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## IgT, a primitive immunoglobulin class specialized in mucosal immunity

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### Abstract

Teleost fish are the most primitive bony vertebrates that contain immunoglobulins. In contrast to mammals and birds, these species are devoid of immunoglobulin A (IgA) or a functional equivalent. This observation suggests that specialization of immunoglobulin isotypes into mucosal and systemic responses took place during tetrapod evolution. Challenging that paradigm, here we show that IgT, an immunoglobulin isotype of unknown function, acts like a mucosal antibody. We detected responses of rainbow trout IgT to an intestinal parasite only in the gut, whereas IgM responses were confined to the serum. IgT coated most intestinal bacteria. As IgT and IgA are phylogenetically distant immunoglobulins, their specialization into mucosal responses probably occurred independently by a process of convergent evolution.

Adaptive immunity first emerged in vertebrates around 550 million years ago along with the appearance of the jawless fish, the most ancient living vertebrate species<sup>1</sup>. In these animals, antigen recognition is mediated by variable lymphocyte receptors<sup>2</sup>. However, these species lack immunoglobulins or T cell antigen receptors, both of which arose in jawed vertebrates<sup>3</sup>. Throughout evolutionary time, immunoglobulins diversified into several isotypes with

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### AUTHOR CONTRIBUTIONS

Y.-A.Z. purified trout IgT, developed polyclonal and monoclonal antibodies to IgT and a polyclonal antibody to trout pIgR, analyzed the biochemical features of IgT and the gene-expression profiles of IgT<sup>+</sup> and IgM<sup>+</sup> cells, measured specific IgT and IgM titers to *C. shasta* and did all experiments involved in cloning and functional studies of trout pIgR; I.S. developed the protocols to obtain leukocytes and bacteria from trout GALT and the immunohistochemistry protocols to detect IgT<sup>+</sup> and IgM<sup>+</sup> cells and *C. shasta* in trout lymphoid tissues and did immunohistochemistry studies and flow cytometry of IgT<sup>+</sup> and IgM<sup>+</sup> cells in trout GALT and gut luminal bacteria; J.L. did the flow cytometry of IgT<sup>+</sup> and IgM<sup>+</sup> cells of trout lymphoid organs, measured the phagocytotic capacity and intracellular bacterial killing of B cells and contributed to the evaluation of total IgT and IgM concentrations in serum and gut mucus, together with Y.-A.Z.; D.P. analyzed IgT and IgM coating on gut bacteria, did the B cell proliferation studies and tested the production of IgT and IgM after stimulation with microbial products; S.B. infected fish with *C. shasta* and provided samples from survivor and control fish; Z.X. contributed to the evaluation of the production of IgT and IgM by cultured trout leukocytes and sorted B cells; S.E.L. and J.B. contributed to the experimental design and discussions related to *C. shasta* infection; J.O.S. designed the overall study, contributed to data analysis and wrote the main body of the paper; and all authors read and commented on the paper. J.L. and D.P. contributed equally to this work.

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specialized roles in innate and adaptive immunity in the mucosal and systemic compartments<sup>3</sup>. However, it is unclear how and when specialization of immunoglobulin isotypes into systemic and mucosal sites occurred in vertebrates. In mammals and birds, the immunoglobulin M (IgM), IgG and IgY isotypes have a predominant role in systemic responses, whereas IgA is the key participant in mucosal surfaces<sup>4,5</sup>. In contrast, cold-blooded vertebrate species lack IgA, although amphibians contain IgX, an isotype expressed mainly in the gut<sup>6</sup>. Teleost fish are the most primitive bony vertebrates that contain immunoglobulins and, in contrast to mammals and birds, they are devoid of IgA<sup>3</sup> or a functional equivalent of IgA. Thus, there is no evidence of immunoglobulin specialization in teleost mucosal and systemic areas and therefore IgM is regarded as the only functional antibody in both compartments<sup>7,8</sup>. This suggests that specialization of immunoglobulin isotypes into mucosal and systemic responses arose during tetrapod evolution.

The sequencing of several genomes from almost every main class of vertebrate has substantially furthered the understanding of the diversity and evolutionary origins of immunoglobulins. In 2005, a previously unknown immunoglobulin isotype, IgT (also called IgZ), was discovered after analysis of the genomes of several teleost fish species<sup>9,10</sup>. This discovery marked a “sobering moment,”<sup>11</sup> as IgT was suggested to represent the final immunoglobulin isotype to be found in vertebrates. Analysis of the locus encoding IgT and IgM heavy chains in trout and zebrafish has showed that although this locus is in a translocon configuration (similar to that of mammalian immunoglobulin heavy-chain loci), its genomic architecture bears a resemblance to that of the locus encoding the T cell antigen receptor  $\alpha$ -chain and  $\delta$ -chain of mammals<sup>9–11</sup>. Such genomic arrangement has allowed the prediction of the existence of two mutually exclusive B cell lineages expressing either IgT or IgM, analogous to the commitment of the T cell lineage into  $\alpha\beta$  or  $\gamma\delta$  cells. Moreover, the genomic structure of the locus encoding IgT and IgM heavy chains rules out possible class-switch recombination events between the genes encoding IgT and IgM. In support of the latter proposal, no evidence has been found of switching of rearranged variable-diversity-joining (VDJ) regions between constant (C) regions of genes encoding IgT ( $C_T$ ) and IgM ( $C_\mu$ ); instead, use of the D and J segments is restricted to the nearby C regions in all analyzed zebrafish and trout genes encoding IgT and IgM<sup>9,10</sup>. In addition, there are no germline  $D_T$  or  $J_T$  segments in IgM heavy-chain cDNAs. Moreover, no switch regions similar to those of amphibians, birds or mammals have been found in the teleost sequences analyzed<sup>9,10</sup>.

Thus far, nothing has been reported about the protein structure of IgT or its distribution and production by putative B cells. More importantly, its function remains an enigma. Here we have characterized IgT at the protein level and we show that it is a monomeric immunoglobulin in serum. However, in the gut mucus, IgT was chiefly polymeric and was expressed more abundantly there than in serum. Notably, we also provide direct evidence for the existence of a previously unrecognized B cell lineage that expressed only surface IgT. This lineage represented the main B cell subset in the gut-associated lymphoid tissue (GALT) of rainbow trout. More critically, our functional studies indicate that IgT acts like a mucosal intestinal immunoglobulin. Thus, we detected rainbow trout IgT responses to an intestinal parasite only in the gut, whereas IgM responses were confined to the serum. Moreover, we found that most trout intestinal bacteria were coated with IgT. Our findings collectively indicate the first nontetrapod immunoglobulin specialized in mucosal immunity, to our knowledge. Therefore, our data challenge the present paradigm that specialization of immunoglobulin isotypes into mucosal and systemic responses arose during tetrapod evolution. As IgT and IgA are phylogenetically distant, their specialization into mucosal compartments must have occurred independently by a process of convergent evolution.

## RESULTS

### Protein characterization of serum and gut mucus IgT

So far, nothing has been reported about the protein structure of IgT. To begin its characterization, we initially raised polyclonal and monoclonal antibodies to rainbow trout IgT (Supplementary Fig. 1), which we used to identify and purify IgT from trout serum. We purified serum IgT by a combination of affinity purification (with an anti-IgT affinity column) and gel-filtration chromatography. The IgT preparation was >95% pure, as judged by SDS-PAGE and Coomassie blue staining. Under nonreducing conditions, the molecular mass of IgT was ~180 kilodaltons (kDa), whereas under reducing conditions, its heavy and light chains had masses of ~75 kDa and ~25 kDa, respectively (Fig. 1a). These masses are in close agreement with the theoretical masses obtained from its amino acid sequence<sup>9</sup>. Liquid chromatography–tandem mass spectrometry confirmed that the purified protein corresponded to IgT (Supplementary Fig. 2). Immunoblot analysis showed that the IgT-specific polyclonal antibody (Fig. 1b) and monoclonal antibody (mAb; Fig. 1c, right) reacted specifically with IgT and that an IgM-specific mAb did not recognize IgT (Fig. 1c, left). The molecular mass of purified serum IgT under nonreducing conditions suggested that it could be a monomeric immunoglobulin. Gel-filtration analysis confirmed that hypothesis, showing that serum IgT eluted only at the position expected for a monomer (~180 kDa), as deduced from the standard curve generated with molecular weight standards (Fig. 1d, left). In contrast, most of the IgT present in the gut mucus was in polymeric form, as it eluted at a position similar to that of trout IgM, a tetrameric immunoglobulin<sup>12</sup> (Fig. 1e, left). However, by SDS-PAGE under nonreducing conditions, gut mucus polymeric IgT migrated as a monomer (Fig. 1e, right), similar to gut or serum monomeric IgT (Fig. 1d,e, right). This finding indicated that the monomeric subunits of gut polymeric IgT are associated by noncovalent interactions. As expected, by SDS-PAGE under nonreducing conditions, serum and gut IgM migrated as a polymer (Fig. 1d,e, right), as most of the IgM subunits are known to be associated through disulfide bonds<sup>12</sup>. We next analyzed the protein concentrations of IgM and IgT in serum and gut mucus and found that whereas the concentration of IgM in serum (2,520 µg/ml) was much higher than that in gut mucus (74.9 µg/ml), the concentration of IgT in gut mucus (7.1 µg/ml) was double that in serum (3.7 µg/ml) (Fig. 1f,g). Thus, the ratio of IgT to IgM was 63-fold higher in the gut mucus than in serum (Fig. 1h), which suggested that IgT might have a role in gut mucosal immunity.

### Identification of a previously unknown IgT<sup>+</sup> B cell lineage

Published studies have demonstrated the occurrence of IgM<sup>+</sup> leukocytes that express IgT transcripts<sup>13</sup>. Here we found direct evidence of the existence of a distinct teleost B cell lineage that expressed only surface IgT. The IgT-specific mAb recognized a population of IgM<sup>+</sup> cells with typical lymphocyte-like morphology (Fig. 2a). Electron microscopy of sorted IgT<sup>+</sup> B cells showed they had a morphology nearly indistinguishable from that of IgM<sup>+</sup> B cells<sup>13</sup>. Thus, IgT<sup>+</sup> B cells were characterized by a large round nucleus, a thin rim of agranular cytoplasm and a varying number of small dendrites surrounding the cell (Fig. 2a, top right). Gene-expression analysis showed that membrane IgT was expressed only in the IgT<sup>+</sup> population and that IgD was expressed only by the IgM<sup>+</sup> subset. In addition, IgT<sup>+</sup> cells lacked expression of T cell–specific genes (those encoding T cell antigen receptor and CD8), the IgE receptor FcεRIγ and the receptor for colony-stimulating factor 1, which indicated an absence of contaminating T cells and monocytes in the sorted IgT<sup>+</sup> B cell population (Fig. 2b). We found the IgT<sup>+</sup> B cell population constituted ~16–28% of all trout B cells in the blood, spleen, head kidney and peritoneal cavity (Fig. 2c,d). In contrast, IgT<sup>+</sup> B cells represented the main B cell subset (54.3% of all B cells) in the gut of trout (Fig. 2d). We used immunofluorescence microscopic analysis of the spleen to further substantiate the presence of the uniquely stained IgT<sup>+</sup> and IgM<sup>+</sup> B cell subsets found by flow cytometry

(Fig. 2e and Supplementary Fig. 3a, isotype control). These images also confirmed the absence of double-positive IgT<sup>+</sup>IgM<sup>+</sup> cells, in agreement with the data reported above (Fig. 2c). Thus, we demonstrated the existence of a previously unknown B cell lineage that expressed only surface IgT and showed that it represented the main B cell population of trout GALT.

### Myeloid and innate features of IgT<sup>+</sup> B cells

To begin to delineate the potential roles of IgT in immunity, we started by evaluating key innate properties of IgT<sup>+</sup> B cells. IgM<sup>+</sup> B cells of teleost fish are highly phagocytic and have intracellular killing abilities<sup>13</sup>. To assess the phagocytic potential of IgT<sup>+</sup> B cells, we incubated peripheral blood leukocytes with fluorescent latex beads and detected phagocytic IgT<sup>+</sup> B cells by flow cytometry with the mAb to IgT. We found a double-positive fluorescence-associated population corresponding to the phagocytic IgT<sup>+</sup> B cell subset (Fig. 3a). In blood (Fig. 3b) and head kidney (data not shown), the percentage of phagocytic IgT<sup>+</sup> B cells was similar to that of IgM<sup>+</sup> B cells. To further assess the degree of bead internalization as well as the ultrastructural features of phagocytic IgT<sup>+</sup> B cells, we sorted these cells and analyzed them by transmission electron microscopy. As expected, the images showed cells with a lymphocyte-like structure (Fig. 3c) identical to that of sorted IgT<sup>+</sup> B cells (Fig. 2a). We found phagocytic IgT<sup>+</sup> B cells containing about one to seven ingested 1-μm beads (Fig. 3c), and we observed many IgT<sup>+</sup> B cells in the process of internalizing beads through pseudopodia formation (Supplementary Fig. 4). Phagocytosis was inhibited in a dose-dependent manner by cytochalasin B (Fig. 3d), which indicated the involvement of microtubules and microfilaments during the internalization process<sup>14</sup>. In addition to phagocytosing beads, IgT<sup>+</sup> B cells also ingested bacteria (data not shown) and were able to kill them intracellularly, as shown by the time-dependent decrease in the survival rate of ingested bacteria (Fig. 3e).

We next assessed the ability of IgT<sup>+</sup> and IgM<sup>+</sup> B cells to undergo rapid proliferation and to secrete immunoglobulin after being incubated with *Vibrio anguillarum* bacterin or *Escherichia coli* lipopolysaccharide. In the presence of the *V. anguillarum* bacterin, head kidney IgT<sup>+</sup> and IgM<sup>+</sup> B cells were proliferating by day 3, although extensive proliferation occurred by day 7 (about five- to sevenfold more than that of control cells; Fig. 4a). As for the production of IgT and IgM, secretion of both immunoglobulin isotypes was significantly greater in response to treatment with *V. anguillarum* bacterin at day 7 (Fig. 4b,c). The enhanced secretion of IgT and IgM at day 7 was associated with a greater percentage of large IgT<sup>+</sup> and IgM<sup>+</sup> cells in the bacterin-exposed leukocytes (Fig. 4d). The greater size of IgT<sup>+</sup> and IgM<sup>+</sup> cells was shown by their greater forward scatter than that of most IgT<sup>+</sup> and IgM<sup>+</sup> B cells from untreated control samples (Fig. 4d). In support of the aforementioned correlation between the increase in immunoglobulin production and greater size of IgT<sup>+</sup> and IgM<sup>+</sup> cells at day 7, we found that sorted large IgT<sup>+</sup> and IgM<sup>+</sup> cells had a significantly greater capacity to produce IgT and IgM, respectively, than did small IgT<sup>+</sup> and IgM<sup>+</sup> B cells (Fig. 4e). Treatment of cells with *E. coli* lipopolysaccharide induced responses similar to those obtained by treatment with *V. anguillarum* bacterin (data not shown). Thus, it seems that both IgM<sup>+</sup> and IgT<sup>+</sup> B cells have similar phagocytic abilities as well as very similar capacities to proliferate and secrete immunoglobulin in response to microbial stimulation.

### IgT responses to a gut parasite restricted to trout GALT

The observation that IgT and IgT<sup>+</sup> B cells were prevalent in the gut (Figs. 1g and 2d) led us to hypothesize a role for IgT in mucosal immunity. To assess our hypothesis, we evaluated the immune responses of fish infected with *Ceratomyxa shasta*, a protozoan parasite with considerable tropism for the gut of salmonids<sup>15</sup>. In agreement with published findings<sup>15</sup>, we found that fish that survived 3 months after infection had obvious signs of inflammation in

the GALT, as shown by extensive leukocytic accumulation in the gut lamina propria (Supplementary Fig. 5). Immunofluorescence microscopy detected the parasite in the gut of all surviving fish analyzed and showed that it was mostly confined to the gut lumen before being excreted in the feces (Fig. 5a–c and d, right). However, there were a few examples in which the parasite was present in the gut epithelium, near the lamina propria (Fig. 5d, left). Notably, immunofluorescence images showed considerable accumulation of IgT<sup>+</sup> B cells in the lamina propria of fish that survived (Fig. 5b). In contrast to uninfected control fish (Fig. 5a), the fish that survived infection had a substantial number of IgT<sup>+</sup> B cells that also permeated the gut epithelium (Fig. 5c, right). In some cases, IgT<sup>+</sup> B cells seemed to be secreting IgT (Fig. 5d). Cell counts of the stained sections showed that surviving fish had ~4.8-fold more IgT<sup>+</sup> cells than did control fish (Fig. 6a). We detected the substantial accumulation of IgT<sup>+</sup> cells in the gut concomitantly with considerably increased (~51-fold) gut mucus IgT (Fig. 6b). In contrast, the number of IgM<sup>+</sup> B cells (Fig. 6a) and the concentration of IgM (Fig. 6b) did not change in the surviving fish. In agreement with the observed greater abundance of IgT at the protein level, we also found considerable upregulation (~733-fold) of expression of the gene encoding IgT in the gut of these fish, in contrast to almost negligible upregulation of IgM expression (Fig. 6c).

Notably, the surviving fish had significant titers of parasite-specific IgM in the serum (~1,264), whereas we did not detect IgM titers in the mucus of most fish (Fig. 6d,e). Conversely, we detected titers of parasite-specific IgT only in the gut mucus (~160) but not in the serum (Fig. 6d,e). These data support the idea that IgT is an immuno-globulin specialized in mucosal immunity.

### A large fraction of gut bacteria coated with IgT

An important property of IgA in the gut of humans is its ability to recognize and coat a large percentage of luminal bacteria<sup>5,16</sup>. This IgA coat is thought to prevent luminal bacteria from attaching and invading the gut epithelium<sup>5,17</sup>. To further support the idea that IgT is involved in gut homeostasis, we tested the hypothesis that like human IgA, IgT coats gut luminal bacteria. For this, we adapted to our fish system a methodology used to assess immunoglobulin coating of human gut luminal bacteria<sup>16</sup>. Flow cytometry showed that most trout gut luminal bacteria stained for IgT (~48%), whereas a smaller proportion (~24%) was positive for IgM (Fig. 7a–c). Immunofluorescence microscopy substantiated the results obtained by flow cytometry, showing a predominance of bacteria with varying degrees of IgT staining (Fig. 7d–g and Supplementary Fig. 6a–d; isotype-matched control antibody, Supplementary Fig. 6e–h). A smaller portion of the bacteria was stained only for IgM (Supplementary Fig. 6c,d), whereas some bacteria were stained for both IgT and IgM (Fig. 7g). Immunoblot analysis confirmed further the presence of IgT and IgM on these bacteria (Fig. 7h).

### Polymeric immunoglobulin receptor in rainbow trout

In mammals, the transport of polymeric IgA from the mucosal epithelium into the gut lumen is mediated by the polymeric immunoglobulin receptor (pIgR)<sup>18,19</sup>. To understand the basis by which IgT and IgM could be found in the gut mucus, we identified and cloned a rainbow trout pIgR-like molecule (Supplementary Fig. 7; primers, Supplementary Table 1) with a very high degree of sequence similarity and phylogenetic relationship to cloned pIgRs of teleost fish and tetrapods (Fig. 8a and Supplementary Fig. 8). Similar to other teleost pIgRs<sup>20,21</sup>, the trout pIgR was a type I transmembrane protein consisting of a ligand-binding extracellular region with two immuno-globulin-like domains (Supplementary Fig. 8). Comparison with mammalian pIgR sequences showed that the two trout pIgR immuno-globulin domains corresponded to mammalian pIgR domain 1 (at the amino terminus of trout pIgR) and domain 5. We raised antibodies to recombinantly produced domain 5 of



trout pIgR (Supplementary Fig. 9) that specifically recognized trout pIgR (Fig. 8b,c). Hence, the antibody to trout pIgR stained only positively transfected Madin-Darby canine kidney (MDCK) cells expressing Flag-tagged trout pIgR (Fig. 8b). In addition, by immunoblot analysis, this antibody reacted with the same protein detected by the Flag-specific mAb in lysates of cells transfected with trout pIgR (Fig. 8c). The secretory component portion of mammalian pIgR is known to be associated with secretory luminal IgA<sup>18</sup>. Using the trout pIgR-specific antibody we detected a trout secretory component-like molecule (tSC) only in the gut mucus, not in the serum (Fig. 8c, left). The molecular mass of the immuno-reactive bands (~38 kDa) was near the theoretical mass obtained from the sequence of trout pIgR, with exclusion of its transmembrane and cytoplasmic domains. To determine whether tSC was associated with IgT and IgM of the gut mucus, we did coimmunoprecipitation studies with IgT- or IgM-specific antibodies. Our results showed that anti-IgT and anti-IgM were able to coimmunoprecipitate tSC in association with the immunoprecipitated IgT or IgM (Fig. 8d). Thus, rainbow trout contain a pIgR whose putative secretory component is associated only with gut mucus IgT and IgM, whereas these immunoglobulins in serum are free of the secretory component.

## DISCUSSION

Immunoglobulins were discovered over a century ago<sup>3</sup>. The study of immunoglobulins in phylogenetically divergent species has greatly contributed to the understanding of their structure, function and evolutionary origins<sup>3</sup>. The discovery of IgT 5 years ago marked a special moment, as it was proposed that this immunoglobulin might represent the final isotype to be found in vertebrates<sup>11</sup>. So far, its function has remained an enigma. Our studies have identified IgT as the most ancient reported immunoglobulin specialized in mucosal immunity.

To characterize trout IgT, we generated polyclonal and monoclonal antibodies that allowed its detection and purification from trout fluids. In contrast to tetrameric IgM, IgT existed as monomer in serum. Notably, in the gut mucus, IgT was present for the most part as a polymer, a situation analogous to that of human IgA, which is found in polymeric form in the gut mucus and as a monomer in the serum<sup>22</sup>. This is not the case, however, for several other mammalian species, in which IgA is also present in polymeric form in the serum<sup>23</sup>. Although the tetrameric IgM of teleosts is found in various redox forms that differ in the number of monomeric subunits associated through disulfide bonds<sup>12</sup>, we found that all subunits from polymeric mucus IgT were associated by noncovalent interactions, a property that seems unique to polymeric IgT. Although IgM was by far the prevailing isotype in the serum, IgT concentrations in the gut mucus were double those in serum, which suggested a possible role for IgT in gut mucosal immunity. Similarly, IgA is the predominant immunoglobulin isotype in mammalian gut mucosa<sup>22</sup>.

The prevalence of IgT in the gut mucus led us to hypothesize the existence of an IgT receptor involved in its transport from trout GALT into the gut lumen. We found that rainbow trout have a pIgR and that its putative secretory component (tSC) was associated with both IgT and IgM in gut mucus, whereas in serum, tSC was absent. This finding supports the idea that like mammalian secretory IgA and IgM, mucosal trout IgT and IgM need to associate with a pIgR for transport into the gut lumen. These data are in agreement with that of a report on pIgR in fugu, another teleost fish, in which a fragment of the pIgR is associated with IgM<sup>20</sup>. However, we were not able to identify a J chain-like molecule in trout or other teleost fish. This apparent lack of a J chain combined with the absence of disulfide bridges holding together the monomers of polymeric IgT are the likely reasons polymeric IgT migrated like a monomer in SDS-PAGE under nonreducing conditions. It is worth mentioning that although amphibians have a J chain, this molecule does not associate

with IgX<sup>6</sup>. Thus, it would seem that the J chain is not required for the interaction of pIgR with bony fish immunoglobulins or amphibian IgX.

It was believed that teleost fish have a single B cell lineage characterized by the surface expression of IgM<sup>7</sup>. In that context, teleost B cells have been compared with the mammalian B-1 lineage. However, in contrast to the B cells of mammals, the IgM<sup>+</sup> B cells of teleosts are found in very large numbers in the blood and spleen, where they can constitute up to ~20–55% of all leukocytes<sup>13,24–26</sup>. Here we have demonstrated the existence of a previously unrecognized B cell lineage that expressed only surface IgT, thus indicating the presence of two main B cell lineages in trout: one that expresses surface IgM and another that expresses surface IgT. Thus, our results confirm the predicted and reported exclusive rearrangements, either V<sub>H</sub>D<sub>τ</sub>J<sub>τ</sub>C<sub>τ</sub> or V<sub>H</sub>D<sub>μ</sub>J<sub>μ</sub>C<sub>μ</sub>, in the immunoglobulin heavy-chain locus of rainbow trout<sup>9</sup>, which indicates the existence of two mutually exclusive B cell lineages that express either IgT or IgM. Given this genomic configuration of the trout immunoglobulin heavy-chain locus, only one immunoglobulin isotype can be expressed at a time in a single B cell. Hence, it would seem that teleosts use a different strategy for generating B cell isotypic diversity than do tetrapods. In addition to the unique IgT<sup>+</sup> B cell population described here, it seems that catfish have an IgD<sup>+</sup>IgM<sup>−</sup> B cell subset<sup>27</sup>, which suggests the existence of at least three different B cell subsets in teleosts.

With the goal of elucidating potential new roles of IgT in immunity, we initially studied the involvement of the IgT<sup>+</sup> B cell subset in innate immunity. Here we have demonstrated that IgT<sup>+</sup> B cells have potent phagocytic and bactericidal abilities similar to those of the IgM<sup>+</sup> B lineage<sup>13</sup>. It has been suggested that the B cell subset that produces IgT could be the equivalent of mammalian B-1 cell lineage<sup>10</sup>. Here we have shown that the capacity with which both IgM<sup>+</sup> and IgT<sup>+</sup> B cells proliferated and secreted immunoglobulin in response to microbial stimulation was essentially very similar. Thus, it seems that both trout B cell lineages have important myeloid traits as well as innate features reminiscent of mammalian B-1 cells. However, an important difference between these two B cell lineages is the prevalence of IgT<sup>+</sup> B cells relative to IgM<sup>+</sup> B cells in the gut, which led us to hypothesize the involvement of IgT in intestinal immunity. In support of our hypothesis, we found that the GALT of fish that survived infection with *C. shasta* (a gut parasite) had more IgT<sup>+</sup> B cells, whereas IgM<sup>+</sup> B cell numbers were not greater than those of control fish. Whether this accumulation of IgT<sup>+</sup> B cells was the result of infiltration of cells into the infected area or was the product of local B cell proliferation will require further investigation. Aggregations of IgA<sup>+</sup> B cells in the gut of mammals are observed mainly in secondary lymphoid follicles, including Peyer's patches and mesenteric lymph nodes<sup>5,19</sup>. The histological features of the trout GALT that contained the groups of IgT<sup>+</sup> B cells in the surviving fish did not resemble those of mammalian Peyer's patches or mesenteric lymph nodes, which reinforces the idea that teleost fish lack similar lymphoid follicles in their GALT<sup>8,28,29</sup>. This indicates that IgT production in the GALT of trout occurs through extrafollicular pathways. It has become apparent that IgA responses in mammals can also be generated independently of Peyer's patches and mesenteric lymph nodes, although the pathways involved in such responses are not well understood<sup>5</sup>. Thus, it is possible that the extrafollicular pathways of IgA production may be reminiscent of those involved in IgT synthesis in teleost GALT. Hence, future studies of the routes involved in the generation of intestinal IgT in fish will probably aid in the analysis of extrafollicular production of intestinal IgA in mammals.

IgM responses in teleost plasma have been studied extensively and are typically characterized as having high titers in response to infection or vaccination<sup>7,30–32</sup>. However, very few reports exist on teleost IgM responses in the gut, and although results in this area are conflicting, reported gut IgM titers have typically been very low<sup>33</sup>. Here we found that IgM-specific responses to the parasite were present only in serum, whereas parasite-specific

IgT titers were confined to gut mucus. These findings demonstrate compartmentalization of the responses of immunoglobulin isotypes into mucosal and systemic areas in response to pathogenic challenge in a cold-blooded vertebrate. Similar IgA-mediated responses to some protozoan parasites in the gut of mammals have been described. For example, IgA<sup>+</sup> B cells represent 95% of all B cells that accumulate in the large intestines of mice infected with the parasite *Eimeria falciformis*<sup>34</sup>. This parasite is also known to induce higher intestinal IgA titers<sup>35</sup>.

One of the hallmarks of IgA in the gut of humans is its ability to coat a large percentage of luminal bacteria. This IgA coating has a key role in immune exclusion at mucosal surfaces<sup>5,17</sup>; thus, the IgA-coated bacteria are prevented from attaching to and invading the gut epithelium<sup>5,17</sup>. Similar to mammalian lumens, the lumens of fish contain high densities of bacteria<sup>36</sup>. We found that the percentage of bacteria coated with IgT was double that of bacteria coated with IgM. These data suggest that IgT and, to a lesser degree, IgM also have a role in immune exclusion. The prevalence of IgT coating in gut bacteria supports further the idea that IgT is a mucosal immunoglobulin. It is likely that IgT and IgM responses to gut luminal bacteria are T cell independent. Future studies of fish that have undergone thymectomy may shed light on the aforementioned hypothesis. In amphibians, both IgM and IgX (but not IgY) are expressed in thymectomized animals<sup>6</sup>, which indicates that these immunoglobulins can be produced in a T cell-independent manner. Therefore, by analogy, IgT and IgM might be present in fish that have undergone thymectomy, although antigen-specific IgT and IgM responses would probably be impaired.

In conclusion, our study has demonstrated unique aspects of the structure and function of IgT that collectively indicate specialization of this ancient immunoglobulin in mucosal immunity. Thus, our findings challenge the present paradigm that specialization of immunoglobulin isotypes into mucosal and systemic responses arose during tetrapod evolution. Notably, although amphibians lack IgA, they contain IgX, an isotype that is unrelated to IgT or IgA yet is expressed mainly in the gut. We thus propose that the specialization of immunoglobulin isotypes into mucosal and systemic compartments is a canonical feature of all jawed vertebrate immune systems required for the maintenance of immune homeostasis in two main body compartments with very different immune needs. As IgT, IgX and IgA are phylogenetically distant, their specialization into mucosal compartments must have occurred independently by a process of convergent evolution driven by similar selective pressures operating on the gut mucosa of teleosts, amphibians and mammals. More particularly, as the gut lumen is rich both in bacterial products derived from commensal bacteria (such as lipopolysaccharide and DNA), as well as food antigens, it is believed that a critical aspect in preserving its mucosal homeostasis relies on mechanisms directed at avoiding or inhibiting acute and chronic inflammatory responses<sup>37</sup>. In this context, secretory IgA is known to be an anti-inflammatory immunoglobulin<sup>22,38</sup>, in contrast to the known proinflammatory abilities of IgM and IgG.

Thus, we predict that a universal property of any intestinal immuno-globulin would be its inability to trigger inflammation in the gut. Consequently, we foresee that mucosal IgT and IgX probably have anti-inflammatory properties, a hypothesis that needs to be addressed in future studies. Finally, we speculate that all classes of vertebrates must have evolved an anti-inflammatory immunoglobulin class or subclass specialized to work in gut mucosal areas. Future comparative studies of mucosal immunoglobulins of species from various phyla will shed light on this hypothesis.



## METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

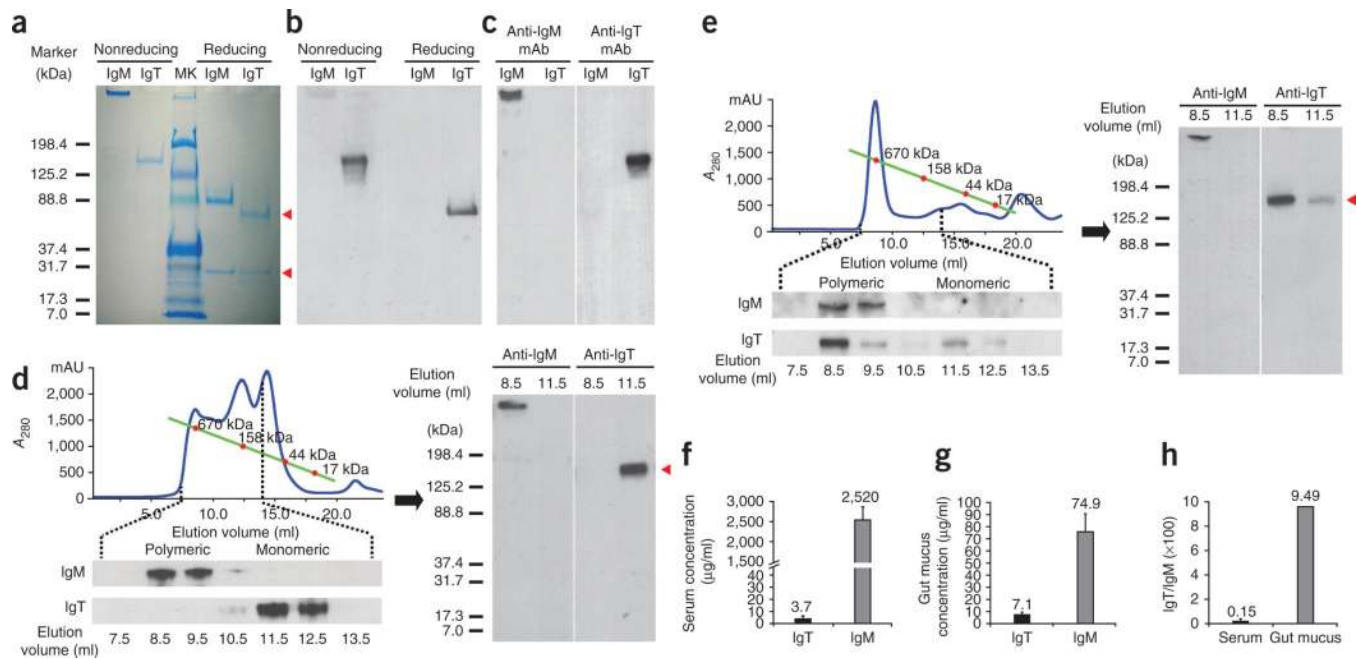
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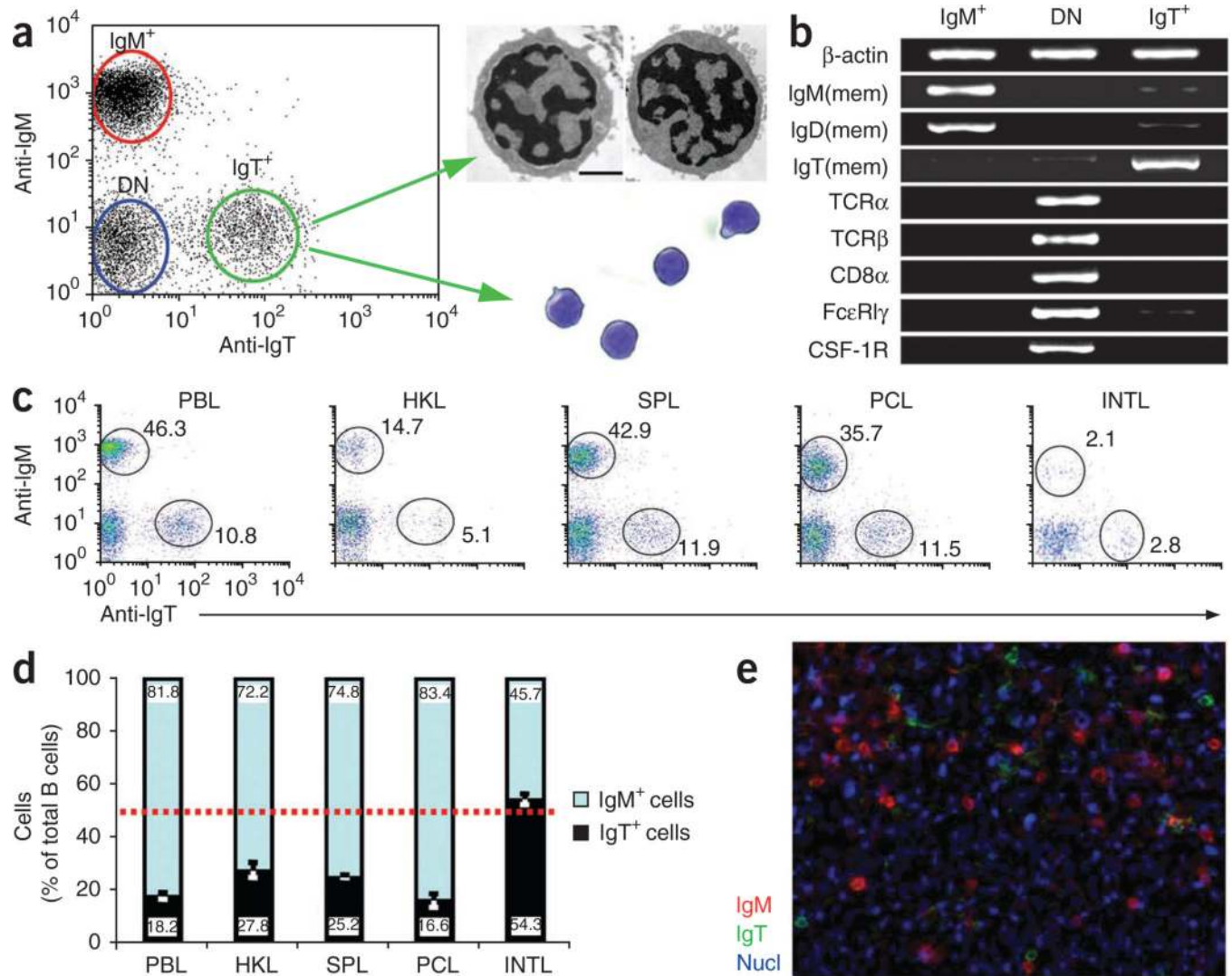
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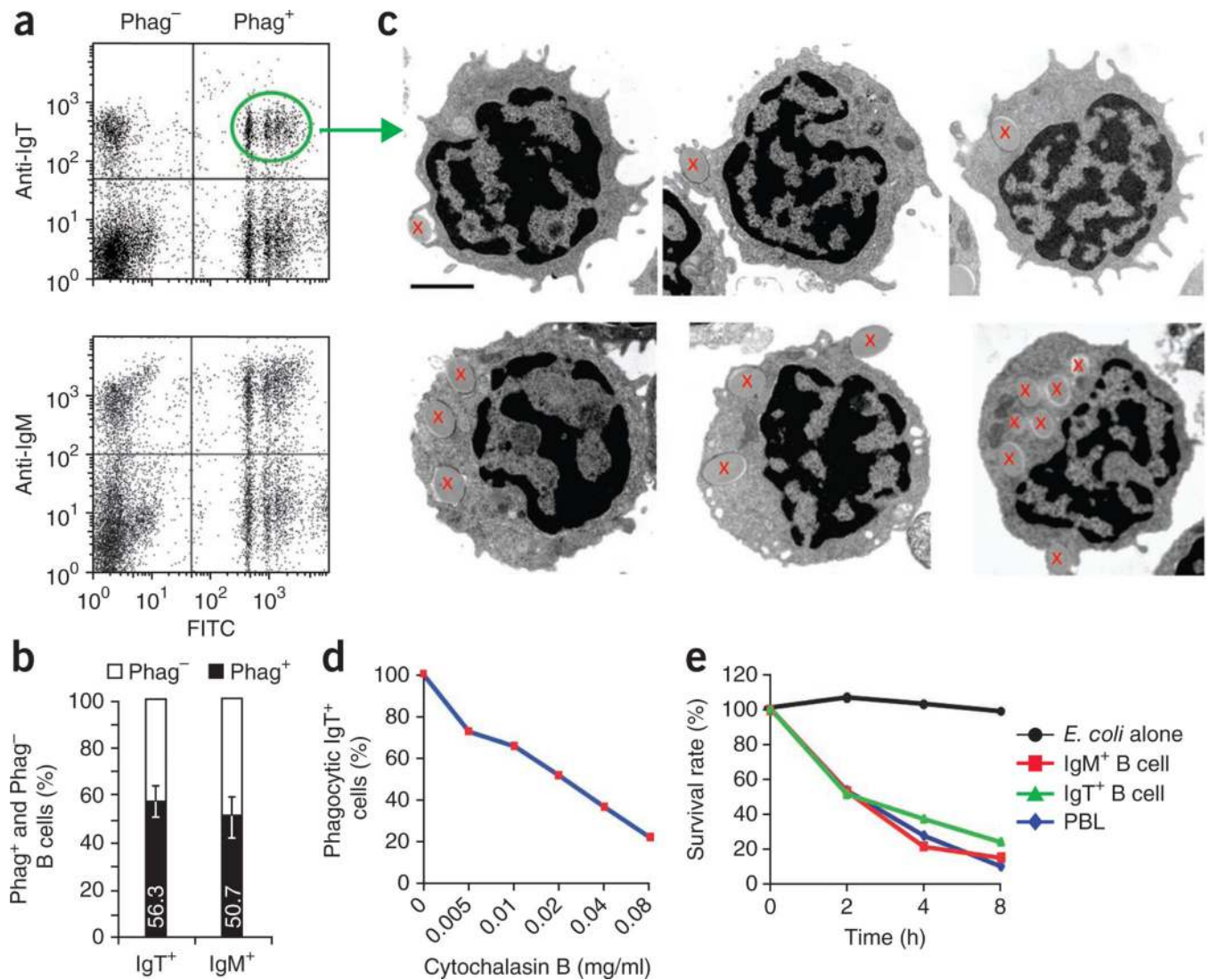
**Figure 1.**

Purification and structural characterization of IgT. **(a)** Coomassie blue staining of purified serum IgM and IgT (2 μg) resolved by SDS-PAGE. Red arrowheads indicate heavy and light chains of IgT. Left margin, molecular size in kilodaltons (kDa). **(b,c)** Immunodetection of IgM and IgT (~0.2 μg) with a polyclonal antibody **(b)** and mAb **(c, right)** to trout IgT or an IgM-specific mAb **(c, left)**. **(d,e)** Fractionation of serum (0.5 ml; **d**) or gut mucus (0.5 ml; **e**) by gel filtration (top left), followed by immunoblot analysis of the fractions with IgM- and IgT-specific mAbs (below). Right, 4–15% SDS-PAGE of gel-filtration fractions corresponding to elution volumes of 8.5 ml and 11.5 ml under nonreducing conditions, followed by immunoblot analysis with mAb to trout IgM or IgT. Red arrowheads indicate monomers.  $A_{280}$ , absorbance at 280 nm. **(f,g)** Immunoblot and densitometric analysis of the concentration of IgM and IgT in serum **(f)** and gut mucus **(g)**;  $n = 10$ –15 fish. **(h)** Ratio of IgT to IgM in serum and gut mucus, calculated from the values in **f** and **g**. Numbers above bars **(f–h)** indicate mean. Data are representative of at least three independent experiments (mean and s.e.m. in **f,g**).

**Figure 2.**

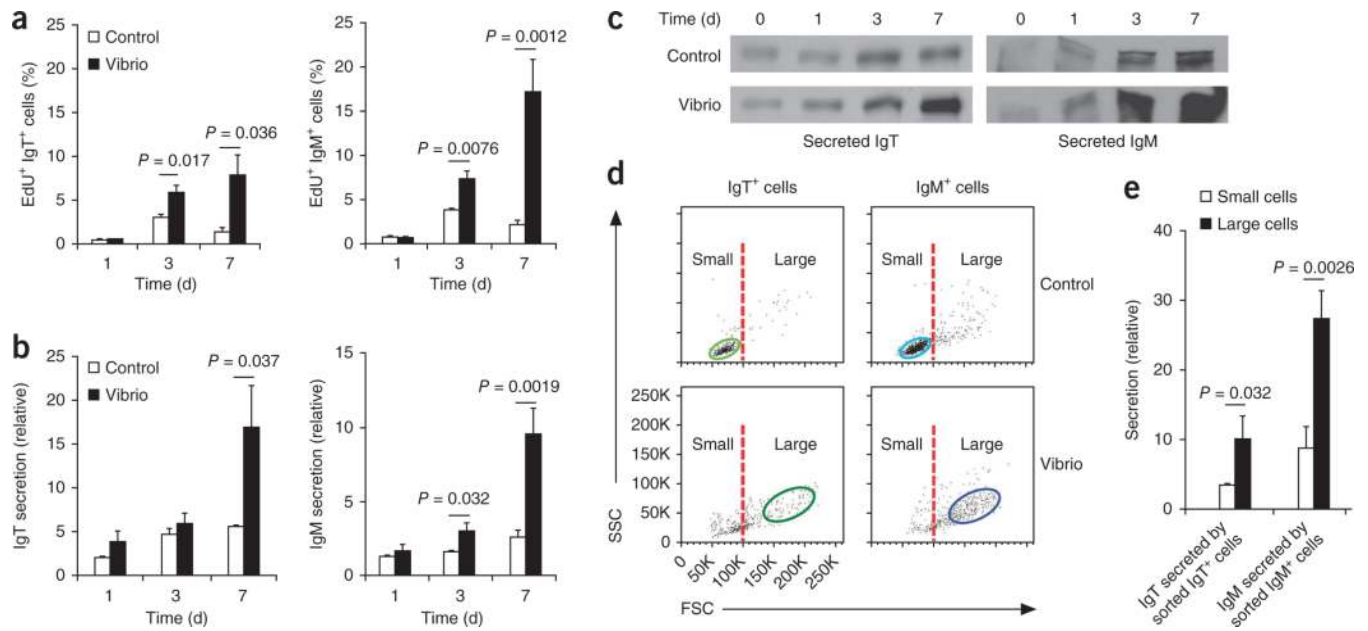
Identification of a previously unknown B cell lineage that expresses only surface IgT. **(a)** Flow cytometry (left) of blood leukocytes double-stained with IgM- and IgT-specific mAbs ( $n = 5$  fish), and transmission electron microscopy (top right) and Giemsa staining (bottom right) of sorted IgT<sup>+</sup> B cells. Scale bar, 2  $\mu$ m (top right); original magnification (bottom right),  $\times 40$ . **(b)** Gene-expression profiles of sorted IgM<sup>+</sup> cells, IgM<sup>-</sup> IgT<sup>-</sup> double-negative cells (DN), and IgT<sup>+</sup> cells among blood leukocytes ( $n = 5$  fish).  $\beta$ -actin, loading control; IgM(mem), IgD(mem) and IgT(mem), membrane forms of IgM, IgD and gT heavy chains; TCR $\alpha$  and TCR $\beta$ , T cell antigen receptor  $\alpha$ - and  $\beta$ -chains; CSF-1R, receptor for colony-stimulating factor 1. **(c)** Flow cytometry of leukocytes from peripheral blood (PBL), head kidney (HKL), spleen (SPL), peritoneal cavity (PCL) and intestine (INTL). Numbers adjacent to outlined areas indicate percent IgM<sup>+</sup> cells (top left) or IgT<sup>+</sup> cells (bottom right). **(d)** Frequency of IgM<sup>+</sup> and IgT<sup>+</sup> cells among total B cells ( $n = 15$  fish). Red dashed line indicates 50%. **(e)** Immunofluorescence staining for IgM (red) and IgT (green) in a cryosection of rainbow trout spleen; nuclei (Nucl; blue) are stained with the DNA-intercalating dye DAPI (isotype-matched control antibodies, Supplementary Fig. 3a). Original magnification,  $\times 20$ . Data are representative of at least three independent experiments (mean and s.e.m. in **d**).





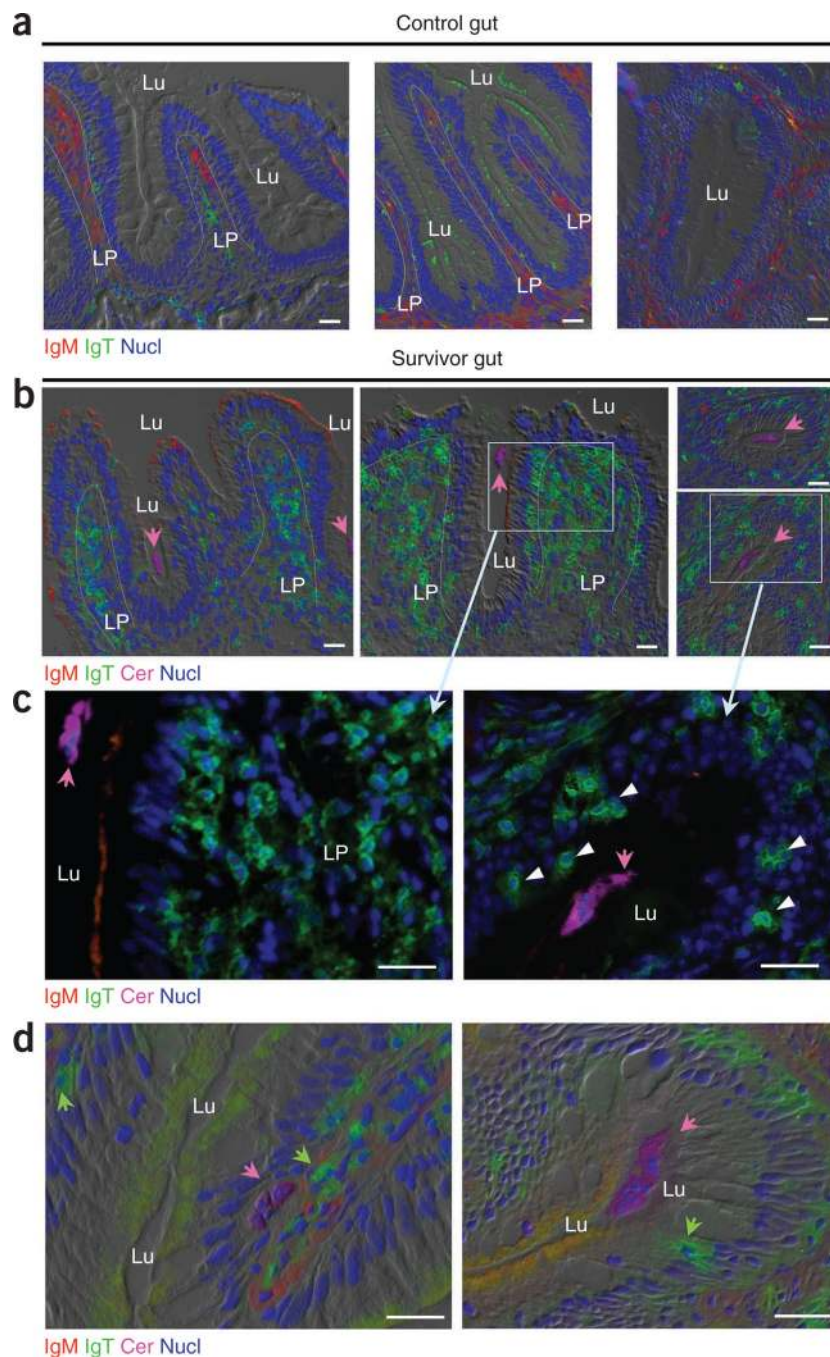
**Figure 3.**

Phagocytic and intracellular killing capacities of IgT<sup>+</sup> B cells. **(a)** Flow cytometry of peripheral blood leukocytes incubated with 1- $\mu$ m fluorescent latex beads (labeled with fluorescein isothiocyanate (FITC)) and then stained with mAb to trout IgT or IgM ( $n = 9$  fish). Phag<sup>-</sup>, nonphagocytic; Phag<sup>+</sup>, phagocytic. **(b)** Phagocytic and nonphagocytic cells in IgT<sup>+</sup> or IgM<sup>+</sup> B cell subsets of peripheral blood leukocytes ( $n = 9$  fish). Numbers in bars indicate mean percent phagocytic cells. **(c)** Transmission electron microscopy of various stages of ingestion of 1- $\mu$ m beads (red 'x') by phagocytic IgT<sup>+</sup> B cells from peripheral blood leukocytes. Scale bar, 2  $\mu$ m. **(d)** Inhibitory effect of cytochalasin B on the phagocytic capacity of IgT<sup>+</sup> B cells, presented as the percentage of phagocytic cells relative to that of PBS-treated control cells. **(e)** Intracellular bacterial killing by sorted IgM<sup>+</sup> and IgT<sup>+</sup> B cells and total peripheral blood leukocytes incubated with live *E. coli* and lysed; lysates were inoculated onto Luria-Bertani agar plates and surviving intracellular bacteria were counted. Results are presented as percent of live bacteria at time 0, set as 100%. Data are representative of at least three independent experiments (mean and s.e.m. in **b**).



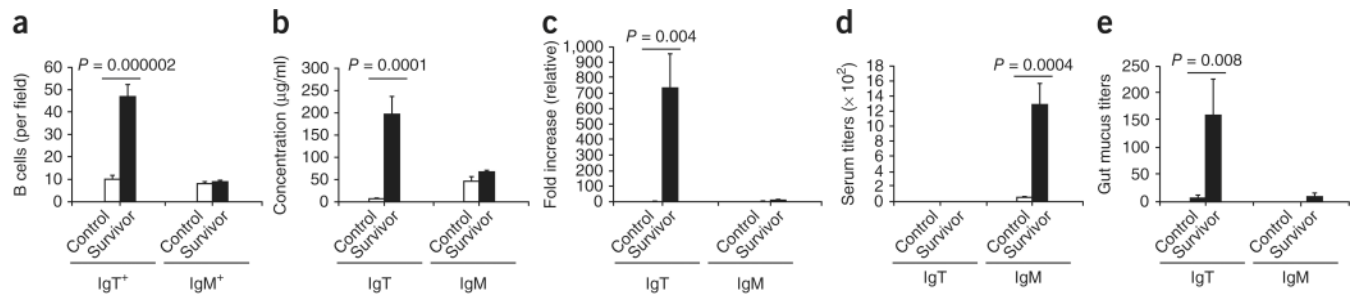
**Figure 4.**

Proliferative and immunoglobulin-secreting capacities of IgT<sup>+</sup> and IgM<sup>+</sup> B cells in response to microbial stimulation. **(a)** Proliferation of IgT<sup>+</sup> and IgM<sup>+</sup> B cells among trout head kidney leukocytes left unstimulated (Control) or stimulated for 1, 3 or 7 d with *V. anguillarum* bacterin (Vibrio), presented as frequency of cells positive for the thymidine analog EdU (5-ethynyl-2'-deoxyuridine) in the IgT<sup>+</sup> or IgM<sup>+</sup> B cell subset ( $n = 10-12$  fish). **(b)** IgM and IgT in supernatants of cells treated as in **a**, assessed by immunoblot and densitometric analysis and presented relative to values on day 0 ( $n = 6$  fish). **(c)** Immunoblot analysis of supernatants of cells treated as in **a**, probed with IgT- and IgM-specific antibodies ( $n = 6$  fish). **(d)** Flow cytometry of IgT<sup>+</sup> and IgM<sup>+</sup> head kidney leukocytes on day 7 in the presence or absence of *V. anguillarum* bacterin; circles outline the predominant small or large B cell populations. FSC, forward scatter; SSC, side scatter. **(e)** IgM and IgT in supernatants of head kidney leukocytes collected 7 d after intraperitoneal injection of a mixture of lipopolysaccharide and *V. anguillarum* bacterin ( $n = 6$  fish), sorted into large and small IgT<sup>+</sup> and IgM<sup>+</sup> cells and cultured for 2 d, assessed by immunoblot and densitometric analysis and presented relative to values at day 0.  $P$  values, unpaired Student's  $t$ -test. Data are representative of three independent experiments (mean and s.e.m. in **a,b,e**).



**Figure 5.** Accumulation of IgT<sup>+</sup> B cells in the GALT of fish that survived infection with *C. shasta*. Differential interference contrast images of immunofluorescence staining of trout gut cryosections from uninfected control fish (**a**) and fish that survived infection with *C. shasta* (**b–d**;  $n = 5$  per group), stained for IgM (red), IgT (green) and *C. shasta* (Cer; magenta); nuclei are stained with DAPI (isotype-matched control antibody staining, Supplementary Fig. 3b). Blue dotted lines in **a,b** outline the border of the lamina propria (LP); magenta arrows point to the parasite located in the gut lumen (Lu). (**c**) Enlarged images of the areas outlined in **b**, without differential interference contrast, showing infiltrating IgT<sup>+</sup> B cells in

the gut lamina propria (left) or epithelium (white arrowheads, right). **(d)** Localization of a parasite in the gut epithelium (left) or lumen (right); green arrows indicate IgT<sup>+</sup> B cells that seem to be secreting IgT. Scale bars, 20  $\mu$ m. Data are representative of at least three independent experiments.

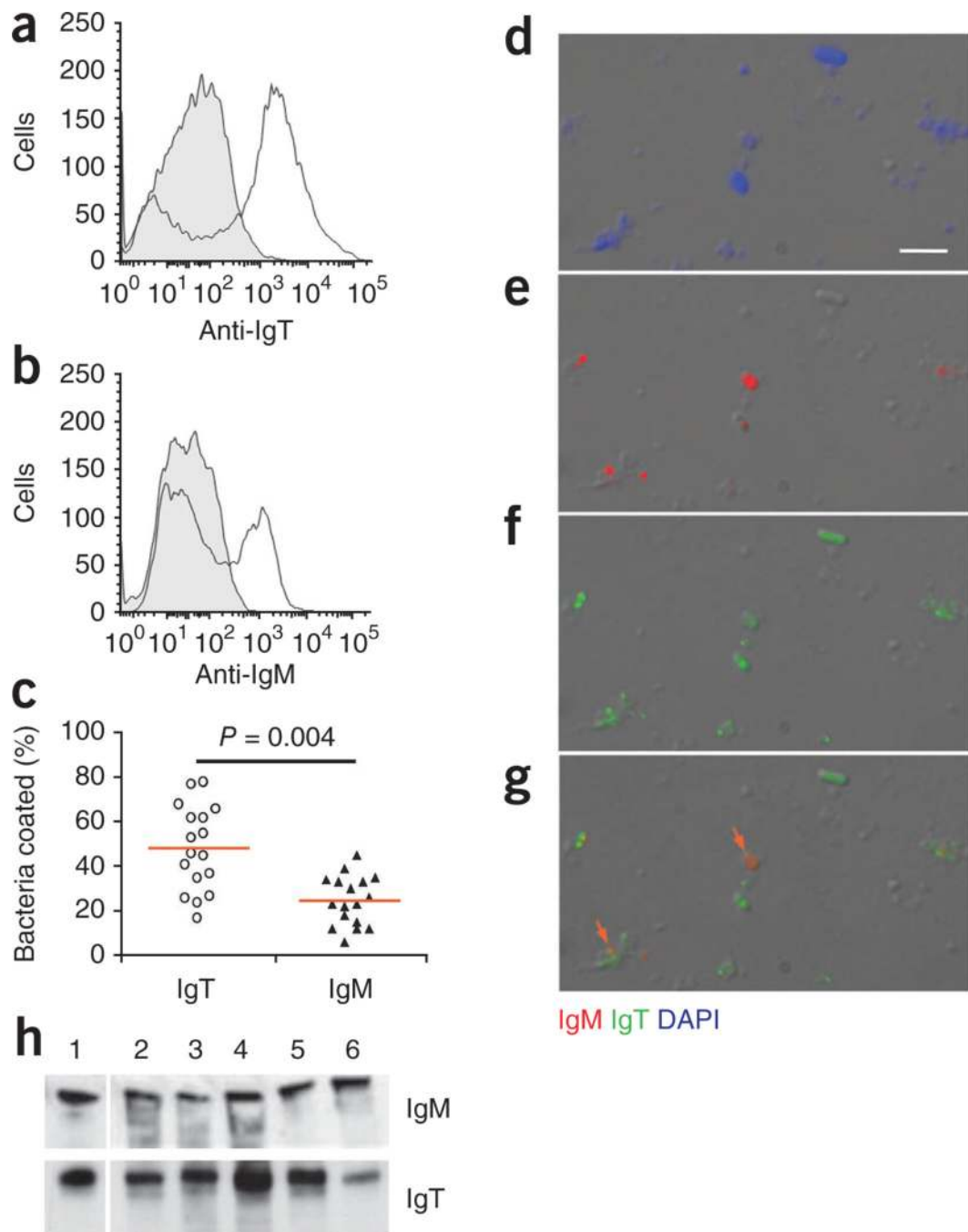


**Figure 6.**

Immune responses in the gut of trout infected with *C. shasta* are mediated by the IgT system.

(a) IgT<sup>+</sup> and IgM<sup>+</sup> B cells in gut cryosections of uninfected control fish and fish that survived infection with *C. shasta* ( $n = 5$  per group), counted in 25 fields (original magnification, ×20). (b) Gut mucus IgT and IgM ( $n = 9$ –12 fish per group). (c) Real-time PCR analysis of IgT and IgM transcripts from the gut, presented relative to expression in control fish, set as 1 ( $n = 7$  fish per group). (d,e) ELISA of specific anti-*C. shasta* IgT and IgM in the serum (d) and gut mucus (e), presented as end-point titers ( $n = 9$  fish per group).  $P$  values, unpaired Student's  $t$ -test. Data are representative of three independent experiments (mean and s.e.m.).

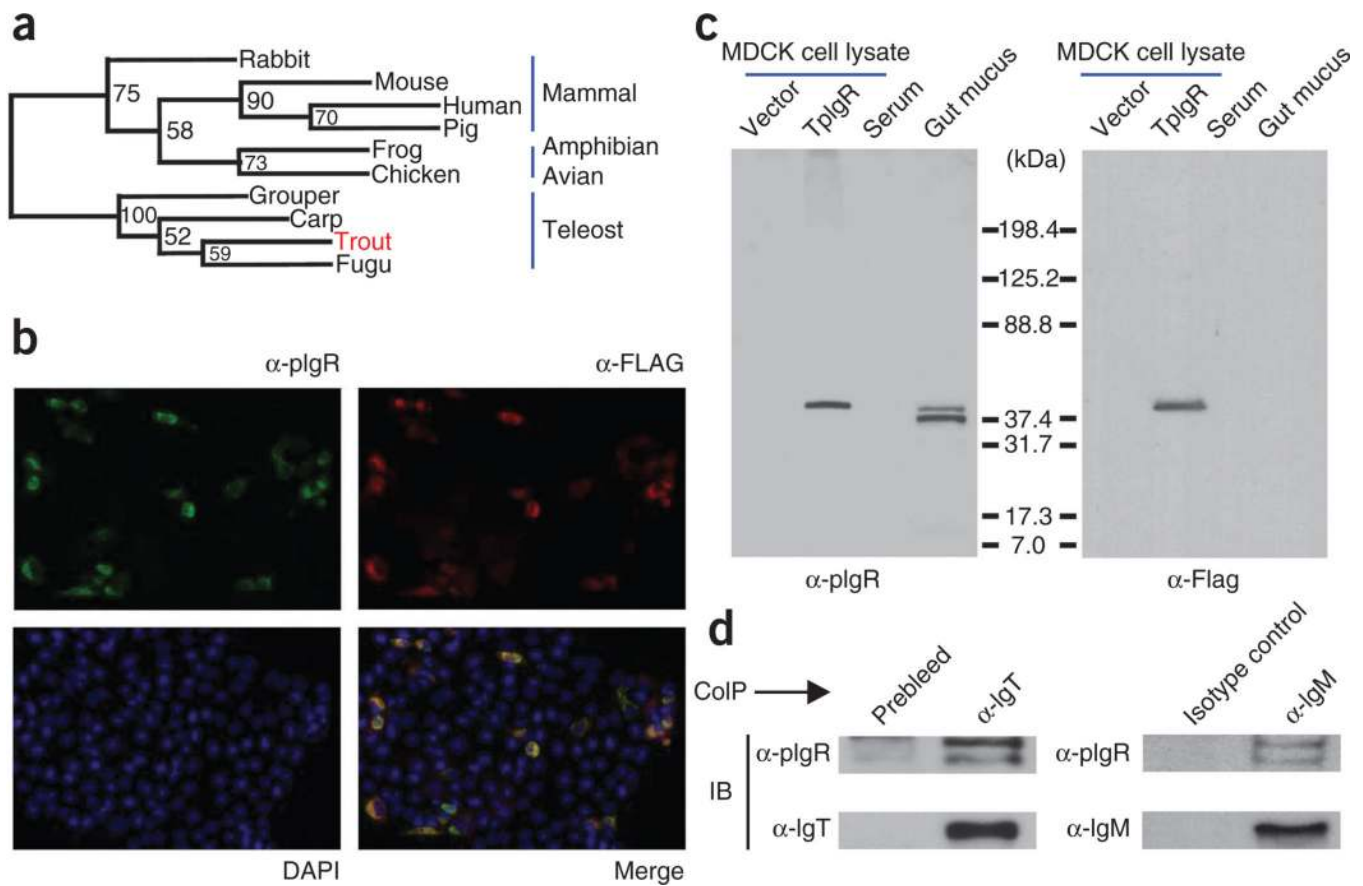




**Figure 7.**

Most trout gut luminal bacteria are predominantly coated with IgT. (**a,b**) Staining of propidium iodide-positive trout gut lumen bacteria ( $3 \times 10^4$ ) with anti-IgT (**a**; solid lines) or anti-IgM (**b**; solid lines) or their respective isotype-matched control antibodies (shaded histograms). (**c**) Coating of bacteria with IgT or IgM. Each symbol represents an individual fish ( $n = 17$ ); red horizontal lines indicate the median.  $P$  value, nonparametric Mann-Whitney test. (**d-g**) Differential interference contrast images of luminal bacteria stained with a DAPI-Hoeschst solution (blue; **d**), anti-IgM (red; **e**) or anti-IgT (green; **f**), or merged IgT and IgM staining (orange; **g**). Isotype-matched control antibody staining, Supplementary

Figure 5f,g. Orange arrows indicate bacteria stained for both IgT and IgM. Scale bar, 5  $\mu\text{m}$ .  
**(h)** Immunoblot analysis of IgT and IgM on luminal bacteria: lane 1, 0.2  $\mu\text{g}$  purified IgM or IgT; lanes 2–6, luminal bacteria ( $n = 5$  fish). Data are representative of at least five independent experiments.

**Figure 8.**

Gut mucus IgT and IgM associate with a trout pIgR. **(a)** Phylogenetic analysis of trout pIgR, constructed on the basis of the amino acid sequences of domain 5 from all pIgR sequences used. Numbers in diagram (percent) represent 1,000 bootstrap replications. GenBank accession numbers: human (*Homo sapiens*), NM\_002644; mouse (*Mus musculus*), NM\_011082; rabbit (*Oryctolagus cuniculus*), NM\_001171045; pig (*Sus scrofa*), NM\_214159; chicken (*Gallus gallus*) NM\_001044644; African clawed frog (*Xenopus laevis*), EF079076; rainbow trout (*Oncorhynchus mykiss*), FJ940682; fugu (*Takifugu rubripes*), AB176853; common carp (*Cyprinus carpio*), GU338410; and orange-spotted grouper (*Epinephelus coioides*), FJ803367. **(b)** Immuno-fluorescence microscopy of trout pIgR expressed on MDCK cells transfected with a plasmid encoding Flag-tagged trout pIgR and stained with rabbit antibody to trout pIgR ( $\alpha$ -pIgR; green), mouse anti-Flag ( $\alpha$ -Flag; red) and DAPI (blue). Original magnification,  $\times 20$ . **(c)** Immunoblot analysis of trout pIgR in cell lysates (5  $\mu$ g) from MDCK cells transfected with empty plasmid (Vector) or plasmid encoding Flag-tagged trout pIgR (TplgR) and in serum ( $\sim 2$   $\mu$ g) and gut mucus ( $\sim 2$   $\mu$ g), separated by SDS-PAGE under reducing conditions. **(d)** Coimmunoprecipitation (CoIP) of tSC from gut mucus with rabbit anti-IgT or mouse anti-IgM, followed by immunoblot analysis (IB) under reducing conditions (tSC detection) or nonreducing conditions (IgT and IgM detection) with anti-pIgR, anti-IgT or anti-IgM; IgG purified from serum obtained from rabbits before immunization (Prebleed) and mouse IgG1 (Isotype control) serve as negative controls for rabbit anti-IgT and mouse anti-IgM, respectively. Data are representative of at least three independent experiments.