Yolk sac-derived Pdcd11-positive cells modulate zebrafish microglia differentiation through the NF-κB-Tgfβ1 pathway
Ruimeng Yang, Ming Zhan, Miaomiao Guo, Hao Yuan, Yi Qin Wang, Yiyue Zhang, Wenqing Zhang, Saijuan Chen, Hugues de The, Zhu Chen, et al.

To cite this version:
Ruimeng Yang, Ming Zhan, Miaomiao Guo, Hao Yuan, Yi Qin Wang, et al. Yolk sac-derived Pdcd11-positive cells modulate zebrafish microglia differentiation through the NF-κB-Tgfβ1 pathway. Cell Death and Differentiation, Nature Publishing Group, 2021, 28 (1), pp.170-183. 10.1038/s41418-020-0591-3. hal-03428097

HAL Id: hal-03428097
https://hal.archives-ouvertes.fr/hal-03428097
Submitted on 25 Nov 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Yolk sac-derived Pdcd11-positive cells modulate zebrafish microglia differentiation through the NF-κB-Tgfβ1 pathway

Ruimeng Yang1,2 · Ming Zhan2,3 · Miaomiao Guo2 · Hao Yuan1 · Yiqin Wang1 · Yiyue Zhang4 · Wenqing Zhang4 · Saijuan Chen1 · Hugues de The1,5,6 ⋅ Zhu Chen1 ⋅ Jun Zhou1 ⋅ Jun Zhu1,5

Received: 3 March 2020 / Revised: 7 July 2020 / Accepted: 9 July 2020 © The Author(s) 2020. This article is published with open access

Abstract

Microglia are the primary immune cells in the central nervous system, which plays a vital role in neuron development and neurodegenerative diseases. Microglial precursors in peripheral hematopoietic tissues colonize the central nervous system during early embryogenesis. However, how intrinsic and extrinsic signals integrate to regulate microglia’s differentiation remains undefined. In this study, we identified the cerebral white matter hyperintensities susceptibility gene, programmed cell death protein 11 (PDCD11), as an essential factor regulating microglia differentiation. In zebrafish, pdcd11 deficiency prevents the differentiation of the precursors to mature brain microglia. Although, the inflammatory featured macrophage brain colonization is augmented. At 22 h post fertilization, the Pdcd11-positive cells on the yolk sac are distinct from macrophages and neutrophils. Mechanistically, PDCD11 exerts its physiological role by differentially regulating the functions of nuclear factor-kappa B family members, P65 and c-Rel, suppressing P65-mediated expression of inflammatory cytokines, such as tnfα, and enhancing the c-Rel-dependent appearance of tgfβ1. The present study provides novel insights in understanding microglia differentiation during zebrafish development.

Introduction

Cerebral white matter hyperintensities (WMH), also known as leukoaraiosis, are common in the aging population and are associated with increased risk of cognitive dysfunction, stroke, and neurodegenerative disorders including Alzheimer’s disease [1]. Perivascular inflammation is a prominent pathological feature in WMH, and WMH burden has been associated with circulating biomarkers of inflammation, including high-sensitivity C-reactive protein and Interleukin-6 [2–4]. Genome-Wide Association studies have helped identify many risk-associated single nucleotide polymorphisms (SNPs) related with WMH development and progression [5–7]. SNPs in PDCD11 have showed significant associations with WMH burden, but their functional role remain undefined [5, 7].
Microglia, the innate immune cells of the central nervous system (CNS), enter the brain during early embryogenesis and play important roles in white matter pathology [8]. As influenced by their environment, microglia assume different molecular patterns ranging from M1 phenotype in secretion pro-inflammatory cytokines to M2 phenotype in inflammation resolution and tissue repair [9]. In the zebrafish embryo, macrophages develop early on the yolk sac (YS) expression L-plastin and spread in the cephalic mesenchyme at about 24 h post fertilization (hpf). After which they invaded brain and differentiated into microglia featured by lower expression of the L-plastin, and higher expression of the neurotrophic lipid carrier, apolipoprotein E at about 60 hpf [10, 11]. Factors like Colony-Stimulating Factor 1 Receptor (CSF1R) and neuronal apoptosis modulate the seeding of microglia precursors in the brain [12, 13]. But how intrinsic and extrinsic signals integrate in microglia differentiation remains largely unknown. Transforming growth factor-beta (TGF-β) family members are cytokine important for both innate and adaptive immune functions [14]. It is required for the maintenance of the microglia-specific homeostatic gene signature. Deletion of the TGF-β receptor in microglia resulted in rapid conversion of microglia toward an inflammatory macrophage phenotype [15, 16]. Although the importance of brain-derived TGF-β1 in shaping immune response in pathogenic conditions has been demonstrated, the origin of embryonic TGF-β1, especially for inducing microglia differentiation, remains unknown.
In this study, helped by zebrafish model organism, we demonstrated that PDCD11, a WHM susceptibility gene, helps promote embryonic microglia differentiation through Tgfβ1. Macrophages in pdcd11 mutants showed increased expression of inflammatory genes and impaired upregulation of microglia signature genes. This unbalanced macrophage generation, likely caused by uncontrolled P65 activation, causes inflammatory gene upregulation and suppresses c-Rel-dependent Tgfβ1 expression under pdcd11 deficiency.

Results

P53 independent inflammatory pathway activation in pdcd11 mutants

Pdc11 is highly conserved between species and mainly harbors two functional domains, including an N-terminal ribosome and Coil (Fig. 1a). To define the role of Pdc11 in embryonic development, we generated a pdcd11 mutant zebrafish line with a 7-bp deletion in the fourth exon by CRISPR-Cas9 nuclease (Fig. S1a). Antibody targeting the ribosome domain of Pdc11 confirmed the complete loss of functional domains in pdcd11 mutants (Fig. 1a). Perturbations of ribosome biogenesis often stabilize and activate genes related to the P53 apoptosis pathway [17–19]. Common with other reported ribosome defects, obvious developmental retardation and craniofacial malformation were observed in 3 dpf pdcd11 mutants (Fig. 1b). Not surprisingly, P53 pathway genes were hyperactivated (Fig. S1b) and acridine orange (AO)-positive dead cells were also significantly increased in pdcd11 deficient embryos from 22 hpf (Fig. S1c). To determine the contribution of the induction of apoptosis in the developmental defects of pdcd11 mutants, pdcd11 mutants in a p53 knockout background were also generated [20]. P53 depletion favorably recovered AO cell numbers and P53 pathway genes in pdcd11 mutants (Fig. S1d, e). However, cranial deformities, shorter body lengths, and survival rates were only partially restored (Fig. S1f, g), possibly suggesting that other P53 independent factors also contribute to the pathology of pdcd11 mutants.

Interestingly, consistent with the perivascularg inflammation a prominent pathological feature in WMH, we found that genes associated with inflammatory responses, like il6, illb, and tnfa, were significantly increased, which otherwise failed to be restored by combined p53 mutation (Fig. 1c).

Using whole-mount in situ hybridization (WISH), the apparent increased expression of inflammatory genes in the brain of 52 hpf pdcd11 mutants were detected, which also could not be recovered by the p53 mutation (Fig. 1d, e).

Macrophage generation imbalance in pdcd11 mutants

Inflammatory gene expression was elevated in pdcd11 mutants as early as 22 hpf. At this time, macrophages and neutrophils were the two major types of immune-related inflammatory cells identified in zebrafish. Macrophages initiate migration in the cephalic mesenchyme at about 24 hpf. Compared with that in WT embryos, pdcd11 deficiency induced increased and earlier accumulation of macrophages in brain tissue as revealed by mfap4 or pan-leukocyte marker l-plastin staining (Fig. 1f). The distribution pattern of mpo-positive leukocytes remained unaltered at this time (Fig. S2a). A previous study showed that apoptotic neuronal death drives the colonization of microglia precursors in the optic tectum [21]. Thus, we speculated that the advanced macrophage migration might be stimulated by increased neural apoptotic signaling. However, the combined p53 mutation failed to restore the increased macrophage accumulation in the brains of pdcd11 mutants, with accumulation actually being exacerbated (Fig. S2b). Interestingly, distinct from nonspecific macrophage markers, the expression of csf1ra, which was previously shown to be required for the migration and proliferation of microglia precursors [13, 22], was contrarily reduced in pdcd11 mutants (Fig. 1f). Consistently, staining by either apoeb or Neutral Red revealed that mature microglia, which are tissue-resident macrophages in the brain, were completely absent in 3 dpf pdcd11 mutants (Fig. 1g). At this time, macrophages stained for mfap4 or l-plastin were abundant in the brain of pdcd11 mutants, with an evidently enlarged and vacuolated morphology, which was also verified using a macrophage transgenic line Tg(mpeg1:GFP) (Fig. 1h, i). Macrophages acquire specific functional phenotypes that are manifest by secretion of inflammatory gene products and their morphological appearance. We found that the expression of tnfa, a well-established marker of M1 macrophages in zebrafish [23], was obviously increased in macrophages sorted from 60 hpf pdcd11-deficient embryos (Fig. 1j). In teleost fish, cxcr3.1 and cxcr3.2 macrophages show functional polarization towards M1 and M2, respectively [24]. We found that in 22 hpf pdcd11 mutants, cxcr3.1 expression was profoundly increased while that of cxcr3.2 was reduced as compared with that in WT controls (Fig. 1k, l).

Similar to the failed restoration of inflammatory genes with p53 mutation, simply inhibiting apoptosis could not restore the macrophage generation abnormalities observed in pdcd11 mutant embryos (Fig. S2c, d). Moreover, no obvious aberrations in proliferation or apoptosis of myeloid progenitors were detected in pdcd11 mutants at 22 hpf (Fig. S2e–h).
Pdcd11-positive cells yield Tgfβ1 required for microglia differentiation

Microglia differentiation requires step-wise-activated intrinsic transcription factors and circumstance-derived inducing factors, like Tgfβ1. Through comparing the RNA-seq data carried between 22 hpf WT and pdcd11 mutants, we noticed that tgfb1a mRNA, a homolog of human TGFB1, was profoundly reduced in 22 hpf pdcd11 mutant embryos (Table S1). The finding was verified by qPCR and western blot (Fig. 2a, b). Using WISH, we found that tgfb1a was highly expressed in cells on the YS at 22 hpf, at a location similar to Pdcd11, and that the expression of tgfb1a was obviously reduced with pdcd11 deficiency (Fig. 2c). Otherwise the expression of tgfb1b, another orthologue of human TGFB1 were lowly expressed at 22 hpf and no difference was detected with pdcd11 depletion (Fig. 2d–i). Moreover, specially enriched Tgfβ1 in Pdcd11 expression cells was revealed using an immunofluorescence assay (Fig. 2d). Tgfβ1-induced signature genes in mouse microglia have been studied [25, 26]. Supporting the role of pdcd11 in mediating Tgfβ1 activation in microglia, brain macrophages sorted from pdcd11-deficient Tg(mpeg1:GFP) line showed reduced expression of Tgfβ1-stimulated genes, including sall1a, tgbbr1a and tgbbr1b, and increased expression of Tgfβ1-suppressed genes like tlr5a and ctsl1 as compared with that in WT embryos (Fig. 2e). The elevation of csf1b and reduction of ctsba in microglia were also verified by WISH (Fig. 2f). Confirming the importance of tgfb1a reduction in contributing to microglia depletion in pdcd11 mutants, tgfb1a overexpression restored both precursor and mature microglia numbers in pdcd11 mutants (Fig. 2g–i). However, this rescue was compromised by the simultaneous repression of Tgfβ1 activation in macrophages

---

**Fig. 2** Reduced TGF-β1 signature genes in pdcd11-deficient macrophages. qPCR (a), western blot (b) and WISH (c) examination of the expression levels of tgfb1a or Tgfβ1 in 22 hpf zebrafish. d Immunofluorescence assessment of Pdcd11 and Tgfβ1 expression in 22 hpf zebrafish embryos. White arrows indicate the co-expressed signals found on the YS. e Fold changes of Tgfβ1-regulated genes expression in macrophages sorted from the brains of 60 hpf pdcd11 mutants as compared with WT controls using Tg(mpeg1:GFP) transgenic line. f Expression of csf1b and ctsba in 60 hpf WT and pdcd11 mutants by WISH. Red arrows indicate the positive cells. g Effect of zebrafish tgfb1a mRNA overexpression on csf1ra expression in WT and pdcd11 mutants. Effect of tgfb1a mRNA or combined mpeg1 driven smad7 overexpression on microglia markers apoeb (h) or Neutral Red staining (i) in WT and pdcd11 mutants. j The effect of LY364947 addition on apoeb expression in WT and pdcd11 mutants with tgfb1a mRNA overexpression. The number positioned in the lower right corner of F–j represent the number of zebrafish embryos shown positive phenotypes versus the total number of embryos examined. Scale bar: 100 μm. Means ± SEM are shown for three independent experiments. *P < 0.05 (Student’s t test).
by either LY364947, a TGFβR-I inhibitor addition, or overexpressing inhibitory cofactor smad7 under control of the mpeg1 promoter, implying the importance of Pdcd11-derived Tgfβ in regulating macrophage development (Fig. 2h–j).

**Elevated P65 transcriptional activity in pdc11 mutants**

Aside from the large N-terminal ribosome domain (Rib), Pdcd11 contains C-Terminal Coil domain (Coil), the function of which has not been defined (Fig. 3a). To investigate the molecular mechanism, we first overexpressed Rib and Coil. While Rib overexpression faithfully restored P53 pathway genes, the disturbances of macrophage generation and inflammation pathway activation could only be reversed with Coil overexpression in pdc11 mutants (Fig. 3b, c and S3a–c). A short C-Terminal fragment (Ter) reportedly could interact with P65 (Fig. 3a), one member of the NF-κB family [27, 28]. The NF-κB signaling pathway participates in modulating tissue immune homeostasis. Under normal circumstances, P65 is retained in the cytoplasm by the inhibitory protein IkappB and is available to the proteasome degradation pathway, which avoids...
uncontrolled inflammation activation. Upon stimulation, P65 is post-translationally modified by phosphorylation at the 536-serine residue, which obviates the interaction with \( \text{I} \kappa \text{B} \). P65 can then translocate into the nucleus to activate the downstream signaling pathway. We found that 536-serine phosphorylated P65 was obviously increased in \( \text{pdcd}11 \) mutants (Fig. 3d). In vitro overexpression of Coil or simply the terminal fragment Ter efficiently reduced nuclear 536-serine phosphorylated P65 (Fig. 3e). Interestingly, we found that in vitro transfected P65 and Coil mostly colocalized in the cytoplasm or in the nucleolus, which correlated with reduced P65 transcriptional activity [29, 30] (Fig. 3f). The cytoplasm and nuclear P65 content with Coil overexpression were then examined respectively by western blot. Increasing Coil transfection obviously increased the P65 cytoplasm content, while correspondingly reducing the nuclear P65 level (Fig. 3g). Moreover, increased 536-serine phosphorylated P65 in \( \text{pdcd}11 \) mutants were restored by either Coil or Ter domain overexpression, but not by Rib domain overexpression (Fig. 3h). To determine whether the rescuing effects of Coil relied on its capacity in suppressing P65 activation, two NF-\( \kappa \)B inhibitors, BMS-345541 and BAY 11-7821, were used [31]. Treatment with either BMS-345541 or BAY 11-7821 reduced 536-serine phosphorylated P65, and inflammatory genes and macrophages also returned to normal in \( \text{pdcd}11 \) mutants (Fig. 3i and S3d, e). A correctly regulated inflammatory cascade is required for the maintenance of microglia homeostasis [32].
However, microglia number or the TGFβ1 level could not be rescued by BMS-345541 or BAY 11-7821 treatment (Fig. 3, k), suggesting that other P65 independent mechanisms might be utilized by Coil in regulating microglia generation.

Interestingly, while Coil overexpression restored both macrophage appearance and inflammatory genes in the pdcd11 mutants, similar efficacy was not detected with either Coil overexpression in pan neuron cells under Huc promoter (also named embryonic lethal, abnormal vision-like, neuron-specific RNA-binding protein 3, elavl3) [33] or macrophages under control of the mpeg1 promoter [34] (Fig. S3c, f).

**Pdcd11 enhances c-Rel/p105 nuclear transport**

Transgenic zebrafish with NF-κB recognition sequences driven fluorescent protein was utilized to study NF-κB activity in zebrafish [35]. Helped by this transgenic line, we were surprised to find that although PDCD11 suppressed P65 activation, NF-κB was otherwise activated in Pdcd11-expressing cells on the YS at 22 hpf (Fig. 4a). In comparison with cytoplasm Coil expressed cells, cells with nuclear Coil expression were prone to activate EGFP under control of the NF-κB recognition sequences (Fig. 4b and S4a).

Among the five members of NF-κB family, P65, c-Rel, and Rel-B harbor the transcription activation domain (TAD), which binds NF-κB recognition sequences and activates the expression of downstream genes [36, 37]. The findings prompted us to speculate that this activation of the NF-κB-binding element relied on the transcriptional activity of other members of the NF-κB family. Indeed, different from P65, Coil colocalized with c-Rel and P105 in the nucleus and Coil overexpression promoted the nuclear retention of c-Rel (Fig. 4c–e and S4b, c). A luciferase assay showed that Coil co-expression enhanced the capacity of c-Rel in activating the NF-κB-binding element (Fig. 4f). Confirming the importance of reduced nuclear c-Rel/P105 in leading the microglia deficiency in pdcd11 mutants, we found that c-Rel and p105 co-overexpression recovered microglia numbers as manifested by either apoeb or Neutral Red staining (Fig. 4g, h). These rescue effects were largely compromised when repressive smad7 was co-expressed in macrophages, suggesting that c-Rel/P105 functions upstream of TGFβ signaling (Fig. 4g, h). Consistently, the expression level of Tgfβ1 was also increased with c-Rel and p105 mRNA overexpression (Fig. 4i, j). Injection of the xanthine derivative pentoxifylline (PTXF), which selectively degrades c-Rel [38], also reduced the number of microglia in WT embryos. The reduction could also be recovered by the transfection on nuclear/cytoplasm P65 content in 293T cells. J Schematic diagram showing the working model for PDCD11-mediated regulation of c-Rel and P65. Under normal circumstances, on one hand, the Ter fragment of PDCD11 (yellow) binds and masks the binding element relied on the transcriptional activity of coil with “M2” properties. However, cells deficient in PDCD11 show increased P65 nuclear translocation, which leads to activation of inflammation-related genes, such as IL-1, IL-6, and TNFa. These cytokines then facilitate “M1” macrophage differentiation.
injection of tgb1a mRNA (Fig. 4k). In a previous study, chromatin immunoprecipitation combined with microarray technology (ChIP/Chip) examination of mitogen-stimulated T cells Rel/NF-kappa-B revealed potential Rel/NF-κB recognition motifs in the promoter region of TGFBI [39]. To further determine the regulatory role on TGFBI by Coil and c-Rel, HEK293 cells were examined using a luciferase reporter assay. Coil co-expression enhanced the capacity of c-Rel in stimulating the TGFBI promoter. This enhancement was abolished by the combined M1 and M2 c-Rel-binding site mutation (Fig. 4l). These results suggest that Coil and c-Rel promotes tgb1 expression in early zebrafish embryos.

Since P65 and c-Rel are structurally similar and both harbor an N-terminal Rel homology domain (RHD) and C-terminal TAD domain, it was of interest to investigate how PDCD11 distinguished the two (Fig. 5a). Co-IP examination revealed that both P65 and c-Rel could interact with the

---

**Fig. 6 Pdcd11 expression pattern examined during zebrafish embryonic development.**

- **a** WISH assessment of pdcd11 expression in zebrafish at 7-ss, 22 hpf, and 32 hpf. The red arrows indicate pdcd11 signals on the YS and pericardial cavity.
- **b** Immunofluorescence staining of Pdcd11 in Tg(pu.1:GFP) zebrafish. The red arrows indicate colocalized Pdcd11 and Pu.1 expressing cells on the YS at 10-ss.
- **c** Pdcd11 expression in 22 hpf and 32 hpf Tg(mpeg1:GFP) zebrafish assayed by immunofluorescence staining.
- **d** WISH examination of pdcd11 expression in pu.1 deficient embryos. The red dotted lines denote the pericardial cavity underneath the brain where pdcd11 is expressed.
- **e** IF assessment of the co-expression between GFP driven by pdcd11 promoter and endogenous Pdcd11. White arrowheads indicate the colocalization signal on the 22 hpf YS.
- **f** The expression pattern of mfap4-positive macrophages in the 52 hpf brain with pdcd11 promoter driven Coil overexpression.
- **g** Rescue effects of apoeb positive microglia with Coil overexpressed under different promoters including Huc, mpeg1, mpo, and pdcd11. The number positioned in the lower left corner of Fig. 6f, g represent the number of zebrafish embryos shown positive phenotypes versus the total number of embryos examined. Scale bar: 100 μm.
Coil domain of PDCD11 (Fig. 5b, c). The Coil domain was further divided into the Coil–coil and Ter fragment, which displayed a different interaction pattern with P65 and c-Rel. Since the RHD domain of P65 and c-Rel interact with the Coil–coil region of PDCD11, the Ter fragment failed to bind to the TAD domain of c-Rel as observed with P65 (Fig. 5d–g). This result was reasonable since, because the RHD domains of NF-κB family members are similar, their TAD domains are much more distinct. The interaction of Ter with the TAD domain of P65 might cover the phosphorylation site required for P65 transcriptional activation. The mere overexpression the Ter fragment could suppress the P65 activation in pdc11 mutants, otherwise the Coil–coil fragment overexpression failed to do so (Fig. 5h). Confirming the importance of Ter fragment in inhibiting P65 activation, the overexpression of this fragment also inhibited P65 nuclear translocation (Fig. 5i). Thus, the differential interactions between the TAD domains and PDCD11 might contribute to the different regulation mode of P65 and c-Rel by PDCD11 (Fig. 5j).

**YS Pdc11-expressing cells are derived from Pu.1-progenitors**

By WISH, we found that aside from the obvious expression signal in the neuron epithelium, scattered cells on the YS were also detected from 7-somite stages (ss) (Fig. 6a). At 32 hpf, pdc11-positive cells had accumulated in the pericardial cavity, the site of inflow of all blood cells (Fig. 6a). In zebrafish, myeloid progenitor cells expressing Pu.1 emerged within the anterior lateral plate mesoderm (ALPM) at the 5-ss and then migrated into the rostral blood island (RBI) and onto the YS. This was followed by differentiation of the cells into macrophages and neutrophils, which then seeded the brain from approximately 24 hpf. Through use of the zebrafish Tg(Pu.1:GFP) transgenic line, we found that at 10-ss in the ALPM region, Pdc11 was activated in a subset of Pu.1-GFP-positive cells (Fig. 6b). However, this co-expression pattern reduced at 22 hpf and thereafter remained absent, suggesting later silencing Pu.1 expression (Fig. 6b). The expression of Pdc11 in macrophages and neutrophils was also examined using Tg(mpeg1:GFP) and Tg(mpo:GFP) transgenic lines, respectively. Mpeg1-positive macrophages appeared to be closely surrounded by Pdc11-positive cells in the pericardial cavity underneath the brain at 32 hpf, but no colocalizations were detected (Fig. 6c). Similarly, no colocalizations between Pdc11 and mpo-positive neutrophils were detected (Fig. S4d). Intriguingly, in pu.1-deficient embryos, while pdc11 expression in the neuron epithelium remained unchanged, its expression in the pericardial cavity was disappeared, implying that the scattered pdc11-expressing cells observed on the YS were Pu.1-dependent (Fig. 6d). To verify the pdc11 expression pattern, the 1.86 kb genomic region upstream of the translation initiation site of zebrafish pdc11 was cloned into a transposon vector containing EGFP. The pdc11 promoter-driven GFP-expressing cells on the YS strongly colocalized with endogenous Pdc11 at 24 hpf in an immunofluorescence assay (Fig. 6e). Overexpression of Coil under the control of the 1.86 kb pdc11 promoter reversed the macrophage imbalance in pdc11 mutants, with the number of inflammatory macrophages reduced and that of microglia increased (Fig. 6f, g). This rescue effect was not observed in embryos overexpressing Coil overexpression under the control of neuron (Huc), macrophage (Mpeg1), or neutrophil (Mpo) specific promoters (Fig. 6g). Collectively, these results suggest that scattered Pdc11-expressing cells may be derivatives of Pu.1-positive progenitors, but that they are distinct from previously identified neutrophils or macrophages.

**Discussion**

WMH examined by magnetic resonance imaging have been used to describe WM changes related with cognitive dysfunction, stroke injury, cerebral small vessel disease, and neurodegenerative disorders [40]. Key cellular components of the gliovascular unit including astrocytes, oligodendrocytes, pericytes and microglia. It was suggested that microglia within the WM be associated functions like surveillance at the blood–brain barrier and clearance debris [41, 42]. In this study, helped by zebrafish model organism, we found that deficiency of pdc11, a WMH susceptibility gene, led to completely lost of mature microglia in the brain. In mouse, helped by in vivo lineage tracing, it was found that adult microglia derive from primitive myeloid progenitors that arise before embryonic day 8 [43]. However, as with zebrafish, multiple waves of microglia exist and originate from distinct hematopoietic precursors were identified [44]. Despite the different ontogeny of primitive and adult microglia between mouse and zebrafish identified, the regulators underlying the differentiation of EMP progenitors toward microglia remain undefined. TGF-β family members are cytokine required for the maintenance of the microglia-specific homeostatic gene signature. Deletion of the TGF-β receptor in microglia resulted in rapid conversion of microglia toward an inflammatory macrophage phenotype [15, 16]. Mice harboring mutated TGFBI exhibit an acute wasting syndrome followed by death [45]. The most prominent lesions are tissue necrosis in specific organs and multifocal, mixed inflammatory cell infiltration into numerous organs [46]. Despite accumulating evidence for local expression of TGF-β1 in the nervous system during inflammation, the cellular source of TGF-β1, which drives microglia differentiation is unknown. Pdc11-deficient
embryos exhibit brain necrosis and organ inflammatory cell infiltration, which is very similar to what is observed with TGFβ1-mutated mice. Moreover, Pdcd11-expressing cells on the YS colocalized with TGF-β1 in Zebra Experimental model.

**Materials and methods**

**Experimental model**

Zebrafish maintenance and staging were performed as previously described [52]. The zebrafish facility and study were approved by the Institutional Review Board of the Institute of Health Sciences, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China), and the methods were carried out in accordance with the approved guidelines. The Tg(pu.1:GFP) [53], Tg(mpeg1:GFP) [54], Tg(mpo:GFP) [55], P53Δfl(zebra) mutants [20, 56], pu.1Δ899 zebrafish lines were used.

**Pdcd11 knock-out zebrafish line generation**

For CRISPR-Cas9 mediated pdcld11 knock-out zebrafish generation, guide RNA (gRNA) targeting exon4 of pdcld11 (5’-GGTCTCCCCAGCCCCGCTG-3’) was designed using the online tool, ZiFiT Targetere software (http://zifit.partners.org/ZiFiT), and was synthesized by cloning the annealed oligonucleotides into the sgRNA expression vector as previously described [57]. The mutant line is screened by Barbi enzyme (NEB) cutting and confirmed by Sanger sequencing.

**mRNA synthesis, and microinjection**

mMACHINE Kit (Ambion) was used for mRNA transcription. For promoter-driven Coil or smad7 expression in zebrafish, 30 ng/μl transposase mRNA was co-injected.

**Chemical treatment**

PTXF (Abcam) was injected (0.5 ng) into one-cell stage embryos. BMS-345541 (Selleckchem, 10 μM), BAY 11-7821 (Selleckchem,10μM), and LY364947 (Selleckchem, 10 μM) were used for treating embryos from 18 hpf.

**Plasmids**

The Tol2(NFκB:EGFP) plasmid was obtained from Addgene [35]. The 1.86-kb sequence immediately proximal to the pdcld11 5’ untranslated region was amplified and cloned into a modified pT2cfosGW vector containing the zebrafish Tol2 transposable element [35]. The resulting construct was injected, together with transposase mRNA, into one-cell stage WT TU embryos [58]. The full length PDCD11 was cloned into vector pCDNA3.1. The C-coil, C-coil–coil, and C-Ter domain of human PDCD11 were amplified and cloned into vector pCS2+. Full-length human c-Rel, P65, and P105 were cloned into pCS2+ with GFP fused in the C-terminus. The RHD and TAD domain of c-Rel and P65 were amplified from the full-length vector and cloned into pCS2+. Full-length zebrafish tgfβ1a was amplified from zebrafish cDNA and cloned into the pCS2+ vector. Mpeg1 promoter driven C-coil and smad7 were cloned by replacing dendra in vector Tol2 (mpeg1:dendra2) (Addgene, 51462). Tol2(mpX:Coil) was cloned by replacing the Lifeact-Ruby (Cla1 and Sal1) with Coil in Tol2(mpX:Lifeact-Ruby) (Addgene, 45246). Tol2(huc:Coil) was also obtained by gateway cloning using p5E-Huc (Addgene, 72640).
WISH

WISH was performed as previously described [59]. Probes for l-plastin, csf1ra, and mpo were used as previously described [10, 60, 61]. Antisense probes, including il6, il1b, mfp4p4, apoeb, pdcd11, csf1a, ctsba, tgfbl1a, tgfbl1b, ecx3r1.1, and ecx3r3.2 were transcribed in vitro from PCR products amplified from zebrafish cDNA with the T7 transcriptase binding sequence added to the reverse primers. Primers used are shown in Table S2.

Neutral Red, AO, TUNEL, pH3, and immunofluorescence assay

For Neutral Red staining, zebrafish larvae collected at 60 hpf and 3 dpf were soaked in Neutral Red (Sigma-Aldrich, N6264; 2.5 mg/mL) overnight at 28.5 °C. For AO staining, zebrafish embryos recovered at 22 hpf were incubated in 10 μg/mL AO stain (Sigma-Aldrich) dissolved in E3 medium in the dark for ~30 min. TUNEL staining was performed as previously described [62]. pH3 and immunofluorescence staining were carried out using the following antibodies: PDCD11 (Sigma-Aldrich; HPA017924; 1:100); pH3 (Santa Cruz Biotechnology; 1:500), TGF-β1 (Sigma-Aldrich, T0438; 25 μg/mL).

qPCR analysis

After treatment with DNAaseI (Ambion), RNA was prepared using Trizol reagent (Invitrogen, H10522) and then subjected to cDNA synthesis using a cDNA synthesis kit (ABI). Real-time PCR was performed using a Fast Start Universal SYBR® Green Master Rox probe (Roche Applied Science, 13800300) and Mastercycler thermal cycler (Eppendorf, 22331). Primers are summarized in Table S2.

Western blot, nuclear extraction, and co-IP

Zebrafish embryos at the indicated developmental stages were de-yolked and homogenized in lysis buffer mixed with proteinase inhibitor as previously described [63]. Nuclear proteins were extracted by the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, 78833). For the co-IP assay, proteins were purified in RIPA buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 5 mM MgCl2, 0.5% NP40, and Roche cocktail protease inhibitor) 48 h after transfection, and mixed with anti-c-Flag agarose affinity gel antibody (Sigma-Aldrich, A220). After incubation at 4 °C overnight, beads were collected for western blotting. Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting according to standard protocols. The antibodies used were anti-GFP (Sigma-Aldrich, G1544), anti-Flag (Sigma-Aldrich, F7425), anti-PDCD11 (Sigma-Aldrich; HPA017924), anti-P65ser536 (Cell Signaling Technology, 3031), anti-α-Tubulin (Sigma-Aldrich, T9026), anti-β-Actin (Cell Signaling Technology, 8457), and anti-LaminB1 (Cell Signaling Technology, 13435).

Cell culture and dual-luciferase reporter assay

Cells were cultured in a humidified incubator at 37 °C in the presence of 5% CO2. 293T and Hela cells obtained from ATCC were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco). Identity of cell lines were validated by STR analysis. Mycoplasma testing was routinely performed on all cells used in the study, and confirmed to test negative. For cell transfection, Effectene transfection reagent (QIAGEN, 1012829) was used according to the manufacturer’s instructions. NF-kB binding motif driven luciferase reporter and human TGFBI 1 (3.1-kb upstream the transcription start site) was used. C-Coil (150 ng) and/or c-Rel (150 ng) as well as Renilla constructs (5 ng) were transfected into 293 T cells (60%–70% confluence per well in 24-well plates) and luciferase activities were assessed according to the manufacturer’s instructions (Promega). Site mutagenesis was carried on the TGFBI promoter (Invitrogen, A13282).

Cell sorting and cytology

Tg (mpeg1-EGFP) transgenic embryos at 60 hpf were connected and digested with 0.5% trypsin (Gibco) for 15 min at 37 °C to analyze microglia gene signature. Sixty hours post fertilization zebrafish brain was cut before digestion. After centrifugation at 400 × g for 5 min, two washes with PBS, and passage through a 40 μm nylon mesh filter, the single cell suspension was subjected to fluorescence-activated cell sorting using a MoFlo device (DakoCytometry). EGFP positive cells were used for downstream qPCR assay.

Cell IF assay

Cell IF were carried as previously described. Cells grown to 60% confluence on coverslips were first fixed and blocked and then incubated with mouse anti-FLAG (Sigma-Aldrich, F7425). Secondary antibody goat anti-mouse, Alexa Fluor 594 (Invitrogen).

Imaging

Zebrafish IF stain images were taken using an Olympus FV1000 scanning confocal microscope. The confocal images were captured with an UPLSAPO 40× or 60× objective. Immuno-stained cell images were collected using a Leica with an UPLSAPO 60× objective.
RNA-sequencing (RNA-seq)

Total RNA from 22 hpf WT and pcd11 mutants was collected respectively and subjected to RNA-seq. Shanghai Novel Bioinformatics Company provided the deep-sequencing service.

Statistical analysis

For all experiments carried, zebrafish embryos were randomized distributed to each group. The outcome of experiments were assessed according to phenotypic changes. Statistical analyses were conducted using the Student’s t test (two-sided) or log-rank tests. Statistical significance was taken to be $P < 0.05$.

Acknowledgements

We are grateful for Min Deng, Mei Dong, Yi Chen, Yi Jin, Zheng Ruan, Juan Chen, and Yan Zhao for their excellent technical support. This work was supported by grants from National Science Foundation of China (No. 81970098, 81803014 and 81802424), China Postdoctoral Science Foundation (No. 2019M651518).

Author contributions

JZ and RY developed the concept of this study. RY and MZ performed most of the experiments in zebrafish and cell lines. RY, MZ, and MG contributed in vector construction and western blot, RY, and YW helped in cell culture, qPCR and the in situ assays. YZ, WZ provide the pu.1Δflfl zebrafish lines. HY provided the ceb-paΔCA zebrafish line. JZ, RY, SZ, CZ HT, and JZ prepared the draft and final version of the manuscript. All authors reviewed the results and approved the manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare no competing financial interests.

Publisher’s note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access

This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

References

1. Debette S, Markus HS. The clinical importance of white matter hyperintensities on brain magnetic resonance imaging: systematic review and meta-analysis. BMJ. 2010;341:c3666.

2. Fornage M, Chiang YA, O’Meara ES, Psaty BM, Reiner AP, Siscovick DS, et al. Biomarkers of inflammation and MRI-defined small vessel disease of the brain: the Cardiovascular Health Study. Stroke. 2008;39:1952–9.

3. Gouw AA, Seewann A, van der Flier WM, Barkhof F, Rozenmuller AM, Scheltens P, et al. Heterogeneity of small vessel disease: a systematic review of MRI and histopathology correlations. J Neurol Neurosurg Psychiatry. 2011;82:126–35.

4. Wright CB, Moon Y, Paik MC, Brown TR, Rabbani L, Yoshita M, et al. Inflammatory biomarkers of vascular risk as correlates of leukoaraiosis. Stroke. 2009;40:3466–71.

5. Fornage M, Debette S, Bis JC, Schmidt H, Ikram MA, Dufouil C, et al. Genome-wide association studies of cerebral white matter lesion burden: the CHARGE consortium. Ann Neurol. 2011;69:928–39.

6. Hofer E, Cavalieri M, Bis JC, DeCarli C, Fornage M, Sigurdsson S, et al. White matter lesion progression: genome-wide search for genetic influences. Stroke. 2015;46:3048–57.

7. Verhaaren FB, Debette S, Bis JC, Smith JA, Ikram MK, Adams HH, et al. Multiethnic genome-wide association study of cerebral white matter hyperintensities on MRI. Circ Cardiovasc Genet. 2015;8:398–409.

8. Lee J, Hamanaka G, Lo EH, Ari K. Heterogeneity of microglia and their differential roles in white matter pathology. CNS Neurosci Ther. 2019;25:1290–8.

9. Orihuela R, McPherson CA, Harry GM. Microglial M1/M2 polarization and metabolic states, Br J Pharmacol. 2016;173:649–65.

10. Herombel P, Thisse B, Thisse C. Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina, and epididymis through a M-CSF receptor-dependent invasive process. Dev Biol. 2001;238:274–88.

11. Mazzolini J, Le Clerc S, Morisse G, Coulonges C, Kuil LE, van Ham TJ, et al. Gene expression profiling reveals a conserved microglia signature in larval zebrafish. Glia. 2020;68:298–315.

12. Kuil LE, Oosterhof N, Geurts SN, van der Linde HC, Meijering E, van Ham TJ. Reverse genetic screen reveals that Il34 facilitates yolk sac macrophage distribution and seeding of the brain. Dis Model Mech. 2019;12:dmn03762.

13. Wu S, Xue R, Hassan S, Nguyen TML, Wang T, Pan H, et al. Il34-Csf1r pathway regulates the migration and colonization of microglial precursors. Dev Cell. 2018;46:552–563e554.

14. Travis MA, Sheppard D. TGF-beta activation and function in immunity. Annu Rev Immunol. 2014;32:51–82.

15. Lund H, Pieber M, Parsa R, Grommisch D, Ewing E, Kular L, et al. Fatal demyelinating disease is induced by monocyte-derived macrophages in the absence of TGF-beta signaling. Nat Immunol. 2018;19:1–7.

16. Zoller T, Schneider A, Kleimeyer C, Masuda T, Potru PS, Pfeifer D, et al. Silencing of TGFbeta signalling in microglia results in impaired homeostasis. Nat Commun. 2018;9:4011.

17. Derenzini M, Montanaro L, Trere D. Ribosome biogenesis checkpoint and stabilizes p53 independent of increased ribosome content. Cancer Res. 2011;71:126–35.

18. Millis EW, Green R. Ribosomopathies: There’s strength in numbers. Science. 2017;358:aam2755.

19. Morcelle C, Menoyo S, Moron-Duran FD, Tauler A, Kozma SC, Thomas G, et al. Oncogenic MYC induces the impaired ribosome biogenesis checkpoint and stabilizes p53 independent of increased ribosome content. Cancer Res. 2019;79:4348–59.

20. Berghmans S, Murphy RD, Wienholds E, Neuberg D, Kutzok JL, Fletcher CD, et al. t(p53) mutant zebrafish develop malignant peripheral nerve sheath tumors. Proc Natl Acad Sci USA. 2005;102:407–12.

21. Xu J, Wang T, Wu Y, Jin W, Wen Z. Microglia colonization of developing zebrafish midbrain is promoted by apoptotic neuron and lysophosphatidylcholine. Dev Cell. 2016;38:214–22.
60. Crowhurst MO, Layton JE, Lieschke GJ. Developmental biology of zebrafish myeloid cells. Int J Dev Biol. 2002; 46:483–92.
61. Willett CE, Cherry JJ, Steiner LA. Characterization and expression of the recombination activating genes (rag1 and rag2) of zebrafish. Immunogenetics. 1997;45:394–404.
62. Finckbeiner S, Ko PJ, Carrington B, Sood R, Gross K, Dolnick B, et al. Transient knockdown and overexpression reveal a developmental role for the zebrafish enosf1b gene. Cell Biosci. 2011;1:32.
63. Link V, Shevchenko A, Heisenberg CP. Proteomics of early zebrafish embryos. BMC Dev Biol. 2006;6:1.