Ontogeny and behaviour of early macrophages in the zebrafish embryo

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SUMMARY

In the zebrafish embryo, the only known site of hemopoiesis is an intra-embryonic blood island at the junction between trunk and tail that gives rise to erythroid cells. Using video-enhanced differential interference contrast microscopy, as well as in-situ hybridization for the expression of two new hemopoietic marker genes, draculin and leucocyte-specific plastin, we show that macrophages appear in the embryo at least as early as erythroid cells, but originate from ventrolateral mesoderm situated at the other end of the embryo, just anterior to the cardiac field. These macrophage precursors migrate to the yolk sac, and differentiate. From the yolk sac, many invade the mesenchyme of the head, while others join the blood circulation. Apart from phagocytosing apoptotic corpses, these macrophages were observed to engulf and destroy large amounts of bacteria injected intravenously; the macrophages also sensed the presence of bacteria injected into body cavities that are isolated from the blood, migrated into these cavities and eradicated the microorganisms. Moreover, we observed that although only a fraction of the macrophage population goes to the site of infection, the entire population acquires an activated behaviour, similar to that of activated macrophages in mammals. Our results support the notion that in vertebrate embryos, macrophages endowed with proliferative capacity arise early from the hemopoietic lineage through a non-classical, rapid differentiation pathway, which bypasses the monocytic series that is well-documented in adult hemopoietic organs.

Key words: Hematopoiesis, Macrophage, DIC video-microscopy, Zebrafish, Yolk sac, Lateral mesoderm, Head mesoderm, Phagocytosis, L-plastin, Draculin

INTRODUCTION

The zebrafish embryo has recently become one of the major model organisms for the study of vertebrate embryogenesis. Its transparency is one of its main assets, allowing one to view the details of embryological processes in the live embryo, using Differential Interference Contrast (DIC, or ‘Nomarski’) microscopy. This tool, together with single-cell tracing experiments using fluorescent dyes, has produced a fairly comprehensive description of zebrafish embryogenesis (Kimmel et al., 1995).

We recently found that when further improved by video-enhancement (Hayden and Allen, 1984), DIC microscopy can give access to a host of previously unapproached details of cell behaviours and even intracellular life in the zebrafish embryo, and reveal unknown aspects of vertebrate embryogenesis. The present paper describes the ontogeny and functional properties of a primitive macrophage lineage in the early zebrafish embryo.

MATERIALS AND METHODS

DIC video-microscopy

Embryos were observed under a coverslip in a depression slide filled with Volvic mineral water containing 0.02% tricaine to prevent embryo twitching. For dorsal viewing during somitogenesis, the embryos were embedded in 0.5% agarose in embryo medium (Westerfield, 1995). Embryos were viewed with a Reichert Polyvar 2 microscope equipped with DIC optics, using ×40 and ×100 oil immersion objectives. Images were captured by a Hitachi HV-C20 tri-CCD colour video camera, in which all contrast-dampening functions were set off, so that with the ×100 objective the final magnification on a 26-cm video screen was ×4000 with excellent contrast, allowing, for instance, sight of mitochondria and intracellular vesicle trafficking in the embryo. Video-sequences were recorded from the Y/C output of the camera either by a Panasonic AG6730 time-lapse tape recorder in S-VHS format, or by a Sony DHR1000 tape recorder in Digital Video (DV) format. Single video images were captured and stored on a PC from the DV videotapes, through the Sony DVK2000 capture board. Digitized images were then processed with the Adobe Photoshop IV software.

Cell labelling in vivo

Single-cell labelling of 16-cell embryos with 2 mDa Dextran-Texas Red (Molecular Probes) was done according to Strehlow et al. (1994).
cDNA clones and whole-mount in situ hybridization

The nucleotide sequences of the cDNAs encoding zebrafish draculin and L-plastin are available from GenBank under accession numbers AF157109 and AF157110, respectively. Whereas draculin appears to be a new zinc-finger protein with no homolog in sequence databases, our zebrafish L-plastin cDNA encodes the 200 C-terminal amino acids of a protein displaying 79% identity to the homologous portion of human L-plastin. The flk-1 cDNA clone was kindly provided by L. Sumoy and D. Kimelman. Whole-mount in situ hybridization was performed according to Thisse et al. (1993). The fully detailed protocol is accessible on the web at the zfin server: http://www-igbmc.u-strasbg.fr/zf_info/zfbook/chapt9/9.82.html.

Injection of bacteria in day2 embryos

The strains used were E. coli K12 DH5 and B. subtilis W168. For intravenous injections, we injected a few nl of a 4x concentrated stationary phase bacterial culture grown overnight, supplemented with 1% Phenol Red to help visualize the injection process, into the axial vein, close to the urogenital opening. Injections in the pericardial cavity or fourth ventricle of the brain were done using the same type of bacterial culture, except that it was not further concentrated 4x.

RESULTS

In the zebrafish, blood circulation starts around 25 hours post-fertilization (hpf). Unlike most fish embryos, the venous blood stream is not contained in any vessel as it approaches the heart; it flows freely over the lateral sides of the yolk, and these two streams join anteriorly to enter the heart (Fig. 1).

A closer look at these blood streams reveals that a fraction of blood cells stop over the yolk, either on the sides of the blood stream or within it, and resist the flow. At higher magnification, two cell types become clear: erythroblasts, as expected, but also amoeboid cells with bean-shaped eccentric nuclei (Fig. 2). High-resolution time-lapse DIC video-microscopy reveals their ever-changing shapes, which can quickly switch from rounded to highly sculpted and vice versa. Fig. 2 shows a selection, although no single plane of focus captures their often exuberant three-dimensional shapes.
Early macrophages in zebrafish

With DIC video-microscopy, we have observed these amoebocytes phagocytosing the corpses or debris from apoptotic erythroblasts in the bloodstream. This behaviour, together with their morphological similarities to macrophages of mammals, qualify these amoebocytes as macrophages. They also show another typical trait of macrophages: a behaviour called ‘frustrated phagocytosis’ (Cannon and Swanson, 1992). So far studied in cultured mammalian cells, this phenomenon occurs naturally in the zebrafish embryo. Apoptotic erythroblasts sometimes inflate to a size larger than the original cell. When a macrophage sticks to such an apoptotic ‘bubble’, it tries to engulf it, unsuccessfully for the prey is too big (Fig. 2D).

These macrophages resist the blood stream by anchoring to the underlying yolk surface (i.e. the plasma membrane of the yolk syncitial layer) or, most often, to the basal lamina of the overlying ectoderm (a thin epidermal layer, itself overlain by a still thinner, protective epithelium, the periderm, see Fig. 1). They stop many erythroblasts that they touch, and then release them back into circulation, often after a close and lengthy interaction (Fig. 2B).

Macrophage development in the yolksac precedes the arrival of erythroblasts

What are the embryonic origins of these early macrophages? They clearly do not originate from the blood island at the trunk/tail boundary, which only provides endothelial cells and proerythroblasts that migrate to the yolksac by 24 hpf (Rieb, 1973; Al-Adhami and Kunz, 1977; Detrich et al., 1995).

We found that several hours before proerythroblast migration, macrophages are already on the anterior yolk, most of them under the hatching gland and along the pericardium (Fig. 3). By 26 somites, each embryo has 80-150 such macrophages, which display two types of morphologies. Some are half-spread and wandering, under the hatching gland and neighbouring epidermis. Fig. 3B shows one of these, rapidly interconverting between spread and more condensed shapes, and bristling with pseudopodia and filopodia. The second type are unspread, rounded cells of homogeneous size (12 μm in diameter), with little cytoplasm, and a typical crown of small blebs (Fig. 3A), which time-lapse recordings show to be highly dynamic. These cells divide frequently, and sooner or later become wandering young macrophages.

By the 29-somite stage, just before proerythroblasts arrive on the yolk, the seemingly random wandering of some young macrophages becomes oriented towards the site of arrival of the proerythroblasts. Several literally meet the proerythroblasts at their entry site (Fig. 3C). Upon meeting, erythroblasts and about one third of the macrophages (about two thirds remain under the hatching gland) interact for approximately 1 hour on the lateral yolksac (Fig. 2C). Then blood circulation slowly begins, taking away erythroblasts and some macrophages, which are then observed in vessels throughout the body (data not shown).
Early macrophage ontogeny can be traced by two leucocyte-specific genes, draculin and leucine-rich glioma inactivated 1 (lrig1).

Our DC microscopic observations suggested that macrophage precursors are born in the ventral side of the early gastrula. We used the expression of two genes, draculin and leucine-rich glioma inactivated 1 (lrig1), to label macrophage precursors. The first gene, draculin, encodes a secreted protein that is expressed in macrophages. The second gene, lrig1, encodes a transmembrane protein that is expressed in a subset of macrophage precursors. We used these genes to label macrophage precursors in vivo.

During gastrulation, macrophage precursors emerge from the mesoderm and migrate towards the midline. This migration occurs in two phases: an initial phase of emigration from the mesoderm, and a later phase of migration within the mesoderm. The initial phase of emigration occurs between the 10- and 20-somite stages, and the later phase of migration occurs between the 20- and 30-somite stages.

During the initial phase of emigration, macrophage precursors are born in the ventral mesoderm and migrate towards the midline. During the later phase of migration, macrophage precursors migrate within the mesoderm and accumulate in the perivascular spaces of the mesoderm. This accumulation is associated with the formation of the vasculature.

The migration of macrophage precursors is mediated by chemotactic factors that are produced by the developing vasculature. These factors attract macrophage precursors towards the midline, where they accumulate in the perivascular spaces of the mesoderm. The accumulation of macrophage precursors in the perivascular spaces is essential for the formation of the vasculature.

In conclusion, macrophage precursors emerge from the ventral mesoderm and migrate towards the midline during gastrulation. This migration is mediated by chemotactic factors that are produced by the developing vasculature. The accumulation of macrophage precursors in the perivascular spaces of the mesoderm is essential for the formation of the vasculature.
Early macrophages in zebrafish

as markers for the ontogeny of early macrophages, from the late blastula stage throughout segmentation and organogenesis.

Both genes were isolated from a systematic in situ expression screen designed to identify genes involved in the early patterning of the zebrafish embryo (C. T. and B. T., unpublished data). Analysis of the predicted amino acid (aa) sequence of the *draculin* (*dra*) cDNA revealed that it codes for a 411-aa-long, poly(zinc finger) protein, containing 13 C2H2-type zinc fingers, preceded by 37 aa at the amino terminus, and 17 aa at the carboxy terminus (data not shown). These amino- and carboxy-terminal domains show no similarity to any sequences in available databases. *Dra* starts to be expressed at the late blastula stage all around the blastoderm margin. At the onset of gastrulation, *dra* expression becomes restricted to the ventral mesoderm territory, the presumptive prechordal plate, and the most dorso-marginal cells of the organizer (data not shown). Later in gastrulation, the dorso-marginal expression disappears, expression in the prechordal plate becomes restricted to its lateral rims, and the ventral mesoderm continues to express *dra* strongly (Fig. 8A,B). Expression persists as these cells form the lateral mesoderm all along the antero-posterior axis of the embryo (Fig. 8C). At the 3-somite stage (Fig. 8C,D,G), *dra* expression becomes stronger in two domains of the lateral mesoderm: a caudal domain, which marks the erythroid lineage, with the same location and dynamics as described for *GATA-1*, a marker of that lineage (Detrich et al., 1995), and a cephalic domain abutting anteriorly the hatching gland primordium (the main derivative of the prechordal plate). These two domains become more sharply defined as all other lateral mesoderm cells cease to express *dra*. At the 8- to 10-somite stages, the anterior domain consists of two thin bands of cells (Fig. 8H), which when compared with Nomarski views of live embryos at the same stage, are clearly cells of the most lateral mesoderm, precisely between the eye and hatching gland anteriorly and the cardiac primordia posteriorly (this was also confirmed by double in-situ hybridization for *dra* and the cardiac marker *nkx2.5*; not shown). Then the cephalic and caudal domains both converge rapidly to the midline. The two sides of the caudal domain fuse at the midline in the antero-posterior sequence typical of erythroid progenitors (Fig. 8E,F). Anteriorly, at 10-11 somites the left and right sides of the cephalic domain start expressing *dra* more strongly and no longer converge as two parallel bands, but as two patches that coalesce at the midline at 15-somites (Fig. 8I,J). During this process, an increasing proportion of these *draculin*-positive cells are found scattered as single cells, at increasing distances from the midline. Once the two patches have coalesced at the midline, this scattering continues to expand anteriorly and laterally, in a pattern indistinguishable from that described above for living macrophage precursors. The fact that the scattering has already begun by 11-somites, before any of these cells has reached the midline, implies that macrophage precursors do not need to reach the midline before emigrating.

**Fig. 5.** Emigration of macrophage precursors from the ventral mesoderm to the yolksac. (A,B,D-F) Dorsal views of an individual embryo, between the 14- and 19-somite stages (medial up, anterior to the left). (A) At the 14-somite stage, cells present on the yolk surface (y) between the eye and cardiac tube (ct), are emigrating from the post-optic ventral mesoderm towards the anterior yolksac. y denotes the part of the yolk surface just lateral to the brain (b) that lies beneath paraxial mesoderm, itself out of focus because it is more dorsal. (B) The same embryo 30 minutes later, showing progression of the emigration. On the left, a group of four emigrating cells have just emerged from beneath the eye. (C) In another embryo, a lateral view shows the emigration of cells in the region lateral to the eye; while the picture is taken at 18-somites, arrows link each cell to its position 30 minutes earlier. (D-F) Same embryo as in A,B; at the 17-somite stage, cells begin to emigrate on the yolk surface beneath the cardiac tubes, the lateral limit of which is indicated by a dotted line. (D) Blue dots point at a macrophage precursor that just emerged laterally from below the cardiac tube, and at two others which are still under it (the other visible cells to the right are cardiac cells that slightly bulge out of the cardiac tube). (E) In the next 5 minutes, the second of the latter three macrophage precursors migrates rapidly out of the cardiac zone, joining the first emigrated one. (F) 1 hour later, a string of at least six cells is now emigrating beneath the cardiac tube towards the yolksac. Bar, 10 μm.
to the yolksac. Once on the yolksac, they progressively stop expressing dra, and this occurs much more quickly in cells on the right side than in those on the left (Fig. 8K).

The flk-1 gene, an early marker of endothelial differentiation, is also expressed in the cephalic lateral mesoderm during the period studied here (Fouquet et al., 1997). We found that by 7-10 somites, flk is expressed anteriorly like dra, in two thin bands anterior to the heart field (data not shown). In the 10-15 somite period, the flk and dra expression patterns become increasingly distinct, with flk-expressing cells soon delineating the bilateral primordia of the arterial head vasculature (Fig. 8M-P). Double in-situ hybridization reveals that at 15 somites (Fig. 8P), the two groups of flk-expressing cells coalesce precisely between the two groups of flk-expressing cells that most likely represent the aortic arch primordia (Fouquet et al., 1997).

By 20-24 hpf, the posterior domain of dra expression becomes the embryonic erythroid blood island centered above the uro-genital opening; dra is strongly expressed in proerythroblasts. As these mature into circulating erythroblasts, they still express dra. Then dra expression in the blood progressively ceases to be detectable by 48 hpf, as erythroblasts become mature erythrocytes (data not shown).

### L-plastin expression and further deployment of early macrophages from the yolksac

To follow later stages of macrophage development we analyzed expression of the zebrafish homolog of L-plastin (see Materials and Methods). L-plastin is an actin-bundling protein which, in the mouse, is specifically expressed in leukocytes, and is involved in their adhesion and activation. As macrophage precursors migrate to the yolksac, they start to express the L-plastin gene (Fig. 8L), and continue to express it as they mature (Fig. 8Q-T).

After their initial accumulation in the anterior part of the yolksac, young macrophages not only join the yolksac circulation valley (Fig. 1), but individually disperse on various tissue surfaces of the yolksac outside the blood flow (Fig. 8Q-S). Many others creep on the basal lamina of the ectoderm and join the head of the embryo, where they colonize the mesenchymal spaces and tissues (Fig. 8R-T, and data not shown). This process starts by the 26-somite stage, and leads to about 100 macrophages in the head at 36 hpf. Prior to blood circulation, L-plastin-positive cells are found exclusively in the yolksac and in the mesenchyme of the head (Fig. 8Q-R). Then at 28 hpf, a distinct group of 20-30 L-plastin-positive cells appears in the caudal part of the axial vein and the mesenchyme surrounding it (Fig. 8S, arrow), and this group expands in the following hours (Fig. 8T). These may represent either yolksac macrophages brought by the blood circulation which settled there, or a second wave of haemopoietic activity from the caudal blood island, in which hemopoietic precursors would now produce leukocytes. Outside this ventro-caudal zone, L-plastin-positive cells remain strikingly scarce in the whole mid-trunk to tail region at 32 and 40 hpf, our latest in-situ hybridization time point (Fig. 8T and data not shown).

### Early embryonic macrophages can eradicate bacterial infections

Eliminating apoptotic corpses (Willett et al., 1999) may not be the sole function of early embryonic macrophages. A day or so after macrophages appear, the embryo hatches and becomes directly exposed to the outer environment. Yet it still has no lymphocytes to neutralize possible microbial invaders (Willett et al., 1999). We therefore set out to test if macrophages alone could cope with a bacterial infection. We injected a large dose of either gram-negative (E. coli) or gram-positive (B. subtilis) live bacteria in the blood of 30 hpf embryos.

Within 15 minutes of E. coli injection, most macrophages were covered with bacteria, which adhered specifically to them.

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**Table 1:** Segregation of macrophage labelling with ventral labelling in embryos single-cell labelled at the 16-cell stage. The tissues considered as strictly ventral were: erythroblasts, pronephric duct, heart, yolk epidermis. Those considered as strictly dorsal were the hatching gland, trunk notochord, neural floor plate. The embryos classified as dorsally labelled were positive for at least one of these dorsal structures, and for none of the ventral structures. The converse criterion defined the ventrally labelled embryos. Other tissues known to be of more ventral (otocyst, lateral line, neural crest) or more dorsal (brain, head endothelium) origin correlated quite well with the above classification. 45 embryos were injected; in addition to the 43 clearly ventrally or dorsally labelled embryos presented above, 2 embryos showed a purely ventro-lateral labelling (otocyst+, neural crest+). Both were MΦ–. Er, erythroblasts; MF, macrophages.

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<th>Ventral labelling</th>
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**Fig. 6.** Segregation of macrophage labelling with ventral labelling in embryos single-cell labelled at the 16-cell stage. The tissues considered as strictly ventral were: erythroblasts, pronephric duct, heart, yolk epidermis. Those considered as strictly dorsal were the hatching gland, trunk notochord, neural floor plate. The embryos classified as dorsally labelled were positive for at least one of these dorsal structures, and for none of the ventral structures. The converse criterion defined the ventrally labelled embryos. Other tissues known to be of more ventral (otocyst, lateral line, neural crest) or more dorsal (brain, head endothelium) origin correlated quite well with the above classification. 45 embryos were injected; in addition to the 43 clearly ventrally or dorsally labelled embryos presented above, 2 embryos showed a purely ventro-lateral labelling (otocyst+, neural crest+). Both were MΦ–. Er, erythroblasts; MF, macrophages.

**Fig. 7.** Single-cell labelling in the 16-cell embryo reveals that macrophages originate from the ventral side of the early gastrula, as erythroblasts and heart cells. (A) 25 hpf: erythroblasts and macrophages both labelled in an embryo single-cell labelled with 2 mM Dextran-Texas Red at the 16-cell stage. (B) Selection of labelled macrophages; (C) low-magnification view of a labelled embryo showing several labelled heart cells and yolksac macrophages (most out of focus, due to yolk curvature), one of which, indicated by a white dot, is shown enlarged in the lower left corner of the picture.

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### Notes

- **L-plastin** is an actin-bundling protein that is specifically expressed in leukocytes and involved in adhesion and activation.
- **Dorsal labelling** includes tissues known to be of more dorsal origin, while **ventral labelling** includes tissues known to be of more ventral origin.
- **Injection experiment** demonstrates the ability of macrophages to clear bacterial infections, confirming their role in early embryonic defense mechanisms.
and not at all to erythroblasts, and phagocytosed them rapidly (Fig. 9A,C,D). Within 3 hours (or less, upon injection of lower doses of bacteria) the blood was cleared from bacteria. Full digestion of the bacteria inside the macrophages was achieved over the next few hours (Fig. 9J,K). All embryos survived and completed their development normally.

Upon injection of an equally high dose of *B. subtilis*, the initial response was the same as with *E. coli*: adhesion of bacteria to macrophages, followed by their rapid ingestion (Fig. 9B,E,F). Then it differed. The vacuoles became much more prominent than with *E. coli*, probably for two reasons: the individual phagosomes rapidly fused into a single one, which remained full of apparently intact *B. subtilis* for several hours (Fig. 9G), probably reflecting greater difficulty in destroying this bacterium than *E. coli*. By 5 hours post injection (p.i.), 95% of the macrophages showed a similar big bacteria-filled vacuole. But only two out of seven injected embryos had cleared their blood of bacteria by this time. For these two, full digestion of the ingested bacteria needed more time than with *E. coli*, but by 17 hours, it was complete, and the two embryos developed normally. The other five embryos, which were not able to clear their blood by 5 hours p.i. despite intense phagocytosis, were overflowed with *B. subtilis* that invaded all the subcutaneous space and apparently proliferated there, and the embryos died soon after. With a fourfold lower dose of bacteria, all embryos could clear the bacteria and survived.

Unexpectedly, once challenged with bacteria, either *E. coli*
or *B. subtilis*, macrophages not only phagocytosed the bacteria, but 3-5 hours after the bacterial injection, they also began to phagocytose living, apparently healthy erythroblasts, something never done in the normal uninfected embryo (Fig. 9G-I). They even engulfed and digested dividing erythroblasts in anaphase/telophase (Figs 9I, 10I). This new behaviour correlated with an overall change in the morphology of most macrophages, towards a highly lobulated, ready-to-engulf cell surface (Fig. 9J,K).

**Attraction of macrophages to sites of bacterial infection**

In the blood, elimination of bacteria does not require active migration from the macrophages; bacteria stick to them, are engulfed and destroyed. We set out to test whether the macrophages could sense the presence of bacteria elsewhere in the embryo, and migrate there to destroy them.

We injected bacteria in one of the two closed body cavities available: in the pericardial cavity, at the 30-somite stage (1-2 hours before the onset of blood circulation), and in the fourth ventricle of the brain, at the 24-somite stage. At these respective stages, there are a few or no macrophages in the pericardial cavity, and none in the fourth ventricle of uninfected embryos.

In all experiments, the outcome was the same: 2 hours after injection of bacteria, the injected cavity was still full of bacteria. In the blood, macrophages have become activated phagocytes that engulf living erythroblasts, although they have not met any bacteria. (G-I) *E. coli* injection into the hindbrain (h) ventricle at 21 hpf. (G) 5 hours p.i., the ventricle (v) is devoid of free bacteria, but contains 25 highly phagocytic macrophages, three of which are visible at this plane of focus: two are anchored to the roof of the ventricle (top), and one to the second rhombomere of the hindbrain (bottom). (H) Close-up of a macrophage bound to the roof of the ventricle. (I) 9 hours p.i.; a macrophage in the blood engulfs an erythroblast in anaphase. (A-D,G) are at the same magnification; (E,F,H,I) are at the same higher magnification. Bars, 10 μm.
bacteria. But 5-6 hours post injection, either the pericardial cavity or the brain ventricle was now cleared of bacteria, and contained instead 25-35 macrophages with big vacuoles containing already partially digested bacteria (Fig. 10). All injected embryos then developed normally.

Quite interestingly, in all experiments, the other macrophages that were still in the blood, even though they did not meet bacteria, had also acquired the activated morphology and behaviour, and were now phagocytosing erythroblasts (Fig. 10F, I).

**DISCUSSION**

We have shown that the zebrafish contains macrophage-like mononuclear phagocytes at an unexpectedly early stage, first in the yolksac by 24 hpf and then in the mesenchyme of the head and in the blood. This progressive dissemination of macrophages from the yolksac all occurs before any other type of leukocyte appears in the embryo.

These early macrophages do not arise from the well-known caudal embryonic blood island that gives rise to erythroblasts and endothelial cells, but from the ventro-lateral mesoderm of the head just anterior to the cardiac field.

At the onset of gastrulation, the progenitors of these two hemopoietic domains map to the ventral side of the late blastula/early gastrula, which we found to express a newly isolated gene, *draculin*, that encodes a zinc-finger, probably DNA-binding protein. As they become part of the converging lateral mesoderm, these two hemopoietic domains become specified by *draculin* expression.

The anterior *dra*-expressing lateral mesoderm initially also expresses *flk-1*, an early marker of the endothelial lineage. Then just as the cells immediately posterior to the *flk/dra*-expressing zone stop converging and start forming the cardiac primordia, the *flk* and *dra* domains become clearly different, with *dra*-expressing cells converging and then emigrating to the yolksac as single scattered cells that will become macrophages, while *flk*-expressing cells continue to converge as two thin bands that soon delineate the bilateral primordia of arterial head vessels. Thus, in its capacity to give rise to both hemopoietic and vasculogenic cells, this small piece of lateral mesoderm anterior to the cardiac field is similar to the caudal lateral mesoderm that produces erythroblasts and endothelial cells, and also to the splanchnopleural lateral mesoderm of birds and mammals (Pardanaud et al., 1996), raising the prospect that so-called hemangioblasts (Pardanaud et al., 1996) may be the common precursors of macrophages and head arterial vessels in the zebrafish embryo.

As they migrate one after another towards the yolksac, macrophage progenitors stop expressing *draculin*. This happens in an asymmetrical way. On the right side, the cells stop expressing *draculin* short after they started emigrating, while on the left side, *draculin*-positive emigrated cells are spread over the dorsal side of the yolk (Fig. 8K, compare to 8L). This asymmetry gives a clue to the external factors that maintain *draculin* expression in macrophage progenitors. At late blastula/early gastrula stages, *draculin* expression appears to be BMP-dependent (B. T. and C. T., unpublished). Later, at the 20-somite stage, BMP4 is expressed in the cardiac territory asymetrically, more strongly on the left than the right (Chen et al., 1997). If BMP4 protein is secreted from the heart primordium it might promote *draculin* expression in neighbouring cells assaymetrically, as we have observed here. This suggests a direct involvement of BMP4 in the maintenance of *draculin* expression in anterior lateral mesoderm and emigrating macrophage progenitors.

As macrophage progenitors emigrate to the yolksac, they start expressing *leucocyte-specific plasin*, and continue to do so as they become mature macrophages. In mammals, *L-plasin* is only expressed in leukocytes; it appears to promote enhanced integrin-mediated adhesion of leukocytes in response to the environmental signals that can activate them (Jones et al., 1998). We note that the first morphological change in zebrafish macrophage precursors as they emigrate away from the midline is their trend to round up during pauses in migration, and stretch again when they carry it further (Fig. 5). This behaviour suggests that they already use *L-plasin* for integrin-based adhesion and motility.

As macrophage precursors accumulate in the anterior yolksac, they adopt homogeneous morphologies, becoming rounded cells with little cytoplasm and unceasing blebbing activity, which divide frequently (Fig. 3A). These cells are strongly reminiscent of mammalian hemopoietic blast cells; we call them 'pre-macrophages', until we learn more about their abilities and the myeloid markers that they express. Then these pre-macrophages asynchronously begin to wander, they contain more cytoplasm, and show characteristic fast motility, with large, highly dynamic lamellipodia, filopodia and complex pseudopodia (Fig. 3B,C). Since these cells already display a typically macrophagic motility but still have no phagocytic experience, we call them 'young macrophages'.

The early cephalic branch of the hemopoietic lineage that we have described as giving rise to macrophages is surprising in at least three respects: (1) the production of macrophages and erythroid cells from such widely separate domains of the lateral mesoderm; (2) the anterior position of the macrophage territory (in vertebrates, the head is not usually considered as a site of hemopoiesis); (3) the rapid differentiation pathway of macrophages from mesenchymal progenitors, plus their retention of proliferative potential once they have differentiated.

Yet, there is evidence for each of these three aspects from previous studies in other species. Evidence for the first two comes from work in *Xenopus* and some teleosts. Using a monoclonal antibody that recognizes all leukocyte types in *Xenopus*, Ohinata et al. (1989) found non-lymphoid, macrophage-like leukocytes spread throughout the embryonic mesenchyme before the onset of blood circulation, and before the ventral blood island starts producing leukocytes. Moreover, with reciprocal grafting experiments in which they exchanged the head and body of marked embryos, as well as in cultured explants, Ohinata et al. (1990) showed that early non-lymphoid leukocytes arise entirely from the head, cut just anterior to the presumptive heart territory.

Likewise, when Colle-Vandevelde (1963) studied the location of hemoglobin-producing blood islands in various teleost embryos, she found a striking diversity among different species. In the Siamese fighting fish, the erythroid blood island was spread on the anterior aspect of the yolksac, in a pattern strikingly similar to young macrophages in zebrafish,
suggesting that in some teleosts, the cephalic hemopoietic progenitors give rise to erythroid cells (leukocytes were not examined in this study).

A third atypical feature we found was the rapid differentiation pathway of early macrophages. New macrophages in adult mammals are produced as the result of a multi-step differentiation pathway in the bone marrow, starting with a multipotential haemopoietic stem cell, that gives rise after some intermediates to a myeloid precursor. This then gives rise to both granulocyte and macrophage lineages, including the monocyctic series that finally gives rise to the differentiated, post-mitotic macrophage. Macrophages are usually thought to be produced in embryonic blood islands through the same differentiation pathway, and to colonize the organs only through the blood, as monocytes, as they do in the adult (Van Furth, 1980) However, over the last decade, three laboratories, working with mouse, rat and chicken embryos, respectively, have challenged this view (Sorokin et al., 1992a,b; Cuadros et al., 1992, 1993; Takahashi et al., 1996 and references therein). Their results are strikingly similar to our findings in zebrafish. In rodents, macrophages appear in the yolksac well before any monocyte or granulocyte, suggesting that they arise from some hemopoietic stem cell through a pathway that bypasses the monocyctic series. So-called 'primitive macrophages', similar to the pre-macrophages that we described here, quickly mature into 'fetal macrophages', which still display a clear proliferative potential (Takahashi et al., 1996). From the yolksac, they quickly invade the mesenchyme of the head, and from there several forming organs, in which they can be seen dividing (Cuadros et al., 1993; Sorokin et al., 1992b).

The early macrophages that we describe in the zebrafish embryo are most likely homologous to these primitive/fetal macrophages described in rodent and avian embryos.

One future goal is to know the long-term fate of these macrophages in the zebrafish. Are they replaced later by 'classical', monocyte-derived macrophages born in the hemopoietic organs of the adult, or are they long-living residents in adult tissues? Then they may constitute a sub-population of tissue macrophages with self-renewal capacity (as opposed to the post-mitotic, monocye-derived macrophages), and possibly with a distinct physiology and sensitivity to various cytokines and chemoattractants (Sorokin et al., 1992a,b; Takahashi et al., 1996).

**Not only scavengers**

We found that zebrafish early macrophages phagocytose apoptotic erythroblasts, as well as apoptotic cells in the head (P. H., unpublished data). Yet it remains uncertain if they eliminate all apoptotic corpses in the embryo. Indeed, apoptotic cells are common in the brain and eyes well before these macrophages become operational. How the embryo eliminates them is unknown.

A day or so after macrophages appear, the embryo hatches, and becomes exposed to the outer environment. Yet it has no immune system. Macrophages provide an essential defence line against microbial invaders. We have found that they efficiently eradicate bacterial populations without the help of lymphocytes, which are born much later (Willett et al., 1999). Upon infection, they become activated, and so phagocytic that they now also phagocytose living erythroblasts. Our findings imply that they have cell surface receptors that can recognize both gram- and gram+ bacteria without the help of antibodies. Such receptors could be related to the mannose receptor of mammalian resident macrophages, which can recognize glycosyl motifs specific of microbial walls (Takahashi et al., 1998).

When the pericardial cavity or the fourth ventricle of the brain was injected with E. coli or B. subtilis, 25-35 macrophages came into the cavity to clear the bacteria. This is much more than the number of resident macrophages that we find on the outer surface of these cavities or inside them in uninfected embryos, especially at the times at which we injected the bacteria (P. H., unpublished data). Therefore, macrophages initially remote from the sites of infection must have been attracted there, either by chemotactic molecules emanating from the bacteria, or by cytokines released by the few macrophages initially residing close to these sites. The latter seems all the more likely that these local infections induced the morphological transformation and aggressive phagocytic behaviour of the entire macrophage population of the embryo.

**Early macrophages and developmental processes**

Macrophages are not only scavengers or fighters, they also secrete an impressive array of growth factors and cytokines in various situations, as well as various proteins capable of remodelling the extracellular matrix (Auger and Ross, 1992). They might therefore be involved in regulating aspects of organogenesis.

Firstly, we observed that macrophages in the yolksac circulation valley interact closely with erythroblasts, sometimes seemingly almost engulfing them, but then releasing them back to the circulation. This intimate interaction resembles the nursing role of mammalian macrophages towards immature erythroid cells, although in the zebrafish embryo it does not take place in a tissue environment as structured as the bone marrow or even the hemopoietic fetal liver of mammals (Crocker and Milon, 1992). The interaction of young macrophages and proerythroblasts in the zebrafish yolksac might be important for the maturation of both cell types.

Secondly, it is known that macrophages can promote angiogenesis (Auger and Ross, 1992). Since we found that they invade the mesenchyme of the head about 4 hours prior to its first vascularization (Rieb, 1973), they could be involved in fostering proper vascularization of the head, which soon becomes much more elaborated than in the rest of the body.

**Towards a cell biology of macrophages in the live animal**

Since we can easily follow individual macrophages in the live zebrafish embryo by video-enhanced DIC microscopy, we have here a unique opportunity to study their cell biology directly in the living vertebrate organism.

We noticed that macrophages in the yolksac circulation valley often adopt a typical shape (Fig. 2G-K) collectively. This may signal a change in cytokines in the microenvironment. We now know that small GTP-binding proteins Rho, Rac and Cdc42, which are key-relays of much cytokine signalling inside the cell, induce definite effects on actin-based cell protrusions. In mouse macrophages, Cdc42 induces the
formation of filopodia, Rac induces lamellipodia and membrane ruffling, while Rho induces cell rounding (Allen et al., 1997). Thus, we may soon become able to read the swiftly changing morphologies of macrophages in these terms, and use these wandering cells as sensitive, readily readable probes of changing morphologies of macrophages in these terms, and use these wandering cells as sensitive, readily readable probes of their fluctuating cytokine environment.

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