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Activating transcription factor 6 α deficiency exacerbates oligodendrocyte death and myelin damage in immune-mediated demyelinating diseases

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Abstract

Endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) play a critical role in immune-mediated demyelinating diseases, including multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE), by regulating the viability of oligodendrocytes. Our previous studies show that activation of the PERK branch of the UPR protects myelinating oligodendrocytes against ER stress in young, developing mice that express IFN- γ , a key pro-inflammatory cytokine in MS and EAE, in the CNS. Several studies also demonstrate that PERK activation preserves oligodendrocyte viability and function, protecting mice against EAE. While evidence suggests activation of the ATF6 α branch of the UPR in oligodendrocytes under normal and disease conditions, the effects of ATF6 α activation on oligodendrocytes in immune-mediated demyelinating diseases remain unknown. Herein, we showed that ATF6 α deficiency had no effect on oligodendrocytes under normal conditions. Interestingly, we showed that ATF6 α deficiency exacerbated ER stressed-induced myelinating oligodendrocyte death and subsequent myelin loss in the developing CNS of IFN- γ -expressing mice. Moreover, we found that ATF6 α deficiency increased EAE severity and aggravated EAE-induced oligodendrocyte loss and demyelination, without affecting inflammation. Thus, these data suggest the protective effects of ATF6 α activation on oligodendrocytes in immune-mediated demyelinating diseases.

Graphical abstract

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Keywords

ATF6α; oligodendrocyte; ER stress; myelin; IFN- γ ; multiple sclerosis; EAE

Introduction

Endoplasmic reticulum (ER) stress activates the unfolded protein response (UPR), an adaptive response that is mediated by three ER-resident transmembrane proteins: pancreatic ER kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor 6 α (ATF6 α) (Marciniak & Ron, 2006; Walter & Ron, 2011). PERK activation inhibits global protein biosynthesis but stimulates certain cytoprotective gene expression by phosphorylating eukaryotic translation initiation factor 2 α (eIF2 α). IRE1 activation induces stress-responsive gene expression by splicing X-box binding protein 1 (XBP1) mRNA. ATF6 α becomes active by transiting to the Golgi complex, where it is cleaved by the proteases S1P and S2P. Cleaved ATF6 α translocates to the nucleus and functions as a transcription factor that enhances the expression of ER chaperones. The primary function of the UPR is to restore ER homeostasis and to adapt cells to stressful conditions. However, if the adaptive measures fail, the UPR triggers apoptosis programs to eliminate stressed cells (Tabas & Ron, 2011; Hetz, 2012).

Each oligodendrocyte must produce enormous amounts of myelin proteins to assemble and maintain myelin sheath. It is known that oligodendrocytes are highly sensitive to disruption of ER homeostasis and that ER stress plays a critical role in various myelin disorders by regulating oligodendrocyte viability (Lin & Popko, 2009; Clayton & Popko, 2016; Volpi et al., 2017). Although the IRE1 branch is the most conserved part of the UPR, activation of this branch in oligodendrocytes is controversial. Some studies show that the IRE1-XBP1 pathway is not activated in oligodendrocytes under normal or disease conditions (Hussien et al., 2015; Stone & Lin, 2015); however, other studies show opposite results (Mhaille et al., 2008; Ni Fhlathartaigh et al., 2013; Naughton et al., 2015). A number of studies demonstrate that the PERK branch can positively or negatively influence oligodendrocyte viability in

myelin disorders (Southwood et al., 2002; Lin et al., 2005; 2013; 2014a; 2014b; Hussien et al., 2014). Nevertheless, despite observation of activation of the ATF6 α branch in oligodendrocytes under normal and disease conditions (Mhille et al., 2008; Naughton et al., 2015; Stone & Lin, 2015), the effects of ATF6 α activation on these cells remains unknown.

Multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) are immune-mediated demyelinating diseases of the CNS (Bauer et al., 2001; Berard et al., 2010; Kipp et al., 2017). It is generally believed that MS and EAE are initiated by an autoimmune reaction against oligodendrocytes and myelin (Frohman et al., 2006; Bradl & Lassmann, 2010). A number of studies show that ER stress is a critical player in regulating oligodendrocyte viability in MS and EAE (Lin & Popko, 2009; Stone & Lin, 2015; Way & Popko, 2016). Data from our laboratory and other groups demonstrate the cytoprotective effects of the PERK pathway on oligodendrocytes during EAE (Lin et al., 2013; 2014a; Hussien et al., 2014). Moreover, we have generated a mouse model that allows for controllable expression of IFN- γ , a key pro-inflammatory cytokine in MS and EAE, in the CNS (Lin et al., 2004). Using the IFN- γ -expressing mouse model, we and other groups demonstrate that the presence of IFN- γ in the CNS of young, developing mice induces myelinating oligodendrocyte apoptosis through ER stress, and that activation of the PERK pathway protects myelinating oligodendrocytes against IFN- γ -induced ER stress in the developing CNS (Lin et al., 2005; 2008; 2010; 2014a; Way et al., 2015).

Data indicate that ATF6 α activation promotes cell survival under stressful conditions (Egawa et al., 2011; Cao et al., 2013; Yoshikawa et al., 2015). In this study, we sought to investigate the role of ATF6 α in oligodendrocytes in immune-mediated demyelinating diseases using the IFN- γ -expressing mouse model and the EAE model. We found that ATF6 α deficiency increased the sensitivity of myelinating oligodendrocytes to IFN- γ -induced ER stress in young, developing mice. We also found that ATF6 α deficiency led to exacerbated oligodendrocyte death and increased disease severity in mice undergoing EAE. Our findings suggest that ATF6 α deficiency increases the vulnerability of oligodendrocytes in immune-mediated demyelinating diseases.

Materials and Methods

Mice and EAE immunization

ATF6a heterozygous knock-out mice (Yamamoto et al., 2007), *GFAP/tTA* mice, and *TRE/IFN- γ* mice (Lin et al., 2004) were on the C57BL/6J background. *ATF6a* heterozygous knock-out mice were intercrossed to get *ATF6a* homozygous knock-out (*ATF6a KO*) mice and control wild type (*ATF6a WT*) mice. *GFAP/tTA* mice and *TRE/IFN- γ* mice were mated with *ATF6a KO* mice, and then the resulting progeny were intercrossed to obtain *GFAP/tTA; TRE/IFN- γ ; ATF6a KO* mice and *GFAP/tTA; TRE/IFN- γ ; ATF6a WT* mice. 0.5 mg/ml doxycycline (Dox) was provided *ad libitum* in the drinking water from conception to repress the expression of the transgene IFN- γ . Genotypes were determined by PCR from DNA extracted from tail tips as described in previous papers (Lin et al., 2004; Yamamoto et al., 2007).

To induce EAE, 8-week-old female mice were injected subcutaneously in the flank and at the tail base with 200 µg of myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide emulsified in complete Freund's adjuvant (BD Biosciences, San Jose, CA, USA) supplemented with 600 µg of Mycobacterium tuberculosis (strain H37Ra; BD Biosciences). Two intraperitoneal injections of 400 ng pertussis toxin (List Biological Laboratories, Denver, CO, USA) were given 24 and 72 h later. Clinical scores (0 = healthy, 1 = flaccid tail, 2 = ataxia and/or paresis of hindlimbs, 3 = paralysis of hindlimbs and/or paresis of forelimbs, 4 = tetraparalysis, 5 = moribund or death) were recorded daily (Lin et al., 2007; 2013; Stone et al., 2017).

All animal procedures were conducted in complete compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Immunohistochemistry (IHC)

Anesthetized mice were perfused through the left cardiac ventricle with 4% paraformaldehyde in PBS. The tissues were removed, postfixed with paraformaldehyde, cryopreserved in 30% sucrose, embedded in optimal cutting temperature compound, and frozen on dry ice. Frozen sections were cut using a cryostat at a thickness of 10 µm. The sections were treated with −20°C acetone, blocked with PBS containing 10% goat serum or horse serum and 0.1% Triton X-100, and incubated overnight with the primary antibody diluted in blocking solution. Fluorescein (1:100, Vector Laboratories, Burlingame, CA, anti-mouse, RRID:AB_2336176; anti-rabbit, RRID:AB_2336197), Cy3 (1:500, Millipore, Temecula, CA, anti-rat, RRID:AB_90854, anti-goat, RRID:AB_92570, anti-rabbit, RRID:AB_92489), or enzyme-labeled secondary antibodies (1:200, Vector Laboratories, anti-rat, RRID:AB_2336202; anti-mouse, RRID:AB_2313581) were used for detection. Immunohistochemical detection of CC1 (APC7, 1:50; EMD Biosciences, Gibbstown, NJ, RRID:AB_2057371), myelin basic protein (MBP, 1:1000; Sternberger Monoclonals, Berkeley, CA, RRID:AB_10120129), CD3 (1:50; Santa Cruz Biotechnology, Dallas, Texas, RRID:AB_627010), CD11b (1:50; Millipore, RRID:AB_92930), immunoglobulin-heavy-chain-binding protein (BiP, 1:100; Santa Cruz Biotechnology, RRID:AB_631616), Glial fibrillary acidic protein (GFAP, 1:1000, Covance, Princeton, NJ, RRID:AB_2314542), Iba1 (1:300, Wako Pure Chemical Industries, Ltd, Osaka, Japan; RRID:AB_2665520), and aspartoacylase (ASPA, 1:1000, kindly provided by Dr. M.A. Aryan Namboodiri at Uniformed Services University of the Health Sciences, Bethesda, MD, Madhavarao et al., 2004) were performed. Fluorescent stained sections were mounted with Vectashield mounting medium with DAPI (Vector Laboratories) and visualized with an Olympus FV1000 confocal microscope. No primary antibody controls were used to confirm the specificity of primary antibodies.

The intensity of BiP fluorescence in oligodendrocytes was measured as described previously (McCloy et al. 2014). Briefly, the mean fluorescence of individual cells was quantified using the NIH ImageJ software (<http://rsb.info.nih.gov/ij/>; RRID:SCR_003070), several readings background fluorescent levels were also taken. The total corrected cellular fluorescence (TCCF) was determined using the formula $TCCF = \text{integrated density} - (\text{area of selected}$

cell $\times$ mean fluorescence of background readings). BiP TCCF levels were normalized to the level of expression in the control mice.

For quantification of cells in EAE mice, we counted immunopositive cells within the anterior funiculus medially next to the anterior median fissure in the lumbar spinal cord, confined to an area of 0.1 mm² as described in our previous paper (Lin et al., 2007, 2013; 2014a). For quantitative MBP IHC analysis in EAE mice, we calculated the percentage of the demyelinated area in the lumbar spinal cord by normalizing the demyelinated area against the total white matter area. The total white matter area and the demyelinated area in the lumbar spinal cord were measured by NIH ImageJ software as described in our previous paper (Lin et al., 2007, 2013).

Toluidine blue staining and electron microscopy (EM) analysis

Mice were anaesthetized and perfused with PBS containing 4% paraformaldehyde and 2.5% glutaraldehyde. The lumbar spinal cord and corpus callosum were processed and embedded. For EAE mice, 1 μ m sections were cut, stained with toluidine blue, and analyzed as previously described (Lin et al., 2007; 2013). The total white matter area and the demyelinated area in the lumbar spinal cord were measured with the NIH ImageJ software. The percentage of the demyelinated area was calculated by normalizing the demyelinated area against the total white matter area. For EM analysis, ultrathin sections were cut, stained with uranyl acetate and lead citrate, and analyzed as described previously (Lin, et al. 2005; Lin et al., 2014a; 2014b; Stone et al., 2017). We counted the total number of axons, the number of myelinated axons, and calculated the percentage of myelinated axons. The diameter of axons and the thickness of myelin sheaths were measured.

Real-time PCR and XBP1 splicing assay

Deeply anesthetized mice were perfused with ice-cold PBS and the spinal cords were extracted and snap frozen with dry ice. RNA was isolated from the whole spinal cord using Trizol reagent (Invitrogen, Carlsbad, CA) and treated with DNaseI (Invitrogen) to eliminate genomic DNA. Reverse transcription was performed using the iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). TaqMan real-time PCR was performed with iQ Supermix (Bio-Rad Laboratories) on the LightCycler 480 System (Roche Diagnostics Corporation, Indianapolis, IN) as described in our previous papers (Lin et al., 2005; 2007; 2013). PCR for XBP1 was performed to detect spliced XBP1 (XBP1s) using Taq DNA Polymerase (Qiagen, Germantown, MD). PCR products were separated by electrophoresis on a 3% agarose gel. Mice heterozygous for *Patched1* (*Ptch1*^{+/-}) with medulloblastoma (MB) were used as a positive control for XBP1 splicing (Ho et al., 2016; Stone et al. 2016).

Western blot analysis

Whole spinal cords harvested from mice were rinsed in ice-cold PBS and were homogenized using a motorized homogenizer as previously described (Lin et al., 2005; 2013). After incubating on ice for 15 min, the extracts were cleared by centrifugation at 14,000 rpm for 30 min twice. The protein content of each extract was determined by DC Protein Assay (Bio-Rad Laboratories). The extracts (40 μ g) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with a primary antibody

against ATF6 α (1:500; B-Bridge, Santa Clara, CA, RRID:AB_10709801), CAATT enhancer binding protein homologous protein (CHOP, 1:1000 Santa Cruz Biotechnology, RRID:AB_783507), or β -actin (1:5000, Sigma-Aldrich, St. Louis, MO, RRID:AB_476694), followed by an HRP-conjugated secondary antibody (1:1000, Vector Laboratories, anti-mouse, RRID:AB_2336177; anti-rabbit, RRID:AB_2336198), and, following incubation with the ECL Detection Reagents (GE Healthcare Biosciences, Pittsburgh, PA), the chemiluminescent signal was detected. The intensity of the recorded chemiluminescence signals of CHOP and actin was quantified using the NIH ImageJ software.

T cell proliferation assay and cytokine assay

Spleen leukocytes were generated from EAE mice at post-immunization day (PID) 10 and plated in 96-well microtiter plates as previously described (Lin et al., 2013; Stone et al., 2017). The cells were treated with MOG 35–55 peptide (0, 1, 10, or 100 μ g/ml), and were incubated at 37°C and 5% CO₂. After 48 h, 20 μ l of BrdU labeling solution (Millipore) was added to the culture media for 24 h. Cell proliferation was determined using the Colorimetric BrdU Cell Proliferation kit (Millipore) according to manufacturer's instructions. We quantified the cytokines in the culture supernatants using the ELISA kits (IFN- γ , IL-4, Thermo Scientific, Grand Island, NY; IL-17A, BioLegend, San Diego, CA) according to the manufacturer's instructions.

Flow cytometry analysis

Deeply anesthetized mice were perfused with 50 ml 0.9% NaCl solution. Whole spinal cord was harvested and single cell suspensions were generated by passing tissues through a 70 μ m cell strainer. Spinal cord cell suspensions were centrifuged in a 70% : 30% discontinuous Percoll (Sigma-Aldrich) gradient at 500 g for 30 minutes. Leukocytes were harvested from the 70 : 30 interface and washed with hanks buffered saline solution (HBSS). Leukocytes were blocked with anti-CD16/32 (1:300, Biolegend, RRID:AB_2103871), and then stained with CD45 (1:5000, Biolegend, RRID:AB_312971), CD11b (1:500, Biolegend, RRID:AB_389305), Gr1 (1:1000, Biolegend, RRID:AB_313376), CD3 (1:100, Biolegend, RRID:AB_2563945), CD4 (1:100, Biolegend, RRID:AB_2563052), CD8 (1:200, Biolegend, RRID:AB_2562609) and DAPI (Sigma-Aldrich). Cells were analyzed using an LSRFortessa flow cytometer (BD Bioscience, San Jose, CA) and the Flowing Software version 2.5.1 (Perttu Terho, Turku Centre for Biotechnology, Finland) as previously described (Fife et al., 2001).

Experimental design and statistical analysis

For EAE experiments only female mice were used due to the well-known sex differences in the EAE model (Voskuhl & Palaszynski, 2001; Rahn et al., 2014; Zhu et al., 2016). EAE clinical score data is presented as mean $\pm$ standard error (SEM) and compared using a two way ANOVA with a Sidak's multiple comparisons test using GraphPad Prism 6 (GraphPad Software, RRID:SCR_002798). All other data are expressed as mean $\pm$ standard deviation (SD). Comparison between two groups was statistically evaluated by *t*-test using GraphPad Prism 6. Multiple comparisons were statistically evaluated by the 1-way ANOVA with a Tukeys posttest using GraphPad Prism 6. Phenotypic differences among the groups were

statistically evaluated by the χ^2 -test using GraphPad Prism 6. *P* values less than 0.05 were considered significant.

Results

ATF6 α deficiency did not affect oligodendrocyte viability or function under physiological conditions

ATF6 α functions as the master regulator of ER chaperone expression during ER stress (Marciniak & Ron, 2006; Walter & Ron, 2011). Recent studies show that mutations of ATF6 α cause the cone dysfunction disorder achromatopsia in human patients (Kohl et al., 2015; Ansar et al., 2015). *ATF6 α KO* mice appear healthy and exhibit normal retinal morphology and function at a young age; however, aged mice display rod and cone dysfunction (Yamamoto et al., 2007; Kohl et al., 2015). A report shows that ATF6 α is activated in oligodendrocytes in young, developing mice and adult mice (Naughton et al., 2015). Moreover, a study shows that deletion of BiP, an ER chaperone and ATF6 α -responsive gene, specifically in oligodendrocytes leads to oligodendrocyte death and myelin loss in both young, developing mice and adult mice (Hussien et al., 2015). Thus, we determined the potential role of ATF6 α in oligodendrocytes under physiological conditions. IHC for CC1, a marker for oligodendrocytes, showed that the number of oligodendrocytes in the CNS of 21-day-old *ATF6 α KO* mice was comparable with *ATF6 α WT* mice (Figure 1A, B, C). MBP IHC showed that ATF6 α deficiency did not alter the degree of myelination in the CNS of 21-day-old mice (Figure 1D, E). Similarly, we found that ATF6 α deficiency did not alter oligodendrocyte numbers or myelin integrity in the CNS of 56-day-old mice (Figure 1F, G, H, I, J). Taken together, these data suggest that ATF6 α is dispensable for the viability and function of oligodendrocytes under physiological conditions.

ATF6 α deficiency rendered myelinating oligodendrocytes susceptible to IFN- γ

Ectopic expression of IFN- γ in the developing CNS stimulates inflammation, including T cell infiltration, microglia/macrophage activation, and upregulation of inflammatory mediators, and leads to myelinating oligodendrocyte death and myelin loss, which recapitulates multiple pathological features of MS demyelinating lesions (Corbin et al., 1996; LaFerla et al., 2000; Lin et al., 2010; 2014a). Importantly, we demonstrate that the presence of IFN- γ in the developing CNS induces myelinating oligodendrocyte apoptosis through ER stress (Lin et al., 2005; 2008; 2010). Moreover, we show that PERK inactivation makes myelinating oligodendrocytes vulnerable to IFN- γ , and that enhanced PERK activation renders myelinating oligodendrocytes resistant to IFN- γ (Lin et al., 2005; 2014a). It is known that ATF6 α activation protects cells against ER stress (Marciniak & Ron, 2006; Walter & Ron, 2011). Therefore, we determined the effects of ATF6 α deficiency on ER-stressed oligodendrocytes in IFN- γ -expressing mice.

We have generated a mouse model that allows for controllable expression of IFN- γ in the CNS using the tetracycline inducible system (Lin et al., 2004). The expression of the transgene IFN- γ in the CNS of *GFAP/tTA; TRE/IFN- γ* transgenic mice is tightly controlled by administration of Dox. *GFAP/tTA* mice and *TRE/IFN- γ* mice were mated with *ATF6 α KO* mice, and then the resulting progeny were intercrossed to obtain *GFAP/tTA; TRE/IFN-*

γ ; *ATF6a* KO mice and *GFAP/tTA; TRE/IFN- γ ; ATF6a* WT mice. These mice were treated with Dox from conception to repress the transgene IFN- γ expression. One group of *GFAP/tTA; TRE/IFN- γ ; ATF6a* WT mice were released from Dox at embryonic day 14 (E14) (IFN- γ +, *ATF6a* WT mice). Another group of *GFAP/tTA; TRE/IFN- γ ; ATF6a* WT mice never released from Dox served as controls (IFN- γ -; *ATF6a* WT mice). One group of *GFAP/tTA; TRE/IFN- γ ; ATF6a* KO mice were released from Dox at E14 (IFN- γ +, *ATF6a* KO mice). Another group of *GFAP/tTA; TRE/IFN- γ ; ATF6a* KO mice never released from Dox served as controls (IFN- γ -; *ATF6a* KO mice). Consistent with previous reports (Lin et al., 2005; 2008; 2014a), we found that a number of IFN- γ +, *ATF6a* WT mice exhibited tremor and ataxia. Interestingly, the tremoring phenotype displayed by IFN- γ +, *ATF6a* KO mice was significantly more severe than IFN- γ +, *ATF6a* WT mice (Table 1).

Despite considerable efforts, we could not find a reliable anti-*ATF6a* antibody for IHC. On the other hand, it is well documented that the chaperone BiP is an *ATF6a*-responsive gene in ER-stressed cells. Thus, we used CC1 and BiP double immunostaining to gauge the activity of *ATF6a* signaling in oligodendrocytes in these four groups of mice. As expected, we found that the presence of IFN- γ markedly increased the expression of BiP in oligodendrocytes of 3-week-old IFN- γ +, *ATF6a* WT mice compared to control IFN- γ -; *ATF6a* WT mice and IFN- γ -; *ATF6a* KO mice, and that *ATF6a* deficiency noticeably reduced the level of BiP in oligodendrocytes of IFN- γ -expressing mice (Figure 2A, B). CC1 IHC, MBP IHC, and EM analysis showed that *ATF6a* deficiency alone did not change oligodendrocyte number, myelin integrity, myelin thickness ($P = 0.9792$) or axon diameter ($P = 0.8807$) in the CNS of 3-week-old mice (Figure 3A, B, E, F, I, J, M, N, O), and that the presence of IFN- γ in the CNS alone led to significant oligodendrocyte loss and myelin loss in the CNS of 3-week-old mice (Figure 3A, B, C, E, F, G, I, J, K, M, N). EM analysis revealed the reduction of the percentage of myelinated axons ($P < 0.0001$), the thickness of myelin ($P = 0.0033$), but not the diameter of myelinated axon ($P = 0.0884$) in the CNS of IFN- γ +, *ATF6a* WT mice compared to IFN- γ -; *ATF6a* WT mice (Figure I, K, O). Importantly, CC1 IHC showed that *ATF6a* deficiency significantly enhanced oligodendrocyte loss in the CNS of IFN- γ -expressing mice (Figure 2, 3C, D, M). MBP IHC showed that *ATF6a* deficiency noticeably increased myelin loss in the CNS of IFN- γ -expressing mice (Figure 3G, H). Moreover, EM analysis showed that *ATF6a* deficiency significantly increased the reduction of the percentage of myelinated axons ($P = 0.0028$) in the CNS of IFN- γ -expressing mice, but did not affect the thickness of myelin ($P = 0.3362$) or the diameter of myelinated axons ($P = 0.3401$) (Figure 3K, L, N, O). Collectively, these data suggest that *ATF6a* deficiency abrogates activation of *ATF6a* signaling in ER-stressed myelinating oligodendrocytes and makes the cells vulnerable to ER stress in the developing CNS of IFN- γ -expressing mice.

Evidence suggests that *ATF6a* deficiency may suppress inflammation (Rao et al., 2014; Ta et al., 2016). We further determined whether *ATF6a* deficiency affects inflammation in the CNS of IFN- γ -expressing mice. CD11b IHC showed that microglia in both IFN- γ -; *ATF6a* WT mice and IFN- γ -; *ATF6a* KO displayed a typical ramified morphology. Interestingly, microglia/macrophages were strongly activated in the CNS of IFN- γ +, *ATF6a* WT mice compared to the control IFN- γ -; *ATF6a* WT mice and IFN- γ -; *ATF6a* KO mice; however, *ATF6a* deficiency did not affect microglia/macrophage activation in the CNS of IFN- γ -

expressing mice (Figure 4A, B, C, D). Microglia/macrophages in both IFN- γ +; ATF6 α WT mice and IFN- γ +; ATF6 α KO mice displayed a typical amoeboid morphology. Moreover, CD3 IHC showed that ATF6 α deficiency did not change T cell infiltration in the CNS of IFN- γ -expressing mice (Figure 4E, F, G, H). Thus, these results rule out the possibility that ATF6 α deficiency increases the vulnerability of myelinating oligodendrocytes to IFN- γ in the developing CNS by enhancing inflammation.

ATF6 α deficiency exacerbated oligodendrocyte loss and demyelination during EAE

It has been demonstrated that ER stress modulates the viability of oligodendrocytes during EAE (Lin & Popko, 2009; Stone & Lin, 2015; Way & Popko, 2016). Enhanced PERK activation in oligodendrocytes attenuates EAE severity and ameliorates EAE-induced oligodendrocyte death and demyelination (Lin et al., 2013). In contrast, PERK inactivation in oligodendrocytes increases EAE severity and enhances EAE-induced oligodendrocyte death and demyelination (Hussien et al, 2014). We assessed the effects of ATF6 α deficiency on oligodendrocytes during EAE. ATF6 α WT mice and ATF6 α KO mice were immunized with MOG35-55 peptide to induce EAE. ATF6 α WT mice displayed a typical EAE disease course, disease onset around PID12 and reaching the peak severity around PID19 (Figure 5A, B). Interestingly, ATF6 α KO mice developed significantly more severe EAE clinical symptoms compared to ATF6 α WT mice (Figure 5A). Moreover, ATF6 α KO mice exhibited a very high rate of mortality (9 out of 21 ATF6 α KO mice vs 1 out of 15 ATF6 α WT mice, Figure 5B).

Western blot analysis showed that the cleaved ATF6 α was undetectable in the spinal cord of naïve ATF6 α WT mice, but was clearly present in the spinal cord of ATF6 α WT mice with EAE (Figure 5C). As expected, the cleaved ATF6 α was not detectable in the spinal cord of ATF6 α KO mice with EAE (Figure 5C). Real time PCR analysis showed a slightly but not significantly increased level of the ATF6 α responsive genes BiP and Suppressor/Enhancer of Lin-12-like (Sel1L) in the spinal cord of ATF6 α WT mice with EAE compared to naïve ATF6 α WT mice (Figure 5D). Importantly, the levels of BiP and Sel1L were significantly decreased in the spinal cord of ATF6 α KO mice with EAE compared to ATF6 α WT mice with EAE (Figure 5D). Moreover, CC1 and BiP double immunostaining showed that the immunoreactivity of BiP was noticeably increased in oligodendrocytes (Figure 5E, F) and other cell types, including astrocytes and microglia/macrophages (Supplemental Figure 1A, B), in the spinal cord of ATF6 α WT mice with EAE compared to naïve ATF6 α WT mice. Similarly, BiP immunoreactivity was markedly decreased in oligodendrocytes (Figure 5E, F) and other cell types, including astrocytes and microglia/macrophages (Supplemental Figure 1A, B), in the spinal cords of ATF6 α KO mice with EAE compared to ATF6 α WT mice with EAE. Thus, these data suggest that ATF6 α is activated in oligodendrocytes during EAE and that ATF6 α activity is abrogated in oligodendrocytes in ATF6 α KO mice with EAE.

It is known that T cells infiltrate the CNS and induce tissue damage before the onset of clinical disease (D'Amelio et al., 1990; Hickey et al., 1991). To assess the degree of demyelination, oligodendrocyte loss and inflammation in the CNS of ATF6 α WT mice and ATF6 α KO mice, CNS tissues were prepared from these EAE mice at PID19 when ATF6 α WT mice were at the peak of disease. Quantitative analysis of MBP IHC showed that the

percentage of area in the white matter of the lumbar spinal cord that was demyelinated in *ATF6a KO* mice was significantly increased compared to *ATF6a WT* mice (Figure 6A, B, C). Quantitative analysis of toluidine blue staining verified that the percentage of demyelinated area in the white matter of the lumbar spinal cord in *ATF6a KO* mice was significantly increased compared to *ATF6a WT* mice (Figure 6D, E, F). Immunostaining for ASPA, a marker for oligodendrocytes (Madhavarao et al., 2004; Lin et al., 2013; 2014a), revealed a few remaining oligodendrocytes in the demyelinated lesions in the lumbar spinal cord of *ATF6a WT* mice (Figure 6G, I). Importantly, there were significantly fewer oligodendrocytes remaining in the demyelinated lesions of *ATF6a KO* mice (Figure 6G, H, I). CD3 immunostaining revealed moderately but not significantly increased numbers of CD3+ T cells in the demyelinated lesions in the lumbar spinal cord of *ATF6a KO* mice compared to *ATF6a WT* mice (Figure 6J, K, L). CD11b immunostaining also revealed a moderately but not significantly increased number of CD11b+ microglia/macrophages in the demyelinated lesions in the lumbar spinal cord of *ATF6a KO* mice compared to *ATF6a WT* mice (Figure 6M, N, O). Moreover, single-cell suspensions were generated from the whole spinal cord of individual EAE mice at PID19. Flow cytometry analysis showed moderately but not significantly decreased numbers of inflammatory cells, including total T cells, CD4+ T cells, CD8+ T cells, microglia, macrophages, and granulocytes/inflammatory monocytes, in *ATF6a KO* mice compared to *ATF6a WT* mice (Table 2). Additionally, real-time PCR analysis showed that *ATF6a* deficiency did not significantly change the expression of the immune cytokines IFN- γ , IL-2, IL-4, IL-5, IL-10, IL-12, IL-17, IL-23, iNOS, and TNF- α in the spinal cord of EAE mice at PID19 (Figure 7A). Taken together, these data suggest that *ATF6a* deficiency exacerbates oligodendrocyte loss and demyelination, but did not significantly alter inflammation in the CNS of mice undergoing EAE.

EAE is initiated by T cell priming in the peripheral immune system. We further performed *in vitro* T cell recall assays to determine the effects of *ATF6a* deficiency on T cell priming during EAE. Spleen leukocytes were harvested from *ATF6a KO* mice and *ATF6a WT* mice at PID10, and re-stimulated with MOG 35-55 peptides. Interestingly, *in vitro* recall assays showed that *ATF6a* deficiency did not change the abilities of T cells to proliferate and to produce the cytokines IFN- γ , IL-17A, or IL-4 (Figure 7B, C, D, E). These data demonstrate that *ATF6a* deficiency does not alter T cell priming in the peripheral immune system during EAE. Collectively, it is unlikely that *ATF6a* deficiency exacerbates oligodendrocyte death and demyelination during EAE by enhancing inflammation.

Finally, we determined whether *ATF6a* deficiency affects the activation of the IRE1 and PERK branches in the CNS of mice undergoing EAE. The PCR-based assays that examine XBP1 splicing are highly sensitive methods for detection of activation of the IRE1-XBP1 pathway (Cox et al., 2011). We found that spliced XBP-1 was undetectable in the spinal cord of either naïve *ATF6a WT* mice or *ATF6a WT* mice with EAE, but became detectable in *ATF6a KO* mice with EAE (Supplemental Figure 2A). These results suggest that *ATF6a* deficiency exacerbates ER stress in CNS cells during EAE, resulting in activation of the IRE1-XBP1 pathway in these cells. Moreover, it is known that induction of CHOP is dependent on activation of the PERK-eIF2 α pathway in ER-stressed cells (Marciniak & Ron, 2006; Lin et al., 2013; 2014a, b). Western blot analysis showed that the level of CHOP was significantly increased in the spinal cord of *ATF6a WT* mice with EAE compared to

naïve *ATF6α* *WT* mice, and that CHOP level was significantly decreased in the spinal cord of *ATF6α* *KO* mice with EAE compared to *ATF6α* *WT* mice with EAE (Supplemental Figure 2B, C). Previous studies have demonstrated activation of the PERK-eIF2α pathway in oligodendrocytes during EAE (Lin et al., 2007; 2013; Hussien et al., 2014). We showed here that *ATF6α* deficiency exacerbated oligodendrocyte loss during EAE. Thus, these data raise the possibility that exacerbation of oligodendrocyte loss contributes to the decreased level of CHOP in the spinal cord of *ATF6α* *KO* mice with EAE.

Discussion

It is well documented that ER stress plays a critical role in various myelin disorders by regulating oligodendrocyte viability (Lin & Popko, 2009; Clayton & Popko, 2016; Volpi et al., 2017). MS and EAE are believed to be initiated by an autoimmune reaction against oligodendrocytes and myelin (Frohmman et al., 2006; Bradl & Lassmann, 2010; Kipp et al., 2017). Recent studies show that ER stress modulates oligodendrocyte viability during EAE, and subsequently influences the disease development (Lin et al., 2007; 2013; Hussien et al., 2014). Importantly, several studies demonstrate that activation of the PERK pathway in oligodendrocytes is cytoprotective, protecting mice against EAE (Lin et al., 2013; Hussien et al., 2014). Nevertheless, the effects of the *ATF6α* pathway on oligodendrocytes in MS and EAE remain unknown.

To explore the effects of *ATF6α* on oligodendrocytes in immune-mediated demyelinating diseases, we first used a mouse model that ectopically expresses IFN-γ in the CNS. The developing CNS of IFN-γ-expressing mice reproduces multiple pathological features of active MS lesions, including inflammation, oligodendrocyte death, and myelin loss (Corbin et al., 1996; LaFerla et al., 2000; Lin et al., 2010; 2014a). Importantly, it has been demonstrated that IFN-γ induces myelinating oligodendrocyte death through ER stress (Lin et al., 2005; 2008; 2010). Our previous studies show that decreased PERK activation exacerbates ER stress-induced myelinating oligodendrocyte death in the developing CNS of IFN-γ-expressing mice, and that enhanced PERK activation protects myelinating oligodendrocytes against IFN-γ-induced ER stress in the developing CNS (Lin et al., 2005; 2008; 2014a). We showed here that *ATF6α* deficiency eliminated IFN-γ-induced *ATF6α* activation in oligodendrocytes and exacerbated IFN-γ-induced myelinating oligodendrocyte death and myelin loss in the developing CNS. Moreover, we found that *ATF6α* deficiency did not alter inflammation in the CNS of IFN-γ-expressing mice. The cytoprotective role of *ATF6α* activation in ER-stressed cells is well documented (Marciniak & Ron, 2006; Walter & Ron, 2011). Thus, these data suggest that *ATF6α* deficiency renders myelinating oligodendrocytes sensitive to IFN-γ-induced ER stress in the developing CNS.

We further explored the effects of *ATF6α* on oligodendrocytes in immune-mediated demyelinating diseases using the EAE model. Recent studies show that ER stress is a critical player in regulating oligodendrocyte viability during EAE (Lin & Popko, 2009; Stone & Lin, 2015; Way & Popko, 2016). Several studies show that PERK activation protects oligodendrocytes against inflammation during EAE, and results in attenuated EAE severity (Lin et al., 2013; Way et al., 2015). In contrast, a report shows that PERK inactivation facilitates oligodendrocyte death during EAE, and leads to exacerbated EAE severity

(Hussien et al, 2014). We showed here that ATF6 α deficiency abrogated ATF6 α activation in oligodendrocytes, increased disease severity, and exacerbated oligodendrocyte death and demyelination in EAE mice. We also showed that ATF6 α deficiency had no significant effect on T cell priming in the peripheral immune system and on inflammation in the CNS during EAE. Although data indicate that the UPR influences innate and adaptive immune responses, there is no evidence that ATF6 α deficiency enhances the activity of inflammatory cells (Garg et al., 2012; Janssens et al., 2014; Rao et al., 2014; Ta et al., 2016). Collectively, these data suggest that ATF6 α deficiency renders oligodendrocytes vulnerable to inflammation during EAE, resulting in exacerbated EAE severity.

During EAE pathogenesis, inflammation causes oligodendrocyte death and demyelination, and then oligodendrocyte death and demyelination promote inflammation, forming a vicious circle (Frohnman et al., 2006; Bradl & Lassmann, 2010; Kipp et al., 2017). While we showed that there was no significant difference in inflammatory cell infiltration in the CNS of *ATF6 α KO* mice and *ATF6 α WT* mice, flow cytometry analysis showed a trend of decreased inflammatory cell infiltration in the whole spinal cord of *ATF6 α KO* mice with EAE. In contrast, IHC analysis showed a trend of increased inflammatory cell infiltration in the demyelinating lesions in the lumbar spinal cord of *ATF6 α KO* mice with EAE. Several lines of evidence suggest that increased oligodendrocyte death and demyelination can lead to enhanced inflammation in the demyelinating lesions during EAE (Hisahara et al., 2000; 2001; Mc Guire et al., 2010). Moreover, recent reports suggest that ATF6 α inactivation can reduce the activity of microglia or macrophages (Rao et al., 2014; Ta et al., 2016). Thus, these data raise the possibilities that: 1) ATF6 α deficiency in inflammatory cells moderately suppress inflammation, resulting in a trend of decreased inflammatory cell infiltration in the whole spinal cord during EAE; 2) exacerbated oligodendrocyte death and demyelination induced by ATF6 α deficiency in oligodendrocytes attract inflammatory cells and concentrate them in active EAE lesions, resulting in a trend of increased inflammatory cell infiltration in the lesion sites. Clearly, the precise role of ATF6 α inactivation in individual cell types, including oligodendrocytes, T cells, microglia, macrophages, or astrocytes, during EAE warrants a further investigation. Unfortunately, the *ATF6 α KO* mice used in this study do not allow us to dissect the cell-autonomous role of ATF6 α in these individual cell types in EAE mice. Cell-specific conditional ATF6 α KO mouse models are required for addressing this important question. Regardless, establishment of the inhibitory effects of ATF6 α deficiency on inflammatory cells in EAE mice will not compromise the conclusion drawn from this study, namely that ATF6 α deficiency renders oligodendrocytes vulnerable to inflammation. Rather, it will provide additional evidence supporting the conclusion.

Interestingly, consistent with our observation regarding the potentially inhibitory effects of ATF6 α deficiency on inflammatory cells, a recent report shows that ATF6 α deficiency moderately but significantly inhibits cultured microglial activation in response to lipopolysaccharide (LPS) (Ta et al., 2016). Nevertheless, in contrast to our study, Ta et al. show that ATF6 α deficiency decreases EAE severity, although the report also shows that ATF6 α deficiency does not affect T cell priming during EAE (Ta et al., 2016). Notably, Ta et al. use a lower concentration of MOG 35-55 peptide and pertussis toxin (100 μ g MOG35-55 peptide + 200 ng pertussis toxin vs. 200 μ g MOG 35-55 peptide + 400 ng pertussis toxin used in this study) to induce EAE in male mice on the C57BL/6 background (vs female mice

on the C57BL/6J background used in this study). Sex difference in both MS patients and EAE animals is well known (Voskuhl & Palaszynski, 2001; Rahn et al., 2014; Zhu et al., 2016). Therefore, the discrepancy of EAE severity between these two studies likely reflects that the immunization regimen used by Ta et al. is not strong enough to induce sufficient inflammation to kill oligodendrocytes in male *ATF6α* *KO* mice at the initiation stage of EAE, so that the contribution of ATF6α deficiency in oligodendrocytes to EAE development cannot be realized. In contrast, the immunization regimen used in this study is strong enough to induce full blown EAE in female *ATF6α* *KO* mice, despite a potentially, moderately anti-inflammatory effect of ATF6α deficiency. The detrimental effects of ATF6α deficiency on oligodendrocytes outweigh the moderately anti-inflammatory effects of ATF6α deficiency on inflammatory cells, resulting in increased EAE severity.

ATF6α functions as the master regulator of ER chaperone expression during ER stress (Yamamoto et al., 2007; Walter & Ron, 2011). Data suggest that ATF6α deficiency diminishes induction of ER chaperones during ER stress, and results in increased cell apoptosis (Cao et al., 2013). A report shows that BiP is required for oligodendrocyte survival under normal condition and that heterozygous deficiency of BiP specifically in oligodendrocytes increases EAE disease severity and exacerbates EAE-induced oligodendrocyte death and demyelination (Hussien et al., 2015). We showed here that ATF6α deficiency diminished BiP induction in oligodendrocytes and enhanced oligodendrocyte death in the CNS of IFN-γ-expressing mice and EAE mice. Thus, it is possible that ATF6α deficiency increases the vulnerability of oligodendrocytes in immune-mediated demyelinating diseases by attenuating induction of BiP.

In summary, using the IFN-γ-expressing mouse model, we showed that ATF6α deficiency renders myelinating oligodendrocytes sensitive to IFN-γ-induced ER stress in the developing CNS. Moreover, using the EAE model, we showed that ATF6α deficiency renders oligodendrocytes vulnerable to inflammation during EAE. The results presented in this study suggest that ATF6α deficiency increases the vulnerability of oligodendrocytes in immune-mediated demyelinating diseases, implying the cytoprotective effects of ATF6α activation on oligodendrocytes in MS. Importantly, in contrast to the PERK pathway that functions as a double-edged sword during ER stress, there is no evidence that ATF6α activation triggers apoptosis programs in ER-stressed cells (Tabas & Ron, 2011; Hetz, 2012). Thus, this study may provide a foundation for developing therapeutic strategies that protect oligodendrocytes against inflammation in MS patients by targeting ATF6α.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Main points

- ATF6 α deficiency exacerbates ER stressed-induced oligodendrocyte death in the developing CNS of IFN- γ -expressing mice.
- ATF6 α deficiency exacerbates EAE severity and EAE-induced oligodendrocyte loss and myelin loss, without affecting inflammation.

Figure 1. ATF6α deficiency had a minimal effect on oligodendrocytes

A, B, C. CC1 IHC showed that ATF6α deficiency did not alter oligodendrocyte numbers in the spinal cord (SC), cerebellum (CB), and corpus callosum (CC) of 21-day-old mice (**A, B**, spinal cord). **D, E.** MBP IHC showed that ATF6α deficiency did not change the degree of myelination in the spinal cord of 21-day-old mice. **F, G, H.** CC1 IHC showed that ATF6α deficiency did not alter oligodendrocyte numbers in the spinal cord (SC), cerebellum (CB), and corpus callosum (CC) of 56-day-old mice (**F, G**, spinal cord). **I, J.** MBP IHC showed that ATF6α deficiency did not change the degree of myelination in the spinal cord of 56-day-old mice. Scale bars: **A, B, D, E, F, G, I, J**, 50 μm. N=4 animals. Error bars represent SD, n.s. Not significant.

Figure 2. ATF6 α deficiency diminished BiP induction in oligodendrocytes of IFN- γ expressing mice

A. CC1 and BiP double immunostaining showed that the level of BiP was markedly increased in oligodendrocytes (arrows) in the corpus callosum of 21-day-old IFN- γ ++; ATF6 α WT mice compared to control IFN- γ -; ATF6 α WT mice and IFN- γ -; ATF6 α KO mice. However, BiP level was noticeably decreased in oligodendrocytes of IFN- γ ++; ATF6 α KO mice compared to IFN- γ ++; ATF6 α WT mice. **B.** Quantification of the relative fluorescence intensity of BiP in oligodendrocytes. Scale bar, 20 μ m. N=4 animals.

Figure 3. ATF6α deficiency exacerbated IFN-γ-induced oligodendrocyte death and myelin loss in the developing CNS

A, B, C, D, M. CC1 IHC showed comparable oligodendrocyte numbers in the corpus callosum (delineated areas) of 21-day-old IFN-γ-; ATF6α WT mice and IFN-γ-; ATF6α KO mice. The number of oligodendrocytes was significantly reduced in IFN-γ+; ATF6α WT mice, and was further reduced in IFN-γ+; ATF6α KO mice. **E, F, G, H.** MBP IHC showed noticeable myelin loss in the corpus callosum of 21-day-old IFN-γ+; ATF6α WT mice compared to control IFN-γ-; ATF6α WT mice and IFN-γ-; ATF6α KO mice, which was exacerbated in IFN-γ+; ATF6α KO mice. **I, J, K, L, N, O.** EM analysis showed a significant reduction of the percentage of myelinated axons and the thickness of myelin in the corpus callosum of 21-day-old IFN-γ+; ATF6α WT mice compared to control IFN-γ-; ATF6α WT mice and IFN-γ-; ATF6α KO mice. Importantly, the percentage of myelinated axons was further reduced in IFN-γ+; ATF6α KO mice, but the thickness of myelin was not altered in IFN-γ+; ATF6α KO mice compared to IFN-γ+; ATF6α WT mice. Scale bar, A–H, 50 μm; I–M, 1 μm. N=4 animals. Error bars represent SD, **P*<0.05, n.s. Not significant.

Figure 4. ATF6 α deficiency did not affect IFN- γ -induced inflammation in the developing CNS
A, B, C, D. CD11b IHC revealed strong activation of microglia/macrophages in the corpus callosum of 21-day-old IFN- γ +; ATF6 α WT mice compared to IFN- γ -; ATF6 α WT mice and IFN- γ -; ATF6 α KO mice. The degree of microglia/macrophage activation in IFN- γ +; ATF6 α KO mice was comparable to IFN- γ +; ATF6 α WT mice. Inset, high magnification image of activated microglia/macrophages (arrows). **E, F, G, H.** CD3 IHC revealed a few infiltrating T cells (arrowheads) in the corpus callosum of 21-day-old IFN- γ +; ATF6 α WT mice compared to IFN- γ -; ATF6 α WT mice and IFN- γ -; ATF6 α KO mice. The number of infiltrating T cells was comparable in IFN- γ +; ATF6 α WT mice and IFN- γ +; ATF6 α KO mice. Scale bars, A–H, 50 μ m. N=4 animals.

Figure 5. ATF6 α deficiency significantly increased EAE disease severity

A. Mean clinical score. Error bars represent SEM. ATF6 α WT mice, N = 15 animals; ATF6 α KO mice, N = 21 animals. **B.** Peak clinical score for individual mice. Error bars represent SD. ATF6 α WT mice, N = 15 animals; ATF6 α KO mice, N = 21 animals. **C.** Western blot analysis revealed that the cleaved ATF6 α was present in the spinal cord of ATF6 α WT mice with EAE at PID19, but not in naïve ATF6 α WT mice or ATF6 α KO mice with EAE. N = 3 animals. **D.** Real-time PCR analysis showed that the levels of BiP mRNA and Sel1L were slightly increased in the spinal cord of ATF6 α WT mice with EAE at PID19 compared to naïve ATF6 α WT mice, but were significantly decreased in ATF6 α KO mice with EAE. N=6 animals. Error bars represent SD. **E.** CC1 and BiP double immunostaining showed that the immunoreactivity of BiP was markedly increased in oligodendrocytes (arrows) in the lumbar spinal cord of ATF6 α WT mice with EAE at PID19 compared to naïve ATF6 α WT mice, but was noticeably decreased in ATF6 α KO mice with EAE. N=4

animals. **F.** Quantification of the relative fluorescence intensity of BiP in oligodendrocytes. N=4 animals. Scale bar, E, 20 μm . * $P<0.05$, n.s. Not significant.

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Figure 6. ATF6 α deficiency exacerbated EAE-induced oligodendrocyte loss and demyelination
A, B, C. MBP IHC showed that the percentage of demyelinated area (delineated areas) was significantly increased in the white matter of the lumbar spinal cord in ATF6 α KO mice at PID19 compared to ATF6 α WT mice. **D, E, F.** Toluidine blue staining showed that the percentage of demyelinated area was significantly increased in the white matter of the lumbar spinal cord in ATF6 α KO mice at PID19 compared to ATF6 α WT mice. **G, H, I.** ASPA immunostaining showed that there were significantly fewer oligodendrocytes in inflammatory lesions in the lumbar spinal cord of ATF6 α KO mice at PID19 compared to ATF6 α WT mice. **J, K, L.** CD3 immunostaining showed that the number of T cells was not significantly changed in the lumbar spinal cord of ATF6 α KO mice at PID19 compared to ATF6 α WT mice. **M, N, O.** CD11b immunostaining showed that the number of microglia/macrophages was not significantly changed in the lumbar spinal cord of ATF6 α KO mice at PID19 compared to ATF6 α WT mice. Scale bar, A, B, J, K, M, N, 50 μ m; D, E, 10 μ m; G, H, 20 μ m. N=5 animals, Error bars represent SD. * P <0.05. n.s. Not significant.

Figure 7. ATF6 α deficiency did not significantly alter immune response during EAE

A. Real-time PCR analysis showed that the levels of immune cytokines IFN- γ , IL-2, IL-4, IL-5, IL-10, IL-12, IL-17A, IL-23, iNOS, and TNF- α were comparable in the spinal cord of ATF6 α KO mice and ATF6 α WT mice at PID19. N=4 animals. **B.** BrdU cell proliferation assay showed that ATF6 α deficiency did not alter T cell proliferation in response to MOG35-55 peptide. N=5 animals. **C, D, E.** ELISA analysis showed that ATF6 α deficiency did not significantly changed the ability of T cells to produce the cytokines IFN- γ , IL-17A, or IL-4 in response to MOG35-55 peptide. N=5 animals. Error bars represent SD, n.s. Not significant.

Table 1

ATF6 α deficiency exacerbated the tremoring phenotype displayed by IFN- γ -expressing mice during development.

	No tremor (%)	Mild tremor (%)	Tremor (%)	Severe tremor (%)
IFN- γ -; ATF6 α WT (N=43)	100	0	0	0
IFN- γ -; ATF6 α KO (N=36)	100	0	0	0
IFN- γ ++; ATF6 α WT (N=14)	28.57	35.71	28.57	7.14
IFN- γ ++; ATF6 α KO (N=15)	0 [*]	6.67 [*]	33.33 ^{n.s.}	60 [*]

Mild tremor = tremor only in tail; tremor = tremor in tail and body; severe tremor = whole body tremor and occasional seizure.

^{*} $P < 0.05$,

^{n.s.}Not significant.

Table 2

ATF6 α deficiency did not significantly change inflammatory cell infiltration in the spinal cord during EAE.

Gate	Cell type	ATF6 α WT	ATF6 α KO
CD45 ^{hi} CD3 ⁺ CD11b ⁻	T cells	19347 $\pm$ 9686.284	9303 $\pm$ 2098.529 ^{n.s.}
CD45 ^{hi} CD3 ⁺ CD11b ⁻ CD4 ⁺	CD4 ⁺ T cells	10663 $\pm$ 4898.063	5000 $\pm$ 304.083 ^{n.s.}
CD45 ^{hi} CD3 ⁺ CD11b ⁻ CD8 ⁺	CD8 ⁺ T cells	6033 $\pm$ 3908.728	3073 $\pm$ 1524.343 ^{n.s.}
CD45 ^{lo} CD11b ⁺	Microglia	14113 $\pm$ 6084.912	13707 $\pm$ 7411.263 ^{n.s.}
CD45 ^{hi} CD11b ⁺ Gr1 ⁻	Macrophages	3513 $\pm$ 1787.593	1840 $\pm$ 1235.664 ^{n.s.}
CD45 ^{hi} CD11b ⁺ Gr1 ⁺	Granulocytes, inflammatory monocytes	20500 $\pm$ 9014.57	15440 $\pm$ 9987.562 ^{n.s.}

Results presented as mean $\pm$ SD. N = 3 animals.

^{n.s.} Not significant.