

ARTICLE

The axillary lymphoid organ is an external, experimentally accessible immune organ in the zebrafish

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Lymph nodes and other secondary lymphoid organs play critical roles in immune surveillance and immune activation in mammals, but the deep internal locations of these organs make it challenging to image and study them in living animals. Here, we describe a previously uncharacterized external immune organ in the zebrafish ideally suited for studying immune cell dynamics in vivo, the axillary lymphoid organ (ALO). This small, translucent organ has an outer cortex teeming with immune cells, an inner medulla with a mesh-like network of fibroblastic reticular cells along which immune cells migrate, and a network of lymphatic vessels draining to a large adjacent lymph sac. Noninvasive high-resolution imaging of transgenically marked immune cells can be carried out in ALOs of living animals, which are readily accessible to external treatment. This newly discovered tissue provides a superb model for dynamic live imaging of immune cells and their interaction with pathogens and surrounding tissues, including blood and lymphatic vessels.

Introduction

Vertebrates use tightly coevolved adaptive and innate immune systems to combat pathogens. The innate immune system and its genetically encoded pathogen recognition receptors respond rapidly to invaders and their cytokine signals, helping tune the adaptive arm of the immune system to the type of pathogen present and influencing effector functions. Adaptive lymphocytes have highly specific somatically recombined receptors that, coupled with powerful effector functions, defend the host and impart memory for previously encountered pathogens. Pathogens are most frequently encountered in barrier tissues, where innate and adaptive immune cells work in concert to surveil the environment and protect the host.

Immune cells are born in primary lymphoid organs such as the bone marrow and thymus in humans and the head kidney

and thymus in fishes, but secondary lymphoid organs (SLOs) are the locations where innate immune cells alert lymphocytes to pathogens and induce an effector response. In higher vertebrates such as humans, the spleen, Peyer's patches, and lymph nodes are sites where lymphocytes are activated by cognate antigens presented by antigen-presenting cells (Neely and Flajnik, 2016). Germinal centers form in these SLOs when lymphocytes respond to antigens and proliferate, with B cells transcribing activation-induced cytidine deaminase (*aicda*) and undergoing affinity maturation with the help of T follicular helper cells to produce high-avidity antibodies (Victoria and Nussenzweig, 2022). Lymph nodes have a variety of characteristic features including a well-defined structure with an outer cortex and inner medulla, connection to draining lymphatic

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vascular networks, a mesh-like three-dimensional internal network of fibroblastic reticular cells (FRCs) facilitating immune cell trafficking, and specific sets of cytokines expressed by their resident cells that help direct lymphocyte homing and migration (Li et al., 2021; Siegert and Luther, 2012; Willard-Mack, 2006). Although fish lack organs that closely correspond to lymph nodes, they do have unique aggregations of adaptive lymphocytes where affinity maturation is thought to occur (Matz and Dooley, 2023). In fishes, the head kidney (anterior kidney) and spleen are sites of adaptive immune activation, complete with the presence of antigen and *aicda* expression (Shibasaki et al., 2023; Waly et al., 2022). A number of recent studies have also identified mucosa-associated lymphoid tissues (MALTs) in bony fishes, including organized nasal-associated lymphoid tissue (O-NALT), diffuse gill-associated tissues (gill-associated lymphoid tissue [GALT], interbranchial lymphoid tissue [ILT], amphibranchial lymphoid tissue [ALT], and Nemausean lymphoid organ [NELO]), and a bursal tissue (Bjorgen and Koppang, 2021; Dalum et al., 2021; Garcia et al., 2022; Løken et al., 2020; Resseguier et al., 2023). The cellular mechanisms and mechanics of lymphocyte activation in vivo remain unclear in part because SLOs are still challenging to image deep within mammals, although advances in intravital multiphoton imaging have led to important new insights from live imaging of immune cell trafficking in murine lymph nodes (De Giovanni et al., 2020; Junt et al., 2007; Shulman et al., 2013).

The adult zebrafish (*Danio rerio*) is an emerging immune model that offers the ability to readily carry out high-resolution optical imaging of immune cell trafficking in living animals (Castranova et al., 2021). In addition to a robust innate immune system, adult zebrafish have orthologous effector CD4 T cell subtypes, cytotoxic CD8 T cells, and B cells that produce IgM and mucosal IgT (Salinas, 2015). They also have a lymphatic vascular system with many of the anatomical, molecular, and functional features of mammalian lymphatics (Küchler et al., 2006; Yaniv et al., 2006). While the known lymphoid organs of adult zebrafish are too deep within the organism to image, more externally located immune cell populations can be visualized in living adult animals, including a recently described tessellated lymphoid network (TLN) in the skin (Robertson et al., 2023) although the anatomy of the TLN is not closely analogous to SLOs of mammals.

Here, we report a previously uncharacterized external lobe located bilaterally just above the base of the pectoral fin. These small translucent external lobes contain a plexus of blood vessels and a network of lymphatic vessels draining to a large adjacent lymph sac. They have an outer cortex containing large numbers of immune cells, and an inner medulla with an FRC network and other features resembling lymph nodes and other SLOs, suggestive of a role in immune surveillance. Based on these and other features described below, we designated these lobes “axillary lymphoid organs” (ALOs). ALOs can be imaged and are readily accessible to external treatment with antigens and pathogens in intact, living animals. They are also easily removed for ex vivo analysis, and they regenerate within a few weeks after removal. Together with a wide array of available zebrafish transgenic reporter lines marking numerous different immune,

vascular, and other cell populations, this newly discovered organ provides a superb model for high-resolution optical imaging of the interaction between immune cells, pathogens, and their surrounding tissues, including the vasculature.

Results

Identification of the ALO

Close examination of postmetamorphic zebrafish reveals a previously undescribed bilateral fleshy lobular structure immediately posterior to the operculum and just dorsal to the base of the pectoral fin, which we have denoted ALOs based on the findings we report below. ALOs are small and translucent, most commonly lacking pigment cells, but they can be readily visualized by confocal microscopy after soaking adult fish in a fluorescent surface stain such as BODIPY 630/650 (Fig. 1, A and B). Although most appear as unilobular appendages (Fig. S1 A), bilobed or multilobed ALOs are occasionally found in some animals (Fig. S1 B), and rarely, ALOs are found that do have pigment cells (Fig. S1 C). ALOs emerge comparatively late in development and are not found in embryonic or larval zebrafish. They first begin to make their appearance when fish reach around 10 mm in standard length, at ~30 days after fertilization, with lobes increasing rapidly in size over the next few weeks before reaching full size (Fig. 1, D–G), after which they persist throughout adulthood. Like many other adult zebrafish organs and tissues, ALOs are able to completely regenerate after amputation, regrowing within about 2 wk (Fig. 1, H–K and Video 1). Similar structures are found in other basal teleost fishes, including additional members of the order Cypriniformes, which, in addition to the zebrafish, includes Afro-Asian minnows (the families Danionidae and Xenocyprididae), loaches (the families Botiidae, Gastromyzontidae, Nemacheilidae, and Vaillantellidae), and their relatives. A survey of fixed teleost specimens revealed a diversity of lobe morphologies, with some fish species possessing substantially larger or more elaborate structures than the ALOs of zebrafish (Fig. 1, L–O; and Table S1).

Anatomical characterization of the ALO

To begin to examine the anatomical structure of the ALO, we collected transverse sections from fixed, paraffin-embedded whole adult zebrafish just caudal to the operculum, through the base of the pectoral fin (Fig. 2 A). External, bilaterally located ALOs are readily observed just dorsal to the base of the pectoral fin in stained sections (Fig. 2 B). Higher magnification images show that ALOs contain a clearly delineated and well-separated cell-rich cortical region and a relatively cell-deficient matrix-rich medullary region (Fig. 2 C). As noted above, most ALOs consist of a single lobe, but fish with lobes containing multiple medullary compartments (Fig. S1, D and E) or with multiple lobes (Fig. S1, F and G) are also noted in histological sections.

Alcian Blue/PAS, Movat's pentachrome, and H&E staining of sectioned ALOs suggest that a variety of different cell types are present in the cortex in distinct surface, mid-level, and basal epithelial layers, including mucus-secreting goblet-like cells near the cortex surface (arrows in Fig. 2, D and E) identifiable by Movat's pentachrome staining (Kotzé and Huyseune, 2020)

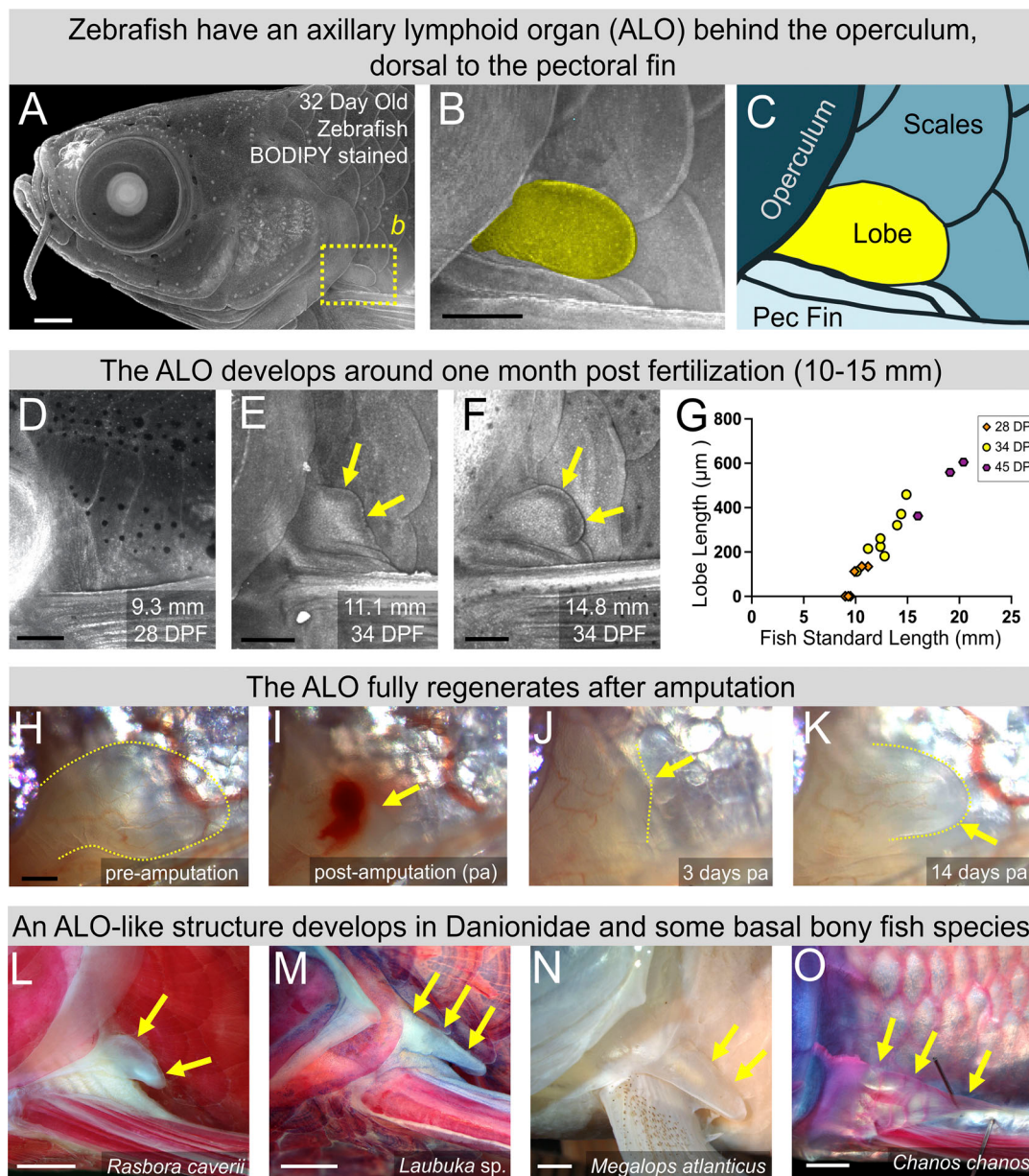


Figure 1. ALO in the zebrafish. (A) Maximum intensity projection confocal micrograph of the red fluorescent surface of a 32-day-old zebrafish soaked in BODIPY 633. (B) Magnified image of the boxed area in panel A, with the ALO pseudocolored yellow. (C) Schematic diagram of the anatomical features shown in panel B. (D–F) Maximum intensity projection confocal micrographs of 9.3-mm (28 days post fertilization [dpf]) (D), 11.1-mm (34 dpf) (E), and 14.8-mm (34 dpf) (F) juvenile zebrafish soaked in BODIPY 633, showing stages prior to ALO emergence, initial ALO budding, and further ALO expansion, respectively. (G) Measurement of ALO length (μm) versus fish standard length (mm) (28 dpf, $n = 6$; 34 dpf, $n = 8$; 45 dpf, $n = 3$). ALOs emerge when fish reach ~ 9 – 10 mm in body length. (H–K) Bright-field images of adult zebrafish ALO pre-amputation (H), immediately after amputation (I), 3 days after amputation (J), and 14 days after amputation (K), with yellow dashed lines and arrows marking the border of the ALO (regeneration, $n = 6$). (L–O) Images of ALOs found on other fish species, with yellow arrows noting the locations of the ALOs. Scale bars = 500 μm (A and H), 250 μm (B), 200 μm (D–F), 1 mm (L and M), and 2 mm (N and O).

(Fig. 2, C–F). We also carried out transmission electron microscopy (TEM) and array tomography on ultrathin sections of ALOs for a more detailed morphological assessment of the structure and ultrastructure of the ALO and its constituent cells (Fig. 2, G–I; and Figs. S1, S2, S3, and S4). Electron microscopy of the ALO cortex confirms its division into surface, mid-level, and basal epithelial layers populated by cells displaying distinct morphologies (Fig. 2 H and Fig. S1). The most superficial cells of the ALO cortex are flattened epithelial cells exposed to the outer environment with abundant

numbers of “microridges,” actin-rich surface protrusions arranged in unique fingerprint-like patterns (Fig. S1, H–J) that have been observed on surface skin cells of many different fish species (Bereiter-hahn et al., 1979). The mid-cortex contains mostly epithelial-like cells, although a few other cell types are also present, notably large cells resembling previously reported club cells (see further discussion of club cells in “Additional resident cells...” section, below). The basal cortical layer contains mostly pyramidal or cuboidal epithelial cells with their apices pointing toward the outside

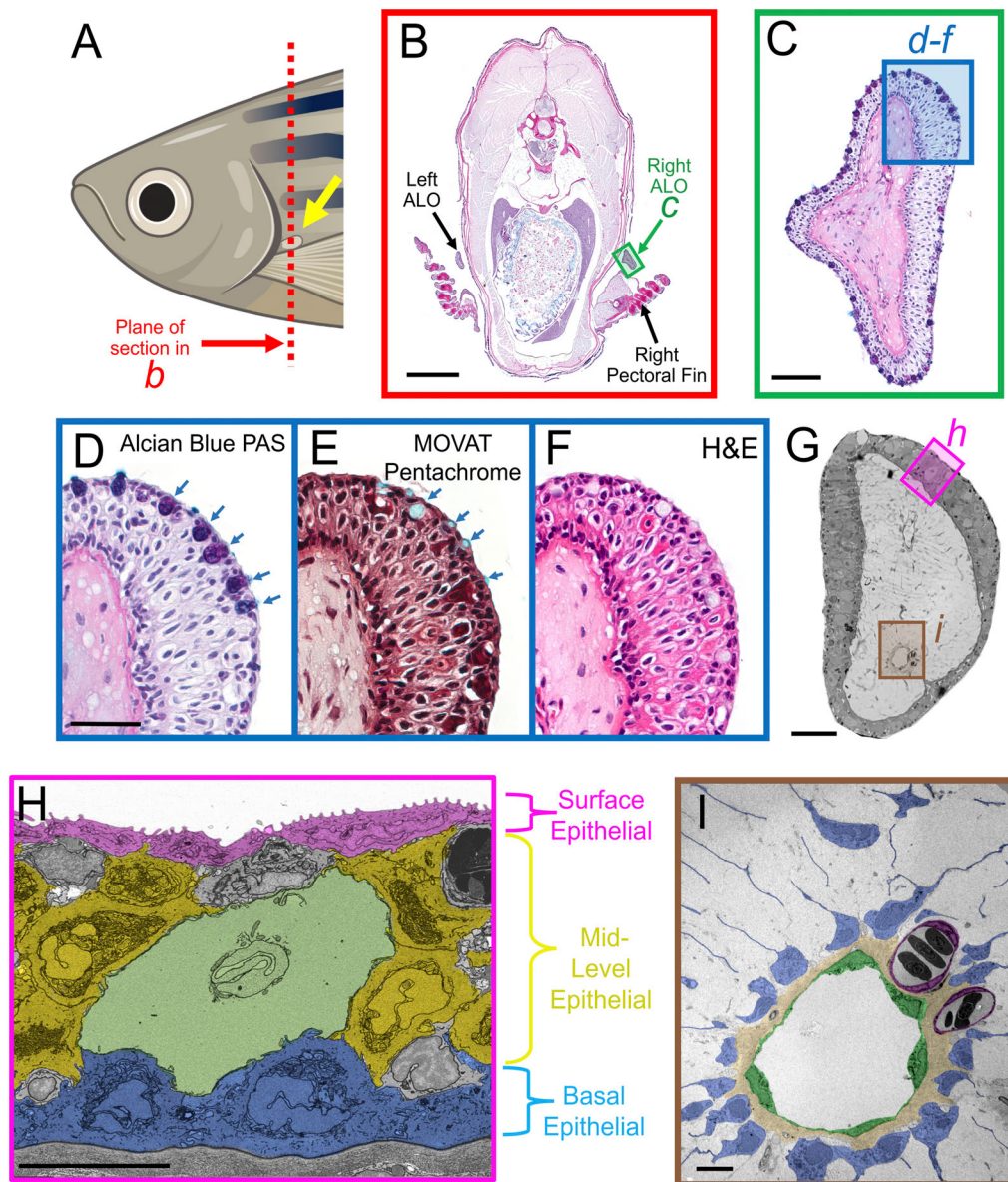


Figure 2. Histology and electron microscopy of the ALO. Histological characterization of ALO morphology. **(A)** Schematic diagram showing the plane of section in panel B. **(B)** Alcian Blue/PAS-stained (stained acid mucins in purple) transverse paraffin section through the anterior trunk of an adult zebrafish. The green box indicates the region shown in panel C. **(C)** Magnified image of the boxed region in panel B, showing the right ALO. The blue box indicates the region shown in panel D. **(D–F)** Magnified images of serial transverse paraffin sections through the anterior trunk of an adult zebrafish stained with Alcian Blue/PAS (D), Movat's pentachrome (E), and H&E (F, also shown in Fig. 8, G and H) with blue arrows highlighting mucus-producing goblet cells (D and E). Panel D shows the boxed region in panel C. **(G)** Transmission electron micrograph of a transverse section through an adult zebrafish ALO. The boxed regions show areas with comparable (h) or actual (i) magnified TEM images in subsequent panels. **(H)** TEM showing the dermal cortex of the ALO with presumptive surface epithelial cells pseudocolored magenta, mid-level epithelial cells yellow, club cell green, and basal epithelial cells blue. **(I)** Magnified TEM image of box i in panel G, showing the medulla of the ALO with lymphatic vessel pseudocolored green, blood vessels magenta, and FRCs blue (I). Histology, $n = 3$; TEM, $n = 2$. Scale bars = 1 mm (B), 50 μm (C), 25 μm (D), 100 μm (G), 10 μm (H and I).

of the ALO, and with their bases immediately apposed to a well-defined basement membrane separating cortical layers of the ALO from the deeper ALO medulla (Fig. S1, K–S). The ALO medulla is composed largely of acellular matrix, but also contains unusual cells with thin, highly elongated, mostly radially arranged sheetlike reticular processes (Fig. 2 I). Many but not all cell bodies of these medullary reticular cells are embedded in a collagenous matrix layer immediately adjacent to endothelial-lined lymphatic vessels that are distinguishable from blood vessels by their lack of red blood cells

(Fig. 2 I, and see discussion of Fibroblastic reticular cells below). In addition to TEM on individual ALO sections, we also carried out volume electron microscopy by array tomography to compile detailed 3D ultrastructural visualization of ALOs and their cell populations (Video 2).

Single-cell analysis of the ALO

To estimate the number of cells present in an average adult ALO, we carried out confocal imaging of 4 Hoechst nuclear dye-

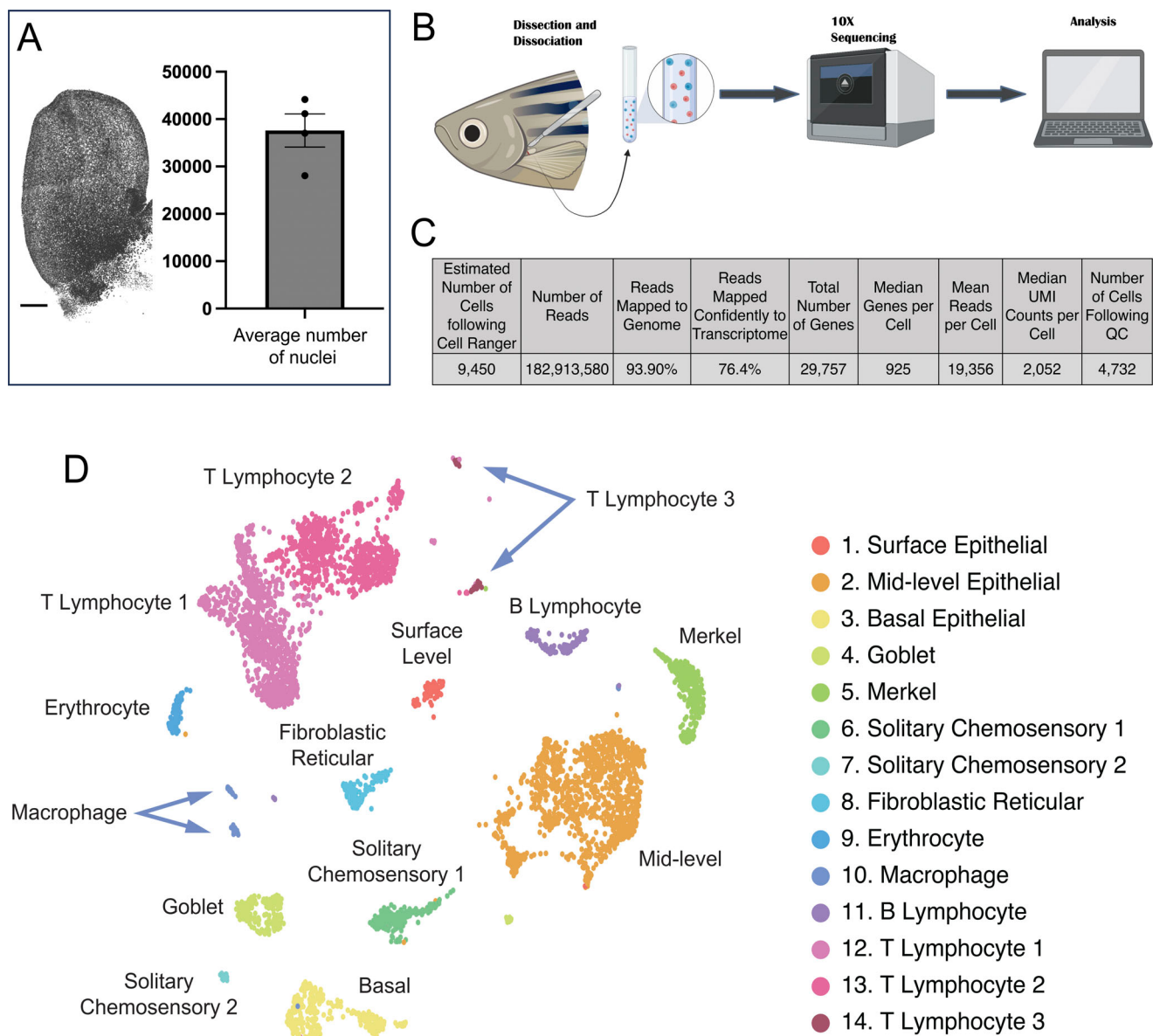


Figure 3. scRNAseq of the ALO. (A) Confocal maximum intensity projection image of an ALO with nuclei labeled with Hoechst and a bar graph showing the average number of nuclei counted in four ALOs. (B) Schematic diagram showing the workflow for ALO scRNAseq; eight ALOs (from two males and two females) were processed. (C) Metrics for the ALO scRNAseq procedure. (D) UMAP plot of data from the ALO scRNAseq procedure, with 14 clusters annotated by cell identity. Scale bar = 300 μ m (A).

stained dissected ALOs followed by automated counting of nuclei. This revealed an average of 37,600 cells per lobe (Fig. 3 A), from which we conservatively estimate there are around 50,000–100,000 cells per ALO considering tissue loss during dissection and undercounting of densely packed cortical nuclei. To better understand the cellular composition of the ALO, we used the 10X Genomics Chromium platform to carry out single-cell RNA sequencing (scRNAseq) on a cell suspension prepared from four ALOs (two from males and two from females) removed from adult zebrafish (Fig. 3 B). An estimated total of 9,450 cells were sampled with 182,913,580 total sequence reads, with 93.9% of reads mapped to the genome and 76.4% of reads mapped to the zebrafish transcriptome. This represented 19,356 mean sequence reads per cell, 2,052 median UMI counts per cell,

and 925 mean genes per cell (Fig. 3 C). Unsupervised clustering using Seurat (Butler et al., 2018) identified 14 separate clusters that could all be definitively annotated as identifiable cell populations (Fig. 3 D) based on their expression of characteristic cell type-specific genes, overall gene expression profiles, comparison with genes expressed in other single-cell data sets, notably DanioCell (Sur et al., 2023), and, importantly, spatial localization of cluster-specific transcripts to morphologically identifiable cells, all as discussed below.

Epithelial cells of the ALO cortex

Clusters 1–3 correspond to resident surface, mid-level, and basal epithelial cell populations in the ALO cortex (Fig. 4, A–C; and Video 2), as confirmed by the methods noted above including

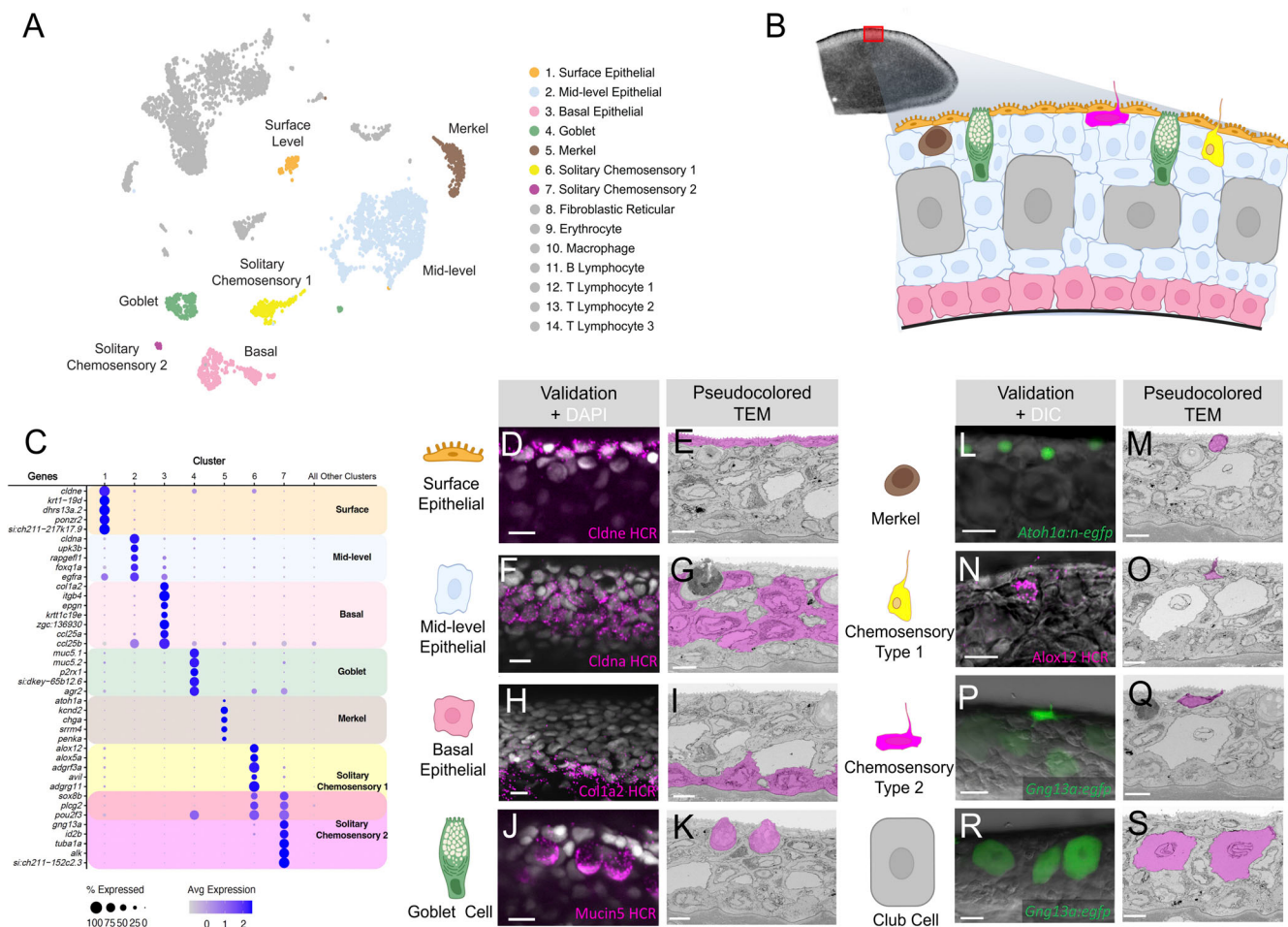


Figure 4. Resident cell types of the ALO dermal cortex. (A) UMAP plot of ALO scRNAseq data highlighting seven clusters that include resident cell types of the ALO cortex. (B) Schematic diagram of the ALO cortex (comparable to the area marked by the red box in the ALO confocal image at upper left), with the cell types represented by each of the highlighted clusters in panel A shown using the same colors. (C) Dot plot showing the relative expression of genes used to identify and characterize clusters corresponding to resident cell types of the ALO cortex. (D, F, H, J, L, N, P, and R) Confocal micrographs of the cortex of HCR stained (D, F, H, J, and N) or transgene-expressing (L, P [also shown in Fig. S3 G], and R) ALOs isolated from adult zebrafish. (E, G, I, K, M, O, Q, and S) Pseudocolored 2D sections from an array tomography section of the ALO cortex with the same cell types shown in the adjacent confocal image panels highlighted in pink. The confocal images and electron micrographs show surface epithelial cells (D and E), mid-level epithelial cells (F and G), basal epithelial cells (H and I), goblet cells (J and K), Merkel cells (L and M), chemosensory type 1 cells (N and O), chemosensory type 2 cells (P and Q), and club cells (R and S). HCR, $n = 4$; transgenic imaging, $n = 4$; TEM, $n = 2$. Scale bars = 10 μm (D, F, H, J, L, N, P, and R) or 5 μm (E, G, I, K, M, O, Q, and S).

in situ hybridization chain reaction (HCR) (Schwarzkopf et al., 2021) using genes highly enriched in each of these cell clusters (Fig. 4 C). Cluster 1 represents surface epithelium (Fig. 4, A and B). This cluster specifically expresses *claudin e* (*cldne*) and *krt1-19d* (Fig. 4 C), both of which are previously reported markers of surface epithelium in the zebrafish fin (Hou et al., 2020). HCR confirms that *cldne* expression is restricted to ALO surface epithelial cells (Fig. 4 D). *dhrs13a.2*, *si:ch211-217k17.9*, and *ponzr2*, genes enriched in periderm in the Daniocell data set (Sur et al., 2023), are also highly specific to this cluster (Fig. 4 C). These cells form a flattened epithelial monolayer on the surface of the ALO, with prominent characteristic apical microridges (Bereiter-hahn et al., 1979) and tight cell-cell junctions (Fig. 4 E, Fig. S1, H–M; and Video 2).

Cluster 2 corresponds to mid-level or intermediate cortical epithelial cells (Fig. 4, A–C; Fig. S1, N–P; and Video 2). The previously characterized intermediate epidermal marker *claudin*

a (*cldna*) (Hou et al., 2020) is highly enriched in this cluster (Fig. 4 C), and HCR confirms *cldna* expression is localized to ALO mid-level epithelial cells (Fig. 4 F). Other genes highly enriched in this cluster include *epidermal growth factor receptor a* (*egfra*), a gene involved in positive regulation of epithelial cell proliferation and inflammatory/immune reactions in mammalian skin (Pastore et al., 2008), and *foxq1a*, a gene found in periderm in the developing zebrafish (Sur et al., 2023) (Fig. 4 C). Mid-level epithelial cells have a varied appearance and morphology in TEM images (Fig. 4 G and Video 2), although most have abundant cell-cell adherence junctions (Fig. S1, N and O) and intracellular fibrillar networks (Fig. S1 P).

Cluster 3 contains basal epithelial cells of the ALO cortex (Fig. 4, A–C; Fig. S1, Q–S; and Video 2). Previously identified zebrafish tail fin basal epidermal markers *colla2* and *krttlc19e* (Hou et al., 2020) are highly enriched in this cluster (Fig. 4 C), and HCR shows *colla2* is restricted to the deepest epithelial layer

of the ALO cortex, adjacent to the basal lamina (Fig. 4 H). Basal epithelial cells also specifically express epidermal-enriched *itgb4* and *epgn* genes (Sur et al., 2023), further confirming their epithelial identity (Fig. 4 C). *Epgn* (epigen or epithelial mitogen) is predicted to enable epidermal growth factor receptor binding activity and growth factor activity, and this cluster is enriched for genes involved in Rho GTPase signaling. Many basal epithelial cells have a roughly pyramidal morphology (Fig. 4 I and Video 2), with a centrally located nucleus with abundant adjacent Golgi localized to the externally facing side of the nucleus. The basal epithelial cell layer is immediately adjacent to a well-defined basal lamina (Fig. S1, Q and R, red arrows) and underlying thick extracellular matrix layers that separate the ALO cortex from the ALO medulla, and together, they form a monolayer with abundant tight cell-cell junctions (Fig. S1 S, yellow arrow).

Additional resident cell types of the ALO cortex

In addition to epithelial cells, several other resident cell types are also present in the ALO cortex (Fig. 4, A and B; and Figs. S2, S3, and S4). Cluster 4 represents mucus-producing goblet cells (Fig. 4, J and K; and Fig. S2, A–H), readily identified by their specific expression of the *muc5.1* and *muc5.2* mucin genes (Jevtov et al., 2014) (Fig. 4 C). Mucins are expressed in intestinal goblet cells (Pelaseyed et al., 2014) and in analogous epidermal mucus-producing cells of other organs and tissues such as the respiratory and reproductive tracts, and in fish skin (Jevtov et al., 2014), where they play an important role in protection from infection. HCR for *muc5.1* confirms the expression of this gene localizes to morphologically distinctive goblet-like cells that display characteristic ultrastructural features of this cell type including internal contents spilling out from the ALO surface (Fig. 4, J and K; Fig. S2, A–E; and Videos 2 and 3). Cluster 4 also strongly and specifically expresses *p2rx1*, *fer1l6*, and *si:dkey-65b12.6* (Fig. 4 C), each of which is highly enriched in epidermal mucus-secreting cells in the Danio cell scRNAseq data set (Sur et al., 2023). The *agr2* gene is most highly expressed in cluster 4, although it is also weakly expressed in clusters 6 and 14. *Agr2* is a protein disulfide isomerase found in mammalian intestinal goblet cells important for proper processing of gel-forming mucins in humans (Al-Shaibi et al., 2021). Cluster 4 is also the cluster most strongly expressing interleukin-13 receptor *il13ra1*, although again it is expressed at lower levels in clusters 6, 7, and 11. IL-13 is a cytokine known to stimulate goblet cell differentiation and goblet cell mucus production (Tukler Henriksson et al., 2015). In addition to their passive role in immune defense via mucus production, goblet cells may also play more active roles in immunity, including taking up and presenting antigens to underlying antigen-presenting cells that induce adaptive immune responses (Yang and Yu, 2021). Interestingly, zebrafish ALO goblet cells also possess a robust capacity to take up exogenous substances (Fig. S2, F–H).

Cluster 5 consists of Merkel cells (Fig. 4, A–C; and Fig. S2, I–O), as confirmed by their previously described highly specific expression of an *atoh1a:nls-Eos* transgene (Brown et al., 2023), and by their characteristic location and morphology (Brown et al., 2023; Pickett et al., 2018) (Fig. 4, L and M). Merkel cells

are mechanosensory cells sensitive to gentle touch stimulation known to express neuroendocrine markers such as *chromogranin-a* (*chga*), which like *atoh1a* is also highly expressed in and highly specific for ALO cluster 5 (Fig. 4 C). Cluster 5 cells also express neural- and sensory-associated genes such as *kcnd2*, *srnm4*, and *penka* (Fig. 4 C). Like their mammalian counterparts (Iggo and Muir, 1969), zebrafish Merkel cells have a number of characteristic morphological and ultrastructural features including their location just under the surface epithelium, small size and relatively round cell shape, and ventral cellular projection (Fig. S2, I–O; and Video 2).

Clusters 6 and 7 correspond to two classes of solitary chemosensory cells (SCCs) (Fig. 4, A–C, Fig. S3, and Video 3). Cluster 6 type 1 “tuft-like” SCCs (SCC1) show highly specific expression of known tuft cell markers such as *avil* and leukotrienes *alox5a* and *alox12* (Fig. 4 C). ALO HCR shows *alox12* is expressed in cells with cell bodies located just under the surface epithelium that have sensory-like apices protruding out through the epithelium (Fig. 4, N and O; and Fig. S3, C and D). Tuft cells sense external stimuli at mucosal barriers, responding by secreting effector molecules such as leukotrienes capable of evoking neural, immune, and other responses (McGinty et al., 2020). Cluster 7 type 2 “taste-like” SCCs (SCC2) show high expression of *gng13a* and *id2b*, which are highly expressed in taste cells in the Danio cell data set (Sur et al., 2023) (Fig. 4 C). Cluster 7 SCC2 cells are specifically marked by the robust expression of a *gng13a:egfp* transgene (Fig. 4, P and Q; and Fig. S3, A, B, and E–G). The two SCC types also share the expression of several characteristic genes such as *sox8b* and *plcg2* (Fig. 4 C). SCCs with apical extensions penetrating through the surface epithelium were readily identified by differential interference contrast (DIC) imaging (Fig. S3, D and G). SCC-like cells were also observed in TEM and array tomography images of the ALO (Fig. S3, H–K; and Video 3), although ultrastructural subtypes corresponding to the *alox12* HCR-positive (SCC1) and *gng13a* HCR- or *gng13a:egfp* transgene-positive (SCC2) cell clusters could not be readily distinguished. Interestingly, TEM array tomography showed that the SCC-like cell invariably had two separate apical projections protruding through the surface epithelium, generally positioned on opposite ends of the cell (Fig. S3, I–K; and Video 3).

In addition to the seven cortical cell types corresponding to the identified scRNAseq clusters noted above, histological and ultrastructural imaging of the ALO also revealed abundant numbers of large, mostly ovoid cells located within the mid-level epithelium (Fig. 4, B, R, and S; Fig. S4 A; and Video 2). These large cells also have a homogeneous-appearing cytoplasm and centrally located, sometimes binucleate or more complex-shaped nuclei (Fig. 4, R and S; Fig. S4, A–C; and Video 2), all features indicative of club cells, an unusual cell type described in mammalian lungs (Zuo et al., 2018) and in fish skin (Henrikson and Matoltsy, 1967), including in zebrafish (Alesci et al., 2022; Chia et al., 2019; Chivers et al., 2007). In fish, club cells are reservoirs for a “fright substance” (Schreckstoff) released when fish are injured that elicits fear responses in nearby conspecific animals (Chia et al., 2019), and they have also been implicated in innate or acquired immunity (Alesci et al., 2022; Chivers et al., 2007). HCR staining with genes specific for each of the clusters

identified in our single-cell analysis (Fig. 3 C) failed to mark the club cell population, suggesting that these large cells were not captured in our single-cell analysis. Interestingly, confocal imaging shows that these cells are very weakly positive for the same *gng13a:egfp* transgene that strongly marks SCC2 cells, allowing them to be visualized by confocal imaging (Fig. 4, R and S; and Fig. S4, D–F).

Vasculature of the ALO medulla

As noted above, the highly cellular, densely packed ALO cortex surrounds a medulla composed of a largely acellular matrix with a sparsely interspersed network of highly elongated cells (Fig. 2). Although vasculature is absent from the ALO cortex, the medulla contains networks of blood and lymphatic vessels readily visualized using *Tg(kdrl:mcherry)^{y206}* and *Tg(mrc1a:eGFP)^{y251}* transgenic reporter lines (Fujita et al., 2011; Jung et al., 2017) labeling blood (magenta) and lymphatic (green) endothelium, respectively (Fig. 5, A–C). Circulating nucleated zebrafish red blood cells marked by intravascular injection of Hoechst nuclear stain into the caudal axial vasculature (blue) circulate robustly through the blood vessels (magenta) but not through the lymphatic vessels (green), confirming their identification (Fig. 5, B–D; and Video 4). The identification of lymphatic vessels is further confirmed by injection of Qtracker 705 Vascular Labels (quantum dots) into the ALO medulla (Fig. 5 E). The quantum dots (magenta) are specifically taken up by the lymphatic vessels and drained efficiently into a large lymph sac (arrow in panel G) located nearby but deeper in the body of the fish (Fig. 5, F and G). Lymphatics in other regions of the fish head and anterior trunk are also linked to the ALO via this large lymph sac, as shown by quantum dot drainage into the sac after injection into the anterior trunk body wall (Fig. 5, H–J). Blood and lymphatic vessels in the ALO medulla can both be visualized ultrastructurally in TEM images (Fig. 5, K–M), with characteristic tight junctions (Fig. 5, L and M). Vessels are enveloped by a matrix-rich layer, and lymphatic vessels in particular are surrounded by numerous cell bodies of unusual medullary reticular cells (Fig. 2, G and I), described below.

FRCs of the ALO medulla

In addition to blood and lymphatic vessels, the medullary core of ALOs contains an elaborate, mesh-like network of cells linked together by long, thin tortuous sheetlike cellular projections that resemble FRC networks found in mammalian lymph nodes and other lymphoid organs, where they help to organize and direct immune cell migration and activity (Li et al., 2021; Siegert and Luther, 2012) (Fig. 2, G and I; Fig. 6; and Video 5). ALO scRNAseq reveals a single distinct cluster (cluster 8) that strongly expresses *platelet-derived growth factor receptor beta* (*pdgfrb*) and *vimentin* (*vim*), both highly enriched in mammalian FRCs (Li et al., 2021) (Fig. 6, A and B), and confocal imaging of ALOs in a *TgBAC(pdgfrb:egfp)^{ncv22}* transgenic reporter line (Boezio et al., 2020) shows that FRCs are strongly EGFP-positive (Fig. 6, C–G; and Video 5), as also are pericytes identified by their location and morphology surrounding ALO blood vessels (Fig. 6, F and G, pseudocolored magenta in Fig. 6 G). Cluster 8 also strongly expresses *spock3*, a gene expressed in the developing zebrafish

thymus (Rubin et al., 2022), and *spock3* HCR specifically marks medullary FRCs in zebrafish ALOs, further validating the identity of these cells (Fig. 6, B, H, and I). In mammalian SLOs, FRCs express chemokine ligands that provide guidance cues to direct and organize lymphocyte migration (Li et al., 2021). Zebrafish ALO FRC cluster 8 similarly strongly expresses several different chemokines including *cxcl12a* and *ccl25b* (Fig. 6 B), which are likely signaling to different immune cell populations present in the ALO-expressing cognate chemokine receptors (see the section below for discussion of ALO immune cells). Live DIC imaging of the ALO medulla shows macrophages and other presumptive immune cells migrating actively along FRCs (Fig. 6 J and Video 6), and TEM images show very close association between FRCs (blue) and presumptive immune cells (magenta) in the medullary matrix (Fig. 6, K and L) and adjacent to medullary lymphatic vessels (Fig. 6, M and N). Interestingly, most FRC bodies are closely juxtaposed to lymphatic vessels and the matrix surrounding these vessels (Fig. 6 O and Video 6), suggesting there may also be communication and cellular interaction between FRCs and lymphatic endothelial cells.

The zebrafish ALO contains large numbers of immune cells

In addition to epithelial, sensory, and stromal cells, scRNAseq of the ALO reveals large numbers of hematopoietic cells in clusters 9–14, including erythrocytes, macrophages, B cells, and several distinct classes of T cells (Fig. 7, A and B). 2,052 of 4,732 cells captured in our scRNAseq data set are blood cells, with lymphocytes alone accounting for 41.6% (1,972/4,732) of the cells, or an estimated 20–40,000 lymphocytes per lobe. Cluster 9 cells are easily identified as erythrocytes by their specific expression of hemoglobins *hbaa1* and *hbba1*, as well as other genes (Fig. 7 B; fish erythrocytes are nucleated and transcriptionally active).

Cluster 10 specifically expresses many well-known diagnostic markers of macrophages, including *macrophage expressed gene 1.1* (*mpeg1.1*), *colony-stimulating factor 1 receptor* (*csflra*), *macrophage receptor with collagenous structure* (*marco*), and *granulin 1* (*grn1*) (Fig. 7, A and B). The Toll-like receptor 2 (*tlr2*) gene is also specifically expressed in the macrophage cluster, in alignment with the role of macrophages as pathogen detectors (Fig. 7 B). Cluster 10 macrophages also strongly express *mhc2dab*, reflecting their function as antigen-presenting cells (Fig. 7 B). Confocal imaging of a *Tg(mpeg1.1:egfp)^{g122}* transgenic line (Cunha et al., 2020) shows that ALO macrophages are found primarily in the cortex (Fig. 7, C–F), although an occasional macrophage can be seen in the medulla migrating along FRCs (Fig. 7 G and Video 6). Live time-lapse imaging of *Tg(mpeg1.1:egfp)^{g122}* transgenics reveals two distinct phenotypes—highly motile cells that can be observed moving between the cortex and medulla, and less motile cells in the cortical layer of the ALO with many cellular extensions protruding in all directions (Fig. 7, E and F; and Video 7).

The large clusters 11–14 include several different lymphocyte cell populations (Fig. 7, A and B). Cluster 11 is a small cluster of B lymphocytes, expressing various immunoglobulin components (*cd79b*, *igllc3*, *iglv5*), as well as *pax5*, known to mark commitment to B cell fate in mammals, and *blnk*, known to be a part of B cell activation signaling (Fig. 7 B) (Fu et al., 1998; Nutt and Kee,

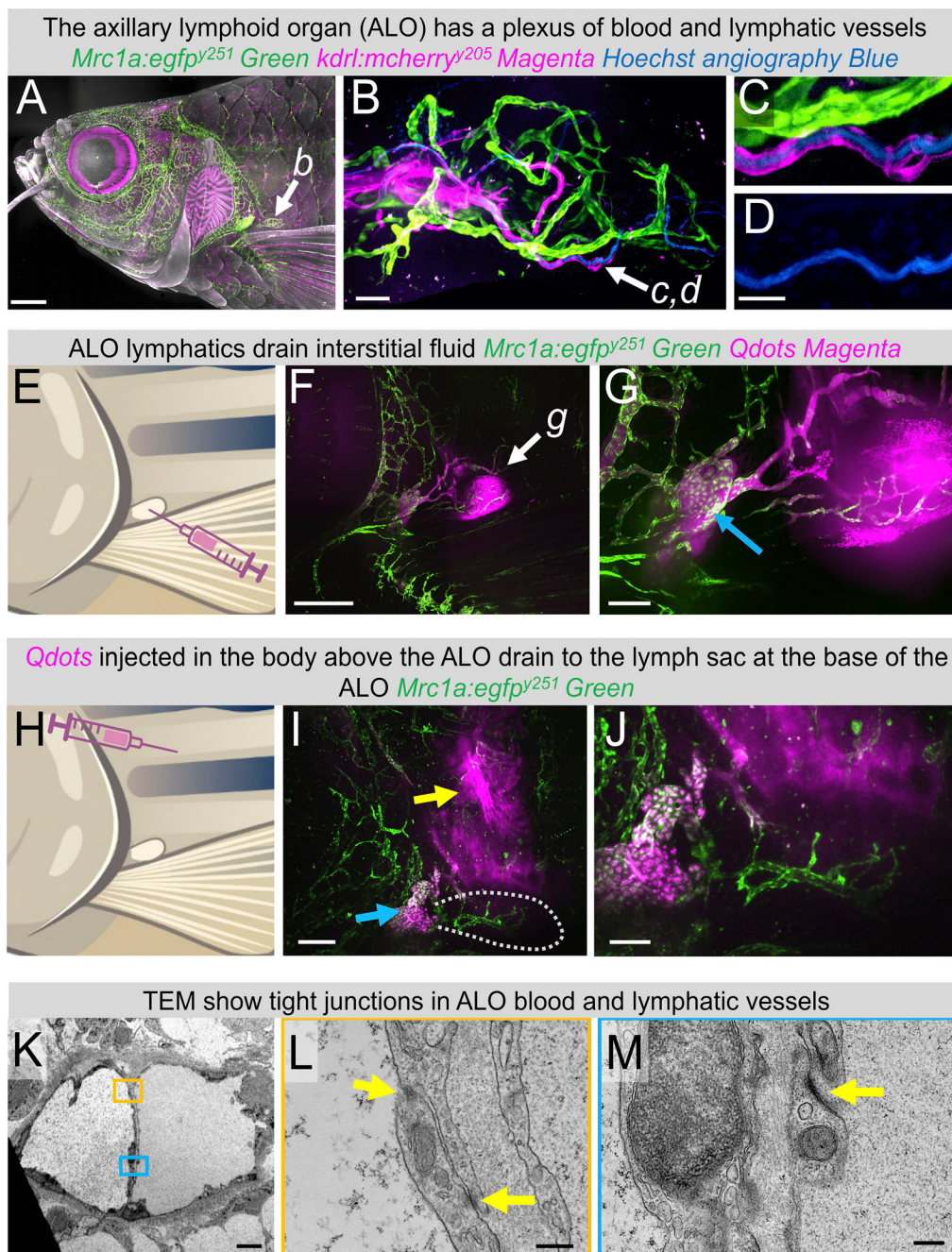


Figure 5. ALO contains a network of blood and lymphatic vessels. (A–D) Confocal micrographs of an adult *Tg(mrc1a:egfp)*^{y251}, *Tg(kdrl:mcherry)*^{y205} double transgenic zebrafish with *mrc1a*-positive lymphatics in green and *kdrl*-positive blood vessels in magenta. This animal was also injected intravascularly with a Hoechst 33342 dye, marking blood cell nuclei with blue fluorescence. Images include (A) an overview image of the head, with the position of the ALO noted; (B) higher magnification image of the area in panel A noted by an arrow, showing a network of blood (magenta) and lymphatic (green) vessels in the ALO, with blood circulation (blue) in blood vessels; and (C and D) higher magnification images of the area noted by an arrow in panel B, showing that blood vessels (magenta) but not lymphatics (green) contain circulating RBCs (blue). (E and H) Schematic diagram of procedure for intralobular (E) and anterior body (H) injection of quantum dots. (F, G, I, and J) Confocal micrographs of an adult *Tg(mrc1a:egfp)*^{y251} zebrafish ALO after intra-organ injection of 705-nm quantum dots (Qdots), with *mrc1a*-positive lymphatics in green and Qdots in magenta. Panels F and I show an overview of the ALO vicinity, noting the higher magnification area shown in panels G and J. The lobe-injected (F and G) and body-injected Qdots (I and J) drain via lymphatics into an adjacent deeper large lymph sac at the base of the ALO, noted with a blue arrow in panels G and I. (K–M) Transmission electron micrographs of ALO vessels, showing an overview of adjacent vessels (K) and higher magnification images of vessel cell–cell junctions (L and M). ALO vasculature imaging, *n* = 8, with Hoechst, *n* = 2, ALO lobe lymphatic drainage, *n* = 5, anterior lymphatic drainage, *n* = 6. Scale bars = 1 mm (A), 500 μm (F), 200 μm (I), 100 μm (G and J), 50 μm (B), 25 μm (D), 5 μm (K), 300 nm (L and M).

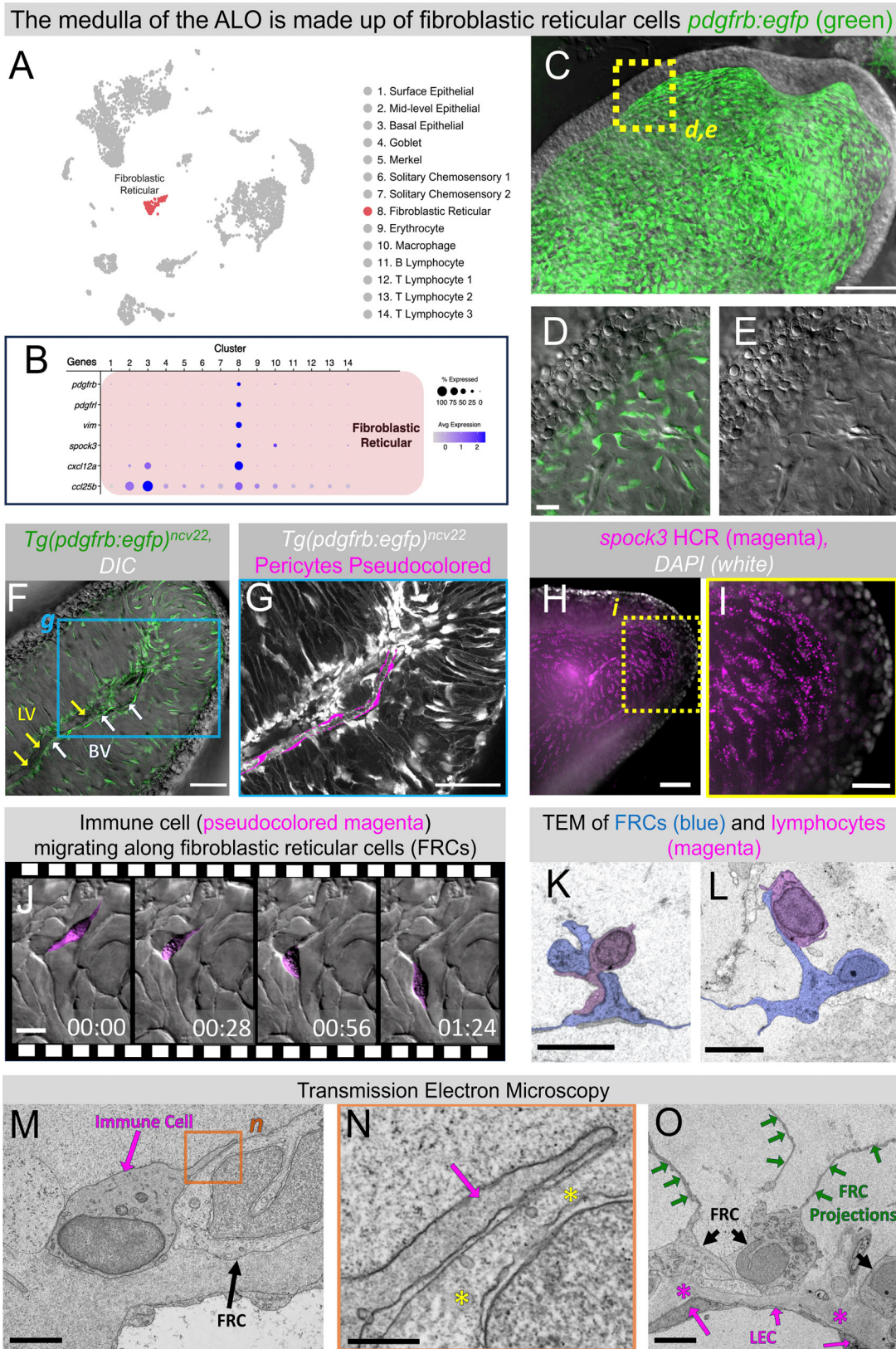


Figure 6. ALO medulla is made up of FRCs. (A) UMAP plot of ALO scRNAseq data highlighting the medullary FRC cluster. (B) Dot plot showing the relative gene expression of genes used to identify and characterize the FRC cluster. (C–G) Confocal (C and D) and DIC (E) micrographs of an ALO excised from an adult *Tg(pdgfrb:EGFP)^{ncv22}* transgenic zebrafish with green fluorescent FRCs. Panel C shows an ALO overview image, and panels D and E show higher magnification images of the yellow boxed area in panel C. Panel F shows an extended depth-of-focus image with DIC, and panel G shows a higher magnification image of the boxed region in panel F, showing FRCs in white and pericytes surrounding a blood vessel (also labeled by the transgene) pseudocolored magenta. (H and I)

Confocal micrographs of the ALO of an adult zebrafish subjected to HCR for *spock3* (magenta), with DAPI counterstain shown in white. Panel I shows a higher magnification image of the boxed area in panel H. **(J)** Successive images from a time-lapse DIC video micrograph of an immune cell (pseudocolored magenta) migrating along the FRC network. Images are selected frames from Video 6. **(K–O)** Transmission electron micrographs of the ALO medulla. Panels K and L show immune cells (pseudocolored magenta) in close apposition to FRCs (pseudocolored blue). Panels M and N show an immune cell (magenta arrow) closely apposed to an FRC body (black arrow) embedded in the matrix surrounding a lymphatic vessel. Panel N shows a magnified image of the boxed region in panel M, with an immune process (magenta arrow) extending across the FRC (yellow asterisks). Panel O shows three FRC bodies (black arrows) surrounding a lymphatic vessel (lymphatic endothelium noted with magenta arrows), with FRC lamellar extensions noted with green arrows and the thick matrix layer surrounding the vessel noted with magenta asterisks. Transgenic imaging, $n = 7$; HCR, $n = 4$; TEM, $n = 2$. Scale bars = 100 μm (C), 20 μm (D and I), 50 μm (F–H), 10 μm (J), 5 μm (K and L), 2 μm (M), 500 nm (N), 4 μm (O).

2007). Confocal imaging with a *Tg(cd79b:egfp)^{fcc89}* transgenic B cell reporter line (Liu et al., 2017) shows B lymphocytes scattered throughout the cortex and medulla at homeostasis (Fig. 7, H and I). Live time-lapse imaging of *cd79b*-positive B lymphocytes reveals multiple distinct cellular behaviors (Video 8). Some B lymphocytes migrate rapidly, sending numerous cellular protrusions in all directions (Fig. 7 J and Video 8) (Pierce, 2009), while other B lymphocytes remain more spherical and stationary, although they can still be observed continuously extending smaller filopodia (Fig. 7 K and Video 8). B lymphocytes are also readily observed migrating actively on the FRC network (Fig. 7 L and Video 8). Relatively few myeloid cells were captured in our scRNAseq data set, and distinct clusters for other myeloid cell types such as neutrophils were not detected, although confocal imaging using a *Tg(lyz:DsRed2)^{nz50}* neutrophil reporter line reveals a few of these cells in each ALO (Fig. S4 G and Video 7), suggesting small numbers of other myeloid cell types may be present.

Clusters 12–14 represent different subsets of T lymphocytes, as confirmed by their common expression of diagnostic markers such as T-cell receptor complex genes *lck* and *zap70* (Fig. 7, A and B). Cluster 12 (T lymphocyte 1) expresses transcription factor *tcf7*, known to be present in both naïve and memory T cells (Pais Ferreira et al., 2020), while cluster 13 (T lymphocyte 2) specifically expresses genes associated with cytotoxic lymphocytes, including *perforin1.1* (*prfl.1*) and *t cell receptor gamma* (*tcrg*) (Fig. 7 B). The final small cluster of cells, cluster 14 (T lymphocyte 3), appears to be proliferating lymphocytes based on the expression of many genes associated with cell cycle activation (*mki67*, *topa*, *pna*). A small subset of all three T lymphocyte clusters also expresses *ccr7*, a gene associated with migratory T cells (Bromley et al., 2005). Confocal imaging of the ALO using T lymphocyte-specific *Tg(lck:egfp)^{cz2}* reporter fish (Langenau et al., 2004) reveals strikingly enriched localization of these cells near the basal cortex of the ALO (Fig. 7, M and N; and Video 9), consistent with CellChat identification of chemokine signaling between ALO basal epithelial cells and lymphocyte cell populations (Fig. 7 V, discussed below). Live time-lapse imaging of *Tg(lck:egfp)^{cz2}* ALOs shows that *lck*-positive lymphocytes are highly active, rapidly moving cells (Video 9). Many cells have elongated cell shapes consistent with streaming T lymphocytes, but even more spherical T cells display numerous active filopodial protrusions (Fig. 7, O and P).

As noted above, immune cells including macrophages, B cells, and T cells closely associate with and actively migrate along the sheetlike FRC network in the central ALO medulla (Fig. 6, J–N; Fig. 7, G, L, and Q; and Videos 6, 7, 8, and 9). CellChat (Jin et al.,

2021) mapping of CXCL signaling based on our scRNAseq data suggests that FRCs and basal epidermal cells both serve as major hubs for signaling to macrophages and lymphocytes, and indicates that FRCs serve as a chemokine signaling hub for all these immune cell types (Fig. 7 V). FRC bodies are most highly concentrated along lymphatic vessels (Fig. 2 I, Fig. 6 O, and Video 5), which are themselves conduits for immune cell trafficking, suggesting FRCs serve as a pathway for immune cell trafficking between ALO lymphatics and the ALO cortex. Interestingly, imaging of a *Tg(lck:mcherry)^{nz107}*, *Tg(cd79b:egfp)^{fcc89}* double transgenic zebrafish (T cells, red; B cells, green) at 5 wk, when many naïve T cells are leaving the thymus, also reveals a dense path of T cells directly connecting the thymus (white arrow) and the ALO (yellow arrow) (Fig. 7, R, S, and U), while high-resolution ex vivo imaging of adult ALOs from the same transgenic line reveals close interactions between T cells and B cells in the dermal cortex (Fig. S4 H and Video 9), consistent with a role of the ALO as an immune surveillance hub. This pathway is also used by large numbers of B cells (Fig. 7, R, T, and U).

T cell leukemia infiltrates the ALO

In further support of its potential role as an immune nexus, the ALO becomes particularly highly infiltrated with leukemic cells in zebrafish leukemia models (Fig. 8 and Video 10). Previous studies using an established zebrafish model of T cell acute lymphoblastic leukemia (T-ALL) have revealed accumulation of lymphocytes in known SLOs such as the head kidney marrow and gill-associated lymphoid tissue (Frazer et al., 2009). In the HLK^{dz102} model of T-ALL, ALOs frequently become preferentially and massively infiltrated with GFP-positive leukemic T cells (Fig. 8, A and B). Leukemic T cells accumulate in large numbers both in the cortex, where substantial numbers of T cells are present in wild-type animals, and in the medulla, where there are usually fewer actively migrating T cells (Fig. 8, C–F). Eventually, infiltrating leukemic cells completely fill most of the ALO, displacing many other cells in the cortex (Fig. 8, G–J; and Video 10). Although the infiltration of the ALO by leukemic T cells is striking, it should be noted that other nonlymphoid tissues also become heavily infiltrated during T cell leukemia (Borga et al., 2019; Langenau et al., 2003).

Discussion

We have identified a new external organ on adult zebrafish, the ALO. The ALO is located behind the operculum of select cypriids, in the path of water flowing outward from the gills. This

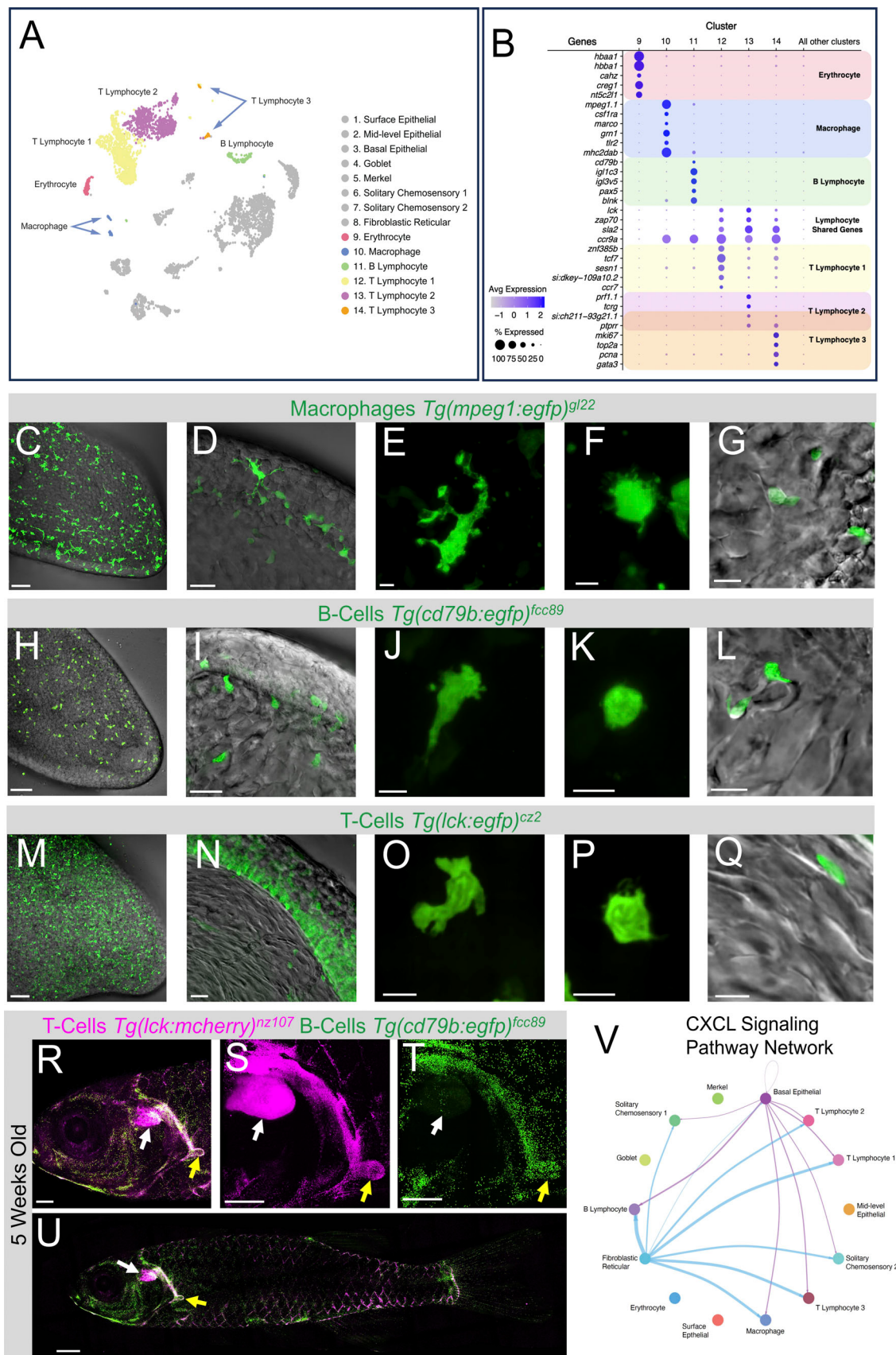


Figure 7. **ALO is an immune cell hub.** (A) UMAP plot of ALO scRNAseq data highlighting blood and immune cell clusters. (B) Dot plot showing the relative expression of genes used to identify and characterize blood and immune cell clusters. (C–Q) Confocal + DIC (C, D, G–I, L, M, N, and Q) or confocal only (E, F, J, K,

O, and P) micrographs of ALOs from adult immune cell transgenic reporter zebrafish. Images include ALO overviews (C, H, and M), side views of the dermal cortex and medulla (D, I, and N), and higher magnification images of individual cells (E, F, J, K, O, and P). Images show *Tg(mpeg1:EGFP)^{g122}*-positive macrophages (C–G), *Tg(cd79b:EGFP)^{fcc89}*-positive B cells (H–L), and *Tg(lck:EGFP)^{c22}*-positive T cells (M–Q). **(G, L, and Q)** Single-plane confocal + DIC images of *Tg(mpeg1:EGFP)^{g122}*-positive macrophages (G), *Tg(cd79b:EGFP)^{fcc89}*-positive B cells (L), and *Tg(lck:EGFP)^{c22}* T cells (Q) migrating on FRCs in adult ALO medullae (see [Videos 6, 7, 8, and 9](#)). **(R–U)** Confocal micrographs of a 5-wk-old *Tg(cd79b:EGFP)^{fcc89}*, *Tg(lck:mcherry)^{h2107}* double transgenic zebrafish with T cells in magenta and B cells in green, showing large numbers of cells migrating between the thymus (white arrow) and the ALO (yellow arrow). Images show an overview of the head (R) and higher magnification images of T cells (S) connecting the thymus and ALO and B cells (T) starting above the thymus and moving down to the ALO, and an overview of the entire fish (U). **(V)** CellChat plot of likely chemokine signaling in the ALO based on the expression of chemokine ligands and receptors in different ALO scRNAseq clusters. Basal epithelial and FRCs both appear to be hubs for chemokine signaling to immune cells. $n = 5$ per transgene combination. Scale bars = 50 μm (C, H, and M), 20 μm (D, I, and N), 5 μm (E, F, J, K, O, and P), 10 μm (G, L, and Q), 500 μm (R, S, and T), and 1 mm (U).

novel organ appears in juvenile fish ~30 days after fertilization, when the fish is around 10 mm long, and regenerates after being amputated. Histological and ultrastructural examination reveals a stratified cortex with three epithelial layers and a reticular medulla containing blood and lymphatic vessels. scRNAseq, in situ hybridization, and transgenic reporter lines were used to identify cell types of the ALO. Enormous numbers of immune cells are present in the ALO, and they can be observed trafficking between lymphatic vessels and the ALO cortex using a network of medullary FRCs as a migration pathway. The basal cortex is densely packed with *lck*⁺ lymphocytes, while B cells and macrophages are more uniformly distributed across the ALO. In animals with T cell leukemias, *lck*⁺ lymphocytes completely infiltrate the cortex and medulla of the ALO. The external location, translucency, and lack of pigment make the ALO ideal for live imaging of immune cells and their surveillance functions.

The small size, transparency, and relatively late appearance in development of the ALO likely contributed to the dearth of previous reports of this organ. On cursory examination, it could easily be mistaken for a scale or an isolated fin abnormality. A few previously published reports have used a pectoral fin axial lobe as a taxonomic character for Cypriniformes fishes ([Kullander and Britz, 2015](#); [Lumbantobing, 2014](#)), and other published reports noted the presence of axillary “spines” in other teleosts, including the Batrachoididae, but these tissues were not investigated beyond gross anatomy ([Greenfield et al., 2008](#); [Lumbantobing, 2010](#)). External examination of specimens of a small sample of different species in the Smithsonian National Museum of Natural History fish collection suggests that ALOs characterize “basal” teleosts including Danionidae species (Table S1). However, the infraclass Teleostei is enormously diverse and species-rich, and we were only able to examine a small, representative sample. Many other teleost fishes likely have ALO-like structures. The fixed teleost specimens in the Smithsonian collection show a diversity of ALO morphologies ([Fig. 1, L–O](#)). Because we did not dissect these specimens as part of this study, we could not ascertain the internal morphology of the ALOs of other species or whether similar internal ALO-like structures are present in other fishes. A full review of the ALO throughout teleost fishes would be needed to better understand the taxonomic distribution and range of morphologies of these structures.

The ALO contains enormous numbers of immune cells, especially lymphocytes, and our findings suggest that it is an SLO that may play an important role as a nexus for immune cell trafficking and immune cell signaling. The ALO cortex includes

three epithelial cell types that localize to separate cortical layers and that have distinct gene expression profiles and morphological features, such as the characteristic microridges of surface epithelial cells. Basal epithelial cells closest to the ALO medulla express copious amounts of transcripts for the chemokine ligands *ccl25a* and *ccl25b*, like gut-associated lymphoid tissue of mammals ([Fig. 6 B](#)) ([Campbell and Butcher, 2002](#)), and appear to be an important signaling hub for immune cell recruitment ([Fig. 7](#)). This is corroborated by live imaging of immune cell transgenic lines, notably the T lymphocyte-specific *Tg(lck:egfp)^{c22}* reporter line, which shows large numbers of highly active T cells aggregating specifically in the basal most area of the ALO cortex ([Video 9](#); see 0:18–0:31). The cortical basal epithelial cells lie in close proximity to the sheetlike cell processes and cell bodies of FRCs, located just on the other side of the basal lamina in the adjacent ALO medulla. FRCs are specialized mesodermal cells found in lymph nodes and other mammalian immune organs that have important and well-documented roles in immune cell trafficking in these organs. In zebrafish, FRC-like cells have been identified in larval kidney and hematopoietic tissues, although it is not clear how closely these resemble mammalian FRCs ([Murayama et al., 2006](#); [Rubin et al., 2022](#); [Stosik et al., 2022](#); [Willett et al., 1999](#); [Xia et al., 2021](#)). ALO FRCs express characteristic markers of mammalian lymph node FRCs ([Fig. 6, A–E](#)), and they have similar thin sheetlike cell processes with dense collagen-rich matrices. Three-dimensional visualization of the FRC network in the zebrafish ALO from confocal images of *TgBAC(pdgfrb:egfp)^{ncv22}* transgenic animals or from array tomography of the ALO ([Video 5](#)) shows that the sheetlike interconnected extensions of FRCs form radial highways that emanate from lymphatic vessels, where many FRC bodies are located ([Fig. 2 I](#); [Fig. 6, F and O](#); and [Video 5](#)), outward to the cortex, similar to the three-dimensional networks of FRCs found in mammalian lymph nodes ([Martinez et al., 2019](#); [Novkovic et al., 2016](#); [Textor et al., 2016](#)). Like cortical basal epithelial cells, medullary FRCs of the ALO also appear to be major hubs for chemokine signaling to immune cells ([Fig. 7 V](#)). Transmission electron micrographs show immune cells closely associating with FRC bodies and processes ([Fig. 6, K–N](#)), and live imaging reveals multiple types of immune cells actively migrating along FRCs, between the ALO cortex and lymphatic vessels in the ALO medulla, although further investigation is needed to determine the nature and function of immune cell migration between the ALO cortex and medullary vessels ([Videos 6, 7, 8, and 9](#)). The lymphatic vessels of the ALO themselves drain to a large adjacent lymph sac ([Fig. 5, E–G](#)), and a dense conduit of T cells can be

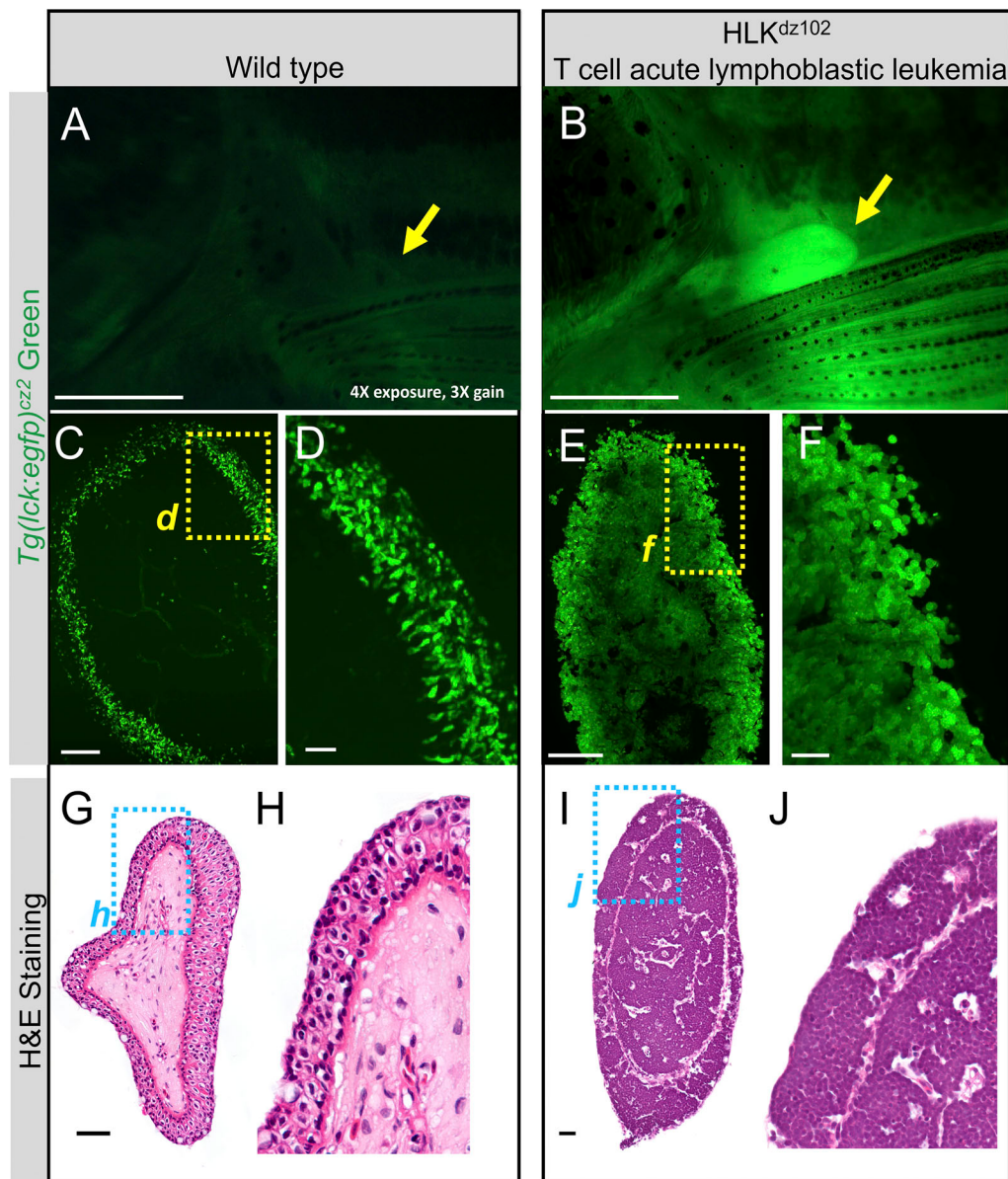


Figure 8. ALO is heavily infiltrated with leukemic T cells in a zebrafish model of T-ALL. (A–F) Fluorescence images of *Tg(lck:EGFP)^{cz2}* wild-type sibling (A, C, and D) or T-ALL HLK^{dz102} mutant (B, E, and F) adult animals. Panels show overview images of the ALO/pectoral area taken with a fluorescent stereomicroscope (A and B), confocal images of most of the ALO (C and E), and higher magnification confocal images of the ALO cortex and underlying medulla (D and F). The images in panels D and F correspond to the boxed regions in panels C and E, respectively. The image in panel A was taken with 4× higher exposure and 3× higher gain than the image in panel B, because this area was much brighter in the T-ALL fish than in the wild-type fish. **(G–J)** H&E-stained paraffin histological sections of ALOs from adult wild-type (G and H also shown in Fig. 2 F) or T-ALL HLK^{dz102} mutant (I and J) adult animals. The images in panels H and J correspond to the boxed regions in panels G and I, respectively. The T-ALL HLK^{dz102} ALO is almost entirely filled with leukemic T cells. Transgenic imaging, *n* = 6; histology, *n* = 3. Scale bars = 1 mm (A and B), 100 μm (C and E), 25 μm (D, F, G, and I).

seen between the nearby thymus and the ALO, especially in juvenile zebrafish (Fig. 7, R–U), connecting the ALO to a recently described path between the suboperculum and thymus (Resseguier et al., 2023). Together, the ALO bears many of the hallmark features of a SLO, and its highly accessible location makes it ripe for further detailed experimental analysis and comparative morphology.

Secondary immune tissues have been described in several teleost species, and although their architecture can differ slightly from those found in higher vertebrates, their

experimental and genetic accessibility makes fish an attractive model for studying adaptive immunity (Bjorgen and Koppang, 2021; Hotez et al., 2024; Zapata, 2022). In both mammals and fish, antigens are concentrated and presented by professional antigen presenters to pools of naïve surveilling lymphocytes in a variety of diverse SLOs across the body such as lymph nodes, Peyer's patches, and adenoids. In teleost fishes, lymphocyte activation and affinity maturation of B cells have been characterized in melanomacrophage centers of the head kidney marrow and the spleen, but these tissues are not easily live-

imaged (Shibasaki et al., 2023; Steinel and Bolnick, 2017; Waly et al., 2022). The high lymphocyte density, tissue organization, and intraepithelial lymphocyte reservoir in the ALO are reminiscent of other teleost MALTs, including the O-NALT, bursa, and gut-associated lymphoid tissue (GALT), where B cells tend to be interspersed with T cells (Dalum et al., 2015; Dalum et al., 2021; Garcia et al., 2022; Løken et al., 2020; Resseguier et al., 2023). Like these other MALTs, the ALO contains large numbers of lymphocytes; 41.6% of the cells captured in our lobe scRNAseq data set, or ~20–40,000 cells per lobe, are lymphoid. This is in contrast to the teleost head kidney (anterior pronephros) and spleen, internal immune organs that have abundance of myeloid lineage cells but far fewer lymphoid cells, only ~8% or 4%, respectively (Robertson et al., 2023), but is similar to the preinvolution thymus (Hasan et al., 2024) to which the ALO shows a robust connection (Fig. 7, R and U). B cells in the ALO display frequent, extensive, and close physical interactions with T cells, further hinting at the similar function of the ALO as germinal centers, although further investigation will be needed to determine whether the ALO is indeed a site of immune activation. The ALO is also likely contiguous with the epidermis, where a TLN was recently described that contains streaming T cells just under the scale junctions (Robertson et al., 2023). Plausibly, all MALTs in the adult zebrafish may be interconnected, although extensive additional imaging and experimental analysis would be needed to examine this.

In addition to three epithelial cell populations (surface, mid-level, and basal), our scRNAseq, in situ hybridization, array tomography, and transgenic reporter line characterization shows that the ALO contains several additional cell types in its cortex, some of which may also have accessory roles in immune defense. Like mammals, fishes have sensory cells that detect external stimuli including touch and waterborne chemicals and molecules. The ALO has Merkel cells that transduce touch (Brown et al., 2023; Maksimovic et al., 2014) and two SCC types, tuft-like cells that sense the environment and secrete leukotrienes to communicate with neurons and immune cells (McGinty et al., 2020), and taste-like sensory cells (Fig. 4, N–Q; Fig. S3; and Videos 2 and 3). As noted previously (Brown et al., 2023), Merkel cells lie very close to the ALO surface and extend a single ventral process (Fig. S2, L and M). Interestingly, our array tomography data reveal that the SCCs each has two separate apical extensions protruding out to the external environment (Video 3), suggesting that individual cells might be able to receive two separate differential external chemosensory signals. Sensory cells such as these could potentially be communicating with immune cells in neuroimmune cellular units (Godinho-Silva et al., 2019). In mammals, dermal sensory neurons in the skin can change the transcriptional states of immune cells in adjacent patches of skin (Cohen et al., 2019), and lymph nodes are innervated and modulated by sensory neurons (Huang et al., 2021). Although it remains unclear whether and to what extent ALO sensory cells interface with immune cells, this organ would provide a superb platform for studying any such interactions in vivo.

The ALO cortex also includes characteristic mucus-producing goblet cells (Fig. 4, J and K; Fig. S2, A–H; and Video 3). These cells produce and secrete mucins in the respiratory, reproductive,

and gastrointestinal tracts of mammals, and in the gut and skin of teleost fishes, where they play an important role in immunity both by creating a passive barrier to infection and by actively participating in immune responses (Zhang and Wu, 2020). Goblet cells in mammals have been shown to actively endocytose soluble substances and pathogens and to transmit these antigens to underlying antigen-presenting cells. Goblet cells in the ALO similarly endocytose and accumulate foreign substances such as bacterial lipopolysaccharide (LPS; Fig. S2, G and H), suggesting the ALO may also serve as a useful model for detailed in vivo dissection of goblet-immune cell interactions. In addition to goblet cells, the ALO cortex contains abundant large ovoid cells with uniformly sparse cytoplasm and complex folded or multi-lobed nuclei, characteristic features of club cells (Video 2). Related to human cells found in the airway epithelium (Zuo et al., 2018), in teleosts these cells are thought to have a role both in conspecific fear responses to injured fish, via release of a “fright substance” (“Schreckstoff”) from injured animals, and in immune responses, possibly via internalization of bacterial-laden mucus (Alesci et al., 2022; Chia et al., 2019; Pandey et al., 2021). Although we were not able to recover a cluster corresponding to these cells in our scRNAseq data, possibly due to their very large size and fragility, we discovered fortuitously that these cells are weakly positive for the same *Tg(gngl3a:eGFP)^{y709}* transgene that we used to visualize chemosensory type 2 cells (Fig. 4 R; and Fig. S4, D and E), making it possible to observe and study them using confocal imaging. Again, the ALO should provide a valuable platform for in vivo investigation of these unusual cells.

In conclusion, we have identified a novel external and highly accessible organ with putative immune surveillance functions in the zebrafish. As a vertebrate species with well-developed genomic resources, a powerful toolkit of methods for imaging of developing and adult animals, and a vast number of transgenic lines available for visualizing almost any cell type of interest in vivo, the zebrafish and its ALO will provide a new powerful model for high-resolution imaging and experimental manipulation of SLO function.

Materials and methods

Fish husbandry and fish strains

Fish were housed in a large zebrafish-dedicated recirculating aquaculture facility (four separate 22,000-liter systems) in 6-liter and 1.8-liter tanks. Fry were fed rotifers, and adults were fed Gemma Micro 300 (Skretting) once per day. Water quality parameters were routinely measured, and appropriate measures were taken to maintain water quality stability (water quality data available upon request). The following transgenic and mutant fish lines were used for this study: *Tg(mr1a:eGFP)^{y251}* (Jung et al., 2017), *Tg(pdgfrb:eGFP)^{ncv22}* (Ando et al., 2016), *Tg(kdrl:mcherry)^{y205}* (Fujita et al., 2011), *Tg(lyz:DsRed2)^{nz50}* (Hall et al., 2007), *Tg(lyve1:DsRed2)^{nz101}* (Okuda et al., 2012), *Tg(CD79b:EGFP)^{fcc89}* (Liu et al., 2017), *Tg(lck:mcherry)^{ns107}* (Amanda et al., 2022), *Tg(mpeg1:EGFP)^{gl22}* (Ellett et al., 2011), *Tg(lck:EGFP)^{cz1}*, *Tg(lck:EGFP)^{cz2}* (Langenau et al., 2004), *Tg(ato1a:nl5-Eos)^{w214}* (Pickett et al., 2018), *HLK^{DZ102/DZ102}* (Rudner et al., 2011), *Tg(lysC:DsRed)^{nz50}* (Hall et al., 2007), and *Tg(gngl3a:eGFP)^{y709}*. Some fish

were maintained and imaged in a *casper* (roy, nacre double mutant [White et al., 2008]) genetic background to increase clarity for visualization by eliminating melanocyte and iridophore cell populations to prevent them from obscuring images. Mutant and transgenic zebrafish were generated on various wild-type backgrounds but were maintained by crossing with EK wild-type zebrafish. This study was performed in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited facility under an active research project overseen by the National Institute of Child Health and Human Development Animal Care and Use Committee (NICHD ACUC), Animal Study Proposal # 21-015.

Taxonomic analysis of ALO phylogeny

Specimens were examined from the formalin-fixed alcohol-preserved collections in the Division of Fishes, National Museum of Natural History, Smithsonian Institution, to determine the presence or absence of the fleshy pectoral lobe. Species were chosen that were closely related to the zebrafish, as well as others in the larger taxon Ostariophysi. The full list of taxa examined for the presence of ALOs is listed in Table S1. Specimens were photographed and their size recorded. Images in Fig. 1, L–O show pectoral lobes on representative Danionidae specimens of *Rasbora caverii* and *Laubuka* sp., as well as examples of lobes from outgroup specimens of Atlantic tarpon (*Megalops atlanticus*) and Milkfish (*Chanos chanos*).

Histology and TEM

Adult zebrafish were euthanized by placing them in an ice bath for 10 min. For histology, the caudal third of the animal was removed to improve fixation penetration and the sample was placed in 4% paraformaldehyde (PFA) shaking overnight. The following day, the fish were rinsed three times with PBS and transferred to 70% ethanol. Samples were then sent to Histoserv, Inc. (<https://histoservinc.com>) for sectioning and histological staining. Slides were imaged on a Leica DMI 6000B inverted compound microscope with a Leica DMC6200 camera, Leica Application Suite X software, and 10× 0.3 NA air, 20× 0.8 NA air, 40× 1.25 NA oil, and 63× 1.32 NA oil Leica objective lenses.

For TEM, zebrafish ALOs were removed after euthanasia and fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. After fixation, samples were inserted into mPrep tissue capsules and loaded onto mPrep ASP-2000 Automated Biological Specimen Preparation Processor (Microscopy Innovations, LLC), which automates all subsequent processing steps including the following: postfixation in 2% osmium tetroxide, en bloc staining in 2% uranyl acetate (aqueous), dehydration in a graded ethanol series followed by further dehydration in 100% acetone, and finally infiltration and embedding in EMbed 812 epoxy resin (Electron Microscopy Sciences). Embedded samples were polymerized in an oven set at 60°C. Samples were then ultrathin-sectioned (90 nm) on Leica EM UC7 Ultramicrotome. Thin sections were picked up and placed on 200-mesh copper grids and poststained with UranylLess (uranyl acetate substitute; Electron Microscopy Sciences) and lead citrate. Imaging was performed on JEOL 1400 Transmission Electron Microscope operating at 80 kV and an AMT BioSprint-29 camera.

Array tomography

ALOs were amputated (described below) from wild-type EK strain zebrafish. Whole ALOs were fixed with Karnovsky's fixative and postfixed in 2% OsO₄ and 1.5% potassium ferricyanide in 0.1 M sodium cacodylate for 1 h at room temperature (RT). Next, whole ALOs were washed with ultrapure water and stained with 1% aqueous uranyl acetate for 1 h at RT. After washing with water, the ALOs were treated with lead aspartate at 60°C for 30 min and washed again with water. The ALOs were dehydrated through a graded ethanol series (10 min each of 35%, 50%, 70%, 95%, and 100% three times) and finally in 100% propylene oxide (PO). After dehydration, the ALOs were infiltrated with increasing amounts of polybed resin with PO (resin: PO; 1:3, 1:1, 3:1, and 100% resin). Finally, the samples were placed in 100% degassed resin in flat beam capsules (EMS) and cured at 65°C for 48 h. After polymerization, resin pieces containing an ALO were trimmed with a Leica EM TRIM2 milling system.

After trimming the block face down, 100 nm serial sections were collected with a 45° ultra-diamond knife (Diatome) using ATUMtome (RMC Boeckeler) on a carbon nanotube PEN tape (Teijin). Tape strips were mounted onto 12-mm double-sided carbon tape with an aluminum base (EMS) and attached to 4-inch type-p silicon wafers (EMS) and grounded with conductive graphene carbon paint (EMS). Overview wafer images were collected using a Canon EOS 1300D camera. Wafers were affixed to a 4-inch stage-decel holder in a Zeiss SEM (GeminiSEM 450; Carl Zeiss) and imaged using ATLAS 5 Array Tomography software (Fibics). Five wafers were imaged using a four-quadrant backscatter detector, with the electron beam operated at 3 kV EHT with 1 kV beam deceleration and 800 pA probe current. Low-resolution overview scans were collected at 3,000-nm pixel resolution, medium-resolution section sets were collected at 150-nm pixel resolution, and high-resolution sites were collected at 25- and 10-nm pixel resolution. Once image acquisition was complete, the image stack was locally cropped and aligned using ATLAS 5 software. The resulting image stack was exported and then processed using Python-based scripts to produce two aligned and contrast/brightness-adjusted image data sets: a stack of images at 10 or 25 nm (xy) × 100 nm (z), and a final binned 100 × 100 × 100 nm isotropic volume. Raw array tomography image data are deposited at EMPIAR.

ALO amputation and ex vivo imaging

Adult zebrafish were anesthetized in buffered tricaine (160 mg/liter) and placed on a moist paper towel under a dissecting microscope (M165; Leica) with gooseneck illumination. Using #55 sharp forceps (11255-20; Dumont) to grab the base of the ALO, the ALO was removed with a gentle tug of the forceps. The amputated ALO was then placed in a MatTek dish (# P35-1.5-14-C) containing 100–150 µl of 10% FBS in PBS. A 25-mm round coverslip was then placed on top of the solution containing the ALO. A similar setup was used for ex vivo imaging on our upright microscope, except we used a 50-mm dish (P50G-1.5-30-F; MatTek) with a 35-mm coverslip (PCS-1.5-35-NON; MatTek), to account for the large diameter of the 20× 1.0 NA water immersion objective.

Live staining and in vivo ALO treatments

Adult and subadult zebrafish were stained with BODIPY membrane stain to visualize external structures by soaking them in 100 ml of 20 ng/ml BODIPY 630/650 (Cat # D10000; Thermo Fisher Scientific) in aquaria system water for 1 h. After soaking, fish were transferred to fresh aquaria system water before anesthesia and live imaging. LPS treatment was carried out by anesthetizing adult zebrafish in 160 mg/liter tricaine solution and placing them on a moist paper towel under a dissecting microscope, then placing 25 μ l of LPS solution (1 mg/ml Cy5-labeled, Cat # LPS-S5-1; Nanocs) directly on the ALO. The fish was then flipped over, and the process was repeated on the other ALO. A Kimwipe (05511; Kimtech Science) wetted with anesthesia water was placed on top of the fish and allowed to sit for 3 min. The fish was transferred to fresh system water for recovery until fully revived (about 2 min), then reanesthetized followed immediately by removal of the ALOs for imaging.

Angiography and lymphatic drainage

ALO lymphatic drainage was assessed by anesthetizing fish in 160 mg/liter tricaine, placing them in a slit cut into a wet sponge, then injecting 50 nl of undiluted (2 μ M) Qtracker 705 Vascular Labels (cat# Q21061MP; Invitrogen) directly into the ALO, or into the dorsal body behind the operculum, above the pectoral fin just under the skin using a Drummond Nanoject III microinjector (# 3-000-207; Item) with pulled glass capillary needles (# 3-00-203-G/X; Drummond item). Fish were then placed back into anesthesia solution and then moved to an imaging dish (#155360; Lab-Tek II) with tricaine solution and gently covered with a sponge to prevent movement. Flow through ALO blood vessels was assessed using angiographic injection of Hoechst 33342 dye to label the nuclei of circulating red blood cells. Injections were performed as noted above but with 250 nl of Hoechst 33342 (1 μ M solution, Cat # H3570, diluted 1:1 with PBS), and with injection into the caudal axial vasculature instead of into the ALO. Fish were placed in fresh aquaria system water for recovery until fully revived (about 2 min), then reanesthetized, and imaged.

Image acquisition

Confocal images were acquired using either a Nikon Ti2 inverted microscope with Yokogawa CSU-W1 spinning disk confocal (Hamamatsu Orca Fusion-BT camera), a Nikon Ti2 inverted microscope with Nikon A1R scanning confocal, or a Nikon FN5P upright microscope with AXR scanning confocal. 405-, 488-, 561-, and 640-nm laser lines were used on all systems. The following Nikon objectives were used: 4 $\times$ Air 0.2 N.A., 10 $\times$ Air 0.45 N.A., 20 $\times$ water immersion 0.95 N.A., 20 $\times$ water immersion 1.0 N.A., 40 $\times$ water immersion 1.15 NA. Stereomicroscopic pictures were taken using a Leica M165 microscope with Leica DFC 7000T camera and Leica Application Suite X software. Images were acquired as 12-bit or 16-bit images.

Image processing

Images obtained from the Nikon A1 and AXR resonant scanners were denoised using Nikon Denoise.ai. A median filter with a kernel size of 3 was applied to some images. A subset of images

were deconvolved using NIS Batch deconvolution. Images were processed using NIS-Elements. Maximum intensity projections of confocal stacks are shown for fluorescent confocal images. When fluorescence and DIC images are shown together, extended depth-of-focus images of stacks are shown. When needed, time-lapse movies were aligned in XY using NIS-Elements. Nonlinear adjustments (gamma) were made to some images to improve the visualization of images with high dynamic range (images adjusted: Fig. 7, D-O; and Videos 7, 8, and 9). Time-lapse movies were made using NIS-Elements and exported to Adobe Premiere Pro CC 2024. Adobe Premiere Pro CC 2024 and Adobe Photoshop CC 2024 were used to add labels and arrows to movies. Schematics were made using Adobe Photoshop CC 2024, Microsoft PowerPoint, and Bio Render software.

Preparation of ALO for cell counting via nuclear stain

ALO were amputated as described above and fixed in 4% PFA in PBS at 4°C overnight. They were then rinsed three times with PBS and placed in a 0.5 mg/ml solution of Hoechst in PBS for 6 h at RT. Samples were then washed three times with PBS and placed in LUCID (PhotonTech Innovations Co., Ltd.) clearing reagent at 4°C for 3 days prior to imaging. Entire ALO volumes were imaged using a 20 $\times$ 1.0 NA water immersion objective on a Nikon A1R confocal, and images were stitched together using Nikon Elements software. Imaris 10.2 software was used to count the nuclei using the spot detection tool selecting an average diameter of 10 μ m, with a quality filter value of 77.2 used to maximize the number of nuclei counted and minimize the number of individual nuclei counted twice.

Preparation of ALO single-cell suspension for scRNAseq

Adult zebrafish were anesthetized with MS-222 prior to removal of the ALOs. ALOs from 4 different *Tg(lck:EGFP)^{cz1}*, *Tg(lysC:DsRed2)^{nz50}* double transgenic fish (two males and two females) were removed with forceps and placed in dissociation media (1: 1 DMEM:Liberase) (A1896701; Gibco/0540119001; Sigma-Aldrich). The tissue was slowly pipetted up and down at RT for 45 min with a 1,000- μ l pipette set to 500 μ l to ensure gentle dissociation. Cell dissociation was stopped by adding an equal volume of STOP solution (5% FBS, 2% BSA in DMEM) (35-010-CV; Corning/A9418-5G; MilliporeSigma/A1896701; Gibco) and gently inverting the tube. Cell suspensions were filtered through a 40- μ m filter and spun down at 500 $\times$ g for 5 min at RT. The supernatant was removed, and cells were resuspended in a 1 $\times$ PBS/2% BSA (10010023; Gibco/A9418-5G; MilliporeSigma) solution. Cells were counted on LUNA-FL (Logos Biosystems) and diluted to the optimum concentration of 700 cells/ml using the same 1 $\times$ PBS 2% BSA solution. 10,000 cells were loaded onto the 10x Genomics Chromium X controller.

Sequence alignment and quality control

Alignment of sequencing reads and processing into a digital gene expression matrix were performed using Cell Ranger version 7.0.0. The data were aligned against GRCz11 release 99 (January 2020) using Lawson Lab Zebrafish Transcriptome Annotation version 4.3.2 (Lawson et al., 2020), available from <https://www.umassmed.edu/lawson-lab/reagents/zebrafish-transcriptome/>.

Cells were processed and analyzed using Seurat version 4.3.0.1 (Hao et al., 2021) and R version 4.2.3. Cells with abnormally high (>2,500) or low (<200) numbers of detected features, or with abnormally high mitochondrial content (>5%) were removed. The remaining cells were normalized to 10,000 transcripts per cell and scaled using the ScaleData function of Seurat with default settings. Following initial clustering, it was noted that one cluster appeared to group with every other cluster instead of forming a unique identity, and it contained an abnormally high percentage of ribosomal protein genes. The cells in this cluster were removed, and the remainder of the data set was used for all subsequent analyses shown. Fig. 3 B provides the cell and read count metrics before and after performing quality control.

Dimensionality reduction, clustering, and visualization

2,000 variable features were identified for our sample using Seurat's FindVariableFeatures function with default parameters. Principal component analysis (PCA) was performed with the RunPCA algorithm using the determined most variable features. To identify the number of significant PCs for downstream analyses, the JackStrawPlot function of Seurat was used. The FindNeighbors and FindClusters functions from Seurat were applied utilizing the number of significant PCs, to identify clusters. After exploring a variety of possible resolutions, 0.15 was selected, generating 14 distinct clusters. Uniform Manifold Approximation and Projection (UMAP) was calculated using the RunUMAP function and visualized using DimPlot. CellChat v2 (Jin et al., 2023, Preprint), an open source R package, was used to produce a cellular communication circle plot inferring chemokine signaling pathways within the data set.

Differential expression analysis

To identify differentially expressed genes between cell types, we used a negative binomial model as implemented in the Seurat FindAllMarkers function, with a $\log_2$ fold change cutoff of 0.25. Genes were considered differentially expressed if the adjusted P value was <0.01. A table of the resulting genes with the highest expression values in each cluster can be found at <https://github.com/nichd-Weinstein/Axillary-Lymphoid-Organ>.

Defining cell types

Each of the 14 clusters was manually annotated based on an extensive survey of well-known tissue- and cell type-specific markers. These markers were identified through a variety of databases (Human Protein Atlas, The Zebrafish Information Network, and Daniocell) and through an extensive literature search. Our search and identification were guided by preliminary confocal imaging and electron microscopy of the ALO. For each cell type, at least five marker genes were identified.

HCR

HCR was performed to visualize and confirm the identities of each cell type/cluster. HCR probesets were designed by Molecular Instruments (Molecular Instruments). ALOs were dissected and fixed in 4% PFA (Cat # 15710; Electron Microscopy Sciences) for 2 h at RT, washed three times for 5 min each time with 1 ml of PBST (1× PBS and Tween-20) (10010023; Gibco/9005-64-5;

MilliporeSigma), and treated with Proteinase K solution (50 µg/ml in PBST; Thermo Fisher Scientific) 10 min at RT. The samples were washed again twice with PBST (5 min each time at RT), before being postfixed with 4% PFA for 20 min at RT. This was followed by another PBST wash cycle (three times for 5 min each time). Fixed ALOs were prehybridized with preheated (37°C) HCR probe hybridization buffer (Molecular Instruments, HCR RNA-FISH Bundle) for 30 min at 37°C, rotating at 30 rpm. Hybridization was performed with 2 µl of each 1 µM probe diluted in 500 µl of probe hybridization buffer at 37°C, rotating at 30 rpm, for 12–16 h. The probe solution was removed by washing with preheated HCR probe wash buffer (Molecular Instruments, HCR RNA-FISH Bundle) four times 15 min each at 37°C, followed by two 5-min washes with 5× SSCT (12.5 ml 20× SSC in 50 µl Tween-20) (Cat # RGF-3240; KD Medical/9005-64-5; MilliporeSigma) at RT before pre-amplification stage. ALOs were pre-amplified with HCR probe amplification buffer (Molecular Instruments, HCR RNA-FISH Bundle) for 30 min at RT. Hairpins (10 µl of 3 µM stock) were pre-annealed (95°C for 90 s and 25°C for 30 min with a ramp rate of −0.1°C per second) to create hairpin secondary structure. Hairpins were then mixed with 500 µl of fresh HCR probe amplification buffer. The pre-amplification buffer was removed from the ALOs before the addition of the newly mixed hairpin solution, followed by an incubation period for 12–16 h in the dark at RT. Excess hairpins were then removed, and samples were washed five times with 5X SSCT at RT and stored at 4°C in the dark until imaging.

Online supplemental material

Fig. S1 shows the morphological variability of ALOs, a close look at microridges on the outside of the ALO, and TEM images of epithelial cells in the ALO. Fig. S2 provides more detailed TEM, transgenic, and HCR images of goblet and Merkel cells in the ALO. Fig. S3 shows additional transgenic and array tomography images of chemosensory cells in the ALO. Fig. S4 shows additional array tomography, TEM, and transgenic images of club cells in the ALO. Table S1 shows a list of teleost specimens examined for the presence of a pectoral lobe. Video 1 shows a time series of the regenerating ALO. Video 2 shows an array tomography scan of the ALO and a close-up of the cortex. Video 3 shows an array tomography scan with a focus on chemosensory cells and goblet cells. Video 4 shows the vascular network in the ALO. Video 5 shows the complex network created by FRCs in the ALO. Video 6 shows presumptive immune cells trafficking on FRCs. Videos 7, 8, and 9 show macrophages, B cells, and T cells in the ALO, respectively. Video 10 shows an ALO infiltrated by T-ALL.

Data availability

Data supporting Fig. 3, B–D; Fig. 4, A–C; Fig. 6, A and B; and Fig. 7, A, B, and V are available as raw and processed 10X scRNAseq data on GEO using the accession number GSE270797. The code used to analyze and visualize the data can be found at <https://github.com/nichd-Weinstein/Axillary-Lymphoid-Organ>. Data supporting Fig. 2 H; Fig. 4, E, G, I, K, M, O, Q, and S; and Videos 2 and 3 array tomography data are

available at <https://www.ebi.ac.uk/empir/EMPIAR-12377/>. Additional data supporting the findings of this study are available by contacting the corresponding author upon request.

Acknowledgments

The authors would like to thank members of the Weinstein and Sheppard laboratories for their critical comments on this manuscript. The authors would also like to thank the Research Animal Branch of the Eunice Kennedy Shriver NICHD and the RAMB contract animal management staff for excellent animal care and husbandry. We also thank Irene Salinas and Benjamin Garcia for helpful discussions.

This work was supported by the NICHD, NIH intramural support ZIA-HD008915 (to B.M. Weinstein), NICHD, NIH intramural support ZIA-HD008808 (to B.M. Weinstein), NICHD, NIH intramural support ZIA-HD001011 (to B.M. Weinstein), National Cancer Institute, NIH contract no. 75N91019D00024 (to K. Narayan), Herbert R. and Evelyn Axelrod Endowment, Division of Fishes, National Museum of Natural History (to D.N. Lumbantobing, L.R. Parenti), Hyundai Hope On Wheels Hope Scholar Award (to J.K. Frazer), University of Oklahoma Health Sciences Center Stephenson Cancer Center Pilot Grant (to J.K. Frazer), Oklahoma Center for Adult Stem Cell Research (to J.K. Frazer), and Canadian Institutes of Health Research MOP77746 (to E. Foley), R35 GM118027 (to A. Huttenlocher), and F32 GM146398 (to T.F. Robertson).

Author contributions: D. Castranova: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, and writing—original draft, review, and editing. M.I. Kenton: conceptualization, data curation, formal analysis, investigation, methodology, software, validation, visualization, and writing—original draft, review, and editing. A. Kraus: conceptualization, data curation, formal analysis, investigation, methodology, visualization, and writing—original draft, review, and editing. C.W. Dell: investigation, methodology, and writing—review and editing. J.S. Park: investigation, methodology, and writing—review and editing. M. Venero Galanternik: investigation and writing—review and editing. G. Park: investigation, methodology, and writing—review and editing. D.N. Lumbantobing: investigation, methodology, visualization, and writing—review and editing. L. Dye: investigation and writing—review and editing. M. Marvel: investigation. J. Iben: data curation, formal analysis, and writing—review and editing. K. Taimatsu: investigation and resources. V. Pham: resources. R.J. Willms: resources. L. Blevens: resources and writing—review and editing. T.F. Robertson: investigation and writing—review and editing. Y. Hou: investigation and writing—review and editing. A. Huttenlocher: conceptualization and writing—review and editing. E. Foley: supervision and writing—original draft. L.R. Parenti: conceptualization, investigation, resources, and writing—review and editing. J.K. Frazer: conceptualization, investigation, resources, supervision, and writing—review and editing. K. Narayan: methodology, supervision, and writing—review and editing. B.M. Weinstein: conceptualization, data curation, formal analysis, funding acquisition, methodology, project administration, resources,

supervision, validation, visualization, and writing—original draft, review, and editing.

Disclosures: The authors declare no competing interests exist.

Submitted: 12 August 2024

Revised: 13 January 2025

Accepted: 3 March 2025

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Supplemental material

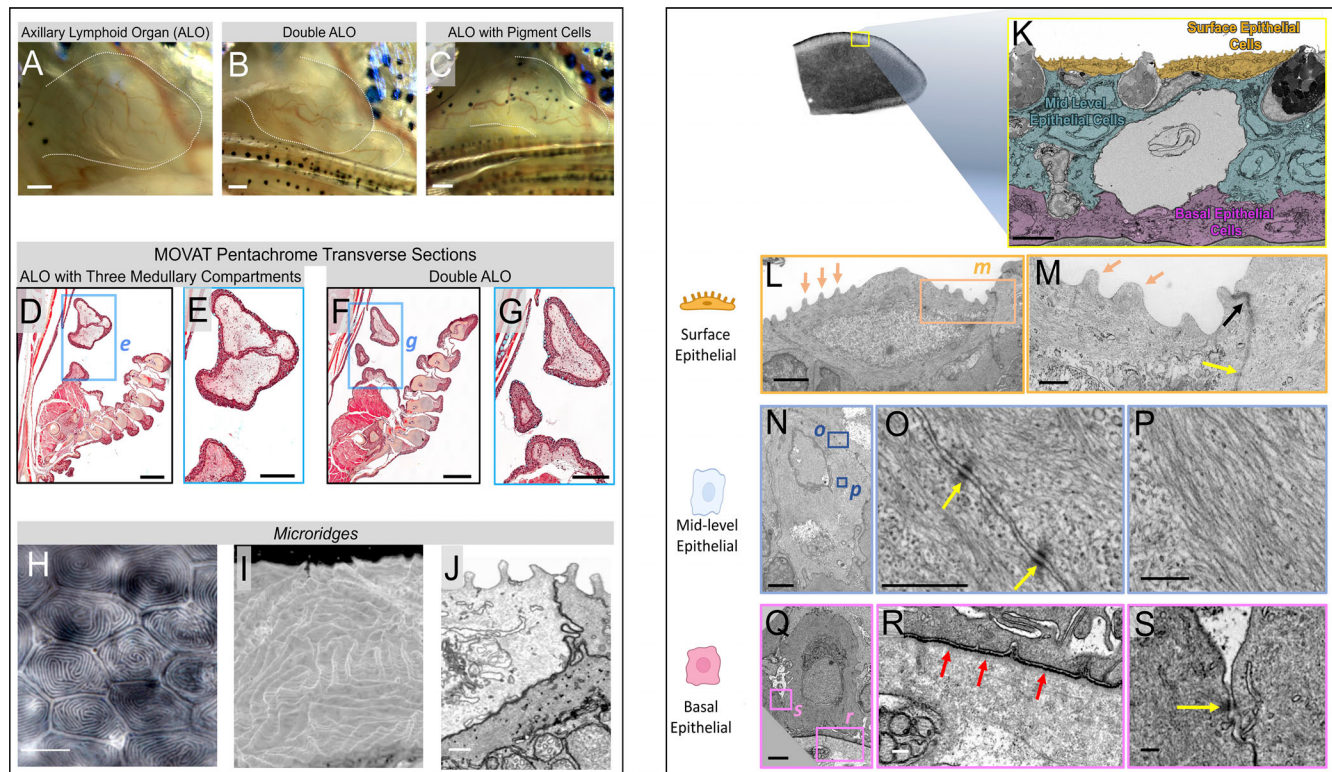


Figure S1. Zebrafish ALO variability, dermal microridges, and cortical epithelial cells. (A–C) Stereomicroscopic images of ALOs from different 10-mo-old EK wild-type zebrafish, with dashed white lines indicating the ALO border, showing a single ALO (A), double ALO (B), and an ALO with melanophores on it (C). (D–G) Movat's pentachrome-stained transverse paraffin sections through the ALOs of two different adult zebrafish showing their morphological variability, including (D and E) a medulla divided into three compartments and (F and G) a fish with two separated ALOs. The blue boxes in panels D and F show the areas magnified in panels E and G, respectively. (H–J) Skin microridges on the outside of the ALO shown in a DIC micrograph (H) and in a rendering of an array tomography 3D image volume reconstruction (I and J), with a 3D reconstruction of the entire array in panel I and part of a single plane shown in panel J (see also Videos 2 and 3). (K) Single pseudocolored section from an array tomography image stack of an adult zebrafish ALO, highlighting the surface, mid-level, and basal epithelial layers. (L and M) Transmission electron micrograph of a microridge (orange arrows) containing surface epithelial cell. Panel M shows a higher magnification image of the orange boxed area in panel L, with a cell–cell tight junction (black arrow) and desmosome (yellow arrow) noted. (N–P) Transmission electron micrograph of a mid-level epithelial cell. Panels O and P show higher magnification images of the blue boxed areas in panel N, with desmosomes (yellow arrows in panel O) and intermediate filaments (panel P) noted. (Q–S) Transmission electron micrograph of a basal epithelial cell. Panels R and S show higher magnification images of the pink boxed areas in panel Q, with the basement membrane separating the dermal cortex and the medulla (red arrows in panel R) and a basal cell–basal cell desmosome (panel S) noted. See Video 2 for additional array tomography images of cortical epithelial cells. Scale bars = 150 μ m (A–C, E, and G), 250 μ m (D and F), 10 μ m (H), 1 μ m (J), 5 μ m (K), 2 μ m (L, N, and Q), 500 nm (M, O, P, R, and S), 200 nm.

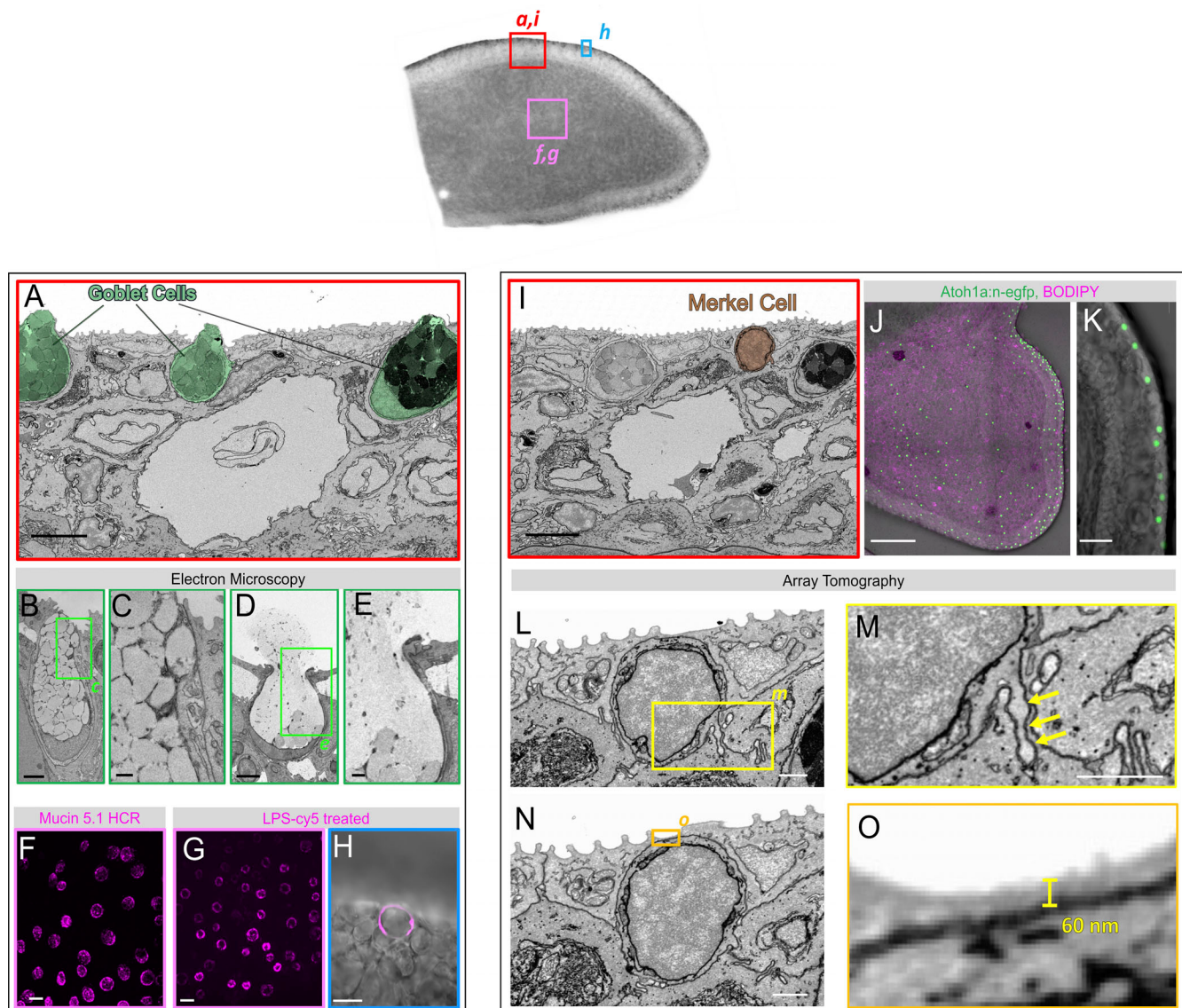


Figure S2. ALO cortical goblet cells and Merkel cells. (A) A single section from an array tomography image stack of an adult zebrafish ALO, with goblet cells pseudocolored green. (B–E) Transmission electron micrographs of goblet cells with intact mucous granules (B and C) or with mucous granules breaking down and mucus being extruded (D and E). Panels C and E show higher magnification images of the green boxed areas in panels B and D, respectively. (F) Confocal micrograph maximum intensity projection showing in situ HCR of an adult ALO probed for mucin 5.1, showing specific labeling of goblet cells. (G and H) Confocal micrographs of ALOs treated in situ with LPS-Cy5 and then excised and imaged ex vivo, showing specific uptake of LPS-Cy5 by goblet cells. Images include a maximum intensity projection (G; comparable view to the image shown in panel F) and a single confocal section with DIC showing a side view of the dermal cortex with a single LPS-Cy5-labeled goblet cell (H). See [Videos 2](#) and [3](#) for additional array tomography images of goblet cells. (I) Single section from an array tomography image stack of an adult ALO, with a Merkel cell pseudocolored brown. (J and K) Confocal/DIC extended depth-of-focus images of an adult *Tg(atoh1a:nls-Eos)^{w214}* transgenic zebrafish ALO showing Merkel cell nuclei in green. Images include (J) an ALO overview (also with BODIPY 633 in magenta) and (K) a side view of the dermal cortex. (L–O) Individual sections from transmission electron microscopic array tomography of an adult zebrafish ALO, showing Merkel cells with large nuclei and small amounts of cytoplasm. Panels M and O show higher magnification images of the yellow and orange boxed areas in panels L and N, respectively. The single inwardly projecting microvilli characteristic of Merkel cells is shown in panel M (yellow arrows). The magnified image in panel O shows that the Merkel cell outer membrane is only 60 nm from the outside surface of the ALO. See [Video 2](#) for additional array tomography images of Merkel cells. Scale bars = 5 μ m (A and I), 2 μ m (B and D), 500 nm (C and E), 10 μ m (F–H), 100 μ m (J), 20 μ m (K), 1 μ m (L–N), 60 nm (O). LPS-Cy5, Cy5-labeled lipopolysaccharide.

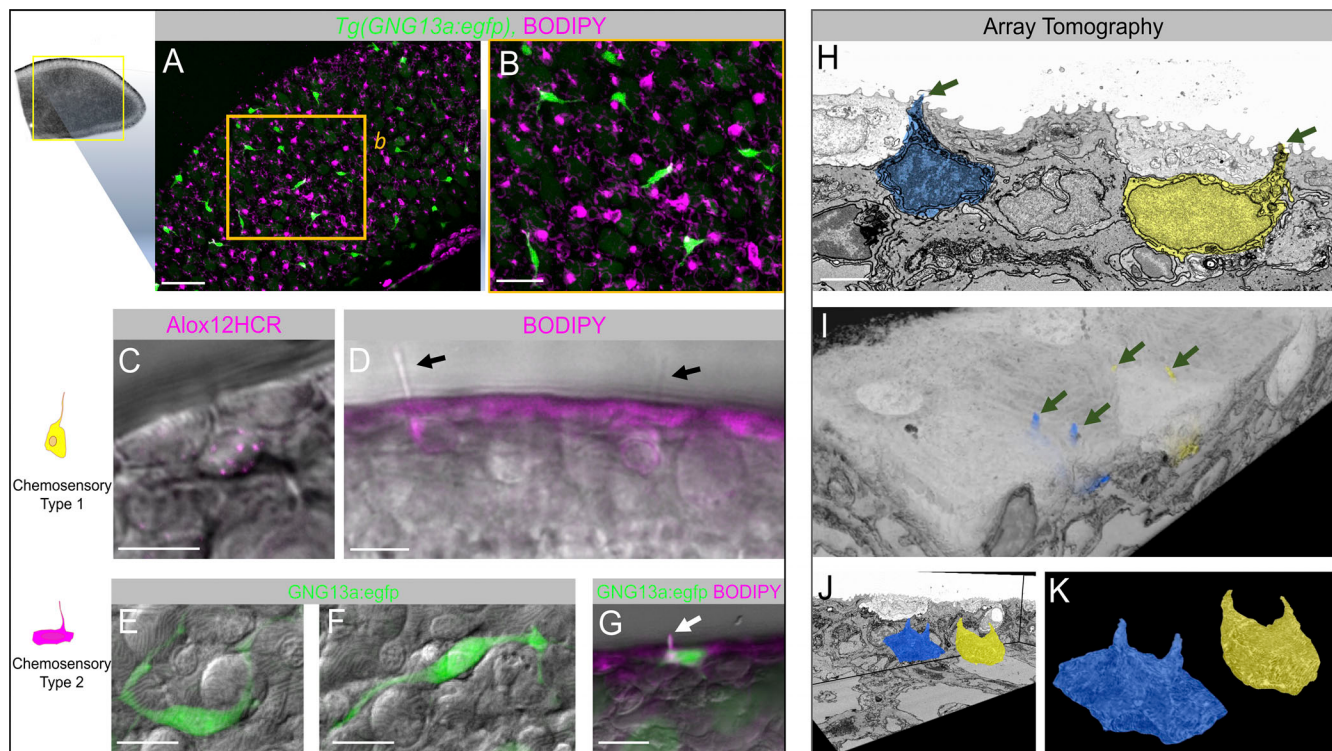


Figure S3. ALO contains two different types of chemosensory cells and has two external microvilli. (A and B) Confocal micrograph maximum intensity projections of an ALO from a *Tg(gng13a:egfp)*⁷⁰⁹ transgenic zebrafish treated with BODIPY 633, showing uptake of the fluorescent dye by chemosensory cells. Panel B shows a higher magnification image of the orange boxed area in panel A, showing only a subset of chemosensory cells are *gng13a*-positive. **(C and D)** Single-plane side-view confocal/DIC micrographs of the cortical surface of wild-type adult zebrafish ALOs either (C) probed for HCR with *alox12* showing expression in a chemosensory cell located just below two surface epithelial cells, or (D) treated with BODIPY 633 showing uptake by chemosensory cells, as well as chemosensory cell microvilli extending into the environment (black arrows). **(E–G)** Confocal micrograph extended depth-of-focus projections of ALOs from a *Tg(gng13a:egfp)*⁷⁰⁹ transgenic adult zebrafish, showing (E and F) overview images of EGFP-positive chemosensory cells with long projections just underneath the surface of the ALO cortex, and (G, also shown in Fig. 4 P) a side view of the ALO cortex of a fish treated with BODIPY 633, showing uptake by the chemosensory cell microvilli (white arrow) and cell body. **(H)** Single section from an array tomography image stack of an adult zebrafish ALO cortex, with two separate chemosensory cells pseudocolored blue and yellow. Single sections such as these show individual chemosensory cell microvilli extending from these cells into the environment (green arrows). **(I–K)** Volume reconstructions of the same array tomography data set of an adult zebrafish ALO cortex from which the image in panel A was taken, with the same two chemosensory cells pseudocolored blue and yellow. Images show volume reconstructions with (I) or without (J) semitransparent fill of non-chemosensory cell portions of the cortex, or with only the segmented chemosensory cells shown (K). The volume reconstructions show that each of the chemosensory cells has two microvilli projecting into the environment (green arrows in panel I). See Videos 2 and 3 for additional array tomography images of chemosensory cells and their paired microvilli. Scale bars = 50 μ m (A), 25 μ m (B), 10 μ m (C–G), 2 μ m (H).

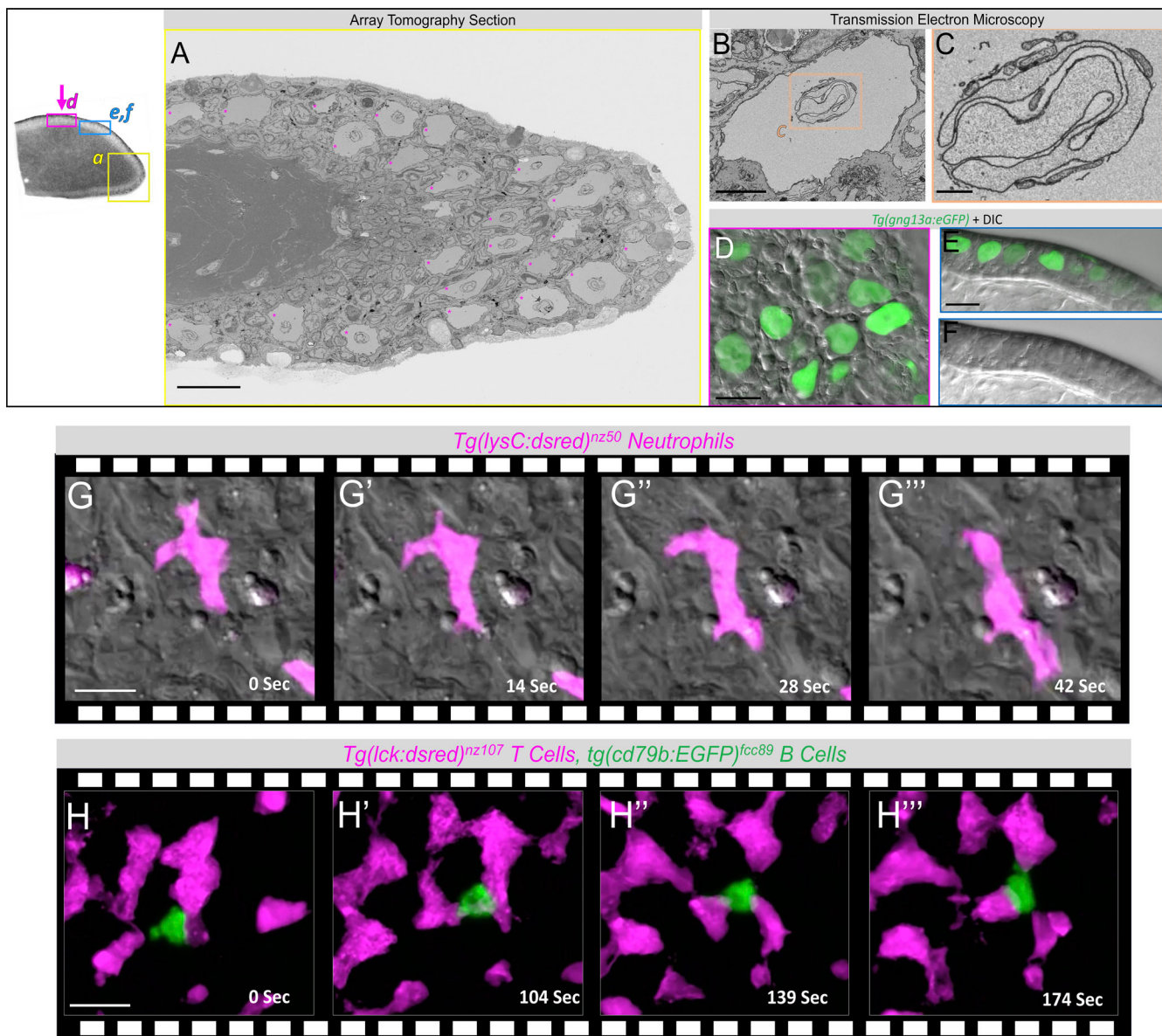


Figure S4. Club cells, neutrophils, and B cell-T cell interactions. (A) Single section from an array tomography image stack of an adult zebrafish ALO, showing an overview tangential section through the edge of an ALO with numerous club cells (magenta asterisks) in the mid-cortex. (B and C) Transmission electron micrograph of an adult zebrafish ALO, showing an individual club cell with homogeneous cytoplasm and complex, folded nucleus. Panel C shows a higher magnification image of the nucleus-containing boxed region in panel B. (D-F) Single section confocal/DIC micrographs of the ALO cortex from a *Tg(gng13a:eGFP)⁷⁰⁹* transgenic zebrafish, with *en face* overview (D) and side-view (E) images of the cortex. Panel F shows the same image as panel E but with only DIC, showing that the unique club cell morphology, a large cell with a central large nucleus, is easily identifiable through DIC alone. See Video 2 for additional array tomography images of club cells. (G-G''') Still images from a time-lapse movie showing a neutrophil *Tg(lysC:DsRed)^{nz50}* crawling in the dermal cortex of an adult ALO (ex vivo) (see Video 7). (H-H''') Still images from a time-lapse movie showing T cell *Tg(lck:DsRed)^{nz107}* and B-cell *Tg(cd79b:egfp)^{fcc89}* interactions (ex vivo) (see also Video 9). Scale bars = 20 μ m (A), 5 μ m (B), 1 μ m (C), 25 μ m (D and E), 10 μ m (G and H).

Video 1. **ALO regeneration.** A combination of still images and live video showing ALO regeneration over 14 days, taken with a stereomicroscope.

Video 2. **Array tomography overview and ALO cortex scan.** Array tomography image Z-stack through the ALO, with a higher magnification scan of the ALO cortex.

Video 3. **Array tomography of chemosensory cells and extruding goblet cells.** Array tomography image Z-stack highlighting chemosensory cells with two microvilli extending into the environment, as well as goblet cells extruding mucus, followed by 3D reconstructions with chemosensory cells segmented.

Video 4. **Adult ALO vasculature.** 3D reconstruction and real-time single-plane live video of confocal micrographs of ALOs on adult *Tg(mrc1a:egfp)^{v251}*, *Tg(kdrl:mcherry)^{v205}* double transgenic zebrafish injected intravascularly with Hoechst 33342 dye, with lymphatic vessels in green, blood vessels in magenta, and circulating Hoechst dye-labeled red blood cell nuclei in blue.

Video 5. **FRCs.** Scroll through Z and 3D reconstructions of ex vivo confocal + DIC imaged ALO from an adult transgenic *Tg(pdgfrb:egfp)^{ncv22}* transgenic zebrafish, with FRCs in green, followed by an array tomography scroll through Z highlighting an immune cell and an FRC.

Video 6. **Immune cells migrate on FRCs.** Ex vivo confocal time-lapse extended depth-of-focus imaging of an ALO from an adult *Tg(mrc1a:egfp)^{v251}* transgenic zebrafish, with a few macrophages in green and many other immune cells migrating along the FRC network.

Video 7. **Live imaging of macrophages and neutrophils in the adult zebrafish ALO.** Ex vivo confocal micrograph extended depth-of-focus and maximum intensity projection time-lapse movies of the ALO of an adult *Tg(mpeg1:egfp)^{gl22}* transgenic zebrafish with macrophages in green, followed by ex vivo confocal micrograph extended depth-of-focus and maximum intensity projection time-lapse movies of the ALO of an adult *Tg(lysC:DsRed)^{nz50}* transgenic zebrafish with neutrophils in magenta.

Video 8. **Live imaging of B cells in the adult zebrafish ALO.** Ex vivo confocal micrograph extended depth-of-focus and maximum intensity projection time-lapse movies of the ALO of an adult *Tg(cd79b:egfp)^{fcc89}* transgenic zebrafish with B cells labeled in green.

Video 9. **Live imaging of T cells, and T cells and B cells interacting in the adult zebrafish ALO.** Ex vivo confocal micrograph extended depth-of-focus and maximum intensity projection time-lapse movies of the ALO of an adult *Tg(lck:egfp)^{c22}* transgenic zebrafish with T cells labeled in green, followed by a time-lapse movie of T-cell *Tg(lck:mcherry)^{ns107}*–B cell *Tg(cd79b:egfp)^{fcc89}* interactions.

Video 10. **ALO in T-ALL.** Ex vivo confocal micrograph scroll through Z of ALOs of adult *Tg(lck:egfp)^{c22}* transgenic wild-type sibling or *HLK^{dz102}* transgenic fish with T-ALL. Normal or leukemic T cells are labeled in green by *lck:egfp* expression.

Provided online is Table S1. Table S1 shows species of bony fishes (teleosts) examined to assess the presence or absence of an ALO-like structure.