-Tag. (**b,c**) The recombinant trout pIgR fragment was produced in *E.coli*, purified with Ni-NTA column, and refolded in a glutathione redox buffer (pH 7.6) by gel filtration using a Superdex 200 FPLC column. The gel filtration fractions containing the refolded recombinant trout pIgR fragment (~2 μg) were resolved on a 4-15% SDS-PAGE under reducing conditions, and stained with Coomassie blue (**d**). The resulting recombinant trout pIgR fragment (~20 kDa, red arrow) was used to raise pAbs in rabbit (**e**). SP, signal peptide; D1 and D5, Domains 1 and 5; TM and CYT, transmembrane and cytoplasm regions.

Supplementary Table 1. *Primers used for trout pIgR cloning and the expression plasmids construction*

Primer	Sequences (5'-3')	Application
pIgR-F1	ACTTGTCACAGCGCCATAGAC	cDNA amplification
pIgR-F2	CACATAGTCACAGTCTGGAACA	cDNA amplification
pIgR-R1	ATACAAAACAAACAGTTACAGTAGA	cDNA amplification
p23b-pIgR(E)-F	ATTGAATTCTCTGTGGTGAACAGCATGGTG	*E.coli* expression
p23b-pIgR(X)-R	ATGCTCGAGTTACTCCCATAGTGGCCTTTGGT	*E.coli* expression
pS-pIgRsig(E)-F	ATTGAATTCATGATGACTCCTCTACTCTTCTT	MDCK cell expression
pS-pIgRsigFLAG-R	CTTATCATCATCATCCTTGTAATCACAGAGAGAGTCTGGCAGACG	MDCK cell expression
pS-FLAGpIgR(N)-F	GATTACAAGGATGATGATGATAAGATGCATAGGGTGACCACCGTGGGTG	MDCK cell expression
pS-pIgRcyt(X)-R	AATCTCGAGTCAGAAACCGTACACCTTCTG	MDCK cell expression

SUPPLEMENTARY METHODS

Development of antibodies against trout IgT and pIgR. The pET-23b expression vector (Novagen) was modified by replacing the N-terminal T7 tag with a 6×His tag and used for constructing the expression plasmids. The DNA fragments encoding for the CH2-4 domains of trout IgT heavy chain were amplified using the primers IgT(E)-F (5'-AATGAATTCCCAGTCCCAAAGTCTCCAAC-3') and IgT(X)-R (5'-AATCTCGAGTCACACACTCATCTCCTTAGG-3') and the proofreading Platinum *Pfx* DNA polymerase (Invitrogen). The resulting amplification was digested with *Eco*R I and *Xho* I and ligated to the modified pET23b vector, which was digested with the same restricted enzymes. The plasmid DNA was prepared and sequenced to confirm that the construct (p23bHis-IgT) encodes the CH2-4 domains of trout IgT heavy chain with an N-terminal 6×His tag for purification. The expression plasmids p23bHis-IgT was subsequently transformed in BL21(DE3) CodonPlus-RIL expression host competent cells (Stratagene). Bacterial cells were cultured in Luria-Bertani broth. Recombinant proteins were expressed by induction with isopropyl-β-D-thiogalactopyranoside (IPTG, Roche) and purified from inclusion bodies as described by us for other recombinant protein purification[1].

Purified recombinant trout IgT fragment was refolded by gel filtration using a Superdex 200 column (GE Healthcare) connected to a FPLC (GE Healthcare) using the same strategy described in[1]. The refolded recombinant trout IgT was used to raise pAbs in rabbits (Cocalico Biologicals, Inc., PA) and mAbs in BALB/c mice (The Hybridoma Facility at The Wistar Institute, PA). Serum titers of both pAbs and mAbs were determined by ELISA. Isotypes and subisotypes of mAbs were determined by ELISA. Antibody specificity for IgT was determined by Western blot, ELISA, and flow cytometry. The positive hybridoma for trout IgT (Clone: 38.5; isotype: IgG2b) was injected intraperitoneally into BALB/c mice to produce ascites (Cocalico Biologicals, Inc., PA). The IgG fraction from the ascites and the polyclonal antiserum were purified using a HiTrap protein G column (GE Healthcare) according to the instructions of the manufacturer. Specific pAbs against IgT were purified by affinity chromatography using the recombinant IgT fragment coupled to NHS-activated Sepharose (GE Healthcare).

To produce Abs against trout pIgR (TpIgR), the expression plasmid p23bHis-pIgR encoding the domain 5 of TpIgR was constructed using the primers p23b-pIgR(E)-F and p23b-pIgR(X)-R

(for primer sequences see Supplementary table I), and the recombinant trout pIgR fragment was produced, purified, refolded, and subsequently used to raise pAbs in rabbits using the same methods described above to generate the polyclonal anti-IgT. The specific pAbs against trout pIgR were purified by affinity chromatography using the recombinant TpIgR fragment coupled to NHS-activated Sepharose (GE Healthcare).

LC-MS/MS analysis of purified trout IgT. Approximately 2 μg of trout IgT purified from serum was resolved by SDS-PAGE on a 4-15% Tris-HCl Gradient Ready Gel (BioRad) under reducing conditions and stained with Bio-Safe Coomassie (BioRad). Mass spectrometry (LC-MS/MS) and protein identification were performed by the Proteomics Facility at the Wistar Institute (PA). In brief, the band of IgT heavy chain was excised from the gel, followed by destaining, reduction, and alkylation. The protein was then digested with 0.02 μg μl^{-1} modified trypsin (Promega) in 40 mM ammonium bicarbonate overnight at 37˚C. Multiple peptide sequences were determined in a single run by loading the digested trout IgT heavy chain to a nanocapillary reverse-phase column (selfpacked New Objective 75 um column terminating in a nanospray 15 um tip) directly coupled to a ThermoElectron LTQ ion trap mass spectrometer. A top six method was used to obtain MS and MS/MS data. The resulting masses and MS/MS spectra were searched against the non-redundant NCBI database using the TurboSEQUEST Browser.

Collection of serum, gut mucus and bacteria. Trout serum was collected and stored as described[2]. To collect trout gut bacteria we adapted to our fish system a protocol reported to obtain human gut bacteria[3]. Briefly, the gut of trout was excised and opened longitudinally, and 0.5 ml of protease inhibitor buffer [1× PBS, containing 1× protease inhibitor cocktail (Roche), 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma), 0.1 mg/ml soybean trypsin inhibitor (Sigma), and 0.5% BSA (Sigma); pH 7.2] was added onto its surface. The mucosal fluid was gently scraped from the inner surface of the gut and transferred to an Eppendorf tube. Following vigorous vortexing, the sample was centrifuged at 40*g* for 10 min to remove large particles. The supernatant was thereafter centrifuged at 400*g* for 10 min to remove trout cells. To separate gut bacteria from mucus, the cell-free supernatant was thereafter centrifuged at 10,000*g* for 10 min. The resulting supernatant (containing gut mucus) was harvested, filtered with 0.45 μm syringe

filter and stored immediately at -80^{o}C, whereas the pellet (containing gut bacteria) was washed three times with 1 ml of PBS (pH 7.2) and resuspended in 50 µl of PBS (pH 7.2).

Isolation of trout leukocytes. Leukocytes from blood, head kidney, spleen, and peritoneal cavity were obtained with 51/34% discontinuous Percoll (GE Healthcare) density gradients as described previously[2]. To obtain trout gut-associated lymphoid tissue (GALT) leukocytes, we adapted to our system a reported protocol to obtain sea bream GALT leukocytes[4] in combination to an existing protocol to obtain gut intraepithelial rainbow trout lymphocytes[5]. Briefly, leukocytes from trout GALT were obtained by means of mechanical agitation of intestine pieces in DMEM medium (supplemented with 5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin) at 4^{o}C for 30 min. Leukocytes were collected, and the aforementioned procedure was repeated four times. Thereafter, the gut pieces were treated with PBS (containing 0.37 mg/ml EDTA and 0.14 mg/ml DTT) for 30 min followed by enzymatic digestion with collagenase (Invitrogen, 0.15 mg/ml) for 2 h at 20 ^{o}C. All leukocyte fractions derived from mechanical and enzymatic treatments were pooled, washed with DMEM medium, and layered over 63/40% discontinuous Percoll gradients. After 30 min centrifugation at 400*g*, cells lying at the interface of the gradient were collected and washed with DMEM medium (supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin).

Flow cytometric, morphological, and gene expression analyses. For flow cytometry studies, trout leucocytes were double stained with monoclonal mouse anti-trout IgM and anti-trout IgT (1 µg/ml each) at 4ºC for 30 min. APC-goat anti-mouse IgG1 (115-135-205; Jackson Scientific) and FITC-goat anti-mouse IgG2b (115-095-207; Jackson Scientific) were used as secondary antibodies to detect IgM and IgT positive cells respectively. Intestinal bacteria were stained with anti-trout IgM or anti-trout IgT or their respective isotype control (Biolegend, 2.5 µg/ml each), at 4ºC for 1 h. After wash, secondary antibodies APC-goat anti-mouse IgG1 or FITC-goat anti-rabbit IgG (111-095-144; Jackson Scientific) were added and incubated for 45 min at 4ºC. To detect bacterial DNA, propidium iodide (PI, Sigma, 5 µg/ml) were added to each sample. Analysis of leukocytes and bacteria were performed with a FACS Canto II (BD Biosciences) and FlowJo software (Tree Star). Morphological (Giemsa staining and TEM analysis) and gene expression analyses of sorted trout IgM^{+} and IgT^{+} B cells were performed as described

previously for IgM^+ B cells[2]. Primer sequences and PCR conditions were reported in[2] except for those of membrane form of IgT, for which the following primer set 5'-ATGCTGGCTCAGACAACAGCA-3' and 5'-AATAACATATCACCACAATGCTTG-3' was used with 42 cycles of PCR amplification. Gene expression analysis of secretory IgT and IgM in trout gut were performed by using quantitative real-time PCR with Power SYBR Green PCR Master Mix (Applied Biosystems) and the primer sets for IgT (5'-CAGACAACAGCACCTCACCTA-3' and 5'-GAGTCAATAAGAAGACACAACGA-3'), for IgM (5'-AAGAAAGCCTACAAGAGGGAGA-3' and 5'-CGTCAACAAGCCAAGCCACTA-3'), and for β-actin (5'- GGACTTTGAGCAGGAGATGG-3' and 5'-ATGATGGAGTTGTAGGTGGTCT-3'). The efficiencies of all of the quantitative PCR primer sets were >95%. Relative expression was determined by the cycle threshold method (C_t) and was normalized to the internal control β-actin.

Gel filtration. To analyze the monomeric or polymeric state of IgT and IgM in trout serum and gut mucus, gel filtration analyses were performed using a Superdex-200 FPLC column (GE healthcare) as described previously for other proteins[1]. Identification of IgM and IgT in the eluted fractions was performed by Western blot analysis using IgM- and IgT-specific mAbs respectively (see below). A standard curve was generated by plotting the elution volume of the standard proteins in a Gel Filtration Standard (BioRad) against their known molecular weight, which was then used to determine the molecular weight of the eluted IgT and IgM by their elution volume.

SDS-PAGE and Western blot. Samples were resolved on 4-15% Tris-HCl Gradient Ready Gels (BioRad) under nonreducing and/or reducing conditions. The gels were either stained with Bio-Safe Coomassie Stain (BioRad) or transferred onto PVDF membranes (Perkin Elmer Life Sciences). For Western blot analysis, the membranes were blocked with 8% skim milk and incubated with monoclonal anti-trout IgT or anti-trout IgM, followed by incubation with peroxidase-conjugated anti-mouse IgG (NA931; GE Healthcare). Immunoreactive bands were visualized using the HyGLO Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific Products). For quantitative analyses of IgT and IgM in serum and gut mucus, Western blot films were scanned and the signal strength of each band was determined by using ImageQuant

TL software (GE Healthcare). Thereafter, the concentration of IgM and IgT were determined by plotting the obtained signal strength values on a standard curve generated for each blot using known amounts of purified trout IgM or IgT.

Phagocytosis and intracellular bacterial killing assays. Phagocytosis (flow cytometric and morphological analyses), and intracellular bacterial killing by trout IgT^+ and IgM^+ B cells were performed as described previously for IgM^+ B cells[2]. Briefly, for phagocytosis, trout leukocytes (2×10^5/well) were incubated in 96-well plates (Nunc) with 1.0 μm fluorescent beads (Polysciences) in the presence or absence of different concentrations of cytochalasin B (0-0.08 mg/ml, Sigma) at a cell/bead ratio of 1:10 for 3 h with 5% CO_2 at 17 ^oC. After incubation, cell suspensions were centrifuged over a cushion of 3% BSA in PBS supplemented with 4.5% D-glucose. The collected cells were stained with anti-IgM and anti-IgT and phagocytosis was evaluated by flow cytometry with a FACSCalibur and CellQuest sofware (BD Biosciences). Phagocytic activity is expressed as the percentage of cells that ingested one or more beads. To assess intracellular bacterial killing, viable *E.coli* were incubated with sorted trout IgT^+ or IgM^+ B cells. Aliquots of cells were pelleted after 0, 2, 4, and 8 h of incubation with 100 μg/ml gentamicin and lysed with 0.9 ml sterilized water. To determine bacterium survival, serial dilutions of the lysates were inoculated onto LB-agar plates[2]. Live bacteria were counted and results are presented as percentage of the number of live bacteria at time 0 h.

B cell proliferation assay. Head kidney leukocytes (HKLs) were isolated as described above. HKLs (4×10^5 cells/well) were plated in flat-bottom 96-well plates (Corning) in a total volume of 0.2 ml of RPMI medium (supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 55 μM 2-Mercaptoethanol, 100 μM Non-essential amino acids and 10 μg/ml nucleosides), and cultured with 5% CO_2 at 17°C. *Vibrio anguillarum-ordalii* bacterin (Vibrogen-2; Novartis Animal Health US Inc., Larchwood, IA) (1/2000 dilution of the commercial bacterial suspension) or a mix of *E. coli* 0111:B4 LPS (Invivogen) and *E. coli* 055:B5 LPS (Sigma) (1:1 ratio, dose 100 μg/ml) were added 2 hours after plating. After 4 hours, 2 days and 6 days of adding the stimulus, leukocytes were incubated with 10 μM of 5-ethynyl-2'-deoxyuridine (EdU) (Invitrogen) for 20 hours before their collection for staining with IgM- or IgT-specific Abs. Stained cells were fixed and EdU^+ cell detection was performed according to the manufacturer's

instructions (Click-iT™ EdU Alexa Fluor 488 Flow Cytometry Assay Kit, Invitrogen) and analyzed by flow cytometry using a FACSCanto with DiVa software (BD Bioscience). Results are presented as percentage of Edu^+ cells within the IgM^+ or IgT^+ B cells populations.

Stimulation of IgT and IgM production by microbial products. HKLs were isolated from naïve trouts as described above and plated in 96-well plates. Culture conditions and timing were the same as previously described in the B cell proliferation assay section. Quantification of IgT and IgM secreted in the cell supernatants was analyzed by Western blotting. Densitometry analysis were performed with ImageQuant TL software (GE Healthcare). To analyze potential differences in the capacity of small or large B cells to produce IgT and IgM, fish were injected with a mix of 100 μg *E. coli* LPS (50 μg *E. coli* 0111:B4 LPS plus 50 μg *E. coli* 055:B5 LPS) and *Vibrio* bacterin (30 μl of 1/3 dilution of the commercial bacterial suspension) in 100 μl of PBS. Control fish were injected with PBS alone. After 7 days HKLs were isolated as described above. The HKLs were stained for IgM and IgT, and small (low FSC) and large (high FSC) IgM^+ or IgT^+ cells were sorted in a DAKO-Cytomation MoFlo High Speed Sorter. Cells (1-8 × 10^4/well) were plated in flat-bottom 96-well plates (Corning) in a total volume of 100 μl of RPMI supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 55 μM 2-Mercaptoethanol, 100 μM Non-essential amino acids and 10 μg/ml nucleosides, and cultured in 5% CO_2 at 17 °C. Supernatants were collected after 4 hours (day 0) and 2 days for their posterior analysis by Western blot. Quantification of IgT and IgM was performed as described above. Results are expressed as fold increase of secreted IgT or IgM relative to their amount at day 0.

Cloning and sequence analyses of trout pIgR. The amino acid sequences for human and fugu pIgR (GenBank accession nos. NM_002644 and AB176853 respectively) were used in TBlastn-based searches to identify orthologous sequences within the trout EST database at NCBI (http://www.ncbi.nlm.nih.gov/). ESTs displaying high similarity to both human and fugu pIgR were retrieved, based on which specific primers pIgR-F1, pIgR-F2, and pIgR-R1 (see Supplementary table I) were designed and used to amplify the cDNA containing complete open reading frame of trout pIgR. Obtained PCR products were cloned and analyzed as described previously[1]. The *O*-glycosylation sites were predicted by the NetOGlyc 3.1 Server (http://www.cbs.dtu.dk/services/NetOGlyc/). Multiple sequence alignments were generated using

ClustalW (Version 1.81) and BioEdit (Version 7.0.9.0), and phylogenetic tree was constructed from the ClustalW-generated alignments using the neighbor joining method within PHILIP program (Version 3.68) and was bootstrapped 1000 times.

Expression of trout pIgR in Madin-Darby Canine Kidney (MDCK) cells. To detect the specificity of the pAb against trout pIgR, an expression plasmid was constructed by overlapping PCR, with the primers listed in Supplementary table I. The resulting plasmid (pCAGGS-FLAG-pIgR) was verified by DNA sequencing to encode the full length of trout pIgR tagged with a FLAG peptide at its N-terminal. MDCK II cells (generous gift from Xiaobo Bai, Drexel University) were grown in DMEM medium (supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37°C and 5% CO_2, and transfected with empty vector pCAGGS (generous gift from Dr. R. Harty, University of Pennsylvania) or plasmid pCAGGS-FLAG-pIgR, by using Lipofectamine 2000 (Invitrogen) according to the manufacture's instruction. Twenty four hours post transfection, cells were lysed with Luciferase Cell Lysis Buffer (New England BioLabs) for Western blot analysis or stained for immunofluorescence microcopy analysis. For Western blot analysis, cell lysates of MDCK cells, transfected with empty vector pCAGGS or plasmid pCAGGS-FLAG-pIgR, were clarified by centrifuging at 14,000*g* for 10 min at 4 ^{o}C, the protein concentrations were measured by Bradford assay (BioRad), and 5 μg of each lysate was used for Western blot analysis. For immunofluorescence analysis, the transfected cells were fixed in cold methanol-acetone (1:1, v/v), stained with polyclonal anti-trout pIgR and monoclonal anti-FLAG (F1804; Sigma) (2.5 μg/ml each), followed by staining with Alexa Fluor 488 donkey anti-rabbit IgG (A21206; Invitrogen) and Alexa Fluor 568 goat anti-mouse IgG (A11004; Invitrogen) (5 μg/ml each). To visualize the nucleus, cells were thereafter stained with DAPI (2 μg/ml). Images were acquired and analyzed with a Nikon E600 fluorescence microscope and NIS-Elements BR imaging software (Nikon).

Co-immunoprecipitation studies. To detect whether polymeric trout IgT and IgM present in the gut mucus were associated to a secretory component-like molecule derived from trout pIgR, we performed co-immunoprecipitating analysis using anti-trout IgT (pAb) or anti-trout IgM (mAb) with the goal to potentially co-immunoprecipitate the secretory component of trout pIgR (tSC). To this end, 10 μg of anti-trout IgT or anti-trout IgM were incubated with 100 μl of trout gut

mucus. As control for these studies, the same amount of rabbit IgG (purified from the prebleed serum of the rabbit) or a mouse IgG1 isotype control (BioLegend) were used as negative controls for anti-IgT and anti-IgM respectively. After overnight incubation at 4 ^{o}C, 20 µl of protein G Agarose (Invitrogen) was added into each reaction mixture and incubated for one hour at 4 ^{o}C. Thereafter, the beads were washed five times with PBS, and subsequently bound proteins were eluted in Laemmli Sample Buffer (BioRad). The eluted material was resolved by SDS-PAGE on 4-15% Tris-HCl Gradient ReadyGels (BioRad) under reducing (for tSC detection) or nonreducing (for IgT or IgM detection) conditions. Western blot was performed with anti-pIgR, anti-IgT, or anti-IgM.

SUPPLEMENTARY REFERENCES

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