

TNF α drives the proliferation and inflammatory response, but not regenerative potential of Müller cells in the mouse retina

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Research

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Abstract

Background Mouse Müller cells, considered dormant retinal progenitors, respond to retinal injury by undergoing reactive gliosis rather than displaying regenerative responses. Tumor necrosis factor alpha (TNF α) is a key cytokine induced after injury, and implicated in mediating inflammatory and regenerative responses. However, the molecular events driving reactive gliosis and regenerative responses in Müller cells, and the role of TNF α in these processes, remain unclear. In this study, we investigated the effects of TNF α on Müller cell responses following injury.

Methods To investigate the involvement of TNF α in retinal injury, adult C57BL/6J mice were subjected to treatment with light (5,000 lux) for 14 consecutive days; induction of TNF α was confirmed by quantitative polymerase chain reaction (qPCR). TNF α effects on Müller-cell proliferation were evaluated via 5-ethynyl-2'-deoxyuridine (EdU) incorporation in culture. TNF α -mediated gene profile changes were examined using Affymetrix microarray, and gene ontology analysis was carried out to define the molecular pathways involved. Gene- and protein-expression changes were further verified by qPCR, western blot, and enzyme linked immunosorbent assay (ELISA).

Results We showed that TNF α induced Müller cell proliferation and the expression of inflammatory and proliferation-related genes, including NFKBIA, Leukemia inhibitory factor, Interleukin-6, Janus kinase (Jak) 1, Jak2, Signal transducer and activator of transcription (Stat) 1, Stat2, Mitogen-Activated Protein Kinase (MAPK) 7, and MAP4K4. Blockade of Jak/Stat and MAPK pathways attenuated TNF α -induced Müller cell proliferation. Moreover, we detected TNF α drove A1 phenotype-reactive gliosis, while Wnt attenuated TNF α -mediated induction of A1 phenotype and promoted an A2-like phenotype.

Conclusion In Müller cells, TNF α triggered primarily inflammatory and reactive gliosis by activating Jak/Stat and MAPK-pathways without inducing progenitor cell/regeneration-related genes. Wnt signaling suppressed inflammation, and induced proliferation and expression of progenitor-cell genes in Müller cells. These results suggest that reactive gliosis and regenerative responses in Müller cells are regulated by independent mechanisms. Our study provides new insights into regulation of inflammatory and regenerative responses of Müller cells in the injured retina

Background

Müller cells are the major glial cells in the retina with cellular processes that span entire retinal layers and contact neurons. Müller cells play key roles in maintaining retinal homeostasis, structure, and function [1-3], and may serve as primary source of retinal progenitors that generate new neurons and contribute to retinal repair following injury. In zebrafish, resident Müller cells participate robustly in retinal regeneration [4, 5]. In contrast, the mammalian retina cannot self-repair [6], and Müller cells respond to retinal damage by undergoing reactive gliosis [7-9]. However, molecular pathways that drive reactive gliosis and regenerative responses in Müller cells are unknown.

In Müller cell, proliferation is associated with their dedifferentiation into progenitor cells and activation of their regenerative potential. Under certain conditions, such as following stimulation with α -aminoadipic acid [10], epidermal growth factor (EGF) [11,12], fibroblast growth factors (FGFs), or insulin [13],¹³ Müller cells of adult mice reenter the cell cycle and generate new retinal neurons. Wnt and Notch signaling are well-known cellular events that participate in Müller-cell proliferation and regenerative processes of the mammalian retina [14-16]. The sonic hedgehog (Shh), and MAPK and Jak/Stat signaling pathways also play important roles in stimulating the proliferation of Müller cells [11, 17-19]. However, induction of Müller-cell proliferation by these factors occurs only in the presence of retinal injury such as that caused by light exposure [20, 21] or treatment with N-methyl-D-aspartate (NMDA) [11, 14] or N-methyl-N-nitrosourea (MNU) [15, 17]. The mechanisms by which these stimulations interact with injury signals to induce Müller cell proliferation remain elusive.

TNF α , a potent cytokine induced after retinal injury, is involved in numerous biological processes such as cellular apoptosis, survival, and proliferation [22]. TNF α activates Stat3-mediated proliferation of Müller cells in the zebrafish retina [5] and in fetal human cortical neural progenitors [23]. In light-damaged zebrafish retina, enhanced proliferation of Müller cells is accompanied by upregulated expression of TNF α . In undamaged retina, administration of TNF α induces proliferation of Müller glia via ASCL1a and Stat3 signaling pathways.⁵ Although TNF α is considered a key inflammatory cytokine in mammals [24, 25], few studies have examined the effect of TNF α on Müller-cell proliferation. It is still unclear whether TNF α serves as key injury signal that promotes Müller-cell proliferation and, thereby, drives retinal neuroregeneration or repair following injury in the mouse retina. In the present study, we used EdU incorporation assay, gene chip analysis, real-time PCR, and western blotting to examine changes in the proliferation and gene expression of Müller cells obtained from the mouse retina and subjected to treatment with TNF α .

Methods

Animals

All procedures involving, and care of, animals adhered to the guidelines of Animal Ethics Committee of Eye and ENT Hospital of Fudan University (Shanghai, China), and conformed to the standards of the National Institute of Health and Association for Research in Vision and Ophthalmology. Adult (6-8 weeks old), postnatal (PN) 3-day-old C57BL/6J mice were purchased from the Chinese Academy of Sciences (Shanghai, China).

Induction of retinal light damage and hematoxylin-eosin (HE) staining

Following a 1-day dark adaptation, C57BL/6J mice over the age of 6-8 weeks were subjected to 5,000 lux of cool white LED light, positioned around and on top of the light box, for 14 consecutive days. Atropine eye drop solution (1%; prepared at the EENT Hospital of Fudan University) was used daily for pupil dilation. The mice were dark-adapted again for 1 day before being returned to a normal environment. Mice in the control group were not exposed to light, and were maintained in a normal environment.

Retinal histology was evaluated using hematoxylin and eosin (HE). Briefly, tissues embedded in paraffin were sectioned at 5 μ m, placed on glass slides, deparaffinized, and rehydrated, after which the retinas were stained with HE performed in accordance with a standard protocol. The thickness of outer nuclear layer (ONL) was quantified under a light microscope (Leica DM 4000B, German).

Müller cell culture

Müller cell cultures were prepared as described previously [26,27]. Briefly, mouse retina was dissected out and digested using a papain-based dissociation system (Worthington Biochemical; Lakewood, NJ) in accordance with the manufacturer's instructions. Dissociated cells were incubated at 37°C in Dulbecco's Modified Eagle's Medium/Ham's F-12 Medium (DMEM/F12) (Invitrogen; Carlsbad, CA) containing 1% penicillin-streptomycin (Invitrogen) and 10% fetal bovine serum (FBS, Invitrogen). Primary Müller cells were cultured, and adherent cells were passaged twice before being used in further experiments. The purity of Müller cells was evaluated by immunocytochemistry using immunolabeling with primary antibodies specific for glutamine synthetase (GS) [28] and vimentin [29], which are biomarkers of Müller cells.

Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF α in mouse Müller-cell cultures were quantified using a TNF α mouse ELISA kit (Invitrogen) in accordance with manufacturer's instructions. Absorbance was read at 450 nm using a microplate reader (please provide manufacturer's information for this instrument).

Cellular proliferation assay

To assess cellular proliferation, Müller cells were dissociated into single-cell suspension using 0.25% EDTA-trypsin (Invitrogen), seeded at a density of 4,000 cells per well in 96-well culture plates containing DMEM/F12 medium supplemented with 10% FBS. TNF α (50 ng/ml; R&D systems, Minneapolis, MN) was added into the culture media of the treated groups of Müller cells, and the cells were allowed to incubate for 24 hours at 37°C. Cell proliferation was evaluated via Click-iT EdU Alexa Fluor 555 Imaging Kit (Invitrogen) [30,31] in accordance with manufacturer's instructions. Briefly, EdU (10 μ M) was added to cell-

culture media, and cells were incubated for 4 hours at 37°C; cells were then fixed and incubated with Hoechst 33342 (1:1000, Invitrogen) used to stain cell nuclei. Rabbit anti-TNF α monoclonal antibody (diluted 1:100; Abcam, Boston, MA) was added to the media used to culture Müller cell obtained from adult light-injured mice to specifically block the effect of TNF α . Five images were obtained per well, and the numbers of EdU+ cells and Hoechst + cells were counted using Image J 2.1.4.7 (NIH, Bethesda, USA). The ratio of EdU+ cells to Hoechst+ cells indicated the rate of Müller-cell proliferation.

RNA isolation

Müller cells were cultured at density of 10^6 cells/25 cm² culture flask. Cells were then treated with 50 ng/ml TNF α for 24 hours, while control cells were treated with PBS. Cells were lysed using Buffer RLT (Qiagen, Valencia, CA) containing 1% β -mercaptoethanol (β -ME, Sigma, USA); RNA was then extracted in accordance with manufacturer's instructions (Qiagen). NanoDrop 2000 (Thermo Scientific, Wilmington, DE) was used to measure RNA concentration.

Microarray hybridization and analysis

Müller cells, obtained from postnatal 3-day-old mice, were treated with TNF α (50 ng/ml) for 24 hours. To acquire biotin-labeled cRNA, two groups of RNA (with three samples per group) were amplified, labeled, and purified using GeneChip 3' IVT PLUS Reagent Kit (Affymetrix, Santa Clara, CA, US) in accordance with the manufacturer's protocol. Array hybridization and washing procedures were performed using Affymetrix Gene Chip Mouse Genome 430 2.0 Hybridization, Wash and Stain Kit (Affymetrix) in Hybridization Oven 645 (Affymetrix) and Fluidics Station 450 (Affymetrix) according to the manufacturer's instructions. Subsequently, GeneChip Scanner 3000 (Affymetrix) and Command Console Software 4.0 (Affymetrix) at default settings were used to scan the slides. After normalization, GeneSpring GX 11.5 Software was used for data analysis and hierarchical clustering. Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) were used for gene annotation and function/pathway analysis.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

Selected gene-expression data, generated via microarray, were confirmed using real-time PCR (qPCR). For qPCR, RNA was reverse-transcribed into cDNA using SYBR Green real-time PCR kit (Takara, Osaka, Japan) according to the manufacturer's instructions. Quantitative PCR was performed using a ViiA 7 Real-Time PCR System (Life Technologies, Pleasanton, CA). Primer sequences were designed and synthesized by Shenggong Company (Shanghai, China) and are shown in Table 1. Relative mRNA expression was normalized to that of β -actin used as endogenous control, and expressed as fold change calculated using

the comparative CT method ($2^{-\Delta\Delta CT}$) with ViiA 7 Software (Life Technologies). The primers shown in Table 1 were also used for conventional RT-PCR, which was carried out at the annealing temperature of 60°C for 32 cycles.

Western blotting

Cells or tissues from 10 mice were pooled together and lysed in radioimmunoprecipitation (RIPA) buffer (Beyotime, Shanghai, China) on ice for 30 min. The lysates were centrifuged at 1000 rpm at 4°C for 10 min to obtain the supernatants. After boiling for 5 min, each 2 µg/µl sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (PVDF, 0.45 µm; Millipore, Bedford, MA). The membrane was blocked using 5% bovine serum albumin (BSA, Beyotime) for 1 hour at room temperature and incubated with the following monoclonal primary antibodies: rabbit anti-TNFα (Abcam, Boston, MA), rabbit anti-GAPDH (CST, Massachusetts State, USA), rabbit anti-Stat3 (CST), and mouse anti-phospho-Stat3 (CST) diluted 1:1000 in 2% BSA) overnight at 4°C. After washing thrice with PBS containing 0.1% Tween-20 (Beyotime, Shanghai, China), the membranes were probed with secondary antibodies (diluted 1:5000 in 2% BSA). Jak inhibitors ruxolitinib or tofacitinib (at 10 µM; Selleck, Houston, USA) were added 2 hours, and exogenous TNFα (R&D systems) was added 30 min, before the cells were harvested. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control.

Simple western analysis

Simple western analysis was performed using 12-230 kDa Wes Separation Module and 8x25 capillary cartridges in accordance with the manufacturer's protocol (ProteinSimple, California, USA). Briefly, after preparing standard pack reagents, samples, and primary antibodies (diluted 1:50) in accordance with manufacturer's instructions, the samples and biotinylated ladder were denatured in a heating block for 5 min and stored on ice until use. Luminol-S and peroxide, supplied in the detection module, were combined in a microcentrifuge tube. The biotinylated ladder, samples, primary antibodies, secondary antibodies, and reagents were dispensed into the assay plate using volumes shown in the plate diagram, and the plate was centrifuged for 5 minutes at 2500 rpm and RT. After completion of the analysis, results were evaluated using Compass software v3.1. The following monoclonal primary antibodies were used in this procedure: rabbit anti-GAPDH (CST), rabbit anti-p44/42 MAPK (Erk1/2, CST), and rabbit anti-Phospho-p44/42 MAPK (CST). ERK inhibitor FR180204 (10 µM, Selleck) was added 2 hours, and exogenous TNFα (R&D systems) was added 30 min, before the cells were harvested.

Statistical analysis

All data were analyzed using SPSS 19.0 (IBM Corporation, Armonk, NY), and Student's t-test was used to compare differences between two groups. All results are shown as mean \pm SD; $p < 0.05$ was considered statistically significant.

Results

Müller cells derived from light-damaged mouse retina show increased proliferation

To investigate the effect of retinal damage induced by over exposure to light, C57BL/6J mice over the age of 6-8 weeks were subjected to 5,000 lux of cool white LED light, for 14 consecutive days. As shown by HE staining (Fig. 1A-C), after 14 days of exposure to light, numerous photoreceptor cells were lost, and the thickness of the outer nuclear layer (ONL) was significantly (** $p \leq 0.01$) decreased in light-damaged retina compared with that of the control retina. Retinas, obtained from mice subjected to light exposure, and from unexposed control mice, were used for primary cell culture. Some of the cells obtained from these retinas were preserved for passaging. The purity of these cells in passage 2 was evaluated by immunocytochemistry using antibodies specific for Müller-cell markers GS and vimentin (Fig 1D-F). More than 90% of cells showed positive labeling for GS and vimentin, indicating that these Müller cells here highly pure. We then used an EdU assay to examine the proliferation capacity of Müller cells from light-treated and control mice. Müller cells obtained from light-treated mice showed a higher EdU+/Hoechst+ ratio (23.0%) than that of Müller cells obtained from controls (17.2%, $p \leq 0.01$, Fig. 1G-I); this finding indicates that the proliferative capacity of Müller cells was increased in a light-damaged mouse retina.

TNF α expression increases in Müller cells obtained from light-treated adult mice

Next, we examined the factors promoting the proliferation of light-damaged Müller cells. For this, we performed RT-PCR to evaluate mRNA expression of growth factors in Müller cells in vitro. Our results indicate that mRNA expression of TNF α and Nfkb β was upregulated 2.06- and 2.50-fold, respectively, in Müller cells cultured from the retina of light-damaged adult mice showing significantly higher levels compared with those of controls ($p \leq 0.001$, Fig. 2A). Other growth factors, including FGF2 and IGF1, did not show increased expression in the light-damaged group. To further investigate the expression of TNF α in Müller cells, we examined supernatants of Müller-cell culture media using ELISA. Our results show that concentration of the TNF α protein in supernatants from the light-damaged group of cells (454.5 ± 29.7 pg/ml) was significantly higher than that in supernatants from controls (372.1 ± 19.2 pg/ml, $p < 0.05$, Fig 2B). In addition, densitometric analyses via western blotting showed that TNF α protein levels were elevated in light-damaged retinas compared with the levels of control retinas (Fig 2C,D). Taken together, these results indicate that TNF α expression increased in Müller cells derived from the retinas of light-damaged mice.

TNF α promotes the proliferation of Müller cells in vitro

Previous studies indicate that Müller cell proliferation is enhanced by TNF α upregulation in the light-damaged retina.⁵ To investigate whether increased TNF α concentration is required for accelerated proliferation of Müller cells in light-damaged mice, we blocked the effect of TNF α by adding a TNF α -neutralizing antibody to the culture medium of Müller cells isolated from adult light-damaged mice; we then evaluated the proliferation capacity of these cells via EdU assays. Our results indicate that the ratio of EdU+ /Hoechst+ cells increased by 33.7 % in the light-damaged group ($p \leq 0.01$; vs. controls groups), and decreased by 16.2% in the cell-culture medium of light-induced mice treated with a TNF α -neutralizing antibody (EdU+/Hoechst+ ratio =17.2% vs. light-damaged group (EdU+ /Hoechst+ ratio =23.0%) (Fig. 3A). This indicates that a neutralizing antibody inhibited Müller cell proliferation in the light-damaged retina.

We then added exogenous TNF α to Müller cell cultures to investigate its effect on cell proliferation in vitro. The results of proliferation assay showed that after treatment with 50 ng/ml TNF α , the relative proliferation rate of Müller cells isolated from PN 3-day mice increased by 42.6% ($p < 0.001$) (Fig 3B)

Microarray analysis of TNF α -regulated genes in Müller cells

Next, we aimed to determine the transcription factors and signaling pathways involved in the proliferation of Müller cells treated with TNF α . For this, we analyzed whole-gene expression using a microarray and evaluated mRNA expression levels of 45,037 transcripts. The results of microarray analysis showed that following stimulation with TNF α , 597 genes showed more than 2-fold changes in expression; this included 389 upregulated genes and 208 downregulated genes. The hierarchical clustering map in Fig. 4A shows all genes with significant expression changes in Müller cells after treatment with TNF α .

KEGG pathway and enrichment analyses were used to further investigate the genes and pathways showing changes in expression after stimulation with TNF α for 24 h ($p < 0.05$, greater than 2-fold change). KEGG pathway analysis showed significant changes in the expression of pathways, such as the TNF signaling pathway, NF-kappa B signaling pathway, MAPK signaling pathway, Jak/Stat signaling pathway, pathways regulating pluripotency of stem cells, those regulating the cell cycle, and Wnt signaling pathway, in Müller cells treated with TNF α ($p < 0.05$) (Fig 4B, Table 2).

We then examined the expression of MAPK and Jak/Stat signaling pathways involved in cellular proliferation. Microarray analysis revealed that six genes in these pathways (*Jak1*, *Jak2*, *Stat1*, *Stat2*, *Map4k4*, *Mapk7*) were upregulated after treatment with TNF α , as shown in Table 3. The results of real-time PCR and RT-PCR were consistent with those of microarray analysis (Fig. 4E, F, Table 3). Expression of inflammatory signaling pathways, including TNF and NF-kappa B signaling pathways, and related

genes (*Nfkb1a*, *Lif*, and *Il-6*) showed significantly enhanced expression after treatment with TNF α (Fig. 4C, F and Table 3).

The mRNA expression of genes related to neuronal stem cells is shown in Fig 6D,F and Table 3. Both microarray and real-time PCR analysis showed that mRNA expression of neuronal stem-cell markers, such as *Hes1*, *Wnt2*, *Sox9*, was significantly downregulated in Müller cells treated with TNF- α . This indicates that neuronal stem-cell signaling pathways may be downregulated by increased expression of TNF- α .

TNF α promotes Müller-cell proliferation via Jak/Stat and MAPK pathways

The results of gene-chip analysis indicate that TNF α stimulated the proliferation of Müller cells via Jak/Stat and MAPK signal pathways. We then used specific signaling-pathway inhibitors, including Jak1/2 inhibitor ruxolitinib, Jak3 inhibitor tofacitinib, and ERK inhibitor FR180204, to examine whether these pathways occur downstream of the TNF α signaling pathway, and whether they participate in regulating the proliferation of Müller cells.

As shown in Fig 5A and C, the proliferation of Müller cells increased after treatment with TNF α (EdU+/Hoechst+ cell ratio from 13.1 to 21.1%, $p < 0.001$); phosphorylation level of Stat3, an important factor in the Jak/Stat signaling pathway, was also significantly elevated. When TNF α was added to Müller cell cultures containing Jak1/2 or Jak3 inhibitors, the phosphorylation level of Stat3 was inhibited, and proliferation of Müller cells decreased compared with that of those treated only with TNF α (EdU+/Hoechst+ cell ratio from 21.1% to 14.1% or 15.9% respectively, $p < 0.001$). These findings indicate that Jak/Stat signaling pathway occurs downstream of the TNF α pathway, and that they both regulate the proliferation of Müller cells.

We additionally found that the phosphorylation level of ERK, an important factor in the MAPK pathway, was elevated after TNF α was added to Müller-cell culture. When TNF α was added to Müller-cell cultures containing an ERK inhibitor, the phosphorylation level of ERK was inhibited, and proliferation of Müller cells decreased compared with that of Müller cells treated only with TNF α (EdU+/Hoechst+ cell ratio from 21.1 to 15.7%, $p < 0.001$, Fig 5B,D). These results indicate that the MAPK signaling pathway acts downstream of the TNF α -pathway to regulate the proliferation of Müller cells.

TNF α drives Müller cells toward A1 phenotype while Wnt counteracts this effect

Neuroinflammation and ischemia can induce two different types of reactive astrocytes called “A1” and “A2,” respectively. The A1 type is induced by activated microglia. A1 astrocytes lack most of the normal astrocyte functions, but gain a new neurotoxic function that induces rapid death in neurons and oligodendrocytes. A2s, which are induced by ischemia, are considered neuroprotective and can regulate

the activity of numerous neurotrophic factors [32]. Because gene chip analysis indicated that neuronal-stem-cell signaling pathways were downregulated in Müller cells treated with TNF α , we hypothesized that TNF α may induce Müller cells toward reactive gliosis rather than toward neurogenesis. Therefore, we performed RT-qPCR to examine the glial phenotype after Müller cells were induced toward reactive gliosis.

After Müller cells were treated with 50 ng/ml TNF α for 24 h, mRNA expression of most genes in cells with A1 phenotype was significantly upregulated compared with that of controls (Fig 6A); mRNA expression of most genes in cells with an A2 phenotype showed no significant changes after treatment with TNF α (Fig 6B). After Müller cells were treated with 100 ng/ml Wnt3a for 24 h, mRNA expression of several genes in cells with an A1 phenotype was significantly downregulated compared with that of controls; other genes in cells with an A1 phenotype showed upregulated expression, but these changes were not statistically significant (Fig 6A). Cells treated with 100 ng/ml Wnt3a and induced toward A2 phenotype showed significant up- and downregulation of several genes, as shown in Fig 6B.

When Müller cells were treated with a combination of 50 ng/ml TNF α and 100 ng/ml Wnt3a for 24 h, most genes in cells with an A1 phenotype showed significantly upregulated expression compared with that of controls, but the fold-change was less than that observed using 50 ng/ml TNF α alone (Fig 6A). The expression of genes in cells with A2 phenotype was significantly upregulated compared with that of controls (Fig 6B). Taken together, these results indicate that treatment with TNF α drove Müller cells toward A1 phenotype, while treatment with Wnt counteracted the effects of TNF α . Treatment with both TNF α and Wnt promoted an A2-like phenotype, but Wnt alone was not sufficient for driving cells toward either an A1 or A2 phenotype.

Discussion

Our present study shows that TNF α expression was increased in mouse light-damaged retina, and that TNF α regulated the proliferation of Müller cells via MAPK and Jak/Stat pathways. Treatment with TNF α alone promoted an inflammatory response and glial-cell proliferation, but did not promote neurogenesis, in the mouse retina.

Subjecting Müller glia of adult rats to light injury drives these cells toward reactive gliosis, which enables them to survive long-term in culture [33]. Studies using retinal explant cultures derived from light-injured rat retina show that Müller glia reenter the cell cycle in response to light injury [15]. In our present study, Müller cells isolated from light-damaged retina of adult mice showed a tendency toward survival and proliferation. Results obtained using an EdU assay demonstrated that Müller cells subjected to light damage showed a higher proliferation ratio than that of controls. The mRNA and protein expression of TNF α increased significantly after induction of light exposure, indicating that TNF α is involved in Müller-cell proliferation in injured mice retina.

TNF α is a pro-inflammatory cytokine involved in inflammation, immune response, synaptic function and cellular proliferation [34-37]. Studies on the role of TNF α in Müller-cell proliferation of zebrafish have shown that TNF α expression increases considerably in light-damaged retina, and that administration of exogenous TNF α induces the proliferation of Müller glia and neurogenesis in undamaged zebrafish retina via ASCL1a and Stat3 pathways [5]. However, limited information is available on the role of TNF α in proliferation of mammalian Müller cells. Previous studies have shown that TNF α expression is increased in injured or degenerated mammalian retinal Müller cells [24, 25]. In this study, we found that TNF α expression was increased after induction of retinal light damage. Administration of a TNF α -neutralizing antibody inhibited the proliferation of light-damaged Müller cells, while treatment with exogenous TNF α promoted the proliferative ability of Müller cells derived from mice of different ages and cultured in vitro. These results indicate that TNF α is a key factor in promotion of Müller-cell proliferation in the light-damaged mouse retina.

Moreover, we found that TNF α may induce Müller-cell proliferation via the Jak/Stat and MAPK pathways in the mouse retina. Previous studies have shown that MAPK signaling pathways are involved in Müller-cell proliferation. Activation of the MAPK pathway occurs commonly in Müller-glia progenitor cells of fish, chicken, and rodent retinas. Wan et al suggested that heparin-binding (HB) EGF/EGF-receptor (EGFR) /MAPK signaling participates in regeneration of injured zebrafish retina by regulating the expression of regeneration-associated genes [19]. Fischer et al. have shown that in damaged chick retina, fibroblast growth factor (FGF) activates the MAPK signaling pathway to promote de-differentiation, and proliferation, of Müller glia into progenitor cells [18]. In the mammalian retina, NMDA-induced damage leads to Müller glia-specific accumulation of pERK1/2 and cFos [13].

In our present study, gene-chip analysis and western blotting demonstrated that mRNA expression of factors involved in the MAPK signaling pathway was upregulated in TNF α -treated Müller cells; ERK phosphorylation was elevated after treatment with TNF α , but TNF α -induced ERK phosphorylation decreased when Müller-cell cultures were pre-treated with an ERK inhibitor. These findings suggest that TNF α participates in regulation of Müller-cell proliferation via MAPK signaling pathways, which are important in regulating the proliferation of Müller glia progenitor cells. Thus, activation of MAPK-signaling may be a requirement for the formation of Müller glia progenitor cells in vertebrate species.

Activation of the Jak/Stat signaling pathway is likely a requirement for the proliferation of Müller cells in zebrafish and in injured mouse retina. David et al. found that exogenous TNF α can induce in-vivo proliferation of Müller glia in undamaged zebrafish retina via Stat3 and ASCL1a signaling pathways [5]. In rodent and chick retinas, ciliary neurotrophic factor (CNTF) /Jak/Stat signaling appears to stimulate glial reactivity and enhance neuroprotection [38, 39]. pStat3 levels are upregulated in HB-EGF-treated Müller glia obtained from NMDA-damaged mouse retinas, indicating that the Jak/Stat pathway is involved in proliferation of mammalian Müller cells [11]. In our present study, we found that numerous genes involved in activation of the Jak/Stat pathways, including *Jak1*, *Jak2*, *Stat 1*, and *Stat2*, were upregulated in TNF α treated Müller cells. The level of phosphorylated Stat3 was also elevated in TNF α -treated Müller cells; however, increased proliferation of Müller glia, induced by treatment with TNF α , was

inhibited when Müller cells were pre-treated with Jak inhibitors. These findings indicate that Jak/Stat pathways may be involved in promoting the proliferation of TNF α -treated Müller cells.

In zebrafish and mammals, ASCL1a is an important factor that promotes the proliferation of Müller glia-derived progenitor cells (MGPCs) and their differentiation into neurons [40, 41]. In our study, TNF α did not upregulate the expression of ASCL1a in Müller cells, indicating that TNF α did not promote the differentiation of MGPCs into neurons.

As mentioned previously, reactive astrocytes are classified into the neurotoxic A1 and the neuroprotective A2 type [32]. Our results indicate that TNF α drove Müller cells toward the A1 phenotype, while Wnt attenuated the A1-promoting effect of TNF α and drove Müller cells toward the A2 phenotype. This finding indicates that the differentiation phenotype promoted by TNF α can be changed by other factors, and that TNF α alone cannot induce the differentiation of mammalian Müller cells into retinal neurons. In mammals, pathways, such as Wnt, Notch, and Shh, likely work in congruence to induce the dedifferentiation and regeneration of Müller cells into retinal neurons

Conclusions

In summary, we detected that Müller-cell proliferation was enhanced and TNF α expression was elevated in light-damaged mouse retinas. Administration of exogenous TNF α induced Müller-cell proliferation via Jak/Stat and MAPK signaling pathways. Our results indicate that TNF α signaling can transiently stimulate the proliferation of Müller cells by activating MAPK and Jak/Stat signaling pathways during early stages of light-induced retinal injury. Our present study had several limitations. TNF α -mediated promotion of mammalian Müller-cell proliferation and differentiation needs to be verified in vivo, which we will address in our future studies. However, this study provides new insights into how light-induced retinal injury regulates the proliferation of mammalian Müller cells, and helps us understand the mechanisms of Müller-cell proliferation in mammalian injured retina.

Abbreviations

TNF α : Tumor necrosis factor alpha; EdU: 5-Ethynyl-2'-deoxyuridine; qPCR: Quantitative polymerase chain reaction; ELISA: Enzyme linked immunosorbent assay; Jak: Janus kinase; Stat: Signal transducer and activator of transcription; MAPK: Mitogen-activated protein kinase; EGF: Epidermal growth factor; FGFs: Fibroblast growth factors; Shh: Sonic hedgehog; NMDA: N-methyl-D-aspartate; MNU: N-methyl-N-nitrosourea; PN: Postnatal; HE: Hematoxylin-eosin; ONL: Outer nuclear layer; DMEM/F12: Dulbecco's Modified Eagle's medium/Ham's F-12; FBS: Fetal bovine serum; GS: Glutamine synthetase; β -ME: β -Mercaptoethanol; KEGG: Genes and genomes; RT-PCR: Reverse transcription-polymerase chain reaction; RIPA: Radioimmunoprecipitation; PVDF: Polyvinylidene fluoride membrane; BSA: Bovine serum albumin;

PBS: Phosphate buffered saline; GAPDH: Glyceraldehyde-3phosphate dehydrogenase; HB-EGF/EGFR: Heparin-binding EGF/EGF-receptor; FGF: Fibroblast growth factor; CNTF: Ciliary neurotrophic factor.

Declarations

Ethics approval and consent to participate

All procedures involving, and care of, animals adhered to the guidelines of Animal Ethics Committee of Eye and ENT Hospital of Fudan University (Shanghai, China).

Consent for publication

Not applicable.

Availability of data and materials

Datasets analyzed during the study are available from the corresponding author on reasonable request.

Competing interests: The authors have no competing interests to declare.

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Authors' contributions

LN, YF, XY, YZ, JW, DC, and XS conceived and designed the experiments; LN, YF, XY, and YZ performed the experiments; LN, YF, XY, and YZ analyzed data; LN, YF, XY, YZ, JW, DC, and XS contributed reagents/materials/analysis tools; LN, YF, XY, YZ, JW, DC, and XS wrote the manuscript

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Tables

Table1. Primer sequences used in quantitative RT-PCR

Gene	Primer	Product length (bp)
<i>Il-6</i>	Forward: 5'GGAGTGGCTAAGGACCAAGA 3'	102
	Reverse: 5' GGCATAACGCACTAGGTTTG 3'	
<i>Wnt2</i>	Forward: 5'CATAGCCCCCACCCTGT 3'	74
	Reverse: 5' AGTTCCTTCGCTATGTGATGTTTCT 3'	
<i>Hes1</i>	Forward: 5'TCGCTCACTTCGGACTCCAT 3'	84
	Reverse: 5' AGGTGGGCTAGGGACTTTACG 3'	
<i>Sox9</i>	Forward: 5'CAAACCTGAAACCTGTCTCTCTC 3'	105
	Reverse: 5' CAAGGTCTATGGCTGAAAACA 3'	
<i>Jak1</i>	Forward: 5'TGTTTGCACAGGGACAGTATG 3'	101
	Reverse: 5' CCCAGGCACTCATTTTCAAT 3'	
<i>Jak2</i>	Forward: 5'CAGACAGATGGGAAGGGAAG 3'	70
	Reverse: 5' ACACGCCTGCTGGTATTCTT 3'	
<i>Stat1</i>	Forward: 5'CTGCTGTGCCTCTGGAATGA 3'	72
	Reverse: 5' CTTGAGCAGAGCGGTTCT 3'	
<i>Stat2</i>	Forward: 5'TGTACTIONCCTTCGCTCCTGA 3'	93
	Reverse: 5'TGTTTTACATGGTGGGCTGA 3'	
<i>Map4k4</i>	Forward:5'GGGAGAGAAGGCAATAGAGATACG3'	77
	Reverse: 5' TCTGAGCCCTTTTGTGCATAAA 3'	
<i>Mapk7</i>	Forward: 5'CCTCCAAGAGCCCTGAAACC 3'	73
	Reverse: 5' GCACCCCTGGACCTTGATG 3'	
<i>Lif</i>	Forward: 5'GCTGCTCTCCCTCTTTCCCTT 3'	81
	Reverse: 5' CCCACAGGGTACATTCATCA 3'	
<i>Nfkbia</i>	Forward: 5'CCATGTAAGAGCCAGGTGTTCA 3'	79
	Reverse: 5' CATTTACAAGAAGGCGACACAGA 3'	
<i>Ascl1</i>	Forward: 5'AGATGAGCAAGGTGGAGACG 3'	166
	Forward: 5'GGAGAACCCGCCATAGAGTT 3'	

A1	primers	Product length (bp)
<u>phenotype</u>		
<i>H2-D1</i>	Forward: 5' TCCGAGATTGTAAGCGTGAAGA 3' Reverse: 5' ACAGGGCAGTGCAGGGATAG 3'	204
<i>Serping1</i>	Forward: 5' ACAGCCCCCTCTGAATTCTT 3' Reverse: 5' GGATGCTCTCCAAGTTGCTC 3'	299
<i>H2-T23</i>	Forward: 5' GGACCGCGAATGACATAGC 3' Reverse: 5' GCACCTCAGGGTGA CTTCAT 3'	212
<i>Ggta1</i>	Forward: 5' GTGAACAGCATGAGGGGTTT3' Reverse: 5' GTTTTGTTGCCTCTGGGTGT 3'	115
<i>ligp1</i>	Forward: 5' GGGGCAATAGCTCATTGGTA 3' Reverse: 5' ACCTCGAAGACATCCCCTTT 3'	104
<i>Gbp2</i>	Forward: 5' GGGGTCACTGTCTGACCACT 3' Reverse: 5' GGGAAACCTGGGATGAGATT 3'	285
<i>Fkbp5</i>	Forward: 5' TATGCTTATGGCTCGGCTGG 3' Reverse: 5' CAGCCTTCCAGGTGGACTTT 3'	194
<i>Psmb8</i>	Forward: 5' CAGTCCTGAAGAGGCCTACG 3' Reverse: 5' CACTTTCACCCAACCGTCTT 3'	121
<i>Amigo2</i>	Forward: 5' GAGGCGACCATAATGTCGTT 3' Reverse: 5' GCATCCAACAGTCCGATTCT 3'	263
<i>Srgn</i>	Forward: 5' GCAAGGTTATCCTGCTCGGA 3' Reverse: 5' TGGGAGGGCCGATGTTATTG 3'	134
<hr/>		
A2	primers	Product length (bp)
<u>phenotype</u>		
<i>Clcf1</i>	Forward: 5' CTTCATCCTCCTCGACTGG 3' Reverse: 5' TACGTCGGAGTTCAGCTGTG 3'	176
<i>S100a10</i>	Forward: 5' CCTCTGGCTGTGGACAAAAT 3' Reverse: 5' CTGCTCACAAGAAGCAGTGG 3'	238
<i>Sphk1</i>	Forward: 5' GATGCATGAGGTGGTGAATG 3' Reverse: 5' TGCTCGTACCCAGCATAGTG 3'	135
<i>Cd109</i>	Forward: 5' CACAGTCGGGAGCCCTAAAG 3' Reverse: 5' GCAGCGATTTTCGATGTCCAC 3'	147
<i>Ptgs2</i>	Forward: 5' GCTGTACAAGCAGTGGCAAA 3' Reverse: 5' CCCCAAAGATAGCATCTGGA 3'	232

<i>Emp1</i>	Forward: 5' GAGACACTGGCCAGAAAAGC 3' Reverse: 5' TAAAAGGCAAGGGAATGCAC 3'	183
<i>Slc10a6</i>	Forward: 5' GCTTCGGTGGTATGATGCTT 3' Reverse: 5' CCACAGGCTTTTCTGGTGAT 3'	217
<i>Tm4sf1</i>	Forward: 5' GCCCAAGCATATTGTGGAGT 3' Reverse: 5' AGGGTAGGATGTGGCACAAG 3'	258
<i>B3gnt5</i>	Forward: 5' CGTGGGGCAATGAGAACTAT 3' Reverse: 5' CCCAGCTGAACTGAAGAAGG 3'	207
<i>Cd14</i>	Forward: 5' GGACTGATCTCAGCCCTCTG 3' Reverse: 5' GCTTCAGCCCAGTGAAAGAC 3'	232
<i>β-actin</i>	Forward: 5'TGGCTCCTAGCACCATGAAGA 3' Reverse: 5' GCCACCGATCCACACAGAGT 3'	71

FGF2: basic fibroblast growth factor 2; IGF1: insulin-like growth factor 1

Table 2. Pathways showing significantly altered expression in Müller cells after stimulation with TNF- α for 24 h

Pathway Name	Gene Ratio	P-value	Representative genes
TNF signaling pathway	39 / 892	0.0000	Lif, Il-6, Cxcl1
NF-kappa B signaling pathway	25 / 892	0.0028	Nfkbia, Nfkb2, Vcam1
MAPK signaling pathway	48 / 892	0.0030	Map4k4, Mapk7
Jak-Stat signaling pathway	31 / 892	0.0132	Jak1, Jak2, Stat1, Stat2
Signaling pathways regulating pluripotency of stem cells	33 / 892	0.0005	Hes1, Il6st, Gsk3b
Wnt signaling pathway	31 / 892	0.0041	Wnt2, Wnt9a, Jun
Cell cycle	25 / 892	0.0188	Sox9, Myc, Smad3

Table 3. Validation of microarray results via qPCR.

Gene	Fold change (Mean± SEM)	
	Microarray (n = 3)	RT-qPCR (n = 3)
<i>Jak1</i>	1.30*	1.46 ±0.14*
<i>Jak2</i>	2.54**	1.62 ±0.21*
<i>Stat1</i>	1.74*	2.82 ±0.76*
<i>Stat2</i>	2.11**	2.51±0.22**
<i>Map4k4</i>	1.88**	2.22±0.10*
<i>Mapk7</i>	1.50*	1.51±0.24*
<i>Nfkbia</i>	6.86***	20.34±0.61***
<i>Lif</i>	4.96**	6.67±0.56***
<i>Il6</i>	3.26**	3.85±0.45**
<i>Hes1</i>	0.64*	0.56±0.02*
<i>Wnt2</i>	0.367**	0.42±0.04*
<i>Sox9</i>	0.35***	0.27±0.09***
<i>Ascl1</i>	0.67	0.17 ±0.05*

* $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, compared with controls.

Figures

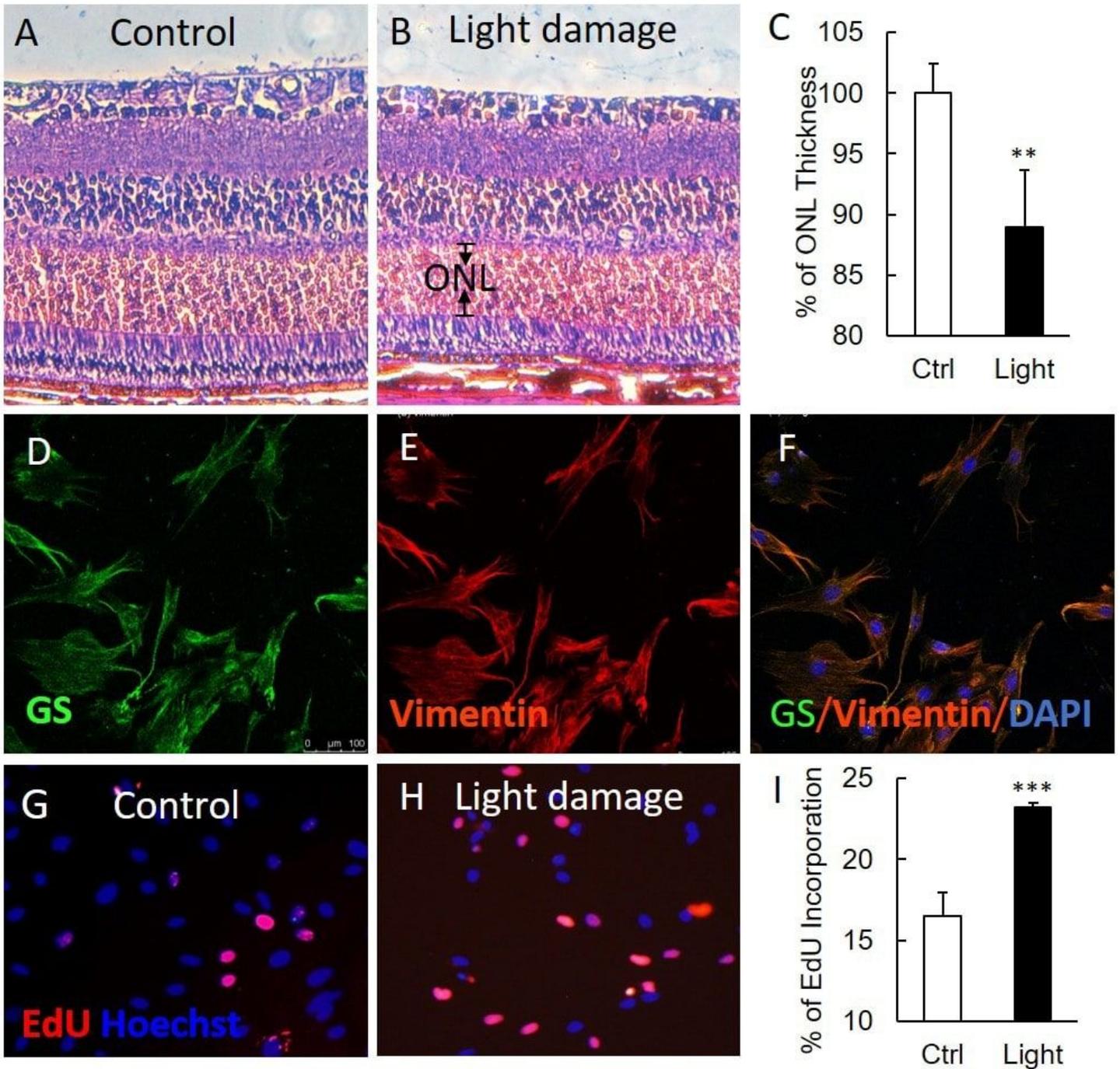


Figure 1

Müller-cell proliferation is enhanced after induction of light damage to mouse retina. (A-C) HE staining shows that the outer nuclear layer (ONL) in the light-damaged mouse retina is thinner than that in controls (** $p \leq 0.01$). (D-F) Identification of Müller cells. (G-I) Müller cells isolated from light-injured mouse retina and control mouse retina were evaluated using a cell proliferation assay. The rates of EdU+/Hoechst+ cells were 17.2% in the controls and 23.0% in the light-damaged group (bars: 200 μ m; ** $p \leq 0.01$; data were analyzed using Student's t-test)

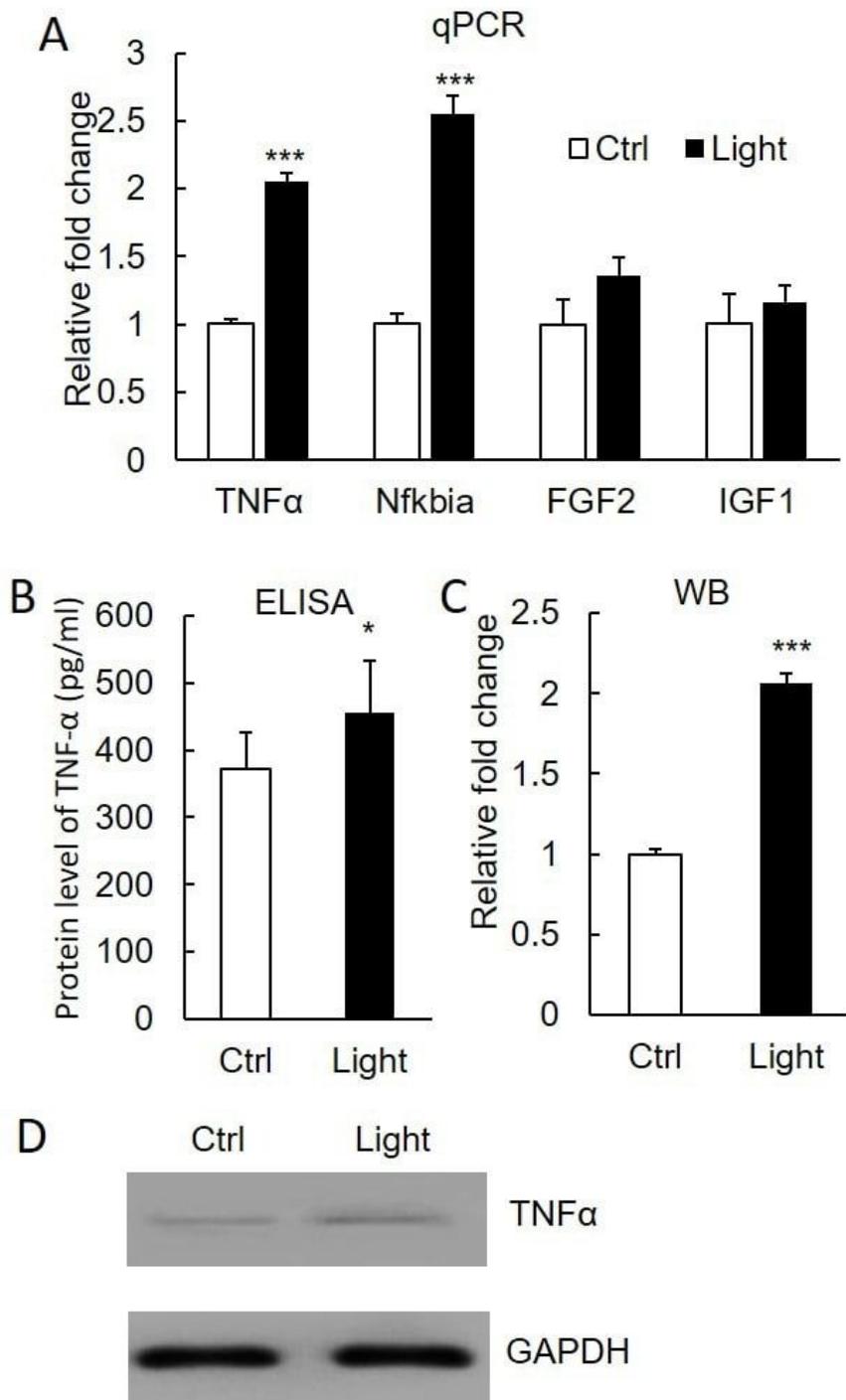


Figure 2

Light-induced retinal damage causes increased expression of TNF- α . (A) Müller cells isolated from light-injured retina and controls were analyzed using RT-qPCR, which showed that expression levels of TNF- α and Nfkb1a mRNA increased significantly in the light-damaged group compared with those of the control group (***) $p \leq 0.001$, compared with controls; data were analyzed using Student's t-test). (B) ELISA immunoassay showed that the levels of TNF- α protein in the media of the light-injured group was

significantly higher (454.5 ± 29.7 pg/ml) than that in the media of the control group (372.1 ± 19.2 pg/ml) (* $p < 0.05$, compared with controls; data analyzed using Student's t-test). (C,D) Western blot shows that the level of TNF- α protein was upregulated in the light-damaged group compared with that of the control group (** $p \leq 0.001$).

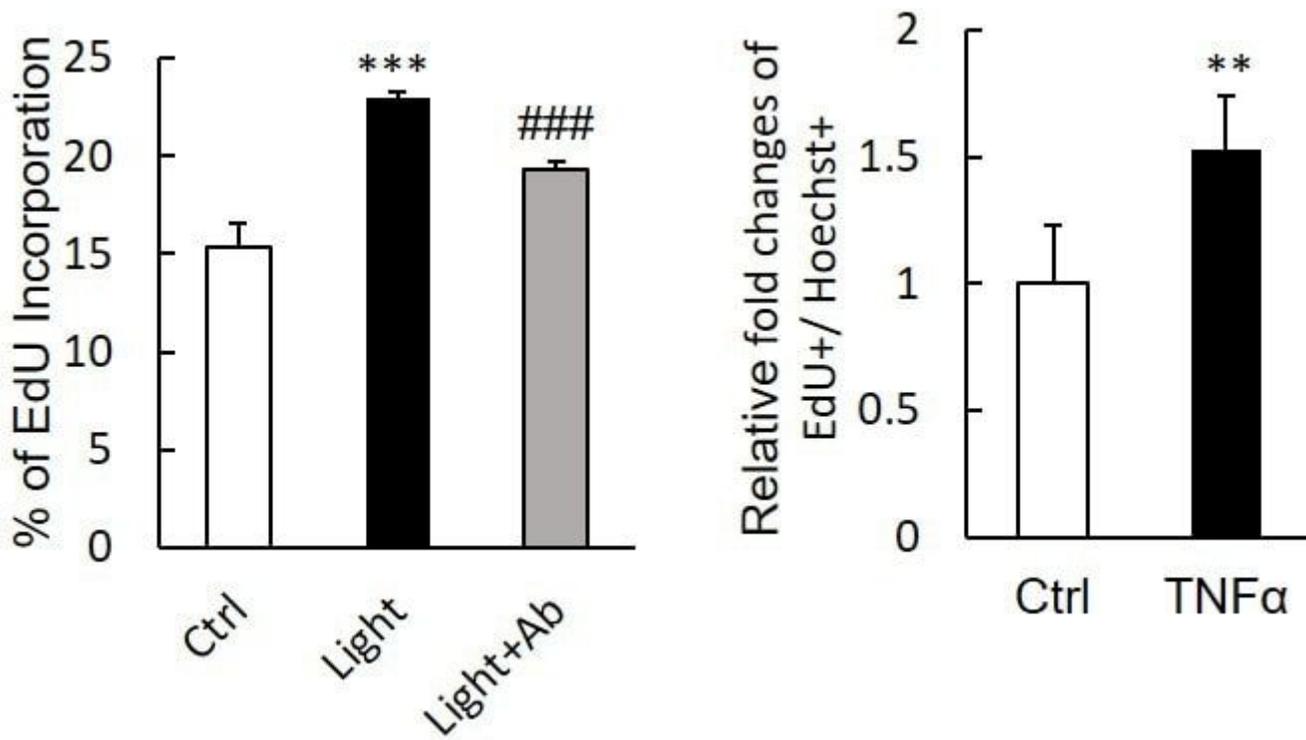


Figure 3

TNF- α promotes proliferation of Müller cells. (A) Müller cells isolated from light-injured mouse retina and control mouse retina were analyzed using a cell proliferation assay. The rates of EdU+/Hoechst+ cells were 17.2% in controls, 23.0% in the light-damaged group, and 19.2% in the light-damaged group treated with a neutralizing antibody specific for TNF- α . (B) Müller cell proliferation followed by TNF- α treatment compared with the control group in PN3 day mice (PN: postnatal; Ctrl=Control, TNF- α = 50ng/ml TNF- α , Light=light damage, Ab= neutralizing antibody specific for TNF- α ; * $p < 0.05$, *** $p \leq 0.001$, compared with controls; ### $p \leq 0.001$, compared with TNF- α -treated group; data were analyzed using Student's t-test).

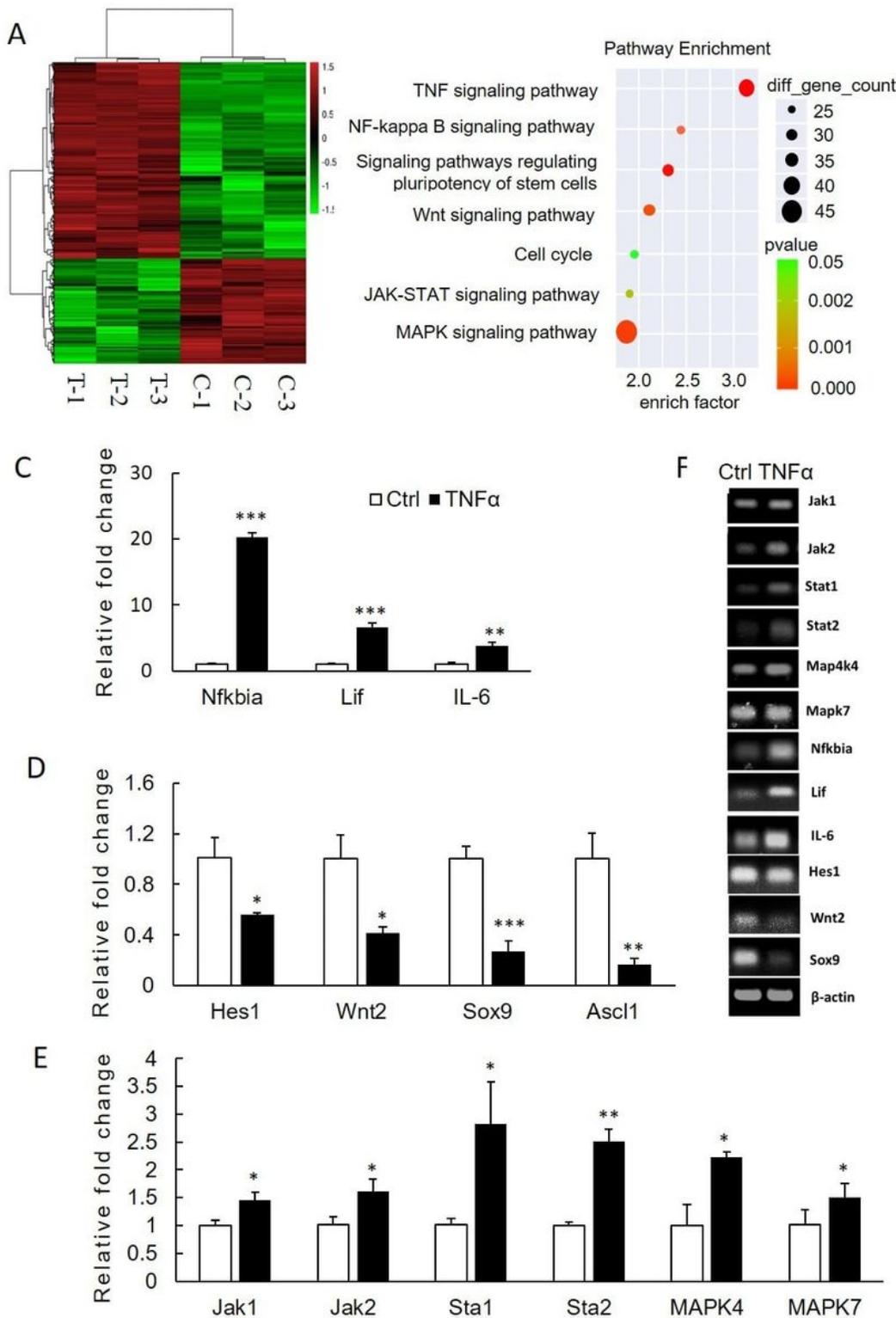


Figure 4

Microarray Analysis Screened TNF α -regulated Genes in Müller cells (A) Hierarchical cluster analysis of differentially expressed genes ($p < 0.05$, more than 2 fold change) followed by TNF- α 50ng/ml stimulation for 24h. Each row represents a probe and each column represents one sample. The values represent the fold changes compared with the corresponding control. Positive and negative fold changes are shown in red and green, respectively, as shown in the color bar. (B) Functional classification into pathways of the

genes differentially expressed between TNF- α -treated Müller cells and untreated controls. The analysis was involved KEGG pathways. (T: TNF- α stimulation group; C: control group). (C-E) Validation of microarray gene expression by PCR. Comparisons were made of mRNA expression of genes related to inflammation(C), stem cells(D) and cell proliferation(E) in mice Müller glia of TNF- α treatment and control group using real-time RT-PCR (Ctrl: control; * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, compared with controls; data were analyzed using Student's t-test.) (F) Representative results of semi-quantitative RT-PCR, performed to analyze TNF- α -treated and control Müller glia, agree with those of real-time RT-PCR.

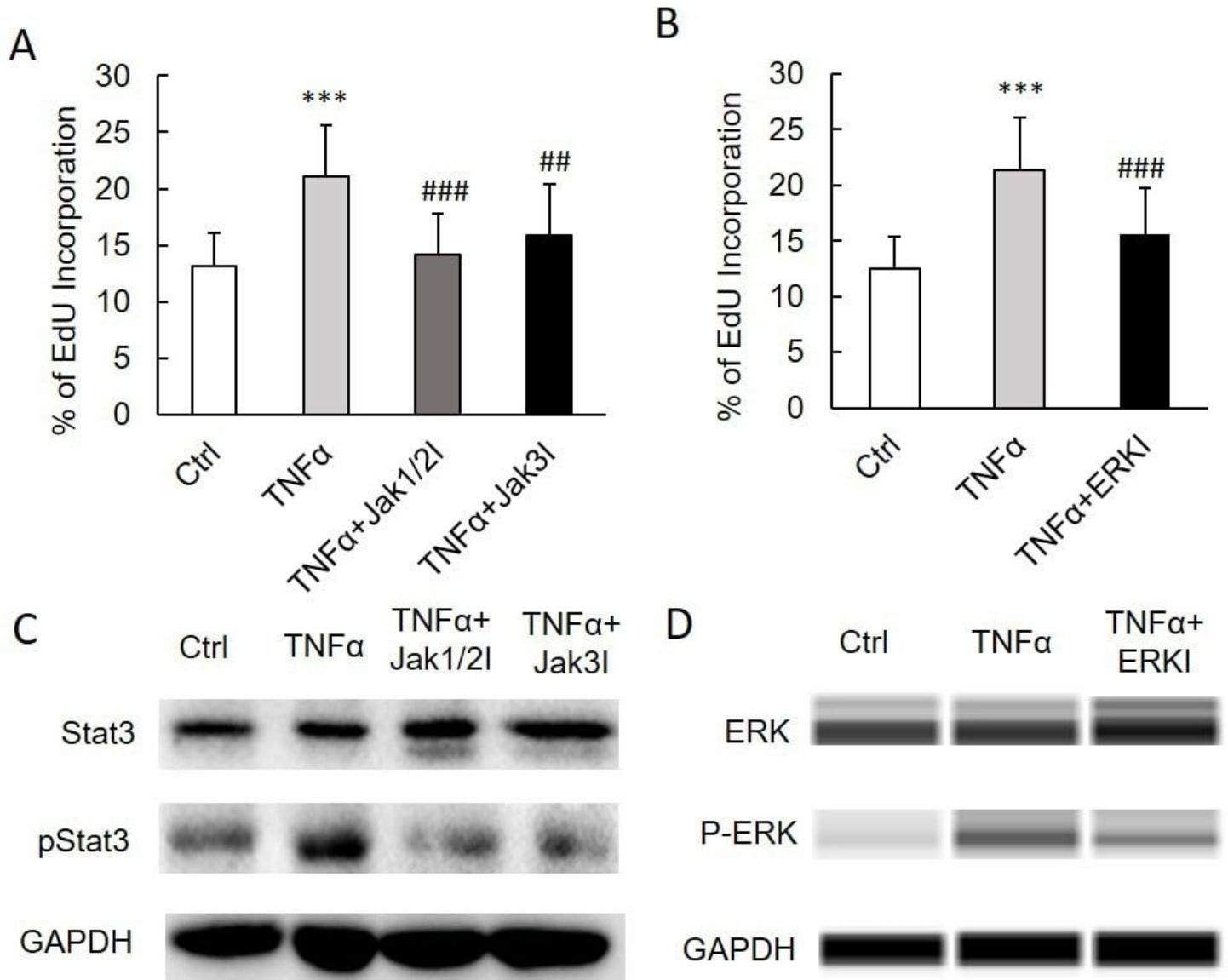


Figure 5

TNF- α induces Müller cell proliferation via Jak-Stat and MAPK pathways in mouse retina. (A,B) Cell proliferation assay of Müller cells shows increased proportion of EdU+ / Hoechst+ cells in the TNF- α -treated groups compared with that of the control group; this increased proliferative capacity of Müller cells, induced by TNF- α , was decreased by pre-treatment with JAK1/2 inhibitor ruxolitinib, JAK3 inhibitor tofacitinib, or ERK inhibitor FR180204. (Jak1/2I= Jak1/2 inhibitor, JAK3I=Jak3 inhibitor; ERKI= ERK

inhibitor; *** ≤ 0.001 , compared with controls; ## $p \leq 0.01$, ### ≤ 0.001 , compared with TNF- α -treated group; data were analyzed using Student's t-test). (C,D) Results of western blotting show that phosphorylation levels of Stat3 and p44/42 MAPK in Müller cells were upregulated by treatment with TNF- α ; this trend was decreased by pre-treating Müller cells with Jak or ERK inhibitors.

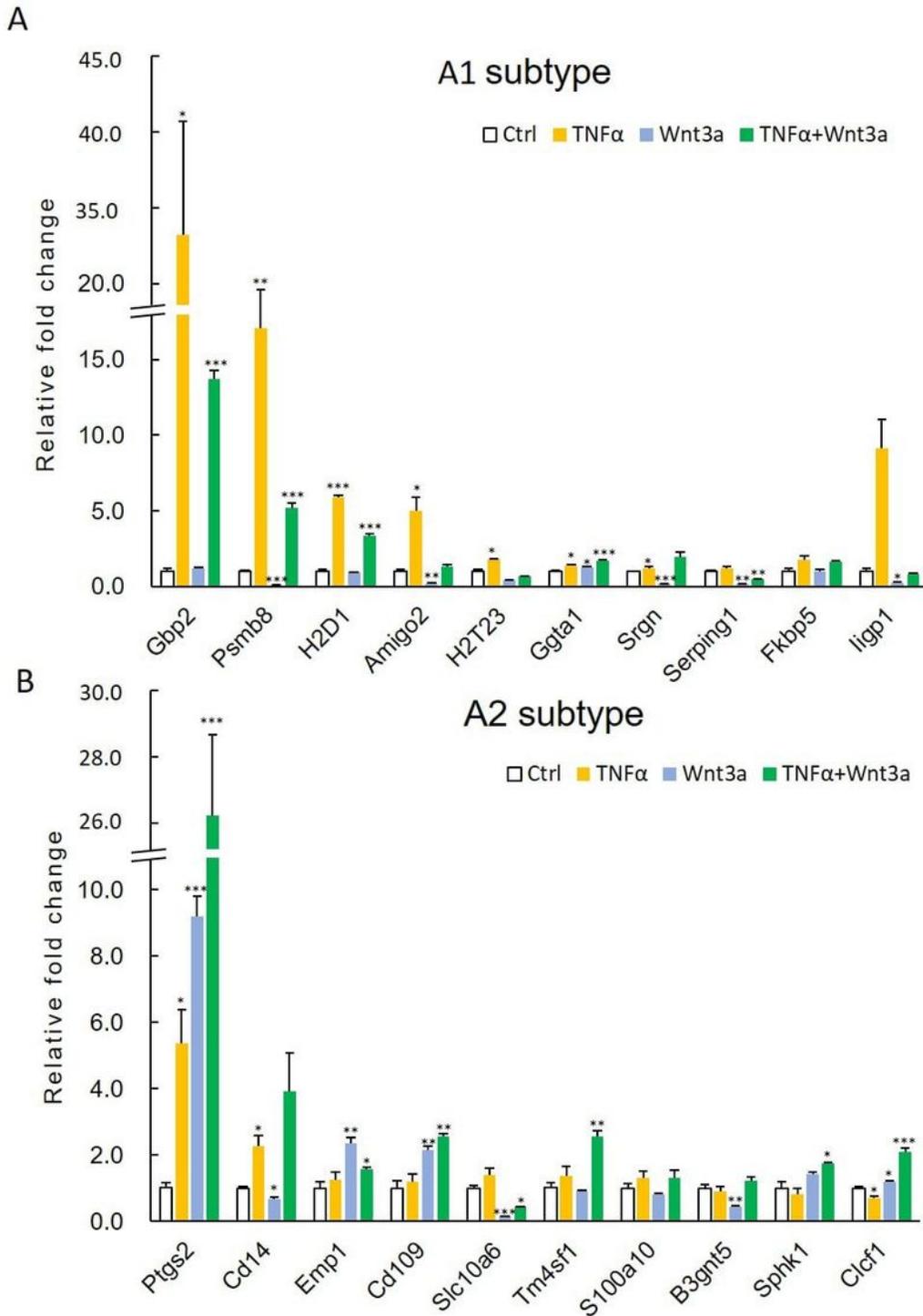


Figure 6

TNF- α drives Müller cells toward A1 phenotype, while Wnt attenuates this effect of TNF- α . (A). To assess the expression of A1 phenotype in Müller cells, the cells were treated for 24 h with 50 ng/ml TNF- α , 100 ng/ml Wnt3a, or a combination of 50 ng/ml TNF- α and 100 ng/ml Wnt3a. (B) To assess the expression of A2 phenotype in Müller cells, the cells were treated for 24 h with 50 ng/ml TNF- α , 100 ng/ml Wnt3a, or a combination of 50 ng/ml TNF- α and 100 ng/ml Wnt3a.