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p38 MAPK signaling acts upstream of LIF-dependent neuroprotection during photoreceptor degeneration

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In many blinding diseases of the retina, loss of function and thus severe visual impairment results from apoptotic cell death of damaged photoreceptors. In an attempt to survive, injured photoreceptors generate survival signals to induce intercellular protective mechanisms that eventually may rescue photoreceptors from entering an apoptotic death pathway. One such endogenous survival pathway is controlled by leukemia inhibitory factor (LIF), which is produced by a subset of Muller glia cells in response to photoreceptor injury. In the absence of LIF, survival components are not activated and photoreceptor degeneration is accelerated. Although LIF is a crucial factor for photoreceptor survival, the detailed mechanism of its induction in the retina has not been elucidated. Here, we show that administration of tumor necrosis factor-alpha (TNF) was sufficient to fully upregulate *Lif* expression in Muller cells *in vitro* and the retina *in vivo*. Increased *Lif* expression depended on p38 mitogen-activated protein kinase (MAPK) since inhibition of its activity abolished *Lif* expression *in vitro* and *in vivo*. Inhibition of p38 MAPK activity reduced the *Lif* expression also in the model of light-induced retinal degeneration and resulted in increased cell death in the light-exposed retina. Thus, expression of *Lif* in the injured retina and activation of the endogenous survival pathway involve signaling through p38 MAPK.

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Throughout our life span, cells have to be repaired or regenerated in order to maintain tissue function. Whereas this happens frequently in most parts of the body, the regeneration capacity of the nervous system is generally very limited in mammals, and the consequences of neuronal disease or injury are mostly irreversible. Therefore, it is crucial to rescue neurons from devastating insults, a task that requires activation of endogenous neuroprotective systems. In the retina, several pathways exist to support the survival of neurons.[1–7] Leukemia inhibitory factor (LIF) controls one of these pathways and is one of the most important endogenous factors for neuroprotection in the retina.[1,2] Photoreceptor injury or degeneration activates a subset of Muller glia cells to express *Lif*, which controls a cascade of neuroprotective signaling between photoreceptors and Muller cells.[1,8] These signaling events include activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway,[9–11] and result in the upregulation of several genes important for neuroprotection, including signal transducer and activator of transcription-3 (*Stat3*), endothelin-2 (*End2*) and fibroblast growth factor-2 (*Fgf2*), and for gliosis (glial fibrillary acidic protein, *Gfap*).[1,12] In the absence of LIF, none of these factors is induced, and photoreceptor cell death is accelerated.[1,12]

Although downstream events of LIF signaling have been studied in the retina,[1,12–14] the signal that initiates *Lif* expression has not been elucidated. One of the hypotheses for the initiation of survival pathways is based on the generation of reactive oxygen species (ROS) in stressed photoreceptors.[15–17] Subtoxic levels of ROS have been shown to be neuroprotective for photoreceptors and ROS may act as signaling molecules for survival pathways in the retina.[15–18] Another hypothesis especially with respect to the involvement of Muller cells includes tumor necrosis factor-alpha (TNF) signaling, as TNF has been recently shown to be the key signaling molecule for Muller cell proliferation and differentiation into a photoreceptor fate in the degenerating zebrafish retina.[19] However, its role during photoreceptor degeneration in the mammalian retina has not been identified in detail.

TNF was shown to regulate expression of several important factors that mediate a proinflammatory response. Likewise, TNF treatment upregulates several cytokines including *Lif* in various cell types.[20,21] The reported neuroprotective effect of TNF is mostly attributed to nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation and the resulting differential regulation of gene expression.[22–27]

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Abbreviations: BSA, bovine serum albumin; Clc, cardiotrophin-like cytokine; Cntf, ciliary neurotrophic factor; End2, endothelin-2; Fgf2, fibroblast growth factor-2; Gfap, glial fibrillary acidic protein; IL-6, interleukin-6; JAK/STAT, Janus kinase/signal transducer and activator of transcription; Lif, leukemia inhibitory factor; Lifr, leukemia inhibitory factor receptor; LIRD, light-induced retinal degeneration; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; Pax6, paired box protein-6; RGC, retinal ganglion cell; rMC-1, rat Muller glia cell line-1; ROS, reactive oxygen species; SEM, standard error of the mean; Stat3, signal transducer and activator of transcription-3; TLR2, Toll-like receptor-2; TNF, tumor necrosis factor; Vim, vimentin
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However, divergent data exist and various reports attribute TNF also a role in the promotion of neurodegeneration (reviewed in detail[22,23]). Blocking TNF in a glaucoma model of retinal ganglion cell (RGC) death, for example, had beneficial effects on RGC survival.[28–30] In contrast, increased survival of RGCs after axotomy of the optic nerve required elevated and not decreased TNF levels.[31]

In addition to NFκB, TNF has been shown to activate the p38 mitogen-activated protein kinase (MAPK) pathway in a variety of models, and a small number of genes have been identified that are regulated through p38 MAPK after TNF induction.[27] However, similar to TNF, no consensus exists on the anti- or pro-apoptotic effects of p38 MAPK activity in neuronal tissues. Active p38 MAPK signaling has been shown to contribute to RGC death after ischemia or optic nerve axotomy.[32,33] In contrast, recent findings suggest that p38 MAPK activity is important for RGC survival after ischemia/reperfusion injury,[34,35] and that active crosstalk between NFκB and p38 MAPK pathways may be an important aspect of this neuroprotection.[35]

To date, the effects of TNF and p38 MAPK on photoreceptor protection and their possible role in the regulation of neuroprotective factors in the retina have not been studied. To gain insight into the mechanisms that regulate LIF-mediated neuroprotection, we investigated the contributions of TNF and p38 MAPK to *Lif* expression both *in vitro* and *in vivo*, and tested the relevance of the findings in the model of light-induced retinal degeneration (LIRD) *in vivo*. The results show that p38 MAPK activity is neuroprotective and required to upregulate expression of *Lif* in the injured retina.

Results

TNF upregulates *Lif* expression through p38 MAPK in cultured Muller cells *in vitro*. Previously, it has been shown that treatment with recombinant TNF induces upregulation of *Lif* expression in fibroblasts and other cells.[20,21] Since LIF is crucial for endogenous neuroprotection in the retina and is expressed by a subset of Muller cells upon photoreceptor injury, we tested whether Muller cells upregulate *Lif* in response to TNF administration *in vitro*. When cultured rat Muller cells (rMC-1)[36] were treated with TNF, *Lif* and *Tnf* were simultaneously upregulated 10.7- and 21-fold, respectively (Figure 1). This transcriptional response was fast and reached its peak at 1 h before it gradually decreased towards basal levels even though TNF was still present in the culture medium. This suggests a transcriptional induction by TNF followed by suppression of expression. The transient upregulation of *Lif* and *Tnf* in Muller cells is consistent with results from previously studied models.[20,21] We also tested expression of genes that are known to be upregulated in activated Muller cells including *Gfap*, ciliary neurotrophic factor (*Cntf*) and *Fgf2*.[1] However, none of these genes was activated by TNF demonstrating a specificity of TNF for the regulation of *Lif* expression in rMC-1 (Figure 1).

Stimulation of cells with TNF has been previously shown to induce p38 MAPK signaling. Thus, we determined the role of p38 MAPK in the regulation of *Lif* expression. Since the expression of *Lif* in cultured Muller cells was robust, we first

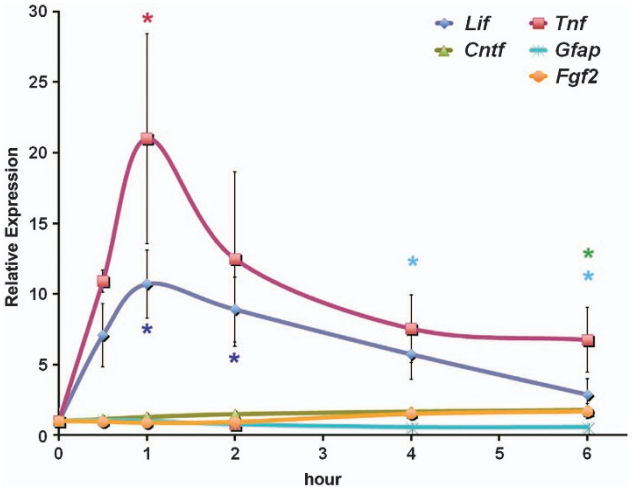

Figure 1 TNF treatment transiently upregulates *Lif* expression in Muller cells *in vitro*. Real-time PCR analysis of gene expression in rMC-1 cells before (0 h) or at various timepoints during TNF treatment as indicated. TNF (10 ng/ml) treatment resulted in a strong upregulation of *Lif* expression as early as 30 min. Peak of expression was at 1 h. *Tnf* expression was upregulated similarly to *Lif*. In contrast, expression of *Cntf*, *Gfap* and *Fgf2* was not affected by TNF. Shown are means ± S.E.M. of $N=3$. ANOVA with Dunnett's post-tests was used to compare control levels (at '0') with expression levels of each gene at all timepoints after TNF treatment. Note that the color of the stars indicating significance match the color of the respective gene. *$P<0.05$

tested the effect of p38 MAPK activity on basal *Lif* expression in the absence of TNF by using two specific chemical inhibitors for p38 MAPK activity, SB239063 and SB202190.[37,38] Treatment with either SB compound downregulated *Lif* expression in a dose-dependent manner within 1 h of treatment and at a similar concentration range (Figures 2a and b). As expected, inhibitor treatment did not block phosphorylation of p38 MAPK (see also Figures 5b and c) but prevented its activity reducing activation of downstream targets like heat shock protein-27 (data not shown). The effect of p38 MAPK inhibition was specific for *Lif* as the expression of *Gfap*, *Cntf* and *Fgf2* was not reduced (Figures 2a and b).

To analyze whether TNF-mediated upregulation of *Lif* expression also involves p38 MAPK signaling, we co-treated Muller cells with TNF and SB239063. Consistent with our results above (Figure 1), TNF treatment induced *Lif* expression (Figure 3a). However, inhibition of p38 MAPK activity by SB239063 completely blocked *Lif* upregulation in the presence of TNF (Figure 3a), suggesting that p38 MAPK activity is crucial not only for basal *Lif* expression but also for TNF-induced *Lif* upregulation.

Another signaling pathway known to be activated by TNF centers around NFκB. Since multiple potential binding sites for NFκB are located in the *Lif* promoter region (not shown) and NFκB was identified to bind the *Lif* promoter upon stimulation of cells with Toll-like receptor-2 (TLR2) agonists,[39] we determined the activation of NFκB in Muller cells upon TNF treatment using a luciferase reporter vector that contains NFκB binding elements. Treatment with TNF strongly increased luciferase levels suggesting that NFκB was activated in Muller cells under these conditions (Figure 3b). Importantly, addition of the p38 MAPK inhibitor SB202190

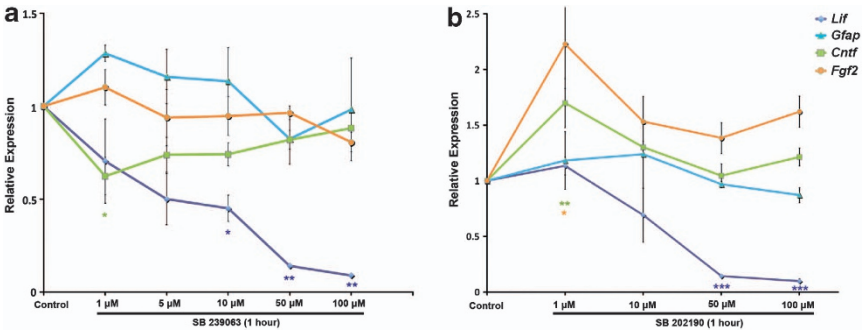

Figure 2 Inhibition of p38 MAPK activity downregulates *Lif* expression in Muller cells *in vitro*. Real-time PCR analysis of gene expression in rMC-1 cells before (control) or at 1 h of treatment with various concentrations (as indicated) of p38 MAPK inhibitors SB239063 (**a**) or SB202190 (**b**). Expression levels of *Lif* inversely correlated with the concentration of p38 MAPK inhibitors. Expression levels of *Cntf*, *Gfap* and *Fgf2* were not affected. Shown are means ± S.E.M. of $N = 3$. ANOVA with Dunnett's post-tests was used to compare control levels with expression levels of each gene after inhibitor treatment. Note that the color of the stars indicating significance match the color of the respective gene. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$

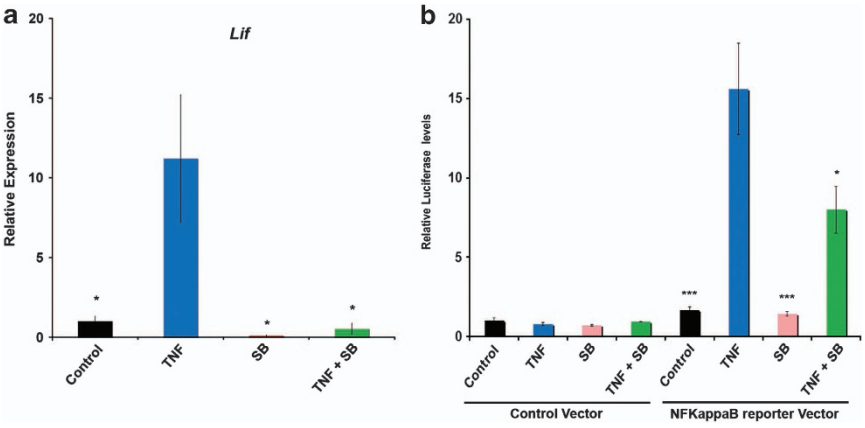

Figure 3 Inhibition of p38 MAPK activity prevents TNF-induced upregulation of *Lif* expression in Muller cells *in vitro*. (**a**) RT PCR analysis of *Lif* expression in rMC-1 cells before (control, black bar) or at 1 h of treatment with TNF (10 ng/ml, blue bar), p38 MAPK inhibitor SB239063 (SB, 100 μM, pink bar) or a combination of both (green bar). Inhibition of p38 MAPK activity downregulated basal *Lif* expression and blocked TNF-induced *Lif* upregulation. Shown are means ± S.E.M. of $N = 3$. ANOVA with Dunnett's post-tests was used to compare *Lif* levels after TNF injection with control levels or levels after SB or SB + TNF treatment. (**b**) Luciferase levels in rMC-1 cells transfected with a control vector or an NFκB reporter vector. Cells were treated for 6 h with TNF (10 ng/ml, blue bars), p38 MAPK inhibitor SB202190 (SB, 100 μM, pink bars), or a combination of both (green bars). Controls (black bars) were not treated. NFκB-mediated luciferase expression was upregulated by TNF treatment. The TNF-mediated upregulation was partially blocked by the inhibitor of p38 MAPK activity. ANOVA with Dunnett's post-tests was used to compare luciferase levels after TNF injection with levels of other treatments. Statistics were calculated seperately for the 'control vector' group and the 'NFκB vector' group. Shown are means ± S.E.M. of $N = 4$

completely inhibited TNF-induced *Lif* upregulation (Figure 3a), but reduced TNF-induced NFκB activity only by 49% leaving it still fivefold above control levels (Figure 3b). This indicates that, although p38 MAPK and NFκB pathways may interact, increased NFκB activity may not be sufficient to induce *Lif* expression in Muller cells in response to TNF treatment (Figure 3b) *in vitro*.

Regulation of *Lif* expression in the neuronal retina *in vivo*. Our results show that TNF induces *Lif* expression via the p38 MAPK pathway in Muller cells *in vitro*. To determine whether *Lif* expression is similarly regulated in the healthy or injured neuronal retina *in vivo*, we injected TNF, a water-soluble p38 MAPK inhibitor (SB220025 trihydrochloride),[40] or a combination of both into the vitreous of wild-type mice that were or were not exposed to damaging levels of white light (Figures 4–7).

Similar to our observations in cultured Muller cells, TNF injections increased the expression of *Lif* and *Tnf* in the retina within 1 – 2 h before levels gradually decreased again (Figure 4). Since *Fgf2*, *End2* and *Stat3* are part of the LIF-controlled endogenous neuroprotective signaling system,[1,12] we also analyzed their expression pattern after TNF injections. Expression of all three genes was significantly upregulated and peaked at around 12 h after injection and thus with a slight delay compared with *Lif* (Figure 4). Although not directly tested, this suggests that LIF may also be important for *Fgf2*, *End2* and *Stat3* expression after TNF injections, as it is in the injured retina.[1,12] Similarly, expression of *Gfap*, which has also been shown to depend on LIF signaling,[1] was upregulated with a similar delay (Figure 4). In contrast, expression of paired box protein-6 (*Pax6*), *Lif* receptor (*Lifr*) and *Cntf*, genes that may not depend on LIF signaling, was not comparably regulated. Although CNTF

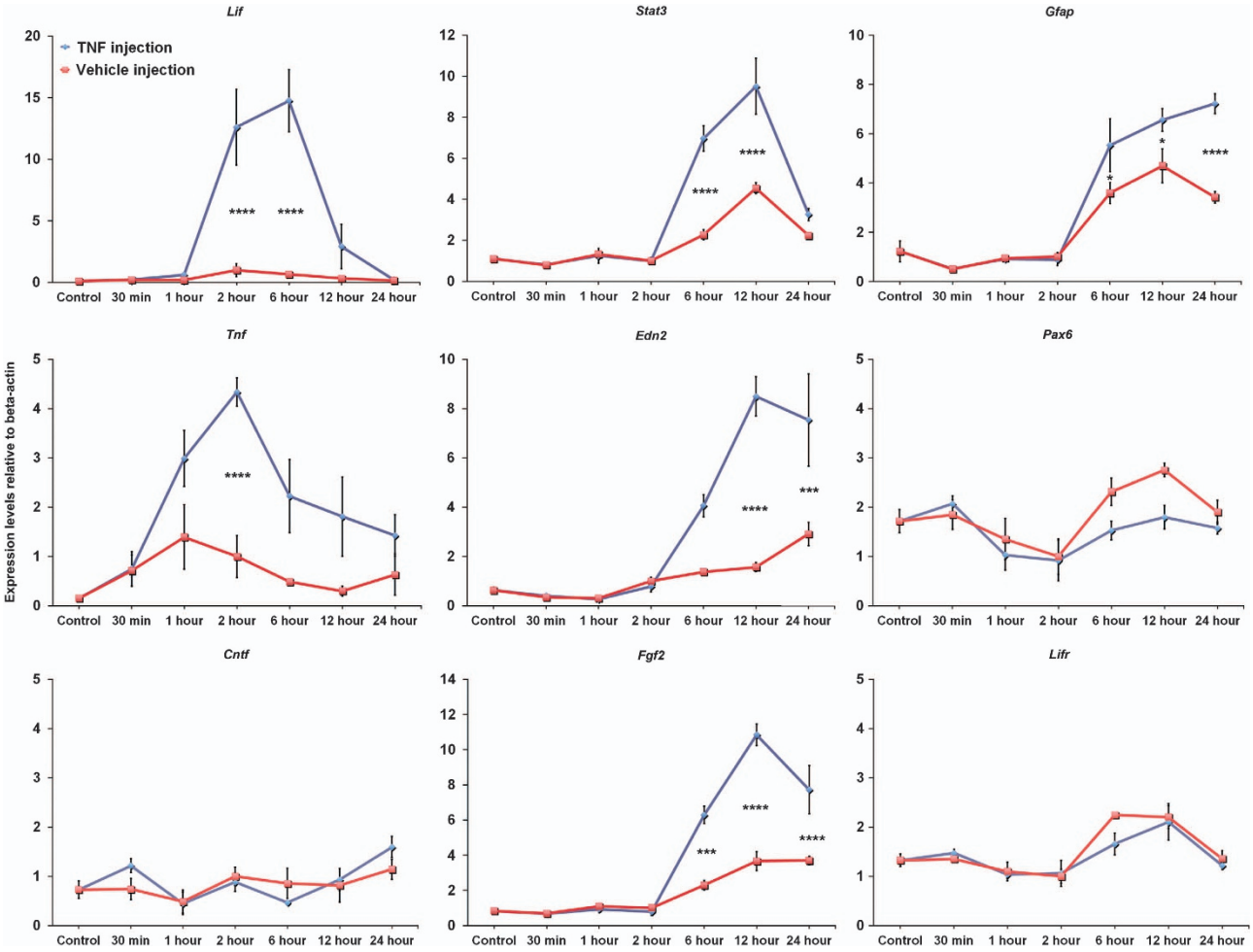

Figure 4 Intravitreal injection of TNF induces Lif gene expression in the neuronal retina *in vivo*. Real-time PCR analysis of gene expression in retinas of C67BL/6 mice before (control) or at indicated timepoints after intravitreal injection of TNF (blue lines) or vehicle (maroon lines). *Lif* and *Tnf* were upregulated as early as 1 h (*Tnf*) or 2 h (*Lif*) after injection, before levels of *Stat3, Edn2, Fgf2* and *Gfap* started to increase at 6 h. Expression of *Cntf*, *Lifr* and *Pax6* was not remarkably affected by the injections. Shown are means ± S.E.M. of $N = 3-4$ per timepoint and treatment. Two-way ANOVA with Bonferroni post-test was used to test for statistical significance between treatments at each timepoint. $^{*}P<0.05$; $^{***}P<0.005$; $^{****}P<0.001$

is strongly neuroprotective in the retina[2,3] and belongs to the same interleukin-6 (IL-6) family of cytokines as LIF, consequences of TNF injections were thus rather specific for the *Lif* gene.

To determine whether p38 MAPK is also involved in TNF-induced *Lif* expression in the retina *in vivo*, we analyzed gene expression at 2 and 6 h after intravitreal injection of TNF and/or the p38 MAPK inhibitor SB220025. As expected, injection of TNF caused increased *Lif* RNA levels at both timepoints (Figure 5a). However, when SB220025 was co-injected with TNF, upregulation of *Lif* expression was reduced by 78 and 73% at the 2 and 6 h timepoints, respectively. Injection of vehicle or SB220025 alone slightly upregulated *Lif* expression probably due to injection-inflicted retinal injury (Figure 5a). Western blots of retinal extracts showed an approximately 7.5-fold increase in phosphorylation levels of p38 MAPK at 2 h after TNF injection as compared with controls, suggesting that TNF increases p38 MAPK activity in the retina (Figures 5b and c). Injection of the p38 MAPK inhibitor (which does not block phosphorylation but the activity of p38 MAPK)[40] either alone or in combination with TNF also resulted in increased p38 phosphorylation levels which may suggest an attempt of inhibitor-treated retinal cells to increase p38 MAPK activity by a positive feedback. Since vehicle injections upregulated phospho-p38 MAPK levels by 3.5-fold, intravitreal injections *per se* may generate a stress response and activate p38 MAPK to a certain degree (Figures 5b and c). In summary, these data imply that the p38 MAPK pathway is also important for TNF-mediated *Lif* regulation in the neuronal retina *in vivo*.

p38 MAPK is an important regulator for increased *Lif* expression after exposure to damaging light *in vivo*. We showed that TNF-induced *Lif* upregulation depends on p38 MAPK activity in the healthy wild-type retina. To test whether *Lif* regulation depends on p38 MAPK also in a disease model, we exposed dark-adapted mice to high-intensity white light that has been shown to cause photoreceptor degeneration and to upregulate *Lif* expression in Muller cells.[1] Six hours after light exposure, *Lif* levels were increased 62-fold

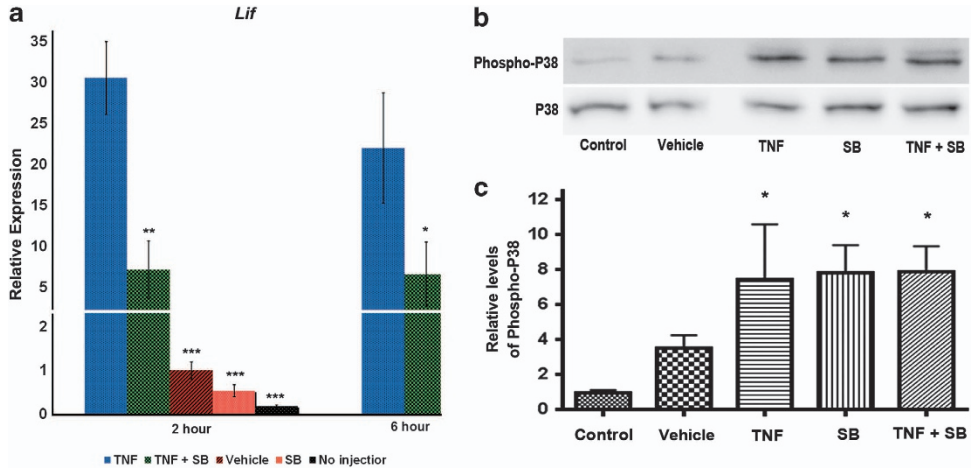

Figure 5 Activation of p38 MAPK is essential for TNF-induced upregulation of *Lif* expression in the neuronal retina *in vivo*. (**a**) RT PCR analysis of gene expression in retinas of C67BL/6 mice before (no injection, black bars) or at 2 and 6 h after intravitreal injection of TNF (blue bars), p38 MAPK inhibitor SB220025 (SB, pink bar), TNF + p38 MAPK inhibitor SB220025 (TNF + SB, green bar) or vehicle (maroon bar). TNF-mediated upregulation of *Lif* expression was significantly attenuated by the p38 MAPK inhibitor. Shown are means ± S.E.M. of $N = 3 - 4$ per timepoint and treatment. ANOVA with Bonferroni post-tests was used to compare expression levels after TNF injection with levels of remaining groups at the 2-h timepoint. Student's *t*-test was used to compare expression at 6 h. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$. (**b**) Detection of phospho-p38 MAPK (upper panel) and p38 MAPK (lower panel) by western blot of protein extracts from retinas before (control) or at 2 h after injection of vehicle, TNF, p38 MAPK inhibitor SB220025 (SB), or TNF + p38 MAPK inhibitor SB220025 (TNF + SB) as indicated. Injection of TNF, SB and TNF + SB increased levels of phospho-p38 MAPK compared with vehicle and uninjected retinas. Shown are representative blots. (**c**) Quantification of signals detected by western blot in (**b**) using Bio1D software. Phospho-p38 MAPK levels were highest after injection of TNF, of p38 MAPK inhibitor SB220025 (SB) and of TNF + p38 MAPK inhibitor SB220025 (TNF + SB). Shown are means ± S.E.M. of $N = 3$–9 per treatment. ANOVA with Bonferroni post-tests was used to compare levels to control. $^{*}P < 0.05$

(Figure 6). Intravitreal injection of vehicle immediately after light exposure slightly further increased *Lif* levels. Importantly, however, injection of p38 MAPK inhibitor SB220025 reduced *Lif* levels by 57% compared with vehicle injections. Similarly to observations made before (Figure 5a), vehicle injection induced *Lif* expression also in non-exposed mice (dark controls). As in the light-exposed retinas, injection of SB220025 reduced this induction by about 63% (Figure 6).

SB-mediated inhibition of *Lif* expression in the light-exposed retina was not as strong as in TNF + SB-injected retinas (Figure 5a), most probably because the SB inhibitor was injected for experimental reasons 2 h after the start of light exposure and thus only after the *Lif*-inducing signaling cascade had been activated by the light stimulus. Cardiotrophin-like cytokine (*Clc*) is another member of the IL-6 family of cytokines that is upregulated in response to light damage.[12] Similarly to *Lif*, induction of *Clc* was partially inhibited by SB treatment (Figure 6). Analysis of *Cntf*, *Gfap* and vimentin (*Vim*), which are expressed in Muller cells,[41–43] did not show any significant changes as a result of SB treatment (Figure 6). These results imply that p38 MAPK is involved in controlling *Lif* upregulation after light-induced photoreceptor injury *in vivo*.

p38 MAPK activity is neuroprotective in the model of light-induced photoreceptor degeneration. If p38 MAPK is indeed important for regulation of neuroprotective *Lif* in the injured retina, inhibition of its activity should increase photoreceptor damage after light exposure. To directly test this hypothesis, we quantified cell death in the retina at 40 h after exposure of wild-type mice to high levels of white light. Although intravitreal injections had a protective effect in general (vehicle), inhibition of p38 MAPK by SB220025 significantly increased cell death as compared with vehicle injections (compare 'SB' to 'vehicle'). In contrast, TNF injections reduced cell death almost to dark control levels (Figure 7). Importantly, cell death showed a distinct negative correlation with levels of *Lif* mRNA after no, SB, vehicle or TNF injections (Figures 5a and 7), corroborating earlier findings that showed that dosage and timing of *Lif* expression is an important factor for retinal physiology and neuroprotection.[13,44,45] These results strongly argue that LIF-mediated endogenous neuroprotection after light damage depends on p38 MAPK signaling in the retina.

Discussion

Regulation of *Lif* expression in Muller cells. Our results demonstrate that p38 MAPK signaling is required for the regulation of *Lif* expression in Muller cells *in vitro* and in the retina *in vivo*. We also show that inhibition of p38 MAPK activity reduces *Lif* expression and increases cell death in a model of photoreceptor injury supporting a direct role for p38 MAPK in LIF-mediated neuroprotection in the retina. Furthermore, treatment with recombinant TNF was sufficient to activate p38 MAPK, to increase *Lif* expression *in vitro* and *in vivo*, and to protect against light damage *in vivo*. However, whether endogenous TNF is involved in regulating *Lif* expression in the injured retina *in vivo* needs still to be determined, even though *Tnf* expression is induced early after light exposure in the eyecup.[46]

p38 MAPK activity has been shown to be one of the most important stress response factors by regulating the expression of several genes through activation of transcription factors such as ATF2, CHOP, CREB and ELK1.[22] Additionally, p38 MAPK affects gene expression at the

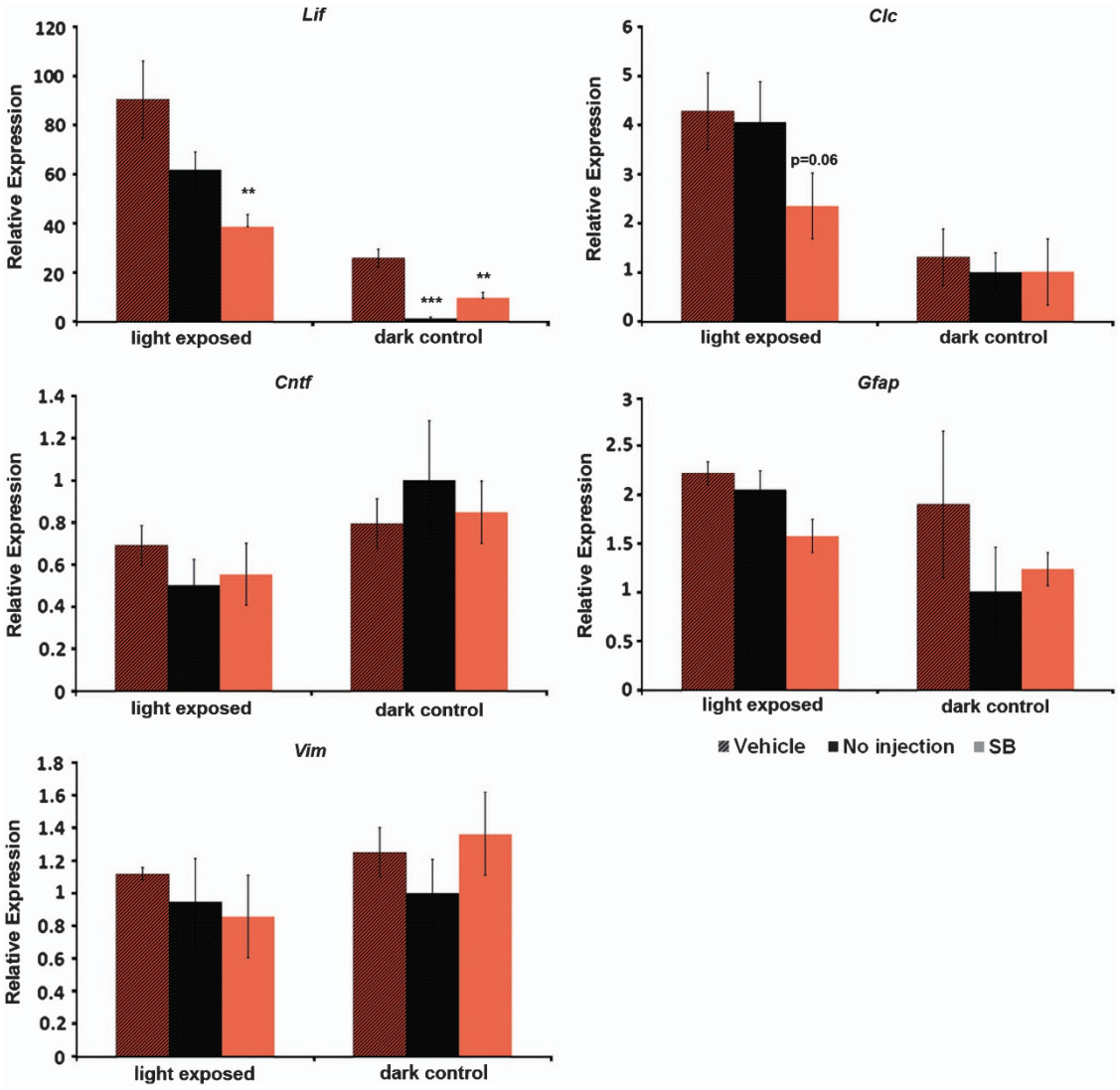

Figure 6 p38 MAPK is involved in light-induced *Lif* upregulation *in vivo*. Real-time PCR analysis of retinal gene expression before (dark controls) or at 6 h after exposure to 2 h of 13 000 lux of white light. Vehicle (maroon bars) or p38 MAPK inhibitor SB220025 (SB, pink bars) was injected into the vitreous immediately after the end of light exposure. Injections in dark-exposed control mice were done at a similar timepoint during the day to control for potential circadian alterations in gene expression. Light exposure significantly upregulated *Lif* expression in non-injected (black bars) and vehicle-injected eyes. Upregulation was prevented by SB injections. Note that *Lif* levels in light-exposed mice after SB injections were not different from dark control mice after vehicle injections. Expression of *Clc* was regulated similarly to *Lif*. Levels of *Cntf, Vim* and *Gfap* were not affected by light exposure or injections. Shown are means ± S.E.M. of $N = 3$–7 per treatment. ANOVA with Bonferroni post-tests was used to compare levels after vehicle injection to levels after SB injections and to levels of mice that received no injection. Statistics were calculated seperately for the light-exposed groups and the control groups. $^{**}P < 0.01$; $^{***}P < 0.005$

post-transcriptional level by enhancing the stability of target mRNAs containing AU-rich elements in their 3′ UTRs.[47] In our experimental systems, however, we focused on the effects of p38 MAPK signaling on the regulation of *Lif* expression at the transcriptional level *in vitro* and *in vivo*.

Activation (phosphorylation) of p38 MAPK by TNF was rapid, as was upregulation of *Lif* expression. The fast upregulation of *Lif* levels was blocked by an inhibitor of p38 MAPK activity, suggesting that the initial role of p38 MAPK involves transcriptional activation of *Lif* expression. Transcription factors involved in this regulatory pathway have not been defined and their identification will need further investigations. However, NFκB is a candidate factor that was upregulated by TNF treatment in a p38 MAPK-dependent manner (Figure 3), and NFκB binding to the *Lif* promoter has recently been shown in response to TLR2 agonists.[39] Furthermore, a connection between p38 MAPK, NFκB and cytokine regulation has been suggested[48] and TNF-mediated activation of p38 MAPK is well documented.[22] Nevertheless, the contribution of NFκB to TNF-mediated *Lif* upregulation may not be major, or else may depend on p38 MAPK. In the presence of TNF and the p38 MAPK inhibitor SB202190, Müller cells retain 50% of NFκB activity but completely lack *Lif* upregulation (Figures 3a and b), at least *in vitro*.

Regulation of *Lif* expression may additionally include a second level of complexity. The fast increase in expression

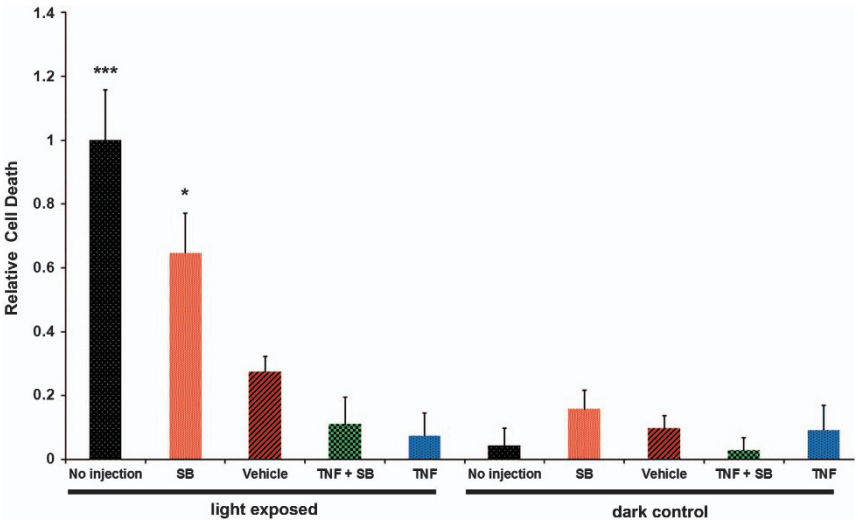

Figure 7 Inhibition of p38 MAPK activity increases cell death in the retina after light exposure *in vivo*. Quantification of cell death in retinas of non-exposed SV129S6 mice (dark controls) or in retinas of mice at 40 h after exposure to 2 h of 13 000 lux of white light (light damage). Twenty hours before light exposure, mice received an intravitreal injection of TNF (blue bars), p38 MAPK inhibitor SB220025 (SB, pink bars), TNF + p38 MAPK inhibitor SB220025 (TNF + SB, green bars) or vehicle (maroon bars). No-injection controls (black bars) were treated equally but did not receive intravitreal injections. The highest level of retinal cell death after light exposure was observed in non injected mice. Vehicle injections induced a protection, which was further strengthened by injection of TNF. p38 MAPK inhibitor SB220025 increased cell death almost to the level of non-injected control mice. Shown are means ± S.E.M. of $N = 3$–7 per treatment. ANOVA with Bonferroni post-tests was used to compare levels of cell death of the various groups to vehicle control. Statistics were calculated seperately for the light damage and the dark control group. $^{*}P < 0.05$; $^{***}P < 0.005$

after TNF treatment *in vitro* (Figure 1) or after light exposure *in vivo*,[11] was followed by a rapid decline of *Lif* mRNA levels towards basal levels. This argues for the presence of a regulatory feedback loop and that transcriptional activation of *Lif* expression might be followed by transcriptional inhibition or by a reduction of *Lif* mRNA stability, or both.

Neuroprotective role of p38 MAPK activity and *Lif* expression. Depending on the concept and experimental setup, activation of p38 MAPK has been shown to promote cell survival or apoptosis. Whereas inhibition of p38 MAPK activity has been reported to protect 661W photoreceptor cells against light damage *in vitro*,[49] our results indicate that inhibition of p38 MAPK activity accelerates light damage *in vivo*. This discrepancy may be due to the lack of intercellular communication between photoreceptors, RPE and Muller cells in the *in vitro* cell culture system. Photoreceptor degeneration induced *in vivo* by high levels of white light depends on RPE65 in the RPE and on the regeneration kinetics of the bleached chromophore in the visual cycle.[50,51] Hence mechanisms of light-induced death in isolated 661W cells *in vitro* and photoreceptors *in vivo* differ and the two experimental systems cannot be directly compared. Of additional importance, photoreceptors injured by light or other stimuli signal to Muller cells, which induce expression of *Lif* to ignite a neuroprotective response in the retina leading to increased photoreceptor survival.[1,8] This intercellular communication is not possible in the cell culture system, and effects of p38 MAPK inhibition in 661W cells may thus not accurately reflect the *in vivo* situation where p38 MAPK activity seems important in Muller cells to upregulate expression of neuroprotective *Lif*.

Despite the neuroprotective role of p38 MAPK, inhibition of its activity in the presence of TNF was still protective against LIRD, in contrast to inhibitor injections alone (Figure 7). Protection correlated with increased levels of *Lif* in retinas of eyes injected with a combination of TNF and inhibitor, and with decreased *Lif* levels after injection of the p38 MAPK inhibitor alone (Figure 5a). This suggests that injection of the p38 MAPK inhibitor was not sufficient to completely block the strong *Lif*-inducing activity of TNF and raises the possibility that a mechanism for TNF-induced *Lif* upregulation in addition to p38 MAPK may be present *in vivo*. Alternatively, TNF may potentially have protective effects through other mechanisms such as NFκB activation and its downstream targets, or the regulation of heat shock proteins.[52] It is interesting to note that p38 MAPK inhibitors only partially blocked TNF-induced NFκB activation whereas they completely abolished *Lif* induction, at least *in vitro* (Figure 5). This may argue that TNF could regulate the fate of photoreceptor cells via several mechanisms.

Due to the inhibition of pro-inflammatory gene expression, inhibitors of p38 MAPK are discussed as potential therapeutic agents in inflammatory diseases like psoriasis and rheumatoid arthriis.[53] Moreover, p38 MAPK inhibitors were shown to block tumor growth and metastases formation.[54] However, our results demonstrating that inhibition of p38 MAPK activity may be detrimental to injured photoreceptor cells ask for precautions when developing p38 MAPK inhibitors for therapeutic use. In cases of open or hidden retinal disease conditions, application of p38 MAPK inhibitors to patients may have adverse effects on vision due to its negative effect on *Lif* expression and LIF-dependent survival factors in the retina. Similar adverse effects may possibly be observed in other neurodegenerative diseases where LIF has a reported protective activity.[55–57] Thus, as a safety measure, p38 MAPK inhibitors may be designed not to cross retina – blood or brain – blood barriers, which may prevent potential neurodegenerative effects.

TNF signaling in the retina. Although most reports attribute TNF a devastating role in the retina and retinal diseases through the modulation of an inflammatory response,[28,29] our results demonstrate that intravitreal injection of TNF before light exposure did not accelerate degeneration. Rather, TNF reduced the relative average cell death by almost fourfold (0.27 ± 0.049 S.E.M., $N=6$ for vehicle *versus* 0.074 ± 0.07 S.E.M. for TNF, $N=5$). Even though these values did not reach statistical significance when tested in context of the additional experimental paradigms using analysis of variance (ANOVA), they showed a tendency for TNF-mediated protection of photoreceptors against light-induced degeneration (Figure 7). At least part of this effect may be explained by increased expression of *Lif* which leads to the upregulation of neuroprotective factors like *Fgf2*, *End2* and *Stat3* (Figure 4).[1,12]

Emerging evidence indicates that TNF may be a signaling molecule of general importance that has differential effects on disease outcome depending on the interaction with its receptors. In a retinal ischemia-reperfusion model, TNF-R2 signaling was neuroprotective whereas TNF-R1 increased neuronal death.[58] Moreover, a recent report suggests that TNF expression in dying photoreceptors and Muller cells is important for Muller cell proliferation and photoreceptor regeneration in a zebrafish model of LIRD.[19,59] Although the mammalian retina diverges from zebrafish in several ways including its limited proliferation and regeneration capacity of Muller cells,[60,61] these results together with our findings may nevertheless indicate a possible role of TNF for the protection of function in the stressed or injured mammalian retina. Clearly, further studies are warranted to elucidate the role of TNF signaling during photoreceptor degeneration in detail.

Materials and Methods

Animals. All experimental protocols were accepted by the Veterinary Authorities of Zurich and experiments were conducted in accordance with the statement of 'The Association for Research in Vision and Ophthalmology' for the use of animals in research. All mice had access to food and water *ad libitum* and were housed in a light–dark cycle of 12 : 12 h with 60 lux at cage level. All experimental conditions and time points were tested with a minimum of $N=3$ mice. All experimental mice were on a C57BL/6 background except for the light damage experiments in which mice of the SV129/S6 strain were used (Figure 7). For intravitreal injections after light exposure Rlbp::GFP mice[62] on a C57BL/6 background were used (Figure 6).

Cell culture assays

TNF and p38 MAPK inhibitor treatment: rMC-1 cells[36] were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Life Technologies), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies), and grown in a humidified 5% CO_2 incubator as described in Sarthy *et al.*[36] Rat recombinant TNF (R&D Systems, Minneapolis, MN, USA) was dissolved in PBS containing 0.1% bovine serum albumin (BSA) (Sigma Aldrich, St Louis, MO, USA), the p38 MAPK inhibitors SB202190 and SB239063 (Sigma Aldrich) were dissolved in water or in DMSO (Sigma Aldrich), respectively. Compounds were added alone or in combinations directly to growth media at concentrations and times indicated in the Results section.

Luciferase assay: rMC-1 cells were transfected using FuGENE6 reagent (Promega, Madison, WI, USA). Renilla luciferase expressing vector, pRL-CMV (Promega), was used as an internal control to normalize the transfection efficiency. Transfection solution was prepared in 91 μl Optimem (Life Technologies) media using 3 μg/ml of total plasmid DNA and 9 μl of FuGENE6 reagent according to manufacturer's instructions. The ratio of renilla to firefly plasmids was 1 : 9. Ten thousand cells in 100 μl growth media were seeded on a 96-well plate and each well was transfected with 3.3 μl of transfection solution after attachment. An EGFP reporter vector, EGFP-C1 (Clontech, Mountain View, CA, USA), was used to assess the transfection efficiency of rMC-1 cells, which was between 15 and 20%. Cells were transfected either with a control vector, pTAL-luc (Clontech), or with an NFκB reporter vector, pNFκB-luc (Clontech). TNF, SB202190 and TNF + SB202190 treatments were performed for 6 h starting at 24 h after transfection. TNF and SB202190 had final concentrations of 10 ng/ml and 100 μM, respectively. Each treatment was performed in triplicates in four independent experiments. Luciferase levels were measured using the Dual Luciferase kit (Promega).

Immunoblotting. Protein homogenates were prepared by sonication (Branson sonifier, 10 strokes of 0.3 s with 30% output) of isolated retinas in Tris-HCl (100 mM, pH 7.5 or pH 8.0). Protein concentrations were determined by Bradford using BSA as standard. Homogenates were mixed with an equal volume of 4X Laemmli sample buffer and a total of 40 μg/ml protein was loaded in each lane of 10% SDS-polyacrylamide gels. Samples were electrophoresed, blotted and probed as described previously in Bürgi *et al.*[12] To detect phospho-p38 MAPK (cat no. 840771; R&D Systems) and p38 MAPK (cat no. 9212; Cell Signaling, Danvers, MA, USA) primary antibodies were used at a dilution of 1 : 500 and 1 : 1000, respectively. The secondary antibody, anti-rabbit IgG peroxidase-linked (cat no. NA934; GE Healthcare, Pittsburgh, PA, USA), was used at a dilution of 1 : 10 000. We have used WesternBright Sirius HRP substrate (Advansta, Menlo Park, CA, USA) for chemiluminescence reaction. Fusion FX7 Advance imaging system (Vilber Lourmat, Torcy, France) with a CCD camera was used for digital signal detection. Recordings were taken at the dynamic range of exposure without binning. Calculations for exposure levels were performed using BioD1 software (Vilber Lourmat) without background subtractions.

RT-PCR analysis. Retinas were collected through a slit in the cornea, and total RNA was prepared and analyzed by real-time PCR (RT-PCR) as described previously in Bürgi *et al.*[12] Briefly, 10 ng cDNA was amplified in a LightCycler instrument (Roche Diagnostics, Basel, Switzerland) using appropriate primer pairs (Table 1) and SybrGreen Master mix (Roche). *Actb* was used as reference gene. For the analysis of gene expression in rMC-1 cells, total RNA was extracted using the Megamax RNA isolation kit (Life Technologies) according to manufacturer's instructions. cDNA was prepared using the high-capacity cDNA reverse transcription kit (Life Technologies). Real-time PCR reactions were conducted using appropriate primer pairs (Table 1) and *Actb* as internal control. Additional internal controls, *Gapdh* and *Rpl32*, were used for each new treatment. Real-time PCR reactions were performed in a StepOne Real-Time PCR system with Fast SybrGreen master mix (Life Technologies). The comparative cycle threshold method was used to calculate relative transcript levels for both mouse and rat experiments.

Intravitreal injections. Intravitreal injections were performed as previously described in Joly *et al.*[1] Rat recombinant TNF (R&D Systems) was reconstituted in sterile PBS containing 0.1% BSA (Sigma Aldrich) and injected at a concentration of 10 μg/ml. SB220025 trihydrochloride (Sigma Aldrich) was dissolved in water and adjusted to 6 mM using sterile PBS containing 0.1% BSA for injections. Sterile PBS containing 0.1% BSA was used for vehicle control injections. For the injection of TNF + SB220025, stock solutions for SB220025 and TNF were mixed and diluted with PBS containing 0.1% BSA to reach final injection concentrations of 10 μg/ml/ml and 6 mM for TNF and SB220025, respectively. Injection volume was 1 μl.

Light damage and cell death assay. Light damage and quantification of cell death by an ELISA-based cell death assay (Roche Diagnostics, Basel, Switzerland) were performed essentially as previously described in Bürgi *et al.*[12] and Samardzija *et al.*[10] with minor modifications. White light intensity for light damage was set to 13 000 lux, and 6–10-week-old animals with dilated pupils[12] were exposed for 2 h. Animals were kept in darkness overnight before and after light exposure. Animals were sacrificed and retinas were isolated for cell death assay at 40 h after light exposure.

Data analysis. Statistical analysis was performed using ANOVA with Dunnett's Multiple Comparison tests where multiple comparisons were made to a single value. ANOVA with Bonferroni post-test was performed for multiple

Table 1 Real-time PCR primer sequences

Gene	Species	Forward primer	Reverse primer
Cntf	Rn	CTCTGTAGCCGTTCTATCTG	GGTACACCATCCACTGAGTC[Rn/Mm]
Fgf2	Rn	GGCTGCTGGCTTCTAAGTGT	TCCGTGACCGGTAAGTGTTG
Gapdh	Rn	ATGACTCTACCCACGGCAAG	GGAAGATGGTGATGGGTTTC
Gfap	Rn	AGTGGTATCGGTCCAAGTTTGC	TGGCGGCGATAGTCATTAGC
Lif	Rn	ATGAAGGTCTTGGCCACAGG	GTATGGCGCAGGTGGCATT
Tnf	Rn	CCACGCTCTTCTGTCTACTGA [RN/MM]	GGCCATGGAACTGATGAGAGG
Rpl32[21]	Rn	AAGCGAAACTGGCGGAAAC	TAACCGATGTTGGGCATCAG
Actb	Rn/Mm	CAACGGCTCCGGCATGTGC[Rn/Mm]	CTCTTGCTCTGGGCCTCG[Rn/Mm]
Clc	Mm	CCCTGGCCCCTCCATCCAGAAA	TGCCCCCAGTCGAGGAGGATTG
Cntf	Mm	CTCTGTAGCCGCTCTATCTG	GGTACACCATCCACTGAGTC[Rn/Mm]
Edn2	Mm	AGACCTCCTCCGAAAGCTG	CTGGCTGTAGCTGGCAAAG
Fgf2	Mm	TGTGTCTATCAAGGGAGTGTGTGC	ACCAACTGGAGTATTTCCGTGACCG
Gapdh	Mm	CAGCAATGCATCCTGCACC	TGGACTGTGGTCATGAGCCC
Gfap	Mm	CCACCAAACTGGCTGATGTCTAC	TTCTCTCCAAATCCACACGAGC
Lif	Mm	AATGCCACCTGTGCCATACG	CAACTTGGTCTTCTCTGTCCCG
Lifr	Mm	ACTGAAGTGGAACGACAGAGG	CTTTACCACTCAGCATTGTGTTG
Pax6	Mm	AGTTCTTCGCAACCTGGCTA	CATCTGAGCTTCATCCGAGT
Stat3	Mm	CAAAACCCTCAAGAGCCAAGG	TCACTCACAATGCTTCTCCGC
Tnf	Mm	CCACGCTCTTCTGTCTACTGA[Rn/Mm]	CCACGCTCTTCTGTCTACTGA
Vim	Mm	TACAGGAAGCTGCTGGAAGG	TGGGTGTCAACCAGAGGAA

Rn, *Rattus norvegicus*; Mm, *Mus musculus*
Primers used for both rat and mouse samples are marked (Rn/Mm)

comparisons against independent controls. Student's *t*-tests were used for individual pairwise comparisons. *P*-values lower than 0.05 were considered to be significant. Error bars represent the standard error of the mean (S.E.M.). We also used the ROUT algorithm, with the coefficient Q value of 0.1, to detect possible outliers related to intravitreal injections. Graph Pad 6 software or Prism 5 (GraphPad Inc., San Diego, CA, USA) were used for all statistical analyses.

Conflict of Interest

The authors declare no conflict of interest.

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