Simultaneous intravital imaging of macrophage and neutrophil behaviour during inflammation using a novel transgenic zebrafish

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Summary

The zebrafish is an outstanding model for intravital imaging of inflammation due to its optical clarity and the ability to express fluorescently label specific cell types by transgenesis. However, although several transgenics labelling myeloid cells exist, none allows distinction of macrophages from neutrophils. This prevents simultaneous imaging and examination of the individual contributions of these important leukocyte subtypes during inflammation.

We therefore used BAC recombineering to generate a transgenic *Tg(fms:GAL4.VP16)i186*, in which expression of the hybrid transcription factor Gal4-VP16 is driven by the *fms* (CSF1R) promoter. This was then crossed to a second transgenic expressing a mCherry-nitroreductase fusion protein under the control of the Gal4 binding site (the UAS promoter), allowing intravital imaging of mCherry labelled macrophages.

Further crossing this compound transgenic with the neutrophil transgenic *Tg(mpx:GFP)i114* allowed clear distinction between macrophages and neutrophils and simultaneous imaging of their recruitment and behaviour during inflammation. Compared with neutrophils, macrophages migrate significantly more slowly to an inflammatory stimulus. Neutrophil number at a site of tissue injury peaked around 6h post injury before resolving, while macrophage recruitment increased until at least 48h. We show that macrophages were effectively ablated by addition of the prodrug Metronidazole, with no effect on neutrophil number. Crossing with *Tg(Fli1:GFP)y1* transgenic fish enabled intravital imaging of macrophage interaction with

endothelium for the first time, revealing that endothelial contact is associated with faster macrophage migration.

Tg(fms:GAL4.VP16)i186 thus provides a powerful tool for intravital imaging and functional manipulation of macrophage behaviour during inflammation.

Keywords

Macrophage, Animal Models, Transgenic animals, Wound healing

Introduction

Mammalian studies have demonstrated a complex partnership between neutrophils and monocyte/macrophages during inflammation (1). This interplay is central to host defence, but is also a major contributor to atherosclerosis (2-5). Unfortunately, intravital imaging of the interaction between monocyte/macrophages, neutrophils and the vasculature is challenging in conventional mammalian models.

The zebrafish has emerged as a powerful model for the intravital study of inflammation (6-10). The early embryo is near transparent, thanks to its small size and limited pigmentation (11). Combining these advantages with the ability to perform mutagenesis and transgenesis has transformed the zebrafish into a unique model for biomedical research (12, 13).

Innate immune responses are evolutionarily ancient, and the functional divergence of macrophages and neutrophils predates the evolutionary split between teleost fish and mammals (14). The myeloid lineage develops rapidly, with functional macrophages

appearing by 12-16h post fertilisation (hpf), and neutrophils by 16hpf (15). Structurally, biochemically and functionally, these cells demonstrate close homology to their mammalian counterparts (15), although macrophages of early zebrafish embryos are tissue resident (16), rather than experiencing a circulating monocyte phase. This is analogous to the primitive macrophages found in the developing mammalian embryo prior to definitive haematopoesis (17).

Intravital imaging of inflammation in zebrafish is reliant upon transgenic lines expressing fluorescent reporters in the cell types of interest, several of which exist. Transgenics using the *pu.1* promoter to drive reporter expression label all myeloid cells (18-20). Several neutrophil transgenics are available, with *Tg(mpx:GFP)i114* being most neutrophil-specific (6, 21). The mych:YFP line generated in an enhancer trap screen is also highly specific for a subset of neutrophils making up 50% of total neutrophil number (22). The *Lysozyme C* promoter labels a subset of both neutrophils and macrophages (23) while *fli1*:GFP labels a poorly-defined subset of myeloid cells, in addition to its predominant expression in endothelial cells (24, 25). Thus, no transgenic is currently available that distinguishes macrophages from neutrophils. The *fms* promoter (CSF1R) has been shown to be macrophage-specific in mice (26, 27) and is not expressed in zebrafish neutrophils (22, 28). It therefore represents a possible strategy to distinguish macrophages from neutrophils.

Using Bacterial Artificial Chromosome (BAC) transgenesis allows highly tissue-specific expression compared with promoter fragment approaches. However, chromosomal integration of the modified BAC is inefficient, and hundreds of embryos need to be injected and screened to maximise the chance of identifying a

single founder. The availability of the Tol2kit (29) provides more efficient integration, generating transgenics more rapidly with higher success rates, but is unable to deliver vectors as large as a BAC (30). We used the *fms* promoter to drive expression of Gal4 (a yeast transcription factor) rather than a fluorescent reporter directly. This has the advantage that once established, a Gal4 transgenic can then be crossed with any other transgenic expressing dominant negative proteins, fluorescent reporters, or combinations of these under the control of the UAS promoter to which Gal4 binds (31). This therefore allows tissue-specific overexpression of any UAS construct, providing a tool that can be applied to a wide range of intravital imaging and mechanistic studies.

In this paper we detail generation and characterization of a novel *Tg(fms:GAL4.VP16)i186* transgenic zebrafish. We demonstrate that this labels a population of migratory phagocytic myeloid cells that are recruited to sites of tissue injury and do not co-express the neutrophil marker myeloperoxidase, indicating these cells are macrophages. Crossing with transgenic lines expressing fluorescent reporters in endothelial cells or neutrophils allowed simultaneous imaging of macrophages with each cell type, and revealed distinct differences in the kinetics of recruitment of neutrophils and macrophages to an inflammatory stimulus. We also observed that macrophages in contact with endothelium migrate more rapidly than macrophages within non-vascular tissue. Finally, expressing nitroreductase selectively in macrophages enabled their ablation without altering neutrophil number. *Tg(fms:GAL4.VP16)i186* therefore allows novel approaches to intravital imaging of inflammation.

Materials and Methods

All experiments were performed under UK Home Office Project License 40/3031 or 40/3325 and conformed to the ethical requirements of our institution. The names first used to describe transgenics within this manuscript conforms with standard nomenclature (32), but some are subsequently shortened to aid clarity.

Generation of a fms:Gal4.VP16 *BAC*

The *fms* gene was identified in the ENSEMBL database (ENSDARG00000007889). BAC libraries (Imagenes) were screened by PCR using the following primer pair:

Forward GGATGCTCTCGATGGCTAAA

Reverse CGTCACATGGACACACTCCT

BAC clone HUKGB735K06247Q (from the keygene 735 BAC library) was found to contain *fms* and appropriate amounts of flanking sequence. A Gal4.VP16 construct was inserted into the *fms* start site by BAC recombineering as described (33). An I-scei digestion site was inserted to allow linearization, by targeting the cmr cassette with an appropriately constructed vector.

Generation of a transgenic line expressing Gal4-VP16 under the fms *promoter*

The BAC construct was linearised and injected into wildtype (AB) zebrafish embryos at the one-cell stage. These were raised to adulthood and founders identified by screening genomic DNA of offspring for Gal4-VP16 by PCR. A single founder was identified and subsequent generations incrossed to produce the line *Tg(fms:Gal4.VP16)i186*.

Breeding of compound transgenic zebrafish.

We generated a *Tg(UAS-E1b:nfsB.mCherry)i149* line expressing a fusion protein of nitroreductase and mCherry under the UAS promoter (the construct was a kind gift of Michael Parsons, Johns Hopkins University). The *Tg(fms:Gal4.VP16)i186* founder was crossed to *Tg(UAS-E1b:nfsB.mCherry)i149* and offspring expressing mCherry were raised to adulthood. The resulting compound transgenic adults (formally termed *Tg(fms:Gal4.VP16)i186; Tg(UAS:nfsB.mCherry)i149*, hereafter referred to as *fms:nfsB.mCherry* for clarity) were crossed to *Tg(mpx:GFP)i114* (6) and *Tg(Fli1:GFP)y1* (25) fish and offspring expressing both mCherry and GFP raised to adulthood to produce stable lines. Compound transgenics expressing both Gal4.VP16, nfsB.mCherry and either *mpx*:GFP or *fli1:*GFP are hereafter referred to as *fms/mpx or fms/fli1* compound transgenics respectively.

Confocal imaging and tailfin injury

Confocal microscopy was performed on anaesthetized embryos immobilized in 1% low melt point agarose (Sigma) within a chamber slide on a Perkin Elmer UltraVIEW Vox spinning disk confocal microscope. Where required, an inflammatory response was induced by tail transection as previously described (6). Volocity software was used to quantify cell number and track cell movement to measure migration speed.

Myeloid/macrophage cell depletion

Myeloid cell depletion was achieved by morpholino antisense knockdown of the transcription factor *pu.1* as previously described (13, 34). Specific macrophage

depletion was achieved by incubation of *fms:mpx* embryos in 5mM of Metronidazole for 15hrs before imaging as previously described (35).

Results

fms:nfsB.mCherry transgenic larvae were observed under fluorescent and confocal microscopy from 16h post fertilization (hpf). No mCherry expression was seen at 16h or 24h, but was easily detectable by 48hpf. **Fig. 1A** shows composite DIC and confocal micrographs of a typical 48hpf *fms:nfsB.mCherry* transgenic embryo. Cells with the morphology and location typical of xanthophores strongly expressed mCherry (**Fig. 1B**) as predicted from their known expression of *fms* (19, 20). In keeping with their role as epidermal pigment cells, timelapse confocal microscopy revealed this xanthophore population to be entirely static over at least 24h of recording.

Characterisation of fms *positive immune cell population*

From 48hpf, timelapse confocal microscopy revealed a second, non-xanthophore population of mCherry expressing cells (**Fig. 1C**) that were likely to represent macrophages. To define their nature, we sought to establish whether these cells were migratory, derived from the myeloid lineage, and whether they expressed *mpx*.

The non-xanthophore cells labelled by the transgene displayed the localisation, morphology, migratory capacity and behaviour of zebrafish macrophages as described in previous reports (16). Timelapse imaging revealed mCherry expressing macrophage-like cells migrating through the tissue (**Fig. 2, Supplemental Movie 1**).

Cells derived from the myeloid lineage are dependent on the transcription factor *pu.1*, loss of which leads to failure of myeloid cell development. Injection of morpholino-modified antisense constructs that knockdown *pu.1* expression (previously shown to efficiently and specifically ablate myeloid cells (13, 18, 34, 36)) abolished the migratory mCherry expressing population, leaving xanthophores unaffected. **Fig. 3** shows representative confocal projections of the *fms:nfsB.mCherry* embryos injected with control or *pu.1* morpholinos imaged over the yolk sac and caudal vein, both sites of predilection for zebrafish macrophages. Although xanthophores persist in *pu.1* morphant embryos (yellow arrowheads) the migratory macrophage-like cells (white arrows) were effectively abolished by *pu.1* knockdown. Thus, the *fms:nfsB.mCherry* labels a motile cell population derived from the myeloid lineage.

To determine whether *fms:nfsB.mCherry* labelled neutrophils, we crossed the *fms:nfsB:mCherry* line with the neutrophil specific *Tg(mpx:GFP)i114* line (6). Triple compound transgenic embryos (*fms/mpx*) contained entirely separate populations of GFP-expressing neutrophils and mCherry-expressing putative macrophages (**Fig. 4 and supplemental Movie 2**). There was no co-localisation of mCherry with GFP. mCherry positive macrophages can be seen phagocytosing cell debris in **Supplemental Movie 2** (arrowed). Thus, the *fms:nfsB.mCherry* transgene labels a migratory, phagocytic myeloid cell entirely distinct from myeloperoxidase-expressing neutrophils. We therefore concluded that these cells are indeed macrophages.

Intravital analysis of macrophage and neutrophil behaviour

To observe recruitment of both neutrophils and macrophages to a site of inflammation simultaneously, we injured compound *fms/mpx* transgenic larvae by tailfin transection as previously described (6, 37). **Fig. 5** shows a representative confocal timelapse acquired up to 14h after injury. **Supplemental Movie 3** shows recruitment of neutrophils and macrophages to the site of injury acquired every 60s over the first 12h. As described previously, neutrophils are rapidly recruited to the site of injury, peaking at 6h post injury, before numbers decline to baseline by 48h. In contrast, recruitment of macrophages occurs over a longer period, progressively increasing until at least 48h after injury (**Fig 6A**). **Supplemental Movie 4** shows a higher power acquisition recorded every 10s for 80min at the site of injury revealing complex interactions between macrophages and neutrophils.

Mammalian neutrophils are thought to migrate more rapidly than macrophages into sites of inflammation (38), though limited data exists on intravital simultaneous comparisons. In order to test whether this holds true for zebrafish myeloid cells, we measured their migration speed to the site of tailfin injury. In keeping with their more rapid recruitment to an inflammatory stimulus, zebrafish neutrophils migrated significantly faster than macrophages. Migration speed of each cell type remained constant between 4-8h after induction of an inflammatory response (**Fig. 6B**). This is in keeping with in vitro data showing that macrophages and neutrophils utilise different mechanisms for migration through extracellular matrix and respond to different chemotactic signals (39).

Intravital imaging of macrophage/endothelial interactions

Macrophages play an important role in development and remodelling of the vasculature (40), though few intravital models exist in which the relationship between these cell types can be studied during vessel formation and remodelling. We therefore generated *fms/fli1* compound transgenics with mCherry-expressing macrophages and GFP-expressing endothelial cells. Macrophages were frequently located in close apposition to the abluminal surface of the caudal vein (**Fig. 7A**), previously demonstrated to be a site of predilection of macrophages (16). Despite the close relationship of macrophages to the vasculature, no evidence of transmigration either into or from the circulation was observed, in keeping with previous reports indicating zebrafish macrophages are analogous to the mammalian primitive macrophage and do not undergo a circulating monocyte-like phase.

Strikingly, macrophages were frequently seen migrating along the abluminal surface of endothelial cells, a manner of migration not previously described to our knowledge (**Fig. 7B** and **Supplemental movie 5**). This raised the possibility that macrophages use endothelial cells as physical or molecular guidance cues to aid migration. We therefore compared migration speeds of macrophages in contact with endothelium with those migrating through the tissue. Macrophages migrating along endothelial cells migrated 63±9% faster than macrophages within tissue (**Fig 7C**), suggesting macrophage/endothelial interaction may facilitate macrophage migration or that macrophage behaviour is altered when in contact with endothelium.

Macrophage ablation using nitroreductase expression

We have demonstrated the capacity of the *fms:nfsB.mCherry* line to express reporter constructs in macrophages *in vivo.* To demonstrate that additional functional

constructs can also be delivered, we sought to utilise the nfsB (nitroreductase) gene fused to mCherry to specifically ablate zebrafish macrophages.

fms/mpx transgenic embryos were incubated in Metronidazole, which is metabolized by nitroreductase to a toxic metabolite, inducing cell death in transgene expressing cells. Metronidazole treatment effectively abolished macrophages, without significantly altering neutrophil numbers (**Fig 8**).

Discussion

In this paper we describe generation of a novel zebrafish transgenic that drives Gal4-VP16 under the control of the *fms* promoter. Although it is rare to identify a single marker that alone defines a specific cell type with complete specificity, we chose fms as the best candidate available. Within the immune system, expression of *fms* appears to be completely restricted to macrophages, and this is critical for studies where distinction between different myeloid cell populations is important. *fms:nfsB.mCherry* also labels xanthophores, but their morphology, static nature and superficial location allowed discrimination between these and the motile macrophages with complete confidence, particularly during timelapse imaging. Since we generated this line, subsequent work has identified a number of other possible macrophage specific genes (41) that represent good candidates to drive macrophage specific transgenes. A panel of transgenic zebrafish using different macrophage specific promoters would allow detailed study of the phenotypes of different macrophage subtypes *in vivo*, and the *fms* transgenic we described would be an important contributor to this approach.

Several pieces of evidence support our conclusion that the putative macrophages identified in *fms:nfsB.mCherry* are indeed macrophages. The expression pattern of the

transgene recapitulates *fms* expression defined by in situ hybridization in earlier studies. Although *fms* expression by in situ hybridization can be detected as early as 19hpf (42), the delay in mCherry expression in the transgenic is likely to represent the time taken to express, transcribe and fold sufficient mCherry for detection by microscopy. Our data shows that these cells arise from a myeloid origin. The labelled cells are clearly not thrombocytes, since we observed no circulating labelled cells. Equally, they are not neutrophils since we have never seen co-expression in compound transgenics containing the *mpx:GFP* transgene, but they are clearly phagocytic. Taken together with their migratory capacity, these data strongly suggest that the labelled cells identified in our transgenic are macrophages, although we cannot rule out the possibility that not all macrophages express the transgene.

In keeping with their distinct identities, we observed marked differences in migration speed and kinetics of recruitment to a site of tissue injury between neutrophils and macrophages. Limited data exists on intravital comparisons of this type, demonstrating the utility of our model for such studies. In addition, examination of high power, fast frame rate timelapse videomicrographs reveals complex interactions between macrophages and neutrophils. Further study of these interactions, made possible using these compound transgenics, is likely to inform our knowledge of immune cell recruitment and cross-talk.

The ability to visualize both endothelial cells and macrophages in the developing embryo revealed a preferred pathway for macrophages along the abluminal surface of endothelial cells. This was reminiscent of the luminal patrolling monocytes described in mice (43), although we never observed zebrafish macrophages transmigrate

endothelium, and the endothelium along which they migrated was frequently of developing vessels that had not yet lumenized and carried no blood flow. Given the role of macrophages in tumour angiogenesis (44), and the impairment of angiogenesis in *pu.1* deficient mice (40) we speculate that this behaviour may contribute to embryonic vascular development in some way.

The explanation for the novel finding that macrophages migrate faster when in contact with endothelium is unclear. These macrophages are unlikely to represent a separate subset as they frequently leave the endothelium (leading to slowed migration speed) or join the endothelium from the tissue (leading to increased migration speed). This suggests that some cue from the endothelium allows macrophages to migrate more rapidly. A preference for using abluminal endothelium to migrate would be compatible with the surveillance functions of macrophages and is noteworthy given the important role of the macrophage in many vascular diseases (3-5).

A major advantage of the Gal4/UAS approach to generating transgenics is the ability to manipulate macrophage function by driving expression of functional proteins in addition to reporter proteins. This is facilitated by the ease by which small construct transgenic lines can be generated using the Tol2 approach (45). We illustrated this approach by using a nitroreductase/metronidazole lineage ablation strategy. In this way, macrophages can be ablated while other immune cell populations are maintained. This opens the way for a wide range of studies expressing other functional constructs such as chemokines in the macrophage.

There are suggestions from the literature that transcriptional silencing of UAS promoters may limit the usefulness of Gal4/UAS zebrafish transgenics (46). However, this has not proved a practical problem in our studies. It may be that subsequent generations of these UAS transgenics may be less effective, but given the ease with which new lines can now be made using the tol2kit system this may not prove overly problematic for future work.

Intravital imaging of the inflammatory response has the ability to inform a wide range of scientific and clinical questions across a number of fields. The addition of zebrafish to existing models broadens the ways in which these questions can be approached. Our data show how imaging of multiple cell populations in vivo can reveal unsuspected interactions, and how delivering functional proteins to macrophages can be used to manipulate defined aspects of the immune response. This is likely to increase our understanding of the behaviour and interaction of these essential cell populations in vivo.

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Figure Legends

Figure 1. The *fms:nfsB.mCherry* transgenic labels two separate populations of cells.

A: Composite DIC (upper panel) and spinning disk confocal micrographs using mCherry filters (lower panel) of a 48hpf *fms:nfsB.mCherry* compound transgenic. Sites of imaging for panels B and C are indicated on DIC micrograph. Autofluorescence can be seen in the yolk sac, but other fluorescence is located in discrete cells. Scale bar = 380um. B: mCherry expression in skin xanthophores, showing their typical morphology (Scale bar = 50um). C: mCherry expression in macrophages in region of mid-trunk (Scale bar = 50um).

Figure 2. the *fms:nfsB.mCherry* transgenic labels a population of motile macrophage-like cells.

A: Maximum intensity projections of 1hr confocal timelapse microscopy of 3dpf *fms:nfsB.mCherry* transgenic embryo, acquired at 15min intervals. Scale bar = 50um.

Macrophages are seen migrating through tissue in the region of the trunk. Two representative cells are arrowed in white and yellow. This sequence is shown in Supplemental Movie 1.

Figure 3. *pu.1* knockdown abolishes macrophage-like cells while sparing xanthophores in the *fms:nfsB.mCherry* transgenic.

A: DIC image of 3dpf *fms:nfsB.mCherry* embryo. Boxed areas indicate regions of panels B and C. Scale bar = 200um. B and C: Representative maximum intensity projections of confocal micrographs of 3dpf *fms:nfsB.mCherry* embryos injected with control or *pu.1* morpholinos imaged over the yolk sac (B) and caudal vein (C). Macrophages are labeled with white arrows, xanthophores with yellow arrowheads. Scale bars = 100um (A) and 200um (B). *Pu.1* knockdown abolishes the macrophage population while leaving the xanthophores unaffected.

Figure 4. The *fms/mpx* transgenic labels separate populations of neutrophils and macrophages.

Confocal microscopy of 3dpf *fms/mpx* transgenic embryo showing GFP expressing neutrophils and mCherry expressing macrophages. Imaging was performed in the region of the trunk. No co-expression of either marker was observed. Scale bar = 17um. This sequence is shown in Supplemental Movie 2.

Figure 5. The *fms/mpx* transgenic allows simultaneous imaging of recruitment of neutrophils and macrophages to a site of tissue injury.

Timelapse confocal microscopy of 3dpf *fms/mpx* transgenic embryo showing migration of GFP-expressing neutrophils and mCherry-expressing macrophages to a

site of tailfin injury from 0-14h after injury. An initial neutrophilic response can be seen before resolving while macrophage recruitment increases more slowly over time. Scale bar = 140um. See also Supplemental Movies 3 and 4.

Figure 6. The *fms/mpx* transgenic reveals marked differences between neutrophil and macrophage migration speed and recruitment kinetics.

A: Timecourse of recruitment of neutrophils and macrophages to a site of tailfin injury in 3dpf *fms/mpx* transgenic embryos. B: Mean migration speed of neutrophils and macrophages recruited to a site of tailfin injury. Data indicates mean+/- SEM, n=74-105 cells/group. *** indicates $p<0.0001$ for comparison of neutrophils versus macrophages.

Figure 7. Macrophages are closely apposed to and migrate along the abluminal surface of endothelium.

A Caudal vein of compound *fms/fli1* embryo. Upper panel; GFP expression in endothelium, Middle panel; Macrophages surrounding caudal vein, Lower panel; merged image (Scale bar = 50um). B: Timelapse confocal microscopy of 3dpf *fms/fli1* transgenic embryo (scale bar = 70um), showing migration along the abluminal endothelium in the trunk. Timestamp indicates minutes between frames shown. Individual macrophages are arrowed in yellow and white. C: Migration speeds of macrophages in contact with endothelium compared with macrophages in the tissue. Data is expressed as mean +/- SEM. $p<0.001$.

Figure 8. Metronidazole specifically ablates macrophages without affecting neutrophils in *fms/mpx* transgenics.

The effect of Metronidazole on macrophage and neutrophil recruitment to a site of tailfin injury in 3dpf *fms/mpx* embryos 7h post injury. A: representative photomicrographs of *fms/mpx* embryos treated with control or 5mM Metronidazole showing abolition of macrophages. Scale bar = 130um. B: Quantification of macrophage and neutrophil recruitment to the site of tailfin injury in *fms/mpx* embryos treated for 15h with 5mM Metronidazole or control. Data indicates mean +/- SEM. *** indicates $p<0.0001$.

Supplemental movie 1. Timelapse confocal microscopy of 3dpf *fms:nfsB.mCherry* transgenic embryo showing migration of macrophages through tissue in region of trunk.

Supplemental movie 2. Timelapse confocal microscopy of 3dpf *fms/mpx* transgenic embryo showing migration of GFP expressing neutrophils and mCherry expressing macrophages. Macrophages can be seen phagocytosing cell debris (example arrowed) No co-expression of either marker was observed. Scale bar = 17um.

Supplemental movie 3. Lower magnification timelapse confocal microscopy of 3dpf *fms/mpx* transgenic embryo showing GFP expressing neutrophils and mCherry expressing macrophages migrating to a site of tailfin injury from 0-12h post injury. Frames acquired every 60sec.

Supplemental movie 4. Higher magnification timelapse confocal microscopy of 3dpf *fms/mpx* transgenic embryo showing GFP expressing neutrophils and mCherry expressing macrophages migrating to a site of tailfin injury. Scale bar = 70um. Frames acquired every 10sec for 80min.

Supplemental movie 5. Timelapse confocal microscopy of 3dpf *fms/fli1* transgenic embryo showing GFP expressing endothelium and mCherry expressing macrophages. Macrophages can be seen migrating along the abluminal surface of the developing vasculature.