INTRODUCTION

Most of the axons in the vertebrate nervous system are surrounded by a lipid-rich membrane called myelin, which promotes rapid conduction of nerve impulses and protects the axon from being damaged. Multiple sclerosis (MS) is a chronic demyelinating disease of the CNS characterized by infiltration of immune cells and progressive damage to myelin and axons. One potential way to treat MS is to enhance the endogenous remyelination process, but at present there are no available treatments to promote remyelination in patients with demyelinating diseases.

Sulfasalazine is an anti-inflammatory and immunomodulating drug that is used in rheumatology and inflammatory bowel disease. Its anti-inflammatory and immunomodulatory properties prompted us to test the ability of sulfasalazine to promote remyelination. In this study, we found that sulfasalazine promotes remyelination in the CNS of a transgenic zebrafish model of NTR/MTZ-induced demyelination. We also found that sulfasalazine treatment reduced the number of macrophages/microglia in the CNS of demyelinated zebrafish larvae, suggesting that the acceleration of remyelination is mediated by the immunomodulatory function of sulfasalazine. Our data suggest that temporal modulation of the immune response by sulfasalazine can be used to overcome MS by enhancing myelin repair and remyelination in the CNS.

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and immune-modulating drug that is mainly used in rheumatology and inflammatory bowel disease (Dale et al., 2007; Plosker and Croom, 2005; Zenlea and Peppercorn, 2014). Its mode of action remains elusive; however, previous reports have shown that sulfasalazine induces immunosuppressive prostaglandins, reduces the synthesis of reactive oxygen compounds, and stabilizes lipid membranes of the cell (Hoult, 1986; Peppercorn, 1984). Its anti-inflammatory and immunomodulatory properties led us to test whether sulfasalazine promotes remyelination, and we found that sulfasalazine promotes oligodendrocyte regeneration and subsequent remyelination in the CNS of a transgenic zebrafish model of NTR/MTZ-induced demyelination. Sulfasalazine treatment did not affect the proliferation of neural precursors in the CNS of wild-type embryos but promoted its proliferation in the CNS of demyelinated zebrafish larvae. Finally, we found that sulfasalazine treatment reduced the number of CNS macrophages/microglia in the CNS of demyelinated zebrafish larvae, suggesting that promotion of remyelination is mediated by the immunomodulatory functions of sulfasalazine.

MATERIALS AND METHODS

Animals
The zebrafish embryos used in this study were Tg(olig2:egfp)vy12 (Shin et al., 2003), Tg(mbp:gal4-vp16;uas:nsfB-mcherry) (Chung et al., 2013), Tg(claudink:gal4-vp16;uas:nsfB-mcherry) (Munzel et al., 2012), and Tg(uas:egfp) (Asakawa and Kawakami, 2008). Embryos were staged according to “days post-fertilization” (dpf) in “hours post-fertilization” (hpf) and morphological criteria (Kimmel et al., 1995).

Metronidazole and sulfasalazine treatment
Metronidazole (Mtz, M1547, Sigma) was dissolved in EM containing 0.2% DMSO to 10 mM as previously described (Chung et al., 2013). For the ablation of myelinating glia, Tg(mbp:gal4-vp16;uas:nsfB-mcherry) and Tg(claudink:gal4-vp16;uas:nsfB-mcherry) larvae were incubated in 10 mM Mtz for 72 h starting at 5 dpf. For sulfasalazine (Sigma) treatment, transgenic larvae were rinsed at 7 dpf in embryo medium (EM; 15 mM NaCl, 0.5 mM KCl, 1 mM CaCl2, 1 mM MgSO4, 0.15 mM KH2PO4, 0.05 mM NaH2PO4, 0.7 mM NaHCO3) and incubated in EM containing 10 μM sulfasalazine for 1 day.

BrdU labeling and immunohistochemistry
Manually-dechorionated embryos were labeled with BrdU (Roche) by incubating them for 1 h 30 min on ice in a solution of 10 mM BrdU and 15% DMSO in EM at 8 dpf. The embryos were then placed in EM, incubated for 1 h at 28.5°C, and fixed using 4% paraformaldehyde in PBS. Embryos were processed for immunohistochemistry, treated for 20 min with 2 M HCl, and were then processed for anti-BrdU immunohistochemistry. For immunohistochemistry, we used the following primary antibodies: mouse anti-BrdU (G3G4, 1:250, Developmental Studies Hybridoma Bank), rabbit anti-Sox10 (1:800, Lifespan bioscience), rat cd11b (1:100, BD bioscience), mouse anti-sodium channel (1:500, Sigma), and mouse anti-acetylated tubulin (1:1000, Sigma). Alexa 488-, 568-, 647-conjugated secondary antibodies were used for fluorescence detection (1:500, Molecular Probe). For histology section, embryos were embedded in 1.5% agar/5% sucrose. Sections (10 μm) were obtained by using a cryostat microtome (Zeiss).

TEM imaging
Tissues were prepared using standard procedures for TEM as...
described previously (Kim et al., 2002). Briefly, zebrafish larva were anesthetized with Tricaine (Sigma) and fixed with 10% paraformaldehyde/2.5% glutaraldehyde/0.1 M phosphate buffer, pH 7.4. Then larva were postfixed in 1% osmium tetroxide, dehydrated, embedded in Eponate-12 resin (Ted Pella). 1-μm-thick sections were obtained using Reichert-Jung Ultracut E ultramicrotome (Leica), stained with toluidine blue, and imaged using a Carl Zeiss Axio microscope. 60-nm-thick sections per block were collected on Formvar-coated slot grids and stained with uranyl acetate/lead citrate, and then recorded on an H-7500 TEM (Hitachi) (80 kV).

**Stab wound**

Adult brain injury was performed as described previously (Marz et al., 2011). Adult zebrafish were anesthetized with Tricaine (Sigma) and a BD U-100 insulin syringe (BD bioscience) was inserted vertically through the skull into the telencephalic region. Then brain damaged fish were transferred into a tank with fresh fish water and fixed in 4% paraformaldehyde at 4-day post injury.

**RESULTS**

**Demyelination by targeted ablation of oligodendrocytes and Schwann cells in the zebrafish nervous system**

To investigate remyelination in vivo, we previously generated an animal model of demyelination using a transgenic zebrafish system in which a bacterial cytotoxin gene, nfsB, which encodes a nitroreductase (NTR) enzyme that can convert prodrugs such as metronidazole (MTZ) into cytotoxins, is expressed in oligodendrocytes under the control of the mbp promoter (Chung et al., 2013).

To examine the effectiveness of the NTR/MTZ system at inducing demyelination in the CNS and PNS via drug-dependent ablation of oligodendrocyte lineage cells, we first crossed Tg(mbp:gal4-vp16) driver lines with Tg(uas:egfp;uas:nfsB-mcherry) fish to generate Tg(mbp:gal4-vp16;uas:gfp;uas:nfsB-mcherry) larvae and exposed bitransgenic larvae to the prodrug, MTZ, at 5 dpf. In the absence of MTZ, bitransgenic larvae do not induce ablation of myelinating glial cells; thus, oligodendrocytes in the spinal cord (Fig. 1A) and Schwann cells in the posterior lateral line (PLL) (Fig. 1C), which express both GFP and nfsB-mCherry, are clearly visible in the Tg(mbp:gal4-vp16;uas:gfp;uas:nfsB-mcherry) larvae. However, exposure of 5 dpf Tg(mbp:gal4-vp16;uas:gfp;uas:nfsB-mcherry) larvae to 10 mM MTZ for three days caused ablation of most of the nfsB-mCherry+ oligodendrocytes. Thus transgenic larvae showed several dying cells with fuzzy red fluorescence in the spinal cord (Fig. 1B, arrows) and red fluorescent cellular debris in the PLL (Fig. 1D, arrows). To confirm demyelination in the spinal cord of transgenic zebrafish, we performed transmission electron microscopy (TEM) imaging. After MTZ treatment for three days, myelin debris was present surrounding Mauthner axons of Tg(mbp:gal4-vp16;uas:gfp;uas:nfsB-mcherry), and the Mauthner axon structure was disrupted following myelin breakdown (Figs. 1F and 1H, arrows). However, wild-type larvae showed compact myelin structure around Mauthner axons and neighboring axons in the ventral spinal cord (Figs. 1E and 1G). Altogether, these data indicate that NTR/MTZ transgenic system efficiently induced demyelination in zebrafish nervous system.

**Sulfasalazine promotes regeneration of myelinating glial cells and remyelination in the demyelinated zebrafish nervous system**

To investigate whether sulfasalazine promotes remyelination in the demyelinated zebrafish nervous system, Tg(mbp:gal4-vp16;uas:gfp;uas:nfsB-mcherry) larvae were first exposed to MTZ at 5 dpf for two days to induce demyelination by ablation.
of oligodendrocytes and Schwann cells. After ablation by MTZ treatment, larvae were washed out to remove MTZ and raised in EM and EM-sulfasalazine media separately to compare remyelination efficiency. After MTZ treatment, the number of GFP-expressing oligodendrocytes and Schwann cells was significantly reduced in Tg(mbp:gal4-VP16;uas:nfsB-mcherry;uas:gfp) larvae (Figs. 2B, 2F, 2I, and 2J) compared with DMSO-treated wild-type larvae (Figs. 2A and 2E), but the number of oligodendrocytes and Schwann cells recovered slightly in the MTZ-treated larvae after one day of recovery time (Figs. 2C, 2G, 2I, and 2J). Interestingly, following sulfasalazine treatment, the number of oligodendrocytes and Schwann cells recovered to levels similar to those of control larvae after one day of recovery, indicating that sulfasalazine promotes regeneration of oligodendrocytes and Schwann cells (Figs. 2D, 2H, 2I, and 2J).

Next, we investigated whether sulfasalazine treatment promotes remyelination following the regeneration of myelinating glial cells in the CNS and PNS. Tg(mbp:gal4-VP16;uas:nfsB-mcherry) larvae were used to test CNS remyelination and Tg(claudinK:gal4-VP16;uas:megfp;uas:nfsB-mcherry) larvae were used to test PNS remyelination. In the ventral spinal cord of DMSO-treated control transgenic larvae, anti-acetylated tubulin antibody labeling shows Mauthner axons surrounded by intact myelin sheath, marked by mbp:GFP fluorescence (Fig. 3A, arrow), and posterior lateral line (PLL) axon fibers surrounded by intact ClaudinK:mGFP fluorescence in the PNS (Fig. 3E, arrow). To compare remyelination efficiency quantitatively, we measured g-ratio, defined as the ratio of the inner axonal diameter to the outer myelinated axonal diameter. Therefore high g-ratio value (≥1) means demyelinated state of axons. After MTZ treatment, GFP+ myelin sheath surrounding Mauthner axons and PLL axon fibers were disrupted following oligodendrocyte and Schwann cell ablation (Figs. 3B and 3F, arrows). However, sulfasalazine treatment promoted remyelination of Mauthner axons (Figs. 3D and 3I) and PLL axon fibers (Figs. 3H and 3J) compared with the transgenic larvae without sulfasalazine treatment (Figs. 3C and 3G), indicating that sulfasalazine promoted remyelination following oligodendrocyte and Schwann cell regeneration in the CNS and PNS.

To investigate whether the regenerated myelinating glial cells in the sulfasalazine-treated larvae reconstructed a functional myelin sheath in the nervous system, we asked if the nodes of Ranvier structure reformed in the regenerated myelin sheath. To do so, we used an antibody against sodium channels (anti-panNavCh, NaCh), which is known to label node structures in axons (Voas et al., 2007). After immunohistochemistry with a NaCh antibody, we observed that NaCh+ node structures were regularly located in the PLL axons of the control larvae (Fig. 4A, arrows). However, sulfasalazine-treated larvae showed an increased number of NaCh+ node structures compared with the control larvae that recovered without sulfasalazine treatment, indicating that sulfasalazine treatment promoted remyelination with functional node structure formation (Figs. 4C-4E, arrows).
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Sulfasalazine promotes precursor proliferation and modulates the immune response
Our previous data showed that sulfasalazine treatment promoted regeneration of oligodendrocytes and Schwann cells and promoted subsequent remyelination in the NTR/MTZ-induced demyelination model. Therefore, we investigated the underlying mechanisms for the promotion of remyelination by sulfasalazine. To test whether sulfasalazine promoted proliferation of spinal cord precursors and thus the formation of oligodendrocyte precursor cells (OPCs) under normal conditions, we exposed wild-type Tg(olig2:egfp) embryos to DMSO and sulfasalazine (S).
sulfasalazine at 24 hpf, prior to OPC specification, and subsequently exposed the embryos to BrdU and performed immunohistochemistry with an anti-BrdU antibody at 36 hpf to detect proliferation and with an anti-Sox10 antibody at 48 hpf to detect OPCs. Since Tg(olig2: egfp) zebrafish express EGFP under the control of the olig2 promoter, EGFP+ cells in this transgenic zebrafish mark neural precursors for motor neuron and oligodendrocyte formation (Shin et al., 2003). Anti-BrdU antibody labeling of the spinal cord sections showed that the number of BrdU+ proliferating precursors is similar between the DMSO-treated control (Fig. 5A, cont: 1.541 ± 1.382 per sections) and sulfasalazine treated embryos (Fig. 5B, sulfasalazine: 1.472 ± 1.133 per sections), indicating that sulfasalazine treatment did not promote precursor proliferation in the wild-type CNS. Consistent with these data, the spinal cords of sulfasalazine-treated embryos showed a similar number of Sox10+ OPCs compared with that of DMSO-treated control embryos (Figs. 5C and 5D, cont: 2.812 ± 1.108, sulfasalazine: 2.65 ± 1.136 per sections).

Next, we exposed Tg(mbp:gal4-vp16;uas:gfp;uas:nfsB-mcherry) larvae to DMSO, MTZ alone, and MTZ in combination with sulfasalazine at 5 dpf for three days to induce demyelination, and after that, we treated demyelinated transgenic larvae with BrdU at 8 dpf to test whether sulfasalazine promoted precursor proliferation during remyelination process. As shown in Fig. 5, sulfasalazine treatment in combination with MTZ increased the number of Brdu+ proliferating precursor cells compared with that of the transgenic larvae treated with MTZ alone (Figs. 5F-5H), indicating that sulfasalazine treatment promotes precursor proliferation under the demyelination status.

In the CNS of MS patients and in animal models of MS, substantial infiltration of macrophages and activation of microglia are observed and have been shown to be associated with disease severity (Lucchinetti et al., 2000; Prineas and Wright, 1978). In addition, inhibition of macrophage/microglia activation or a reduction in their density suppressed and attenuated progression of experimental autoimmune encephalomyelitis (EAE), a common immune-mediated animal model of MS (Bhasin et al., 2007; Heppner et al., 2005; Martiney et al., 1998). Altogether, these studies suggest that macrophages/microglia play a detrimental role in the remyelination process. Because sulfasa-

![Figure 6](http://molcells.org)

**Fig. 6.** Sulfasalazine modulates immune response by inhibiting recruitment of macrophages/microglia in the injured forebrain and demyelinated CNS. (A-D) Transverse sections of the injured telencephalic region of the three-month-old adult fish (dorsal at the top). Brain sections of the DMSO- (A, B) and sulfasalazine-treated (C, D) adult fish were labeled with anti-CD11b antibody to detect macrophages/microglia (red color) at 4 dpf (days-post-lesion). (B, D) Higher magnification images of the circled areas in (A) and (C). (E) Quantification of the number of CD11b+ macrophages/microglia at the stab lesion site. Data were obtained from each of five adult fish treated with DMSO and sulfasalazine, respectively. Error bars represent s.d. (**p < 0.01). (F-J) Dorsal views of the brain of Tg(mbp:gal4-vp16;uas:nfsB-mcherry;uas:gfp) larvae to DMSO, MTZ alone, and MTZ in combination with sulfasalazine at 5 dpf for three days to induce demyelination. Brain sections of the DMSO- (F) and MTZ (G) at 5 dpf for two days and recovered for one day in EM (H) and sulfasalazine solution (I). (J) Quantification of the number of apoe+ microglia in the brain. Data were obtained from each of the five larvae. Error bars represent s.d. (***p < 0.001, *p < 0.05). Scale bars: 200 μm in (A, B), 50 μm in (E-H).
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Sulfasalazine is thought to be an anti-inflammatory and immune-modulating drug (Hoult, 1986; Peppercorn, 1984), we hypothesized that sulfasalazine treatment promotes remyelination process by modulating the behavior macrophages/microglia in the demyelination animal model.

To test this idea, we first induced injury in the forebrain of adult zebrafish and treated the injured brain with either DMSO or sulfasalazine. Next, we compared the number of macrophages/microglia in the injured forebrain of DMSO- and sulfasalazine-treated adult zebrafish following labeling with an anti-CD11b antibody, which marks macrophages/microglia in zebrafish (Fantin et al., 2010). In the injured forebrain of sulfasalazine-treated adult zebrafish, the number of anti-CD11b-positive macrophages/microglia clearly decreased around the lesion site (Figs. 6C-6E) compared with that in the DMSO-treated control zebrafish forebrain (Figs. 6A and 6B), indicating that sulfasalazine treatment reduced the number of macrophages/microglia in the injured forebrain.

To test whether sulfasalazine modulates macrophages/microglia in the demyelination animal model, we next labeled Tg(mbp:gal4-VP16;uas:gp;uas:nfsB-mCherry) larvae by fluorescent in situ RNA hybridization with an apoE mRNA probe, which was used to label macrophages/microglia (Tedde et al., 2008). After demyelination by MTZ treatment at 5 dpf for three days, the number of macrophages/microglia markedly increased in the brain of MTZ-treated Tg(mbp:gal4-VP16;uas:gp;uas:nfsB-mCherry) larvae compared with that in the DMSO-treated control, indicating that ablation of oligodendrocytes and subsequent demyelination recruited macrophages/microglia to the CNS (Figs. 6F and 6G). Interestingly, after sulfasalazine treatment, the number of macrophages/microglia are dramatically reduced in the brain of the MTZ-induced demyelination model compared with that of transgenic larvae that is recovered without sulfasalazine treatment (Figs. 6G-6I), indicating that sulfasalazine treatment reduced macrophages/microglia in the demyelinated brain. Altogether, these data indicate that promotion of oligodendrocyte regeneration and subsequent remyelination by sulfasalazine might be mediated by the immunomodulatory functions of sulfasalazine.

DISCUSSION

Macrophages are myeloid cells derived from hematopoietic stem cells in the bone marrow and circulate the peripheral vasculature to survey tissue environments for damage or infection (Miron and Franklin, 2014). Microglia are the resident macrophages in the CNS and are generated from erythromyeloid precursors in the embryonic yolk sac (Rawji and Yong, 2013). In this study, we have shown that sulfasalazine treatment promotes oligodendrocyte and Schwann cell regeneration and subsequent remyelination in the nervous system of a transgenic zebrafish model of demyelination, and sulfasalazine function in remyelination is mediated by modulation of the immune response. Thus, sulfasalazine treatment reduces the number of macrophages/microglia in the CNS of the transgenic zebrafish model of demyelination, suggesting that modulation of the immune response, especially macrophages/microglia, is important for the remyelination process.

In the CNS of MS patients, infiltration of macrophages and activation of resident microglia are observed, and these immune cells have been shown to be associated with disease severity (Lucchinetti et al., 2000; Prineas and Connell, 1979). From studies using several models of CNS demyelination such as EAE, cuprizone- and lysolecithin-induced demyelination, macrophages/microglia have been shown to play a negative role in remyelination (Franklin and Ffrench-Constant, 2008; Miron and Franklin, 2014; Rawji and Yong, 2013). Inhibition of macrophages/microglia activation or a reduction in their density suppressed and attenuated EAE progression (Bhasin et al., 2007; Heppner et al., 2005; Martiney et al., 1998). Administration of the microglia inhibitor minocycline to the cuprizone-induced demyelination model caused a decrease in demyelination and an improvement in motor coordination behavior (Pasquini et al., 2007; Skripuletz et al., 2010). In addition, treatment with corticosteroid enhanced remyelination associated with a reduction in the number of macrophages/microglia at the lesion site (Pavelko et al., 1998), and activation of macrophages/microglia using zymosan resulted in a loss of OPCs and oligodendrocytes in the lysolecithin-induced demyelination model (Schonberg et al., 2007). Altogether, these data indicate that macrophages/microglia play a negative role for remyelination in various models of demyelination in the CNS. Consistent with these studies, our data showed that reductions in macrophages/microglia are observed in the injured and demyelinated brain following sulfasalazine treatment, which resulted in the acceleration of the remyelination process (Fig. 6). This suggests that removal of macrophages/microglia from the demyelinated CNS promotes remyelination.

However, in contrast to its negative role in remyelination, several studies have shown that macrophages/microglia play a positive role in remyelination. First, in response to injury, OPCs near the lesion are activated from a normal quiescent state to a regenerative phenotype, which is the first step in remyelination, and macrophages/microglia are considered to be the major source of the factors that induce OPC activation (Franklin and Ffrench-Constant, 2008; Schonrock et al., 1998; Wilson et al., 2006). Second, after the activation and recruitment of OPCs to the demyelinated region, the next step in remyelination is the differentiation of OPCs into mature, myelinating oligodendrocytes. For the differentiation of OPCs, it is crucial to remove myelin debris that is generated during demyelination because CNS myelin contains proteins that inhibit OPC differentiation (Franklin and Ffrench-Constant, 2008; Kotter et al., 2006). In the cuprizone-induced demyelination model, removal of myelin debris by the phagocytic function of macrophages/microglia has been shown to be important for remyelination (Jurevics et al., 2002; Olah et al., 2012; Skripuletz et al., 2013). In the lysolecithin-induced demyelination model, depletion of macrophages reduced oligodendrocyte remyelination and delayed recruitment of OPCs to the lesion site by the lack of phagocytic function of macrophages, which is required for the removal of myelin debris (Kotter et al., 2001; 2005; 2006).

Because these reports and the discrepancies regarding whether the removal of macrophages/microglia promoted remyelination in our study, we reasoned that after demyelination, macrophages/microglia play a positive role in activating OPCs and removing myelin debris, but a prolonged presence of macrophages/microglia in a demyelinated region play a negative role in remyelination. In our experiment, we showed that the number of macrophages/microglia increased after demyelination by MTZ treatment. Thus, these cells have a chance to remove myelin debris and stimulate OPC activation for remyelination (Fig. 6F). Next, because we treated with sulfasalazine after demyelination occurred, sulfasalazine reduced the number of macrophages/microglia from the lesion after promoting recovery, and thus, the demyelinated CNS escaped from the negative effects caused by the sustained presence of macrophages/microglia. Our hypotheses are supported by the previous reports showing that the depletion of macrophages/microglia at the initial stage of demyelination significantly
reduced remyelination but macrophage depletion at later stages in the remyelination phase does not change remyelination, suggesting that the beneficial role of macrophages/microglia was time-dependent. Kotter et al. (2005). Altogether, our data suggest that temporal regulation of the immune response of macrophages/microglia by sulfasalazine is important for the promotion of remyelination.

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