Tweak regulates astrogliosis, microgliosis and skeletal muscle atrophy in a mouse model of amyotrophic lateral sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that primarily affects motoneurons in the brain and spinal cord. Astrocyte and microglia activation as well as skeletal muscle atrophy are also typical hallmarks of the disease. However, the functional relationship between astrocytes, microglia and skeletal muscle in the pathogenic process remains unclear. Here, we report that the tumor necrosis factor-like weak inducer of apoptosis (Tweak) and its receptor Fn14 are aberrantly expressed in spinal astrocytes and skeletal muscle of SOD1G93A mice. We show that Tweak induces motoneuron death, stimulates astrocytic interleukin-6 release and astrocytic proliferation in vitro. The genetic ablation of Tweak in SOD1G93A mice significantly reduces astrogliosis, microgliosis and ameliorates skeletal muscle atrophy. The peripheral neutralization of Tweak through antagonistic anti-Tweak antibody ameliorates muscle pathology and notably, decreases microglial activation in SOD1G93A mice. Unexpectedly, none of these approaches improved motor function, lifespan and motoneuron survival. Our work emphasizes the multi-systemic aspect of ALS, and suggests that a combinatorial therapy targeting multiple cell types will be instrumental to halt the neurodegenerative process.

Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating and incurable neurodegenerative disease. Once symptomatic, the median survival of patients is <3 years. Clinical manifestations typically occur in mid-life, followed by the rapid and progressive wasting of muscles and subsequent paralysis (1). While mostly sporadic, ALS can also be genetically inherited and these forms represent 17–23% of all cases (2). Both sporadic and familial ALS patients present similar symptoms and pathophysiology. Mutations within superoxide dismutase 1 (SOD1) account for ~12% of familial and 1% of sporadic cases (3). Transgenic mice overexpressing human mutated SOD1 recapitulate the main pathological traits of ALS and remain to date the most reliable and informative disease model. While the primary pathological target of ALS is undeniably the motoneuron, both cell-autonomous and non-cell-autonomous processes are mediated by mutant SOD1 and contribute to motoneuron degeneration. The toxic action of mutant SOD1 in astrocytes and microglia influences the progression of the disease and later stages of ALS pathogenesis (4,5). Similarly, muscle-restricted expression of mutant SOD1 results in skeletal muscle atrophy
and motoneuron degeneration (6,7) pointing to intrinsic defects within ALS muscle. It is therefore becoming evident that a combined therapeutic approach targeted at neuronal, glial and muscular cell types is the most pertinent approach for treating ALS.

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (Tweak, Tnfsf12) is a member of the TNF family (8), which signals through the fibroblast growth factor-inducible 14 (Fn14, Tnfrsf12a) receptor (9). While Tweak is also bound and scavenged by the monocyte- and macrophage-specific CD163 receptor (10), the signaling cascades initiated by this interaction have yet to be determined. Interestingly, Tweak plays a dual role in health and disease. Indeed, it promotes tissue repair following an acute injury, while it becomes a pathogenic pro-inflammatory effector in chronic states (11). Importantly, Tweak is expressed in glial cells and potently induced by the pro-inflammatory cytokine interferon-γ (IFNγ), which we have shown to induce a motoneuron selective death pathway and be up-regulated in ALS mice and patients (12,13). Further, Tweak activates an Fn14-dependent neuronal death, specifically of cortical neurons (14). Finally, the Tweak-Fn14 pathway has also been ascribed a prominent role in the regulation of proliferation and differentiation as well as neuron-sourced pro-inflammatory cytokines, which we have shown in motoneurons to facilitate their visualization once in culture (22,23). We therefore added a previously validated antagonistic anti-Fn14 antibody (14) to motoneuron cultures prior to Tweak treatment. We next investigated mRNA levels of Fn14 and CD163 by quantitative PCR (qPCR) and show that the spinal cord of 150-day-old SOD1G93A mice expresses significantly more Fn14 mRNA than that of wildtype mice (Fig. 1D). Expression of CD163 was previously detected in resident microglial cells and motoneurons of ALS mice (19,20) where levels of CD163 mRNA did not change between isolated wildtype and SOD1G93A microglia (20). In support of these previous reports, we also observe that CD163 mRNA levels are similar between groups (Fig. 1E). Since both Tweak and CD163 can be cleaved and thus also act as circulating soluble proteins (8,21), we quantified them by ELISA assays in the serum of wildtype and SOD1G93A mice during the course of the disease. In both groups, soluble Tweak is found in relatively low levels and soluble CD163 in relatively high levels (Fig. 1F and G) at all three analyzed time-points, without any significant differences between wildtype and SOD1G93A mice. Combined, our results suggest that astrocytic Tweak in its transmembrane form as well as Fn14 mRNA is increased in symptomatic SOD1G93A ALS mice.

**Tweak induces a CD163-dependent motoneuron death**

Since astrocytic Tweak expression is increased in ALS mice, we sought to determine if and how Tweak could trigger motoneuron death. We isolated embryonic motoneurons from Hb9-GFP mice that express GFP under the motoneuron-selective Hb9 promoter to facilitate their visualization once in culture (22,23). We first evaluated the effect of Tweak on motoneuron survival and found that adding a soluble recombinant human or mouse form of Tweak to the culture media for 48 h leads to motoneuron death (Fig. 2A). We chose to pursue future experiments with the murine isoform shows that blocking the Fn14 receptor does not prevent Tweak-dependent death. The antagonistic Fn14 antibody alone suggests that blocking the Fn14 receptor does not prevent Tweak-dependent death. The antagonistic Fn14 antibody alone did not impact motoneuron survival (Fig. 2C). However, quantification of surviving motoneurons shows that blocking the Fn14 receptor does not prevent Tweak-dependent death. The antagonistic Fn14 antibody alone did not impact motoneuron survival (Fig. 2C). These results suggest that the protective effect of antagonistic Fn14 is specific to a subset of neuronal cells and that Tweak promotes apoptosis of primary motoneurons in an Fn14-independent manner. This led us to investigate the implications of CD163 in Tweak-induced motoneuron death. We thus similarly blocked CD163 with a previously validated antagonistic anti-Fn14 antibody (14) to motoneuron cultures prior to Tweak treatment. We then set out to identify the Tweak receptor that participates in the observed neurotoxicity. The functional interaction of Tweak with Fn14 is best characterized (9), and we observed an increased expression of both Tweak and Fn14 in the spinal cord of 150-day-old SOD1G93A mice. Further, Tweak-induced cortical neuron death occurs through its binding with Fn14 (24). We therefore added a previously validated antagonistic anti-Fn14 antibody (14) to motoneuron cultures prior to Tweak treatment. We next investigated Fn14 mRNA levels of Fn14 and CD163 by quantitative PCR (qPCR) and show that the spinal cord of 150-day-old SOD1G93A mice expresses significantly more Fn14 mRNA than that of wildtype mice (Fig. 1D). Expression of CD163 was previously detected in resident microglial cells and motoneurons of ALS mice (19,20) where levels of CD163 mRNA did not change between isolated wildtype and SOD1G93A microglia (20). In support of these previous reports, we also observe that CD163 mRNA levels are similar between groups (Fig. 1E). Since both Tweak and CD163 can be cleaved and thus also act as circulating soluble proteins (8,21), we quantified them by ELISA assays in the serum of wildtype and SOD1G93A mice during the course of the disease. In both groups, soluble Tweak is found in relatively low levels and soluble CD163 in relatively high levels (Fig. 1F and G) at all three analyzed time-points, without any significant differences between wildtype and SOD1G93A mice. Combined, our results suggest that astrocytic Tweak in its transmembrane form as well as Fn14 mRNA is increased in symptomatic SOD1G93A ALS mice.

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### Results

**Tweak is up-regulated in spinal cord astrocytes of symptomatic ALS mice**

We first explored the expression profile and levels of Tweak in SOD1G93A ALS mice over the course of the disease. We analyzed spinal cord and serum from non-symptomatic (30 days), early symptomatic (90 days) and symptomatic (150 days) SOD1G93A mice. While we did not detect an aberrant expression of Tweak in the spinal cord of non-symptomatic and early symptomatic SOD1G93A mice (Supplementary Material, Fig. S1), we observed a significant up-regulation of Tweak in the spinal cord of symptomatic SOD1G93A mice compared with wildtype littermates (Fig. 1A). Using markers for motoneurons (SMI32), astrocytes (GFAP) and activated microglia (Mac-1), we demonstrate that increased Tweak expression occurs specifically in astrocytes (Fig. 1A). Via immunoblot analysis, we further confirm the increased expression of Tweak in the spinal cord of symptomatic SOD1G93A mice (Fig. 1B and C).
endocytosis and this entry into neurons is prevented by blocking CD163 but not by inhibiting Fn14 (Fig. 2F and Supplementary Material, Videos S1–S3). This is in accordance with previous studies reporting an internalization of Tweak by CD163 (27).

Immunocytochemistry with an anti-cleaved caspase-3 antibody, a key mediator of apoptosis, shows that Tweak-treated cultures contain significantly more caspase-3 positive motoneurons compared with untreated cells (Fig. 2G and H). To confirm a caspase-3-dependent action of Tweak, we added the caspase-3 inhibitor, z-DEVD-fmk (DEVD), to the culture media prior to Tweak administration (Fig. 2I). We demonstrate that blocking caspase-3 activity prevents motoneuron death induced by Tweak. Combined, our results support a role for Tweak in a CD163-, endocytosis- and caspase-3-dependent motoneuron death that could participate in the neurodegenerative process in ALS.

Figure 1. Tweak and Fn14 are up-regulated in symptomatic SOD1G93A mice. (A) Representative images of ventral horn region of the lumbar spinal cord of 150-day-old wildtype and SOD1G93A mice immunolabeled with SMI32, GFAP, Mac-1 and Tweak. Scale bar, 50 μm. (B) Representative immunoblot of Tweak expression in the spinal cord of 150-day-old wildtype and SOD1G93A mice. Upper band corresponds to membrane-bound protein and lower band to intact transmembrane domain. Actin served as loading control. (C) Quantification of total Tweak/actin from spinal cord immunoblots of 150-day-old wildtype and SOD1G93A mice (n = 3) (a.u., arbitrary units). (D) Fn14 qPCR in the spinal cord of 150-day-old SOD1G93A mice compared with age-matched wildtype littermates (n = 3). Total mRNA expression levels were first normalized to the relative expression level of P0. (E) qPCR analysis of CD163 mRNA expression in the spinal cord of 150-day-old SOD1G93A mice compared with age-matched wildtype littermates (n = 3). Total mRNA expression levels were first normalized to the relative expression level of P0. (F) ELISA analysis of Tweak levels in the serum of 30-, 90- and 150-day-old wildtype (30 days, n = 20; 90 days, n = 9 and 150 days, n = 13; for SOD1G93A, 30 days, n = 8; 90 days, n = 3 and 150 days, n = 9) mice. (G) ELISA analysis of CD163 levels in serum of wildtype (30 days, n = 20; 90 days, n = 10 and 150 days, n = 12) and SOD1G93A (30 days, n = 8; 90 days, n = 8; 90 days, n = 3 and 150 days, n = 6) mice. Data are means ± standard deviation (SD), *P < 0.05; ns, not significant; C–E, unpaired two-tailed t test; F and G, two-way ANOVA.
Figure 2. Tweak induces death of primary motoneurons via CD163, endocytosis and caspase-3. (A) Motoneurons were cultured for 24 h and treated with indicated concentration of human or mouse recombinant Tweak. Motoneuron survival was determined 48 h later and expressed relative to survival in the absence of any treatment (n = 3). (B) Cortical neurons were treated or not with Tweak (100 ng/ml) and antagonistic anti-Fn14 antibody (150 ng/ml). Survival was determined 48 h later. (C) Motoneurons were treated with antagonistic anti-Fn14 antibody (150 ng/ml) 1 h prior to addition of Tweak. Survival was determined 48 h later (control, n = 5; Tweak, n = 6; Fn14, n = 3). (D) After 24 h of culture, motoneurons were treated with antagonistic anti-CD163 antibody (100 ng/ml) 1 h prior Tweak addition. Survival was determined 48 h later (n = 4). (E) Representative images of GFP-positive Hb9::GFP motoneurons immunolabeled with CD163. Scale bar, 25 μm. (F) Representative images from live imaging videos tracking the movement of recombinant Tweak (red) as it interacts with motoneurons (green). Tweak endocytosis is observed in red, while motoneuron death is depicted by arrows. Scale bar, 20 μm. (G and H) Motoneurons were treated with mouse Tweak (100 ng/ml) and processed for cleaved caspase-3 immunolabeling 24 h later. (G) Representative images of GFP-positive primary motoneurons immunolabeled with cleaved caspase-3 (red). (H) The percentage of cleaved caspase-3-positive motoneurons was then determined (n = 4). Scale bar, 20 μm. (I) Motoneurons were treated or not with Tweak (100 ng/ml) and DEVD (10 μM). Survival was determined 48 h later (n = 3). Data are means ± SD; **P < 0.01; ***P < 0.001, H, unpaired two-tailed t-test; B–D and I, one-way ANOVA.
Tweak induces proliferation and IL-6 production in SOD1 mutant astrocytes

Our in vivo analysis uncovers a significant up-regulation of Tweak in spinal cord astrocytes of symptomatic SOD1G93A mice (Fig. 1A). To further understand the implications of this increased expression, we analyzed expression of Tweak and its receptors in primary spinal cord astrocytes. Analysis by qPCR shows that Tweak mRNA levels are similar between wildtype and SOD1G93A astrocytes (Fig. 3A). Given that IFNγ potently stimulates Tweak activity and expression (28,29), we also assessed Tweak transcript levels in astrocytes 48 h after IFNγ addition to the culture media. We show similar levels between wildtype and SOD1G93A astrocytes and between IFNγ-treated astrocytes compared with untreated cells (Fig. 3A). We also measured by ELISA soluble Tweak released by astrocytes in the culture media. Untreated wildtype and SOD1G93A astrocytes release similar low levels of soluble Tweak and IFNγ treatment induces Tweak release by astrocytes, without any significant difference between groups (Fig. 3B). Our results thus suggest that the impact of IFNγ on Tweak in primary spinal astrocytes is predominantly on the release of the soluble form. We next evaluated expression of CD163 and Fn14 in primary astrocyte cultures. Using an ELISA to quantify soluble CD163 released in the media, we show no significant difference between wildtype and SOD1G93A astrocytes (Fig. 3C). However, qPCR analysis reveals a significant up-regulation of Fn14 mRNA in SOD1G93A astrocytes compared with wildtype cells (Fig. 3D). Adding soluble Tweak to wildtype astrocytes promotes proliferation as well as release of certain pro-inflammatory effectors such as IL-6 (30,31). We therefore explored whether increased expression of Fn14 in SOD1G93A astrocytes could render them more sensitive to Tweak-dependent pathways. Treatment of astrocytes with Tweak for 48 h leads to similar increased release of IL-6 in the media of both wildtype and SOD1G93A astrocytes (Fig. 3E). Finally, we evaluated the effect of Tweak on astrocytic proliferation by calculating the number of Ki-67-positive nuclei 24 h following treatment. We find that SOD1G93A astrocytes proliferate significantly more than...
wildtype cells in the presence of Tweak (Fig. 3F). Together, our results suggest that SOD1<sup>G93A</sup> spinal cord astrocytes overexpress Fn14 very early in development and are more sensitive to Tweak-induced proliferation.

**The Tweak–Fn14 pathway is aberrantly expressed in skeletal muscles of SOD1<sup>G93A</sup> mice**

Both Tweak and Fn14 also play important roles in the regulation of skeletal muscle in both health and disease. Further, Fn14 has recently been demonstrated to be up-regulated in the tibialis anterior (TA) muscle of end-stage SOD1<sup>G93A</sup> mice, correlating with the atrophy status of the muscle (32). We therefore sought to determine the expression profiles of Tweak and Fn14 mRNA by qPCR at various time points during the course of the disease (30 (non-symptomatic), 90 (early symptomatic) and 150 (symptomatic) days). In SOD1<sup>G93A</sup> mice, fast-twitch muscles are typically more compromised than slow-twitch muscles (33,34). We thus assessed Tweak and Fn14 expression in the TA (fast-twitch), soleus (slow twitch) and gastrocnemius (mixed-twitch) skeletal muscles.

In the TA, we find that Tweak mRNA levels are not significantly different between wildtype and SOD1<sup>G93A</sup> mice during the course of the disease (Fig. 4A), where Tweak levels are highest at 30 days with a subsequent decrease over time, suggesting a

![Figure 4](https://example.com/figure4.png)

**Figure 4. Aberrant regulation of Tweak and Fn14 expression in the skeletal muscle of SOD1<sup>G93A</sup> mice.** (A) qPCR analysis of Tweak expression in the TA muscle of 30-, 90- and 150-day-old wildtype (n = 4 for 30 days and 3 for 90 and 150 days) and SOD1<sup>G93A</sup> mice (n = 3 for 30 and 90 days and 5 for 150 days). Total mRNA levels were normalized to the relative expression level of PolJ. (B) qPCR analysis of Tweak expression in the gastrocnemius muscle of wildtype (n = 4 for 30 days and 3 and 150 days) and SOD1<sup>G93A</sup> mice (n = 3 for 30 and 90 days and 4 for 150 days). (C) qPCR analysis of Tweak expression in the gastrocnemius muscle of 30-, 90- and 150-day-old wildtype (n = 3 for 30 and 90 days and 5 for 150 days) and SOD1<sup>G93A</sup> mice (n = 3 for 30 and 90 days and 5 for 150 days). (D) qPCR analysis of Fn14 expression in the TA muscle of wildtype (n = 3 for 30 and 90 days and 4 for 150 days) and SOD1<sup>G93A</sup> mice (n = 3 for 30 and 90 days and 4 for 150 days). (E) qPCR analysis of Fn14 expression in the gastrocnemius muscle of 30-, 90- and 150-day-old wildtype (n = 3 and SOD1<sup>G93A</sup> mice (n = 3). (F) qPCR analysis of Fn14 expression in the soleus muscle of wildtype (n = 3 for 30 and 90 days and 4 for 150 days) and SOD1<sup>G93A</sup> mice (n = 3 for 30 and 90 days and 4 for 150 days). All data represented are means ± SD; two-way ANOVA.
developmental regulation of Tweak in the TA. In the gastrocnemius and soleus (Fig. 4B and C), Tweak mRNA also appears to be developmentally regulated but in a different manner, as we observe a significant up-regulation in the muscle of 90-day-old mice. In addition, at 90 days, Tweak is significantly lower in the gastrocnemius of SOD1	extsuperscript{G93A} mice compared with wildtype muscle. As for Fn14, we observe a significant up-regulation of Fn14 mRNA in the TA and gastrocnemius of symptomatic ALS mice compared with control littermates (Fig. 4D and E), while Fn14 mRNA levels are not significantly different between groups in the soleus (Fig. 4F).

Our results thus suggest that muscles composed of fast-twitch fibers display an increased expression of Fn14 during the symptomatic stages of ALS, most likely due to their selective vulnerability to denervation (18,33,34). Further, we have uncovered that Tweak expression is significantly increased at 90 days in muscle containing slow-twitch fibers (gastrocnemius and soleus) and at 30 days in muscle containing solely fast twitch fibers (TA), suggesting a muscle- and time-dependent regulation of Tweak expression. Combined, our data show that both Tweak and Fn14 are aberrantly expressed in the skeletal muscle of SOD1	extsuperscript{G93A} mice.

**Genetic deletion of Tweak in SOD1	extsuperscript{G93A} mice does not prevent motor deterioration or increase life expectancy**

Our analysis of the spinal cord and skeletal muscle in SOD1	extsuperscript{G93A} mice reveals a dysregulation of the Tweak–Fn14 pathway in both of these ALS target tissues. We thus set out to better define the contribution of Tweak to ALS pathology by crossing SOD1	extsuperscript{G93A} mice with Tweak	extsuperscript{−/−} mice. These mice are fertile, viable and without any signs of motor deficit (35). Wildtype, Tweak	extsuperscript{−/−}, SOD1	extsuperscript{G93A}, Tweak	extsuperscript{+/+} and SOD1	extsuperscript{G93A};Tweak	extsuperscript{−/−} mice were weighed weekly from 28 to 56 days and thrice-weekly after that (Fig. 5A). Analysis of weight curves shows no significant difference between wildtype and Tweak	extsuperscript{−/−} mice, suggesting that Tweak deletion alone does not influence weight. Similarly, both SOD1	extsuperscript{G93A};Tweak	extsuperscript{+/+} and SOD1	extsuperscript{G93A};Tweak	extsuperscript{−/−} mice are not significantly different, displaying similar typical weight loss over time. Next, we determined disease onset, defined as the age that peak weight was reached (12), and find no significant difference between SOD1	extsuperscript{G93A}; Tweak	extsuperscript{+/+} and SOD1	extsuperscript{G93A}; Tweak	extsuperscript{−/−} mice (mean onset SOD1	extsuperscript{G93A}; Tweak	extsuperscript{−/−} mice, 107 ± 18 days and for SOD1	extsuperscript{G93A}; Tweak	extsuperscript{−/−} mice, 109.6 ± 16 days, means ± SD) (Fig. 5B). In addition, the deterioration of muscular strength, as evaluated by a grid test, was comparable between SOD1	extsuperscript{G93A} mice bearing or not the genetic deletion of Tweak (Fig. 5C). Further, we observe that the deletion of Tweak does not influence the lifespan of SOD1	extsuperscript{G93A} mice (median survival of 173 days for SOD1	extsuperscript{G93A}; Tweak	extsuperscript{+/+} mice and 165.5 days SOD1	extsuperscript{G93A}; Tweak	extsuperscript{−/−} mice) (Fig. 5D), Consistently, the SM32 neurofilament marker to visualize motoneurons, we did not find any significant difference in the number of surviving motoneurons between 150-day-old SOD1	extsuperscript{G93A}, Tweak	extsuperscript{+/+} and SOD1	extsuperscript{G93A}; Tweak	extsuperscript{−/−} mice (Fig. 5E and F).

**Genetic deletion of Tweak in SOD1	extsuperscript{G93A} mice markedly reduces astrogliosis and microgliosis**

Tweak acts on astrocyte proliferation and pro-inflammatory cytokine, suggesting a role in maintenance and/or amplification of the neuroinflammatory status in ALS spinal cord. We therefore assessed the effect of Tweak deletion on spinal cord pathology of 150-day-old mice, where neurodegenerative, astrogliosis and microgliosis processes have already taken place and are easily detectable. We evaluated the extent of the neuroinflammatory response with specific markers for astrocytes (GFAP), activated microglia (Mac-1) and all microglia (Iba1). Quantification of GFAP fluorescent signal intensity shows that Tweak deletion significantly reduces astrogliosis in SOD1	extsuperscript{G93A};Tweak	extsuperscript{−/−} mice compared with SOD1	extsuperscript{G93A};Tweak	extsuperscript{+/+} mice (Fig. 6A and B). Strikingly, both Mac-1 and Iba1 signal intensities are also significantly decreased in Tweak-deleted SOD1	extsuperscript{G93A} mice (Fig. 6A–D), suggesting that the loss of Tweak affects overall microglia number or transition into an activated state. We thus further characterized individual SOD1	extsuperscript{G93A};Tweak	extsuperscript{+/+} and SOD1	extsuperscript{G93A};Tweak	extsuperscript{−/−} microglia (Fig. 6B). While total surface area between SOD1	extsuperscript{G93A};Tweak	extsuperscript{+/+} and SOD1	extsuperscript{G93A};Tweak	extsuperscript{−/−} microglia is not significantly different (Fig. 6F), we find that SOD1	extsuperscript{G93A};Tweak	extsuperscript{−/−} microglia display smaller soma area (Fig. 6G), decreased Iba1 signal intensity in total cell (Fig. 6H) and soma alone (Fig. 6I) and an increased number of primary processes (Fig. 6J) compared with SOD1	extsuperscript{G93A};Tweak	extsuperscript{−/−} microglia. Thus, Tweak deletion markedly reduces activation of astrocytes and microglia in SOD1	extsuperscript{G93A} spinal cord.

**Genetic deletion of Tweak in SOD1	extsuperscript{G93A} mice increases myofiber and neuromuscular junction endplate area**

SOD1	extsuperscript{G93A} mice are characterized by severe muscle atrophy and neuromuscular junction (NMJ) defects with the first observed NMJ abnormalities arising within the post-synaptic compartment (36). Given the role of Tweak in denervation-induced skeletal muscle atrophy (18) and the dysregulation of the Tweak–Fn14 pathway in skeletal muscle reported herein (Fig. 4), we evaluated the effect of the genetic ablation of Tweak on atrophy and post-synaptic NMJ apparatus of TA from 150-day-old SOD1	extsuperscript{G93A};Tweak	extsuperscript{+/+} and SOD1	extsuperscript{G93A};Tweak	extsuperscript{−/−} mice. Myofiber area was quantified on TA cross-sections and comparison of myofiber size distribution shows that muscle atrophy is reduced in SOD1	extsuperscript{G93A};Tweak	extsuperscript{−/−} mice compared with SOD1	extsuperscript{G93A};Tweak	extsuperscript{+/+} mice (Fig. 7A and B).

We next used a tetramethyldihydroamine-conjugated α-bungarotoxin (α-BTX) to label acetylcholine receptor (AChR) and assess NMJ endplate pathology. We first measured the endplate area and find that SOD1	extsuperscript{G93A};Tweak	extsuperscript{−/−} endplates are significantly bigger than those of SOD1	extsuperscript{G93A};Tweak	extsuperscript{+/+} mice (Fig. 7C and D). Finally, we categorized the morphological maturation of endplates as either normal pretzel shape, perforated with bright lines or completely disorganized (37) (Fig. 7E). Here, we demonstrate that there is no significant difference between SOD1	extsuperscript{G93A};Tweak	extsuperscript{+/+} and SOD1	extsuperscript{G93A};Tweak	extsuperscript{−/−} mice, where both groups predominantly have disorganized endplates (Fig. 7F). Our results thus show that deleting Tweak from SOD1	extsuperscript{G93A} mice partially reduces muscle pathology.

**Tweak immunodepletion in symptomatic SOD1	extsuperscript{G93A} mice reduces microgliosis and improves muscle pathology without preventing motoneuron degeneration**

To further assess the contribution of Tweak on disease progression without potential developmental compensation, we evaluated the effect of neutralizing Tweak activity. To do so, we treated SOD1	extsuperscript{G93A} mice at onset (100 days) with an antagonistic murine anti-Tweak antibody (mP2D10 clone). This antibody has successfully been used and validated in an experimental model of multiple sclerosis (38). The Tweak antibody (10 μg/g) or the IgG control (10 μg/g) was administered (or not) by intraperitoneal (IP) injection twice-weekly in SOD1	extsuperscript{G93A} mice. Consistent with the genetic approach, we find that treating mice with the antagonistic Tweak antibody does not improve lifespan, weight loss or muscular strength of SOD1	extsuperscript{G93A} mice (Fig. 8A–C). Further, we
also show that treating SOD1<sup>G93A</sup> mice with the Tweak antibody does not prevent motoneuron loss in the lumbar spinal cord (Fig. 8D and E). We next used GFAP and Mac-1 markers to analyze neuroinflammation in the spinal cord. Quantification of GFAP fluorescent signal intensity shows that astrogliosis is not significantly different between Tweak-treated, IgG-treated and untreated SOD1<sup>G93A</sup> mice (Fig. 8F and G). However, we observe a significant decrease in Mac-1-positive microgliosis in SOD1<sup>G93A</sup> mice treated with antagonistic anti-Tweak antibody compared with IgG-treated and untreated littermates (Fig. 8F and H). Our results thus suggest that immunodepletion of Tweak in symptomatic ALS mice reduces microgliosis without improving motor functions.

We next assessed the effect of the neutralizing anti-Tweak antibody on muscle pathology. Myofiber area was quantified on cross-sections of TA (Fig. 9A) and myofiber size distribution reveals that Tweak-treated SOD1<sup>G93A</sup> mice display a greater proportion of large myofibers compared with IgG-treated and untreated mice (Fig. 9A and B). We also determined the effect of Tweak depletion on NMJ endplate size and morphology. Measuring...
Figure 6. Genetic ablation of Tweak decreases astrocytosis and microgliosis in SOD1G93A mice. (A) Representative images of the ventral horn area of the lumbar spinal cord from 150-day-old SOD1G93A;Tweak+/+ and SOD1G93A;Tweak−/− mice co-labeled for astrocytes (GFAP), activated microglia (Mac-1) and microglia (Iba1). Scale bar, 100 μm.

Quantification of GFAP (B), Mac-1 (C) and Iba1 (D) fluorescent signal intensity in 150-day-old SOD1G93A;Tweak+/+ and SOD1G93A;Tweak−/− mice (n=3). (E) Representative images of magnified Iba1-positive microglia from the lumbar spinal cord of 150-day-old SOD1G93A;Tweak+/+ and SOD1G93A;Tweak−/− mice. Scale bar, 50 μm.

Quantification of total surface area (F), soma surface area (G), total surface Iba1 signal intensity (H), soma Iba1 signal intensity (I) and number of primary processes (J) in spinal cord microglia of 150-day-old SOD1G93A;Tweak+/+ (63 microglia from 3 mice) and SOD1G93A;Tweak−/− mice (49 microglia from 3 mice). All data represented are means ± SD; ****P < 0.0001; unpaired two-tailed t-test.
endplate areas in the TA shows that Tweak-treated SOD1G93A mice have significantly bigger endplates than IgG-treated and untreated littermates (Fig. 9C and D). Finally, when categorizing endplate morphology as pretzel-shaped, perforated with bright lines or completely disorganized, we find there is no significant difference between groups, with a predominance of immature looking end-plates (Fig. 9E and F). Therefore, treating symptomatic SOD1G93A mice with an antagonistic anti-Tweak antibody significantly improves certain muscle and post-synaptic NMJ parameters.

Discussion

Here, we identified Tweak as a molecular determinant acting at the intersection of astrocytes, microglia and skeletal muscle in ALS mice. Indeed, Tweak expression is specifically increased in spinal cord astrocytes of late symptomatic SOD1G93A mice and primary SOD1G93A spinal cord astrocytes display an increased expression of Fn14 and Tweak-dependent proliferation compared with wildtype cells. Further, the Tweak-Fn14 pathway is aberrantly expressed in skeletal muscle during the course of the disease. In primary motoneurons, Tweak induces a CD163-,
endocytosis and caspase-3-dependent death. Importantly, we show that depletion of Tweak in SOD1G93A mice via a genetic and a pharmacological approach significantly reduces astrocytosis and microgliosis as well as ameliorates skeletal muscle atrophy and post-synaptic NMJ pathology. Despite these significant effects, there was no alteration in the number of surviving motoneurons or lifespan in SOD1G93A mice.
Figure 8. Immunodepletion of Tweak in symptomatic SOD1<sup>G93A</sup> mice reduces microgliosis. (A) Starting at 100 days of age, SOD1<sup>G93A</sup> mice were administered an antagonistic Tweak antibody or an IgG control (10 μg/g) twice-weekly by an IP injection. The Kaplan–Meier survival curves of uninjected (n = 10), IgG-treated (n = 9) and anti-Tweak-treated (n = 9) SOD1<sup>G93A</sup> mice. (B) Uninjected (n = 10), IgG- (n = 9) and Tweak-treated (n = 9) SOD1<sup>G93A</sup> mice were weighed thrice-weekly starting at 84 days of age. (C) Muscular strength, evaluated via grid test, was evaluated in uninjected (n = 10), IgG- (n = 9) and anti-Tweak-treated (n = 9) SOD1<sup>G93A</sup> mice weekly starting at 84 days of age. (D) Representative images of the ventral horn area of the lumbar spinal cord of 150-day-old uninjected, IgG- and anti-Tweak-treated SOD1<sup>G93A</sup> mice immunolabeled with SMI32 to visualize the motoneurons. Scale bar, 50 μm. (E) Quantification of the number of motoneurons in the lumbar spinal cord of 150-day-old uninjected, IgG- and anti-Tweak-treated SOD1<sup>G93A</sup> mice (n = 3). (F) Representative images of the ventral horn region of the lumbar spinal cord of 150-day-old uninjected, IgG-treated or Tweak antibody-treated SOD1<sup>G93A</sup> mice labeled for GFAP and Mac-1. Scale bar, 50 μm. (G) Quantification of GFAP fluorescent signal intensity in the spinal cord of uninjected, IgG-treated and Tweak antibody-treated SOD1<sup>G93A</sup> mice (n = 3). (H) Quantification of Mac-1 fluorescent signal intensity in the spinal cord of uninjected, IgG-treated and Tweak antibody-treated SOD1<sup>G93A</sup> mice (n = 3). Data are means ± SD; A and B, two-way ANOVA; E, G and H, one-way ANOVA.
We therefore propose a model whereby Tweak contributes to ALS pathology in both the spinal cord and periphery (Fig. 10). In the spinal cord, increased IFN\(^\gamma\) expression (12,13), a potent activator and upstream effector of Tweak, may induce up-regulation of astrocytic Tweak in late disease stages, which can subsequently participate in the maintenance of neurodegenerative and neuroinflammatory events. Indeed, our in vitro experiments show that Tweak promotes neurotoxicity and astrocytic proliferation, both typical ALS hallmarks. In the periphery (skeletal muscle), Tweak could influence pathology by directly modulating muscle atrophy (18), or indirectly by influencing the activation state of microglia. Indeed, skeletal muscle–microglia communication has previously been proposed to occur via reactive oxygen species released by atrophied muscle that travel retrogradely within the motoneuron and subsequently activate microglia (6,39,40).

Combined, our results suggest that both peripherally and centrally expressed Tweak may contribute to the sustained activation of microglia in ALS.

One surprising finding is that in light of the significant astrogliosis and especially microgliosis rescue in Tweak-depleted mice, disease progression was not halted. Growing evidence indicates that SOD1 mutant has both cell- and non-cell-autonomous effects on motoneurons. The excision of mutant SOD1 in astrocytes slows disease onset and/or progression (5,41) while targeted deletion of SOD1 mutant in microglia modulates late phase of disease (4). However, the ablation of proliferating microglia during the symptomatic stage (85 days), a later intervention compared with the genetic approaches discussed, did neither prevent motoneuron degeneration nor motor function deterioration (42), which may have been due to the ablation of beneficial effects of microglia on motoneurons.
microglia of an M2 phenotype that eventually shift to M1 detrimental microglia during the course of the disease (20, 43). While not investigated here, it is possible that depletion of Tweak may also have impacted the activity and function of beneficial M2 microglia. Additionally, Tweak being a downstream effector of IFNγ, depleting Tweak and reducing microgliosis may not impact the deleterious effect of Tweak-independent IFNγ signaling on astrogliosis and motoneuron death (12). As for the role of astrocytosis in disease progression, selective ablation of GFAP-positive proliferating astrocytes in SOD1 mutant mice does not alter motor decline, neuronal loss or survival (44). In fact, the total number of astrocytes did not change following ablation of proliferative astrocytes, suggesting that newly derived astrocytes from NG2-positive glial progenitors, which were not depleted in these mice, might contribute to the pathogenic progress (44, 45). Furthermore, the identification of region-specific astrocytes, which determine survival and electrical properties of α-motoneurons, suggests that subpopulations of astrocytes may be selectively neurotoxic (46). Thus, the overall effect of Tweak on astrocytosis may not be selective toward the contributory NG2-positive glial progenitors and region-specific astrocytes (35). All together, our results emphasize the multi-systemic aspect of ALS while reinforcing that rescuing the primary target, that is motoneurons, is instrumental for significant lifespan and overt phenotype rescue.

There is a significant microgliosis reduction in the spinal cord of SOD1G93A;Tweak−/− mice, which could in fact be mediated indirectly by the specific up-regulation of astrocytic Tweak. Indeed, IL-6 promotes microgliosis and we have observed an increased production of this cytokine following Tweak activation (47). However, the systemic administration of anti-Tweak neutralizing antibody in SOD1G93A mice decreases microgliosis but not astrocytosis, suggesting that peripheral Tweak activity may specifically influence microgliosis activation (47, 48). Tweak does appear to act preferentially on microglia over astrocytes (48). Alternatively, the Tweak antibody may have been administered at a time-point at which astrocytosis was already initiated and therefore could not be halted but at which microgliosis could still be manipulated.

Here, we find an aberrant modulation of the Tweak–Fn14 pathway in various skeletal muscles in a type- and time-dependent manner. Our analysis also reveals that misregulation of Fn14 and not Tweak is more prominent in vulnerable muscles such as the TA and gastrocnemius. Combined, these observations could appear to mitigate the importance of Tweak and Fn14 in the skeletal muscle of SOD1G93A mice. However, two crucial pieces of information point to the contrary. First, both genetic and pharmacological ablation of Tweak significantly improves muscle atrophy and NMJ endplate size in SOD1G93A mice. Second, increased Fn14 expression following skeletal muscle denervation, similar to what we have observed in SOD1G93A muscle, has previously been demonstrated to be the main mediator behind the persisting subsequent atrophy (18). While the present study has focused on Tweak, future studies investigating the effect of Fn14 modulation on muscle and NMJ pathology in SOD1G93A mice will be essential to fully understand the contribution of the Tweak–Fn14 pathway on neurodegeneration-induced muscle atrophy in ALS.

Considering Tweak’s prominent role in regulating muscle atrophy (49), the modulation of muscle parameters observed herein is not as dramatic as expected. While both Tweak and Fn14 contribute to skeletal muscle function, it has been suggested that the receptor acts as the limiting factor in the activation of the Tweak–Fn14 signaling cascade (18). Tweak depletion may therefore not impact the aberrant expression of Fn14 in the skeletal muscle, thus maintaining the activation of certain atrophic pathways, especially as Fn14 can function in a Tweak-independent manner in muscle (16). Further, Tweak positively regulates various muscle pathways such as myoblast proliferation during regeneration and myotube formation (50). Depleting Tweak may therefore hinder certain aspects of myogenesis in SOD1G93A;Tweak−/− mice and of proliferation/regeneration in SOD1G93A mice treated with the antagonistic Tweak antibody. Finally, analysis of post-synaptic NMJ apparatus shows a specific effect of Tweak depletion on endplate size but not morphological maturation. Seeing as endplate maturity and neuromuscular transmission are tightly connected (51), an improvement in both the pre- and post-synaptic NMJ compartments is most likely required to significantly ameliorate endplate morphology. This suggests a selective effect of Tweak depletion on the post-synaptic NMJ apparatus. Indeed, it has recently been demonstrated that administration of a neutralizing anti-Tweak antibody in a mouse model of myotonic dystrophy type 1, which unlike ALS is an inherited muscular dystrophy, led to significant benefits in motor function and muscle histology (52). Thus, to optimize the muscle benefits in ALS, the timing, dosing and frequency of Tweak depletion needs to be further evaluated and most likely combined with central nervous system-specific therapeutic candidates.

Our work highlights the potential of an anti-Tweak immunotherapy approach to alleviate neuroinflammation and muscle atrophy in ALS as well as the therapeutic potential of Tweak modulation for other disorders characterized by these symptoms. Importantly, our work reinforces the complex interplay between different tissues and/or cell types that occurs in ALS, a disorder that will undoubtedly require a comprehensive combinatorial therapeutic approach.

Figure 10. Proposed model on how Tweak contributes to neuroinflammation and skeletal muscle atrophy in ALS. Increased production of IFNγ by astrocytes and motoneurons promotes astrocytic Tweak expression. Tweak subsequently participates in neurodegenerative and neuroinflammation processes by activating motoneuron death, stimulating astrocytic proliferation and inducing astrocytic IL-6 release, which in turn promotes microgliosis. In ALS muscle, an aberrant regulation of Tweak and its receptor Fn14 contributes to maintenance of denervation-induced skeletal muscle atrophy. The atrophic state of the muscle also retrogradely communicates via the motoneuron to microglia and further adds to the neuroinflammatory environment.
Materials and Methods

Animals

All animal experiments were approved by the national ethics committee on animal experimentation, and were done in compliance with the European community and national directives for the care and use of laboratory animals (authorization A34–506). SOD1<sup>G93A</sup> and Hb9::GFP mice were maintained on a C57BL/6 background and purchased from Jackson Labs. Tweak−/− mice were also maintained on a C57BL/6 background and obtained from Dr Avi Ashkenazi (Genentech) (35). For weight curves, mice were weighed weekly starting at 28 days of age and thrice-weekly starting at 56 days of age. For the grid test (53), mice were evaluated weekly starting at 56 days. Starting with a 40 g metal grid (followed by 30, 20 and 10 g grids), we measured the time the animal held on to the grid before dropping it, with a platinum-metal grid (followed by 30, 20 and 10 g grids), we measured the time point at which the animal remained longer than 15 s without turning over when placed on its back. For the immunotherapy experiments, 100-day-old mice were administered the time point at which the animal remained longer than 15 s without turning over when placed on its back. For the immunotherapy experiments, 100-day-old mice were administered the Tweak antibody or the IgG control by IP injection twice a week for 8 weeks (10 µg/g). Mixed-sex cohorts of mice per experimental group were used as the SOD1<sup>G93A</sup> males and females in our colony display similar median survivals (SOD1<sup>G93A</sup> males, 173 days; SOD1<sup>G93A</sup> females, 173 days; not significant, log-rank test). Similarly, no gender effects on the median survival were observed in either genetic or pharmacological approaches (SOD1<sup>G93A</sup>; Tweak−/− males, 169 days; SOD1<sup>G93A</sup>; Tweak−/− females, 160 days and SOD1<sup>G93A</sup> anti-Tweak-treated males, 163.5 days; SOD1<sup>G93A</sup> anti-Tweak-treated females, 155 days; not significant, log-rank test). Further, for all histological analyses, all experimental groups were evenly sex-matched to avoid any sex-dependent influences.

Antibodies and reagents

Rabbit polyclonal antibodies for mouse Tweak and CD163 were raised using the following peptide EPPELNPQTEESQD (Tweak) and VTNAPGEMKKELR (CD163) conjugated to KLH. Anti-sera were purified on peptide-Sepharose affinity column (AbiLiance). The primary antibodies used were as follows: mouse anti-SMI32 (1:1000, Sternberger Monoclonals), mouse anti-GFAP (1:600, Millipore), rabbit anti-Tweak (1:50, AbiLiance), rat anti-Iba1 (1:500, Wako Chemical Industries); chicken anti-GFP curve. Further, for all histological analyses, all experimental groups were evenly sex-matched to avoid any sex-dependent influences.

ELISA assays

The ELISA kits used were as follows: mouse IL-6 (R&D Systems), mouse Tweak and mouse CD163 (CUSABIO Biotech Co). All ELISAs were implemented according to the manufacturer’s instructions. For IL-6, supernatants were diluted 1:2, recombinant mouse IL-6 standard was serially diluted from 500 to 7.8 pg/ml and a control sample was used in order to validate the experiment and quantify. For Tweak, sera were diluted 1:4 and recombinant mouse Tweak standard was serially diluted from 1000 to 15.6 pg/ml. For CD163, sera were diluted 1:50, supernatants were undiluted, recombinant mouse CD163 standard was serially diluted in sample diluents ranging from 2000 to 31.25 pg/ml. For all ELISA assays, the absorbance at 450 nm was recorded using a microplate reader and the wavelength was corrected for optical imperfections by subtracting the reading at 540 nm. Standard curves were plotted and final concentrations were calculated by interpolations from the standard curve.

Primary cultures

Motoneuron cultures were prepared from Hb9::GFP embryonic day (E)12.5 embryos as previously described (54). Briefly, cells were dissociated mechanically after trypsin treatment of the dissected spinal cords. The largest cells were isolated using iodixanol density gradient purification (OptiPrep, Sigma-Aldrich). After a BSA cushion, motoneurons were plated onto poly-l-ornithine- and laminin-coated wells in supplemented Neurobasal (Life Technologies) medium in the presence of 1 ng/ml BDNF, 100 pg/ml GDNF and 10 ng/mlCNTF, completed with 2% horse serum, B27 supplement (Life Technologies), 50 µM l-glutamine, 25 µM l-glutamate, 25 µM β-mercaptoethanol and 0.5% penicillin/streptomycin (pen/strep). Motoneurons were then incubated at 37°C and 7.5% CO<sub>2</sub>. For survival assays, we used phase contrast microscopy to count motoneurons characterized by a set of defined morphological criteria (54).

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Primary astrocytes were isolated from individual spinal cords from neonatal pups. Briefly, following treatment with trypsin, cells were mechanically dissociated from the dissected spinal cords. After a BSA cushion, astrocytes were plated onto 60 mm tissue culture plates coated with poly-L-ornithine in DMEM [10% fetal bovine serum (FBS), 1% pen/strep]. Astrocytes were incubated at 37°C until confluency and split only once for experimental use. For proliferation assays, culture media were changed to DMEM (1% pen/strep) without FBS 24 h prior to addition of recombinant Tweak to astrogial proliferation and synchronize all cells. Upon addition of recombinant Tweak, culture media were restituted to DMEM (10% FBS, 1% pen/strep).

Cortical neurons were isolated from E17.5 embryos as previously described (12,54). Cells were plated on poly-L-ornithine- and laminin-coated wells in Neurobasal media (1 mM sodium pyruvate, 2% B27 supplement) and incubated at 37°C and 7.5% CO2. For survival assays, we used phase contrast microscopy to count healthy neuronal cells in a pre-determined field of view (12).

Live imaging
Motoneurons (20 000) were plated on a glass-bottom 35 mm culture dish (World Precision Instruments) and treated with recombinant Tweak 24 h later. Media were gently aspirated and replaced 3 h later, at which point the NTA-Atto 550 was added. For the receptor inhibition experiments, the antagonistic Fn14 and CD163 antibodies were added 1 h before adding recombinant Tweak. Live imaging was performed immediately, using an Inverse1 Zeiss Axioobserver/LSM 5 LIVE DUO confocal microscope, equipped with filters suitable for FITC/Cy3 fluorescence. Cells were maintained in a 37°C 5% CO2 chamber and images were taken every 30 min during a 16 h period. Videos were subsequently processed in the exact same manner using the Imaris software (Bitplane) by applying a Gaussian filter (0.32) on the green channel to smooth the image and by setting the transparency of the green channel at 87% to better visualize the endocytosis of the Alexa555-tagged Tweak.

Immunocytochemistry
For immunocytochemistry of motoneurons and astrocytes, cells were plated on coated glass coverslips. First, 4% paraformaldehyde (PFA) in PBS was added to the culture media (1:1) for 10 min followed by 4% PFA for 15 min. Cells were then washed with PBS and incubated 1 h with blocking solution at room temperature (RT) (4% BSA, 4% donkey serum, 0.1% Triton X-100). This was followed by an overnight incubation with the primary antibodies at 4°C. After PBS washes, cells were incubated with secondary antibodies dilute in blocking solution for 1 h at RT. Finally, cells were washed in PBS, with the last wash containing Hoescht. Images were taken with an Inverse1 Zeiss Axioobserver/LSM 5 LIVE DUO confocal microscope or an upright Zeiss AxioImagerZ1/Apotome microscope. For astrocyte proliferation assays, the percentage of Ki67-positive Hoescht-positive cells was quantified with ImageJ software.

For immunohistochemistry, mice were anesthetized and perfused transcardially with 4% PFA. Lumbar spinal cords were removed and post-fixed in 4% PFA, flash-frozen and cut at a 12 μm thickness. The sections were then rinsed 5 min in PBS and incubated for 2 h at RT in blocking solution (Tris-buffered saline, 0.9% L-Lysine and BSA, 20% goat serum, 0.3% Triton X-100). This was followed by overnight incubation at +4°C with primary antibodies. Subsequently, sections were incubated 1 h with secondary antibodies. All washes were done with PBS. Slides were mounted in Mowiol. Images were taken with an Inverse1 Zeiss Axioobserver/LSM 5 LIVE DUO confocal microscope. Quantification of GFAP and Mac-1 signal intensity and measurement of microglia surface area was done with ImageJ software. For quantification of motoneurons, astrocytes and microglia, an average of nine cross-sections was used per animal.

Hematoxylin and eosi staining of TA sections
Whole TA were dissected and placed overnight in 4% PFA, then transferred to 70% ethanol solution. Tissues were embedded in paraffin, cut at a thickness of 5 μm, deparaffinized and stained for hematoxylin and eosi. Images were taken with a Leica DMRB microscope. Muscle fiber area was measured using the NIH ImageJ software.

NMJ labeling
TA muscle sections were labeled by immunohistochemistry to allow quantification of NMJ endplate area and morphology as described previously (37). Whole TA muscles were dissected and fixed in 4% PFA for 15 min. Following removal of connective tissue, TA muscles were incubated with αBTX for 30 min at RT, followed by PBS washes. Finally, 2–3 thin files per TA were sliced and mounted in Mowiol. All files were single sections of the surface of the TA muscle. Images were taken with an Inverse1 Zeiss Axioobserver/LSM 5 LIVE DUO confocal microscope. ImageJ software was used to calculate the area of NMJ endplates.

Statistical analysis
All experiments were done at least three times independently. All statistical analyses were done with the Graphpad Prism software. When appropriate, a Student’s unpaired two-tail t-test, a one-way or two-way analysis of variance (ANOVA) followed by a Holm–Sidak’s post hoc tests were used. For the Kaplan–Meier survival analysis, the log-rank test was used and survival curves were considered significantly different at P < 0.05.

Supplementary Material
Supplementary Material is available at HMG online.

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