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A macrophage relay for long distance signaling during post-embryonic tissue remodeling

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Abstract

Macrophages have diverse functions in immunity as well as development and homeostasis. Here we identify a function for these cells in long distance communication during post-embryonic tissue remodeling. Ablation of macrophages in zebrafish prevented melanophores from coalescing into adult pigment stripes. Melanophore organization depends on signals provided by cells of the yellow xanthophore lineage via airinemes, long filamentous projections with vesicles at their tips. We show that airineme extension from originating cells, and vesicle deposition on target cells, depend on interactions with macrophages. These findings identify a role for macrophages in relaying long range signals between non-immune cells. It will be interesting to see if this signaling modality functions in the remodeling and homeostasis of other tissues during normal development and disease.

Main Text

Macrophages are phagocytic cells with essential roles in immunity including recognition and disposal of infectious microbes, dying cells and debris. Yet, non-immune activities have also been identified. Macrophages are now known to function during development and homeostasis, including blood vessel and mammary duct morphogenesis, pancreatic cell specification, hematopoietic stem cell maintenance, and lipid metabolism (1–4). To investigate potential roles for macrophages in post-embryonic tissue remodeling, we examined the larval-to-adult transformation of zebrafish, a period of morphogenesis, patterning and growth, with similarities to human fetal and neonatal development (5, 6).

We depleted macrophage populations by expressing bacterial nitroreductase (NTR) in these cells. NTR kills cells by converting metronidazole (Mtz) to toxic metabolites (7, 8) (fig. S1). Fish treated with Mtz between mid-larval and juvenile stages had a severe defect in the adult pattern of neural crest derived pigment cells. Untreated juvenile zebrafish exhibit dark stripes of black melanophores dorsal and ventral to a light "interstripe" of yellow–orange xanthophores and iridescent iridophores. Yet, macrophage-depleted fish retained numerous melanophores in the interstripe (Fig. 1A; fig. S2). This pattern resembled a phenotype that results from a defect in long distance communication between melanophores and

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Author Contributions

D.S.E. performed experiments. D.S.E. and D.M.P. designed the experiments, analyzed data and wrote the manuscript.

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xanthophore precursors (9). During stripe development, progenitors of adult melanophores migrate to the skin and begin to differentiate widely over the flank (10). At this stage, precursors to adult xanthophores are already located in the prospective interstripe, where they differentiate as xanthophores, and also in prospective stripes, where they remain as unpigmented xanthoblasts (6) (Fig. 2A). Interactions between melanophores and cells of the xanthophore lineage are required for pattern formation and maintenance (11–14); e.g., xanthophores repel melanophores at short range (15, 16). By contrast, xanthoblasts extend thin projections, "airinemes," that contact dispersed melanophores and melanoblasts (9). Airinemes arise from surface blebs, can reach up to several cell diameters (>150 μm), and have large (~1 μm) membrane-bound vesicles at their tips that harbor the Notch pathway ligand DeltaC and potentially other factors. When xanthoblasts are ablated, or when airineme production or Delta–Notch signaling are inhibited, melanophores fail to organize and many persist in the interstripe (9). Given the similarity of this phenotype to that of macrophage-depletion, we speculated that macrophages contribute to airineme-dependent signaling.

To test for correspondence in macrophage and airineme behaviors, we used time-lapse imaging. The haphazard wanderings of macrophages, revealed by an *mpeg1* reporter (8), were qualitatively similar to the haphazard paths taken by airineme vesicles, revealed by a membrane-targeted *aox5* reporter (6, 9) (Fig. 2B; fig. S3). To see if macrophages and airinemes interact, we examined both reporters simultaneously (fig. S1D). Of 178 airinemes, 168 (94%) were associated with macrophages, though not all macrophages may have been visible owing to limits on temporal resolution and detection in deeper tissues. Airinemes extended in association with macrophages traversing xanthoblasts and, while airinemes were extending their vesicles remained associated with macrophages for up to 189 μm (Fig. 2C; fig. S4A; movies S1–4). Interactions occurred between macrophages and airineme vesicles rather than filaments. In 3 of 168 instances, portions of macrophage-associated vesicles detached from trailing filaments and airineme extension continued in association with a second macrophage (fig. S4B; movie S5).

To better understand macrophage–airineme relationships, we used an *mfap4* membrane-targeted reporter of macrophages (17) (fig. S1B), which revealed intact airineme vesicles engulfed by macrophages, with filaments trailing back to source xanthoblasts (Fig. 3A; movie S6). These observations suggested the hypothesis that macrophages drag airineme vesicles and filaments from surface blebs of xanthoblasts, much as optical tweezers can pull tethered surface blebs from cells in culture (18). If so, we predicted that depleting the macrophage population should reduce the incidence of airineme extension. Supporting this idea, Mtz-depletion of NTR+ macrophages dramatically reduced airineme incidence (Fig. 3B,D; fig. S5; movie S7). As a second paradigm, we used cell transplantation to construct fish with xanthoblasts but not macrophages. Mutants for *colony stimulating factor-1 receptor a* (*csf1ra*) are deficient for macrophages but also xanthophores and xanthoblasts (19, 20). We therefore transplanted cells from wild-type embryos carrying lineage reporters into *csf1ra* mutant embryos, and reared chimeras that developed xanthoblasts but not macrophages (*aox5*+, *mpeg1*–). These larvae exhibited far fewer airinemes than macrophage-intact wild-type hosts (Fig. 3C; fig S6A; movie S8). We observed similar macrophage dependencies even in a background with particularly exuberant airinemes (fig. S6B; movies S9, S10) (9).

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In all paradigms, the few airinemes extended were associated with residual macrophages (movie S11). Thus, macrophages are essential for airineme extension.

Macrophages provide a variety of signals to target cells (2–4, 21) but did not influence the competence of xanthoblasts to initiate airineme formation, as surface blebs from which airinemes arise (9) were similarly abundant in control and macrophage-depleted backgrounds (fig. S7A). Likewise, neither onset nor cessation of peak airineme activity were associated with specific changes in macrophage abundance (fig. S7B).

We therefore asked how macrophages recognize airineme-initiating blebs when they are present. DeltaC localizes to these blebs (9), yet airinemes developed normally in *delta c* mutants suggesting roles for other factors (fig. S7C). On apoptotic cells, the phospholipid phosphatidylserine (PS) occurs in the outer leaflet of the plasma membrane where it serves as an "eat me" signal for macrophages (21, 22). Accordingly, we hypothesized that xanthoblast blebs present PS to macrophages. To detect PS, we delivered a secreted form of PS-binding Annexin V, secA5-mCherry, into the tissue environment of xanthoblasts by expressing it in nearby melanophores or iridophores (10, 23–25). We found strong secA5 labeling of airineme-initiating blebs on xanthoblasts, and an absence of labeling on xanthophores, which form neither blebs nor airinemes (Fig. 3E; fig. S8). If PS is required for macrophage–bleb recognition, we predicted that PS–secA5 interaction should block airineme extension. Indeed, airinemes were produced less often in the vicinity of secA5+ cells than secA5– cells (Fig. 3F). These observations suggest that a macrophage–PS recognition system has been co-opted for airineme extension and long distance communication.

Our observations indicate that macrophages may relay signals—associated with airineme vesicles—from xanthoblasts to melanophores. We therefore sought to verify that macrophage-associated airineme vesicles can be deposited on melanophores, and to test if macrophages remain with vesicles after delivery, and so utilized fish transgenic for a membrane-targeted *tyrp1b* reporter (6) of melanophores. Macrophage-associated airineme vesicles were deposited frequently on melanophores, without indications of membrane fusion or internalization; macrophages did not cease their movements after vesicle "hand-off" and instead continued to wander (Fig. 4; movies S12, S13). Because patterning can depend on attenuation of signals we also asked whether macrophages might dispose of previously extended airineme vesicles. We observed macrophages phagocytosing vesicles that had stabilized on melanophores, as well as vesicles that had been extended without stabilizing (movies S14, S15). Thus, macrophages not only mediate signaling to melanophores, they may regulate the duration and specificity of signaling.

Communication at a distance is fundamentally important to patterning yet its mechanisms remain incompletely understood. Considerable attention has been given to signaling via long actin-based filopodia, or "cytonemes" (26, 27), yet additional classes of cellular projections have been identified, the properties of which are only beginning to be explored (28, 29). Our analyses show that macrophages are key players in long-range, airineme-dependent communication between xanthoblasts and melanophores. Indeed, macrophage wanderings may allow for diffusion-like dissemination, envisaged by mathematical models (13, 30),

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despite the large size of airineme vesicles and the long distances involved. Our findings add to the increasingly diverse recognized functions of macrophages, defined classically for their phagocytic capabilities. It remains to be determined whether macrophages passively carry signals, actively process them, or assist in discriminating among target cells (9). Identifying additional features of macrophage–airineme interactions in this, and potentially other contexts, will shed light on the evolution and generality of this system, and could also suggest novel approaches for delivery of therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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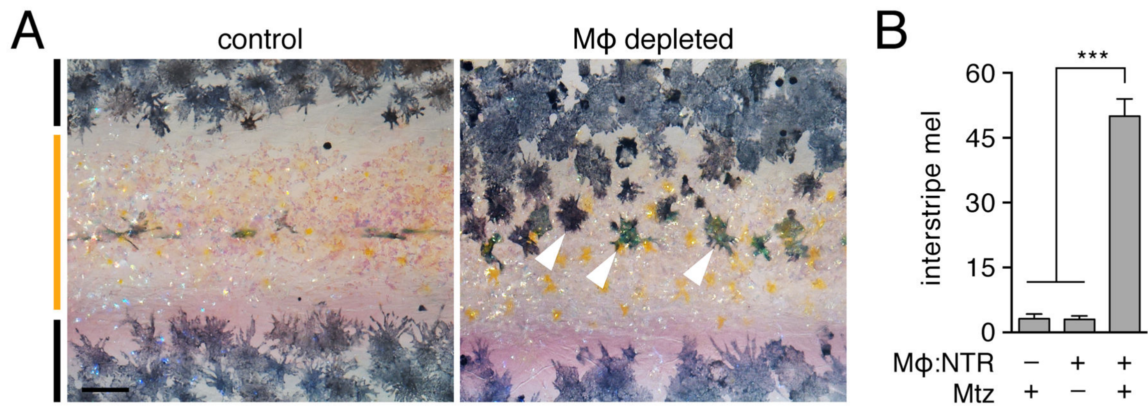

Fig. 1. Macrophage depletion resulted in melanophore pattern defect

(**A**) In controls, melanophores in stripes (black bars at left) bordered the interstripe (orange bar). Macrophage-depletion resulted in ectopic melanophores (arrowheads). (**B**) Macrophage-depleted fish (NTR+, Mtz+) had many more melanophores in the interstripe than controls ($F_{2,17}$=95.7, P<0.0001; N=20 larvae total; plot, means±SE), though total melanophore numbers did not differ ($F_{2,17}$=1.0, P=0.4). Stage 13 SSL (5). Scale bar: 100 μm (C).

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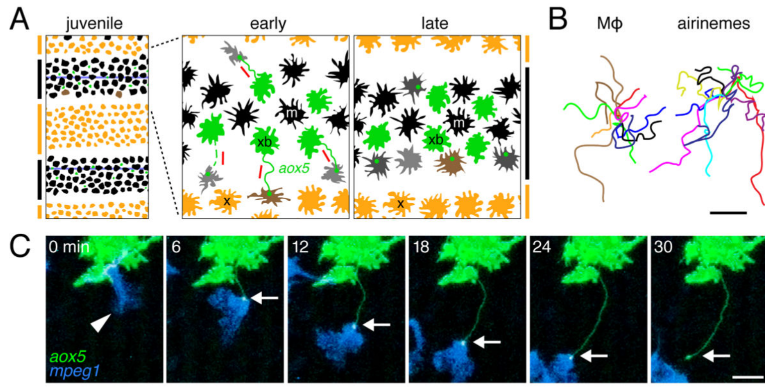

Fig. 2. Correspondence of macrophage movements with xanthoblast-derived airineme projections

(**A**) Consolidation of melanophore stripes depends on interactions with cells of the xanthophore lineage. Juvenile pattern showing region corresponding to Fig. 1A (outlined in blue) with events early and late in stripe formation. Xanthoblasts (green; xb marked by *aox5*) among melanophores (m) extend airinemes (red bars) that contact differentiating melanophores (grey) or embryonic melanophores persisting in the interstripe (brown); airineme vesicles can persist on melanophores for several hours, even after filaments have fragmented. Several days later, airinemes are no longer produced and melanophores have consolidated into definitive stripes. Xanthophores (orange; x) occur in the interstripe. (**B**) Similar wanderings of macrophages and airineme vesicles. (**C**) A macrophage (blue, arrowhead) that has traversed a single xanthoblast (green; 0 min) associated with an airineme vesicle (arrow) and trailing filament (6–24 min); airineme extension ceased upon macrophage–vesicle dissociation (24–30 min). Scale bars: 40 µm (B); 20 µm (D).

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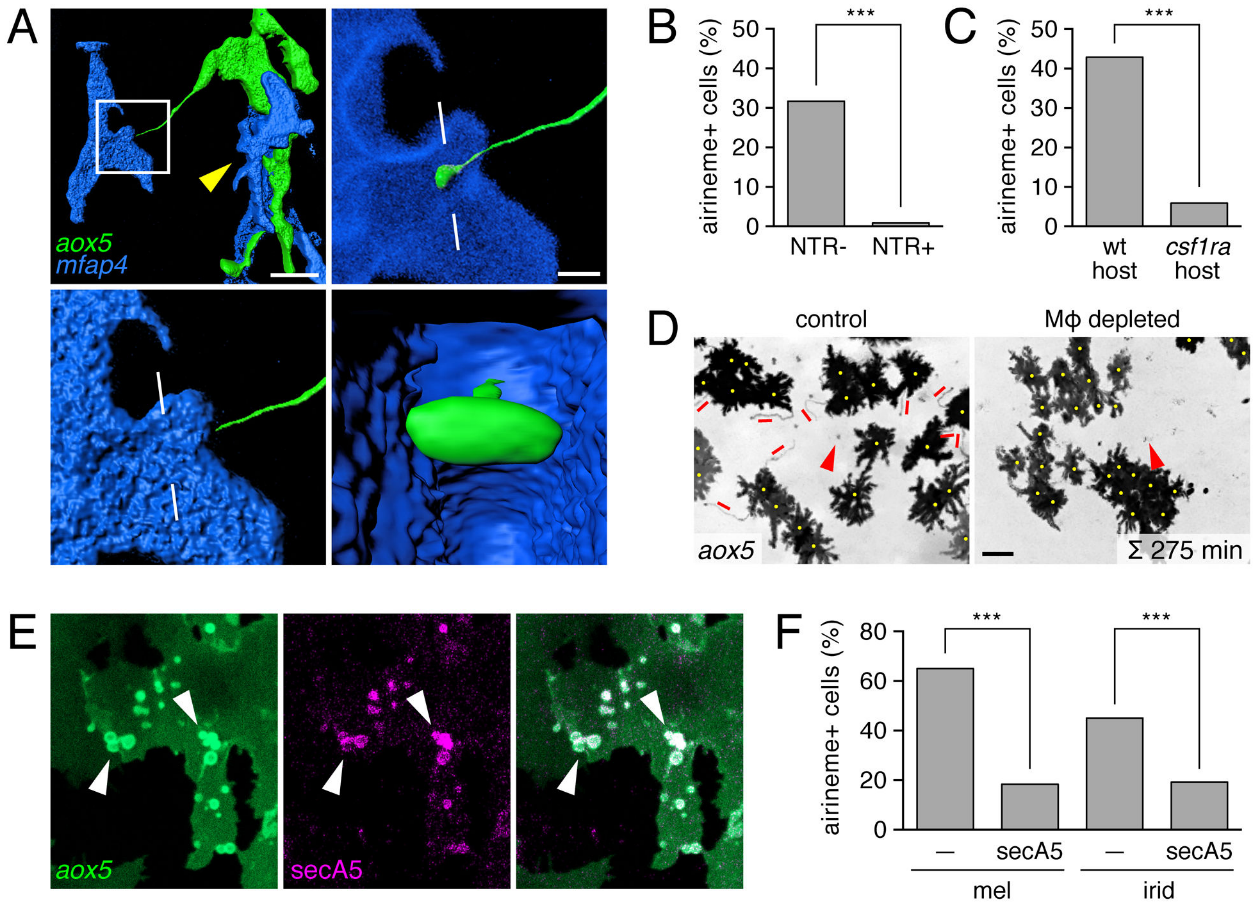

Fig. 3. Macrophage requirements for airineme extension

(**A**) Xanthoblast (green) and macrophages (blue) illustrating close association (arrowhead) and engulfment of airineme vesicle with filament. White boxed region shown with and without surface rendering of macrophage in lower right and upper left 9 hatch marks, cut plane inside macrophage at lower right). (**B**) Xanthoblasts of macrophage-depleted fish (NTR+) were less likely to extend airinemes than controls (NTR–; χ^2=52.0, d.f.=1, P<0.0001, N=284 cells, 14 larvae). (**C**) Xanthoblasts in macrophage-deficient *csf1r* mutant hosts similarly had fewer airinemes than wild-type hosts (χ^2=23.8, d.f.=1, P<0.0001, N=198 cells, 9 larvae). (**D**) Merged time-lapse frames illustrate airinemes (red dashes) in control (NTR–) but not macrophage-depleted (NTR+) trunks. Yellow dots, approximate cell centroids; arrowheads, autofluorescence in cells not carrying *aox5* reporter. (**E**) PS localization at airineme-originating blebs (arrowheads) of a single xanthoblast detected by secA5. (**F**) Airineme extension was repressed in the vicinity of secA5 expressing melanophores (mel; χ^2=146.4, d.f.=1, P<0.0001, N=626 cells, 15 larvae) and iridophores (irid; χ^2=16.7, d.f.=1, P<0.0001, N=215 cells, 8 larvae), though macrophage motility (μm/min) did not differ between secA5– and secA5+ regions (t_{54}=0.8, P=0.4; N=56 cells, 6 larvae). Scale bars: 10 μm (A, upper left); 2 μm (A, upper right), 50 μm (D).

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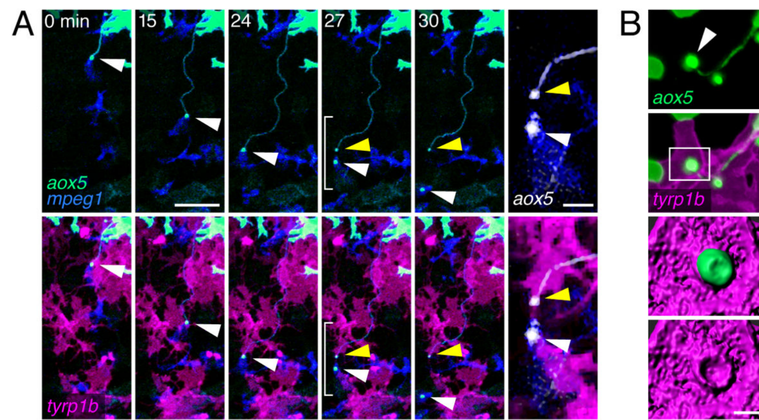

Fig. 4. Macrophages relay airineme vesicles to melanophores

(**A**) Airineme vesicle (green) associated with a macrophage (blue; 2-color merge, upper panels) and melanophores (magenta; 3-color merge, lower panels). The airineme extended as the macrophage migrated (0–24 min, white arrowhead) but a portion of the vesicle and its filament then stabilized on the melanophore (27 min, yellow arrowhead) as the macrophage continued on with some vesicular material (30 min). Far right, bracketed region at 27 min. (**B**) Airineme vesicle associated with melanophore membrane. Boxed region surface-rendered in lower panels. Scale bars: 50 μm (A); 10 μm (A, closeup); 2 μm (B, closeup).