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Characterization of the mononuclear phagocyte system in zebrafish

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The evolutionarily conserved immune system of the zebrafish (*Danio rerio*), in combination with its genetic tractability, position it as an excellent model system in which to elucidate the origin and function of vertebrate immune cells. We recently reported the existence of antigen-presenting mononuclear phagocytes in zebrafish, namely macrophages and dendritic cells (DCs), but have been impaired in further characterizing the biology of these cells by the lack of a specific transgenic reporter line. Using regulatory elements of a class II major histocompatibility gene, we generated a zebrafish reporter line expressing green fluorescent protein (GFP) in all APCs, macrophages, DCs, and B lymphocytes. Examination of *mhc2dab:GFP*; *cd45:DsRed* double-transgenic animals demonstrated that kidney *mhc2dab:GFP^hi^*; *cd45:DsRed^hi^* cells were exclusively mature monocytes/macrophages and DCs, as revealed by morphologic and molecular analyses. Mononuclear phagocytes were found in all hematolymphoid organs, but were most abundant in the intestine and spleen, where they up-regulate the expression of inflammatory cytokines upon bacterial challenge. Finally, *mhc2dab:GFP* and *cd45:DsRed* transgenes mark mutually exclusive cell subsets in the lymphoid fraction, enabling the delineation of the major hematopoietic lineages in the adult zebrafish. These findings suggest that *mhc2dab:GFP* and *cd45:DsRed* transgenic lines will be instrumental in elucidating the immune response in the zebrafish. (*Blood*. 2011;117(26):7126-7135)

Introduction

In recent years, the zebrafish (*Danio rerio*) has proven to be a unique vertebrate model for the study of hematopoiesis.[1] The use of the zebrafish to study the ontogeny of leukocyte subsets,[2] immune cell migration,[3,4] and host-pathogen interactions[5] has provided new insights into our understanding of innate immunity in the developing vertebrate embryo. A major focus of previous studies was on the neutrophil response, because several transgenic reporter lines have been generated that mark this granulocyte subset. Whereas neutrophils generally constitute the first line of defense against invading pathogens, the role of other immune cell subsets in the innate immune response has received less attention. In addition to neutrophils, macrophages are key in the response to pathogen challenge. In the zebrafish embryo, primitive macrophages have been demonstrated to be capable of clearing injected bacteria by phagocytosis.[6] However, the absence of markers specific to macrophages has limited the study of this myeloid cell subset in the zebrafish.

The mononuclear phagocyte system (MPS) comprises monocytes, tissue macrophages, and dendritic cells (DCs), as well as their lineage-committed progenitors.[7] The primary function of mature MPS cells is the clearance of pathogens by phagocytosis. This activity is crucial during immune challenge to clear invasive pathogens. Mononuclear phagocytes also play an important role in the removal of apoptotic cell corpses, especially during embryonic development. In mice, embryonic macrophages colonize several structures to be removed during development, including interdigital tissues, the hyaloid vasculature, and pupillary membranes.[8,9] In addition, macrophages residing in hematopoietic tissues support erythroblast proliferation and differentiation and engulf dying erythrocytes in the spleen.[10,11] The capacity to engulf a wide array of particles relies on the existence of different surface receptors, such as scavenger receptors (Marco; CD163) or TLRs that recognize pathogen-associated molecular patterns.[12,13] After phagocytosis, macrophages and DCs can activate antigen-specific T lymphocytes, a process dependent on MHCII molecules.

Monocytes and macrophages have been identified in zebrafish based on morphology, cytochemistry, and gene expression.[1] During development, the first macrophages originate from the anterior lateral plate mesoderm at approximately 20 hours postfertilization (hpf) and migrate over the yolk ball before colonizing other tissues.[6,14] These primitive macrophages can be visualized in vivo using a variety of transgenic lines in which green fluorescent protein (GFP) expression is driven by myeloid-specific promoter sequences, including *pu.1:eGFP*, *lyz:eGFP*, and *mpx:eGFP*.[15-19] However, these promoters are only specific to macrophages during a short, defined window of embryogenesis; each is also subsequently expressed in granulocytic cells, precluding the prospective isolation of mononuclear phagocytes after 48 hpf. Although a novel macrophage-specific reporter line was recently generated using the *mpeg1* promoter, it appears that the transgene is active only in the embryo and larvae, precluding its use for the study of macrophages in adult fish.[20] Another important subset of the MPS is the DC. DCs are professional APCs and constitute a rare leukocyte population in mammals. We and others recently described the identification of DCs in adult zebrafish and medaka (*Oryzias latipes*).[21,22] Zebrafish DCs, detected through their phagocytic ability and further enriched

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by their affinity for the lectin peanut agglutinin, displayed morphologic and cytochemical characteristics reminiscent of those exhibited by their mammalian counterparts. However, the presence of a high proportion of immature myeloid cells within peanut agglutinin–positive cells demonstrated the need for improved enrichment strategies.[22]

To address these issues and to fully characterize the MPS of zebrafish, we generated a transgenic line that specifically marks APCs using the regulatory elements from the MHCII *dab* gene to drive GFP. We demonstrate that, when used in combination with a previously generated *cd45:DsRed* transgenic animal (also referred to as *ptprc:DsRed*), the *mhc2dab:GFP* transgenic line can be used to identify and isolate a pure population of mononuclear phagocytes. This has enabled characterization of gene expression, tissue distribution, and functional properties of monocytes, macrophages, and DCs in the zebrafish. We also show that, when combined with light-scattering characteristics, the expression of the *mhc2dab:GFP* and *cd45:DsRed* transgenes delineates each of the major hematopoietic lineages in the adult zebrafish.

Methods

Zebrafish strains and maintenance

Animals were maintained in accordance with University of California San Diego Institutional Animal Care and Use Committee guidelines, and all animal procedures were performed with their approval. The transgenic lines Tg(*ptprc:DsRed*)*sd3*,[23] Tg(*lyz:DsRed*)*nz50*,[15] and Tg(*lck:lck-EGFP*)*cz2*[24] were used.

Generation of Tg(*mhc2dab:GFP-LT*)*sd6* and Tg(*mhc2dab:mCherry*)*sd7* transgenic animals

A 3.8-kb fragment upstream of the *mhc2dab* transcriptional start site was amplified from the bacmid AL928944 using the following primers: FP-GAGCGGCCGCCTTAGTGTATGTACGAGTGTATAGATGTTTCCC and RP-GAGGATCCGAGTCTTTGAATGTGTCAAATGAAGAACTTTC (with 5′-NotI and 3′-*Bam*HI cloning sites added [underlined sequences]). This fragment was cloned into GFP-LT/Tol2 and mCherry/Tol2 vectors, and the resulting constructs were coinjected with transposase mRNA into zygotes to generate transgenic founders.[25] Throughout the text, transgenic animals are referred to without allele designations for clarity.

Fluorescent microscopy

Transgenic lines were imaged using an inverted confocal microscope (SP5; Leica). GFP and DsRed were excited by 488- and 543-nm laser lines, respectively.

Flow cytometry

Hematopoietic cells isolated from adult organs were processed as described previously.[26] Sytox Red (Invitrogen) was used to exclude dead cells and debris. FACS was performed using a FACSAria flow cytometer (Becton Dickinson), flow cytometry was performed with an LSRII flow cytometer (Becton Dickinson), and data analyses were performed using FlowJo Version 9.2 software (TreeStar). For in vivo phagocytosis assays, 2 μg of pHrodo E. coli BioParticles (Molecular Probes) was injected intravenously in *mhc2dab:GFP* adult fish. Splenocytes were collected 18 hours after injection for analysis.

Real-time quantitative PCR

For real-time quantitative PCR (Q-PCR) analyses, RNA was isolated from sorted cells using the RNeasy Kit (QIAGEN), and cDNA was obtained using qScript cDNA Supermix (Quanta BioSciences). Q-PCR was performed with the Mx3000P System (Stratagene) according to the manufacturer's instructions. Each sample was tested in duplicate. For each independent experiment, elongation-factor 1-α (*ef1a*) expression was scored for each population. The signals detected for each transcript were normalized to *ef1a*, and data were analyzed by the ΔΔCt method according to the manufacturer's recommended protocol (Stratagene). Primers are listed in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Results

Generation of the *mhc2dab:GFP* reporter line

Our previous studies revealed the presence of APCs, including DC cells, in adult zebrafish.[22] Because further biologic characterization has been hindered by our inability to specifically mark these cells, we speculated that the promoters of genes orthologous to mammalian APC-specific genes would constitute potential candidates for the generation of novel zebrafish transgenic lines marking all APC populations. Among others, MHCII genes are required for antigen presentation and T-cell activation and are constitutively expressed by APCs.[27] The structure of MHCII proteins is highly conserved among vertebrates. Each MHCII molecule is a heterodimer composed of an α and a β chain. In mice, 4 class II genes, Aα, Aβ, Eα, and Eβ, pair to form the classic MHCII molecules I-A and I-E, respectively. The human MHCII locus is more complex and encodes α and β chains for HLA-DP, HLA-DQ, and HLA-DR. Understanding the genomic organization of the MHC locus in zebrafish has been complicated by the fact that, unlike in mammals, it appears to be fragmented,[28] a trait shared among other teleosts, including trout, stickleback, guppy, and cichlids.[29] Consequently, the exact number of MHC genes in the zebrafish is unknown. One class II α (*mhc2daa*) and 6 class II β (*mhc2dab* to *mhc2dfb*) genes have been identified by genomic library screens.[30,31] Most of these appear to be pseudogenes, however, because only the *mhc2daa* and *mhc2dab* genes are expressed.[31] Preliminary analysis of the zebrafish *mhc2dab* gene suggested that it might be expressed in APCs, because robust expression was observed in adult organs relevant to immunologic function, such as the thymus, gut, and spleen (data not shown).

To generate a transgenic zebrafish line that specifically marks APCs, we analyzed the upstream regulatory elements of the *mhc2dab* gene. In mammals, *mhc2* expression is regulated at the transcriptional level by the class II transactivator, which is recruited to the *mhc2* promoter by proteins selectively bound to the X1, X2, and Y promoter sequences that are required for optimal constitutive and cytokine-induced expression in both humans and mice.[27] Analysis of the zebrafish *mhc2dab* proximal promoter revealed the presence of these conserved elements within a 4-kb fragment upstream of the transcriptional start site (data not shown). This 4-kb regulatory domain of the *mhc2dab* gene was cloned upstream of GFP-LT and flanked by Tol2 recombination sequences. The *mhc2dab:GFP* construct was injected into zygotes and the resulting potential founders were raised to maturity and mated to produce F_1 embryos.

Characterization of *mhc2dab:GFP* transgenic embryos

Transgenic progeny of *mhc2dab:GFP* founders were identified by fluorescent microscopy, in which reporter gene expression was first observed at 5 days postfertilization (dpf) within the thymus and 4 days later in scarce cells within the skin (data not shown). Widespread transgene expression was not observed until after 12 dpf, when GFP^+ cells increased in number and became brightly

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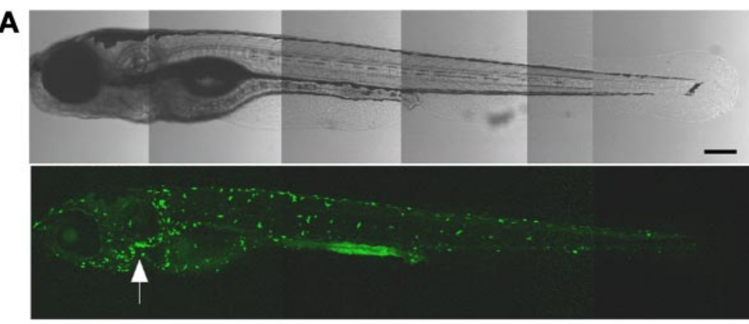

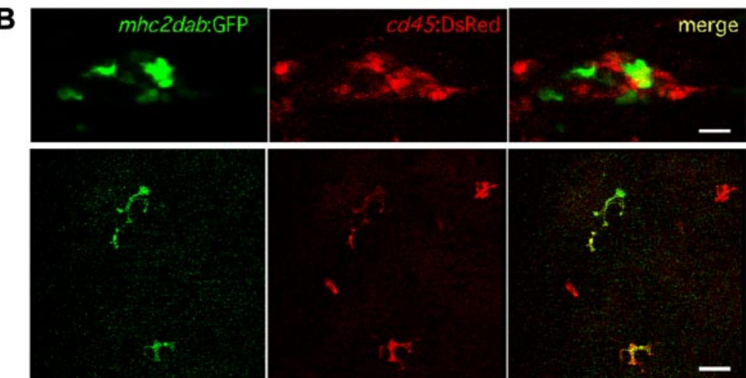

Figure 1. Expression of the *mhc2dab:GFP* and *cd45:DsRed* transgenes during zebrafish development. (A) Lateral view of a 14-dpf *mhc2dab:GFP* transgenic fish showing reporter expression in cells scattered throughout the skin and in the thymus (arrowhead). The scale bar indicates 200μm. (B) Thymic (top panel) and skin (bottom panel) reporter expression in a double *cd45:DsRed*; *mhc2dab:GFP* transgenic fish at 12 dpf. For each location, the GFP (left), DsRed (middle), and merged (right) images are shown. The scale bar indicates 20μm.

fluorescent (Figure 1A). To assess which *mhc2dab:GFP*$^+$ cells were of hematopoietic origin, *mhc2dab:GFP* animals were mated to *cd45:DsRed* transgenic fish, which we described previously to label definitive blood cells at 72 hpf.[23] Confocal analyses performed on *cd45:DsRed*; *mhc2dab:GFP* double-transgenic animals at 12 dpf showed that most thymic *mhc2dab:GFP*$^+$ cells did not express the hematopoietic-specific *cd45:DsRed* transgene (Figure 1B top panel). This finding, along with the morphology and expression of the *forkhead box N1* and *E-cadherin* genes, suggests that early thymic mhc2dab$^+$ cells are thymic epithelial cells (supplemental Figure 1), a result that is consistent with observations in mammals.[27] Interestingly, both *cd45:DsRed* and *mhc2dab:GFP* expression colocalized to stellate cells within the skin (Figure 1B bottom panel), suggesting that the *mhc2dab* promoter also marked APCs derived from the blood-forming system. Fluorescent microscopic analyses demonstrated persistent transgene expression in thymic epithelial cells and skin leukocytes throughout adulthood. However, at approximately 45 dpf, GFP expression also became evident in keratinocytes (data not shown). This may have resulted from a lack of regulatory sequences in the 4-kb *mhc2* promoter or it may accurately reflect expression in epithelial cells, which can serve as "nonprofessional" APCs in mammals.[27] Epithelial GFP expression did not impair the ability to visualize GFP^{hi} skin leukocytes by confocal imaging.

Mhc2dab expression was not observed earlier during embryogenesis, particularly within primitive macrophages specified by 20 hpf.[6] Analysis of *cd45:DsRed* animals between 24 and 28 hpf showed transgene expression in hematopoietic cells on the yolk ball and within surrounding tissues, including the retina and brain (supplemental Figure 2). Based on their tissue distribution and behavior, these cells likely represent primitive macrophages.[6] DsRed$^+$ cells were purified by flow cytometry at 24 hpf and analyzed for gene expression by Q-PCR. Sorted cells expressed high levels of canonical macrophage-associated genes, including *csf1r*, *mpeg1*, and *marco* (data not shown). In contrast, the expression of *mhc2dab* and *mhc2daa* was undetectable. These findings are consistent with murine embryonic macrophages.[32] Although highly phagocytic, macrophages in the early mammalian embryo do not appear to be capable of antigen presentation via MHCII molecules; rather, it appears that these cells exist primarily to remodel developing tissues,[7,8] serve as immune sentries,[6] and seed nascent sites of the MPS, including microglia of the brain, with phagocytes.[33]

Expression of *mhc2dab:GFP* in the adult animal

Our initial results indicated that the *mhc2dab:GFP* transgenic line marked developing APCs in the zebrafish embryo, making it a suitable tool for the study of APC ontogeny and early function. To assess the potential of our transgenic line for immunobiologic investigations in adult animals, we examined the persistence of transgene expression in 3-month-old *mhc2dab:GFP* animals using flow cytometry. Because all hematopoietic lineages are produced in the adult kidney, we analyzed whole kidney marrow (WKM). Using light-scattering characteristics, we and others have previously shown that each of the major hematopoietic lineages segregate into unique subsets.[26,34] Figure 2A shows the gates used to discriminate between erythroid, lymphoid, precursor, myeloid, and eosinophilic cells in the WKM. In the mammalian hematopoietic system, expression of MHCII proteins is mainly confined to B lymphocytes, macrophages, and DCs.[27] Whereas no expression was observed in erythroid cells, 70% of kidney lymphoid cells expressed the *mhc2dab:GFP* transgene (Figure 2B top panels). To determine the cell types marked by the *mhc2dab:GFP* transgene, we purified GFP$^+$ and GFP$^-$ subsets from the lymphoid fraction and surveyed each for B- and T-lymphocyte markers. Lymphoid *mhc2dab:GFP*$^+$ cells specifically expressed *pax5*, a master regulator of the B-cell lineage,[35] whereas expression of the T-cell receptor gene *tcr-a* was restricted to the *mhc2dab:GFP*$^-$ fraction (Figure 2C). These results suggested that B cells, but not T cells, are marked with the *mhc2dab:GFP* transgene. Within the precursor fraction, *mhc2dab:eGFP* marked approximately 70% of cells.

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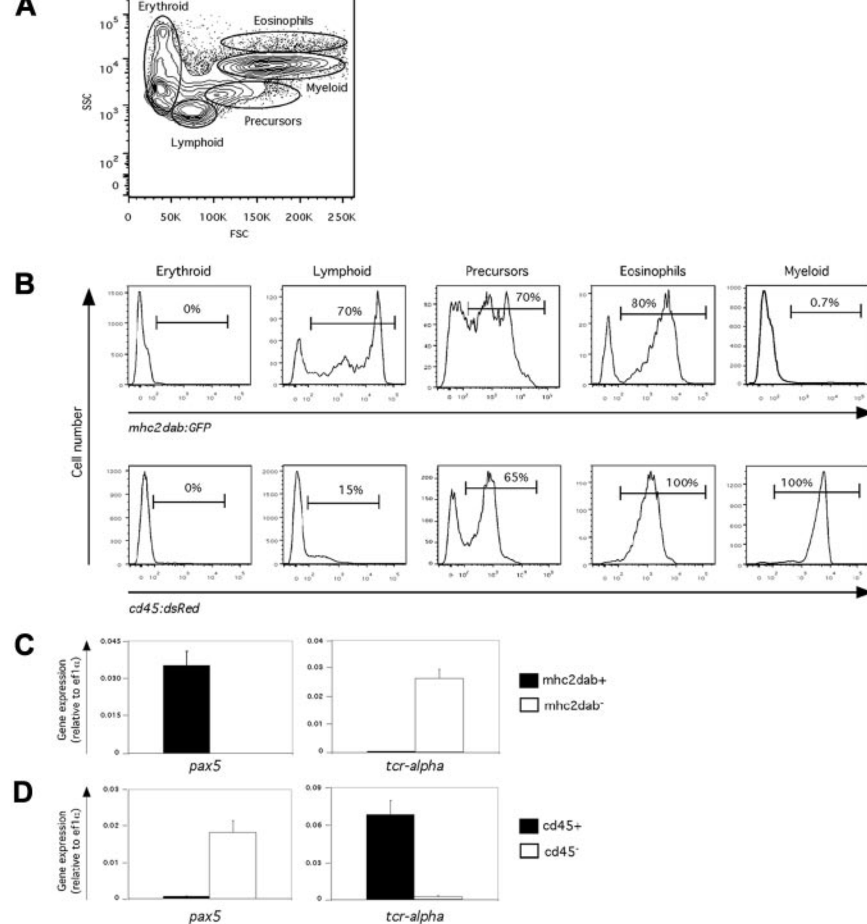

Figure 2. Expression of the *mhc2dab:GFP* and *cd45:DsRed* transgenes in adult WKM. (A) Gating strategy to isolate the main hematopoietic lineages—erythroid, lymphoid, precursor, myeloid, and eosinophils—using light-scattering characteristics (B) For each gate, the expression of *mhc2dab:GFP* (top panels) and *cd45:DsRed* (bottom panels) is presented as a histogram plot; percentages are indicated. (C-D) Gene expression in *mhc2dab:GFP* (C) and *cd45:DsRed* (D) kidney lymphoid cells examined for the presence of B-cell (*pax5*) and T-cell (*tcr-a*)–specific transcripts. Units on the y-axis represent transcript expression normalized to *ef1a* transcript levels. Error bars indicate SD.

Purified *mhc2dab:GFP*$^+$ precursor cells expressed both B-cell and myeloid genes, suggesting that precursors of both lineages are present in this fraction. Within the eosinophil gate, approximately 80% of cells were *mhc2dab:GFP*$^+$, which is consistent with our previous observation that *gata-2:eGFP*^{high} eosinophils expressed high levels of *mhc2dab*[36] and with studies showing MHCII expression in mammalian eosinophils.[37] Finally, within the myeloid scatter fraction, $<$ 1% of cells expressed *mhc2dab:GFP*. Because the majority of cells found within this gate are neutrophils, we crossed *mhc2dab:GFP* animals with the neutrophil-specific *lyz:DsRed* line to more precisely assess the lineal affiliation of *mhc2dab*. Expression of these 2 transgenes was largely mutually exclusive, demonstrating that neutrophils do not express the *mhc2dab:GFP* transgene (supplemental Figure 3). Our results demonstrate that the *mhc2dab* promoter is active in adult zebrafish, driving expression in B lymphocytes, eosinophils, and a minor population of myeloid cells that is distinct from neutrophils.

We similarly analyzed expression of the *cd45:DsRed* transgene to further refine our studies. Whereas no expression was observed in erythrocytes, 100% of cells within the myeloid and eosinophil gates expressed high levels of DsRed (Figure 2B bottom panels); however, only 65% and 15% of precursor and lymphoid cells, respectively, expressed the DsRed transgene. Based on the presence of erythrocyte precursors, we did not expect the *cd45:DsRed* transgenic line to mark the entire precursor fraction.[26,34] However, it was surprising that only a subset of lymphoid cells was positive. We therefore sorted DsRed-positive and DsRed-negative cells from the lymphoid fraction for gene-expression analysis. Q-PCR showed that lymphoid *cd45:DsRed*$^+$ cells expressed high levels of the *tcr-a* gene but were negative for *pax5* (Figure 2D). Purified lymphoid *cd45:DsRed*$^-$ cells showed the opposite expression pattern. These results demonstrate that the *cd45:DsRed* reporter line efficiently marks all myeloid cells and T lymphocytes in the WKM, but is not expressed in the B-cell lineage. The lack of transgene expression in B lymphocytes is not reflective of the endogenous expression of *cd45*, because lymphoid *cd45:DsRed*$^-$ cells are positive for *cd45* transcript expression (data not shown).

Isolation of a pure population of mononuclear phagocytes using combined expression of *mhc2dab:GFP* and *cd45:DsRed* transgenes

Because we expected the *mhc2dab* promoter to mark cells of the MPS, we investigated the nature of the rare *mhc2dab:GFP*$^+$ population within the myeloid scatter fraction. To better define this population, we used a combination of *mhc2dab:GFP* and *cd45:DsRed* reporter lines. As described in the previous paragraph, the combined expression pattern permits exclusion of B cells (*mhc2dab*$^+$; *cd45*$^-$). Therefore, we used this combination to focus on candidate APCs within the myeloid lineage. As shown in Figure 3Ai, separation of cells based on both transgenes revealed distinct populations of myeloid cells: approximately 87% were *cd45:DsRed*$^+$ only, whereas 0.7% expressed high levels of both *mhc2dab:GFP* and *cd45:DsRed* transgenes. To confirm our hypothesis that the myeloid *mhc2dab:GFP*$^+$ population represents a leukocyte subset distinct from neutrophils, we performed May-Grünwald-Giemsa staining on myeloid *cd45:DsRed*$^+$; *mhc2dab:GFP*$^+$ cells isolated by flow cytometry. Morphologic analyses revealed that the

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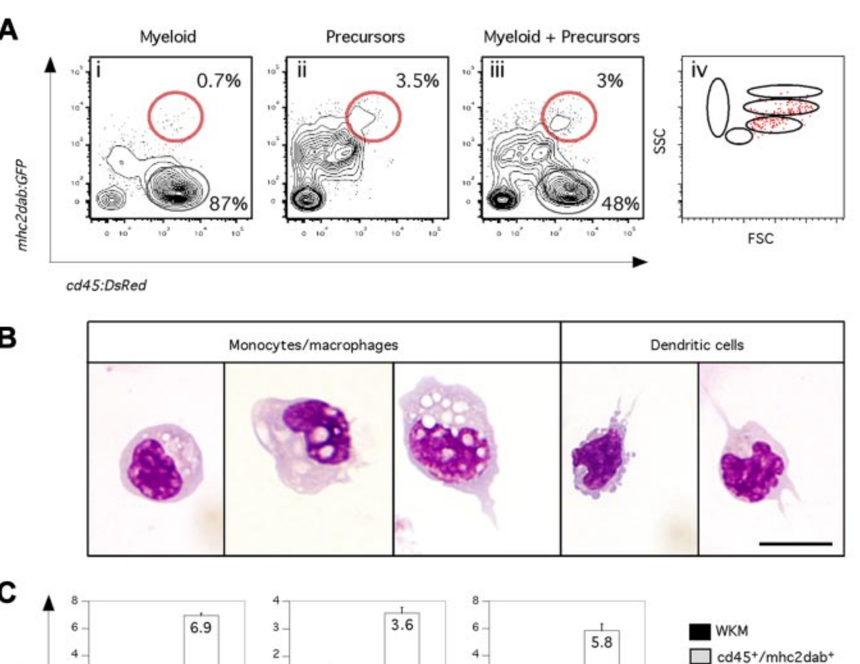

Figure 3. Isolation of a pure population of mononuclear phagocytes in WKM. (A) Distribution of *cd45:DsRed* and *mhc2dab:GFP* fluorescence in the myeloid gate (i), in the precursor gate (ii), and in a gate encompassing the myeloid and precursor gates (iii). *cd45:DsRed*^{high}; *mhc2dab:GFP*^{high} mononuclear phagocytes (red gate) were reanalyzed by forward and side scatter, showing that these cells overlap the myeloid and precursor fractions in WKM (iv). (B) Morphology of *cd45:DsRed*$^{+}$; *mhc2dab:GFP*$^{+}$ double-positive cells isolated from the myeloid fraction in WKM. Cells were cytospun and stained with May-Grünwald-Giemsa. The majority of the cells show the characteristics of macrophages, with kidney-shaped nuclei and vacuoles. Rare cells presented a different morphology with dendrites and a higher nuclear/cytoplasm ratio. The scale bar indicates 20 μm. (C) Q-PCR expression for genes specific to the neutrophil lineage (top panels) and the MPS (bottom panels). Whereas *cd45:DsRed*$^{+}$; *mhc2dab:GFP*$^{-}$ cells were highly enriched for neutrophil genes (top panels), *cd45:DsRed*$^{+}$; *mhc2dab:GFP*$^{+}$ cells expressed macrophage-specific genes only (bottom panels). Units on the y-axis represent changes (fold) above WKM. Error bars indicate SD.

majority of cells exhibited the characteristics of monocytes/macrophages, namely low nuclear to cytoplasm ratios and a high number of cytoplasmic vacuoles (Figure 3B). Interestingly, a few *cd45:DsRed*$^{+}$; *mhc2dab:GFP*$^{+}$ mononuclear phagocytes presented branched projections emanating in all directions from the cell body and a clear cytoplasm devoid of large granules (Figure 3B). These cytologic features resemble those we described previously for zebrafish DCs.[22]

We performed Q-PCR on sorted cells to assess the gene-expression profile of the 2 different cell subsets isolated from the myeloid scatter fraction. As expected, myeloid *cd45:DsRed*$^{+}$; *mhc2dab:GFP*$^{-}$ cells expressed high levels of neutrophilic genes, including *csf3r*, *mpx*, and *lyz* (Figure 3C). In contrast, purified myeloid *cd45:DsRed*$^{+}$; *mhc2dab:GFP*$^{+}$ cells showed little or no expression of *mpx*, *csf3r*, or *lyz*. Rather, they expressed high levels of *marco*, *csf1r*, *b7r*, and *interleukin-12p40* transcripts, suggesting that these cells were mononuclear phagocytes. We also investigated the expression of *ptpn6*, *mpeg1*, and *cxcr3.2*, which were previously reported as being macrophage specific in the zebrafish embryo.[20,38] Whereas we confirmed the specific expression of *mpeg1* in macrophages, *cxcr3.2* and *ptpn6* transcripts were present in both myeloid populations (supplemental Figure 4).

The existence of cells expressing high levels of *mhc2dab:GFP* within the precursor fraction (Figure 2) suggested that mononuclear phagocytes may not exclusively localize within the myeloid fraction, so we aimed to better define the scatter profile of this novel population. Indeed, mature *cd45:DsRed*$^{+}$; *mhc2dab:GFP*$^{+}$ mononuclear phagocytes found within the myeloid fraction were also detected in the precursor fraction, demonstrating double-positive cells overlapping the standard myeloid and precursor gates in WKM (Figure 3Aii). This was in contrast to mature *lyz:DsRed*$^{+}$ or *mpx:eGFP*$^{+}$ neutrophils, which localized entirely within the myeloid scatter fraction (data not shown). Interestingly, the precursor fraction of *cd45:DsRed*; *mhc2dab:GFP* double-transgenic animals also contained 2 additional populations with lower levels of transgene expression (Figure 3Aii). These populations, defined as *cd45:DsRed*$^{-}$; *mhc2dab:GFP*^{low} and *cd45:DsRed*^{low}; *mhc2dab:GFP*^{low}, likely represent less differentiated leukocyte precursor subsets. Finally, because kidney mononuclear phagocytes displayed a specific scatter profile overlapping the myeloid and precursor fractions (Figure 3Aiv), we determined that the most effective way to isolate these cells was to include both scatter fractions. As shown in Figure 3Aiii, kidney mononuclear phagocytes, referred to as *cd45:DsRed*^{high}; *mhc2dab:GFP*^{high} cells, accounted for 3% of the cells in the combined myeloid and

Table 1. Mononuclear phagocyte cell counts in adult hematolymphoid organs

Organ	Mononuclear phagocytes, %	Isolated cell number
Liver	0.6 ± 0.2	560 ± 182
Brain	0.3 ± 0.1	3503 ± 542
Gut	2.8 ± 0.9	3492 ± 97
Spleen	2.2 ± 1.1	3210 ± 830
WKM	1.4 ± 0.2	3669 ± 142
Thymus	1.4 ± 1.0	1102 ± 376
Skin	0.9 ± 0.3	4858 ± 1402

Numbers are presented as means ± SD. Percentages were obtained by analyzing cell suspensions from each organ by flow cytometry (n = 3). Cell numbers correspond to the number of mononuclear phagocytes obtained following cell sorting (n = 3), which, in the case of WKM, is approximately 50% of the total cells.

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Organ	Dendritic Cells	Monocytes/ macrophages
Liver	0%	100%
Brain	0%	100%
Gut	3%	86%
Spleen	5%	95%
WKM	6%	94%
Thymus	10%	90%
Skin	15%	85%

Figure 4. Distribution of DCs and monocytes/macrophages in adult tissues. For each tissue investigated, *cd45:DsRed*, *mhc2dab:GFP* double-positive cells were sorted, cytospun, and stained with May-Grünwald-Giemsa. Differential cell counts were obtained by identifying at least 200 cells per organ. Except for the gut, in which presumptive mast cells were also found, 2 different and separable morphologies were observed: those of DCs and those of monocytes/macrophages. The scale bar indicates 20 μm.

precursor fractions. Attempts at sorting mononuclear phagocytes using this purification strategy reproducibly resulted in the recovery of approximately 3500 cells per adult WKM.

Distribution of mononuclear phagocytes among adult tissues

Because the *mhc2dab:GFP*; *cd45:DsRed* transgene combination enabled the isolation of mononuclear phagocytes, we initiated a detailed examination of their tissue distribution in the adult zebrafish. Using flow cytometry, we performed cell counts of the myeloid *mhc2dab:GFP*$^+$; *cd45:DsRed*$^+$ cells within each hematolymphoid organ to establish the abundance and types of mononuclear phagocytes present. These analyses demonstrated that mononuclear phagocytes are abundant in the kidney, spleen, gut, and skin (Table 1). Consistent with other vertebrate animals, mononuclear phagocytes accounted for a small fraction of the leukocyte population in all organs investigated (1%-2%). Myeloid *cd45:DsRed*$^+$; *mhc2dab:GFP*$^+$ cells were sorted from each tissue presented in Table 1 and stained with May-Grünwald-Giemsa to assess morphology. In the liver and brain, sorted cells uniformly exhibited the characteristics of monocytes/macrophages (Figure 4). Whereas monocytes and macrophages were the predominant cell types isolated from gut, spleen, kidney, thymus, and skin, we also detected DCs within the myeloid *cd45:DsRed*$^+$; *mhc2dab:GFP*$^+$ fraction of these organs (Figure 4). Dendritic cells were scarce within the gut, spleen, and kidney, but accounted for approximately 10% and 15% of the mononuclear phagocytes in the thymus and skin, respectively. Morphologic analyses showed that sorted cells from all organs constituted a pure population of mononuclear phagocytes, with the exception of the gut. Indeed, 10% of the gut myeloid *cd45:DsRed*$^+$; *mhc2dab:GFP*$^+$ population displayed classic morphologic features of mast cells, including single-lobed nuclei and multiple dense granules in the cytoplasm (supplemental Figure 5).

The skin contained the highest number of mononuclear phagocytes and the highest ratio of DCs. To visualize how these cells are spatially organized under steady-state conditions, we performed confocal analyses on the skin of *mhc2dab:GFP*; *cd45:DsRed* adult animals. Imaging revealed a network of double-positive DC-like cells reminiscent of the Langerhans cell network found in the mammalian epidermis (Figure 5A). In addition to double-positive cells, single-positive cells were also observed (Figure 5A). Purification of each of the 3 subsets by flow cytometry showed that *mhc2dab:GFP*$^+$; *cd45:DsRed*$^+$ cells specifically expressed *marco* and *csf1r* by Q-PCR (Figure 5B), which is consistent with our results suggesting that double-positive cells are mononuclear phagocytes. In contrast, purified *mhc2dab:GFP*$^-$; *cd45:DsRed*$^+$ cells appeared to be neutrophils, as evidenced by the high expression of *mpx* (Figure 5B). Finally, *mhc2dab:GFP*$^+$; *cd45:DsRed*$^-$ cells appeared to be B lymphocytes based on their unique expression of *igm*. These results demonstrate that the combined expression of *mhc2dab:GFP*; *cd45:DsRed* transgenes uniquely marks mononuclear phagocytes regardless of the tissue source. Furthermore, double-transgenic animals can be used to discriminate between additional leukocyte populations, including B lymphocytes and neutrophils.

Zebrafish mononuclear phagocytes become activated upon antigen challenge

A key feature of APCs is their ability to recognize and uptake pathogen-derived antigens for presentation to T lymphocytes. To begin to functionally characterize zebrafish APCs, we investigated their phagocytic properties in vivo using pHrodo-marked, heat-inactivated bacteria in which the rhodamine derivative pHrodo fluoresces only upon acidification of the phagosome, making it a useful phagocytosis sensor.[39] We injected labeled bacteria intravenously into adult *mhc2dab:GFP* zebrafish and collected spleens

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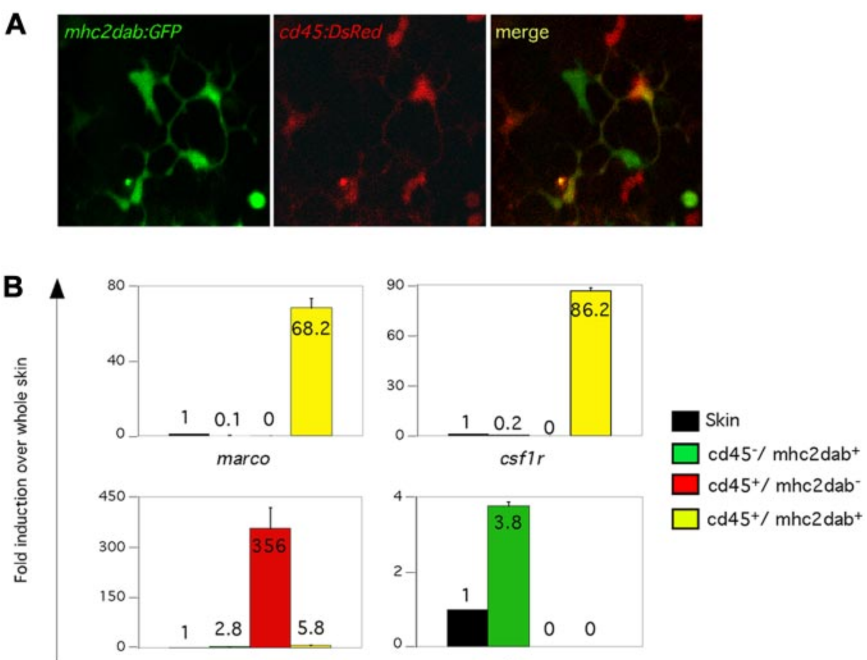


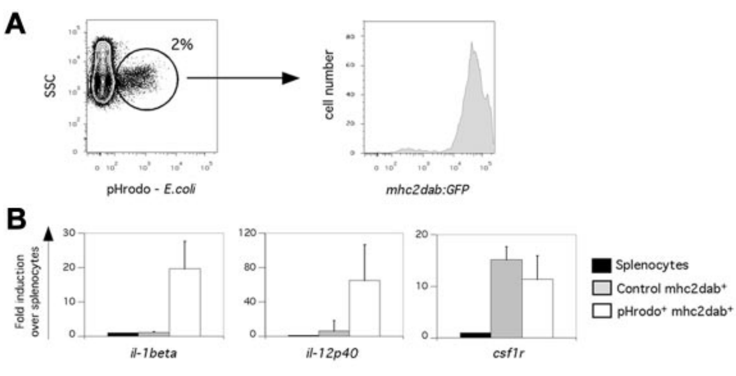
Figure 5. Visualization of DC-like cells in adult skin. (A) Skin from a *cd45:DsRed*, *mhc2dab:GFP* double-transgenic adult fish imaged with confocal microscopy. The GFP (left), DsRed (middle), and merged (right) images are shown. Three populations of cells could be isolated based on their expression of the 2 transgenes and their different morphologies. (B) Q-PCR expression for genes specific to macrophages and DCs (*marco*, *csf1r*), neutrophils (*mpx*), and B cells (*igm*) for sorted cells from the skin. Units on the y-axis represent changes (fold) above whole skin. Error bars indicate SD.

18 hours after injection for analysis by flow cytometry. As presented in Figure 6A, approximately 2% of total splenocytes were pHrodo$^+$ phagocytes. Interestingly, all pHrodo$^+$ phagocytes expressed the *mhc2dab:GFP* transgene (Figure 6A). These cells were myeloid, based on their light-scattering characteristics (data not shown). We then isolated these phagocytic *pHrodo*$^+$, *mhc2dab:GFP*$^+$ cells and unchallenged myeloid *mhc2dab:GFP*$^+$ splenocytes by flow cytometry. Both populations were compared for gene expression by Q-PCR. Zebrafish APCs specifically up-regulated the expression of inflammatory cytokines after phagocytosis. As shown in Figure 6B, the expression of *il-1beta* and *il-12p40* was increased 20- and 60-fold, respectively, whereas *csf1r* expression was unchanged. These results demonstrate that zebrafish MPS cells display functional properties similar to their mammalian counterparts.

Discussion

Until recently, there have been limited studies investigating the morphologic and functional characterization of immune cells in the zebrafish. Recent efforts, facilitated by the generation of lineage-specific transgenic reporter lines, have permitted the characterization of neutrophils and eosinophils.[15,17,18,36] The biology of other hematopoietic lineages, however, remains unclear because of the lack of specific reporter lines. Monocytes/macrophages and DCs, collectively referred to as mononuclear phagocytes, were previously identified in zebrafish based on their morphology and ultrastructural characteristics.[1,22] However, our attempts to isolate mononuclear phagocytes to purity using basic methodologies have been unsuccessful so far. In the present study, we demonstrate that the combined expression of *cd45:DsRed* and *mhc2dab:GFP*, along with light-scattering characteristics, can be used to isolate these lineages to purity in the zebrafish. This advance now enables the thorough characterization of mononuclear phagocyte identity, localization, and function.

Our results have demonstrated that all cell types comprising the mammalian MPS were identified within the myeloid *cd45:DsRed*$^+$; *mhc2dab:GFP*$^+$ double-positive population: monocytes, macrophages, and DCs. Cytochemical analyses demonstrated that with the exception of the gut, this marker combination was sufficient to isolate pure populations of mononuclear phagocytes from any adult organ. Examination of myeloid *cd45:DsRed*$^+$; *mhc2dab:GFP*$^+$ cells in immune-related tissues showed that mononuclear phagocytes were the most abundant in the intestine and spleen, followed



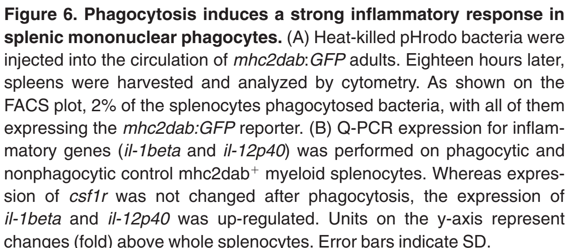


Figure 6. Phagocytosis induces a strong inflammatory response in splenic mononuclear phagocytes. (A) Heat-killed pHrodo bacteria were injected into the circulation of *mhc2dab:GFP* adults. Eighteen hours later, spleens were harvested and analyzed by cytometry. As shown on the FACS plot, 2% of the splenocytes phagocytosed bacteria, with all of them expressing the *mhc2dab:GFP* reporter. (B) Q-PCR expression for inflammatory genes (*il-1beta* and *il-12p40*) was performed on phagocytic and nonphagocytic control mhc2dab$^+$ myeloid splenocytes. Whereas expression of *csf1r* was not changed after phagocytosis, the expression of *il-1beta* and *il-12p40* was up-regulated. Units on the y-axis represent changes (fold) above whole splenocytes. Error bars indicate SD.

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by the kidney and thymus. Although the sites of antigen presentation to lymphocytes have yet to be defined in the zebrafish, these observations are in agreement with these tissues functioning as secondary lymphoid organs in mammals.[40] Quantification of the different mononuclear phagocyte subsets by cytochemical staining indicated that zebrafish DCs are scarce among adult tissues, a trait shared with their mammalian counterparts. We estimate that the number of DCs isolated by flow cytometry ranges from 100-700 DCs per tissue, with the spleen and the skin presenting the lowest and highest yields, respectively. In the skin, DCs accounted for up to 15% of the mononuclear phagocyte population. Confocal imaging of transgenic adult skin showed the presence of a dense network of double-positive cells with a dendriform morphology. These cells were likely Langerhans cells, a DC subtype found in the mammalian epidermis, which we recently characterized in the zebrafish.[22]

In the present study, we performed the first gene-expression analyses in purified mononuclear phagocytes and observed expression of genes associated with macrophage and DC development and function. It was recently reported that embryonic macrophages and neutrophils could be separated in the *mpx:eGFP* line on the basis of transgene expression levels, macrophages being GFP^{low}.[41] In the adult animal, our results indicate that mononuclear phagocytes lack expression of myeloid peroxidase. Rather, we found that *mpx:GFP*^{low} cells in the adult kidney exhibited the morphology of myeloid precursors (data not shown), an observation consistent with the early expression of *mpx* during mammalian myelopoiesis.[42] The *lyz:DsRed* transgenic line was originally described to mark both neutrophils and macrophages.[15] However, subsequent reports have suggested that the transgene becomes restricted to neutrophils after 2 dpf.[43] In support of these findings, we show herein that mononuclear phagocytes do not express *lyz* transcripts and that the expression of *mhc2dab:GFP* is mutually exclusive to *lyz:DsRed* in the adult kidney. Finally, because our transgenic lines allow for the distinction between macrophages and neutrophils, we have investigated the expression of several genes recently reported to be macrophage specific in the zebrafish embryo.[38] Whereas *mpeg1* was found to be macrophage specific, *cxcr3.2* and *ptpn6* were equally expressed in both kidney neutrophils and macrophages. Although we cannot exclude the possibility that *cxcr3.2* and *ptpn6* are differentially expressed during development, our findings are in agreement with mammalian studies demonstrating expression in lineages outside of the MPS.[44]

Our analyses indicated that *mhc2dab:GFP* marked the 3 hematopoietic lineages that act as APCs in mammals: macrophages, DCs, and B lymphocytes. The identification of B cells within the *mhc2dab:GFP*$^+$ fraction is of great relevance for the study of zebrafish immunity because there are no transgenic lines that mark the B-cell lineage. In addition, these findings strongly suggest that the function of antigen presentation by B lymphocytes may be evolutionarily conserved in zebrafish. Combined with existing transgenic lines, including *rag2:DsRed*,[45] the *mhc2dab:GFP* reporter will allow a better understanding of B-cell differentiation in teleosts. In particular, the ability to track and study the ontogeny of B cells during embryonic development will likely lead to new insights into B-cell biology in the zebrafish, an issue that remains to be thoroughly investigated.

We also observed expression of *mhc2dab:GFP* within the WKM eosinophil fraction, a result consistent with our previous findings that *gata-2:*eGFP$^+$ eosinophils express *mhc2* transcripts.[36] The role of eosinophils as APCs to induce Th2 responses in mammals has been documented previously.[37] In addition to eosinophils, we observed the expression of *mhc2dab:GFP* in another

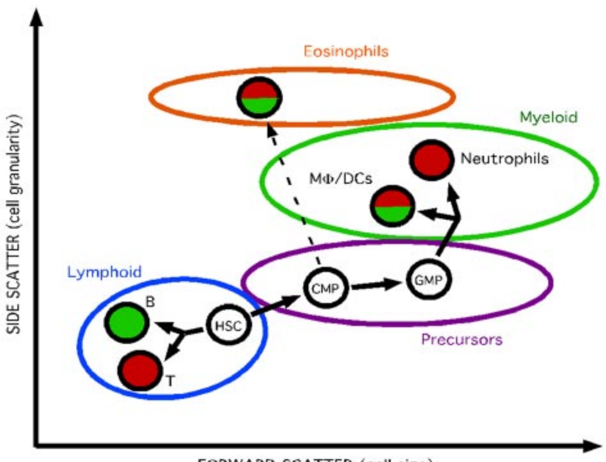

Figure 7. Separation of the major hematopoietic lineages from kidney using the *cd45:DsRed* and *mhc2dab:GFP* transgenic lines. By combining light-scattering characteristics and expression of the fluorescent reporter genes, each of the major blood cell lineages could be resorted and purified by flow cytometry.

granulocyte subset in the intestine. Purified *mhc2dab:GFP*$^+$ cells from the gut demonstrated similar morphologic characteristics to mast cells described previously in the zebrafish.[46] Whereas the exact nature of these cells needs to be validated, the expression of *mhc2* has been described previously in mammalian mast cells.[47]

The *cd45:DsRed* transgenic line that we describe in this study marks the majority of leukocytes. Gene-expression and morphologic analyses showed that all myeloid cells—monocytes/macrophages, DCs, eosinophils, neutrophils, and mast cells—expressed the transgene. However, we have determined that within the lymphoid lineages, only T cells were marked. Whereas B cells express at least one variant of the endogenous *cd45* gene, the promoter fragment that we used to generate our transgenic line was not expressed in this lineage. Accordingly, it was shown that the murine *cd45* promoter contains 3 distinct transcriptional initiation start sites that are differentially active in distinct hematopoietic lineages.[48] Overall, the combined unique expression patterns of *cd45:DsRed* and *mhc2dab:GFP* transgenic lines now permits the specific identification and purification of the major immune cell lineages in the zebrafish by flow cytometry: monocytes/macrophages/DCs (FSC^{hi}, SSC^{int}, *cd45*$^+$, *mhc2dab*$^+$), neutrophils (FSC^{hi}, SSC^{int}, *cd45*$^+$, *mhc2dab*$^-$), eosinophils (FSC^{hi}, SSC^{hi}, *cd45*$^+$, *mhc2dab*$^+$), T cells (FSC^{low}, SSC^{low}, *cd45*$^+$, *mhc2dab*$^-$), and B cells (FSC^{low}, SSC^{low}, *cd45*$^-$, *mhc2dab*$^+$; Figure 7). Because these identification criteria are applicable to a variety of different tissues, our transgenic lines will allow a more precise characterization of the cellular composition of each hematolymphoid organ in the zebrafish.

The generation of an adaptive immune response in mammals requires interactions between APCs and lymphocytes. These interactions occur in specialized secondary lymphoid organs such as the spleen, lymph nodes, and Peyer patches.[49] Despite the presence of a lymphatic system, zebrafish (and teleosts in general) do not appear to possess organized secondary lymphoid structures, and the sites of immune recognition remain to be determined. Because the localization of different APC subsets within immune tissues is an important determinant of their function, our transgenic lines will be instrumental in defining the sites of immune recognition in teleosts. Our observations that mononuclear phagocytes are abundant in the spleen and intestine suggest that these organs might serve as the preeminent sites for antigen presentation. Because histologic analyses have indicated that zebrafish do not form germinal centers

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in the spleen, and because fish have gut-associated lymphoid tissues scattered throughout their intestinal mucosa,[50] it is generally believed that the gut may act as the predominant site of immune cell interaction in teleosts. The ability to mark and follow zebrafish APCs now permits elucidation of this open question.

In conclusion, the ability to specifically identify and isolate cells of the MPS will advance studies of immunology in the zebrafish. The identification of gene-regulatory elements targeting expression to cells of the MPS now enables lineage tracing of the first mononuclear phagocytes to appear in the embryo via fate-mapping approaches. Recent studies have demonstrated that murine microglia are seeded by primitive macrophages that first appear in the extra-embryonic yolk sac.[33] It will be interesting to determine whether other adult phagocyte subsets initiate from this population during embryogenesis.

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Authorship

Contribution: V.W., J.Y.B., and D.T. designed the experiments; V.W. and J.Y.B. performed the research and analyzed the data; P.W.G. performed aspects of the research; and V.W., J.Y.B., and D.T. wrote the paper.

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