Cell Death and Differentiation (2011) 18, 754–768

www.nature.com/cdd

IFNγ triggers a LIGHT-dependent selective death of motoneurons contributing to the non-cell-autonomous effects of mutant SOD1

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Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disease that primarily affects motoneurons in the brain and spinal cord. Dominant mutations in superoxide dismutase-1 (SOD1) cause a familial form of ALS. Mutant SOD1-damaged glial cells contribute to ALS pathogenesis by releasing neurotoxic factors, but the mechanistic basis of the motoneuron-specific elimination is poorly understood. Here, we describe a motoneuron-selective death pathway triggered by activation of lymphotoxin-β receptor (LT-βR) by LIGHT, and operating by a novel signaling scheme. We show that astrocytes expressing mutant SOD1 mediate the selective death of motoneurons through the proinflammatory cytokine interferon-γ (IFNγ), which activates the LIGHT-LT-βR death pathway. The expression of LIGHT and LT-βR by motoneurons *in vivo* correlates with the preferential expression of IFNγ by motoneurons and astrocytes at disease onset and symptomatic stage in ALS mice. Importantly, the genetic ablation of *Light* in an ALS mouse model retards progression, but not onset, of the disease and increases lifespan. We propose that IFNγ contributes to a cross-talk between motoneurons and astrocytes causing the selective loss of some motoneurons following activation of the LIGHT-induced death pathway.
Cell Death and Differentiation (2011) **18,** 754–768; doi:10.1038/cdd.2010.143; published online 12 November 2010

Amyotrophic lateral sclerosis (ALS) is a devastating motoneuron disease, characterized by the selective and gradual degeneration of both upper and lower motoneurons. Approximately, 10% of ALS cases are inherited and among these, 20% are caused by dominant mutations in the *superoxide dismutase-1* (*SOD1*) gene. Mice expressing human SOD1 mutations develop a motor syndrome with features of the human disease.[1] Both cell-autonomous and non-cell-autonomous processes contribute to motoneuron degeneration: a toxic action of mutant SOD1 within motoneurons has been documented as crucial for the onset and the early phase of disease progression,[2] whereas a non-cell-autonomous component, involving damage to astrocytes and microglia is determinant for disease progression.[3] Astrocytes have a pivotal role in the pathogenic process by determining the extent of the inflammatory response from microglia,[3] but also by releasing soluble factors selectively toxic for motoneurons.[4–8] The specificity of this toxicity toward motoneurons might be explained by the activation of a motoneuron-specific death pathway; a hypothesis that has been tested in several studies.

Active killing of neurons by death receptors of the tumor necrosis factor (TNF) receptor superfamily, including TNFR1, p75^{NTR} or Fas has been documented.[9,10] TNFα can efficiently trigger the death of cultured motoneurons,[11] but may not directly participate to motoneuron degeneration in disease.[12] Nerve growth factor in combination with nitric oxide (NO), produced by reactive astrocytes, has been proposed to induce a p75^{NTR}-dependent motoneuron death *in vitro*,[13] but conflicting results have not yet demonstrated a functional relevance of p75^{NTR} in the direct killing of motoneurons in ALS models.[9] We previously demonstrated that Fas triggers a motoneuron-restricted death pathway, which is exacerbated in a cell-autonomous manner by mutant SOD1.[14,15] Interestingly, a functional involvement of the Fas death pathway in motoneuron degeneration in mutant SOD1 mice has been shown.[15–17] Regarding the pathogenic processes, the mutant astrocyte-mediated toxicity to motoneurons would occur independently of the Fas death pathway,[8] suggesting that other sources, such as microglia or serum, activate Fas.[14,18] Our understanding of the selective degenerative process integrating external death triggers remains, however, incomplete.

LIGHT (TNFSF14) is a type II transmembrane protein of the TNF superfamily that can engage the lymphotoxin-β receptor (LT-βR), the herpes virus entry mediator (HVEM) and the decoy receptor 3. LIGHT, which is expressed by immature dendrocytes, activated lymphocytes, monocytes and natural killer cells, and is important for both innate and adaptive

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Keywords: amyotrophic lateral sclerosis; interferon-γ; LIGHT; astrocytes; motoneurons

Abbreviations: ALS, amyotrophic lateral sclerosis; SOD1, superoxide dismutase-1; LT-βR, lymphotoxin β receptor; IFNγ, interferon-γ; TNF, tumor necrosis factor; NO, nitric oxide; HVEM, herpes virus entry mediator; GFP, green fluorescent protein; NOS, nitric oxide synthase; IFNγR, IFNγ receptor; VAChT, vesicular acetylcholine transferase; GFAP, glial fibrillary acidic protein; PGD_2, prostaglandin D_2; ROS, reactive oxygen species; CSF, cerebrospinal fluid; IL, interleukin

Received 10.5.10; revised 22.9.10; accepted 05.10.10; Edited by L Greene; published online 12.11.10

immune processes.[19] Remarkably, LIGHT can function with the immunomodulatory cytokine interferon-γ (IFNγ) to induce a singular slow apoptotic death in tumor cells,[20] reminiscent of the progressive nature of motoneuron degeneration in the disease.

Here, we report that the activation of LT-βR by LIGHT triggers a novel motoneuron-selective death pathway, which shows an additive killing potency with the activation of Fas. We demonstrate that IFNγ selectively induces death of motoneurons through the LIGHT-LT-βR pathway and mediates the neurotoxic effect of astrocytes expressing mutant SOD1. LIGHT and LT-βR are expressed by motoneurons both in control and mutant SOD1 mice, whereas expression of IFNγ is observed in motoneurons and astrocytes at the onset and symptomatic stage in ALS mice. Finally, deficiency of *Light* in ALS mice delays the progression, but not the onset of disease and extends life expectancy. We propose that besides its proinflammatory activity, IFNγ induces a motoneuron-specific LIGHT-dependent death pathway that contributes to the loss of motoneuron in ALS.

Results

LIGHT triggers a motoneuron-selective death pathway. To investigate the potential role of LIGHT in triggering death of motoneurons, we first asked whether cultured motoneurons express LIGHT, LT-βR and HVEM. We isolated embryonic motoneurons from mice expressing the green fluorescent protein (GFP) under the control of the motoneuron-selective *Hb9* promoter (*Hb9*::*GFP*) to facilitate motoneuron tracing.[8] We found that all motoneurons cultured for 24 h express LIGHT, LT-βR and HVEM (Figure 1a–d). We next exposed motoneurons for 48 h to increasing concentrations of mouse or human soluble LIGHT (sLIGHT) and assessed survival by counting phase-bright neurons using morphological criteria,[14] or GFP-positive neurons isolated from *Hb9*::*GFP* embryos. In both cases, we observed that mouse and human sLIGHT induce death of about 50% of motoneurons in a dose-dependent manner (Figure 1e and not shown). We next investigated motoneuron survival with respect to sLIGHT in a time-dependent manner. Cell survival was not significantly altered 24 h following sLIGHT addition, was diminished by about half after 48 h and was unchanged after 72 or 96 h of LIGHT treatment (Supplementary Figure 1a).

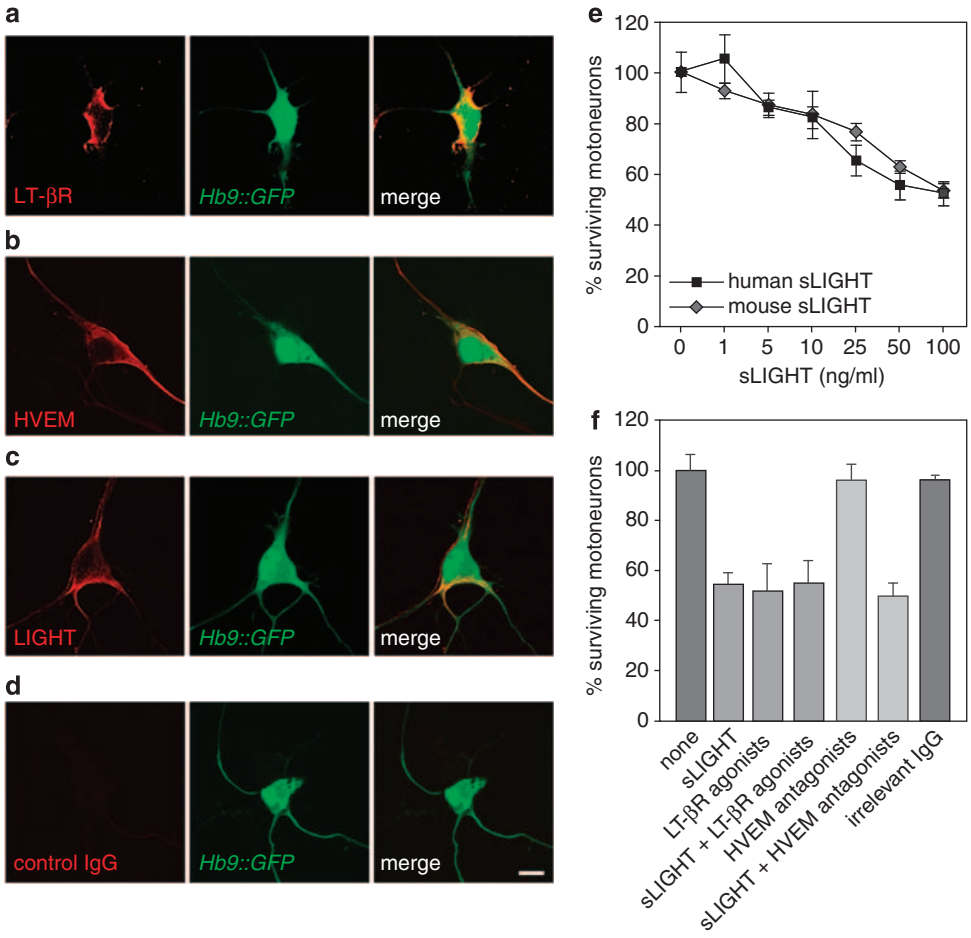
To ensure that the LIGHT killing effect functions specifically through LT-βR and/or HVEM, sLIGHT was challenged with soluble decoy receptors, including LT-βR-, HVEM-, Fas- or TNFR1-Fc chimeras. Although Fas- or TNFR1-Fc inhibited motoneuron death induced by their respective ligands, they did not show any inhibitory activity on sLIGHT. LT-βR-Fc and HVEM-Fc, both of which compete with sLIGHT, however, efficiently rescued motoneurons from LIGHT-induced death (Supplementary Figure 1b and c), supporting the specificity of the death signal triggered by LIGHT.

As LIGHT can engage both LT-βR and HVEM, it raised the question whether these receptors function cooperatively, or if not, which of both triggers motoneuron death. We show that agonistic anti-LT-βR antibodies show the same killing activity as sLIGHT, without unspecific additive toxicity when combined with sLIGHT (Figure 1f). On the contrary, antagonistic anti-HVEM antibodies[21] did not inhibit LIGHT-induced death (Figure 1f). These results suggest that LT-βR activation is sufficient to induce death of motoneurons and that HVEM does not cooperate with LT-βR to mediate LIGHT killing effect.

The identification of LIGHT as a novel death-inducing ligand in motoneurons prompted us to investigate its target-cell specificity. When we examined whether cortical, hippocampal, sensory or striatal neurons were sensitive to LIGHT, we observed that none of these neuronal types was killed by sLIGHT (Figure 1g). This motoneuron-specificity to LIGHT cannot be attributed to the absence of expression of the death receptor, as all these neuronal types express substantial levels of LT-βR (Figure 1h), but is likely due to an intrinsic property of motoneurons.

Fas and LT-βR-triggered death are additive and differ in their signaling. The activation of Fas that triggers death of about 50% of motoneurons,[14] raised the question about its functional complementarity with LIGHT. Interestingly, LIGHT and FasL added together induced death of about 70% of motoneurons (Figure 2a). We next investigated whether LT-βR and Fas use the same signaling network. The p38 kinase controls crucial steps in Fas death signaling, including NO production through upregulation of the neuronal NO synthase (NOS) and cytochrome *c* release from mitochondria.[14] We first determined the phosphorylation levels of nuclear p38 by quantitative confocal miscroscopy.[15,22] Treatment of motoneurons with sLIGHT, as well as sFasL, resulted in a significant increase in levels of p38 phosphorylation (Figure 2b and Supplementary Figure 2a). SB203580, a pyridinyl imidazole inhibitor of p38, prevented phosphorylation of p38, consistent with its inhibitory effect on the autophosphorylation-dependent activation of p38.[23] In addition, we found that SB203580 blocked degeneration of motoneurons induced by sLIGHT (Figure 2c), supporting the role of p38 in the LIGHT death pathway. Interestingly, inhibition of NO production by the broad-spectrum NOS inhibitor L-NAME did not provide protection against LIGHT-induced death (Supplementary Figure 2b), although it saved motoneurons from death induced by trophic factor deprivation (Supplementary Figure 2c).

We then examined the contribution of the mitochondrial pathway in the death program. Fas activation leads to a redistribution of cytochrome *c* from a punctuated mitochondrial to a diffuse cytoplasmic immunoreactivity in motoneurons.[14] Interestingly, we did not observe a cytoplasmic redistribution of cytochrome *c* following sLIGHT treatment at 24, 30 or 36 h (Figure 2d and e and not shown). We next inhibited Bax, a major effector of cytochrome *c* release, using the inhibitory pentapeptide VPMLK (V5). Although V5 efficiently rescued motoneurons from Fas-induced death, V5 did not protect motoneurons against LIGHT-killing effect (Figure 2f). We also investigated the functional impact of the overexpression of two other factors acting at the mitochondrial checkpoint: Bcl-Xl and t^n-Bid, a dominant negative form of Bid.[24] Consistently, LIGHT-induced death signaling was not affected by the overexpression of either Bcl-Xl or t^n-Bid in motoneurons in contrast to the Fas death pathway (Figure 2g). Thus, the LIGHT-LT-βR death pathway appears



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	% cell survival	
neuronal type	none	sLIGHT
cortical	100 ± 9.2	96,9 ± 5,2
hippocampal	100 ± 6.9	104.4 ± 11.4
sensory	100 ± 7.1	96.6 ± 5.3
striatal	100 ± 7.8	92.5 ± 6.1

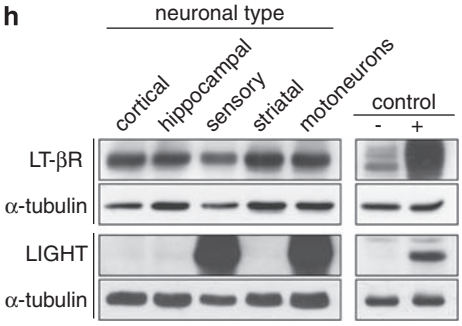


Figure 1 sLIGHT selectively induces death of motoneurons. (**a**–**d**) *Hb9*:: *GFP* motoneurons were cultured for 24 h and immunostained with anti-LT-βR (**a**), anti-HVEM (**b**) and anti-LIGHT (**c**) antibodies. Goat (**d**) or rabbit (not shown) irrelevant IgGs were used as control. Scale bar, 10 μm. (**e**) Mouse motoneurons were cultured for 24 h and then incubated with increasing concentrations of human or mouse sLIGHT. Motoneuron survival was determined 48 h later and expressed relative to non-treated cells. Henceforth, sLIGHT will refer to the human form of the recombinant protein. The distance between the x-axis values is arbitrary. (**f**) After 24 h in culture, motoneurons were treated (or not) with agonistic anti-LT-βR antibodies (100 ng/ml), antagonistic anti-HVEM antibodies (100 ng/ml) or irrelevant goat IgG (100 ng/ml) in combination (or not) with sLIGHT (100 ng/ml). Cell survival was determined 48 h later. (**g**) Twenty four hours after plating, cortical, hippocampal, sensory and striatal neurons were treated (or not) with sLIGHT (100 ng/ml). Neuron survival was determined 48 h later. (**h**) Protein extracts from cortical, hippocampal, sensory, striatal and motoneurons cultured for 24 h were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by Western blotting with specific antibodies to LT-βR and LIGHT. The loading control was α-tubulin. Lysates from Cos-7 cells transfected (+) or not (−) with LT-βR or LIGHT expression vectors were used as controls. Graphs in (**e**), (**f**) and table in (**g**) show means values of three independent experiments, each done in triplicate

to proceed independently of cytochrome *c* release, suggesting that a previously unknown cascade of events is involved.

LIGHT triggers an unconventional caspase-dependent death process. Caspases are pivotal components in the apoptotic death program. We, therefore, explored the protective effect of inhibiting prototypic initiator (caspase-8 and -9) and effector (caspase-3, -7 and -6) caspases. We observed that motoneuron death induced by sLIGHT was not blocked by the caspase-8 inhibitor, z-IETD-fmk, which effectively blocked death induced by trophic factor deprivation or Fas activation (Figure 3a, Supplementary

Figure 3a and not shown). However, Ac-LEHD-cmk, that inhibits caspase-9, rescued motoneurons from LIGHT-induced death (Figure 3a). Surprisingly, when we examined executioner caspases, we found that z-DEVD-fmk, which inhibits the caspase-3/-7 axis, did not save motoneurons from the sLIGHT killing effect (Figure 3a), whereas it efficiently rescued motoneurons from death induced by trophic factor deprivation or Fas activation (Supplementary Figure 3a and not shown). We also found that the caspase-6 inhibitor, z-VEID-fmk blocked death of motoneurons triggered by sLIGHT (Figure 3a). We further confirmed the requirement of caspase-9 independently of caspase-3 by examining their respective activation using specific antibodies against cleaved forms (Figure 3b and Supplementary Figure 3b). We detected a significant increase in caspase-9 activation, but not of caspase-3, in motoneurons following sLIGHT treatment (Figure 3c). We further examined whether the LIGHT-LT-βR pathway was functionally dependent on caspase-9 and -6 but independent of caspase-7 by overexpressing their catalytically inactive mutants that function as dominant-negative forms.[25] Consistently, we showed that the caspase-9 and -6, but not caspase-7 mutants saved motoneurons from LIGHT-induced death (Figure 3d), whereas caspase-7 mutant saved motoneurons from Fas-induced death (Supplementary Figure 3c). Implication of caspase-6, but not of caspase-3/-7, was further confirmed using agonistic anti-LT-βR (Supplementary Figure 3d). Finally, we confirmed the functional implication of caspase-6 by probing its processed specific substrate, lamin A.[26] Results showed that, although sLIGHT increased lamin A cleavage in motoneurons (Figure 3e and f), it was prevented by caspase-6, but not by caspase-3, inhibitor (Supplementary Figure 3e). Thus, LIGHT triggers a novel caspase-dependent signaling pathway in motoneurons.

IFNγ induces motoneuron death in a LIGHT-dependent manner. IFNγ is a cytokine known to function synergistically with LIGHT to activate caspases in tumor cells. Therefore, we investigated the influence of IFNγ on the modulation of the LIGHT-LT-βR system in motoneurons. We first demonstrated the expression of IFNγ receptor (IFNγR) chain 1 and 2 in nearly all motoneurons *in vitro* (Figure 4a–c). We next tested whether IFNγ was functionally relevant to LIGHT-induced death. By exposing motoneurons to a suboptimal dose of sLIGHT and increasing doses of IFNγ, we found that IFNγ substantially potentiates the LIGHT-killing effect (Supplementary Figure 4a), while having no effect on sFasL-induced death (Supplementary Figure 4b).

We then found that IFNγ induced death of about 50% of motoneurons in a dose-dependent manner (Figure 4d). The similarity with the loss of half of the motoneurons and the slow death kinetics of LIGHT-induced death prompted us to examine whether IFNγ-triggered death involved the LIGHT-LT-βR pathway. We thus competed endogenous LIGHT with LT-βR or HVEM-Fc and showed that both decoys saved motoneurons from the killing effect of IFNγ, whereas neither Fas- nor TNFR1-Fc conferred any protective effect (Figure 4e). To further argue for a LIGHT-dependent mechanism, we showed that inhibition of caspase-6, but not caspase-3, saved motoneurons from IFNγ-induced death (Supplementary Figure 4c). Finally, we investigated whether the IFNγ responsiveness was altered in *Light*-deficient motoneurons.[27] We found that motoneurons isolated from *Light*$^{-/-}$ embryos are resistant to IFNγ-killing effect (Figure 4f), confirming that IFNγ triggers motoneuron death in a LIGHT-dependent manner.

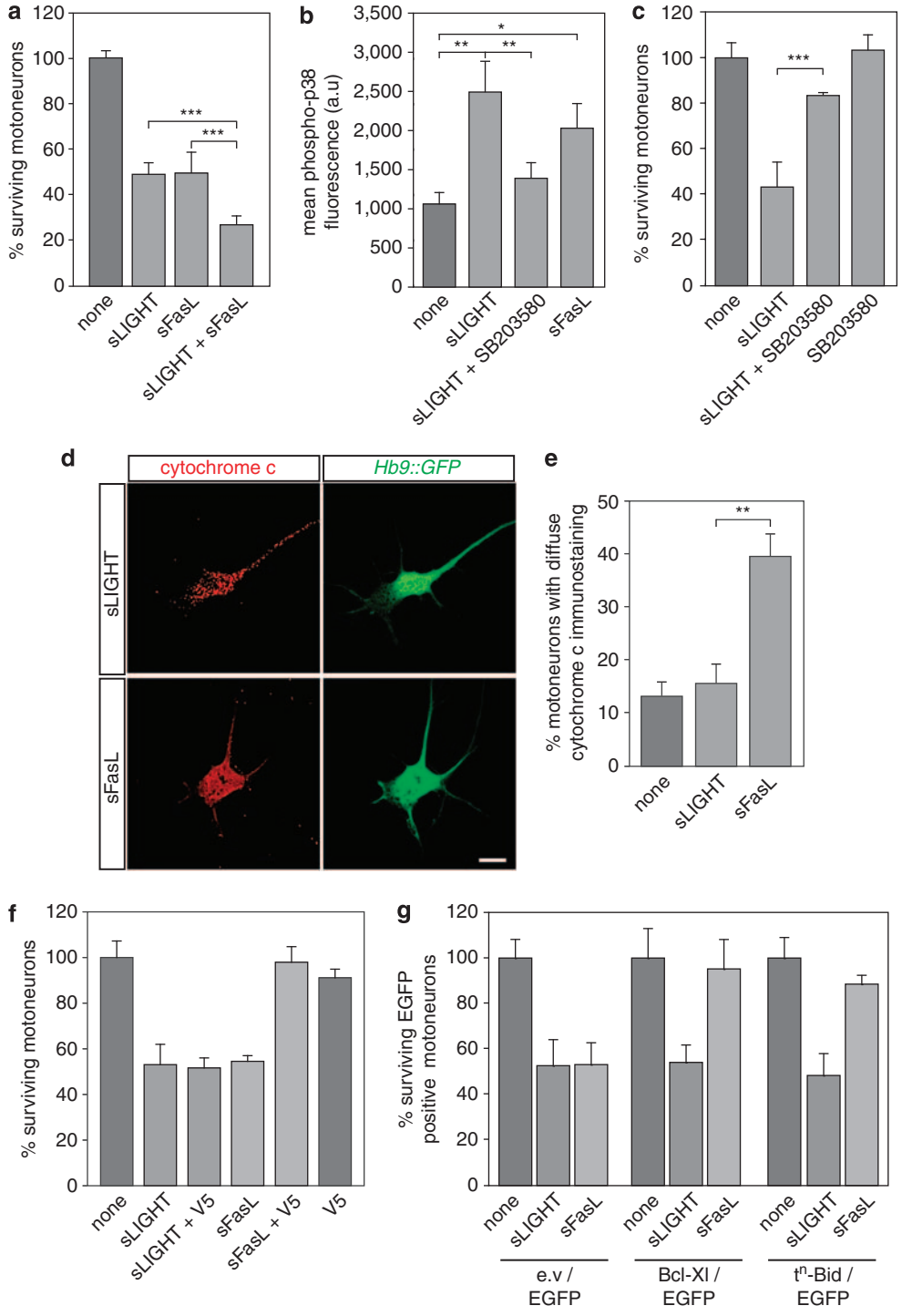
To assess whether IFNγ could trigger death of other neuronal types through a LIGHT-independent pathway, we examined the responsiveness of cortical, hippocampal, sensory and striatal neurons to IFNγ. After ensuring that all these neurons express both IFNγ receptors (Figure 4g), we exposed them to an efficient dose of IFNγ or to the potent combination of IFNγ with sLIGHT, and observed that none of them were sensitive to IFNγ (Figure 4h and Supplementary Figure 4d).

The ability of IFNγ to activate the LIGHT-LT-βR pathway raised the possibility that IFNγ might enhance expression levels of LIGHT and/or LT-βR. Densitometric analysis of LIGHT and LT-βR immunoblots revealed that IFNγ significantly increased levels of LIGHT, but not of LT-βR, in motoneurons (Figure 4i and j). Altogether, these findings implicate a mechanism in which IFNγ specifically triggers a death program in motoneurons by eliciting the LIGHT-LT-βR pathway.

ALS mutant astrocytes selectively kill motoneurons through the IFNγ/LT-βR pathway. The motoneuron selectivity of IFNγ-triggered LIGHT-mediated death prompted us to investigate whether it has a function in the selective loss of motoneurons in a pathological context. We cultured motoneurons from mice overexpressing the G93A SOD1 mutation and asked whether mutant motoneurons show a differential susceptibility to IFNγ and LIGHT compared with wild-type motoneurons. Dose–response curves of IFNγ or its synergistic combination with sLIGHT on mutant motoneurons did not differ from those of wild-type motoneurons (Figure 5a and b), showing that the SOD1^{G93A} mutant does not exacerbate the IFNγ and LIGHT effect.

Coculture systems have demonstrated that mutant astrocytes release factors that selectively trigger death of motoneurons, independently of the expression of mutant SOD1 in neurons.[5,7,8] Interestingly, we found that levels of IFNγ, as determined by ELISA, were significantly augmented in *SOD1*^{G93A} astrocytes and in their corresponding conditioned media compared with wild-type cells (Figure 5c and d). We, therefore, investigated the involvement of the IFNγ-induced LIGHT-mediated death pathway in mutant astrocyte-conferred neurotoxicity. We used a coculture system of highly purified wild-type rat motoneurons and rat astrocyte monolayers of different genotypes.[4] Firstly, we demonstrated that rat motoneurons are as responsive as mouse motoneurons to sLIGHT, agonistic anti-LT-βR antibodies and IFNγ (Figure 5e). We next found that, in contrast to the wild type, *SOD1*^{G93A} astrocytes express substantial levels of IFNγ, whereas we did not observe any difference in LIGHT levels (Figure 5f). We then investigated the effect of mutant astrocyte-derived IFNγ on motoneuron survival. After 48 h of coculture, about 50% of wild-type motoneurons plated on

SOD1^{G93A} astrocytes died compared with motoneurons cultured on wild-type astrocytes (Figure 5g). Motoneurons cocultured with wild-type and *SOD1*^{G93A} astrocytes were then treated with neutralizing anti-IFNγ antibodies that we had first validated as functional by saving motoneurons from IFNγ-induced death (Figure 5e). We found that inhibition of IFNγ activity substantially prevented the death of motoneurons induced by mutant astrocytes (Figure 5g). Consistently, inhibition of the LIGHT-LT-βR death pathway significantly increased the percentage of surviving motoneurons cultured with *SOD1*^{G93A} astrocytes (Figure 5g). Collectively, these results suggest that an IFNγ-induced motoneuron-selective death is involved in the astrocytic neurotoxicity conferred by mutant SOD1.



Evidence of a role for IFNγ in the cross-talk between motoneurons and astrocytes in ALS. We next sought evidence for the involvement of the IFNγ-LIGHT-LT-βR pathway in *SOD1^{G93A}* transgenic mice. We examined the expression of LT-βR and LIGHT by lumbar spinal cord motoneuron of *SOD1^{G93A}* at presymptomatic (75 days), early onset (90 days) and symptomatic (110 days) stages and of age-matched wild-type mice. We observed that in wild-type and *SOD1^{G93A}* mice the vast majority of motoneurons, identified by their selective expression of vesicular acetylcholine transferase (VAChT), constitutively express both LT-βR and LIGHT (Figure 6a and Supplementary Figure 5a and b).

We next monitored the expression of IFNγ during the course of the disease. We observed by western blot analysis that, although barely detectable at presymptomatic stage and indistinguishable from those seen in non-transgenic mice, levels of IFNγ in the spinal cord of *SOD1^{G93A}* significantly increased at early disease onset and were further enhanced at symptomatic stages compared with wild-type mice (Figure 6b and Supplementary Figure 5c). As measured by ELISA, IFNγ was found at higher levels in dissociated spinal cords of symptomatic *SOD1^{G93A}* mice compared with wild type or with mice overexpressing non-pathogenic SOD1^{WT} (Figure 6c). We then questioned the identity of cells expressing IFNγ in *SOD1^{G93A}* mice. Consistent with previous results (Figure 6b), we were not able to detect IFNγ in the spinal cord of both wild-type and presymptomatic mutant *SOD1* mice. Interestingly, at early onset and symptomatic stages, IFNγ was readily detected in astrocytes (GFAP+), but not in microglial cells (Iba1+) (Figure 6d and e). It is to be noted that, IFNγ was also detected in spheroid GFAP+ cells reminiscent of degenerative astrocytes in spinal cords of *SOD1^{G93A}* mice at 90 and 110 days of age[28] (Supplementary Figure 5d). The only other cell type positive for IFNγ at early onset and symptomatic stages were motoneurons (Figure 6f). Consistently, we found that the percentage of motoneurons immunoreactive for IFNγ increased significantly at 90 and 110 days compared with 75-day-old *SOD1^{G93A}* and age-matched wild-type mice (Figure 6g). A similar increase in IFNγ was observed in symptomatic *SOD1^{G85R}* mice, whereas *SOD1^{WT}* showed no increase in IFNγ either in motoneurons or glial cells (Supplementary Figure 5e). Expression of IFNγ by mutant astrocytes and motoneurons, at onset and symptomatic stages, suggests that IFNγ potentially contributes to the progression rather than the onset of motoneuron disease.

Genetic ablation of *Light* in *SOD1^{G93A}* mice retards disease progression. To evaluate the functional involvement of the IFNγ-LIGHT-LT-βR pathway in ALS pathogenesis, we genetically deleted *Light* in mice overexpressing *SOD1^{G93A}* by cross-breeding. *Light*-deficient mice are viable and fertile with no behavioral abnormalities.[27] We ensured that the expression levels of SOD1 mutant in the spinal cords of *SOD1^{G93A}/Light$^{+/+}$* and *SOD1^{G93A}/Light$^{-/-}$* mice were similar (not shown). The time of disease onset in *SOD1^{G93A}/Light$^{+/+}$* and *SOD1^{G93A}/Light$^{-/-}$* was determined by the time of peak weight (Supplementary Figure 6).[2] We did not observe any significant difference between *SOD1^{G93A}/Light$^{+/+}$* and *SOD1^{G93A}/Light$^{-/-}$* mice (97 ± 1.7 days and 97.2 ± 1.6 days, respectively, mean ± S.E.M., log-rank test $P = 0.7765$) (Figure 7a). To investigate the impact of *Light* deletion on disease progression, the swimming performance was evaluated weekly in the cohorts of *Light$^{+/+}$*, *Light$^{-/-}$*, *SOD1^{G93A}/Light$^{+/+}$* and *SOD1^{G93A}/Light$^{-/-}$* mice.[29] We found that *Light* deficiency significantly retarded the decline of motor function in ALS mice (Figure 7b). Furthermore, the loss of body weight was significantly slowed in *SOD1^{G93A}/Light$^{-/-}$* compared with *SOD1^{G93A}/Light$^{+/+}$* mice (Supplementary Figure 6). We also observed that deletion of *Light* increases the lifespan of SOD1 mutant mice by 17.9 days (*SOD1^{G93A}/Light$^{+/+}$*, 135.5 ± 2.8 days and *SOD1^{G93A}/Light$^{-/-}$*, 153.4 ± 2.9 days, mean ± S.E.M., log-rank test $^{***}P = 0.0002$) (Figure 7c). We next investigated whether the amelioration in motor performance of *Light*-deficient SOD1 mice was associated with an increase in motoneuron survival. We quantified the number of surviving motoneurons on VAChT-immunostained sections, taken from the lumbar region of 120-day-old mice spinal cords of different genotypes. We observed that *Light* ablation in *SOD1^{G93A}* mice leads to a significant increase in the number of surviving motoneurons compared with the marked loss seen in SOD1 mutant mice (Figure 7d). These results indicate that LIGHT

Figure 2 sLIGHT and sFasL show additive killing effect but LIGHT in contrary to FasL functions independently of mitochondrial cytochrome *c* release. (**a**) Mouse motoneurons were cultured for 24 h and treated (or not) with sLIGHT (100 ng/ml), recombinant human soluble FasL (sFasL, 100 ng/ml in the presence of 1 μg/ml enhancer antibody), or a combination of both. Motoneuron survival was assessed 48 h after treatment. Note that the enhancer antibody used to cross-link sFasL had no effect on motoneuron survival when added alone or with sLIGHT (not shown)($n = 4$, $^{***}P < 0.001$). (**b**) Quantification of phospho-p38 kinase fluorescence in *Hb9::GFP* motoneurons treated (or not) with sLIGHT in the presence or the absence of SB203580 (10 μM) for 1 h. Treatment with sFasL served as a control.[15] Confocal fluorescence imaging and ImageJ image analysis were used to determine the nuclear mean fluorescence intensity of p38 in *Hb9::GFP* neurons (a.u, arbitrary unit, $^{*}P < 0.05$, $^{**}P < 0.01$). (**c**) Motoneurons were maintained in culture for 24 h and treated or not with sLIGHT (100 ng/ml) and SB203580 (10 μM). Survival was determined 48 h later and expressed relative to survival in the absence of any treatment. (**d** and **e**) *Hb9::GFP* motoneurons were incubated or not with 100 ng/ml of sLIGHT or sFasL, fixed and immunostained with anti-cytochrome *c* antibody 30 h later. sLIGHT-treated *Hb9::GFP* motoneuron shows the same punctuate labeling of mitochondria as non-treated motoneurons (not shown), whereas following Fas activation a proportion of motoneurons show diffuse labeling of cytochrome *c*. Scale bar, 10 μm. (**e**) The percentage of *Hb9::GFP* motoneurons showing diffuse *versus* punctuated cytochrome *c* labeling was determined by direct counting under fluorescence microscope ($n = 4$). (**f**) Survival assay was done as in (**c**). The Bax inhibitory peptide V5 was used at the concentration of 50 μM. (**g**) Mouse motoneurons were co-electroporated with an equimolar ratio of a vector coding for EGFP and a vector coding for either Bcl-Xl, a dominant negative form of Bid (t^n-Bid) or an empty vector (e.v) and cultured for 24 h before being treated or not with sLIGHT or sFasL. The survival of EGFP-positive motoneurons for each combination of vectors was expressed as a percentage of surviving motoneurons in the absence of sLIGHT or sFasL. Histograms show mean values ± S.D. of at least three independent experiments, each done in triplicate

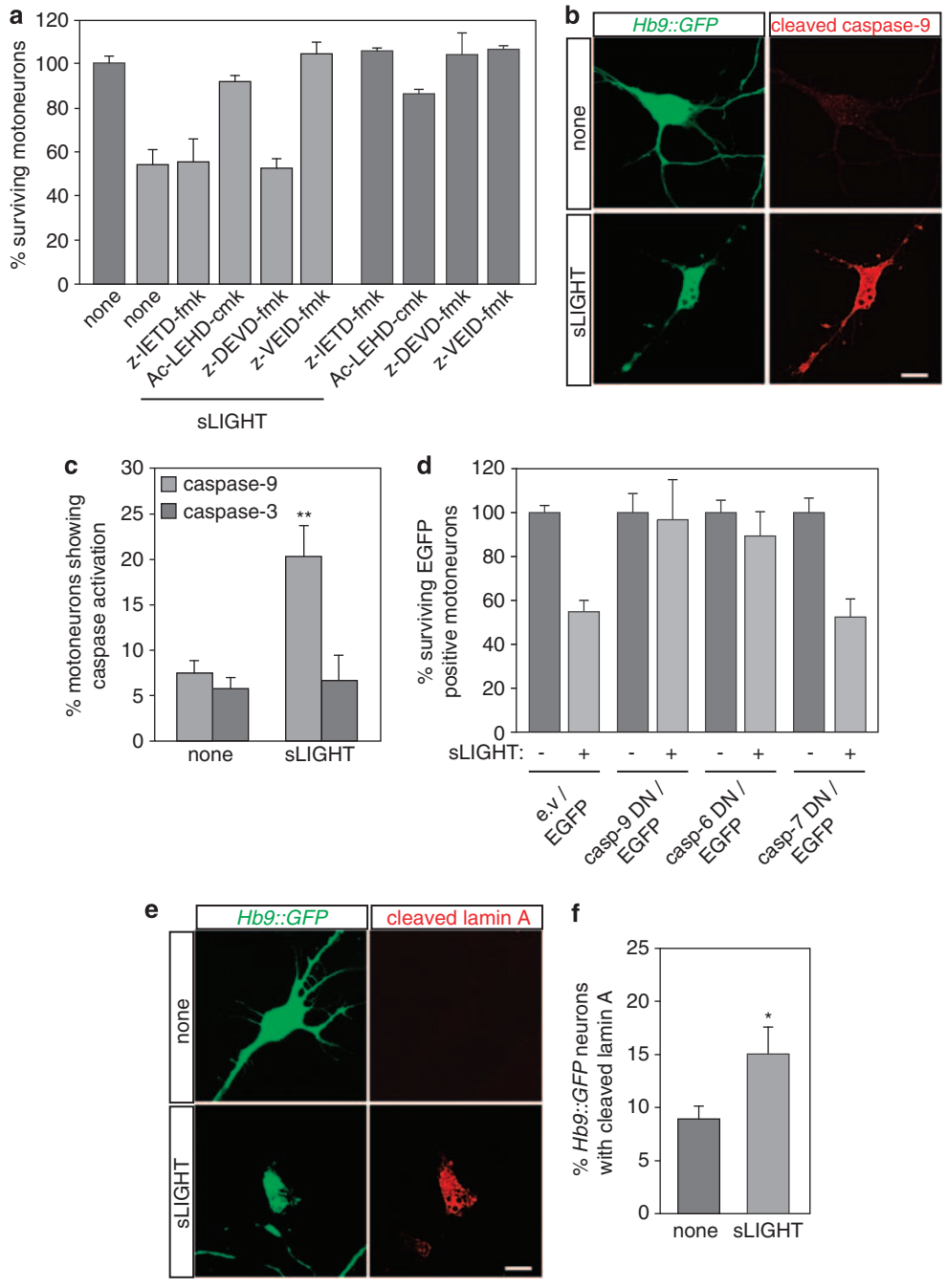
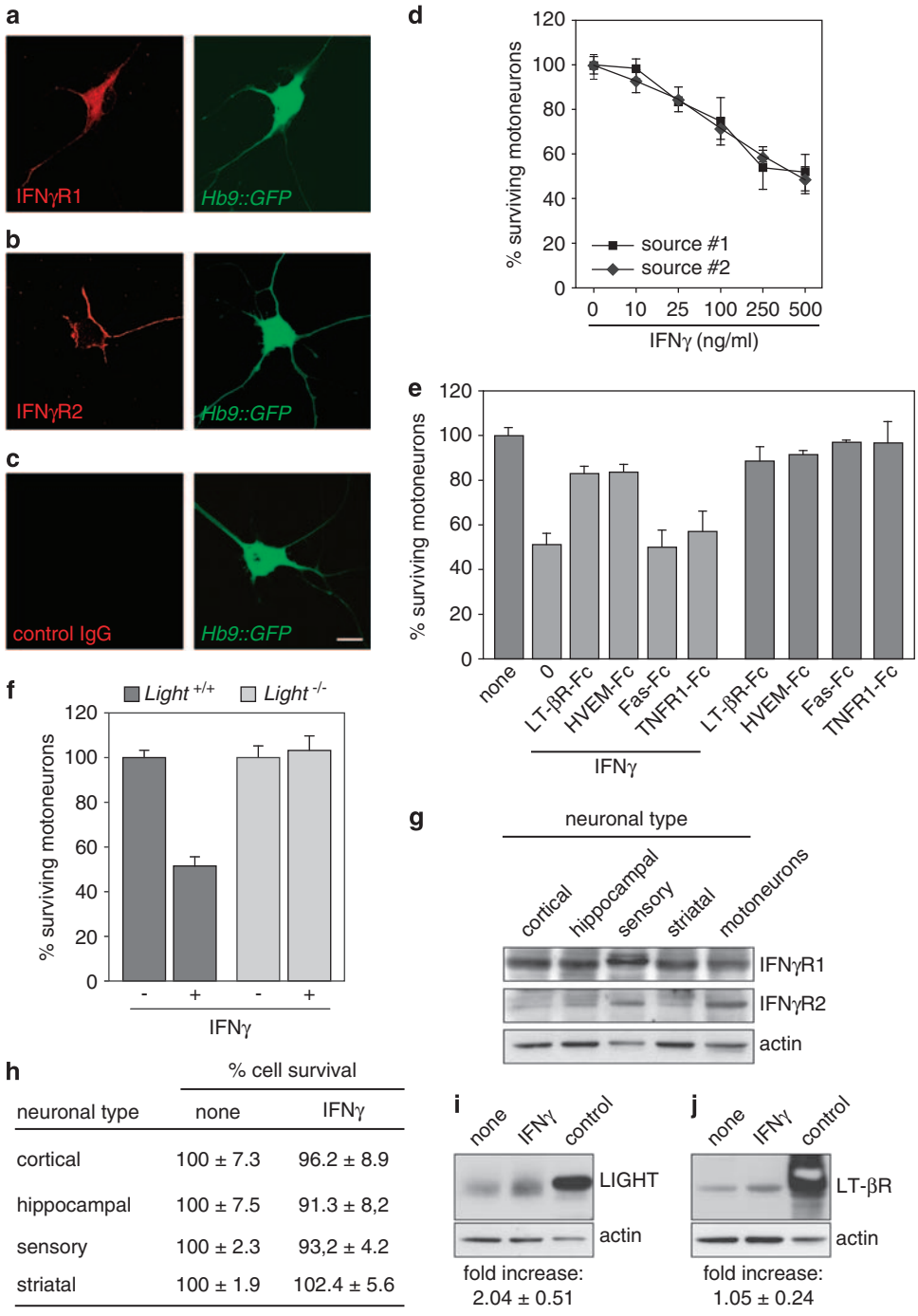


Figure 3 sLIGHT triggers motoneuron death through an unconventional caspase cascade. (**a**) Motoneurons were incubated with z-IETD-fmk (10 μM), Ac-LEHD-cmk (1 μM), z-DEVD-fmk (10 μM) or z-VEID-fmk (10 μM) added together with sLIGHT (100 ng/ml). Motoneuron survival was determined 48 h later and expressed relative to non-treated condition. (**b**) Activation of caspase-9 was visualized in sLIGHT-treated *Hb9*:: *GFP* motoneurons with antibodies specific to the cleaved form of caspase-9. (**c**) The percentage of *Hb9*:: *GFP* motoneurons immunopositive for active caspase-9 (**b**) or -3 (see Supplementary Figure 3b) was determined 30 h later following addition of sLIGHT by direct counting using fluorescence microscopy. Statistical attribute is shown for none *versus* sLIGHT-treated cells ($n=3$, $^{**}P<0.01$). (**d**) Motoneurons were co-electroporated with a combination of expression vectors encoding EGFP and catalytically inactive mutants of caspase-9 (casp-9 DN), -6 (casp-6 DN) or -7 (casp-7 DN), and incubated in the presence or absence of sLIGHT. The effect of the dominant negative caspases on motoneuron survival was determined by counting the EGFP-positive motoneurons 48 h after treatment. e.v, empty vector. (**e**) The experimental procedure was performed as in (**b**) only that the percentage of motoneurons positive for cleaved lamin A was determined 38 h after treatment using an anti-cleaved lamin A antibody. Scale bars, 10 μm. (**f**) The percentage of cleaved lamin-A-positive *Hb9*:: *GFP* motoneurons for LIGHT-treated or untreated conditions was determined by counting under fluorescent microscope ($n=3$, $^{*}P<0.05$). All values are expressed as the means ± S.D. of three independent experiments



h

	% cell survival	
neuronal type	none	IFNγ
cortical	100 ± 7.3	96.2 ± 8.9
hippocampal	100 ± 7.5	91.3 ± 8,2
sensory	100 ± 2.3	93,2 ± 4.2
striatal	100 ± 1.9	102.4 ± 5.6

Figure 4 IFNγ selectively kills motoneurons in a LIGHT-dependent manner. (**a**–**c**) Isolated *Hb9::GFP* motoneurons were immunostained 24 h after seeding with antibodies directed against IFNγR1 (**a**), IFNγR2 (**b**) or with hamster (**c**) or mouse (not shown) irrelevant IgG as control. Scale bar, 10 μm. (**d**) Motoneurons were cultured for 24 h and incubated with increasing concentrations of soluble mouse recombinant IFNγ from two different sources. The percentage of surviving motoneurons was determined 48 h later. The distance between the x-axis values is arbitrary. (**e**) Motoneuron survival was determined 48 h following treatment or not with LT-βR-Fc (100 ng/ml), HVEM-Fc (10 ng/ml), Fas-Fc (1 μg/ml) or TNFR1-Fc (100 ng/ml) in combination or not with 250 ng/ml of IFNγ. The number of surviving motoneurons is expressed as a percentage of the number of motoneurons in the control condition (none). (**f**) Motoneurons were isolated from E12.5 embryos of indicated genotype and cultured for 24 h before being treated with 250 ng/ml of IFNγ. Motoneuron survival was determined 48 h later and expressed relative to the non-treated condition for each genotype. (**g**) Immunoblot analysis of cortical, hippocampal, sensory, striatal and motoneurons proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Actin was used as a loading control. (**h**) Cell survival assay was performed as in (**d**), with neuronal cells being treated with 250 ng/ml of IFNγ. (**i** and **j**) Motoneurons were cultured for 24 h and incubated with 250 ng/ml of IFNγ. Eight hours later, cells were lysed and expression levels of LIGHT (**i**) and LT-βR (**j**) were determined by western blotting with indicated antibodies. Fold-increase of LIGHT and LT-βR over non-treated conditions was determined by densitometric analysis of immunoreactive bands, normalized to their respective actin signals ($n = 3$, means ± S.D.). Results shown in (**d**, **e**, **f** and **h**) are the mean values ± S.D. of three independent experiments performed in triplicate

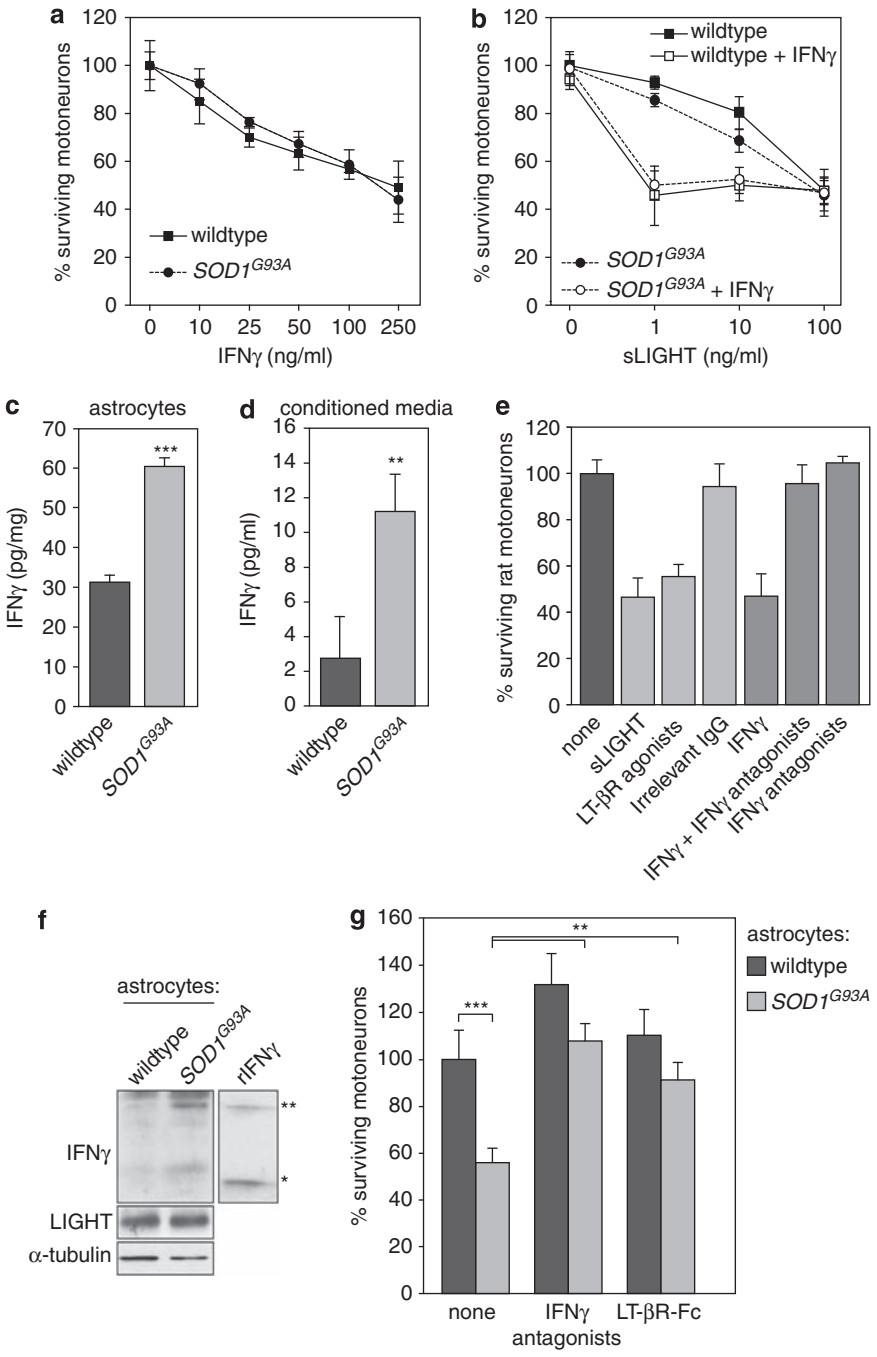


Figure 5 SOD1 mutant astrocytes kill motoneurons in an IFNγ/LIGHT-dependent pathway. (**a**) Twenty four hours after plating, indicated concentrations of mouse recombinant IFNγ were added to motoneurons isolated from *SOD1*^{G93A} or wild-type embryos of the same littermate. Motoneuron survival was determined 48 h later and expressed relative to non-treated condition of corresponding genotype. (**b**) Mutant *SOD1*^{G93A} and wild-type motoneurons were treated after 24 h in culture with increasing concentrations of sLIGHT in combination or not with IFNγ (10 ng/ml). Motoneuron survival was determined 48 h after treatment. (**c** and **d**) IFNγ levels in extracts (**c**) and conditioned media (**d**) of astrocytes of indicated genotype were quantified by ELISA ($n = 4$, means ± S.D.). (**e**) Immunopurified E14 rat motoneurons were treated or not with sLIGHT (100 ng/ml), agonistic anti-LT-βR antibodies (100 ng/ml), irrelevant IgG (100 ng/ml), recombinant soluble rat IFNγ (250 ng/ml) in combination or not with antagonistic anti-IFNγ (500 ng/ml) or anti-IFNγ antibodies alone. Survival of motoneurons was determined 48 h after treatment by direct counting. (**f**) Total protein extract of wild-type and *SOD1*^{G93A} rat astrocytes were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by immunoblotting with anti-IFNγ specific antibodies. Recombinant rat IFNγ was used as a control. Asterisks indicate the monomeric (*) form of IFNγ and the apparent stable dimeric (**) biologically active form of IFNγ. (**g**) Wild-type motoneurons were plated on astrocyte monolayer of indicated genotype (wild type, *SOD1*^{G93A}) and incubated or not with function-blocking anti-IFNγ antibodies (500 ng/ml) or LT-βR-Fc (100 ng/ml) for 48 h. Survival of motoneurons is expressed as the percentage of the number of motoneurons surviving on wild-type astrocyte monolayer in the absence of any treatment. The graphs show the mean values ± S.D. of at least three independent experiments performed in triplicate

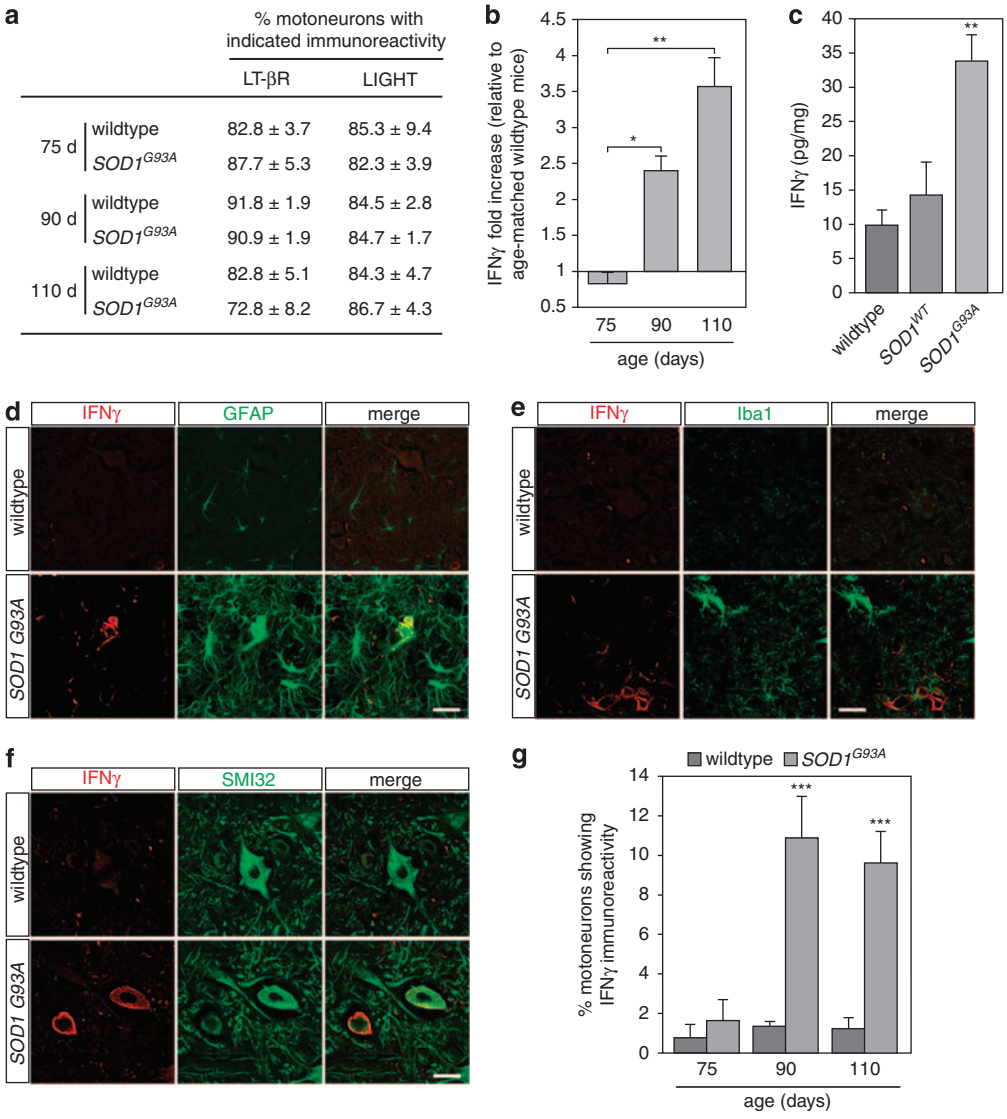


Figure 6 IFNγ is upregulated in spinal cords of ALS mice. (**a**) The percentage of motoneurons, as identified with VAChT immunostaining on adjacent sections (Supplementary Figure 5a and b), immunoreactive for LIGHT and LT-βR was determined in lumbar spinal cord of wild-type and *SOD1*^{G93A} mice at 75, 90 and 110 days (d) of age. (**b**) Total protein extracts from lumbar spinal cords of wild-type and *SOD1*^{G93A} mice at indicated age were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and probed with antibodies to IFNγ and actin (Supplementary Figure 5c). IFNγ signals were quantified, normalized to actin signals and expressed as the ratio of *SOD1*^{G93A} to wild-type values. (**c**) ELISA quantification of IFNγ levels in dissociated spinal cord of 110-day-old wild-type and *SOD1*^{G93A} mice and 365-day-old *SOD1*^{WT} mice. Values in (**a**–**c**) are means ± S.D., $n = 3$. (**d** and **f**) Immunostainings of wild-type and *SOD1*^{G93A} mice lumbar spinal cord sections at 90 days of age using antibody against IFNγ in combination with either GFAP (**d**), Iba1 (**e**) or SMI32 (**f**). Scale bar, 30 μm. (**g**) Lumbar spinal cord sections of wild-type and *SOD1*^{G93A} mice were immunostained as in (**f**) at 75, 90 and 110 days of age and the percentage of IFNγ immunoreactive motoneurons was determined by counting under fluorescent microscope (75 days, $n = 3$; 90 days, $n = 4$; 110 days, $n = 5$, values are means ± S.D.)

contributes to disease progression, but not disease onset, in ALS mice.

Discussion

Our study reveals a novel death signaling pathway triggered by the activation of LT-βR by LIGHT, which appears restricted to motoneurons and can be triggered by IFNγ delivered by astrocytes expressing mutant SOD1. We provide evidence that strongly support the potential role of this death pathway in the progression of motoneuron disease.

The selective vulnerability of motoneurons to mutant astrocyte-derived toxicity has been recapitulated in cocultures of rat, mouse or human embryonic stem-cell-derived motoneurons and astrocytes expressing different SOD1 mutations.[4,5,7,8] Among the potential actors of the neurotoxicity of mutant astrocytes, proinflammatory molecules have been proposed. In particular, it has been suggested that prostaglandin D_2 (PGD_2) contributes to astrocyte-derived toxicity.[5] Interestingly, it has been demonstrated that the genetic or pharmacological inhibition of the PGD_2/PGD_2 receptor signaling markedly reduced astrogliosis,[30] raising the ques-

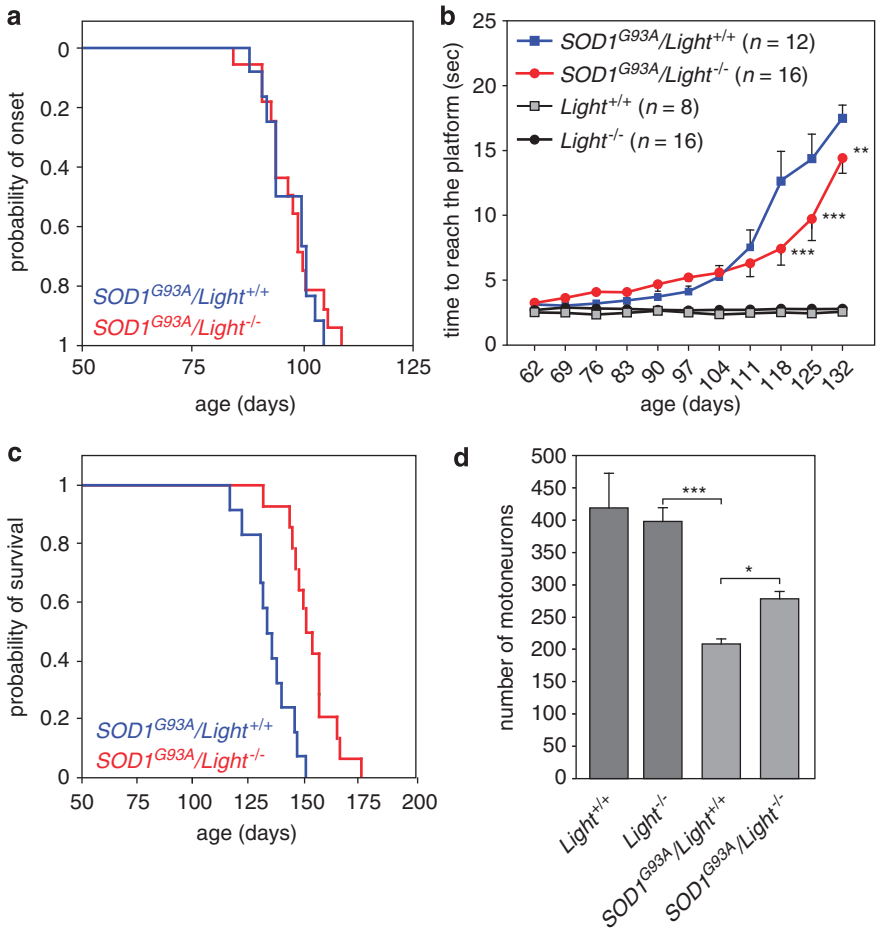


Figure 7 Targeted deletion of *Light* in ALS mice delays progression but not onset of the disease. (**a**) The cumulative probability of onset of $SOD1^{G93A}/Light^{+/+}$ ($n=12$) and $SOD1^{G93A}$; $Light^{-/-}$ ($n=16$) was determined by the peak of weight curve. (**b**) The progressive motor deficit of $SOD1^{G93A}/Light^{+/+}$, $SOD1^{G93A}/Light^{-/-}$, $Light^{+/+}$ and $Light^{-/-}$ was determined by evaluating weekly the swimming performance of mice (values are means ± S.E.M.). Statistical attributes are shown only for $SOD1^{G93A}/Light^{+/+}$ *versus* $SOD1^{G93A}/Light^{-/-}$ (Supplementary Table). (**c**) Kaplan–Meier survival curves for $SOD1^{G93A}/Light^{+/+}$ ($n=12$) and $SOD1^{G93A}/Light^{-/-}$ ($n=14$) mice. (**d**) The mean motoneuron survival was determined by counting the number of VAChT-immunostained motoneurons in 28 sections of lumbar spinal cord from 120-day-old $Light^{+/+}$ ($n=3$), $Light^{-/-}$ ($n=3$), $SOD1^{G93A}/Light^{+/+}$ ($n=4$) and $SOD1^{G93A}/Light^{-/-}$ ($n=4$) (values are means ± S.D.)

tion whether PGD_2 function directly on motoneurons as a death trigger and/or indirectly by consolidating the inflammatory phenotype of mutant astrocytes. In the present study, we show that inhibition of IFNγ activity by antagonist antibodies greatly improves the survival of motoneurons cocultured with mutant astrocytes, whereas blocking the interaction between LIGHT and LT-βR by LT-βR-Fc produced a more moderate improvement in motoneuron survival. This difference in efficacy could be attributed to the proinflammatory activity of IFNγ in astrocytes. Accumulating evidence suggests that IFNγ induces and/or functions in combination with reactive oxygen species (ROS) to potently activate the astrocytic reaction. It has been demonstrated that IFNγ induces NO production and increases levels of GFAP in primary astrocytes.[31] Expression of $SOD1^{G93A}$ or $SOD1^{G37R}$ in astrocytes is accompanied by increased levels of ROS, including NO,[4,7] and expression of $SOD1^{G93A}$ in glioblastoma leads to the production of IFNγ.[32] How IFNγ and ROS cooperate in the acquisition or the consolidation of the reactive status of astrocytes expressing SOD1 mutations remains to be elucidated.

In this study, we show that astrocytes derived from the ALS rat or mouse models also produce IFNγ, which can directly trigger death of motoneurons. In contrast, it has been shown that exposure of freshly isolated motoneurons to IFNγ fails to alter neuron survival.[33] In our survival experiments, motoneurons were cultured for at least 24 h before treatment and indeed we observed that freshly isolated motoneurons were resistant to IFNγ or LIGHT (not shown), suggesting that motoneurons need at least 24 h to acquire competence to die through IFNγ and LIGHT. In the light of these studies, IFNγ can be proposed as a key player of toxicity of mutant astrocytes by directly promoting death of motoneurons through LIGHT and by promoting release of other glial-derived neurotoxic factors.

Toxic mechanisms elicited by mutant astrocytes to promote death of motoneurons might involve additional effectors, such as those acting through Bax activation.[10] Indeed, Nagai *et al.*,[8] showed that mutant astrocytes kill embryonic stem-cell-derived motoneurons in a Bax-dependent manner. The discrepancies observed between our coculture system and

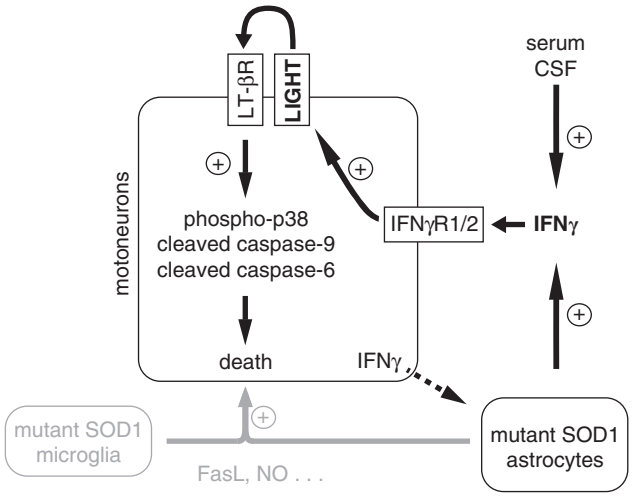


Figure 8 Model for the non-cell-autonomous effect of mutant SOD1 on the activation of motoneuron selective death pathways. IFNγ produced by mutant astrocytes can selectively trigger death of some motoneurons through activation of the LIGHT-LT-βR pathway. IFNγ from circulating blood (serum) and the cerebrospinal fluid (CSF)[40] may also contribute to this neurodegenerative process. Motoneurons might represent an additional source of IFNγ that could participate to the inflammatory process. In grey, neurotoxic factors, including NO, or members of the TNF family, such as FasL, produced by mutant microglial cells and/or astrocytes, could also participate in the elimination of motoneurons in the disease.[10,14] Vulnerable motoneurons, having distinct intrinsic features, might be differentially susceptible to these non-cell-autonomous death triggers

that of those authors could be related to the different species origin (mouse or rat) and motoneuron sources (primary or ES), as well as the use of different culture conditions (timeframe, cell density and so on). The implication of a Bax-dependent pathway is consistent with some evidence demonstrating that the classical mitochondrial pathway has a function in the pathogenic process of motoneuron disease.[1,18] However, several arguments indicate that alternative cell death mechanisms may participate in the neurodegenerative process: overexpression of Bcl-2 in mutant *SOD1* mice delays the onset but has no effect on the duration of the disease;[34] the targeted deletion of *caspase-11* in *SOD1^{G93A}* mice, which results in a marked reduction of caspase-3 activity, failed to prevent neurodegeneration;[35] *Bax* deletion in mutant SOD1 mice does not prevent neuromuscular denervation and the fatal outcome of the disease.[36] Here, we show that ablating LIGHT, which triggers death of motoneurons following a differential mitochondrial execution phase, delays progression of the disease and increases motoneuron survival in *SOD1^{G93A}* mice. We, therefore, propose that the diversity of motoneurons regarding their functional identity or their selective vulnerability to disease may be related to the way in which they execute a death sentence.

Around 20 years ago, IFNγ immunoreactivity was observed in motoneurons after axotomy and was proposed to induce glial reaction.[37] In our experimental conditions, motoneurons and astrocytes were the only identifiable intraparenchymal sources of IFNγ, which strikingly attests to the cross-talk between motoneurons and astrocytes in motoneuron disease. Our results are also corroborated by a study showing an increase in IFNγ levels in *SOD1^{G93A}* spinal cords at end-stage.[38] It is to be noted that, increased levels of IFNγ have been detected in the serum of ALS patients as the disease progresses,[39] and we found increased serum levels of IFNγ in *SOD1^{G93A}* mice at symptomatic stage of the disease compared with age-matched wild-type or *SOD1^{WT}* mice (Supplementary Figure 7). Recently, IFNγ levels were also found significantly increased in the cerebrospinal fluid (CSF) of sporadic ALS patients compared with patients with non-inflammatory neurological diseases.[40] This evidence supports a potential role of IFNγ, provided by motoneurons, serum, CSF and/or astrocytes, in the pathogenic process (Figure 8). IFNγ could participate in an inflammatory cascade that would also include the activation of microglial cells, as IFNγ has been shown to enhance microglial cell activity *in vivo*.[41] Whether IFNγ directly activates microglial cells or mediates astrocytic-dependent microglia activation remains to be determined. Moreover, activated microglia could participate with reactive astrocytes in the chronic production of neurotoxic molecules that could function through different mechanisms in different motoneuron populations (Figure 8). Recently, interleukin (IL)-1β has been proposed to promote a microglial-mediated neuroinflammation process contributing to disease progression in *SOD1^{G93A}* mice.[42] In our study, the timing of IFNγ expression in *SOD1^{G93A}* mice suggests that IFNγ participates in the progression rather than the onset of disease, and *Light* deletion in *SOD1^{G93A}* mice delayed progression, but not onset, of motoneuron disease. Cooperation between IFNγ and IL-1β provides a potential pathological mechanism for initiating and/or expanding the neuroinflammatory response in ALS.

Collectively, the motoneuron-selective IFNγ-induced LIGHT-mediated death pathway we describe would represent a non-cell-autonomous pathogenic mechanism that contributes to the progression phase of the disease. To complete our understanding of the role of the IFNγ-LIGHT pathway in motoneuron disease and the development of pertinent therapeutic strategies, it will be important to conduct a detailed analysis of the cell-type-specific genetic ablation of IFNγ in mutant SOD1 mice, as well as a correlative study between expression pattern/levels of IFNγ and clinical parameters in ALS patients.

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. *Hb9::GFP* mice (TM Jessell's laboratory, Columbia University, NY, USA) were maintained on a CD1 background (Charles River laboratories, Wilmington, MA, USA).[43] *SOD1^{G93A}* mice were maintained on a B6SJL background (Jackson laboratories, Bar Harbor, ME, USA).[44] *SOD1^{G85R}* (DW Cleveland's laboratory, University of California, San Diego, CA, USA), *SOD1^{WT}* (S Przedborski's laboratory, Columbia University, NY, USA) and *Light$^{-/-}$* mice (K Pfeffer's laboratory, Heinrich-Heine University, Düsseldorf, Germany) mice were maintained on a C57BL/6 background.[27,45,46] Sprague-Dawley *SOD1^{G93A}* L26H rats (DS Howland, Wyeth Research, Princeton, NJ, USA) were maintained as described.[4]

Cell cultures. Motoneurons from E12.5 spinal cord of CD1, *Hb9::GFP*, *Light$^{-/-}$* or *SOD1^{G93A}* embryos were isolated as described[47] modified by,[14] using iodixanol density gradient centrifugation. Motoneurons were plated on poly-ornithine/laminin-treated wells in the presence (or not when mentioned) of a cocktail of neurotrophic factors (0.1 ng/ml glial-derived neurotrophic factor (GDNF), 1 ng/ml brain-derived neurotrophic factor (BDNF) and 10 ng/ml ciliary neurotrophic factor (CNTF)) in supplemented neurobasal medium. When needed, motoneurons were

electroporated before plating with the indicated expression constructs as we previously described.[14] For survival assays, between 60 and 130 motoneurons in 2-cm diameters of each well were counted as described previously.[14,47] In our conditions, the number of living motoneurons remained relatively constant between 24 and 96 h of culture: if 24 h is set at 100% surviving motoneurons, 48 h, 96.1 ± 6%; 72 h, 91.2 ± 4.5%; and 96 h, 92.9 ± 6.6% (means ± S.D., $n = 4$). To allow for comparison of values from different experiments, survival values were expressed relative to the value in the presence of neurotrophic factors only (taken as 100%). Motoneurons from E14 rat embryos were immunopurified using Ig192 mouse monoclonal anti-p75 antibody as previously described[4] and cultured in supplemented neurobasal medium in the presence of GDNF (0.1 ng/ml). Motoneuron cultures were free of GFAP1+ or Iba1+ glial cells. Cortical, hippocampal, dorsal root ganglion neurons and striatal neurons were isolated from E17.5 embryos as described.[14,48] Cortical, hippocampal, sensory and striatal neurons were plated on poly-ornithine/laminin-treated wells and cultured in neurobasal medium complemented with 1 mM sodium pyruvate, 2% B27 supplement (Invitrogen, Carlsbad, CA, USA) at the exception of sensory neurons that were maintained in the same supplemented neurobasal medium used for motoneurons but in the presence of 100 ng/ml nerve growth factor instead of GDNF, BDNF and CNTF. Unless otherwise indicated, cell survival experiments were done on neurons isolated from CD1 mice. All neuronal types were seeded at the density of 1500 cells/cm^2 and surviving neurons were directly counted under light or fluorescence microscopy. Cos-7 cells were maintained in Dulbecco Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS). For expression analysis of FLAG-tagged LT-βR and HA-tagged LIGHT (D Olive's laboratory, Inserm U891, Marseille, France), cells were transfected using Fugene 6 following manufacturer's instruction (Roche diagnostics, Indianapolis, IN, USA).

Proteins and chemicals for survival assay. Soluble human recombinant LIGHT and FasL, enhancer antibodies used for aggregating tagged sFasL, human HVEM-Fc, LT-βR-Fc, Fas-Fc, TNFR1-Fc, L^G-nitro-L-arginine-methyl ester.HCl and L^G-nitro-D-arginine-methyl ester.HCl were purchased from Alexis Biochemicals (San Diego, CA, USA). Soluble mouse recombinant LIGHT, functional goat polyclonal anti-LT-βR anti-HVEM antibodies were purchased from R&D systems (Minneapolis, MN, USA). Soluble mouse recombinant IFNγ (source no. 1), the pyridinyl imidazole p38 inhibitor SB203580, and the caspase inhibitors z-IETD-fmk, Ac-LEHD-cmk, Z-DEVD-fmk were purchased from Calbiochem (San Diego, CA, USA). Recombinant rat TNFα was from BD Biosciences (Franklin Lakes, NJ, USA). Soluble mouse recombinant IFNγ (source no. 2) and soluble recombinant rat IFNγ were from PBL Biomedical laboratories (Piscataway, NJ, USA). z-VEID-fmk, Bax inhibitor peptide V5, neutralizing goat polyclonal anti-IFNγ antibodies (I5027) were purchased from Sigma-Aldrich (St Louis, MO, USA).

Expression constructs. Expression of enhanced GFP (EGFP), Bcl-Xl, t^n-Bid, the catalytically inactive caspase-9^{C287A} and caspase-7^{C186A}, were placed under the control of the cytomegalovirus promoter. The active site mutant caspase-6^{C163S} was introduced by PCR-based site-directed mutagenesis with the following primer (sense): 5′-TTATCATTCAGGCATCTCGGGGAAAC-3′ using the pcDNA3.1-caspase-6 as template (Quickchange site-directed mutagenesis kit, Stratagen, La Jolla, CA, USA). The construct was checked by sequencing.

Immunocytochemistry. *Hb9::GFP* motoneurons were seeded on poly-ornithine/laminin-treated glass coverslips at the density of 5000 cells/cm^2 and cultured in the supplemented neurobasal medium as above. At indicated time, neurons were processed for immunocytochemistry as previously described.[14,15] Primary antibodies we used were: anti-LT-βR (sc-8376, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1 : 50), anti-HVEM (AF2516, R&D systems, 1 : 50), anti-LIGHT (sc-28880, Santa Cruz Biotechnology, 1 : 50), anti-phospho-p38 kinase (V1211, Promega, Madison, WI, USA, 1 : 500), anti-cytochrome *c* (6H2.B4, BD Biosciences, 1 : 500), anti-cleaved caspase-9 (9509, Cell Signaling Technology, Beverly, MA, USA, 1 : 200), anti-cleaved caspase-3 (9661, Cell Signaling Technology 1 : 200), anti-cleaved lamin A (30H5, Cell Signaling Technology, 1 : 200), anti-IFNγR1 (559911, BD Biosciences, 1 : 250) and anti-IFNγR2 (ab31606, Abcam, Cambridge, MA, USA, 1 : 2000). Alexa Fluor 555-conjugated donkey anti-goat, anti-rabbit or anti-mouse antibody was used as secondary antibodies (Invitrogen). Images were taken using a Zeiss LSM510 laser scanning confocal microscope. Images of phospho-p38 immunostaining were collected with an Olympus BX50WI confocal laser-scanning microscope. Fluorescence analysis was performed using the NIH ImageJ software on *Hb9::GFP* motoneurons that were selected on GFP native fluorescence. Analysis of nuclear mean fluorescence intensity was done on 100–200 motoneurons per culture condition.[22]

Western blot. Neurons were plated at the density of 20 000 cells/cm^2 in 6-cm diameter dishes containing corresponding complemented neurobasal medium (see above). Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting were carried out on neurons, astrocytes and mouse lumbar spinal cords using the protocol previously described.[29] Primary antibodies were anti-LT-βR (sc-8377, 1 : 500), anti-LIGHT (sc-28880, 1 : 500), anti-mouse IFNγ (sc-52557, 1:500) from Santa Cruz Biotechnology, anti-rat IFNγ (I5027, Sigma-Aldrich, 1 : 500), anti-IFNγR1 (559911, BD Biosciences, 1 : 500), anti-IFNγR2 (ab31606, Abcam, 1 : 2000), anti-α-tubulin (B-5-1-2, Sigma-Aldrich, 1 : 20 000) and anti-actin (AC-40, Sigma-Aldrich, 1 : 20 000). Proteins were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized with the chemiluminescent HRP substrate (Millipore, Bedford, MA, USA). Where indicated, immunoblot images were quantified and normalized relative to the α-tubulin or actin levels using ImageJ software (National Institutes of Health, USA).

IFNγ ELISA. We prepared primary spinal cord astrocytes from mouse P1-P2 pups as previously described.[8] At the first passage, astrocytes were cultured in supplemented neurobasal medium until confluency. Media was then changed and 8 h later, conditioned media were collected and cleared by centrifugation at 1500 × *g* for 5 min. Astrocytes were then washed and homogenized into in 100 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Triton-X100. Lumbar spinal cords of wild-type, *SOD1^{WT}* and *SOD1^{G93A}* mice were dissociated in 100 mM Tris-Hcl pH 7.4, 150 mM NaCl and cleared by centrifugation at 1500 × *g* for 5 min. Levels of IFNγ were determined using OptEIA mouse IFNγ ELISA Kit II according to manufacturer's instructions (BD Biosciences).

Motoneuron–astrocyte cocultures. Primary astrocyte cultures were prepared from spinal cord of P1-P2 wild-type and *SOD1^{G93A}* rats as previously described.[4] Dissociated cells were plated into tissue culture flasks and maintained in 5% CO_2 at 37 °C in DMEM containing 10% FBS until they reach confluency. After 7–9 days, the flasks were shaken at 37 °C for 48 h to eliminate weakly adherent microglial cells. Adherent cells were then removed by Trypsin and plated at a density of 2 × 10^4 cells/cm^2 and maintained in DMEM supplemented with 10% FBS, HEPES (3.6 g/l), penicillin (100 IU/ml) and streptomycin (100 μg/ml) until confluency. Astrocyte monolayers were 98% pure as determined by GFAP immunoreactivity and devoid of OX42-positive microglial cells. Wild-type motoneurons, purified as above, were plated on rat astrocyte monolayer of different genotypes at the density of 300 cells/cm^2 and maintained for 48 h in L15 medium (Invitrogen) supplemented with 2% horse serum, 0.63 mg/ml bicarbonate, 5 μg/ml insulin, 0.1 mg/ml conalbumin, 0.1 mM putrescine, 30 nM sodium selenite, 20 nM progesterone, 20 mM glucose, 100 U/ml penicillin, 100 μg/ml streptomycin.

Immunohistochemistry. Immunostaining of lumbar spinal cord sections was performed as we described previously.[29] The following antibodies were used: anti-mouse IFNγ (I5027, Sigma-Aldrich, 1 : 100), anti-non-phosphorylated neurofilament (SMI32, Sternberger Monoclonals, Covance, Princeton, NJ, USA, 1 : 500), anti-GFAP (MAB360, Millipore, 1 : 500), anti-Iba1 (Wako Chemical Industries, Osaka, Japan, 1 : 100), anti-LT-βR (sc-8376, 1 : 50), anti-LIGHT (sc-28880, 1 : 50) from Santa Cruz Biotechnology and anti-VAChT (V5387, Sigma-Aldrich, 1 : 2500). Proteins were detected using either fluorochrome-conjugated secondary antibodies (Alexa Fluor 488 or 555) or the peroxidase/DAB detection system following the manufacturer's instruction (Dako, Glostrup, Denmark).

Analysis of *Light* and *SOD1^{G93A}* mutant mice. *Light$^{-/-}$* and *SOD1^{G93A}* mice were genotyped as previously described.[27,49] *Light$^{-/-}$* males were crossed with *SOD1^{G93A}* females to obtain *SOD1^{G93A}/Light$^{+/-}$* mice. *SOD1^{G93A}/Light$^{+/-}$* male mice were then backcrossed with *Light$^{+/-}$* female mice. Following the double cross-breeding, only *Light$^{+/+}$*, *Light$^{-/-}$*, *SOD1^{G93A}/Light$^{+/+}$* and *SOD1^{G93A}/Light$^{-/-}$* mice were chosen for the behavioral assays. To define the onset of disease, we measured the body weight twice a week and determined the time mice reached their peak body weight before muscle atrophy and weight loss.[3] To assess progression of motor decline, we weekly performed a swimming tank test starting at the age of 50 days and measured swimming speed as we previously described.[29] For statistical purposes, we set the maximum swimming latency at 20 s. The mortality was defined as the point in time when the mice are unable to right

themselves within 30 s after being placed upon their back. All behavioral studies were done in a blinded manner.

Statistical analysis. Statistical significance was determined by unpaired two-tailed *t*-test or by a one-way analysis of variance (ANOVA) followed by a Tukey's *post hoc* tests. Statistical analysis of swimming performance was done using a two-way (group $\times$ time) repeated measures ANOVA followed by a Newman-Keuls's *post hoc* test. A log-rank test was used to calculate the statistical differences in the onset and survival of the different mouse cohorts. Kaplan–Meier survival curves were plotted using GraphPad Prism Software. GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and StatSoft Statistica software (Statsoft, Tulsa, OK, USA) were used for calculations. Significance was accepted at the level of $P<0.05$. Please see Supplementary Table for statistical details.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements. We thank K Dudley and P Bomont for their critical reading of the manuscript, and all members of the Avenir team for their helpful comments throughout the work. We are grateful to D Olive who provided the LIGHT and LT-βR constructs, DE Bredesen who provided the caspase-9 and -7 constructs, H Esumi who provided the caspase-6 construct, AO Hueber for Bcl-Xl construct and J-C Martinou for Bid construct. We thank K Pfeffer and S Scheu for the gift of *Light*-deficient mice. We thank J-C Bensadoun for help with statistical analysis and I Medina for help with confocal image analysis. This work was supported by a PACA conseil regional – Trophos SA Ph.D fellowship to JA and grants from the Institut National de la Santé et de la Recherche Médicale (Inserm), Association Française contre les Myopathies (AFM), Association Française pour la Recherche sur la SLA (ARS) and the Thierry Latran foundation.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)