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Supplementary Materials for

Cold-blooded vertebrates evolved organized germinal center like structures

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MATERIALS AND METHODS

Immunofluorescence microscopy

Spleen tissues were sampled and embedded in Tissue-Tek OCT compound (Sakura Finetek). Spleen cryoblocks were sectioned at a thickness of 5 μm and then fixed for 10 min in 4% paraformaldehyde (PFA; Wako Chemicals). For the detection of trout IgM^+ B cells and $CD4^+$ T cells, tissue cryosections were immunostained as previously described by us (*19, 49*). Briefly, we used mouse anti-trout IgM monoclonal antibody (mAb) (clone: 1.14, mouse IgG1; 1 μg/ml) (*53*) and affinity purified guinea pig (gp) anti-trout CD4 polyclonal antibody (pAb) (1 μg/ml) to incubate the cryosections overnight at 4 °C. For the detection of cells expressing activated caspase 3, a rabbit anti-human caspase 3 (ab13847-100; 1:300 dilution; Abcam) which cross-reacts with several species, including rainbow trout, was used following the methodology described in (*29*). As antibody controls for anti-trout IgM mAb, anti-trout CD4 pAb, and anti-human caspase 3, we used mouse IgG1 (clone MOPC-21, Biolegend), guinea pig IgG purified from pre-immune sera and rabbit IgG isotype control (Invitrogen), respectively. To detect anti-trout IgM mAb, anti-trout CD4 pAb, and anti-human caspase 3, cryosections were incubated for 2 h at room temperature (RT) with Alexa Fluor 488-conjugated goat anti-mouse IgG1 (Invitrogen), Alexa Fluor 568-conjugated goat anti-guinea pig IgG (Invitrogen) and Alexa Fluor 647-conjugated goat anti-rabbit IgG (Invitrogen) respectively at 2.5 μg/ml each. Cell nuclei were stained with DAPI (1 μg/ml; Sigma-Aldrich) and the slides were mounted with Fluoroshield (Abcam). Images were acquired by using a Leica DM6000 fluorescence microscope at the Penn Vet Imaging Core (the University of Pennsylvania), or a Leica SP8 confocal microscope at General Research Institute (Nihon University) and analyzed with LAS X software. When required, the area of images was measured by ImageJ as described in (*54*). IgM^{hi} and IgM^{low} B

cells were analyzed using LAS X software (IgM positively stained cells were determined as IgM^{hi} cells when cells exhibit saturated signal using the the Over /Underexposure mode).

Proliferation of IgM^+ B cells and $CD4^+$ T cells on cryosections was performed as previously reported by us in (*19, 49*). Briefly, fish were anesthetized with MS-222 (100 mg/l) and i.p. injected with 200 µg of 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen) in 100 µl of PBS. After 24 h, animals were euthanized as described above, and spleen organs were harvested and embedded in Tissue-Tek OCT compound (Sakura Finetek). The cryoblocks were sectioned at a thickness of 5 µm and then fixed for 10 min in 4% PFA. EdU^+ cells were stained with Click-iT EdU Alexa Fluor 647 Imaging Kit (Invitrogen) according to the manufacturer's instructions. EdU-stained spleen cryosections were then stained with anti-trout IgM mAb and anti-trout CD4 pAb as described above to detect IgM^+ B cells and $CD4^+$ T cells, respectively. Cell nuclei were stained with DAPI (1 µg/ml) before mounting with Fluoroshield. Images were acquired and analyzed using a Leica DM6000 fluorescence microscope or Leica SP8 confocal microscope and LAS X software.

Quantification of cells on tissue sections

To quantify the number of proliferating cells or other cell types in splenic MMCs and non-MMC areas, we adapted a methodology used to count cells in GCs reported in (*55, 56*). Briefly, images from stained spleen sections were acquired by using a Leica DM6000 fluorescence microscope at the Penn Vet Imaging Core (the University of Pennsylvania), or a Leica SP8 confocal microscope at General Research Institute (Nihon University). On these images, the exact perimeter containing aggregates of proliferating cells within and nearby a given MMC was first drawn using Fiji ImageJ software (*57*), and proliferating cells within that perimeter were then

counted using the same software. To normalize the number of proliferating cells per area of tissue, number of proliferating cells were expressed per 0.1 mm^2. To count proliferating cells in non-MMC areas of the same spleen section, we counted proliferating cells within non-MMC areas equivalent in size to those counted for MMCs. For instance, if for one spleen section we counted proliferating cells in 3 different MMC areas (each measuring 0.2, 0.1 and 0.3 mm^2 respectively), we then counted proliferating cells in 3 different non-MMC regions containing the same areas of those measured for MMCs (0.2, 0.1 and 0.3 mm^2). The aforementioned approach was also used to count the number of IgM^{hi}, IgM^{low} cells in splenic MMCs and non-MMC areas, as well as the number of proliferating B and T cells in splenic M-LAs and non-M-LA areas from control and infected fish.

Analysis of trout DNP-KLH-specific IgM titers by ELISA

DNP-KLH or KLH (Millipore) were used as the antigen for coating the ELISA plates. Antigen (20 µg/ml in 0.05M carbonate-bicarbonate buffer, pH9.6; 50 µl per well) was absorbed overnight at 4 °C onto MICROLON microplates (Greiner). Nonspecific binding sites were then blocked with 8% skim milk in PBS. Plates were washed with 0.05% PBS-Tween. Serum samples from control or DNP-KLH immunized fish were serially diluted in PBS. For analysis of the binding of IgM to antigens, biotinylated anti-trout IgM (0.5 µg/ml in 1% PBS-BSA) was added to each well. After 2 h of incubation at RT, plates were washed and bound antibodies were detected by incubation with Pierce High Sensitivity Streptavidin-HRP (0.5 µg/ml; Thermo Fisher Scientific), followed by color development with TMB ELISA Substrate (Abcam) as a substrate. After 30 min, the color reaction was stopped with a solution of 0.6 N sulfuric acid and absorbance was measured at 450 nm by microplate reader (InfiniteF50 Plus, TECAN). DNP-specific antibody endpoint titers are presented as the reciprocal of the highest serum dilution that

provided an average absorbance exceeding twofold the average background absorbance at 405 nm.

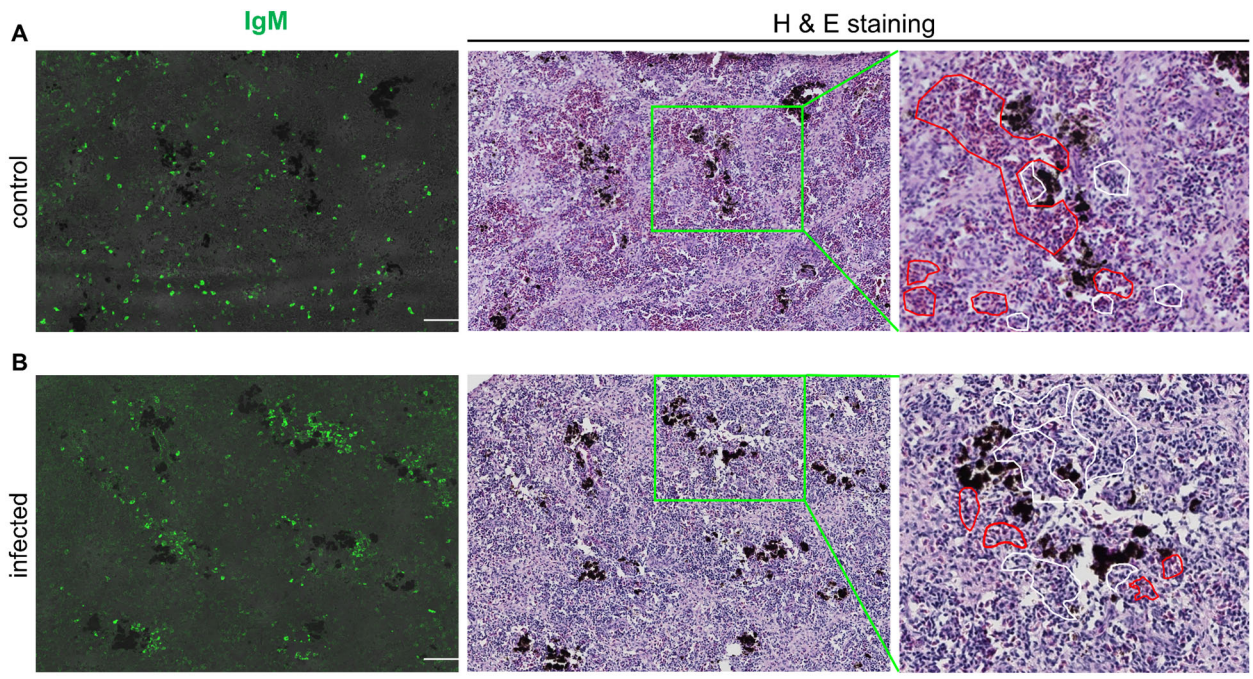

Fig.S1 Red and white pulp from spleen of control and infected fish. (**A** and **B**) Histological analysis of spleen from control (A) and infected (B) fish. Spleen cryosections were first stained for IgM (green), followed by H&E staining. Right panels of (A and B) show enlarged images of the areas outlined in middle panels, each containing an MMC and its surrounding tissue. White and red lines in right panels of A and B outline representative areas of white and red pulp respectively. As seen in these images, red and white pulp of trout spleen intermingle throughout the spleen.

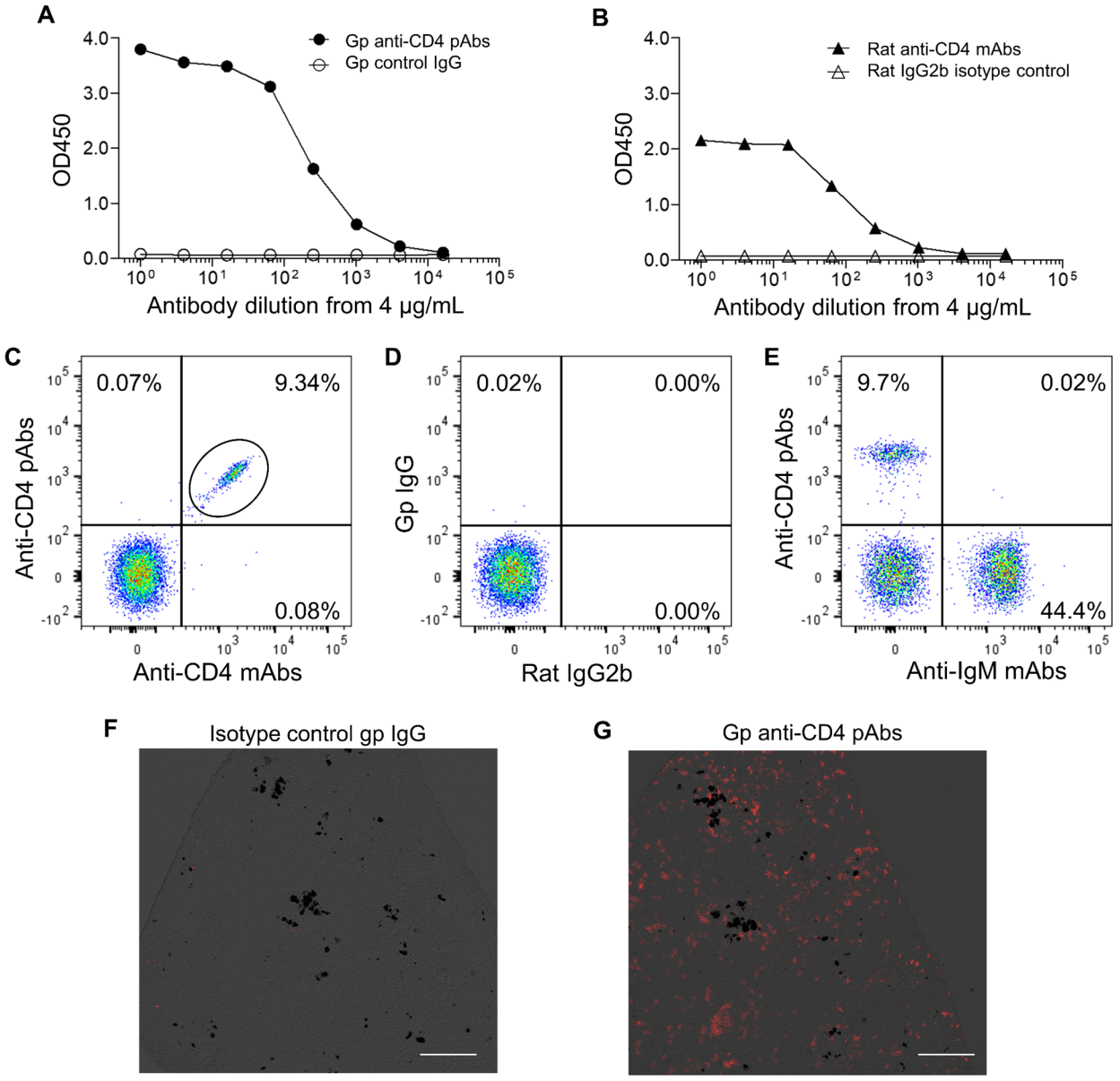

Fig.S2 Validation of a newly developed guinea pig anti-trout CD4 pAb reagent. (**A** and **B**) Representative ELISA showing the reactivity of guinea pig (gp) anti-trout CD4 pAb (A) and rat anti-trout CD4 mAb (B) against recombinant trout CD4. ELISA plates were coated with recombinant trout CD4 which was detected with gp anti-trout CD4 pAb (A) or rat anti-trout CD4 mAb (B**)** or their respective isotype control Abs (gp IgG and rat IgG2b). (**C** to **E**) Flow

cytometry of trout splenic leukocytes double-stained with: gp anti-trout CD4 pAb and rat anti-trout CD4 mAb (C), isotype control for gp IgG and rat IgG2b (D), and gp anti-CD4 pAb and mouse anti-trout IgM mAb (E). (**C** to **E**) show representative dot plots of the lymphocyte gate. Numbers in quadrants in each dot plot represent percentage of positively stained cells. (**F** and **G**) Immunofluorescence analysis of spleen from a naïve fish. Consecutive cryosections were stained with isotype gp IgG (F) or gp anti-trout CD4 pAb (G). Data are representative of at least three independent experiments (*n* = 3-4 fish/experiment). Results show that gp anti-CD4 pAb (A) recognizes recombinant trout CD4 to a higher degree than the rat anti-CD4 mAb (B). Moreover, gp anti-trout CD4 pAb and rat anti-trout CD4 mAb double-stain the same $CD4^+$ T cells (black circle in(C)) thus indicating the correct specificity of the gp anti-trout CD4 pAb. As expected, this new gp anti-trout CD4 pAb did not cross-react with IgM^+ B cells (E). Isotype control gp IgG did not produce any significant staining (D and F).

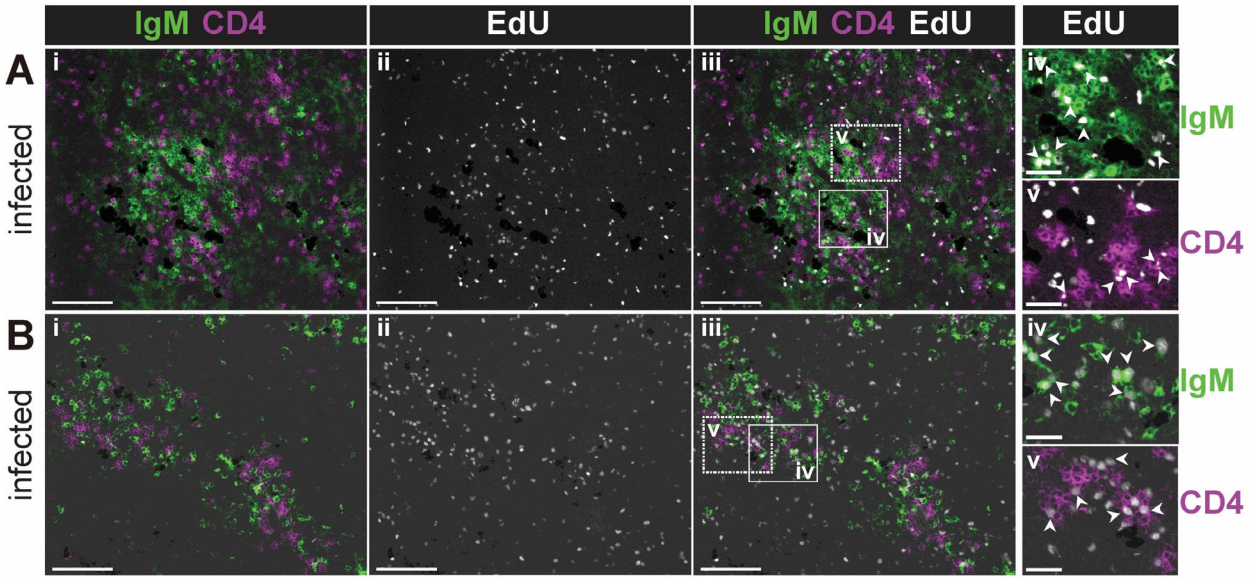

Fig.S3 Boundaries between B and T cell aggregates are not clear in some M-LAs from infected fish. (**A** and **B**) Immunofluorescence analysis of EdU incorporation by IgM^+ B cells and $CD4^+$ T cells from M-LAs of spleen of infected. Spleen cryosections were stained for IgM (green), CD4 (magenta), EdU (white). Image in (i) shows a representative M-LA stained for IgM (green) and CD4 (magenta); (ii and iii) show the same tissue area of (i) stained for EdU (white) (ii), IgM (green), CD4 (magenta) and EdU (white) (iii); (iv and v) show enlarged images of the areas outlined in (iii) displaying proliferating (EdU^+) IgM^+ B cells (iv) and proliferating (EdU^+) $CD4^+$ T cells (v). White arrowheads point to examples of proliferating IgM^+ B cells (iv) and $CD4^+$ T cells (v). Scale bars, 100 μm (i to iii), and 20 μm (iv and v). Groups of dark cells in all images represent MMCs. Data is representative of at least three independent experiments.

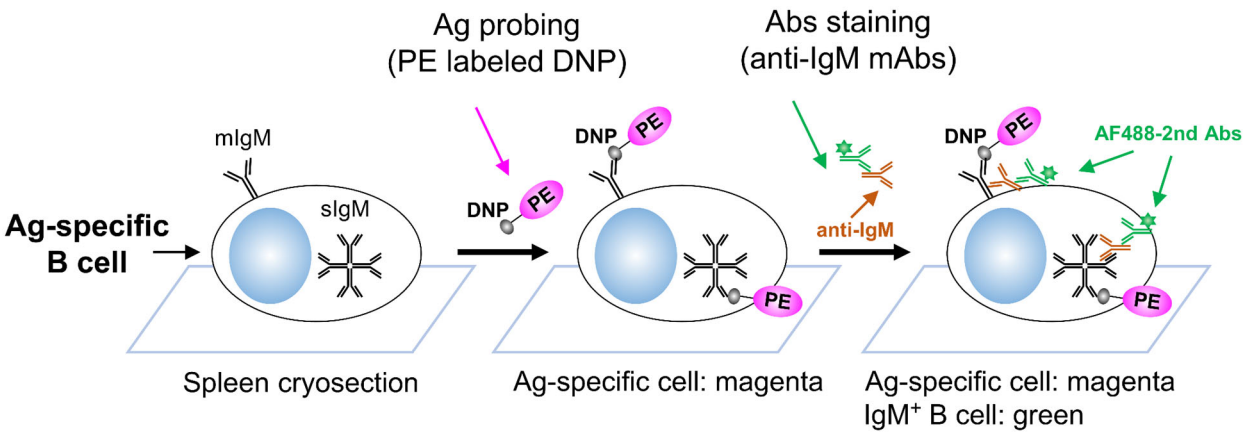

Fig.S4 Detection of antigen(Ag)-specific B cells on spleen cryosections. Scheme of the strategy used to detect Ag-specific B cells on spleen cryosections from control or immunized fish. Cryosections were first stained with the antigen probe (DNP-PE), followed by anti-trout IgM mAb and anti-trout CD4 pAb and their corresponding secondary Abs. The cryosections were analyzed by confocal microscopy. mIgM: Membrane IgM; sIgM: Secreted IgM.

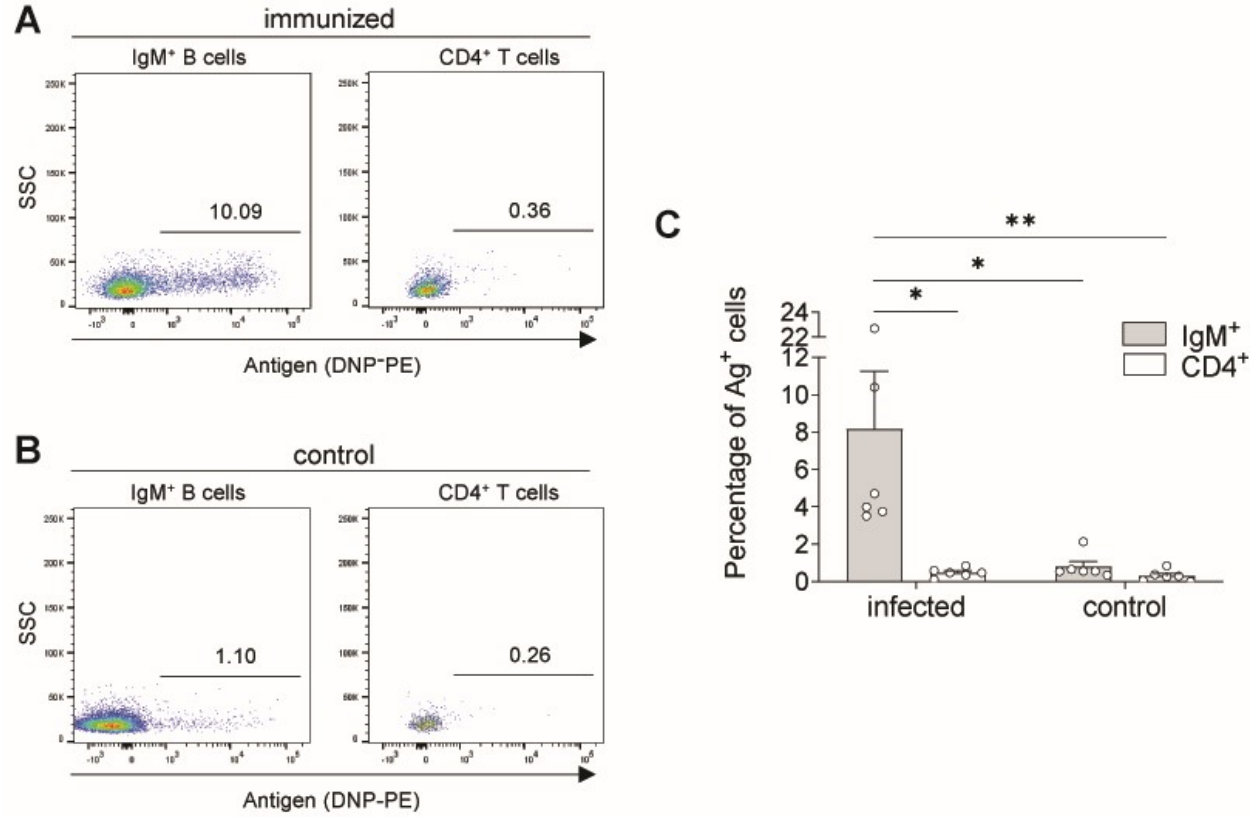

Fig.S5 Detection of antigen-specific B cells by flow cytometric analysis. Leukocytes from the spleen of control or DNP-KLH immunized fish were co-stained with the antigen probe (DNP-PE) and the anti-trout IgM mAb or anti-trout CD4 pAb and their corresponding secondary Abs. (**A** and **B**) Representative dot plots showing the binding of DNP-PE to IgM^+ B cells (left) and $CD4^+$ T cells (right) from immunized fish (A) or control fish (B). (**C**) Percentage of IgM^+ B cells and $CD4^+$ T cells binding DNP-PE. (**A** to **C**) 6 immunized and 6 control fish were analyzed. Empty cycles represent values from individual fish. Statistical analyses were performed by two-way ANOVA followed by Tukey's test. *$P < 0.05$, **$P < 0.01$.

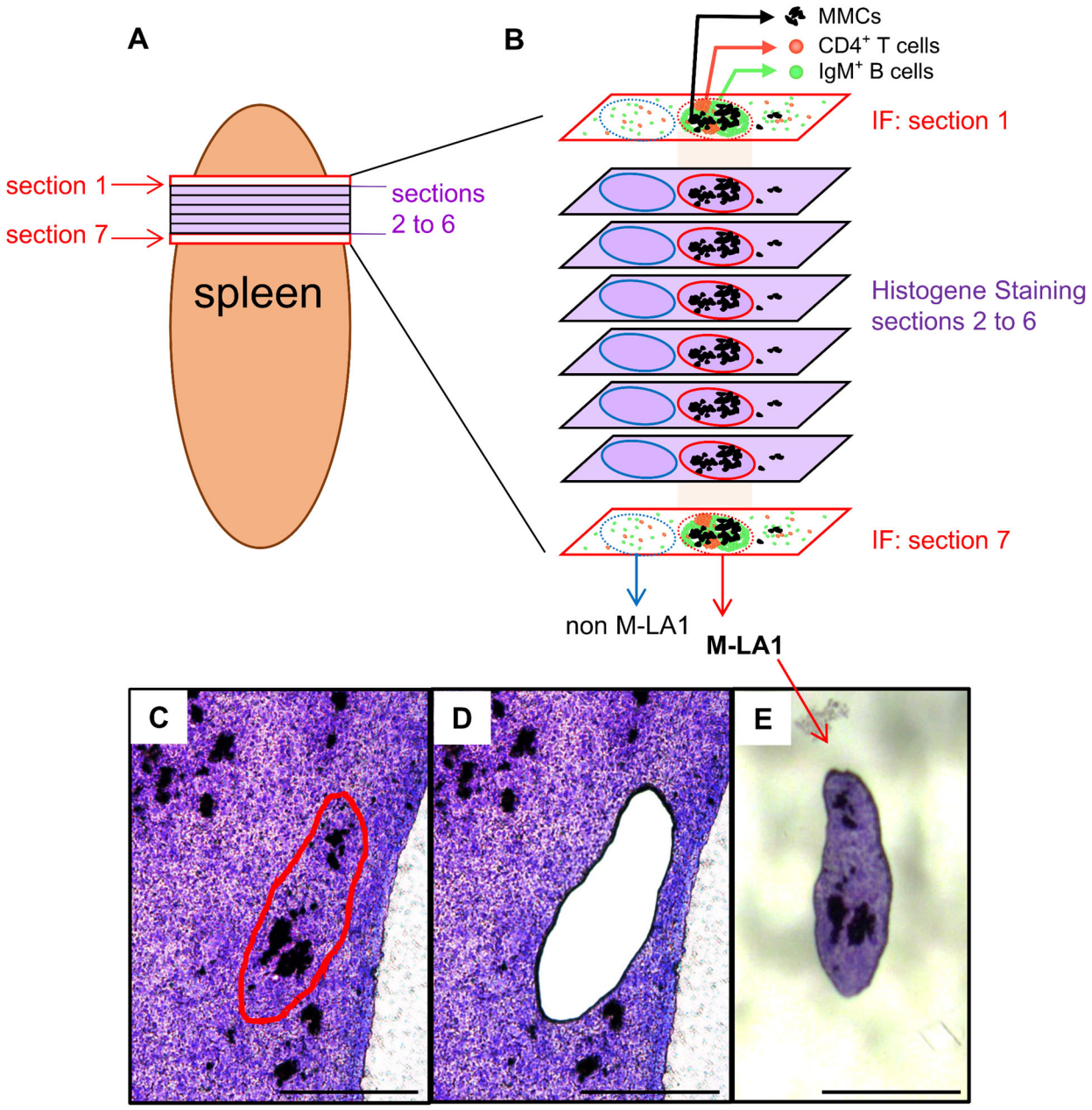

Fig.S6 Strategy to harvest splenic M-LA and non M-LA areas by laser capture microdissection (LCM). (**A**) To identify M-LA and non M-LA areas from sections of the same spleen, a first section (top red) from the spleen is stained for IgM, CD4 and EdU as described in (Fig. 2) with the goal to identifying M-LAs and non M-LA areas. Once identified, five consecutive sections are then obtained and the seventh section (bottom red) is stained again with

the same Abs to confirm that the M-LA is still in that region and that the chosen non M-LA areas remain devoid of M-LAs. This process may be repeated several times in these same areas, depending on the thickness of that specific M-LA or non M-LA area (B shows a graphic representation of a spleen section depicting an M-LA and a non M-LA area [black areas of the section represent MMCs]). (**C**) To microdissect M-LA and non M-LA areas from the non-stained spleen sections (sections 1 to 5 of B), that section is first stained with snap H&E staining (Histogene Staining Solution) for easy identification of melanomacrophages (dark cells). As an example, we show the laser capture microdissection of an M-LA from a snap H&E-stained section (C to E). Using the Leica software from the LCM apparatus we can quantify the exact area of the M-LA that has been microdissected, and these data is then used to harvest the same amount of area from a non M-LA region of the same spleen. Scale bar, 200μm.

Supplementary Table 1. Primer sequences used in this study.

Primer	Sequences (5'-3')	Used for
IGMR1	TAAAGAGACGGGTGCTGCAG	First strand cDNA synthesis
IGV5	TGARGACWCWGCWGTGTATTAYTGTG	Analysis of IgHμ CDR3 diversity
IGC3	GGAACAAAGTCGGAGCAGTTGATGA	
Forward VH-specific primer cocktail:		Cocktail primers used for hypermutation analysis
IGHV1-10_F	ACTAACTGAGTCTGGGCCAG	
IGHV1_F	ATTGACKGAGTCTGGAYCAK	
IGHV2_F	ACTMTMAGTCTSTCCTGTAAG	
IGHV3_F	TGCAGCCTGGACAGCCTCTGAC	
IGHV4_F	GGATCAGTCWCCTYCTCARGTG	
IGHV6_F	GACTGTCCWGCCAGGTCAACC	
IGHV7_F	GTAAAGCCTGGAGAGTCTCTG	
IGHV8_F	AAACTCACATGTGCCTGTAGTG	
IGHV9_F	GAGTCAGTAACASTGTCMTGT	
IGHV10_F	CCAGGAGAGTCTKTCASCATC	
IGHV11_F	GTGAAACTATCTTGCCAAGT	
IGHV12_F	CTTTGAGCATCACCTGTAAGG	
IGHV14_F	TTGACTATCAGTTGTGCAATC	
IGHV15_F	AGAAACTCTCAAYCTMTCYTG	
IGHV16_F	CTCAGCCTCTCCTGTAAGGGA	
Cμ1_R	CACATTGCGCAAGAGGGAACAA	Reverse primer used for hypermutation analysis

Supplementary Table 2. Probe sequences used for *in situ* hybridization.

Gene	Used for	Sequences (5'-3')
Aid	anti-sense probe	TCACAGTCCAAGTAGCTCAAAAGCATCTCCCCAATCTTCTGCC TCACCAGGCTGGAGGATGTCGTTAAGTTTCCTGGCCAGTTGA ACAGAGTTCTGATGCAGTCCGTCCCAGGCCTTAAACACACGC TGTCTGCAAGCTACAAAGGTCTGCCAACAGTAGAAATAGTCT TCATAGTTCATGACAGTGATCTGCACCCCGGCTCTCTGCAGCA TGCGGAGACCCTCTCTCGCACTGCTGTCCTCCGGGTCACAGA AGTAGAGCCTGGAGACATAGATCCTGAGGCGGAGGTTGGGG GTCTGGCTGAGGAACTGGGCCAGCCTGTAGGAGCAGTCTGAG CAGGGGGACCAGGAACAGAACCAGGTGACTGAGTAACACAG TCCCACACTGTCTGGAGCTCCATAACCCCACAGGCCTGGACA CAGGGCGCCTGCTTCCAGGAGGCGCAGGAACAGCAGCTCAA CATGACAGCCGGACCGGTTGCGCAGGTGTCCAAAGTCGAAGG AGAGTGAGTTTGGTCCCACCCGCCTCTTGACCACAAAGCACA GGTAGGTTTCGTGTCGGCCCTTGGCCCAGCGCATGTTCTTATA GTGGTAGATAAACTTCTTCTGGGCCAACAGAACACTGTCAAA TTTGTTGATCAT
	sense probe (control)	ATGATCAACAAATTTGACAGTGTTCTGTTGGCCCAGAAGAAG TTTATCTACCACTATAAGAACATGCGCTGGGCCAAGGGCCGA CACGAAACCTACCTGTGCTTTGTGGTCAAGAGGCGGGTGGGA CCAAACTCACTCTCCTTCGACTTTGGACACCTGCGCAACCGGT CCGGCTGTCATGTTGAGCTGCTGTTCCTGCGCCTCCTGGAAGC AGGCGCCCTGTGTCCAGGCCTGTGGGGTTATGGAGCTCCAGA CAGTGTGGGACTGTGTTACTCAGTCACCTGGTTCTGTTCCTGG TCCCCCTGCTCAGACTGCTCCTACAGGCTGGCCCAGTTCCTCA GCCAGACCCCCAACCTCCGCCTCAGGATCTATGTCTCCAGGC TCTACTTCTGTGACCCGGAGGACAGCAGTGCGAGAGAGGGTC TCCGCATGCTGCAGAGAGCCGGGGTGCAGATCACTGTCATGA ACTATGAAGACTATTTCTACTGTTGGCAGACCTTTGTAGCTTG CAGACAGCGTGTGTTTAAGGCCTGGGACGGACTGCATCAGAA CTCTGTTCAACTGGCCAGGAAACTTAACGACATCCTCCAGCC

		TGGTGAGGCAGAAGATTGGGGAGATGCTTTTGAGCTACTTGG ACTGTGA
Igmμ heavy chain	anti-sense probe	GGCCGCGAATTCACTAGTGATTATGCATCTCTGAGGCACATT CAAGCTGAGGTTAACTAGGTTGGGTTGGTTTGAGGTTCTGTC AATGGTTCTCATAAGAATTTTTGTGGACTTGATCATGCTTTCG TGGTAAACTACACAGCTATAGACCACTTCTTCGTTCTTCCACA AGTCATTGCTAAATGTGAGCTGACTGTAGACAGAGTANGTCC TTCCTGATTGAATCTGGCTAGTGGTGTTGAATTGGTACAATGC TGAACTGCTCGTTCTCTCCACCGGCTCATCGTCAACAAGCCAA GCCACTAAAACGTCCTTGGGGTAGAAATCTTTGACGTACCAA GTCAGGGTCACCGTATTATCACTAGTTTGTTCTGCTGGAGCCA GCAGAAAGACAGATGGACGCTGTGGAACTCCTCCGGTCTCCC TCTTGTAGGCTTTCTTTACCAAGTCCCCCAGGTTTTCCATGTG ATCTACAGCGCAGTAGAATACTGTCCCATTGCTCCAGTCCTCA TAAGTGATGTCAAGTATGGCAATTTTGTCAGTGACACCCTTTC GGCTGGTTAAGGTCTTTCCATTGTCATTTTCCCATTTGACGCT CAGGAAGCCAGGAACTAGTTCATTGACATCACACACAAGCTG CGCTTTTTTGTTCATAAGCATATCCTCAAGAGACGGCTCGATG ATCGTAATGACTACTGAATGTCCATGGACTGGACCATCTGAT GAAGTGTAGCCCACAGTTCTCCTCACATTTCCAGCTTTGTTCT CAAACACGCAAGTGAATGTTACTTCTTCACTCTTCCACTCACT CTCATTGACTCTGAGATAGCTGGTTGTGCTGTACAGAGTTGTC TCACTCTTCTTCTCACTCTCACAARAACTCTTGAAATCAGATA CAACTTCTTGTTCTGTTCCTTTTTCCATCCTCATCCATTTGATT GTGTGTGTACGGGGTGAAAAGTCATTGGCAAAGCAGGCGAA GGAAGCCGTCTTATTTTCTGACATCTCCTCTTTASAGGGGGTC ATTACGTAAAGAGACGGCTGCTGCAGATATTCCGGTTGTTTTT TCACTGGTACTTTCTTTGATCCAGCAGAATGTTCCACGGCGCA CTCAAATTTTTTACTGTCCCAGTCTGCTCTCTTTACACGGAGT TGACTGACTCCCATGTARCTTCCACCGGTTTGGACCGCAGGGT ACTGAACGAAATCARTCAGGGAATTCCCGCCTTCGTCATTCC ATTTGAAGGTGAGGGAGGCAGGCGTGAAGCCAGTGGCAATG CAACCCAGAGTCATCATATCTCCGGTCCCGGAGCCACATTGC

		GCAAGAGGGAACAAAGTCGGACCAGTTGAWGAGGSTGTGGA CACGGTCACCATGGTCCCTTTCCCCCAGTAGTCAAAGTAGTTG TAGCTCCCAGTGTATCTGGCACAATAATACACAGCAGAGTCT TCAGTTTTCAGGCTGTTCATCTGGAGATACACCTGCTTCATGA TGTTGTGTCTGGAGATGGTGAACCGACCTTGAACAGACTGAG AGTAGGCAATATTTCTAATGTCATAATGCGCTGCCACAAATT NTAGTCCTTTACCAGGAGCCTGTATGATCCATGCAATCCAAT AGCCATTTATGTTCAAATTAGAGGCAGTACAGGTCAGTTTGT GGGTTTNTAAAGGCTTTTTAACCATTGGTCCAGACTCAGTCAG TTTTTGACTATGAACACCTATCAGTCTTATTATCATCAGTAAA AACACAGTAGTAAATGAATC
	sense probe (control)	GATTCATTTACTACTGTGTTTTTACTGATGATAATAAGACTGA TAGGTGTTCATAGTCAAAAACTGACTGAGTCTGGACCAATGG TTAAAAAGCCTTTANAAACCCACAAACTGACCTGTACTGCCT CTAATTTGAACATAAATGGCTATTGGATTGCATGGATCATAC AGGCTCCTGGTAAAGGACTANAATTTGTGGCAGCGCATTATG ACATTAGAAATATTGCCTACTCTCAGTCTGTTCAAGGTCGGTT CACCATCTCCAGACACAACATCATGAAGCAGGTGTATCTCCA GATGAACAGCCTGAAAACTGAAGACTCTGCTGTGTATTATTG TGCCAGATACACTGGGAGCTACAACTACTTTGACTACTGGGG GAAAGGGACCATGGTGACCGTGTCCACASCCTCWTCAACTGG TCCGACTTTGTTCCCTCTTGCGCAATGTGGCTCCGGGACCGGA GATATGATGACTCTGGGTTGCATTGCCACTGGCTTCACGCCTG CCTCCCTCACCTTCAAATGGAATGACGAAGGCGGGAATTCCC TGAYTGATTTCGTTCAGTACCCTGCGGTCCAAACCGGTGGAA GYTACATGGGAGTCAGTCAACTCCGTGTAAAGAGAGCAGACT GGGACAGTAAAAAATTTGAGTGCGCCGTGGAACATTCTGCTG GATCAAAGAAAGTACCAGTGAAAAAACAACCGGAATATCTG CAGCAGCCGTCTCTTTACGTAATGACCCCCTSTAAAGAGGAG ATGTCAGAAAATAAGACGGCTTCCTTCGCCTGCTTTGCCAAT GACTTTTCACCCCGTACACACACAATCAAATGGATGAGGATG GAAAAAGGAACAGAACAAGAAGTTGTATCTGATTTCAAGAG TTYTTGTGAGAGTGAGAAGAAGAGTGAGACAACTCTGTACAG

		CACAACCAGCTATCTCAGAGTCAATGAGAGTGAGTGGAAGA GTGAAGAAGTAACATTCACTTGCGTGTTTGAGAACAAAGCTG GAAATGTGAGGAGAACTGTGGGCTACACTTCATCAGATGGTC CAGTCCATGGACATTCAGTAGTCATTACGATCATCGAGCCGT CTCTTGAGGATATGCTTATGAACAAAAAAGCGCAGCTTGTGT GTGATGTCAATGAACTAGTTCCTGGCTTCCTGAGCGTCAAAT GGGAAAATGACAATGGAAAGACCTTAACCAGCCGAAAGGGT GTCACTGACAAAATTGCCATACTTGACATCACTTATGAGGAC TGGAGCAATGGGACAGTATTCTACTGCGCTGTAGATCACATG GAAAACCTGGGGGACTTGGTAAAGAAAGCCTACAAGAGGGA GACCGGAGGAGTTCCACAGCGTCCATCTGTCTTTCTGCTGGCT CCAGCAGAACAAACTAGTGATAATACGGTGACCCTGACTTGG TACGTCAAAGATTTCTACCCCAAGGACGTTTTAGTGGCTTGGC TTGTTGACGATGAGCCGGTGGAGAGAACGAGCAGTTCAGCAT TGTACCAATTCAACACCACTAGCCAGATTCAATCAGGAAGGA CNTACTCTGTCTACAGTCAGCTCACATTTAGCAATGACTTGTG GAAGAACGAAGAAGTGGTCTATAGCTGTGTAGTTTACCACGA AAGCATGATCAAGTCCACAAAAATTCTTATGAGAACCATTGA CAGAACCTCAAACCAACCCAACCTAGTTAACCTCAGCTTGAA TGTGCCTCAGAGATGCATAATCACTAGTGAATTCGCGGCC