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Alsin and SOD1^{G93A} Proteins Regulate Endosomal Reactive Oxygen Species Production by Glial Cells and Proinflammatory Pathways Responsible for Neurotoxicity*[S]

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Recent studies have implicated enhanced Nox2-mediated reactive oxygen species (ROS) by microglia in the pathogenesis of motor neuron death observed in familial amyotrophic lateral sclerosis (ALS). In this context, ALS mutant forms of SOD1 enhance Rac1 activation, leading to increased Nox2-dependent microglial ROS production and neuron cell death in mice. It remains unclear if other genetic mutations that cause ALS also function through similar Nox-dependent pathways to enhance ROS-mediate motor neuron death. In the present study, we sought to understand whether alsin, which is mutated in an inherited juvenile form of ALS, functionally converges on Rac1-dependent pathways acted upon by SOD1^{G93A} to regulate Nox-dependent ROS production. Our studies demonstrate that glial cell expression of SOD1^{G93A} or wild type alsin induces ROS production, Rac1 activation, secretion of TNFα, and activation of NFκB, leading to decreased motor neuron survival in co-culture. Interestingly, coexpression of alsin, or shRNA against Nox2, with SOD1^{G93A} in glial cells attenuated these proinflammatory indicators and protected motor neurons in co-culture, although shRNAs against Nox1 and Nox4 had little effect. SOD1^{G93A} expression dramatically enhanced TNFα-mediated endosomal ROS in glial cells in a Rac1-dependent manner and alsin overexpression inhibited SOD1^{G93A}-induced endosomal ROS and Rac1 activation. SOD1^{G93A} expression enhanced recruitment of alsin to the endomembrane compartment in glial cells, suggesting that these two proteins act to modulate Nox2-dependent endosomal ROS and proinflammatory signals that modulate NFκB. These studies suggest that glial proinflammatory signals regulated by endosomal ROS are influenced by two gene products known to cause ALS.

Amyotrophic lateral sclerosis (ALS) is a lethal degenerative neurological disorder characterized by progressive degeneration of motor neurons in the brain and spinal cord (1, 2). The majority of ALS patients have onset of disease between 40 and 50 years of age and about 50% of patients die within 3 years. The majority of ALS cases are categorized as sporadic with no family history of disease. In this context, the causative genes and environmental factors that initiate the disease process remain poorly defined. Only ~10% of ALS cases have a clearly inherited genetic component and hence are classified as familial ALS (1, 2).

The best-characterized forms of familial ALS include those caused by mutations in the gene encoding Cu/Zn-superoxide dismutase (*SOD1*)[2] (3). Approximately 20% of familial ALS cases are caused by a variety of dominant *SOD1* mutations (1, 3). There remains great uncertainty as to the primary mechanism(s) by which mutant SOD1 leads to pathology observed in ALS (1, 4). Proposed mechanisms include toxicity associated with misfolding of mutant SOD1, such as ER stress and inhibition of the proteasome, enhanced proinflammatory ROS production, altered axonal transport, excitotoxicity caused by glutamate mishandling, and mitochondrial damage (1, 4). Relevant to the studies in this report are findings demonstrating that SOD1 mutations induce NADPH oxidase-dependent ROS production in microglia of *SOD1*^{G93A} mice leading to motor neuron death (5, 6). These studies have demonstrated that deletion of the Nox2 isoform of NADPH oxidase, and to a lesser extent also the Nox1 isoform, can prolong survival in *SOD1*^{G93A} transgenic mice. The importance of SOD1 in regulating cellular ROS production was first revealed by studies demonstrating that SOD1 can directly associate with endosomal Rac1 to regulate its activity (7). Rac1 is an essential activator of several NADPH oxidases and SOD1 binding to Rac1 slows the hydrolysis of GTP bound to Rac1 in a redox-dependent manner. Thus, SOD1 association with Rac1 is proposed to be a redox-dependent sensor for regulating redox-active NADPH oxidase containing endosomes (called redoxosomes) (7, 8). Redoxosomes are important signaling endosomes that regulate proinflammatory receptor signals in a redox-dependent manner through NADPH oxidases (8–11). Redoxosomes are a subpopulation of early endosomes that produce ROS and have been shown to contain early endosomal markers (Rab5 and EEA1), redox effectors (Nox2 or Nox1, Rac1, p47^{phox}, p67^{phox}, and SOD1), and certainly ligand-activated cytokine receptors (TNFR1 or IL-1R1) (8–11). ROS produced by the redoxosome facilitate the redox-dependent recruitment of TRAFs to TNFR1 and IL-1R1 and in this manner facilitate proinflammatory signaling such as

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[2] The abbreviations used are: SOD, superoxide dismutase; ROS, reactive oxygen species; H_2HFF, dihydro-2′,4,5,6,7,7′-hexafluorofluorescein; DHE, dihydroethidium; hESC, human embryonic stem cells.

NFκB activation (8–11). Interestingly, mutant forms of SOD1 have enhanced redox-independent binding to Rac1 and this has been proposed as a mechanism for enhanced ROS production in microglia of ALS mice (7, 12).

Seven known NADPH oxidase catalytic subunits exist (Nox1, Nox2^{gp91phox}, Nox3, Nox4, Nox5, Duox1, and Duox2) (13, 14). The most widely characterized of these is phagocytic gp91^{phox} (also known as Nox2). Nox2 is also expressed in microglia (6) and a variety of other nonphagocytic cell types. Rac, a small GTPase, is an essential activator of Nox1 and -2, and along with several other subunits (p22^{phox}, p40^{phox}, p47^{phox}, p67^{phox}, NoxO1, and NoxA1) can act to promote Nox complex activation in a cell type-specific fashion (13, 14). In certain cell systems, Rac1 has also been shown to be required for ROS production by Nox4 (15–17). Because NADPH oxidases (Nox) generate the O_2^- substrate of the SOD1 dismutation reaction ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$), this class of Nox enzymes has recently generated considerable interest in studies of ALS.

It is presently unclear if familial and sporadic forms of ALS have common or overlapping molecular mechanisms of disease pathogenesis. A recent genomewide association study in sporadic ALS patients has begun to shed light on this topic (18). Several genes that regulate endosomal trafficking, Rac1, and NADPH oxidases were identified in this study, including *Nox4*, *TIAM2*, *IQGAP2*, *PTPRT*, *RAP1GAP*, and *MAGI2* (12). Interestingly, the *ALS2* gene product alsin, which when mutated causes juvenile ALS, has also been shown to influence endosomal trafficking and Rac1 activity (19–23). Alsin appears to serve as a GEF for Rab5 and an effector of Rac1 GTPase activity (24–26). These findings are of considerable interest because SOD1 also regulates Rac1 GTPase and NADPH oxidase activity in Rab5-bound early endosomes (7, 8, 11, 12). Both the Rab5-GEF and Rac1-effector functions of alsin appear to influence endocytic mechanisms and endosomal dynamics (20, 26) and alsin appears to protect from motor neuron degeneration in certain *SOD1* mutant mice (20) and motor neurons expressing SOD1 mutants in culture (27, 28). Given the association of Nox1, Nox2, and Nox4 with disease progression in ALS mice (5, 6) and humans (18), these findings suggest the intriguing hypothesis that alsin and SOD1 both influence the dynamics of Rac1-dependent, NADPH oxidase-mediated, ROS production by redoxosomes that may impact proinflammatory signaling in ALS. In support of this hypothesis, alsin has been shown to bind three components of the redoxosome (Rac1, Rab5, and SOD1).

To test this hypothesis, we investigated whether alsin expression influences SOD1^{G93A}-mediated ROS production by glial cells. Three NADPH oxidases were evaluated as sources of cellular ROS (Nox1, -2, and -4) using shRNA knockdown, based on their association with disease severity in ALS models. Findings from our studies demonstrated that wild type alsin attenuates SOD1^{G93A}-mediated Rac1 activation, ROS production by Nox2, NFκB activation, and TNFα secretion by glial cells and protects neurons from toxicity in co-culture studies. SOD1^{G93A} expression enhanced TNFα-dependent redoxosomal ROS production by Nox2 and this was attenuated by alsin expression. Taken together, our results suggest a potential role for alsin in regulating redox-dependent proinflammatory signals via redoxosomes that are enhanced by SOD1^{G93A}.

EXPERIMENTAL PROCEDURES

Recombinant Expression Vectors and Small Hairpin RNA (shRNA)—Glial cells (MO59J, obtained from ATCC) or neuronal cells (NSC-34, a kind gift from Dr. Neil Cashman, University of British Columbia) were infected with recombinant adenoviruses (1000 particles per cell) as previously described (29). MO59J and NSC-34 were cultured in DMEM with 10% FBS. The following E1-deleted recombinant adenoviral vectors were used in the experiments: Ad.SOD1^{G93A}, which encodes the SOD1^{G93A} mutant (7); Ad.Alsin, which encodes wild type full-length alsin (25) (A kind gift from Dr. Horazdovsky, Mayo Clinic); Ad.NFκBLuc, which encodes an NFκB-responsive promoter driving firefly luciferase (30); Ad.shNox1 (31), Ad.shNox2 (32), and Ad.shNox4 (32, 33) encoded shRNAs against mouse Nox1, Nox2, and Nox4, respectively. In addition, Ad.Empty, an empty vector with no transgene insert, and Ad.LacZ (which encodes a β-galactosidase expression cassette) were used as controls for viral infection (29). For NFκB transcriptional assays using infection with two recombinant adenoviruses, a slightly modified sequential infection method was used (30). In this case, cells were infected with experimental vectors (*e.g.* Ad.SOD1^{G93A}, Ad.Alsin, Ad.shNox, etc.) 12 h before infection with Ad.NFκB, and were then used for assays at 36 h after initial infection.

NFκB and NADPH Oxidase Activity Assays—NFκB transcriptional activity was assessed using the previously described NFκB-inducible luciferase reporter vector (Ad.NFκBLuc) (30). Luciferase activity was assessed with 2 μg of cell lysate. NADPH-dependent superoxide production (*i.e.* NADPH oxidase activity) was analyzed by a lucigenin-based chemiluminescent assay. Prior to the initiation of the assay, 1 μg of endomembrane protein (100,000 pellet, see below) was combined with 5 μM lucigenin (Sigma) in PBS and incubated in the dark at room temperature for 10 min. The reaction was initiated by the addition of 100 μM NADPH (Sigma), and changes in luminescence were measured over the course of 3 min. The slope of the luminescence curve (relative light units per minute) was used to calculate the rate of ROS formation as an index of NADPH oxidase activity (9, 11). In the absence of NADPH, background levels of lucigenin-dependent luminescence were always >1,000-fold less than maximally induced values in the presence of NADPH. Additionally, background levels of luminescence in the absence of NADPH did not significantly vary between samples.

Cellular Endomembrane Collection and Western Blotting—Cells were scraped into ice-cold PBS and centrifuged. Cell pellets were resuspended in 0.5 ml of homogenization buffer (0.25 M sucrose, 10 mM triethanolamine, 1 mM phenylmethylsulfonyl fluoride, and 100 μg/ml of aprotinin). Samples were lysed by nitrogen cavitation (650 p.s.i. for 5 min) and then centrifuged at 3,000 × *g* at 4 °C for 20 min. The supernatant was designated post-nuclear supernatant. The post-nuclear supernatant was subsequently centrifuged at 100,000 × *g* for 2 h at 4 °C to pellet the endomembranes (100,000 pellet), which were then resuspended in 100 μl of homogenization buffer and used immediately for ROS production assays or frozen for later analysis. Western blotting

was performed using standard protocols and protein concentrations were determined using the Bio-Rad protein quantification kit. Immunoreactive proteins were detected using an Odyssey Infrared Imaging System (LI-COR Biotech). Anti-alsin antibody was purchased from Sigma.

Redoxosome Detection—Intra-endosomal ROS production was detected using OxyBURST Green dihydro-2′,4,5,6,7,7′-hexafluorofluorescein (H_2HFF)-BSA (Invitrogen). Stock solutions (1 mg/ml) were generated immediately prior to use by dissolving H_2HFF-BSA in PBS under nitrogen and protected from light. Cells were preincubated in the presence of 50 μg/ml of OxyBurst Green H_2HFF-BSA and TNFα (0.1 ng/ml) for 20 min at 37 °C and then fixed in 4% paraformaldehyde. Cells were then immediately imaged using a Leica spinning-disc fluorescence microscope. For samples infected with adenoviral vectors, the infections were done 36 h prior to assays. When SOD/catalase proteins were added to the medium, this was done at a protein concentration of 0.1 mg/ml at the time of TNFα treatment.

Detection of Cellular ROS Using DHE—Eight hours prior to addition of DHE (dihydroethidium), cell cultures were switched to a serum-deprived (0.2% serum) medium. Cell samples were stained with DHE (1 μM) for 30 min in the dark at 37 °C (34). Cells were then rinsed with PBS and evaluated using a spinning-disc fluorescence microscope. For flow cytometry, cells were harvested by trypsinization and filtered through a 70-μm nylon cell strainer (Falcon) and then assessed by FACScan flow cytometer (BD Biosciences).

Collection of Conditioned Glial Cell Medium—Glial cells cultured in DMEM, 10% FBS were infected with Ad.G93A, Ad.Empty, or Ad.shNox vectors as indicated. At 8 h postinfection, virus-containing media were discarded, glial cells were washed with fresh media twice and subsequently cultured in fresh media. At 48 h postinfection, culture media were collected as conditioned glial cell medium and immediately applied to naive neuronal cell or glial cell cultures.

Cell Labeling with DiO or DiI—Glial or neuronal cells were incubated with Vybrant DiO or Vybrant DiI (Invitrogen) for 30 min at 37 °C at a final concentration of 1 μM (35). To gain a universal cell labeling, this labeling step was performed every other day for a total of 3 times before adenoviral infections and combining the two separately labeled cell types into one co-culture. Co-cultures were initiated by mixing 1.0×10^6 DiI-labeled glial cells and 0.5×10^6 DiO-labeled neurons. At 12 h and 5 days after initiating the co-cultures, cells were harvested by trypsinization, filtered through a 70-μm nylon cell strainer (Falcon), and the cell numbers were assessed with a BD Biosciences LSR II Violet Flow Cytometer with 405-, 488-, 561-, and 639-nm lasers.

Cytokine Measurements and Rac1 Activity Assays—The concentrations of cytokines IL-6 and TNFα in the culture medium were determined with the mouse IL-6 ELISA kit or the mouse TNFα ELISA kit (BD Biosciences), respectively. Rac1 activity assays were performed using a Rac1 G-LISA^{TM} Activation Assay Kit (Cytoskeleton) following the manufacturer's protocol.

RESULTS

Alsin Modulates ROS Production by SOD1^{G93A} Expressing Glial Cells—We and others have previously demonstrated that expression of SOD1^{G93A} enhances ROS production in spinal cords of ALS mice (5–7). The source of this ROS production by SOD1^{G93A} or SOD1^{G37R} expressing glial cells has been predominantly thought to be Nox2 (5–7, 36). However, genome-wide association studies in sporadic ALS patients have also linked Nox4 to the progression of disease (18). To this end, we were interested in systematically evaluating whether Nox1, Nox2, and/or Nox4 contribute to excessive ROS production by SOD1^{G93A} expressing glial cells and whether alsin (another gene known to cause juvenile ALS) also regulates ROS through any of these Nox isoforms. To approach this question, we used recombinant adenoviruses capable of overexpressing SOD1^{G93A} and alsin, or inhibiting Nox1, -2, and -4 through expressed shRNAs. As anticipated, expression of SOD1^{G93A} in a glial cell line (MO59J) led to a significant increase in ROS production as detected by DHE fluorescence and FACS analysis (Fig. 1A). SOD1^{G93A}-induced ROS production was inhibited by the addition of two Nox inhibitors, diphenylene iodonium and apocynin (Fig. 1*B*). Analysis of NADPH-dependent ROS production by the endomembrane fraction, using the chemiluminescence lucigenin probe, substantiated these findings demonstrating that SOD1^{G93A} expression induced ROS (likely superoxide based on the specificity of lucigenin) production that was inhibited by treatment of cells with diphenylene iodonium and apocynin (Fig. 1*C*).

We next sought to evaluate whether Nox1, -2, and/or -4 was responsible for the enhanced ROS production in SOD1^{G93A}-expressing glial cells using expressed shRNAs. Analysis of Nox1, -2, and -4 mRNA levels in MO59J cells using quantitative PCR demonstrated that only Nox2 and Nox4 were expressed at detectable levels. Expression of Nox2 or Nox4 shRNAs demonstrated significant and specific knockdown of their respective cellular target mRNAs by ~50% (supplemental Fig. S1). Because endogenous Nox1 mRNA was undetectable, we chose to utilize Nox1-shRNA as a negative control in selected studies. The ability of these Nox-shRNAs to attenuate SOD1^{G93A}-induced ROS production by glial cells was then evaluated by co-infection with the respective adenoviral vectors. Results from these experiments demonstrated that only Nox2-shRNA significantly inhibited ROS production (Fig. 1*D*); no significant decrease in ROS was seen with either Nox1- or Nox4-shRNA, although there was a minor decrease noted in Nox4-shRNA expressing cells.

Deletions in the alsin gene (*ALS2*), a large 1651-amino acid protein, causes a juvenile form of ALS (23). It has been hypothesized that alsin could potentially regulate Nox activity like SOD1 (12), but no direct information on this exists to date. We therefore sought to investigate whether alsin expression influences Nox-dependent ROS production in glial cells. Indeed, expression of full-length wild type alsin in glial cells significantly induced ROS production as detected by DHE in whole cells and by lucigenin with endomembranes (Fig. 2, *A–D*). Surprisingly, coexpression of alsin with SOD1^{G93A} significantly reduced ROS production by glial cells from that induced by

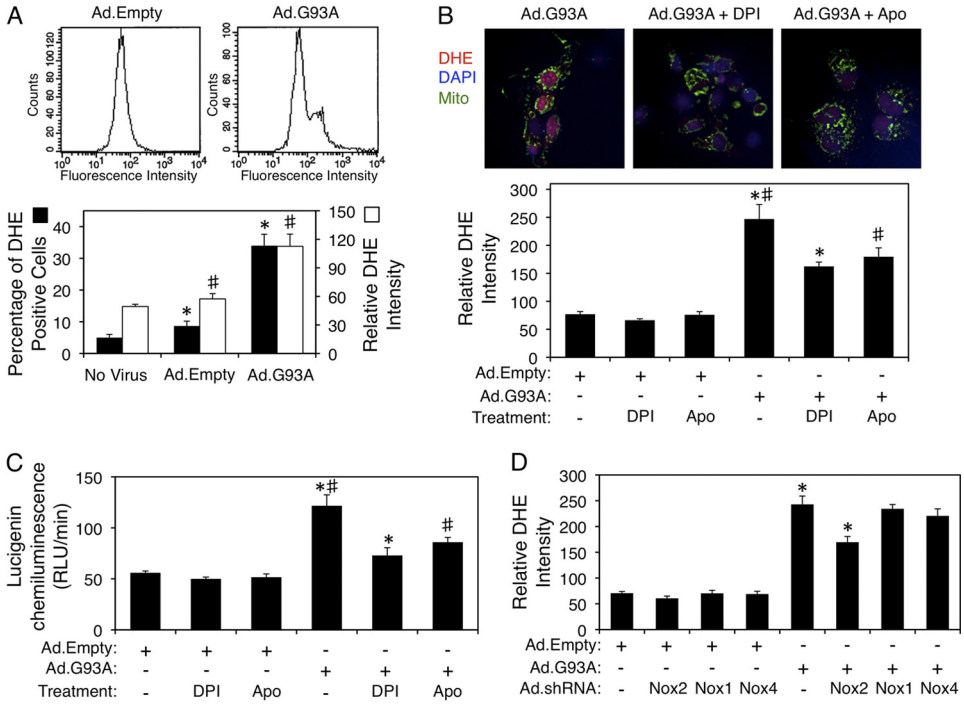

FIGURE 1. **NADPH oxidase inhibitors decrease SOD1^{G93A}-induced ROS generation in mouse glial cells.** *A*, mouse glial cells were infected with Ad.Empty or Ad.SOD1^{G93A} virus for 28 h prior to changing to serum-deprived (0.2% serum) medium for 8 h, and then incubated with DHE (1 μM) for 30 min at 37 °C. Cells were then assessed by FACScan flow cytometry. *Top panel*, two representative graphs for samples with Ad.Empty and Ad.SOD1^{G93A}; *bottom panel,* quantification of flow cytometry data as either the percentage of total cell population (*left y* axis) or relative DHE intensity (*right y* axis). *B*, mouse glial cells were infected with Ad.SOD1^{G93A} or Ad.Empty for 28 h prior to changing to serum-deprived (0.2% serum) medium for 8 h. Diphenylene iodonium (*DPI*, 10 μM) or apocynin (*Apo*, 100 μM) were added 60 min before washing with PBS. Cells were then incubated with DHE (1 μM) for 30 min at 37 °C before fluorescence microscopy (*top panel*) or FACScan flow cytometry (*bottom panel*). In the fluorescent images, the mitochondria are stained *green* and the nucleus is stained *blue*. *C*, mouse glial cells were infected with Ad.G93A or Ad.Empty for 36 h prior to cell harvesting. A 1-μg portion of each 100,000 endomembrane pellet was used to evaluate NADPH-dependent production of O_2^- by lucigenin-based chemiluminescence. *D*, mouse glial cells were infected with the indicated adenoviral vectors for 28 h prior to changing to serum-deprived (0.2% serum) medium for 8 h. Cells were then incubated with DHE (1 μM) for 30 min at 37 °C before FACScan flow cytometry. Data in all graphs represent the mean ± S.E. with $n = 3$; marked comparisons (* or #) demonstrate significant differences as determined by one-way analysis of variance followed by Student's *t* test ($p < 0.05$).

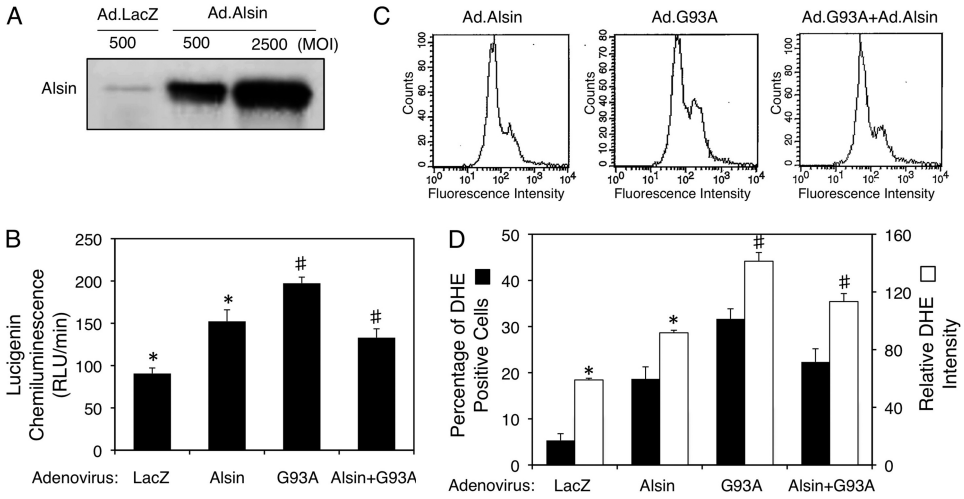

FIGURE 2. **Alsin decreases ROS generation induced by SOD1^{G93A}.** *A*, mouse glial cells were infected with Ad.LacZ or Ad.Alsin virus at the indicated multiplicity of infections for 36 h. Cell lysates were then evaluated by Western blotting with anti-Alsin antibody. *B*, mouse glial cells were infected with the indicated vectors for 36 h. A 1-μg portion of endomembranes from each sample was used to evaluate NADPH-dependent production of O_2^- by lucigenin-based chemiluminescence. *C*, mouse glial cells were infected with the indicated vectors for 28 h prior to changing to serum-deprived (0.2% serum) medium for 8 h, and then incubated with DHE (1 μM) for 30 min at 37 °C. Cells were then assessed by FACScan. *D*, mouse glial cells were infected and treated as in *C* and quantified as either the percentage of total cell population (*left y* axis) or relative DHE intensity (*right y* axis). Data represent the mean ± S.E. with $n = 3$; marked comparisons (* or #) demonstrate significant differences as determined by one-way analysis of variance followed by Student's *t* test of the comparisons ($p < 0.05$).

SOD1^{G93A} expression alone (Fig. 2). These results demonstrate for the first time that alsin has the ability to modulate glial cell ROS production in a fashion that is altered by the presence of SOD1^{G93A}; alsin increases ROS production in otherwise healthy glial cells, but decreases ROS production in the presence of SOD1^{G93A}.

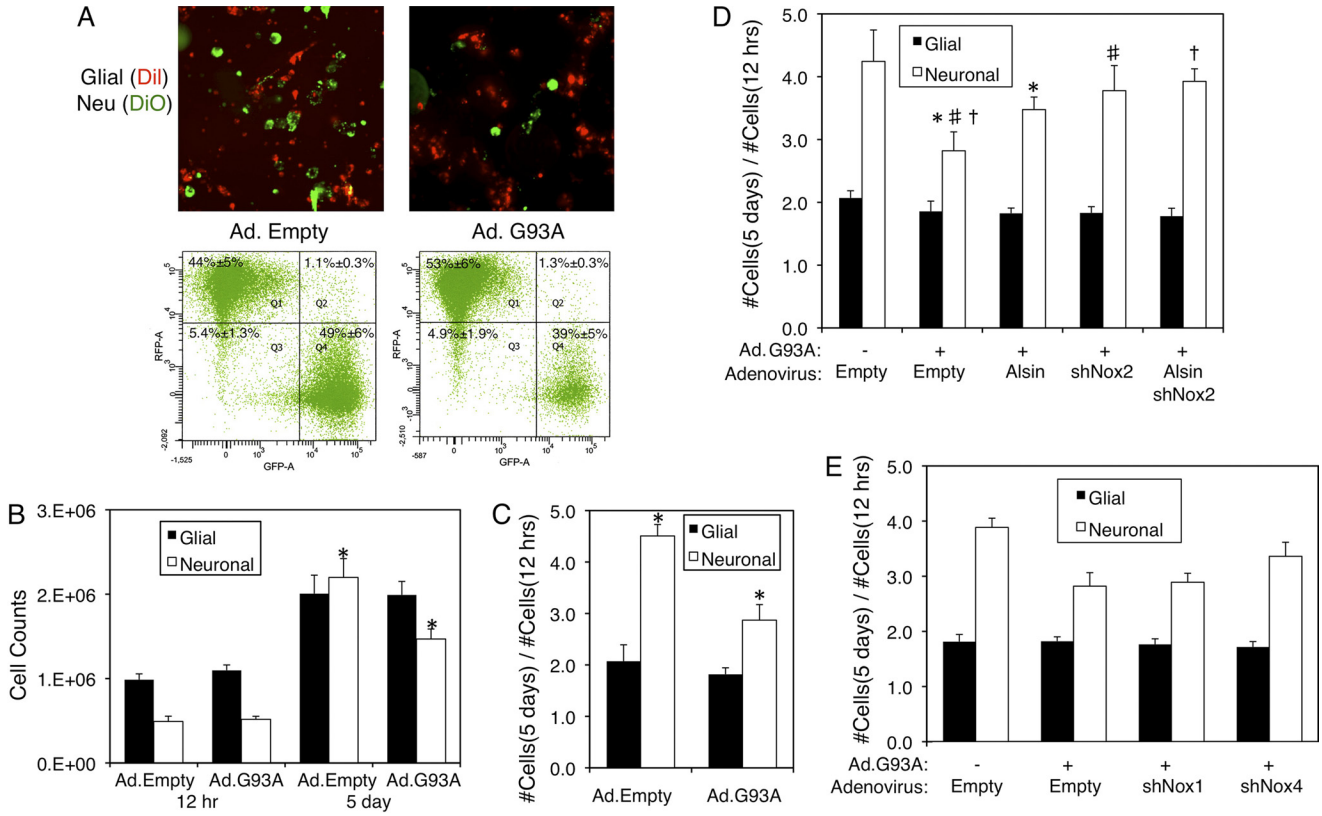

FIGURE 3. **Alsin or Nox-shRNA expression in SOD1^{G93A}-expressing glial cells decreases neurotoxicity in co-culture.** *A*, mouse glial cells and neuronal cells were labeled with DiI or DiO, respectively. Glial cells also received infections with Ad.Empty or Ad.G93A 36 h prior to their co-culture with neuronal cells. After 5 days, the co-cultures were evaluated by fluorescence microscopy (*top panel*) or flow cytometry using a BD Biosciences LSR II instrument (*bottom panel*). *B*, glial and neuronal cell numbers as determined by flow cytometry. *C*, ratio of 5-day cell number/12-h cell number to reflect cell growth. *D* and *E*, mouse glial cells and neuronal cells were labeled with DiO or DiI, respectively. Glial cells received the indicated infections 36 h prior to the initiation of co-culture with neuronal cells. The cells were co-cultured for 5 days, and then assessed by flow cytometry using a BD Biosciences LSR II instrument. Raw cell numbers for the calculations shown in *panels D* and *E* can be found in supplemental Fig. S3. Data in all graphs represent the mean ± S.E. with $n = 3$; marked comparisons (* or #) demonstrate significant differences from one-way analysis of variance followed by Student's *t* test of the comparisons ($p < 0.05$).

Alsin Coexpression in SOD1^{G93A}-expressing Glial Cells Protects Neurons from Cell Toxicity in Co-culture—Motor neuron cell death is diagnostic for ALS pathology. Previous studies have demonstrated that glia expressing SOD1 ALS mutants secrete Nox2-dependent factors that kill motor neurons (36, 37). To investigate the relevance of SOD1^{G93A}-induced glial ROS production to the mechanism of neuronal cell death in ALS, we developed a co-culture method using MO59J glial cells and the NSC-34 motor neuron-like cell (a hybrid cell line produced by fusion of motor neuron-enriched, embryonic mouse spinal cord cells with mouse neuroblastoma (38–40)). To differentiate glial cells from neuronal cells in co-culture, two fluorescent lipophilic dyes, DiO (green) and DiI (orange), were chosen to mark membranes of each cell type. Each dye is highly fluorescent and photostable when incorporated into cell membranes. Following selective labeling of MO59J cells (with DiI) and NSC-34 cells (with DiO) for a week in culture, nearly all cells were labeled. No evidence for toxicity was noted from each of these dyes after prolonged culture (2 weeks). Co-culture for 1 week after labeling led to no detectable inter-cell translocation of the dyes (supplemental Fig. S2).

We next sought to investigate the influence of SOD1^{G93A}-expressing MO59J glial cells (labeled with DiI) on co-cultured NSC-34 motor neuron-like cells (labeled with DiO). Glial cells were infected with Ad.SOD1^{G93A} or Ad.Empty viruses 36 h prior to co-culture. Five days after the initiation of co-culture, cells were assessed by fluorescence microscopy (Fig. 3*A*, *top panel*) or by flow cytometry (Fig. 3, *A, bottom panel*, and *B* and *C*) to count the neuronal (*green*) and glial (*red*) cells. From 12 h to 5 days of co-culture, both glial and neuronal cells proliferated in control conditions (*i.e.* Ad.Empty-infected glial cells) leading to a 2.1- and 4.5-fold amplification of these cell types, respectively, over the 5-day period (Fig. 3, *B* and *C*). By contrast, co-culture of Ad.SOD1^{G93A}-infected glial cells with neuronal cells led to a significant reduction in the number of neuronal cells in the co-culture after 5 days, without a significant change in the number of glial cells (Fig. 3, *B* and *C*); neuronal cells cultured with Ad.SOD1^{G93A}-infected glial cells for 5 days expanded 54% less than the neuronal cells cultured with control Ad.Empty-infected glial cells. These results demonstrate that SOD1^{G93A}-expressing glial cells impair growth of neurons and/or lead to neuronal cell death. Although these results do not specifically distinguish between impaired neuronal growth and neuronal death, they do clearly demonstrate neurotoxicity caused by SOD1^{G93A}-expressing glial cells. Such findings are similar to previous studies using human ES cells (hESC)-derived glial cells/astrocytes expressing SOD^{G37R} and SOD1^{G93A} in co-culture with hESC-derived motor neurons (36, 37).

Having established our glial/neuronal cell co-culture system, we examined whether expression of alsin and/or Nox shRNAs

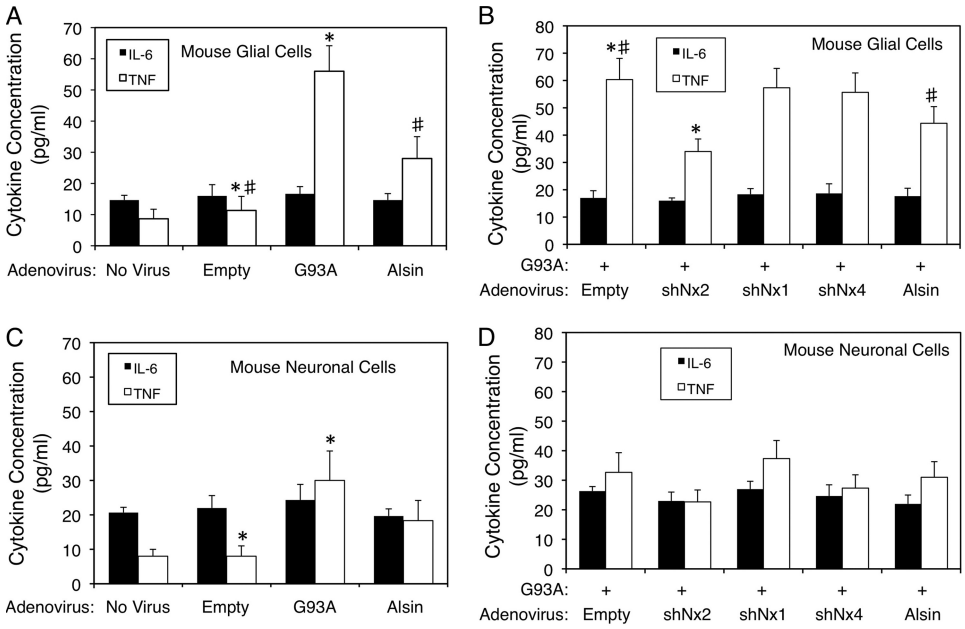

FIGURE 4. **Alsin and Nox2 regulate production of TNFα by SOD1^{G93A}-expressing glial cells.** *A* and *B*, mouse glial cells were infected with the indicated vectors for 48 h prior to medium collection. The concentrations of cytokines IL-6 and TNFα in the culture media were determined as described under "Experimental Procedures." *C* and *D*, mouse neuronal cells were infected with the indicated vectors for 48 h prior to medium collection. The concentrations of cytokines IL-6 and TNFα were determined as above. Data represent the mean ± S.E. with $n = 3$; marked comparisons (* or #) demonstrate significant differences as determined by one-way analysis of variance followed by Student's *t* test of the comparisons ($p < 0.05$).

in SOD1^{G93A}-expressing glial cells could influence the level of neurotoxicity observed in co-culture. To this end, labeled MO59J glial cells were infected with Ad.SOD1^{G93A} together with either Ad.Alsin or Ad.shNox2 and these cells were cultured with labeled NSC-34 motor neuron-like cells. Flow cytometry demonstrated that coexpression of alsin with SOD1^{G93A} in glial cells significantly increased the total number of neuronal cells in co-culture as compared with glial cells expressing SOD1^{G93A} alone (Fig. 3*D* and supplemental Fig. S3*A*). Similarly, coexpression of shNox2 with SOD1^{G93A} in glial cells also led to a significant increase in neuronal cell number in co-culture (Fig. 3*D* and supplemental Fig. S3*A*). Interestingly, expression of alsin and shNox2 together with SOD1^{G93A} in glial cells did not have a synergistic effect on neurotoxicity (Fig. 3*D* and supplemental Fig. S3*A*), suggesting that alsin and Nox2 act within the same pathway to inhibit neurotoxicity induced by SOD1^{G93A}-expressing glial cells. As a control for shRNAs that did not significantly change ROS production in SOD1^{G93A}-expressing glial cells, we also tested whether coexpression of Nox1-shRNA or Nox4-shRNA together with SOD1^{G93A} in glial cells would influence neurotoxicity in co-culture (Fig. 3*E* and supplemental Fig. S3*B*); as expected, no significant changes in neuronal cell survival/growth rate were seen in these studies. In comparison to earlier studies, these findings demonstrate a correlation between the level of ROS production by SOD1^{G93A}-expressing glial cells (with and without alsin or Nox2shRNA) and the extent of neurotoxicity in co-culture. Additionally, neurotoxicity induced by SOD1^{G93A}-expressing glial cells was inhibited by treatment of cell co-cultures with the Nox2 inhibitor apocynin (supplemental Fig. S4). These findings provide further evidence for the Nox2 dependence of neurotoxicity.

Enhanced Production of TNFα, but Not IL-6, by SOD1^{G93A}-expressing Glial Cells Is Influenced by Nox2 and Alsin—Enhanced production of proinflammatory factors in the CNS, such as TNFα and IL-6, is thought to be centrally important to inflammation in a number of neurodegenerative diseases including ALS (4). For example, increased TNFα in the serum and CSF of ALS patients and mouse models has been correlated with the severity of disease (41–43). Similarly, enhanced IL-6 production in the serum and CSF has also been noted in ALS patients (44–46), but others have also noted no differences (47, 48). We hypothesized that enhanced production of these proinflammatory cytokines by SOD1^{G93A}-expressing glial cells might be linked to ROS production and the mechanism of neuronal cell death in co-culture. To this end, we examined the effects of SOD1^{G93A}, alsin, and/or Nox-shRNA expression in glial and neuronal cells on the ability of these cell types to produce TNFα and IL-6 in the culture medium.

Results from these experiments demonstrated that secretion of TNFα was significantly increased in both glial (4.9-fold) and neuronal (3.8-fold) cells expressing SOD1^{G93A} (Fig. 4, *A* and *C*). Interestingly, alsin expression alone also induced a modest and significant level of TNFα production from glial cells (2.5-fold, Fig. 4*A*), however, the low level induction in neuronal cells (2.3-fold) did not reach significance (Fig. 4*C*). No differences in IL-6 production were noted in either glial or neuronal cells under these conditions (Fig. 4, *A* and *C*). We further examined whether alsin or Nox-shRNA expression could influence SOD1^{G93A}-dependent TNFα secretion. TNFα production by SOD1^{G93A} glial cells was significantly reduced by the expression of alsin or shNox2, but not shNox1 or shNox4 (Fig. 4*B*). None of these conditions altered IL-6 production by SOD1^{G93A}-expressing glial cells. These observed reductions in

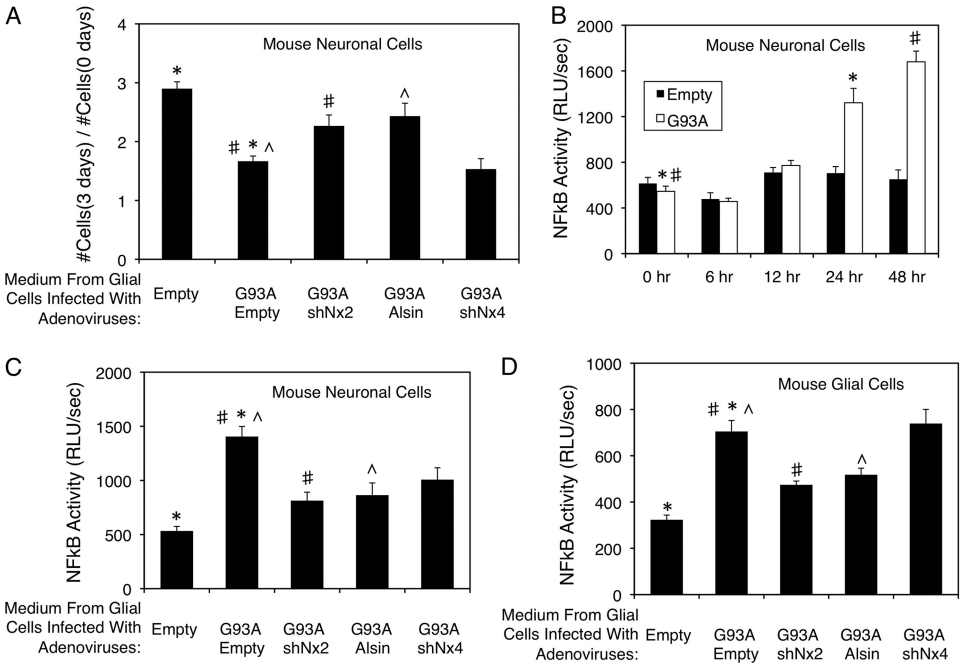

FIGURE 5. **Conditioned medium collected from SOD1^{G93A}-expressing glial cells is neurotoxic and causes activation of NFκB in neuronal and glial cells.** *A*, mouse glial cells were infected with the indicated vectors. At 8 h post-infection, virus containing media were washed off and switched to fresh media. At 48 h postinfection, glial cell-conditioned media were collected and exposed to neuronal cell cultures. The change in the number of neuronal cells after 3 days of exposure was then quantified. *B*, mouse glial cells were infected with either Ad.Empty or Ad.SOD1^{G93A} virus and conditioned media were generated as in *A*. These glial cell-conditioned media were then placed onto neuronal cells preinfected with Ad.NFκBLuc. At each indicated time point post-glial cell medium exposure, neuronal cells were harvested for luciferase assay to determine NFκB activation. *C*, mouse glial cells were infected with the indicated vectors and conditioned media were generated as in *A*. These glial cell-conditioned media were then placed onto neuronal cells preinfected with Ad.NFκBLuc. After 24 h of exposure to glial cell-conditioned media, neuronal cells were harvested for luciferase assay. *D*, glial cell medium collected as in *A* were exposed to naive glial cells preinfected with Ad.NFκBLuc. After 24 h of exposure to glial cell-conditioned media, glial cell cultures were harvested for luciferase assay. Data in all graphs represents the mean ± S.E. with $n = 3$; marked comparisons (*, #, and ∧) demonstrate significant differences using one-way analysis of variance followed by Student's *t* test of the comparisons ($p < 0.05$).

TNFα secretion by SOD1^{G93A} glial cells, facilitated by alsin or shNox2 expression, correlated with protection of neuronal cells in co-culture (Fig. 3*D*). Furthermore, neither alsin nor any of the Nox-shRNAs altered TNFα or IL-6 production in SOD1^{G93A}-expressing neuronal cells (Fig. 4*D*). Taken together, these studies demonstrate that both Nox2 and alsin can influence TNFα production by SOD1^{G93A}-expressing glial cells. It therefore appears probable that TNFα plays an important role in neurotoxicity resulting from co-culture with SOD1^{G93A}-expressing glial cells.

SOD1^{G93A}-expressing Glial Cells Secrete Factors That Activate NFκB in Motor Neuron-like Cells—To better establish that secreted factors (such as TNFα) from SOD1^{G93A}-expressing glial cells influence survival of neuronal cells, we performed experiments with glial cell-conditioned media and evaluated both neuronal cell survival and activation of the proinflammatory transcription factor NFκB. Indeed exposure of NSC-34 neuronal cells to conditioned medium from SOD1^{G93A}-expressing MO59J glial cells led to a significant reduction in the number of neuronal cells in culture over a 3-day period, as compared with conditioned medium from control cells (Fig. 5*A*). These results confirmed that cell-cell contact between glial and neuronal cells is not necessary for neuronal cell toxicity associated with SOD1^{G93A}-expressing glial cells. Furthermore, we found that coexpression of alsin or Nox2-shRNA, but not Nox4-shRNA, in SOD1^{G93A}-expressing glial cells diminished neuronal cell toxicity of conditioned medium, leading to a significantly larger neuronal cell population after 3 days in culture (Fig. 5*A*). These results are consistent with our findings that alsin and shNox2 reduce the production of ROS (Figs. 1 and 2) and TNFα (Fig. 4) in glial cell cultures and suggest that these redox-mediated proinflammatory changes may be responsible for neuronal cell toxicity.

Although activation of NFκB has been linked to both survival and death signaling, in microglia and astrocytes NFκB activation is associated with enhanced ROS production and the proinflammatory state that leads to motor neuron cell death in ALS (4). Furthermore, NFκB activation by TNFα in motor neurons has been demonstrated to potentiate glutamate-induced spinal cord motor neuron death (49). Nox-dependent ROS-mediated signaling is also important for the activation of NFκB by TNFα and IL-1 (9, 10, 29), two factors thought to be important in the progression of ALS disease. Therefore, we next examined the status of NFκB transcriptional activity in both neuronal and glial cells exposed to conditioned medium harvested from SOD1^{G93A}-expressing glial cells and how modulation of alsin or Nox2 expression influenced this process. To this end, neuronal and glial cells were preinfected with a recombinant adenovirus containing an NFκB-luciferase reporter, followed by exposure to conditioned medium harvested from glial cells infected with adenoviruses expressing SOD1^{G93A}, Nox2-shRNA, Nox4-shRNA, and/or alsin. Results from these experiments demonstrated that conditioned medium from SOD1^{G93A}-expressing glial cells significantly induced NFκB transcriptional activity in neuronal cells by 24 h postexposure over that

seen with control glial cell-conditioned medium (Fig. 5*B*). Coexpression of alsin or Nox2-shRNA with SOD1^{G93A} in glial cells significantly reduced the ability of glial cell-conditioned medium to activate neuronal NFκB (Fig. 5*C*). Interestingly, conditioned medium from Nox4-shRNA expressing SOD1^{G93A}-glial cells attenuated NFκB activation following exposure to neuronal cells, but this did not reach statistical significance (Fig. 5*C* and data not shown). These findings demonstrate that glial cell secreted factors influence NFκB activation in neuronal cells in a paracrine fashion. More importantly, they also demonstrate that alsin and Nox2 expression influence several features of SOD1^{G93A}-expressing glial cells in a similar fashion including: 1) glial cell ROS production, 2) toxicity to neuronal cells, 3) TNFα secretion by glial cells, and 4) paracrine activation of NFκB in neuronal cells.

Excessive inflammation in ALS has been thought to involve amplification of a proinflammatory state exacerbated by excessive Nox-dependent ROS production (4, 12). This interplay likely involves dysregulated production of inflammatory cytokines and neurotrophic factors that act in both a paracrine and autocrine fashion between activated microglia, activated astrocytes, and motor neurons. Our findings in Fig. 5, *A–C*, demonstrate the importance of alsin and Nox2 in regulating paracrine factors from SOD1^{G93A}-expressing glial cells and their effects on neuronal cell health. We sought to evaluate whether factors secreted by SOD1^{G93A}-expressing glial cells might also act in an autocrine fashion to activate other glial cells. To this end, we harvested conditioned media from glial cells expressing SOD1^{G93A}, Nox2-shRNA, Nox4-shRNA, and/or alsin, and exposed this media to naive glial cells expressing the NFκB-luciferase reporter. Results from these experiments demonstrated a striking similarity to that observed in neuronal cell experiments (Fig. 5*D*). Conditioned medium from SOD1^{G93A}-expressing glial cells induced NFκB activation in naive glial cells and this was attenuated by alsin or Nox2-shRNA, but not Nox4-shRNA expression. These findings clearly demonstrate that factors secreted by SOD1^{G93A}-expressing glial cells induced NFκB activation in an autocrine fashion and that this pathway is modulated by alsin and Nox2.

SOD1^{G93A} Expression in Glial Cells Enhances Redoxosomal ROS Production Induced by TNFα—Our studies demonstrate that SOD1^{G93A} expression enhances ROS and TNFα production by glial cells. TNFα is known to induce Nox2-dependent ROS production in redoxosomes that harbor the ligand-bound TNFα receptor (TNFR1) (8, 10). In this context, ROS production by the redoxosome signals redox-dependent activation of TNFR1-TRAF2 complex formation required for endosomal IKK-dependent NFκB activation (10). SOD1^{G93A} has also been demonstrated to enhance endosomal ROS production by inhibiting GTP hydrolysis by Rac1 (an activator of Nox2) (7, 12). Therefore, we hypothesized that enhanced redoxosomal ROS production in SOD1^{G93A}-expressing glial cells might amplify responsiveness to proinflammatory cytokines such as TNFα. To investigate this hypothesis, we used a previously described method for detecting superoxide accumulation within intracellular vesicular compartments (11, 50). This approach involved loading endosomes with the membrane-impermeable fluorochrome H_2HFF (Oxyburst green) conjugated to BSA followed by fluorescent microscopy to detect endosomal ROS production.

Our initial studies, which evaluated changes in H_2HFF-detectable endosomal ROS in SOD1^{G93A}-expressing and control glial cells, revealed no significant difference in endosomal ROS production (data not shown). Reasoning that detection limits for this technique likely require synchronization of receptor activation in redoxosomes, we altered our approach to compare the responsiveness of redoxosomes to low levels of TNFα (0.1 ng/ml) stimulation. To this end, we infected glial cells with Ad.SOD1^{G93A} or Ad.Empty virus and then treated these cells with TNFα and H_2HFF-BSA prior to evaluating endosomal ROS at 20 min post-stimulation. This alternative approach demonstrated that indeed SOD1^{G93A} expression in glial cells significantly elevated both the number (5-fold) of TNFα-induced redoxosomes and their fluorescent intensity of ROS production (1.8-fold), as compared with control TNFα-stimulated cells (Fig. 6, *A*, *B*, and *E*). These findings are consistent with previous studies that localized SOD1 on the surface of redoxosomes as a Rac1-bound redox sensor for Nox2-mediated ROS production (7, 8). For example, ALS mutants of SOD1 bind more tightly to Rac1-GTP than wild type SOD1, and inhibit the redox-dependent hydrolysis of GTP-bound Rac1 on the surface of redoxosomes leading to enhanced Nox2-dependent ROS production (7). Additionally, wild type SOD1 recruits to the surface of TNFα-activated redoxosomes containing TNFR1 to facilitate Rac1/Nox2-dependent ROS production and the redox-dependent recruitment of TRAF2 to the receptor complex, which is required for NFκB activation (10). Thus, these new findings (Fig. 6, *A*, *B*, and *E*) demonstrate for the first time that ALS mutant SOD1^{G93A} functionally enhances redoxosomal ROS production in the context of a proinflammatory signaling pathway important in ALS (*i.e.* TNFα). Such findings help to explain why SOD1^{G93A} expression in glial cells enhances Nox2-dependent proinflammatory signals that lead to NFκB activation (Fig. 5*D*).

SOD1 recruits to the surface of endosomes following stimulation with several proinflammatory cytokines (8, 10, 11, 50) where it binds to Rac1 and regulates Nox-dependent activation in the redoxosome. Rac1-GTP is essential for Nox2 activation and SOD1 binding to Rac1-GTP decreases GTP hydrolysis in a redox-dependent fashion (7). In this manner, SOD1 binding to Rac1-GTP enhances Nox2-dependent ROS production in redoxosomes under reducing conditions, whereas under oxidizing conditions SOD1 uncoupling from Rac1-GTP leads to enhanced GTP hydrolysis and inactivation of the Nox2 complex (7, 8). In this manner, SOD1 acts as a sensor for Nox-dependent redoxosomal ROS production (7, 8). The finding that FALS mutants of SOD1 demonstrate diminished redox-dependent uncoupling from Rac1-GTP has been one mechanism proposed for enhanced proinflammatory ROS production in FALS (7, 12), a finding supported by our present studies. The mechanism by which alsin attenuates Nox2-dependent ROS production in SOD1^{G93A}-expressing glial cells (Fig. 2) remains unclear. Based on the previous observations that alsin is also an effector that binds Rac1 (12), we hypothesized that alsin may directly modulate proinflammatory Rac1-dependent ROS production

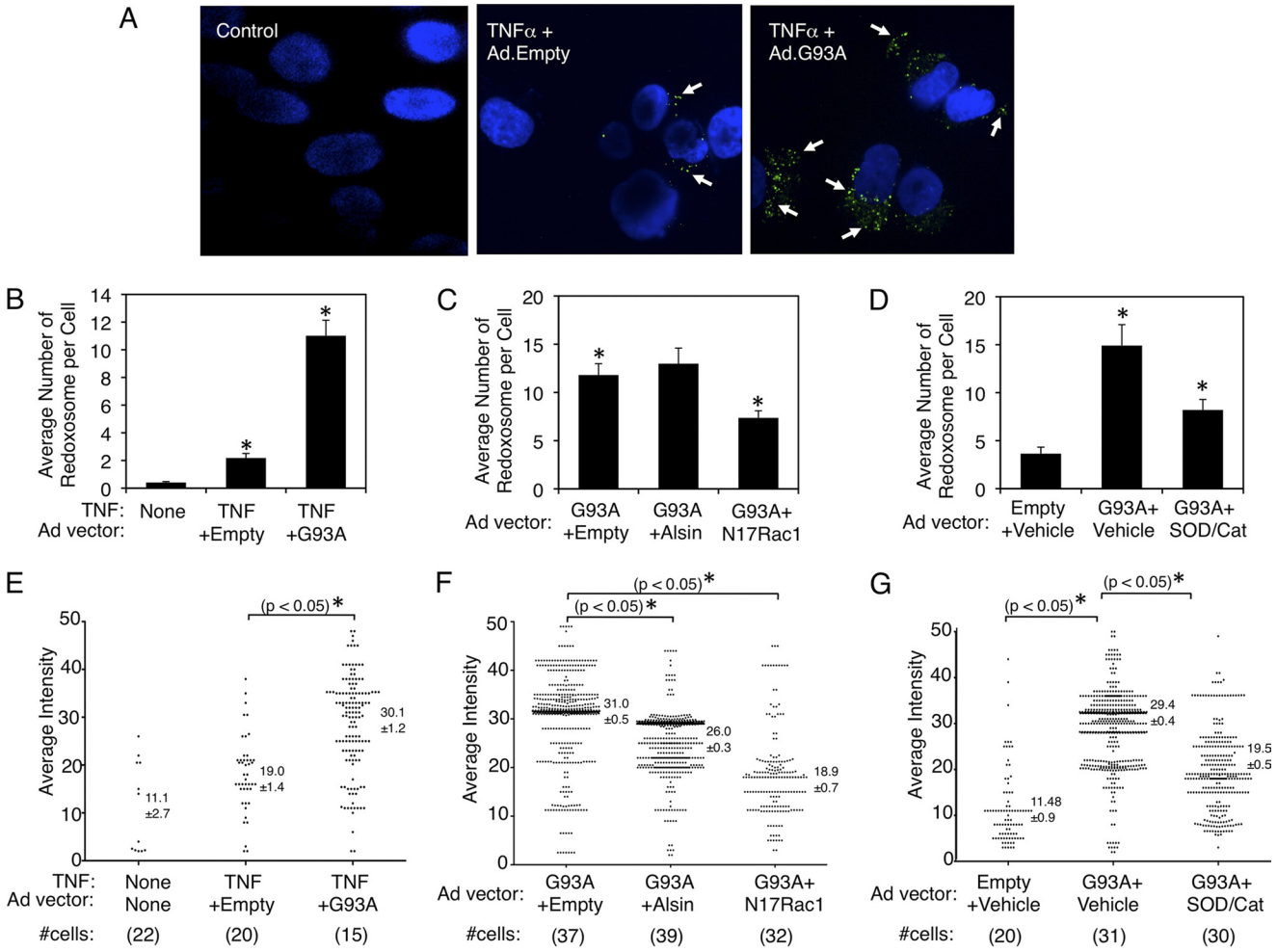

FIGURE 6. **SOD1^{G93A} expression in glial cells induces redoxosomal ROS production.** *A*, glial cells were infected with the indicated adenoviral vectors 36 h prior to TNFα (0.1 ng/ml) treatment in the presence of H_2HFF-BSA (50 μg/ml). Cells were then washed and fixed at 20 min post-treatment and mounted in DAPI containing anti-fadent prior to fluorescence microscopy. The *green* fluorescence (*white arrows*) represents oxidized H_2HFF resulting from ROS production in the endosomal compartment, and DAPI *blue* fluorescence marks nuclei. *B–G*, glial cells were infected with the indicated adenoviral vectors (marked *below* each graph) at 36 h prior to TNFα (0.1 ng/ml) treatment in the presence of H_2HFF-BSA (50 μg/ml). All conditions in *panels C*, *D*, *F*, and *G* were exposed to TNFα. In *panel D* and *G*, cells were also simultaneously treated with 0.1 mg/ml of SOD and 0.1 mg/ml of catalase proteins at the time of TNFα treatment. Cells were then washed and fixed at 20 min post-TNFα treatment and mounted in DAPI-containing anti-fadent prior to fluorescence microscopy. *Panels B–D* depict the average number of redoxosomes per cell. *Panels E–G* depict the average fluorescence intensity for data in *B–D* (each *dot* represents the average fluorescence intensity of an individual redoxosome). Fluorescence intensities were quantified by Metamorph software. *Numerical values* within all graphs represent the mean ± S.E., with the number of cells analyzed (*n*) for each condition marked in *parentheses* at the *bottom* of *panels E–G*. Marked comparisons (*) demonstrate significant differences ($p < 0.05$) as analyzed by one-way analysis of variance followed by Bonferroni's post-test.

by the redoxosome. To test this hypothesis we examined ROS production by TNFα-stimulated redoxosomes in glial cells expressing alsin and/or SOD1^{G93A}. Interestingly, coexpression of alsin with SOD1^{G93A} in glial cells significantly attenuated the amount of endosomal ROS production following TNFα stimulation without altering the number of formed redoxosomes (Fig. 6, *C* and *F*). These findings suggest that alsin can modulate TNFα-induced redoxosomal ROS that is enhanced by SOD1^{G93A}. Furthermore, expression of a dominant-negative form of Rac1 (N17Rac1) had an even stronger effect, by decreasing both the number of redoxosomes and the intensity of redoxosomal ROS production following TNFα stimulation of SOD1^{G93A} glial cells (Fig. 6, *C* and *F*), consistent with the fact that Rac1 is an essential activator of Nox2. Importantly, when purified bacterial SOD and bovine catalase proteins (which degrade superoxide and H_2O_2, respectively) were added to the medium with TNFα and H_2HFF-BSA to degrade intra-redoxosomal ROS as previously described (10, 11), the intensity of endosomal H_2HFF-BSA fluorescence and number of detectable redoxosomes were significantly reduced (Fig. 6, *D* and *G*). These results demonstrated that indeed the fluorescent signal detected by H_2HFF-BSA was superoxide and/or H_2O_2 derived. These findings demonstrate for the first time that alsin can functionally regulate SOD1^{G93A}/Rac1-dependent ROS production by TNFα signaling redoxosomes. These findings also help to explain how alsin can decrease ROS production (Fig. 2) and NFκB activation (Fig. 5*D*) in SOD1^{G93A}-expressing glial cells.

Enhanced Redoxosomal ROS Production by SOD1^{G93A}-Expressing Glial Cells Is Proinflammatory—Our studies thus far have demonstrated that enhanced ROS production in SOD1^{G93A}-expressing glial cells correlates with enhanced TNFα secretion and NFκB activation. Furthermore, enhanced ROS production by SOD1^{G93A}-expressing glial cells appears to originate from enhanced redoxosomal activity. To definitively

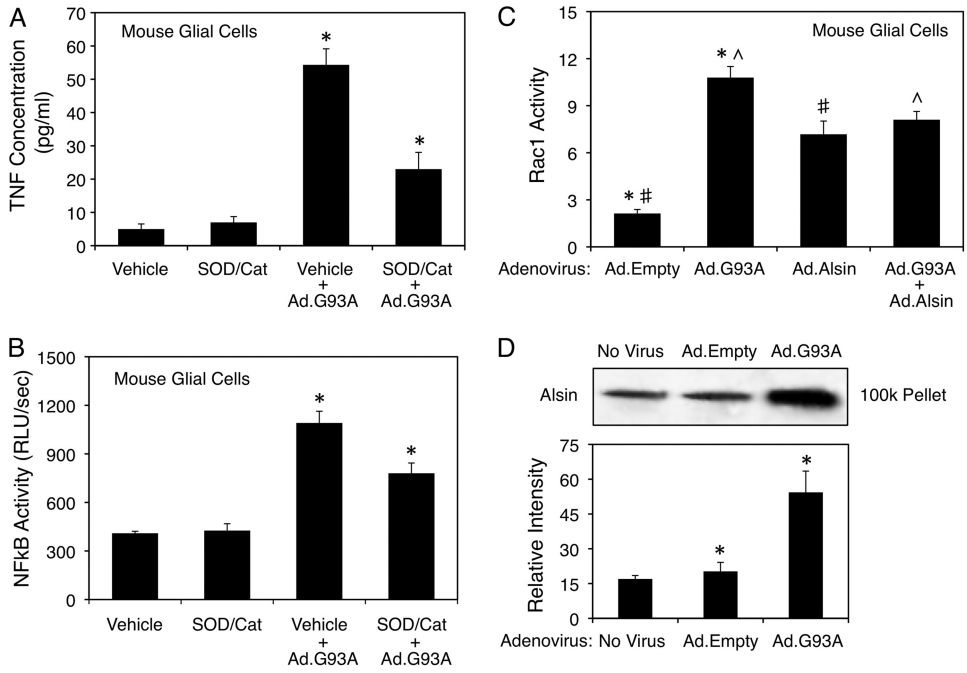

FIGURE 7. **Redoxosomal ROS induced by SOD1^{G93A} augments TNFα production and NFκB activation in glial cells, whereas alsin attenuates these end points by inhibiting SOD1^{G93A}-induced Rac1 activation.** *A* and *B*, mouse glial cells were infected with Ad.SOD1^{G93A} and treated with 0.1 mg/ml of SOD and 0.1 mg/ml of catalase proteins at 18 h post-infection. Cells were then infected with Ad.NFκBLuc at 24 h. At 48 h post-Ad.SOD1^{G93A} infection, culture medium and cells were harvested separately and concentrations of TNFα in the media were determined (*A*) and NFκB activity in the cells was assayed (*B*). *C*, mouse glial cells were infected with the indicated vectors for 36 h prior to collection of the cells for Rac1 activity assays. *D*, mouse glial cells were infected with Ad.Alsin plus the vectors indicated for 36 h prior to obtaining 100,000 $\times g$ endomembrane pellets. These samples were then used for Western blotting for alsin (*top panel*). The intensity of the alsin bands was quantified (*bottom panel*). Data represent the mean $\pm$ S.E. with $n = 3$; marked comparisons (*, #, or $\wedge$) demonstrate significant differences from one-way analysis of variance followed by Student's *t* test of the comparisons ($p < 0.05$).

demonstrate that increased redoxosomal ROS production in the presence of SOD1^{G93A} is indeed responsible for activation of NFκB and TNFα expression, we evaluated whether quenching of ROS within the endosomal compartment would attenuate activation of NFκB and secretion of TNFα in SOD1^{G93A}-expressing glial cells. To this end, SOD1^{G93A} glial cells were incubated with purified SOD/catalase proteins under conditions that significantly neutralized redoxosomal ROS (Fig. 6, *D* and *G*) and the level of TNFα secretion and NFκB activation was quantified. Results from these studies demonstrated that SOD/catalase endosomal loading significantly lowered TNFα levels in the medium 2.4-fold (Fig. 7*A*). Similarly, the rise in NFκB activation induced by SOD1^{G93A} expression was diminished by ~50% in the presence of SOD/catalase in the medium (Fig. 7*B*). These findings provide strong support that redoxosomal ROS production induced by SOD1^{G93A} expression enhances the proinflammatory phenotype of glial cells by inducing NFκB and TNFα.

SOD1^{G93A} and Alsin Both Induce Rac1 Activation and SOD1^{G93A} Expression Enhances Recruitment of Alsin to Endomembranes—Rac1 activation is required for Nox2-dependent superoxide production (11, 51). Studies thus far suggested that Rac1 plays an important role in glial ROS production induced by SOD1^{G93A} expression (Fig. 6, *C* and *F*). Alsin has the unique ability to induce glial cell ROS in the absence of SOD1^{G93A}, whereas inhibiting ROS in the presence of SOD1^{G93A} (Figs. 2 and 6, *C* and *F*). We hypothesized that alterations in Rac1 activity induced by alsin and/or SOD1^{G93A} expression might control Rac1-dependent ROS production by glial cells. To test this hypothesis, we evaluated the abundance of activated Rac1 (Rac1-GTP) in glial cells expressing SOD1^{G93A} and/or alsin. Results indicated that SOD1^{G93A} and alsin expression can independently activate Rac1 in glial cells 5.1- and 3.4-fold, respectively (Fig. 7*C*). Consistent with the ability of alsin to reduce Nox2-dependent ROS in SOD1^{G93A}-expressing glial cells, coexpression of alsin and SOD1^{G93A} in glial cells significantly attenuated Rac1 activation compared with SOD1^{G93A} expression alone (Fig. 7*C*). Together with earlier findings, these results provide strong support that alsin and SOD1^{G93A} both converge on Rac1 to regulate endosomal Nox2-dependent ROS production.

Previous studies have demonstrated that SOD1 recruits to endomembranes following activation of proinflammatory receptors such as TNFR1 and IL-1R (10, 11, 50). In this context, SOD1 binds Rac1 to regulate Nox-dependent endosomal ROS. ALS mutant SOD1 proteins that have a higher affinity for Rac1-GTP enhance endosomal ROS production (7, 12). We reasoned that because alsin overexpression attenuates SOD1^{G93A}-induced Rac1 activation (Fig. 7*C*), alsin might recruit to the endosomal compartment in a SOD1^{G93A}-dependent manner to regulate Rac1/Nox2-dependent ROS production. To test this hypothesis, we evaluated the recruitment of alsin to the endomembrane fraction in cells overexpressing alsin and/or SOD1^{G93A}. Findings from these studies demonstrated that alsin recruitment to endomembranes was significantly increased 2.6-fold under conditions of SOD1^{G93A} expression (Fig. 7*D*). These and our earlier findings (Fig. 6, *C* and *F*) demonstrate that the ability of alsin to regulate redoxosomal ROS

induced by SOD1^{G93A} correlates with changes in its location to the endosomal compartment.

DISCUSSION

Enhanced ROS production has been proposed to be an important pathophysiologic component for several types of neuronal degenerative diseases including ALS (4). This is perhaps not surprising because most neuronal degenerative diseases have significant components of inflammation. The challenge in understanding the importance of enhanced ROS production in ALS has been to determine whether primary defects in ROS regulation incite pathophysiologic events responsible for disease progression. The first report to implicate Nox2 as a source of altered ROS production in a FALS (SOD1^{G93A}) mouse model, described by Wu and colleagues (6), was later substantiated by others (5). Additionally, work using hESC differentiated into astrocytes demonstrated that expression of SOD1^{G37R}-induced Nox2 protein production and ROS-mediated killing of hESC-derived motor neurons in co-culture (36). Importantly, inhibition of Nox2 with apocynin rescued motor neuron survival in the presence of SOD1^{G37R}-expressing astrocytes (36). These *in vitro* studies by Marchetto and colleagues (36) were key to demonstrating that Nox2 plays an important intrinsic role within FALS astrocytes to cause paracrine killing of motor neurons. Together with the finding that SOD1 ALS mutants can also directly regulate Rac1 and Nox2-dependent ROS production, these findings point to dysregulation of NADPH oxidases as an important potential mechanism in the pathology observed in FALS.

Studies have also demonstrated that Nox2 is up-regulated in spinal cord microglia of sporadic ALS patients (6), suggesting that the mechanisms of enhanced ROS production through NADPH oxidases may not be limited to SOD1 mutations found in FALS. The potential involvement of NADPH oxidases in FALS disease progression is supported by genomewide association studies demonstrating that Nox4 and several Nox regulators are closely linked to the disease (12, 18). Furthermore, studies on the juvenile form of ALS caused by mutations in the *ALS2* gene encoding for alsin (22) have demonstrated that alsin regulates Rac1 activity (21, 25, 26) and thus also has the potential to regulate NADPH oxidases that require Rac1 (*i.e.* Nox1, -2, and -4). These findings suggest that multiple independent ALS disease-causing and disease-associated genes may converge on regulatory pathways that influence NADPH oxidase-dependent ROS production. In the present study, we sought to determine whether both SOD1^{G93A} and alsin may both regulate the activity of NADPH oxidases in glial cells and the effect of this regulation on neuronal survival.

As previously reported for SOD1^{G37R}-expressing astrocytes (36), our studies demonstrate that SOD1^{G93A}-expressing glial cells hyperactivate Rac1- and Nox2-dependent ROS production leading to an enhanced proinflammatory state (TNFα and NFκB) and toxicity of a motor neuron-like cell line in co-culture. Nox1 and Nox4 appeared not to play a major role in these processes. The ability of SOD1^{G93A} to activate Rac1 is consistent with previous studies demonstrating that SOD1 binds Rac1-GTP and reduces the rate of GTP hydrolysis and that mutant forms of SOD1 have a higher affinity for Rac1-GTP (7). Interestingly, expression of full-length wild type alsin together with SOD1^{G93A} in glial cells led to protection of neuronal cells in co-culture by decreasing proinflammatory activation of glial cells (*i.e.* Rac1 activation, Nox2-dependent ROS production, TNFα production, and NFκB activation). These findings suggest that alsin plays a protective role and is consistent with reports demonstrating alsin knockdown induces motor neuron cell death (20, 28) and that expression of full-length alsin protects against neurotoxicity caused by SOD1 mutations (27). Interestingly the RhoGEF domain of alsin was required for this protective effect (27), a finding consistent with alsin mediating protection through the Rho-GTPase Rac1.

Our studies demonstrate for the first time that alsin can attenuate proinflammatory pathways regulated by Nox2 in SOD1^{G93A}-expressing glial cells and that this effect in turn influences survival of motor neuron-like cells. Previous studies have focused on the function of alsin in motor neurons and have demonstrated similar protective effects by alsin overexpression on SOD1^{G93A}-associated toxicity (27). The mechanism of alsin-mediated protection of motor neurons remains unclear, but previous studies have suggested that protection may be mediated through alsin binding to mutant SOD1 (27) and/or through Rac1 modulation (28). Our studies, demonstrating for the first time protective functions of alsin in glial cells, add to this growing body of literature. Interestingly, however, our studies also demonstrate that overexpression of alsin alone in glial cells (in the absence of SOD1^{G93A}) also drives similar amplification of proinflammatory pathways as seen following expression of SOD1^{G93A} alone (alsin overexpression induced Rac1, glial cell ROS, TNFα production, NFκB activation, and toxicity to motor neuron-like cells in co-culture). Thus, our findings suggest that alsin is not simply a protective modulator of SOD1^{G93A} toxicity in glial cells, but rather that both alsin and SOD1 can act to directly regulate proinflammatory signals by glial cells.

Alsin is known to localize to the endosomal compartment where it can serve as a GEF for Rab5, an early endosomal effector GTPase (19, 25, 28). Alsin is also an effector of Rac1 that has been shown to control endocytic mechanisms at the cell membrane (26, 52) and endolysosomal trafficking (20). Thus, alsin is a key regulator of endosomal dynamics. We hypothesized that alsin may regulate proinflammatory pathways through redox-active signaling endosomes (*i.e.* redoxosomes) known to facilitate redox-mediated activation of proinflammatory receptors such as TNFR and IL-1R (8, 10, 11). Our findings demonstrating that alsin recruitment to endomembranes is enhanced by SOD1^{G93A} expression supports its potential function on redoxosomes. The recruitment of SOD1 to redoxosomes, following TNFα or IL-1β stimulation, is important for controlling Rac1-dependent NADPH oxidase activation, redox-dependent TRAF recruitment to receptor complexes, and ultimately activation of NFκB (8). Our novel findings that SOD1^{G93A} expression induces redoxosomal ROS production by TNFα, and that this ROS is attenuated by alsin overexpression, provide a mechanistic link for anti-inflammatory effects of alsin in the presence of SOD1^{G93A}. Thus, our findings are consistent with alsin being a modulator of proinflammatory Nox2-dependent redoxosomal activation.

In summary, our studies demonstrate that SOD1^{G93A} expression in glial cells modulates proinflammatory signaling

through redoxosomes in a Nox2-dependent fashion. This elevated proinflammatory state in turn leads to secreted factors that are toxic to neurons. Alsin appears to directly attenuate glial cell-dependent neurotoxicity by reducing Nox2-mediated signaling by redoxosomes. This protective effect appears to be mediated by the ability of alsin to decrease Rac1 activation in the presence of SOD1^{G93A}. Such findings provide insights into potential common regulatory pathways controlled by NADPH oxidases that influence proinflammatory signaling and neurotoxicity in two independent genetic forms of ALS.

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