Expression of ALS-linked SOD1 mutant increases the neurotoxic potential of microglia via TLR2

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Running head: SOD1 mutant increases microglial neurotoxicity

Amyotrophic lateral sclerosis (ALS) is a progressive motor neuron disease, in which activated microglia overexpressing ALS-linked SOD1 mutants (mSOD1) are known to contribute to neuronal death. However, it is unclear how mSOD1 expression affects microglial activation and subsequently damage neurons. In this study, we created mSOD1-overexpressing BV-2 microglial cell lines. Following TLR2, but not TLR4 stimulation, we observed that overexpression of human SOD1 G93A, L8Q or G10V mutant, as compared to the wild-type SOD1 or a mock control, significantly enhanced microglial secretion of a neurotoxic cytokine, tumor necrosis factor-alpha (TNF-α), which was dependent on the NADPH-oxidase-mediated increased generation of reactive oxygen species (ROS). In further experiments, we demonstrated that mSOD1 expression regulated TNF-α secretion at a post-transcriptional level and involved ROS-sensitive TNF-α converting enzymes, e.g. ADAM10 and 17, which shed TNF-α from its membrane-anchored precursor. Together with a recent report that the function of SOD1, as a self-regulating redox sensor in NADPH oxidase-dependent ROS production, is lost due to its genetic mutations, we conclude that mSOD1 expression in ALS facilitates microglial neurotoxic inflammatory responses via TLR2, which is mediated by an uncontrolled ROS generation. The link, between mSOD1, innate immunity and NADPH oxidase, offers new opportunities in ALS therapies.

Amyotrophic lateral sclerosis (ALS) is a progressive paralytic disease that usually leads to death within 3 to 5 years. It is pathologically characterized by degeneration of motor neurons in the spinal cord, brain stem and motor cortex. In 20% of the familial ALS patients, mutations in the human Cu, Zn superoxide dismutase (SOD1) gene can be found (1). Transgenic mice overexpressing high levels of mutated human SOD1 (mSOD1) can model this disease (2). Analysis of postmortem tissues from ALS patients and of mSOD1 transgenic mice revealed a marked microglia-dominated neuroinflammation (3, 4, 5, 6). Microglial activation can also be visualized in patients by positron emission tomography using 11c-labelled (R)-PK11195, a radioligand of microglial peripheral benzodiazepine receptors (7). This neuroinflammatory response coinciding with neuronal death and disease progression argues for a causal relationship. In mSOD1 transgenic mice, mSOD1 expression in microglial cells has been proven to be crucial for neuronal survival: 1) mSOD1-expressing neurons are protected from cell death by neighboring non-neuronal cells lacking mSOD1 expression (8); 2) the disease progression in the late stage can be blocked by specifically switching off mSOD1 expression in microglia (9); and 3) reconstruction of bone marrow of mSOD1 transgenic mice with wild-type (wt) SOD1-expressing precursor cells, which creates a pool of wtSOD1-expressing microglial cells, extends the life time of the mutant mice (10). However, non-specific ablation of proliferating microglia in the mSOD1 transgenic mice did not prevent motor neuronal death (11). In a recent clinical trial, minocycline, a drug with wide-spectrum of anti-inflammatory effects, also failed to be effective in ALS patients (12). Thus, detailed mechanisms of how mSOD1 expression affects microglial activation, and subsequently damages
neurons in ALS, are far from being clear. Specific therapeutic targets against microglial detrimental effects in ALS need to be identified.

Microglia, as resident innate immune effector cells of the central nervous system, are armed with an extensive repertoire of pattern recognition receptors including CD14 and toll-like receptors (TLRs) that are necessary for detection and rapid elimination of invading microorganisms (13, 14, 15, 16). Upon ligand binding, the CD14/TLR signalling cascade via a common adaptor, MyD88, induces activation of AP-1 and NF-κB transcription factors, both of which subsequently regulate the expression of numerous immune response genes including inflammatory cytokines and chemokines (16). Recently, CD14 and TLRs have been recognized also to respond to endogenous ligands (16, 17). Several reports indicated the involvement of TLR2 and TLR4 in oxidative stress-related cell activation (18, 19, 20, 21), which has been discussed to be relevant in ALS pathogenesis (22, 23). Moreover, CD14, TLR2 and TLR4 on microglia were shown in the pathophysiology of Alzheimer disease to interact with aggregated β amyloid peptide and to induce microglial inflammatory activation (24, 25, 26, 27). Interestingly, mSOD1 protein was observed also to aggregate in ALS pathogenesis (28, 29, 30), and to be secreted into extracellular space by neurons (31). Thus, after observing a marked up-regulation of TLR2 and CD14 in an ALS mouse model (32), we hypothesized that in ALS pathogenesis microglia could be activated by endogenous ligands via innate immune receptors, which modulate microglial effects on neuronal death.

In this study, we investigate how mSOD1 expression affects innate immune receptor-initiated microglial inflammatory activation, and observed that mSOD1 enhances NADPH oxidase-dependent production of reactive oxygen species (ROS), and subsequently increases the secretion of TNF-α, which is related to TNF-α converting enzymes, e.g. a disintegrin and metalloproteinase (ADAM) 10 and 17 (33, 34).

**EXPERIMENTAL PROCEDURES**

**Construction of knock-down and transgenic vectors** - pcDNA6.2-GW/EmGFPmiR (Invitrogen, Karlsruhe, Germany) was engineered to contain different select hairpins targeting p47-phagocyte oxidase (p47-PHOX), ADAM10 and ADAM17 genes. The single-stranded DNA oligomers targeted to p47-PHOX, ADAM10 and ADAM17 genes were synthesized by Invitrogen, annealed and inserted into the linearized backbones according to the manufacturer’s instructions. Sequences of the DNA oligomers are listed in Table I. After transfection, the vector will transcribe artificial miRNAs which have 100% homology to the gene sequence of interest, resulting in target RNA cleavage.

pCEP4-wtSOD1, mSOD1(L8Q) and mSOD1(G10V) were constructed by ligating PCR products into pCEP4 vector (Invitrogen) between KpnI and XhoI. The PCR was performed using cDNA encoding wtSOD1 (kindly provided by Dr. Kopito, Stanford University, ref. 35) and mSOD1(L8Q or G10V) (kindly provided by Dr. Engelhardt, University of Iowa Carver College of Medicine, ref. 36) as the templates and the following primers (Invitrogen) containing KpnI or XhoI restriction sites (underlined): 5′-GGGGTACCCCACCATGGCGACGAAGGCCGT-3′ and 5′-GGGCTCGAGTTATTGGGCGATCCCAAT-3′.

**Cell culture and cell line establishment** - BV-2 cells, derived from an immortalized microglial cell line (37) (kindly provided by Prof. K. Frei, University Hospital Zürich, Zürich, Switzerland), were cultured in Dulbecco's Modified Eagle medium (Invitrogen) supplemented with 10% fetal calf serum (PAN Biotech, Aidenbach, Germany) under a humidified atmosphere of 5% CO2 at 37°C.

To establish human SOD1-overexpressing cell lines, BV-2 cells were transfected with pcDNA3.1-wtSOD1, pcDNA3.1-mSOD1(G93A) (kindly provided by Dr. Kopito, ref. 35), pCEP4-wtSOD1, pCEP4-mSOD1(L8Q), pCEP4-mSOD1(G10V) and relative mock control vectors using lipofactamine LTX (Invitrogen). In order to silence the p47-PHOX, ADAM10 and ADAM17 gene expression, human wtSOD1 or mSOD1(G93A)-expressing and mock control cells were further transfected with pcDNA6.2-GW/EmGFPmiR RNAi expression vectors targeting interested genes. All transfected cells were selected with G418 (Sigma, Schnelldorf, Germany), hygromycin B (Sigma) or with both.
G418 and blasticidin (Invitrogen) until the stable expression of investigated genes.

**Western blot analysis of SOD1 expression in cell lines**- Cultured cells were briefly washed with PBS and then lysed in 50 mM HEPES/0.1 mM EDTA (pH 7.2) buffer containing 100 mM iodoacetamide (Sigma) / 2.5% SDS. The samples were incubated at 37°C for 1 hour and centrifuged at 14,100g for 10 minutes to remove insoluble materials. Clarified protein was then prepared in Laemmli buffer with 2-mercaptanol, boiled and resolved using 12% SDS/PAGE gels. After electrophoresis, proteins were electroblotted on nitrocellulose membranes (Whatman GmbH, Dassel, Germany). After membranes were blocked with 3% BSA for 1 hour, they were incubated with sheep anti-superoxide dismutase (Cu/Zn) human erythrocyte (1:5,000 dilution; EMD Biosciences, Darmstadt, Germany), followed by HRP-conjugated rabbit anti-sheep IgG (1:10,000 dilution; Jackson ImmunoResearch Laboratories, Suffolk, UK). Blots were developed with the ECL Western Blotting Detection System (GE Healthcare, Munich, Germany). Subsequently, membranes were re-blotted with mouse anti-actin (1:1000 dilution; Millipore GmbH, Schwalbach/Ts, Germany) and HRP-conjugated rabbit anti-mouse IgG (1:10,000 dilution; Jackson ImmunoResearch Laboratories) to confirm equivalent protein loading.

**Cell challenge and ELISA detection of TNF-α, IL-6 and IL-1β**- Stably transfected BV-2 cells (overexpressing wt or mSOD1, or a mock control) were plated in 48-well plates at the density of 1×10⁵/well. Cells were treated with Pam3Cys-SKKKK (TLR2 ligand, EMC, Tübingen, Germany), purified lipopolysaccharide (LPS, from E. coli, Serotype R515(Re), TLR4 ligand, Alexis Biochemicals, Lörrach, Germany) and Polyinosinic-polycytidylic acid (poly(I:C), TLR3 ligand, Alexis Biochemicals) at different concentrations as indicated in the results for 24 hours. Supernatants were then collected for detection of TNF-α, IL-6 and IL-1β by ELISA kits (all from R & D Systems, Wiesbaden-Nordenstadt, Germany). In order to analyze the effects of ROS on TNF-α release after TLR2 stimulation, part of the cells were co-treated with antioxidant reagents, N-Acetyl-L-cysteine (NAC) at 2 or 5mM, and 1000U/ml catalase (both from Sigma). To analyze the role of p47-PHOX, ADAM10 and ADAM17 on cytokine secretion, cells co-transfected with different SOD1 expression vectors and interested gene-silencing vectors were used for different stimulations described above.

**Griess assay for NO detection**- To evaluate NO release after cell challenges, stably transfected BV-2 cells were plated and treated using the same protocol for cytokine analysis as described in the last section. Modified Griess reagents (Sigma, working range: 0.43-65 μM nitrite) were used to measure the levels of nitrite (NO2-) in supernatants of the cultured cells.

**Reverse transcription and quantitative PCR for analysis of gene transcripts**- To measure transcripts of inflammatory genes (e.g. TNF-α, IL-6, IL-1β and iNOS), different SOD1-overexpressing BV-2 cells were plated and treated with 0.1µg/ml Pam3Cys-SKKKK as described above. Total RNA was isolated by the RNEasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized by priming total RNA with random primers (Invitrogen) and using M-MLV reverse transcriptase according to manufacturer’s instructions (Sigma). For quantification, real-time (RT)-PCR was performed using the Applied Biosystems 7500 Fast real-time PCR system (Foster City, CA) according to our established protocol (38). To confirm successful gene knocking down, transcripts of silenced genes in cells transfected with different RNAi expression vectors were also measured by quantitative RT-PCR. The oligonucleotides (Invitrogen) used for PCR amplification are listed in table II.

The amount of double-stranded PCR product synthesized in each cycle was measured using SYBR green I dye. Threshold cycle (Ct) values for each test gene from the replicates PCRs was normalized to the Ct values for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control from the same cDNA preparations. The ratio of transcription of each gene was calculated as 2^(ΔCt), where ΔCt is the difference Ct (test gene) - Ct (GAPDH).

**NF-κB-luciferase reporter assays**- Different human SOD1-overexpressing cells were cultured in 24-well plates at the density of 1×10⁵/well and
transfected with 0.4 µg of luciferase NF-κB reporter construct (Stratagene, La Jolla), and 0.1 µg of pECDNA3.1/His/LacZ (Invitrogen) as an internal transfection control per well using 1.25 µl of Lipofectamine LTX (Invitrogen) in accordance with the manufacturer's instructions. Twenty-four hours after transfection, cells were treated with 0.1µg/ml Pam3Cys-SKKKK or 10ng/ml LPS as described above for another 24 hours. Luciferase and β-galactosidase activity was then assayed with Bright-Glo Luciferase Assay System and β-galactosidase Enzyme Assay Systems (both from Promega, Mannheim, Germany). Luciferase reporter activity was normalized for transfection efficiency with the galactosidase activity from the same sample.

Assessment of H2O2 and anti-oxidant capacity- For assessment of H2O2, the cells were plated in 48-well plates as described above. After treatment with 0.1µg/ml Pam3Cys-SKKKK or 10ng/ml LPS for 24 hours, the supernatants were collected and immediately detected with the Fluoro H2O2® detection kit (Bachem, Heidelberg, Germany) according to the protocol provided by the manufacturer. In this assessment, the non-fluorescent reagent 10-acetyl-3,7-dihydroxyphenoxazine was oxidized by H2O2 in a 1:1 stoichiometry to produce a fluorescent product resorufin (excitation at 530-571 nm, emission at 590-600 nm) and measured by a fluorescence plate reader (Safire®™, TECAN, Männedorf, Switzerland).

For assessment of anti-oxidant capacity, the cells were plated in 6-well plates at the density of 1×10^5/well and treated with 0.1µg/ml Pam3Cys-SKKKK or 10ng/ml LPS for 24 hours. The concentration of thiol groups in cell lysates (lysate buffer: 10mM Tris pH 8.0, 150mM NaCl, 1% Triton X-100 and protease inhibitor cocktail from Roche Applied Science, Mannheim, Germany) was determined using a colorimetric assay using Ellman’s reagent (10mM dithio-bis 2-nitrobenzoic acid (DTNB) + 10mM cystamine, both from Sigma) as the substrate, and the color reaction was monitored at 412 nm (39).

Statistics- Data in figures is presented as mean ± SEM. One-way ANOVA followed by Tukey's HSD or Tamhane's T2 post hoc test (dependent on the result of Levene's test to determine the equality of variances) was used for multiple comparisons. Two-independent-samples t test was used to compare means for two groups of cases. All statistical analysis was performed using SPSS 15.0 for Windows (SPSS, Chicago, IL). Statistical significance was set at p < 0.05.

RESULTS

Generation of microglial cells that overexpress human SOD1. In order to investigate the effects of ALS-linked mSOD1 on microglial inflammatory activities, we transfected BV-2 cells with the wild type (wt) and G93A, L8Q or G10V mutated (m) human SOD1 genes, and with a mock control as outlined in the Experimental Procedures. To verify the expression of human SOD1 in the different transfected cells, cell lysates were prepared and analyzed by Western blot. As shown in Fig.1, human SOD1 protein expression was markedly increased in wtSOD1 and mSOD1(G93A)-transgenic cells as compared to the mock control.

SOD1 G93A mutation enhances TLR2 stimulation-induced microglial TNF-α secretion. Since we recently reported that TLR2 and CD14 are two highly up-regulated innate immune receptors in the spinal cord of an ALS mouse model (32), we first asked whether mSOD1 affects microglial activation via these two receptors. We stimulated wtSOD1 and mSOD1(G93A)-overexpressing and the mock control BV-2 cells with Pam3Cys-SKKKK (TLR2 ligand) and LPS (CD14/TLR4 ligand), as well as with poly(I:C) (TLR3 ligand) as a stimulating control. The cells were strongly activated by these stimuli as analyzed by measuring the cytokine secretion (Fig. 2). Interestingly, we observed that after Pam3Cys-SKKKK stimulation at lower concentrations (0.1 or 1 µg/ml), mSOD1(G93A)-overexpressing cells released significantly more TNF-α than wtSOD1-overexpressing or mock control cells (Fig. 2A, p<0.05). This difference in cytokine release between different cell types became smaller when TLR2 ligand concentration was increased (10µg/ml). Surprisingly, IL-6 secretion was markedly reduced in mSOD1(G93A)-overexpressing cells as compared to wtSOD1-expressing cells, especially upon stimulation of Pam3Cys-SKKKK at higher concentrations (1 and...
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10 µg/ml) (Fig. 2B, p<0.05). Following LPS stimulation, there was no significant difference in TNF-α secretion between mSOD1(G93A)-overexpressing and other two cell types (Fig. 2C). Upon stimulation of TLR3 with poly(I:C), mSOD1(G93A)-overexpressing cells responded with a significantly higher TNF-α secretion than mock control, but not wtSOD1-expressing cells (Fig. 2E, p<0.05). The increase in IL-6 secretion following TLR4 stimulation using LPS was significantly reduced in mSOD1(G93A)-expressing cells as compared to the wtSOD1-overexpressing, but not mock control cells (Fig. 2D, p<0.05). No elevated IL-6 release was detected following TLR3 stimulation (Fig. 2F). IL-1β and NO secretion were also measured in this study. Upon TLR2 stimulation with Pam3Cys-SKKKK at 0.1µg/ml, mSOD1(G93A)-overexpressing cells tended to increase NO release, although not reaching statistical significance (Fig. 2G, p>0.05). The secretion of IL-1β from all studied cell lines was not significantly increased by the TLR2 stimulation (data not shown).

In order to confirm the effects of mSOD1(G93A) expression on microglial activation, we further created new cell lines overexpressing human mSOD1 (L8Q or G10V) mutant and wtSOD1. As shown in Fig. 2H, after TLR2 stimulation, mSOD1 (L8Q or G10V)-overexpressing cells secreted significantly more TNF-α than wtSOD1-overexpressing or mock control cells (p<0.05), but did not enhance the IL-6 secretion (data not shown).

*SOD1 G93A mutation does not enhance transcription of TNF-α.* After we observed that mSOD1-overexpression enhances TLR2-induced microglial secretion of TNF-α but reduces IL-6 release, we aimed to clarify the mechanism of how mSOD1 modulates TNF-α and IL-6 production. We measured gene transcripts of the inflammatory molecules (TNF-α, IL-6, IL-1β and iNOS) by quantitative PCR. As shown in Fig. 3A-D, the transcription of all studied inflammatory genes was significantly increased by TLR2 stimulation in the different cell lines (p<0.05). When comparing the gene transcriptional levels between different cell types, we observed that mSOD1(G93A)-overexpression, compared to mock or wtSOD1 controls, reduced transcription of TNF-α and IL-1β while increasing transcription of IL-6 and iNOS, though all mSOD1(G93A)-induced transcriptional changes of inflammatory genes did not reach statistical significance (Fig. 3A-D, p>0.05). Furthermore, we observed that mSOD1(G93A)-overexpression significantly increased the basal level activity of NF-κB signaling compared to the other two controls (Fig. 3E, p<0.05). However, following Pam3Cys-SKKKK and LPS stimulations, the difference in NF-κB activation among the different cell types was reduced. Thus, the regulation of mSOD1(G93A) on TNF-α and IL-6 production might occur at a post-transcriptional level.

*SOD1 G93A mutation enhances microglial oxidative stress and subsequent TNF-α secretion following TLR2 stimulation.* Since oxidative stress is well recognized as an important pathogenic event in ALS patients and animal models (22, 23), we further studied the role of ROS on microglial secretion of TNF-α. As shown in Fig. 4A and B, in response to Pam3Cys-SKKKK and LPS stimuli, mSOD1(G93A)-overexpressing and mock control cells significantly increased H2O2 release and their anti-oxidative capacity was reduced as indicated by the amount of thiol group in the cell lysate (p<0.05). WtSOD1-overexpressing cells released more H2O2 only after LPS stimulation and did not have reduced protein thiol levels. When the levels of ROS production were compared between different cell types, it could be seen that LPS stimulation equally increased the intensity of oxidative stress, whereas, Pam3Cys-SKKKK stimulation differentially modified it. Specifically, mSOD1(G93A)-overexpressing cells released significantly more H2O2 and had a significantly reduced anti-oxidant capacity than the other two studied cell types upon TLR2 stimulation (Fig. 4A and B, p<0.05). Thus, we pretreated microglial cells with anti-oxidant regents, e.g. NAC and catalase, before incubating cells with Pam3Cys-SKKKK. Interestingly, the mSOD1(G93A)-overexpression-associated enhancement of TNF-α secretion was reversed (Fig. 4C). However, the mSOD1(G93A)-overexpression-related reduction of IL-6 secretion upon TLR2 stimulation was not significantly affected (data not shown).

P47-PHOX mediates SOD1 G93A mutation-enhanced oxidative stress and
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subsequent TNF-α secretion following TLR2 stimulation. Very recently, it has been reported that deficiency of the NADPH oxidase complex component, gp91-PHOX, or inhibition of NADPH oxidase assembly by apocynin can significantly prolong the life span of mSOD1(G93A) transgenic mice (40, 41). Therefore, we tested the effect of NADPH oxidase on mSOD1(G93A)-mediated regulation of microglial inflammatory activation. Following transfection of knock-down vectors, p47-PHOX gene expression was clearly reduced, and subsequently, the anti-oxidative capacity in mSOD1(G93A)-overexpressing microglial cells was not decreased by TLR2 stimulation (Fig. 5A and B). Accordingly, the mSOD1(G93A)-overexpression-induced enhancement of TNF-α secretion upon TLR2 activation was reversed (Fig. 5C). LPS stimulation could equally reduce anti-oxidant capacity in all different cell types, and the silencing of p47-PHOX gene expression did not specifically decrease TNF-α release from the mSOD1(G93A)-overexpressing microglial cells upon LPS activation (data not shown).

**ADAM10 and 17 are involved in SOD1 G93A mutation-enhanced TNF-α secretion following TLR2 stimulation.** Since we had observed that expression of mSOD1 regulates TLR2-induced microglial TNF-α release at a post-transcriptional level, we hypothesized that TNF-α converting enzymes, e.g. ADAM10 and ADAM17 could play a causal role since they can be activated by ROS (42). Thus, we further tested the effect of ADAM10 and ADAM17 on mSOD1(G93A)-induced enhancement of TNF-α secretion by knocking down ADAM10 or ADAM17 gene expression in different human SOD1-overexpressing cells. ADAM10 and ADAM17 expressions were markedly decreased by transfection with relevant gene silencing vectors (Fig. 6A). Due to technical limitations, we were unable to measure the ADAM activity on cell surface. As seen in Fig.6B, in the gene silencing control group, mSOD1(G93A)-overexpression could significantly increased microglial TNF-α release (p<0.05) following TLR2 activation as compared to wtSOD1-overexpressor or the mock control. Following ADAM10 or ADAM17 gene silencing, the mSOD1(G93A)-induced enhancement of microglial TNF-α release was reversed.

**DISCUSSION**

Recently, evidence has accumulated suggesting that ALS-linked mSOD1-expressing microglia contribute to motor neuronal death and promote disease progression of ALS (8, 9, 10). However, the mechanisms of how mSOD1 affects microglial activation and subsequently injures neurons are unclear. Here, we observed that overexpression of the ALS-linked G93A, L8Q or G10V SOD1 mutant markedly enhances microglial secretion of the neurotoxic proinflammatory cytokine, TNF-α, in a clear TLR2-dependent manner. Increase of NADPH oxidase-mediated ROS production plays an important role in this event.

Innate immune receptors, such as TLR2 and TLR4, have been discussed to be involved in ALS pathogenesis. Expression of TLR2 and CD14, the co-receptor of TLR4, is markedly up-regulated in an ALS mouse model (32). A chronic challenge with LPS at the pre-symptomatic stage of the disease exacerbates motor neuronal degeneration (43). Furthermore, deficiency of MyD88, the signaling molecule downstream to TLR2 and 4, in bone marrow cells accelerates disease onset and reduces survival of the mSOD1-transgenic mice (44). Because of the potential existence of endogenous ligands for these receptors in the central nervous system (ref. 16, 17, although the ligands still need to be identified), we hypothesize that toxic effects of microglial activation in ALS pathogenesis are regulated by innate immune receptors. Expression of SOD1 mutants could, then, contribute to microglial neurotoxicity via interacting with the receptor-mediated inflammatory signaling.

In this study, we observed that, as compared to wtSOD1-expressing microglia, mSOD1-expressing cells increase the secretion of neurotoxic TNF-α, but reduce release of a rather protective cytokine, IL-6, upon TLR2 activation. This result, with regards to cytotoxic secretion, is similar to a previous report using microglia derived from mSOD1-transgenic mice (45). TNF-α may contribute to motor neuron death in ALS since it has been shown that inhibition of TNF-α function can reduce the disease progression (46, 47). IL-6 is a multifunctional member of the neuropoietic cytokine family (48) and has been
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suggested to promote neuronal survival, reduce neurotoxicity and enhance axonal regeneration (49, 50, 51). Interestingly, our study showed that the modulating effects of mSOD1-expression on microglial neurotoxic inflammatory activities depend on TLR2, but not TLR4 stimulation.

What could be the link between TLR2 receptor activation and mSOD1 expression? Several authors recently reported the pathogenetic role of NADPH oxidase complex in ALS, which generates ROS in response to inflammatory signals (36, 40, 41). The NADPH oxidase complex contains two constitutively associated transmembrane proteins (p22-PHOX and gp91-PHOX) and three cytosolic components (p47-PHOX, p67-PHOX and p40-PHOX). Upon cell activation, p47-PHOX becomes phosphorylated, thereby initiating translocation of the p47-PHOX/p67-PHOX/p40-PHOX complex to the membrane. The small GTPase Rac1 is an important activator of this oxidase (52). Interestingly, SOD1, rather than a cytosolic enzyme that facilitates the conversion of superoxide to H2O2, can bind to Rac1 and serve as a self-regulating redox sensor for NADPH-dependent ROS production. ALS-linked mutations in the SOD1 gene damage this self-limiting mechanism, which leads to more ROS release (36). Thus, gene ablation of gp91-PHOX or inhibiting the assembly of NADPH complex by apocynin was able to increase the lifespan of a mSOD1(G93A) transgenic ALS mouse model (40, 41). In our study, we observed that overexpression of mSOD1 enhances TLR2 stimulation-induced microglial oxidative stress and subsequent TNF-α secretion in a clear p47-PHOX-dependent manner. Therefore, we extend the knowledge on the pathogenetic effect of NADPH oxidase in ALS by demonstrating the potential upstream activating mechanism to this enzyme.

Our results further indicate that TNF-α secretion is regulated by mSOD1-overexpression in microglial cells at a post-transcriptional level. In addition, we observed that gene silencing of TNF-α converting enzymes, especially ADAM17, reduces mSOD1-induced enhancement of TNF-α secretion following TLR2 stimulation. Because ADAM 17 are responsible for TNF-α shedding from the cell surface and sensitive to ROS (33, 42), our study provides further insights into the mechanisms of how mSOD1 expression adversely affects neuronal death through NADPH oxidase.

Indeed, oxidative stress has been long discussed in the pathologic processes of ALS. Because of the multiple sources of ROS in the cell and the broad and pleiotropic targets of ROS (53), the pathophysiology of mSOD1-enhanced ROS production in ALS is complicated (22). ROS have been related to excitotoxic injury of motor neurons. They also play a role in pathways involved in neuronal apoptosis, mitochondrial damage and SOD1 aggregation. ROS are mainly produced by inflammatory cells and, on the other way round, modulate inflammatory activities. For example, we observed down-regulated transcription of TNF-α by mSOD1 expression in this study (although not statistically significant), which could be due to the impaired mRNA stability after enhancement of NADPH-mediated ROS production (38). Given the complex interactions between oxidative stress and other pathogenetic pathways in ALS, it is unlikely that a single therapeutic intervention will arrest disease progression. Several studies showed that preclinical consumption of the antioxidant, vitamin E, can prevent ALS (54, 55). However, a meta-analysis based on all randomized or quasi-randomized (e.g. alternate allocation) controlled clinical trials of antioxidants up to now still shows the inefficacy of antioxidants in ALS (56).

In summary, our study demonstrates that mSOD1-expression in microglia enhances secretion of the neurotoxic cytokine, TNF-α, in a TLR2-dependent fashion. Increased p47-PHOX-dependent production of ROS is likely to mediate this cellular response (see schematic figure). Such a link between the ALS-associated SOD1 mutant, innate immunity and oxidative stress may be relevant for design of future ALS therapies.
REFERENCES

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FOOTNOTES
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The abbreviations used are: ALS, amyotrophic lateral sclerosis; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-alpha; PHOX, phagocyte oxidase; SOD1, Cu, Zn superoxide dismutase; TLRs, toll-like receptors; NAC, N-Acetyl-L-cysteine; DTNB, dithio-bis 2-nitrobenzoic acid; TACE, TNF-α converting enzyme; ADAM, A disintegrin and metalloproteinase

FIGURE LEGENDS
Fig. 1, Generation of microglial cells overexpressing human SOD1. BV-2 cells were stably transfected with a mock control, wtSOD1 and SOD1 G93A mutant (mSOD1) expression vectors. Cell lysates were prepared and analyzed by Western blot. Human SOD1 protein expression was markedly increased in wtSOD1 and mSOD1-transgenic cells as compared to the mock control cells.

Fig. 2, Expression of mSOD1 increased TNF-α secretion upon TLR2 stimulation. Wt and mutated (G93A, L8Q or G10V) SOD1-overexpressing and mock control cells were treated with Pam3Cys-SKKKK, LPS and Poly(I:C) at different concentrations as indicated in the figure for 24 hours. Supernatant was then collected for ELISA analysis of TNF-α and IL-6, or for NO detection. Cytokine secretion or NO release was significantly increased in each cell line upon different stimuli (A-E, G-H, p<0.05), except that IL-6 secretion was not activated by poly(I:C) (F). ANOVA with post-hoc test, a: vs. wtSOD1 transgene, b: vs. the mock control, p<0.05, n≥6 per each group. The scale for concentrations of Pam3Cys-SKKKK and LPS in the x-axes is logarithmically transformed.

Fig. 3, Expression of mSOD1 did not alter inflammatory gene transcription. As seen in A-D, the gene transcripts of TNF-α, IL-6, IL-1β and iNOS in different SOD1-expressing cells as measured by quantitative PCR were significantly increased following treatment with 0.1μg/ml Pam3Cys-SKKKK for 24 hours, compared to their basal untreated levels (t test, n≥5 per each group). However, there is not a significant difference in the different inflammatory gene transcription between different cell types following stimulation (A-D, ANOVA, p>0.05). E, NF-κB report activity was assayed in different cell types after 0.1μg/ml Pam3Cys-SKKKK and 10ng/ml LPS stimulations for 24 hours. Expression of mSOD1(G93A) induced significantly higher NF-κB activity than wtSOD1 and the mock control at basal level, whereas, after stimulation with TLR2 or TLR4 ligand, the difference of NF-κB activation between different cell types was reduced. ANOVA with post-hoc test, n=4 per each group. a: vs. wtSOD1 transgene, p<0.05; b: vs. the mock control, p<0.05.

Fig. 4, Expression of mSOD1 enhanced microglial oxidative stress and subsequently TNF-α secretion following TLR2 stimulation. The different microglial cell lines were treated with either 0.1μg/ml
Pam3Cys-SKKKK or 10ng/ml LPS for 24 hours. As seen in A and B, compared to the basal level, significantly more H2O2 release or less thiol in the cell lysate was detected in the mock control and mSOD1(G93A)-overexpressing cells after TLR2 or TLR4 activation. WtSOD1-overexpressing cells produced more H2O2 only after LPS stimulation. When oxidative stress upon TLR2 or TLR4 stimulation was compared between different cell types, mSOD1(G93A)-overexpression was observed to result in significantly higher levels of H2O2 and lower concentrations of thiol groups in the cell lysate. C: Following co-treatment with anti-oxidants (NAC at 2 and 5 mM, and 1000U/ml catalase), mSOD1(G93A)-overexpression-induced enhancement of TNF-α secretion was reversed. The basal TNF-α secretion from different cell lines is set at 1, and the TNF-α secretion after stimulation is shown as the fold increase as compared to its relevant basal level. *: t test vs. basal level for each cell line, p<0.05, n=3 per each group. ANOVA with post-hoc test, a: vs. wtSOD1 transgene and b: vs. the mock control, p<0.05, n=4 per each group.

Fig. 5. Knock-down of p47-PHOX expression reversed mSOD1-overexpression-induced enhancement of TNF-α secretion. BV-2 cells co-transfected with human SOD1-encoding and p47-PHOX gene silencing vectors were treated with 0.1μg/ml Pam3Cys-SKKKK for 24 hours. As seen in A, the gene transcript of p47-PHOX, as measured by quantitative PCR, was markedly reduced in p47-PHOX gene silencing cell lines. Accordingly, mSOD1(G93A)-overexpression-associated reduction of thiol groups (see B) and enhancement of the TNF-α secretion were both reversed (see C). The basal TNF-α secretion from different cell lines is set at 1, and the TNF-α secretion after stimulation is shown as the fold increase as compared to its relevant basal level. *: t test vs. basal level for each cell line, p<0.05, n=4 per each group. ANOVA with post-hoc test, a: vs. wtSOD1 transgene and b: vs. the mock control, p<0.05, n=4 per each group.

Fig. 6. Knock-down of ADAM10 or ADAM17 gene expression reversed mSOD1-overexpression-induced enhancement of TNF-α secretion. BV-2 cells co-transfected with human SOD1-encoding vector and gene silencing vector targeting mouse ADAM10 or 17 were activated by 0.1μg/ml Pam3Cys-SKKKK for 24 hours. As seen in A, the gene transcripts of ADAM10 or ADAM17, as measured by quantitative PCR, were clearly reduced in the relevant gene silencing cells. Accordingly, mSOD1(G93A)-overexpression-induced enhancement of TNF-α secretion was reversed by silencing ADAM gene expression. The basal TNF-α secretion from different cell lines is set at 1, and the TNF-α secretion after stimulation is shown as the fold increase as compared to its relevant basal level. *: t test vs. basal level for each cell line, p<0.05, n=6 per each group. ANOVA with post-hoc test, a: vs. wtSOD1 transgene and b: vs. the mock control, p<0.05, n=6 per each group.

Fig. 7. Schematic figure. Upon stimulation of TLR2, the p47-PHOX-containing NADPH oxidase complex is activated and produces reactive oxygen species (ROS, O2-) (A). ROS, in turn, activate TNF-α converting enzymes (TACE), such as ADAM10 and ADAM17 (B), which subsequently cut membrane-anchored precursor of TNF-α and release TNF-α (C). SOD1 binding to Rac1 serves as a self-regulating redox sensor for NADPH oxidase-dependent ROS generation. In ALS, but not in the healthy condition, the mutation in SOD1 gene impairs this mechanism to limit ROS production, which leads to enhanced TNF-α shedding (D).
Table I, Sequences of DNA oligomers inserted into pcDNA6.2-GW/EmGFPmiR to construct knock-down vectors

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<th>Oligomers</th>
<th>Sequences</th>
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<td>Mmi515226_top_p47PHOX</td>
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<td>ACTGACTGACAAAGCGTACTGCTGTGTTGAA</td>
</tr>
<tr>
<td>Mmi515226_bot_p47PHOX</td>
<td>CCTGTTCAACAGCGTACGCTTTTTGTTTGCC</td>
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<tr>
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<td>GCCAAAACAAAGCGTACTGCTGTGTTGAA</td>
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<tr>
<td>Mmi503668_top_ADAM10</td>
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<td>ACTGACTGACAATGGGAGTGCAGGTTTCT</td>
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<tr>
<td>Mmi503668_bot_ADAM10</td>
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<td>GCCAAAACAAATGGGAGTGCAGGTTTCT</td>
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### Table II, Sequences of oligonucleotides used for the real-time quantitative PCR

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<tr>
<th>Gene</th>
<th>Sense</th>
<th>Anti-sense</th>
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<td>P47-PHOX</td>
<td>5'-GCCCAAAGATGGCAAGAATA-3'</td>
<td>5'-TGTTCCCGAACTCTTCTCGT-3'</td>
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<td>ADAM10</td>
<td>5'-AAGGGATATGCAATGGCTTC-3'</td>
<td>5'-TTGCCCATTAATGCACACTT-3'</td>
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<td>ADAM17</td>
<td>5'-CCAGCTGAGCATCAACACTT-3'</td>
<td>5'-TGGACAAGAATGCTGAAAGG-3'</td>
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<td>TNF-α</td>
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<td>5'-CTCCACTTTGGTGTTTGCTA-3'</td>
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<td>IL-6</td>
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<td>5'-ATTTCCACGATTTCCCAGAG-3'</td>
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<td>IL-1β</td>
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<td>5'-TCATCTCGGAGCCTGTAGTG-3'</td>
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<td>5'-CATTCCCAATGTGCTTGTTC-3'</td>
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<td>GAPDH</td>
<td>5'-ACAACTTTGGCATTGGAA-3'</td>
<td>5'-GATGCAGGGATGATGTTCTG-3'</td>
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</table>
Liu et al., Fig. 1
Liu et al. Fig. 2
Liu et al., Fig. 3
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Yang Liu, Wenlin Hao, Alik Dawson, Shirong Liu and Klaus Fassbender

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