Role of tumor necrosis factor–alpha in zebrafish retinal neurogenesis and myelination

Xu-Dan Lei, Yan Sun, Shi-Jiao Cai, Yang-Wu Fang, Jian-Lin Cui, Yu-Hao Li

Key Laboratory of Tumor Microenvironment and Neurovascular Regulation, Nankai University School of Medicine, Tianjin 300071, China

Co–first authors: Xu-Dan Lei and Yan Sun

Correspondence to: Yu-Hao Li; Jian-Lin Cui. Key Laboratory of Tumor Microenvironment and Neurovascular Regulation, Nankai University School of Medicine, Tianjin 300071, China. liyuhao@nankai.edu.cn; cuijianlin@nankai.edu.cn

Received: 2015-08-27 Accepted: 2016-02-23

Abstract

• AIM: To investigate the role of tumor necrosis factor–alpha (TNF–α) in zebrafish retinal development and myelination.

• METHODS: Morpholino oligonucleotides (MO), which are complementary to the translation start site of the wild–type embryonic zebrafish TNF–α mRNA sequence, were synthesized and injected into one– to four–cell embryos. The translation blocking specificity was verified by Western blotting using an anti–TNF–α antibody, whole-mount in situ hybridization using a hepatocyte– specific mRNA probe ceruloplasmin (cp), and co–injection of TNF–α MO and TNF–α mRNA. An atonal homolog 7 (athoh7) mRNA probe was used to detect neurogenesis onset. The retinal neurodifferentiation was analyzed by immunohistochemistry using antibodies Zn12, Zpr1, and Zpr3 to label ganglion cells, cones, and rods, respectively. Myelin basic protein (mbp) was used as a marker to track and observe the myelination using whole–mount in situ hybridization.

• RESULTS: Targeted knockdown of TNF–α resulted in specific suppression of TNF–α expression and a severely underdeveloped liver. The co–injection of TNF–α MO and mRNA rescued the liver development. Retinal neurogenesis in TNF–α morphants was initiated on time. The retina was fully laminated, while ganglion cells, cones, and rods were well differentiated at 72 hours post–fertilization (hpf). mbp was expressed in Schwann cells in the lateral line nerves and cranial nerves from 3 days post–fertilization (dpf) as well as in oligodendrocytes linearly along the hindbrain bundles and the spinal cord from 4 dpf, which closely resembled its endogenous profile.

• CONCLUSION: TNF–α is not an essential regulator for retinal neurogenesis and optic myelination.

• KEYWORDS: tumor necrosis factor–alpha; retina; neurogenesis; myelination; zebrafish

DOI:10.18240/ijo.2016.06.07

INTRODUCTION

Tumor necrosis factor–alpha (TNF–α) is a pleiotropic inflammatory cytokine that is chiefly produced by activated macrophages. TNF–α is a general signal produced by apoptotic neurons that initiates Müller glial proliferation through the Ascl1a and STAT3 proteins in the damaged zebrafish retina [1]. TNF–α mediates de– and re-myelination. On the one hand, TNF–α is up–regulated during demyelination and may act as a primary neurotoxin in progressive forms of multiple sclerosis (MS)[2,3]. On the other hand, TNF–α depletion leads to a significant delay in remyelination, which suggests that it also has a reparative role in oligodendrocyte proliferation and regeneration[4]. As in vertebrates, the zebrafish retina differentiates from a sheet of neuroepithelial cells that then develops in a programmed spatiotemporal pattern to produce the mature laminated retina. The retina has a limited number of cell types arranged in evolutionarily and highly conserved spatial patterns and functional circuits. Experimental alterations in retinal development are, therefore, easily visualized microscopically [5]. The myelin sheath is the membrane structure that protects, supports, and nourishes axons. The myelin structure is formed by oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). The myelin structure, myelin synthesis, and gene expression patterns are highly conserved between zebrafish and mammals[6]. Therefore, the zebrafish is an ideal model for investigating the mechanisms that control neurogenesis and myelination.

In this study, embryonic and larval zebrafish were used. The translation of TNF–α gene was successfully inhibited to generate a zebrafish model for evaluating the effects of TNF–α in neurogenesis, neurodifferentiation and myelination. Our study will contribute to comprehensive understanding the role of TNF–α in the neural development.
Tumor necrosis factor–alpha and retinal development

MATERIALS AND METHODS

Experimental Animals Wild-type AB zebrafish were maintained in the Zebrafish Research Center at Nankai University. Embryos and larvae were incubated with E3 medium (pH 7.2) under a 14/10-h light/dark cycle at 28.5°C [10]. All animal protocols were approved by the Nankai University Animal Care and Use Committee and were in compliance with Chinese Association for Laboratory Animal Sciences guidelines.

Morpholino Oligonucleotides, RNA Synthesis, and Microinjections Morpholino oligonucleotides (MO; Gene Tools, LLC, Philomath, OR, USA) used in this study were either complementary to the translation start site of the zebrafish TNF-α (GenBank NM_212859) or containing a 5-base mismatch. The sequences were: TNF-α MO, 5'-AA AGCGCCCGACTCTCAAGCTTCAT-3' (antisense start codons underlined); TNF-α mismatch control (MM), 5'-AA AcCcCCcCACTCTGacAACCCTCAT-3' (mismatched bases underlined). Both TNF-α MO and TNF-α MM were suspended in 1× Danieau’s solution (58 mmol/L NaCl, 0.7 mmol/L KCl, 0.4 mmol/L MgSO4, 0.6 mmol/L Ca(NO3)2, 5 mmol/L HEPES; pH 7.1-7.3) at a concentration of 1 ng/µL. Embryos were injected with 4 ng TNF-α MO or TNF-α MM at one- to four-cell stage, respectively [81].

For the mRNA rescue injections, TNF-α full-length coding sequences were subcloned into a pCS2 vector. Next, mRNA was synthesized using an SP6 mMESSAGE mMACHINE kit (Thermo Fisher Scientific, Waltham, MA, USA). Embryos were co-injected with 10 pg of TNF-α mRNA and 4 ng TNF-α MO at the one- to four-cell stage.

Western Blot Analysis At 72 hours post-fertilization (hpf), Western blot was performed as described previously [12]. A polyclonal anti-TNF-α (1:500; AnaSpec, Fremont, CA, USA) was used as the primary antibody in this study. An anti-GAPDH (1:3000; Millipore, Billerica, MA, USA) was used as a loading control.

Enzyme–linked Immunosorbent Assay An enzyme-linked immunosorbent assay (ELISA) was carried out to quantify the TNF-α expression among 50 embryos from each group (uninjected, mismatch control, TNF-α morphant and TNF-α rescue) at 72 hpf. Samples were prepared according to the manufacturer’s protocol. TNF-α content was measured using a TNF-α Mouse ELISA Kit (ab100747; Abcam, Cambridge, MA, USA). The ELISA detection protocol described above was repeated three times.

Whole-mount in situ Hybridization 1-phenyl-2-thiourea (PTU, Sigma) was used on the embryos or larvae to block pigmentation at a final concentration of 0.003% until 96 hpf. Whole-mount in situ hybridization was performed according to a standard protocol [13-14]. Hepatocytes were specifically labeled using a ceruloplasmin (cp; GenBank NM_131802) mRNA probe. An atonal homolog 7 (ato7; GenBank NM_131632) mRNA probe was used as a marker to explore the retinal neurogenesis. Schwann cells and oligodendrocytes were labeled using an mRNA probe for myelin basic protein (mbp; GenBank AY860977). Probes were added to Eppendorf tubes at a concentration of 2 ng/µL.

Immunohistochemistry and Whole Mount Immunostaining Immunohistochemistry was performed using standard procedures [14]. Three primary antibodies were used in this study: Zn12, Zpr1 and Zpr3 (all diluted at 1:200; Zebrafish International Resource Center, Eugene, OR, USA). A fluorescent labeled Cy3 (diluted at 1:500, Millipore) was used as the secondary antibody. The nuclei were counterstained by 4’,6-diamidino-2-phenylindole (DAPI, diluted at 1:1000; Sigma).

Real-time Quantitative Polymerase Chain Reaction At 4 days post-fertilization (dpf), total RNA was extracted from 20 larvae in each group (uninjected, mismatch control, and TNF-α morphant) using TRIZOL according to the manufacturer’s protocol (Life Technologies, Carlsbad, CA, USA). Total RNA was then reverse-transcribed by M-MLV reverse transcriptase (Promega, Madison, WI, USA) using oligo (dT) primers. qRT-PCR was performed using the SYBR Green Labeling System (Promega). Reaction procedures included a denaturing step at 95°C for 5 min followed by 40 cycles of 95°C for 15s, 55°C for 20s and 72°C for 30s. Primer sequences included the following: mbp (GenBank AY860977), forward 5’-GGGCAGAAAGAAGAAGGC-3’, reverse 5’-CGGGTGGAAGAGTGGTG-3’; actin (GenBank AY222742), forward 5’-TTCACCACACAGCGGAAG-3’, reverse 5’-TACCGCAAGATTCCATACCCA-3’.

The qRT-PCR experiment described above was repeated three times.

Photography and Image Analysis Images of immunohistochemistry were captured with an FV 1000 confocal microscope (Olympus, Japan). Images of whole-mount in situ hybridization were photographed with a DP72 digital camera mounted on an SZX16 fluorescence dissecting microscope (Olympus). Images of the Western blots were converted to eight-bit grayscale and then performed densitometric analysis using Image J software (1.42X; NIH, http://rsb.info.nih.gov/ij/). The ratio between the area of TNF-α and to area of GAPDH was calculated in uninjected, mismatch, and TNF-α morphant groups, respectively.

RESULTS

Knockdown of Tumor Necrosis Factor–alpha The translation of the TNF-α gene was inhibited by an injection of the TNF-α-targeted MO. At 72 hpf, the expression of TNF-α protein was specifically reduced (Figure 1A, 1B). The gross development in TNF-α morphants was similar to those in uninjected and mismatch embryos at 72 hpf. In zebrafish, cp mRNA is expressed in early hepatocytes from 32-34 hpf and considered as a specific marker of developing...
Figure 1 Embryonic phenotype and liver development following TNF-α knockdown at 72 hpf

A: The Western blotting resultsof the TNF-α antibody at 72 hpf. B: TNF-α protein expression was significantly suppressed in the TNF-α-morphant (MO) embryos. C: The gross development of uninjected (UI), mismatch control (MM) and MO embryos. MO embryos showed no apparent morphological change. D: The quantification of TNF-α protein expression in embryos from UI, MO, MM and TNF-α-rescue (MO+mRNA) groups by ELISA. Note that the TNF-α is significantly decreased in MO group (ANOVA, *P<0.05). E-H: Whole-mount in situ hybridization with the riboprobe cp. Compared to the uninjected (E, arrowhead) and mismatch control (F, arrowhead), the TNF-α morphant showed a severely underdeveloped liver (G, asterisk). Note the restoration of liver development in the rescue embryos (H). Dorsal is up and rostral is left in C and E-H. Scale bar (E-H)=200 μm.

Therefore, we verified the targeted knockdown of TNF-α by cp whole-mount in situ hybridization. Compared to the uninjected (Figure 1E, arrowhead) and mismatch control (Figure 1F, arrowhead), the TNF-α morphant showed a severely underdeveloped liver that was almost undetectable at 72 hpf (Figure 1G, asterisk). To further prove the specificity of TNF-α knockdown, TNF-α mRNA was used for the rescue experiment. We co-injected the TNF-α MO with TNF-α mRNA into embryos and quantified the expression level of TNF-α protein by ELISA at 72 hpf. Following TNF-α knockdown, the TNF-α was significantly decreased (Figure 1D; ANOVA, P<0.05). The liver development was again analyzed by in situ hybridization with the cp probe. The co-injection of TNF-α MO and mRNA restored the liver to a size comparable to that of the uninjected embryos (Figure 1H, arrowhead). Taken together, these results indicate that TNF-α -MO injection (4 ng) was able to specifically knockdown TNF-α.

Expression of Myelin Basic Protein and Myelination in the Nervous System

Myelin basic protein is one of the main protein components of the myelin sheath which is specifically expressed in oligodendrocytes in the CNS and Schwann cells in the PNS. Therefore, we used mbp as a marker to assess the myelination in TNF-α morphants. In the PNS, mbp was expressed strongly in Schwann cells linearly along the lateral line of the trunk in uninjected (Figure 3A, 3D, arrowheads), mismatch control (Figure 3B, 3E, arrowheads), and TNF-α morphant (Figure 3C, 3F, arrowheads) embryo at 3 dpf. The mbp mRNA was also detected in the cranial nerves (Figure 3, arrows) and the distribution of mbp -expressing cells shared a unanimous pattern in embryos from uninjected (Figure 3A, 3D),
mismatch control (Figure 3B, 3E), and TNF-α morphant (Figure 3C, 3F) groups. By 4 dpf, more mbp-expressing Schwann cells were found along the lateral line nerves (Figure 4A-4C, arrowheads) and cranial nerves (Figure 4A-4C, arrows) in larvae from all the three groups. In the CNS, the mbp-expressing cells were detected symmetrically along the hindbrain bundles as well as the lateral spinal cord (Figure 4A-4C, open arrowheads), which matched the location of myelinated axons at this stage. Quantification of mbp mRNA revealed that the mbp expression was similar in larvae from the uninjected, mismatch control, and TNF-α morphant groups at 4 dpf (Figure 4D). No significant difference was found in myelination among all the three groups. These findings suggest that the axons are myelinated in the PNS and CNS following TNF-α knockdown.

**DISCUSSION**

TNF-α mediates a broad range of cellular activities, including proliferation, survival, differentiation, and apoptosis, and is considered essential for the induction and maintenance of the inflammatory immune response24-25. Here we used a TNF-α -targeted MO to inhibit TNF-α gene translation. The specific knockdown was verified in three
Figure 3 Expression of mbp mRNA in Schwann cells of the PNS at 3 dpf A-F: Images of mbp mRNA expression in embryos from uninjected (UI; A and D), mismatch control (MM; B and E) and TNF-α morphant (MO; C and F) groups at 3 dpf. Note that the mbp-expressing cells of the TNF-α morphants were distributed linearly along the lateral line nerves (arrowheads) and cranial nerves (arrows). A-C: The dorsal view. Dorsal is up and rostral is left in D-F. Scale bar=200 μm.

Figure 4 mbp expression in Schwann cells and oligodendrocytes using whole-mount in situ hybridization at 4 dpf A-C: Dorsal view of mbp mRNA expression in larvae from uninjected (UI; A), mismatch control (MM; B), and TNF-α morphant (MO; C) groups at 4 dpf. More mbp-expressing Schwann cells were found linearly along the lateral line nerves (arrowheads) and in cranial nerves (arrows). In the CNS, mbp-expressing oligodendrocytes were distributed symmetrically along the hindbrain bundles and lateral spinal cord (open arrowheads). D: The relative expression of mbp mRNA in the three groups at 4 dpf. Scale bar (A-C)=200 μm.

ways. First, Western blotting showed that the injection of TNF-α MO resulted in specific suppression of TNF-α protein expression in the TNF-α morphants at 72 hpf (Figure 1A, 1B). Second, a severely underdeveloped liver was verified by whole-mount in situ hybridization with the hepatocyte-specific mRNA probe cyp. The pro-inflammatory cytokine TNF-α is a key regulator of liver homeostasis in vertebrates and required for liver development in zebrafish. MO knockdown of TNF-α reportedly resulted in defective hepatocyte proliferation and reduced liver size. In uninjected and mismatch control animals, cyp was expressed specifically and strongly in the liver at 72 hpf (Figure 1E, 1F). After TNF-α knockdown, the liver was severely underdeveloped (Figure 1G), whereas the embryonic phenotypes remained similar to those of the uninjected and mismatch controls (Figure 1C). Third, TNF-α MO and TNF-α mRNA were co-injected to test whether liver development was rescued. The expression of TNF-α was significantly decreased in TNF-α morphants; following co-injection, the TNF-α expression level increased, although it is still slightly lower than the expression in uninjected and mismatch controls (Figure 1D). Moreover, the liver size was
Tumor necrosis factor–alpha and retinal development

restored (Figure 1H). These results indicate that TNF-α knockdown was successful, creating a model to evaluate the role of TNF-α in retinal development and myelination. Similar to vertebrates, the zebrafish retina differentiates from neuroepithelium. The neurogenesis in zebrafish retina is initiated in a small and discrete patch which is close to the optic stalk. Then the retina develops in a spatiotemporal pattern. Therefore, zebrafish retina is a suitable model to investigate neurogenesis and neurodifferentiation. \(^{28-30}\) atoh7 is expressed in ganglion cells immediately after they exit from the mitosis around 28 hpf. At 48 hpf, the retina starts to laminate while most neurons in the inner nuclear layer become differentiated. The cells in the outer nuclear layer begin to differentiate 10h later; photoreceptors, including rods and cones, are well-developed at 72 hpf. \(^{31}\) In situ hybridization revealed that atoh7 expression matched that described previously at 28 hpf in un.injected, mismatch control, and TNF-α morphant retinas\(^{[32]}\) (Figure 2A-2C). At 72 hpf, the ganglion cell, inner nuclear, and outer nuclear layers were fully laminated. Differentiated ganglion cells, cones, and rods were present in retinas from TNF-α morphants (Figure 2F, 2I, 2L). Therefore, following TNF-α knockdown, the differentiation of ganglion cells was initiated as scheduled. Also, no disruption was found in the differentiation of ganglion cells, cones and rods. We believe that TNF-α may not be essential for the regulation of neurogenesis and differentiation in the zebrafish retina. The gene expression patterns, myelin structure and myelin synthesis in zebrafish are very similar to mammals. At 2 dpf, a relatively loose structure is appeared first. Then the myelin sheath is formed at 4 dpf. After 3d, the myelin structure becomes compact by the tunica vaginalis. Furthermore, most myelin-associated genes in mammals have their homologies in zebrafish, such as mhp, sox10, etc.\(^{[33-34]}\). Myelin basic protein, a highly conserved protein between zebrafish and mammals, was expressed in oligodendrocyte lineage cells. In the present study, an mhp mRNA probe was used to track the myelination. In the PNS, TNF-α morphants showed mhp-positive signals at 3 dpf in Schwann cells along lateral line and cranial nerves (Figure 3C). These signals increased in strength with the developmental period (Figure 4C). In the CNS, axons started myelination later. Until 4 dpf, mhp-positive signals were detected in the oligodendrocytes along the hindbrain bundles and the lateral spinal cord in TNF-α morphants (Figure 4C), corresponding to the location of myelinated axons from the larvae of uninjected and mismatch controls.\(^{[35]}\) Also at 4 dpf, no significant difference was detected in mhp mRNA expression among all the three groups (Figure 4D). Therefore, in the PNS and CNS, the distribution of mhp-positive signals was spatiotemporally consistent with the formation of zebrafish myelin under physiological conditions\(^{[36]}\). MS is an autoimmune disease as well as the most common demyelinating disease caused by a combination of genetic susceptibility and environmental factors. During clinical treatment, some patients undergo partial remyelination, especially during the early disease stages \(^{[37-38]}\). Our findings may partially explain why TNF-α plays a conflicting role in MS and why use of the monoclonal anti-TNF-α antibody was ineffective in MS clinical trials \(^{[39]}\). TNF-α in the CNS is important for oligodendrocyte regeneration. However, our results show that TNF-α has little effect on myelination. Therefore, TNF-α may not directly govern myelination and is probably downstream of other key molecules.

ACKNOWLEDGEMENTS

The abstract of this article was published as a meeting abstract in Invest Ophthalmol Vis Sci 2015;56(7):1494.

Foundations: Supported by the National Natural Science Foundation of China(No.81301080); the Tianjin Natural Science Foundation (No.15JCYBJC24400, No.15JQNCJ10900); the Scientific Research Foundation for the Returned Overseas Chinese Scholars (No.2012-1707).

Conflicts of Interest: Lei XD, None; Sun Y, None; Cai SJ, None; Fang YW, None; Cui JL, None; Li YH, None.

REFERENCES


