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B Cell Heterogeneity in the Teleost Kidney: Evidence for a Maturation Gradient from Anterior to Posterior Kidney[1]

Patty Zwollo,[2]* Suzanne Cole,* Erin Bromage,† and Stephen Kaattari†

The fish immune system is quite different from the mammalian system because the anterior kidney forms the main site for hematopoiesis in this species. Using transcription factor-specific Abs derived from the murine system, together with anti-trout Ig Abs and Percoll gradient separation, we analyzed B cells from trout kidney sections and compared them to those from spleen and blood. For this study, immune cells were separated by Percoll gradients, and the resulting subpopulations were defined based on expression of B cell-specific transcription factors Pax-5 and B lymphocyte-induced maturation protein-1, as well as proliferative and Ig-secreting properties. Comparison of kidney, blood, and spleen B cell subsets suggest that 1) the anterior kidney contains mostly proliferating B cell precursors and plasma cells; 2) posterior kidney houses significant populations of (partially) activated B cells and plasmablasts; and 3) trout blood contains resting, non-Ig-secreting cells and lacks plasma cells. After LPS induction of resting B cells in vitro, the kidney and spleen have a high capacity for the generation of plasma cells, whereas the blood has virtually none. Our results indicate that trout B cell subsets are profoundly different among blood, anterior kidney, posterior kidney, and spleen. We hypothesize that developing B cells mature in the anterior side of the kidney and then migrate to sites of activation, either the spleen or the posterior kidney. Lastly, our data support the notion that the trout kidney is a complex, multifunctional immune organ with the potential to support both hemopoiesis as well as humoral immune activation. ***The Journal of Immunology,*** **2005, 174: 6608–6616.**

The immune system of teleosts, including the rainbow trout, is unusual in the sense that it does not possess bone marrow or typical lymph nodes (1, 2). Instead of bone marrow as the primary site for hemopoiesis, teleosts use the anterior kidney (2, 3). The trout kidney is located ventrally across the backbone extending from the base of the cranium (anterior kidney) to the caudal region (posterior kidney) (4), as shown in Fig. 1. The anterior portion of the kidney has no renal function and lacks nephrons; it houses the site for hemopoiesis, which is interdigitated with adrenal-like tissue. The mid- and posterior kidney possess both renal and immune tissues (2, 5). Furthermore, the teleost kidney houses a system of macrophages that concentrate and separate phagocytosed material, the so-called reticuloendothelial system (6). Thus, the trout kidney is a highly unusual, complex organ that houses four structurally and functionally distinct systems, including the hemopoietic, reticuloendothelial, endocrine, and excretory systems (2). Little is known about the level of interactions among these systems, although effects of cortisol levels on immune cell survival have been shown to influence both developing and activated lymphocytes (7–10).

Evidence for B cell development at the anterior kidney is supported by the expression of RAG-1/2 (11, 12), TdT (13), and transcription factor Ikaros (14). Progenitor B cells generated in this site undergo several developmental stages, resulting in (im)mature B cells with membrane Ig on their cell surface (15, 16). As in mammalian bone marrow, anterior kidney also stores Ig-secreting long-lived plasma cells (17, 18). Recently, we have shown that in the absence of immune activation, anterior kidney is the major site for the storage of Ig-secreting cells, presumably through continuous Ab secretion by long-lived plasma cells (18).

Although B cells are also present in the mid- and posterior kidney, it is unclear whether they possess comparable developmental and functional roles as observed in the anterior kidney. Thus, it is unclear whether posterior kidney functions as a primary or secondary immune organ. The spleen is considered to be the main secondary immune organ, because trout possess no lymph nodes (1).

In mammalian species, activation stages for B cells during immune responses are well defined. Resting B cells express high levels of membrane Ig and MHC class II, as well as the B transcriptional master regulator B cell-specific activator protein, also named Pax-5 (19, 20). In the presence of Ags or mitogens, cells enter the activated B cell stage, distinguishable from resting B cells based on the appearance of low levels of secreted Ig (19), increased expression of MHC class II (20), and the induction of protooncogene c-*myc* (21). Once activated, B cells begin to proliferate to become plasmablasts, dividing cells characterized by a decrease in MHC class II and membrane Ig expression (22, 23). During this stage, expression of the transcription factor B lymphocyte-induced maturation protein-1 (Blimp-1)[3] is induced and is expressed through all subsequent stages (24, 25). Blimp-1 is known to down-regulate expression of the *Pax-5* gene (26, 27). Because Pax-5 is known to suppress Ig expression (28, 29), down-regulation of Pax-5 leads to greatly increased Ig expression. Blimp-1 also suppresses c-*myc* expression (25), thereby reducing cell proliferation. At this stage, plasmablasts leave the cell cycle

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[3] Abbreviations used in this paper: Blimp-1, B lymphocyte-induced maturation protein-1; TCM, tissue culture medium; PVDF, polyvinylidene difluoride; RT, room temperature.

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and become plasma cells, characterized by the absence of membrane Ig and MHC class II proteins, high Ig secretion, expression of Blimp-1, and absence of both c-*myc* and Pax-5 (22, 23).

In this study, we explored the location and frequency of mature B cells, plasmablasts, and plasma cells in the trout kidney, spleen, and blood. Due to a paucity of serological reagents for distinguishing trout B cell populations, we began addressing this question, relying upon physical separation of B cell subsets based on cell density, using Percoll gradients. For this study, five linear segments of trout kidney (from anterior to posterior kidney), as well as blood and spleen samples, were analyzed using such gradients, and the resulting "subpopulations" were characterized based on expression of B cell-specific transcription factors Pax-5 and Blimp-1, as well as proliferative and Ig-secreting properties.

Data obtained here show that distinct patterns of mature B, proliferating B, and plasma cell populations exist in anterior and posterior kidney, blood, and spleen of the trout. Furthermore, we show that Percoll profiles acquired from LPS-stimulated mature B cells can reveal important information on the immune state of the rainbow trout.

Materials and Methods

Animals and facilities

Three-year-old rainbow trout (mean weight of 2 kg) were maintained in 300-gallon tanks within a recirculating system, using biologically filtered, dechlorinated, chemically balanced, and UV-treated city water. Fresh water exchange was ~4% per day, with 75% of the volume recirculating through the biofilter per hour. Water temperature was maintained at 10°C, and the photoperiod was adjusted to match seasonal change. Fish were fed dry pellet feed (ASD2; Ziegler Brothers).

Isolation of immune cells

Fresh cells were collected from blood, kidney, or spleen. Kidneys were divided into five sections (K1–K5) based on anatomical location and were analyzed separately, as shown in Fig. 1. K1 corresponded to the most anterior site of the kidney (site of hemopoiesis), residing below the most anterior seven vertebrae (anterior kidney). Each progressive section consisted of the kidney below the next seven posterior vertebrae (anterior kidney). Each progressive section consisted of the kidney below the next seven posterior vertebrae with K5 terminating the posterior kidney. A total of seven immune sites were analyzed: kidney sites K1–K5, PBLs, and spleen.

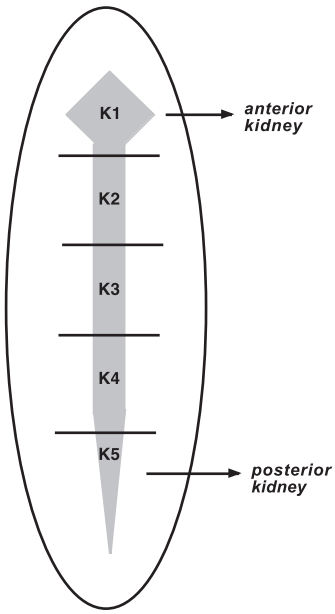

FIGURE 1. Schematic drawing of the trout kidney to indicate the location of segments K1–K5. The kidney was divided into five sections, K1–K5, based on anatomical location. K1 corresponds to the most anterior site of the kidney, residing below the most anterior seven vertebrae (anterior kidney). Each progressive section consists of the kidney below the next seven posterior vertebrae, with K5 terminating the posterior kidney.

Blood was collected in heparinized tubes. Kidney and spleen tissues were collected in 5 ml of sterile HBSS (137 mM NaCl, 5.6 mM D-glucose, 5 mM KCl, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$, and 20 mM HEPES at pH 7.05), and single-cell suspensions were obtained by repeated uptake and release through a 10-ml syringe followed by forcing cells through a 40-nm nylon cell strainer (BD Biosciences). Numbers of immune cells of a specific density at each immune site were counted. Erythrocytes, chromaffin, and steroidogenic cells were not included.

Cell culture and mitogens

Cells were cultured in tissue culture medium (TCM) (30), consisting of RPMI 1640 with 10 mM L-glutamine, 10% FCS, 50 μg/ml gentamicin, 50 μM 2-ME, and the nucleosides adenosine, uracil, cytosine, and guanosine (10 μg/ml; Sigma-Aldrich). B lymphocytes were activated using the B cell mitogen LPS (from *Escherichia coli* 055:B5, pasteurized for 30 min at 70°C in distilled water) at 100 μg/ml.

Percoll gradients

Cell suspensions were washed and resuspended in cold HBSS. Percoll (Pharmacia) was prepared from stock by adding 1/10 of a volume of sterile 10× PBS (19 mM $NaH_2PO_4{\cdot}H_2O$, 81 mM $Na_2HPO_4{\cdot}7H_2O$, 1.37 M NaCl, and 26 mM KCl; pH 7.4) to a density of 1.13 g/ml. Percoll gradients in the range of 50–70% with HBSS as diluent were prepared in 50-ml Falcon tubes with a maximum of five 5-ml layers, according to the manufacturer's instructions. Freshly prepared gradients were allowed to equilibrate for 30 min on ice. Five milliliters of each cell suspension ($\leq 2 \times 10^8$ cells) were layered carefully on top of each gradient, and the tubes were centrifuged for 20 min at 1900 × *g* at 4°C in a clinical centrifuge. Each layer was removed carefully and washed in 40 ml of ice-cold HBSS followed by a 20-ml HBSS wash. Cell samples from each layer were stained with trypan blue to determine cell viability and counted using a hemacytometer.

Antibodies

Two antisera were used as primary Abs: a rabbit anti-mouse Pax-5 polyclonal antiserum (ED-1; Ref. 31) against 148 aa of the paired domain of the murine Pax-5 protein, which also reacts with the human, zebrafish, and *Xenopus* 53-kDa Pax-5 protein (19, 32, 33). The rabbit anti-mouse Blimp-1 polyclonal antiserum was a gift from Dr. M. Davis (Stanford University, Stanford, CA; Ref. 24) and was directed against aa 1–350 of the murine Blimp-1 open reading frame, which does not include the zinc finger region and which also reacts with the human, *Caenorhabditis elegans*, and *Xenopus* 95-kDa Blimp-1 protein (34, 35).

Western blot analysis

Cells were collected in aliquots of 2×10^6 cells, and pellets were either quick-frozen at −80°C or used immediately. Whole-cell protein lysates were prepared by resuspending cells in 40 μl of a sample buffer containing 5% 2-ME, and proteins were separated by size using denaturing 12% SDS-PAGE gels. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore). Membranes were incubated in blocking solution of 5% dry milk in PBS (1.9 mM $NaH_2PO_4{\cdot}H_2O$, 8.1 mM $Na_2HPO_4{\cdot}7H_2O$, 137 mM NaCl, and 2.6 mM KCl; pH 7.4) for 1 h, followed by a 1-h incubation in blocking solution in the presence of primary Ab (1:2000). Three 10-min washes in PBS were then followed by a 1-h incubation with secondary Ab goat anti-rabbit IgG-HRP conjugate (Zymed Laboratories) in blocking solution, and membranes were washed three more times in PBS and developed using a chemiluminescence kit (ECL; Amersham Biosciences).

EMSAs

Nuclear extracts were prepared from fresh, Percoll-purified cells as described elsewhere (31). Procedures for nuclear extract preparation were conducted on ice in a cold room at 6°C. For mobility shift assays, standard binding assays were conducted for 20 min at 30°C in 10- to 15-μl reactions, containing 60 mM KCl, 12 mM HEPES (pH 7.9), 4 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 30 ng of BSA, 12% glycerol, 1 μg of nuclear extract, 2–4 fmol of ^{32}P-labeled DNA probe, and 2 μg of poly(dI:dC) (31). The double-stranded oligonucleotide probes CD19/Pax5 (5′-CAGACACCCATGGTTGAGTGCCCTCCAG-3′) and c-*myc*/Blimp-1 (5′-TTTCGCGTACAGAAAGGGAAAGGACTAGCG-3′) were labeled with [α-^{32}P]dCTP and [α-^{32}P]dATP, respectively, as described previously (31, 19, 25). Sequence of mutant probes were as follows: *CD19*/mutPax5, 5′-CAG ACA CC***G*** ***G***TG GTT ***T***AG TGC CCT CCA G-3′; and

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c-*myc*/mut-Blimp-1, 5′-TTT CGC GTA CAG A***TT*** GGG ***TT***A GGA CTA GCG-3′ (mutations are in bold italics). The ratio of nuclear extract to poly(dI:dC) (in micrograms) was kept constant at 1:2 in all experiments. In Ab or competition EMSAs, nuclear extracts were preincubated in the presence of 1 μl of Ab (1/5 diluted) or with excess unlabeled oligonucleotides without probe for 10 min at 30°C. Products were separated by electrophoresis on 5% nondenaturing polyacrylamide gel in buffer containing 33 mM Tris-HCl, 33 mM boric acid, and 0.74 mM EDTA. Gels were dried and exposed to Eastman Kodak X-OMAT-AR film.

Cell supernatants and ELISA

Cells were plated at 1 million cells/well in TCM using 96-well cell culture plates (Corning Glass) and incubated for 24 h at 18°C under blood gas (10% O_2, 10% CO_2, and 80% N_2). Supernatants were collected and stored until use at −20°C. Wells of a 96-well ELISA plate (Costar) were coated overnight with 100 μl of 2 μg/ml mouse anti-trout Ig mAb 1-14 (Warr's; Ref. 36). Wells were then blocked with 100 μl of 1% BSA in 1% Tween in TBS (TTBS) for 1 h at room temperature (RT). Plates were washed three times with TTBS, and 50 μl of diluted cell supernatants in 1% BSA-TBS were added to wells. Samples were incubated for 1 h at RT followed by three washes in TTBS. Standards of purified trout Ig were included for each plate. Fifty microliters of biotinylated 1-14 mAb (1 μg/ml) in blocking buffer were then added, and samples were incubated for 1 h at RT. Samples were washed three times in TTBS, 50 μl of streptavidin-HRP was added, and samples were incubated for 1 h at RT. After three TTBS washes, 50 μl of substrate (0.04% ABTS citrate buffer; pH 4.0) was added, and OD_{405} were read.

Nonspecific ELISPOT

Assays were done as described previously (18, 37): PVDF membranes were coated with 1-14 mAb at 1 μg/ml, and remaining sites were blocked for 1 h with a solution of 3% casein and 4% sucrose. Cells in RPMI 1640 were added to wells in the range of 10^6, 10^5, and 10^4, and incubated for 16 h at 18°C in the presence of blood gas. Secreted Ab bound on the PVDF filter was then detected using biotinylated 1-14 mAb (1 μg/ml) in combination with streptavidin-HRP (1 μg/ml) and the substrate 3-amino-9-ethylcarbazole (Sigma-Aldrich).

Cellular proliferation assay

Cells were diluted to 2×10^7 cells/ml in TCM. Fifty microliters were aliquoted in quadruplicate and pulsed with 10 μl of tritiated thymidine (100 μCi/ml in TCM). Cells were incubated at 18°C in the presence of blood gas for exactly 24 h and harvested with distilled water onto glass fiber filters with a Skatron cell harvester. The filters were then dried, placed in scintillation vials with mixture, and counted on a Beckman liquid scintillation counter.

RNA isolation and RT-PCR

Total cellular RNA was isolated from Percoll-separated cell fractions from kidney, spleen, or PBLs, using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. cDNA was prepared using the GeneAmp RNA-PCR kit (Applied Biosystems) according to the manufacturer's instructions and using 0.3 μg of RNA. Reaction primers used in PCR analysis included the following: sense primer, tHC-S (5′-CCTTAACCAGC CGAAAGGG-3′; nt 1094–1112 on CH3) and antisense primers, tHCm-AS (5′-CCAACGCCATACAGCAGAG-3′; on exon TM1, nt 1447–1466, specific to the membrane form of Ig; Ref. 38) and tHs-AS-2, (5′-TGAGGTTCTATCAATGGTTCTC-3′; C-terminal exon, specific to secreted Ig, nt 1670–1691; Ref. 38). Each reaction was denatured at 94°C for 1 min, annealed at 60°C for 1 min, and extended at 72°C for 1.5 min. The samples were amplified over 28 cycles. The PCR products were analyzed by gel electrophoresis using 2% agarose gels and quantified using NIH Image analysis software.

Results

Pax-5 and Blimp-1 expression in Percoll-separated lymphocytes

To characterize trout B cell subpopulations, cells from anterior kidney (which is developmentally the most heterogeneous tissue; reviewed in Ref. 16) were physically separated into subpopulations based on their cell density, using Percoll in the range of 50–70%. In humans and mice, 70 and 66% layers typically contain resting B cells, the 62% layer contains partially activated B cells, the 60% layer contains activated B cells and plasmablasts, and the 50% layer contains plasma cells (39). Cells with densities of 55% and lower contain the APCs (see Fig. 2*A*, and Ref. 39).

Trout cells were separated over Percoll gradients of 50, 60, 62, 66, and 70%, and cells in each layer were counted. To test whether the Percoll method was successful in separating B cell subpopulations, we used Western blot analysis. Two antisera were used, each recognizing a specific, differentially expressed, B cell-specific transcription factor, Pax-5 and Blimp-1. These two transcription factors display distinct and partially overlapping expression patterns during B cell differentiation. Because transcription factors typically contain highly conserved domains with high homology between species, they provide excellent developmental markers. The 53-kDa Pax-5 protein is expressed specifically in mature B cells, activated B cells, and plasmablasts of adult vertebrate animals but is absent from plasma cells and non-B cells (19, 32, 33). In mice and humans, Blimp-1 is expressed in activated B cells, plasmablasts, and plasma cells but not in resting mature B cells, B cell precursors, or non-B cells (24, 25).

A rabbit polyclonal antiserum against the paired domain (DNA binding domain) of murine, B cell-specific transcription factor Pax-5 (ED-1; Ref. 31) was first tested for its ability to recognize Pax-5 in trout. Western blot analyses using this antiserum showed that an ~50-kDa protein species, similar in size as mouse and zebrafish Pax-5, was present in trout anterior kidney (shown in Fig. 2*A*), PBL, and spleen cell lysates (data not shown), thus confirming that the anti-Pax-5 serum is capable of detecting trout Pax-5. This was not unexpected because the protein sequence encoded by exons 2–4 of trout Pax-5 (covering the paired domain) has a 98% homology with the murine paired box for Pax-5 (P. Zwollo, unpublished data) recognized by ED-1. Western blot analysis of cell lysates from Percoll layers displayed specific patterns of Pax-5 expression: 70 and 66% layers had the highest signals, 62 and 60% had the low signals, whereas no Pax-5 was detected in the 50% layer (Fig. 2*A*). This distribution is in agreement with Pax-5 expression during all B developmental stages except the plasma cell stage (19).

Similarly, a rabbit anti-mouse Blimp-1 antiserum (a gift from Dr. M. Davis, Stanford University) was tested on trout anterior kidney cell lysates. An ~90-kDa band was strongly detectable in 50 and 55% layers, very faintly detectable in 60 and 62% layers, and undetectable in 66–70% layers (Fig. 2*A*), in agreement with reported size and expression pattern of the (~95-kDa) mouse Blimp-1 protein (24). Thus, Pax-5 and Blimp-1 display partially overlapping patterns of expression, providing important B cell differentiation markers.

To provide further evidence that the two marker proteins represent trout Pax-5 and Blimp-1, EMSAs were performed. Such assays define specificity of a transcription factor based on its ability to recognize specific and highly conserved DNA binding sequences, using DNA probes containing DNA binding sites for Pax-5 (*CD19*/Pax5) or Blimp-1 (c-*myc*/Blimp-1).

Nuclear extracts were prepared from the 50 and 70% Percoll layers of anterior kidney cells and incubated with the probes. A protein present in 70% anterior kidney nuclear extracts bound specifically to the *CD19*/Pax-5 probe. This probe contains a Pax-5 DNA binding site of the murine *CD19* gene, a known target gene for murine Pax-5 (Fig. 2*B*). Furthermore, addition of the anti-Pax-5 serum ED-1 (which recognizes the DNA binding domain of Pax-5) specifically blocked DNA binding of this protein species (Fig. 2*B*), supporting evidence that this protein represents trout Pax-5. When nuclear extracts from anterior kidney 50% Percoll-purified cells were used, no protein species capable of binding to the *CD19*/Pax5 probe were detectable (data not shown). These data are in agreement with the known expression pattern of Pax-5 (19). To provide

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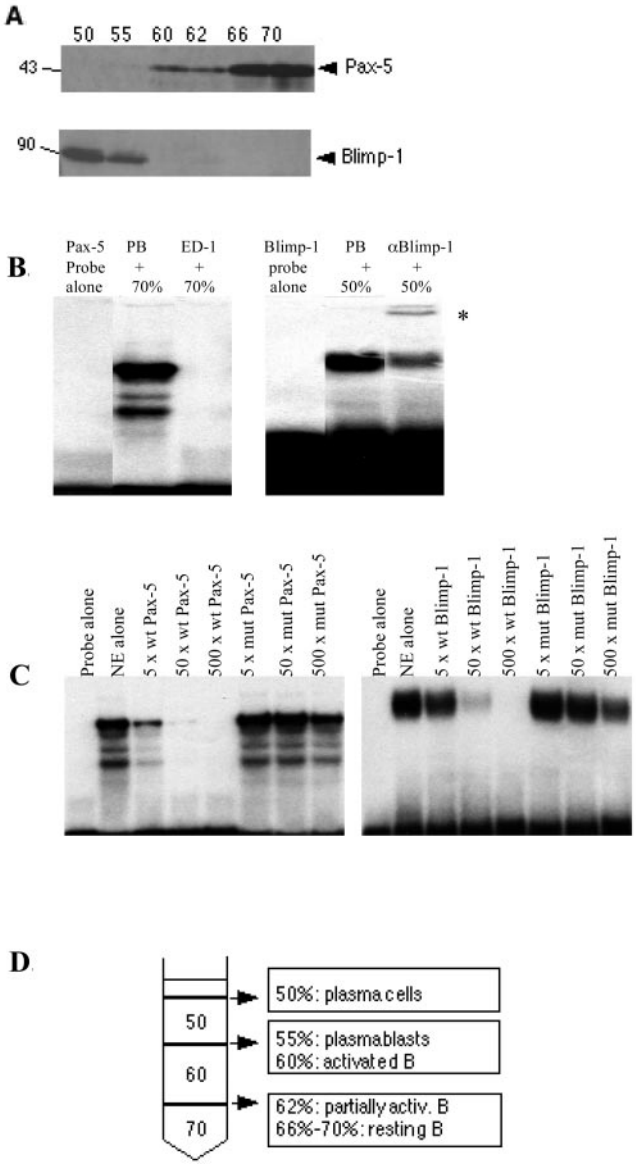

FIGURE 2. Analysis of B cell subsets using Percoll gradients. *A*, Western blot analysis of cell lysates from 1 million cells collected from different Percoll densities as indicated (*top*) in % Percoll. The m.w. markers are indicated on the *left*. *Top panel*, Using anti-Pax-5 antiserum Ed-1. *Bottom panel*, Same membrane stripped and reprobed using anti-Blimp-1 antiserum. *B*, EMSA using Pax-5-specific (CD19/Pax5) and Blimp-1-specific (c-*myc*/Blimp-1) probes. *Left panel*, Using the CD19/Pax5 probe: *lane 1* represents probe alone; *lanes 2* and *3* contain 1 μg of nuclear extract from 70% Percoll anterior kidney cells; sample 2 was preincubated with prebleed rabbit serum; and *lane 3* with ED-1 serum. The *right panel* used the c-*myc*/Blimp-1 probe. *Lane 1*, Probe alone; *lanes 2* and *3* contain 1 μg of nuclear extract from 50% Percoll anterior kidney cells; *lane 2* was preincubated with prebleed rabbit serum; and *lane 3* with anti-Blimp-1 antiserum. Supershift on the *right panel* is indicated by an asterisk (*). *C*, Competition shift assays as in Fig. 2*B* using labeled CD19/Pax5 or c-*myc*/Blimp-1 probes. *Left panel*, Unlabeled, wild-type CD19/Pax-5 or mutant CD19/mutPax5 oligonucleotides were preincubated in the absence of labeled probes in 5, 50, or 500× molar excess, indicated on *top*. *Right panel*, Same approach but with c-*myc*/Blimp-1 and c-*myc*/mutBlimp-1 double-stranded oligonucleotides. *D*, Schematic overview of B cell distribution by Percoll density. To facilitate comparison between the experiment shown in Fig. 1*A* and subsequent experiments, cell subsets from Percoll densities in Fig. 1*A* are grouped into 50, 60, and 70% sets, corresponding to P50, P60, and P70.

further evidence for the identity of Pax-5 in the resolved complexes, nuclear extracts were preincubated in the presence of 5, 50, or 500× excess unlabeled, wild-type *CD19*/Pax5 double-stranded oligonucleotides or with double-stranded oligo *CD19*/mutPax5, in which the Pax-5 site had been mutated (Fig. 2*C*, *left panel*). As expected, wild-type, unlabeled *CD19*/Pax5 double-stranded oligonucleotides were able to compete for binding with Pax-5, whereas mutated, unlabeled double-stranded oligonucleotides (*CD19*/mutPax5) were not.

Similarly, a mobility shift assay was performed for Blimp-1. A DNA probe containing the Blimp-1 DNA binding site on the murine c-*myc* promoter was incubated with trout 50% nuclear extracts. As shown in Fig. 2*B*, a specific complex is detected, indicating that trout Blimp-1 recognizes this DNA binding site specifically. Incubation in the presence of the anti-Blimp-1 antiserum (specific to a region outside of the DNA binding domain of Blimp-1) resulted in the formation of a supercomplex (see Fig. 2*B*), verifying that the DNA-protein complex contained Blimp-1. In contrast to 50% extracts, only a very weak complex was detected when 70% extracts were used in this experiment (data not shown). This pattern is in agreement with the expression pattern of Blimp-1, being highest in plasma cells and very low in early activated B cells (25). Lastly, mobility shift assays were performed in which 50% nuclear extracts were preincubated in the presence of 5, 50, or 500× excess unlabeled, wild-type (c-*myc*/Blimp-1) or mutated (c-*myc*/mutBlimp-1) double-stranded oligonucleotides (Fig. 2*C*, *right panel*). As expected, wild-type, unlabeled double-stranded oligonucleotides were able to compete for binding with Blimp-1, whereas mutated, unlabeled double-stranded oligonucleotides (c-*myc*/mutBlimp-1) were not.

Together, the Western blot and mobility shift assays provide strong evidence that both Pax-5 and Blimp-1 transcription factors are expressed in specific trout B subpopulations and that trout B cell subpopulations can be separated based on their cell density.

Based on this pattern, we designed subsequent experiments using three Percoll densities: 70% (containing 70–61% cell densities), 60% (containing 60–51% densities), and 50% (containing 50% and lower density cells); shown in Fig. 2*D*. We tested the hypothesis that mature (resting and early activated) B cells (Pax-5^+Blimp$^-$) reside in the 70% layer, proliferating B cells reside in the 60% layer (Pax-5^+Blimp$^+$), and plasma cells (Pax-5^-Blimp$^+$) reside in the 50% layer. Several different methods were used to establish Ab production and B cell proliferation patterns in each density layer. We will refer to Percoll-separated cells as P50 (50%), P60 (60%), and P70 (70%) cells.

Ratio of membrane vs secreted Ig in Percoll-separated cells

To test the correlations between B cell differentiation stages and Percoll density layers P50, P60, and P70, we measured the relative levels of membrane vs secreted forms of Ig mRNA using a RT-PCR approach on freshly isolated, unstimulated cells. In general, it would be predicted that the P50 cells containing the plasma cells would express almost exclusively the secreted form, whereas the P70 cells (which include both resting and early activated B cells) would express significantly more membrane than secreted Ig. P60 cells, containing plasmablasts, were expected to contain both forms of Ig mRNA.

Cells were analyzed from the three major B cell compartments, namely kidney (K1–K5), PBLs, and spleen. As shown in Fig. 3, P50 cells in all sites possessed mostly secreted Ig, because the secreted to membrane ratios (S/M) are all well above 1.0. P50 cells from anterior kidney (K1) had the highest secreted to membrane Ig ratio, and those from spleen and K3 had the lowest. This suggests that anterior kidney contained the most Ig-secreting plasma cells

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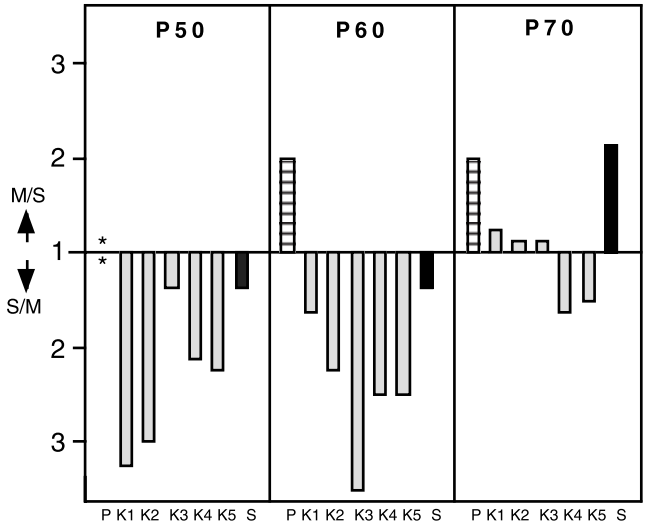

FIGURE 3. Results from RT-PCR on membrane and secreted forms of heavy chain Ig. Amplified DNA fragments were separated by agarose gel, and relative intensities were quantified using NIH Image analysis software. *Top three panels*, M>S: ratios of membrane to secreted values (M/S) for cell subsets with M>S. *Bottom three panels*, S/M: ratios of secreted to membrane values (S/M) for samples with S<M. $n = 3$. ▤, PBLs (P); ▨, kidney (K); ■, spleen (S).

and/or that these cells secrete the highest amount of Ig per cell compared with all other sites. For P60 cells, all immune sites except the PBLs showed significantly more secreted than membrane Ig mRNA (Fig. 3, S/M). PBLs at this density (60%) appear to have much more membrane than secreted Ig mRNA (Fig. 3, M/S), indicative of many P60 PBLs being in an earlier phase of activation compared with all other subpopulations at this density (closer to 60% compared with P60 cells from other tissues). Lastly, P70 cells showed great variation among sites, with PBL and spleen showing mostly membrane Ig, and K4 and K5 showing slightly more secreted than membrane Ig mRNA. This could suggest that PBL and spleen contain more resting B cells compared with K4 and K5.

Comparing the five kidney segments, it is noteworthy that K1 has the lowest ratio of secreted to membrane Ig ratio in P70. This ratio slowly increases toward more secreted Ig when going toward the posterior (K5) kidney. Thus, there is a gradient from very few partially activated B cells in K1 with increased numbers toward posterior kidney, with K5 containing the highest number of partially activated B cells.

Correlation between levels of Ab secretion and Percoll density

It has been shown that trout (18) and other fish (40) possess Ig-secreting cells in the absence of an immune response, and this phenomenon has been explained through the presence of long-lived plasma cells (18). To determine the Ig quantities secreted from P50, P60, and P70 cells, equal numbers of freshly enriched Percoll cells from nonimmunized trout were cultured for 24 h in the absence of cell activators, and total Ig levels were determined using ELISAs. Fig. 4*A* compares the Ig quantities between the five kidney sections and spleen. Blood contained too few P50 cells to be included in this analysis.

In agreement with our hypothesis, the highest Ig levels were present in supernatants from P50 cells, independent of the tissue from which they originated (Fig. 4*A*). Much lower but significant Ig levels were detected in the P60 layers in all tissues. P70 supernatants expressed insignificant or undetectable levels of Ig in all sites except the posterior side of the kidney (regions K3–K5; Fig. 4*A*); this subset of high density cells are not expected to contain plasmablasts or plasma cells based on presence of developmental markers as shown in Fig. 2*A*. However, it is possible that posterior kidney contains a small subpopulation of high density (P70) cells that have the ability to become Ig-secreting cells within the 24 h of culturing as part of the ELISA, displaying rapid differentiation

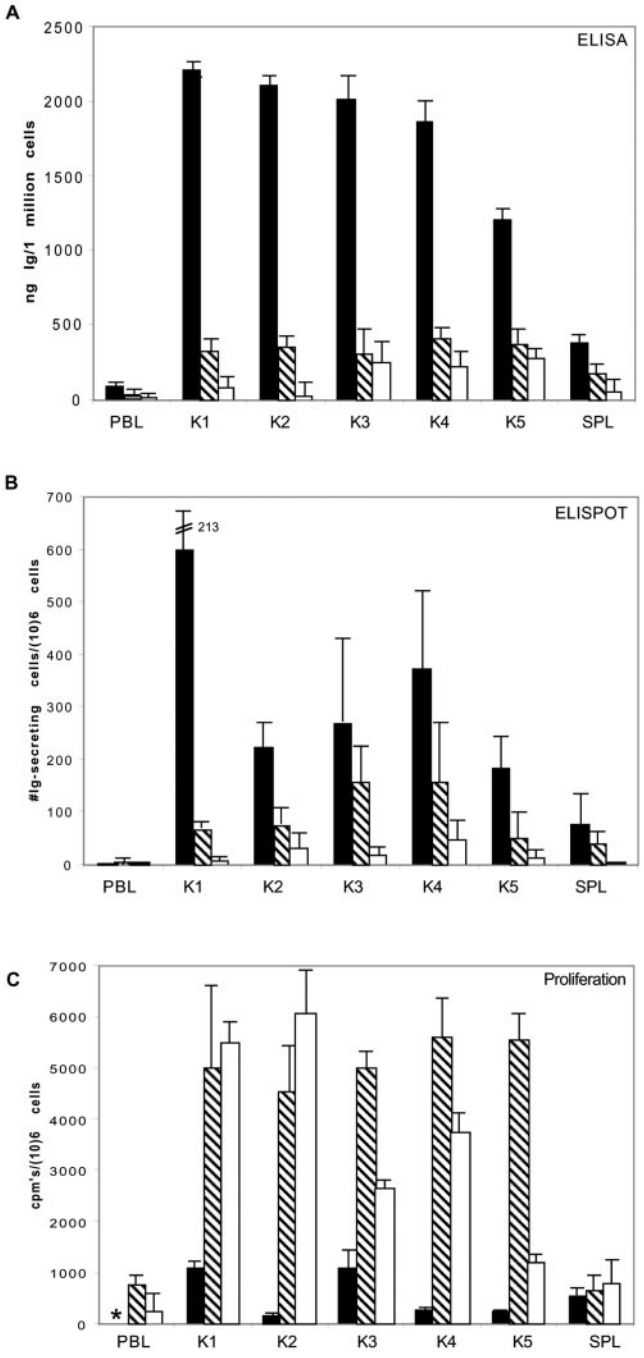

FIGURE 4. Comparison of Ig secretion and proliferation between immune sites. ■, P50; ▨, P60; □, P70. *A*, Ig titers (in nanograms of Ig per 1 million cells) from unstimulated cell supernatants using nonspecific ELISA. Fish ($n = 4$), except for spleen and K3 ($n = 2$). *B*, Number of Ig-secreting cells pre 1 million cells after 24-h incubation in culture in absence of mitogens using ELISPOT ($n = 4$). *C*, Cell proliferation rates using [^{3}H]thymidine incorporation measured after 24-h incubation in culture in absence of mitogens. ∗, P50 for PBL not done for lack of enough cells ($n = 4$).

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characteristics of memory B cells. Alternatively, such cells may be partially activated B cells (see Fig. 2*D*).

It should be noted that, in the absence of stimulation, the total levels of Ig secreted from the cells was low; for example, the average Ig titers for P50 from spleen were 384 ng of Ig per 1 million cells, whereas blood had even lower values of 85 ng per 1 million cells. Strikingly, the Ig concentration of P50 kidney cells (K1-K4) was much higher than that of P50 spleen cells, with the highest value of 2.2 μg per 1 million cells in the anterior kidney (K1; Fig. 4*A*). The presence of Ig-secreting cells in the absence of immune activation suggests that these are either natural (constituent) Ig secretors or long-lived plasma cells.

In conclusion, ELISA data show that the P50 cells in both kidney and spleen are responsible for the majority of Ig secretion, whereas P60 cells from the same tissues secrete lower but significant amounts of Ig, and P70 cells do not significantly contribute to Ig secretion. This suggests either that the P50 layers contain the majority of Ig-secreting cells or that P50 cells secrete more Ab per B cell than P60 cells. This question was addressed using ELISPOT assays.

Correlation between number of Ab-secreting cells and Percoll density

The number of Ig-secreting cells per 1 million cells was determined in each tissue and for each Percoll layer using ELISPOT (Fig. 4*B*). From the data, it is clear that P50 cells for both kidney (K1–K5) and spleen represented the majority of Ig-secreting cells. Strikingly, the P50 cells of blood did not contain any detectable numbers of Ig-secreting cells, suggesting that blood contains very few or no plasma cells (Fig. 4*B*). As expected, P70 cells in all tissues contained very few or no detectable numbers of Ab-secreting cells. P60 layers of kidney and spleen contained intermediate numbers of Ig-secreting cells, whereas virtually no Ig-secreting cells were found in P60 of blood. The anterior kidney possessed the highest relative number of Ig-secreting P50 cells (591 per 1 million cells); strikingly, the number of Ig-secreting cells in the anterior kidney (K1) was ~10 times higher compared with the spleen (Fig. 4*B*). Another unique feature of K1 was that this site had very few Ig-secreting P60 cells relative to P50 cells, suggesting that almost all Ig-secreting cells in the K1 site are plasma cells.

To determine whether P50 B cells secrete more Ig per cell than P60 cells, we calculated the relative Ig secretion per cell for both P50 and P60 cells of kidney and spleen. This was done by dividing the Ig concentration values from supernatants (from ELISAs) by the number of Ig-secreting cells (from ELISPOT). Despite considerable variation, on the average P50 cells generate 1.8 times ($\pm$1.2) more Ig per cell compared with P60 cells, suggesting that the trout plasma cell secretes more Ab compared with the trout plasmablast.

Correlation between proliferation rates and Percoll density

Cell proliferation rates of immune cells were determined from freshly isolated and Percoll-purified cells. This assay has some limitations because it does not identify the proliferating cells type(s). However, this assay is useful in that it identifies Percoll samples that do not show any cell proliferation, from which it can then be concluded that the lymphocytes in such samples do not proliferate.

Equal numbers of cells were cultured in medium without mitogens for 24 h, in the presence of tritiated thymidine, and DNA was collected as described in *Materials and Methods*. Under these conditions, P50 cells did not show proliferation, as shown in Fig. 4*C*. This is in agreement with our hypothesis, which states that plasma cells, but not plasmablasts, are present in P50 layers (Fig. 2*D*). Furthermore, very low proliferation rates (<1000 cpm per 1 million cells) were detectable in P60 and P70 layers of blood and spleen. Very low proliferation rates are to be expected for the spleen in the absence of stimulation, because this is a secondary immune organ, whereas PBLs also would not be expected to contain proliferating cells. Within the kidney, high cell proliferation rates were observed in all P60 layers (K1–K5), whereas P50 cells displayed very low proliferation, in agreement with our hypothesis. However, anterior kidney (K1 and K2) also displayed high proliferation rates for P70 cells (Fig. 4*C*), but this is perhaps not surprising given that anterior kidney is the major site for hematopoiesis in trout, containing very heterogeneous and highly proliferative cell populations. The posterior side of the kidney showed lower proliferation in P70 cells, most strikingly in K5, supporting the notion that no hemopoiesis takes place in the posterior kidney.

We conclude that, with the exception of the anterior side of the kidney (K1 and K2), no significant cell proliferation is present in P50 and P70 subsets, with the majority of proliferating B cells residing in the P60 layers. These results are supported by our ELISA and ELISPOT data as follows: 1) P50 cells are nonproliferating, Ig-secreting cells or plasma cells; and 2) P60 cells contain Ig-secreting, proliferating cells (plasmablasts).

Use of Percoll gradients to measure immune responses in vitro

Next, we wished to test the use of Percoll purification as an approach to characterize in vitro immune responses. To obtain a starting profile for each tissue, the relative number of cells in each Percoll layer in the absence of an immune response was determined. As shown in Fig. 5*A*, the most striking observation from this analysis is the virtual absence of P50 cells in PBLs, consistent with our ELISA and ELISPOT data and indicative of the absence of plasma cells in blood. P50 cells (including both plasma cells and APCs; Ref. 39) were most abundant in posterior kidney (K5) and the spleen. Within the kidney going from anterior (K1) to posterior kidney (K5), there is a trend of increased relative number of P50 cells and a concomitant decrease of P70 cells (Fig. 5*A*).

P70 cells include resting and partially activated B and T lymphocytes, but this high density subpopulation presumably lacks APCs (39). Furthermore, the B cell mitogen LPS does not activate T cells. Thus, LPS activation of P70-purified cells is expected to result in a population of activated B cells, plasmablasts, and plasma cells, but not T cells, macrophages, or other non-B cells.

To investigate potential benefits of Percoll gradients as a means of analyzing humoral immune responses, P70 cells from freshly isolated spleen, blood, K1, and K5 were purified by Percoll separation. This subpopulation of cells was then cultured for 7 days in the presence or absence of B cell mitogen LPS. On day 7, cells were collected, again separated through 50, 60, and 70% Percoll gradients, and cell distribution was determined (outlined in Fig. 5*B*).

One representative experiment using this approach is shown in Fig. 6*C* (the experiment was repeated twice). In the absence of LPS, the majority of cells from all sites remain high density P70 cells. Immune activation as a result of LPS was apparent with a shift from P70 toward P60 for all sites, and P50 populations for some sites, with distinct patterns for each organ. For anterior kidney (K1), LPS resulted in a strong induction of P60 cells and a weak induction of P50 cells. The generation of so many P60 cells in K1 is likely to include the maturation of various immune cell precursors in this site. In the presence of LPS, kidney section K5 has a much greater shift toward P50 cells compared with K1. Of interest, K5 has the most dramatic shift toward P50 cells of all four sites examined (comparing number of P70 cells to number of P50 cells), suggesting that the posterior kidney has great capacity for

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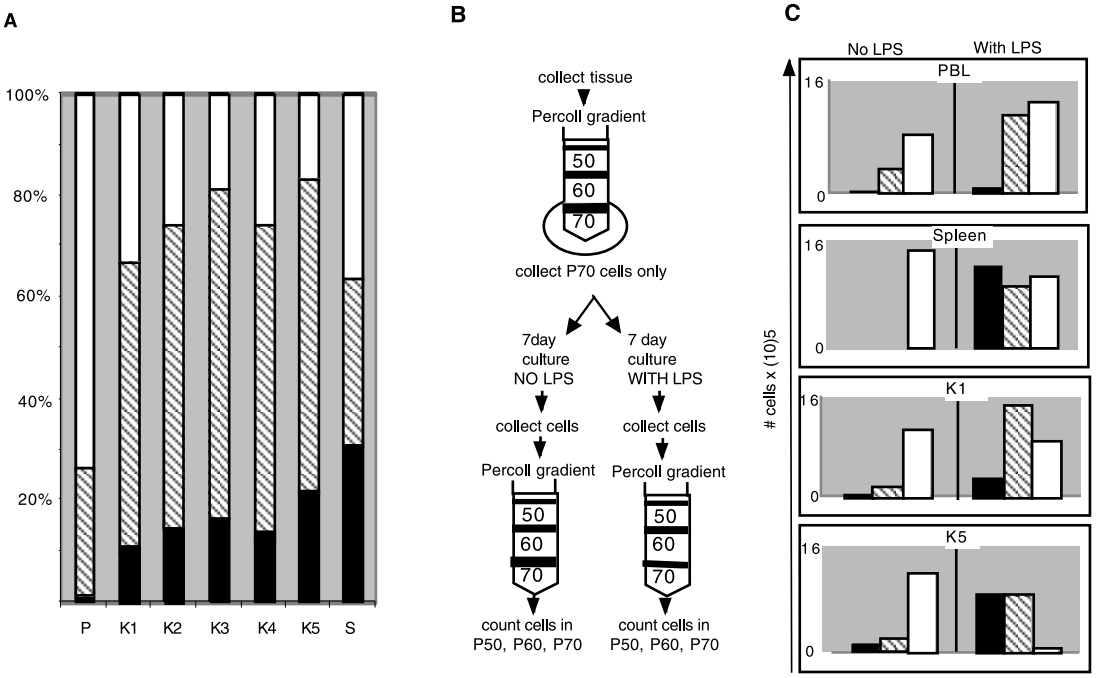

FIGURE 5. Analysis of in vitro immune responses. Percoll-purified P70 cells after 7 days in culture in the presence or absence of LPS. SPL (spleen), PBL, K1, and K5, ■, P50; ▨, P60; □, P70. *A*, Comparison of cell numbers for each immune site and for P50, P60, and P70, before LPS activation in vitro. Relative frequency of P50, P60, and P70 cells within each immune site to a total of 100% ($n = 9$). *B*, Outline of approach used for the LPS activation of P70 cells. *C*, Cell distribution for each of the four tissues from one representative experiment. *Top panel*, PBLs; *second panel*, spleen; *third panel*, kidney (K1); *bottom panel*, kidney K5. *Left boxes* within each panel, cultures without LPS; *right boxes*, in presence of 100 μg/ml LPS.

immune activation, including plasmablast and plasma cell formation. PBLs showed the ability to shift to P60 cells under influence of LPS; however, very few P50 (plasma cells) were detected, in agreement with our ELISA, ELISPOT, and RT-PCR data. Spleen was interesting because in the absence of LPS, the P70 cells remained in the high density resting state over the period of 7 days, suggesting that P70 of the spleen contains mostly mature resting B cells. In contrast, LPS induced a strong shift toward both P60 (including plasmablasts) and P50 (including plasma cells) in this tissue, similar to the pattern for posterior kidney (K5).

Discussion

In this study, we physically separated unstimulated leukocytes from rainbow trout immune tissues using density gradients and examined the resulting cell subsets for various parameters of B cell function. Five distinct regions of the kidney, including the anterior and posterior kidney, were analyzed and compared with blood and spleen subsets. The data indicate that the 70% Percoll layer contains resting B and partially activated B cells, the 60% layer contains plasmablasts, and the 50% layer contains the plasma cells. Additionally, interesting and unexpected B cell distribution patterns were found within the trout kidney. In vitro LPS stimulation of Percoll-separated high density (P70) cells from different sites revealed unique and site-specific patterns of immune responses.

The current literature is not entirely clear on whether in mammalian species plasma cells secrete more Ig per cell as compared with plasmablasts. Ectopic expression of Blimp-1 into mature B cells or B cell lines indicates that this transcription factor can induce several changes in B cells including increase in Syndecan-1, increases in cell size and granularity, as well as secretion of IgM, although such cells do not become "full-fledged" plasma cells (24, 25). Furthermore, the Ig levels secreted by such Blimp-1-induced B cells are lower than those secreted by plasma cell (lines) (24, 25). Ultrastructural evidence indicates that plasma cells have a larger, better-developed endoplasmic reticulum than plasmablasts, which would be consistent with higher Ig secretion rates in plasma cells (41). Furthermore, an increased level of secreted Ig would be expected in plasma cells because such cells lack Pax-5, thus having completely de-repressed *Ig* gene, which results in greatly increased Ig production (21, 27, 28).

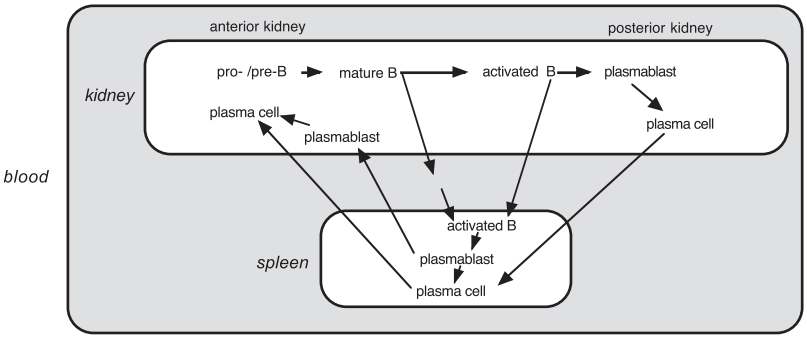

FIGURE 6. Model outlining trout kidney function during B cell development and activation and immunological relationships with spleen and blood. In this model, B cell stages and developmental capacity were determined experimentally. Migratory routes are hypothetical. B cell precursors develop in the anterior side of the kidney, whereas mature B cells migrate to the posterior kidney or the blood. Posterior kidney and spleen are both sites for B cell activation. Plasmablasts formed in posterior kidney or spleen differentiate into plasma cells. Plasma cells migrate to the anterior kidney where they may become long-lived plasma cells (see *Discussion*).

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Comparison of P50 and P60 cells for all seven immune sites indicated that, on the average, trout plasma cells (P50) secrete ~1.8 times more Ig per cell than plasmablasts (P60), but there is significant variation between and within sites. This variation can be explained partly by the fact that outbred animals were used for this study. Additionally, the rather complex pattern of ELISPOT sizes was not taken into consideration, which may reveal additional patterns. A more accurate and quantitative approach to calculating the amount of secreted Ig per cell is being developed currently as part of a different study (E. Bromage and S. Kaattari, unpublished data).

Results from PBLs show that this immune site contains few to no P50 cells (plasma cells), because the majority of PBLs are P70 or "high density" cells. Furthermore, the majority of unstimulated leukocytes in the blood do not proliferate spontaneously. Lastly, culture supernatants from P60 cells of PBLs contain the lowest levels of Ig compared with spleen and kidney, and both P50 and P60 layers contain very few to no Ig-secreting cells. RT-PCR studies also indicate that blood B cells for all three Percoll densities have the lowest ratios of secreted to membrane Ig. Our in vitro LPS activation studies indicate that even after LPS stimulation of P70 PBLs in vitro, very few if any plasma cells are generated from such cells after 7 days, although plasmablasts are generated at levels similar to spleen and kidney. This is in agreement with our recent results using hydroxyurea in PBL cultures, reporting the presence of plasmablasts but not plasma cells in trout blood (18). This could be caused by the absence of essential cytokine-producing cells in PBL cultures, thus preventing differentiation into or maintenance of plasma cells in the blood. Mammalian studies have reported a virtual absence of plasma cells in blood, in agreement with our findings (42–44).

The trout kidney is an extraordinarily heterogeneous organ containing distinct, location-dependent patterns of B cell subsets. From the data presented here, we conclude that the majority of immune cells in the kidney are P60 cells, containing plasmablasts and other, highly proliferative cells. There are a number of consistent phenotypic shifts when analyzing kidney sites from the anterior to posterior kidney. First, using RT-PCR, an increase in the ratio of secreted to membrane Ig was found in P70 cells. This translates into an increase in secreted Ig and a decrease in membrane Ig in partially activated B cells and is in agreement with the ELISA data, showing low levels of Ig secretion in P70 subsets from kidney sections K3–K5 but not K1 or K2. Thus, it appears that posterior kidney has a significant number of partially activated B cells, whereas anterior kidney has few or none. Second, there is a significant decrease in proliferation rates of P70 cells comparing sites from K1 to K5, with K5 having the lowest proliferation rates, which are similar to those of P70 of the spleen. This pattern supports the hypothesis that both spleen and posterior kidney represent secondary immune "organs," whereas anterior kidney represents a primary immune organ. Third, experiments using LPS activation in vitro show that posterior kidney has the highest capacity for production of plasma cells from a purified set of P70 B cells, compared with anterior kidney, spleen, and blood. Together, these shifts suggest that the anterior kidney contains few or no early activated B cells, whereas posterior kidney has a clear subpopulation of partially activated B cells. We hypothesize that B cells develop in anterior kidney and then migrate within the kidney to sites of Ag presentation in the posterior kidney.

The hypothesis presented in Fig. 6 provides a working model for the role of trout kidney in immune development and activation. We hypothesize that the anterior kidney contains Ag-independent sites or niches, thus representing a primary immune organ where B cell progenitors mature into Ag-responsive B cells. In this model, the posterior kidney acts as a secondary immune site, a site where mature B cells can be activated by APCs and possibly T cells, resulting in differentiation into plasmablasts and plasma cells. The spleen is strictly a secondary immune organ involved in B cell activation and subsequent differentiation into plasma cells. Subsets of plasmablasts from either spleen or posterior kidney home back to the anterior kidney were they may become (long-lived or short-lived) plasma cells. In this model, the blood serves as a reservoir to maintain or store mature B cells and to provide a means of transporting plasmablasts and/or plasma cells to their various homing sites, as suggested by other groups (45–47).

The notion that both B cell development and activation can occur in the kidney is supported by studies in zebrafish. In this teleost species, B cells develop in the pancreas as well as the anterior kidney, as shown using in situ hybridization on tissue sections (48). In adult zebrafish, expression of both Igμ and RAG-1 was seen in the pronephros (anterior kidney), whereas the trunk kidney (posterior kidney) showed expression of Igμ but not of rag1. These data suggest that developing B cells are present in the anterior kidney but absent from the posterior kidney, whereas mature, membrane Igμ-positive B cells are present in posterior kidney (48).

A recent study (49) in the clearnose skate provides further evidence that the mammalian pattern of transcription usage is fundamental to the definition of lymphocytes. Authors show a clear conservation of expression of several lymphoid-specific transcription factors in the skate, including Pax-5. Pax-5 expression is highest in the spleen of the skate, whereas two elasmobranch-specific organs, the Leydig and epigonal organs, are B cell rich but express lower levels of Pax-5. The Leydig organ contains mostly B cells, whereas the epigonal organ is also rich in granulocytes. These two organs presumably provide the main sites for hemopoiesis in adult animals and may be the anterior kidney equivalent of the trout. Interestingly, Ig-expressing cells in epigonal and Leydig organs do not express Pax-5, suggesting that both organs contain (Pax-5^+Ig^-) developing B cells and plasma cells but no or few mature (Pax-5^+Ig^+) B cells. Leydig and epigonal organs may thus house the predominant Ig^+Pax-5^- plasma cell population in the skate (49), a function observed in the anterior trout kidney as well. The study suggests that although hemopoietic organ sites have shifted in phylogeny, the functional association between specific transcription factors and lymphoid development remains strong (49).

In this study, we show that Percoll separation is a powerful method for the analysis of structurally and functional distinct B cell populations in the trout. Western blot and electrophoretic mobility shift analyses revealed that trout Pax-5 and Blimp-1 provide excellent markers for the different stages of B cell activation: Pax-5 detects resting B cell through the plasmablast stage, whereas Blimp-1 detects plasmablast through plasma cell stages. Ig-secreting cells are found mostly in P50 and are absent from P70 cells in all immune sites tested. To distinguish between few cells expressing high levels of marker protein or many cells expressing low levels, as well as to distinguish between a population of double-positive cells (Pax-5^+Blimp-1^+) vs single-positive cells (either Blimp-1^+ or Pax-5^+), we are now in the process of generating flow cytometric approaches with both Pax-5 and Blimp-1.

In summary, the Percoll approach allows for thorough analysis of B cell subpopulations in the absence of flow cytometric facilities, as well as for the physical separation of large numbers of functionally distinct, live B cell subsets. The findings described in this study are in agreement with our hypothesis that the trout kidney is a complex, multifunctional immune organ supporting both primary and secondary immune functions in this animal.

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Disclosures

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