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Role of SODD in Regulation of Tumor Necrosis Factor Responses

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Signaling from tumor necrosis factor receptor type 1 (TNFR1) can elicit potent inflammatory and cytotoxic responses that need to be properly regulated. It was suggested that the silencer of death domains (SODD) protein constitutively associates intracellularly with TNFR1 and inhibits the recruitment of cytoplasmic signaling proteins to TNFR1 to prevent spontaneous aggregation of the cytoplasmic death domains of TNFR1 molecules that are juxtaposed in the absence of ligand stimulation. In this study, we demonstrate that mice lacking SODD produce larger amounts of cytokines in response to in vivo TNF challenge. SODD-deficient macrophages and embryonic fibroblasts also show altered responses to TNF. TNF-induced activation of NF-κB is accelerated in SODD-deficient cells, but TNF-induced c-Jun N-terminal kinase activity is slightly repressed. Interestingly, the apoptotic arm of TNF signaling is not hyperresponsive in the SODD-deficient cells. Together, these results suggest that SODD is critical for the regulation of TNF signaling.

Studies of tumor necrosis factor receptor (TNFR) superfamily signaling bear important physiological implications, as these receptors play critical roles in the processes of programmed cell death, immune responses, organ development, and metabolism (3, 13). The initial theory of how TNFRs are triggered proposed that unliganded monomeric receptors are trimerized upon binding to their respective ligands, which also function as trimers (8, 19, 25). TNFR type 1 (TNFR1) belongs to a subset of the TNFR superfamily that possesses death domains in the cytoplasmic regions of the receptors (23). The death domain of TNFR1 is crucial for recruiting signaling proteins, transducing downstream signaling pathways leading to both cell death and NF-κB activation. It was thought that ligand-dependent trimerization of TNFR1 brings together cytoplasmic death domains, which then initiate downstream signaling pathways by recruiting the TNFR-associated death domain protein (TRADD) (7).

One potential danger of such a receptor-triggering mechanism is that TNFR1 molecules might oligomerize accidentally if they are in close proximity, leading to inappropriate intracellular signaling without ligand stimulation. Indeed, when TNFR1 is artificially overexpressed, the receptor spontaneously self-associates and signals independently of ligand binding (7, 27). In a physiological setting, unwarranted TNFR1 signaling must also be prevented. Discovery of the protein silencer of death domain (SODD) led to the proposal that SODD directly binds to TNFR1 and inhibits the recruitment of TRADD to the death domain of TNFR1 (9). Upon TNF binding to TNFR1, SODD has been shown to be transiently released from the TNFR1 receptor complex, thus permitting signal transduction from TNFR1. After ~10 min of TNF stimulation, SODD is recruited back to the receptor complex, resulting in a dampening of the intensity of TNFR1 signal transduction (9).

Recently, the theory of ligand-induced trimerization of TNFRs has been challenged by studies showing that receptor trimers can be assembled independently of ligands. A pre-ligand-binding assembly domain located in the extracellular domain of the receptor is required for such ligand-independent trimer formation and, more importantly, for efficient signaling upon ligand stimulation (2, 18). This new model implies that certain conformational changes occur in receptors as a result of ligand binding. In the context of the new model, the in vivo significance of SODD in TNFR1 signaling warrants further investigation.

In addition to TNFR1, SODD can also associate with the cytoplasmic region of death receptor 3 (DR3) (9). However, SODD does not interact with other death receptors, such as Fas, DR4, and DR5, or with intracellular signaling proteins, such as TRADD, the Fas-associated death domain (FADD) protein, or receptor-interacting protein. SODD has a characteristic protein binding domain called the BAG domain that is required for binding to TNFR1 and the ATPase domain of heat shock protein 70 (Hsp70). In vitro, SODD is able to disassemble aggregated TNFR1 in the presence of ATP, suggesting that SODD may function to modulate conformational changes in TNFR1 in a manner analogous to the function of the nucleotide exchange factor BAG-1 in the ATPase cycle of Hsp70 (15).

To investigate the physiological function of SODD in TNFR1 signaling in vivo, we generated *SODD*$^{-/-}$ null mutant mice. SODD is dispensable for normal embryogenesis and organ development. However, when challenged with TNF, *SODD*$^{-/-}$ mice displayed increased cytokine production compared to their wild-type counterparts. Our study demonstrates the importance of SODD in the regulation of the TNFR1 signaling pathway.

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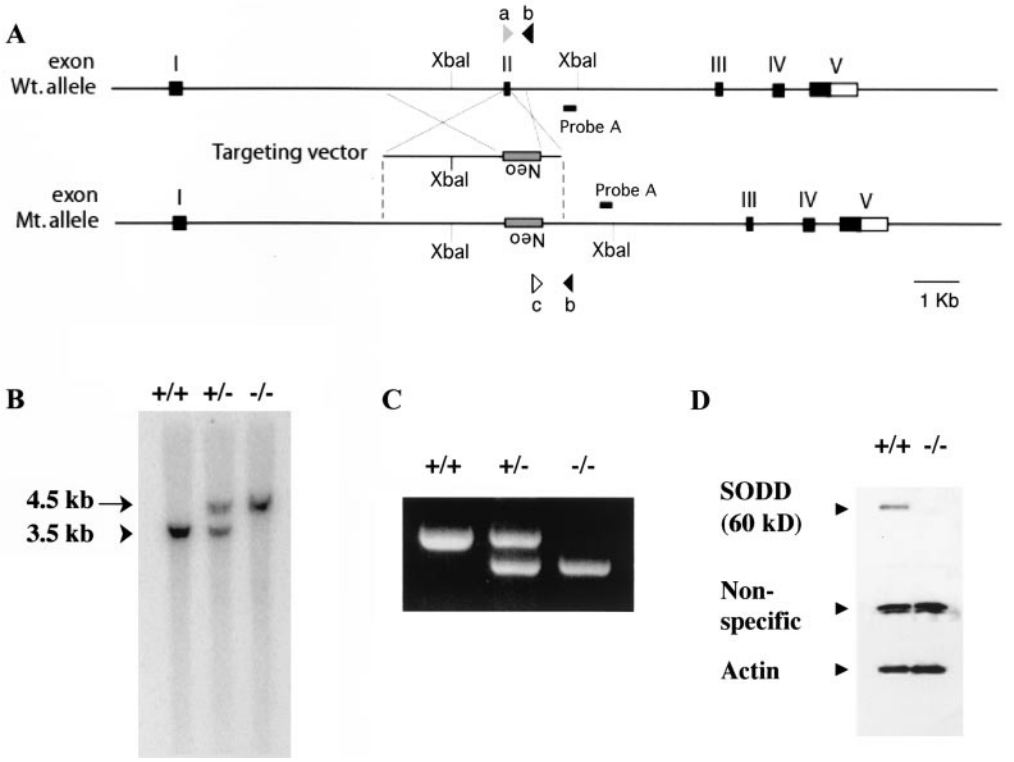

FIG. 1. Generation of SODD-null mutant. (A) Targeting vector to create SODD mutation. The genomic structures of the SODD gene and the exon that was targeted are indicated. Probe A indicates the position of the diagnostic probe in the endogenous flanking region outside of the targeting vector. a, b, and c, positions of primers that were utilized for genotyping. Wt., wild-type; Mt., mutant. (B) Southern blot analysis of mouse tail DNA. The genomic DNA was digested with *Xba*I, fractionated on an agarose gel, blotted, and probed with probe A. +/+, wild type; +/−, heterozygous mutant; −/−, homozygous mutant. (C) PCR genotyping of mouse tails. Primers a, b, and c were utilized in the same PCRs that yielded products of wild-type (top) and mutant (bottom) alleles. (D) Western blot analysis of proteins from primary EF. Total cellular proteins were fractionated on an SDS-PAGE gel, blotted, and probed with a specific antibody against SODD. The blot was then stripped and reprobed with actin for a loading control.

MATERIALS AND METHODS

Generation of *SODD*$^{-/-}$ mice. A genomic clone containing the mouse *SODD* gene was isolated from the screening of a 129/Ola mouse genomic DNA phage library with a full-length cDNA probe. The targeting vector was constructed by replacing a *SODD* coding exon (corresponding to cDNA nucleotides 274 to 378) with the neomycin resistance cassette in the reverse orientation. The targeting vector was linearized with *Sac*I and electroporated into E14K ES cells (Bio-Rad Gene Pulser; 0.34 kV; 250 μF). After G418 selection (200 μg/ml; GIBCO-BRL), homologous recombinants were identified by PCR and confirmed by Southern blot analysis with an external probe, as depicted in Fig. 1A. Four clones heterozygous for the targeted mutation were injected into 3.5-day C57BL/6 blastocysts, which were subsequently transferred into pseudopregnant foster mothers. The chimeric mice were crossed with C57BL/6 mice to produce heterozygous *SODD*$^{+/-}$ mice. The heterozygotes were intercrossed to generate homozygous *SODD*$^{-/-}$ mice. The genotypes of the mutant mice were determined by PCR and confirmed by Southern blot analysis of genomic DNA from tail biopsy samples. As shown in Fig. 1A, primers a and b were used to detect the wild-type allele, and primers b and c were used to detect the mutant allele. The sequences of these primers were as follows: primer a, 5′-GCT CCA TAC CCA GGC TCC TAC T-3′; primer b, 5′-CCT TTA AGA GCA GCT GGC CAA CAC AAA C-3′; primer c, 5′-AAG CGC ATG CTC CAG ACT GCC TTG GGA A-3′.

Western blot analyses. Total cell lysates were fractionated by gel electrophoresis on 10% Tris-glycine polyacrylamide gels (Novex) and transferred to polyvinylidene difluoride membranes (Millipore). The blots were subsequently incubated with anti-SODD (N-19; Santa Cruz Biotechnology), anti-IκBα (Cell Signaling), anti-anti-phospho-c-Jun (KM-1; Santa Cruz Biotechnology), and anti-actin (Sigma) antibodies. Immunoblot analyses were performed with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse (Amersham-Pharmacia) secondary antibodies, and proteins were visualized with the ECL Plus enhanced chemiluminescence system (Amersham-Pharmacia) according to the manufacturer's instructions.

NO and cytokine measurements. Resident macrophages were isolated from peritoneal lavage fluid, counted, plated (2.5×10^4 cells/well), and incubated with various concentrations of recombinant mouse TNF-α plus 100 U of recombinant mouse gamma interferon (IFN-γ) (R&D Systems)/ml for 24 h. In experiments where embryonic fibroblasts (EF) were used, 10^5 cells were plated in 24-well plates for TNF treatment for 24 h. The supernatants were assayed for nitrite, the stable end product of NO, using the Griess Reagent System (Promega). The interleukin 1 (IL-1) and IL-6 levels in sera or culture supernatants were measured using enzyme-linked immunosorbent assay kits (R&D) following the manufacturer's instructions.

Flow cytometric analyses. Single-cell suspensions were prepared from thymus, spleen, lymph nodes, and bone marrow. For each cytometric analysis, 5×10^5 cells were used. All antibodies (including those detecting CD4, CD8, CD25, CD44, and CD3) were purchased from BD PharMingen. After the cells were stained, fluorescent signals on the cell surfaces were analyzed with a FACSCalibur flow cytometer (Becton Dickinson) and CellQuest software.

Thymocyte and EF death asssay. Freshly isolated thymocytes were cultured in RPMI supplemented with 10% fetal calf serum at a concentration of 10^6/ml in 48-well plates. The thymocytes were treated with mouse TNF-α (10 ng/ml), cycloheximide (10 μg/ml; Sigma), Fas ligand (10 μg/ml; FasL-CD8 fusion protein), or z-VAD (50 μM; Sigma). After 20 h of incubation at 37°C, the cells were harvested and stained with fluorescein isothiocyanate-CD8α, phycoerythrin-CD4, and 7-AAD (1 μg/ml; Sigma) and analyzed by flow cytometry as previously described. Viable (7-AAD-negative), double-positive ($CD4^+$ $CD8^+$) cells were identified. In the primary EF death assay, viable cells were determined by trypan blue exclusion.

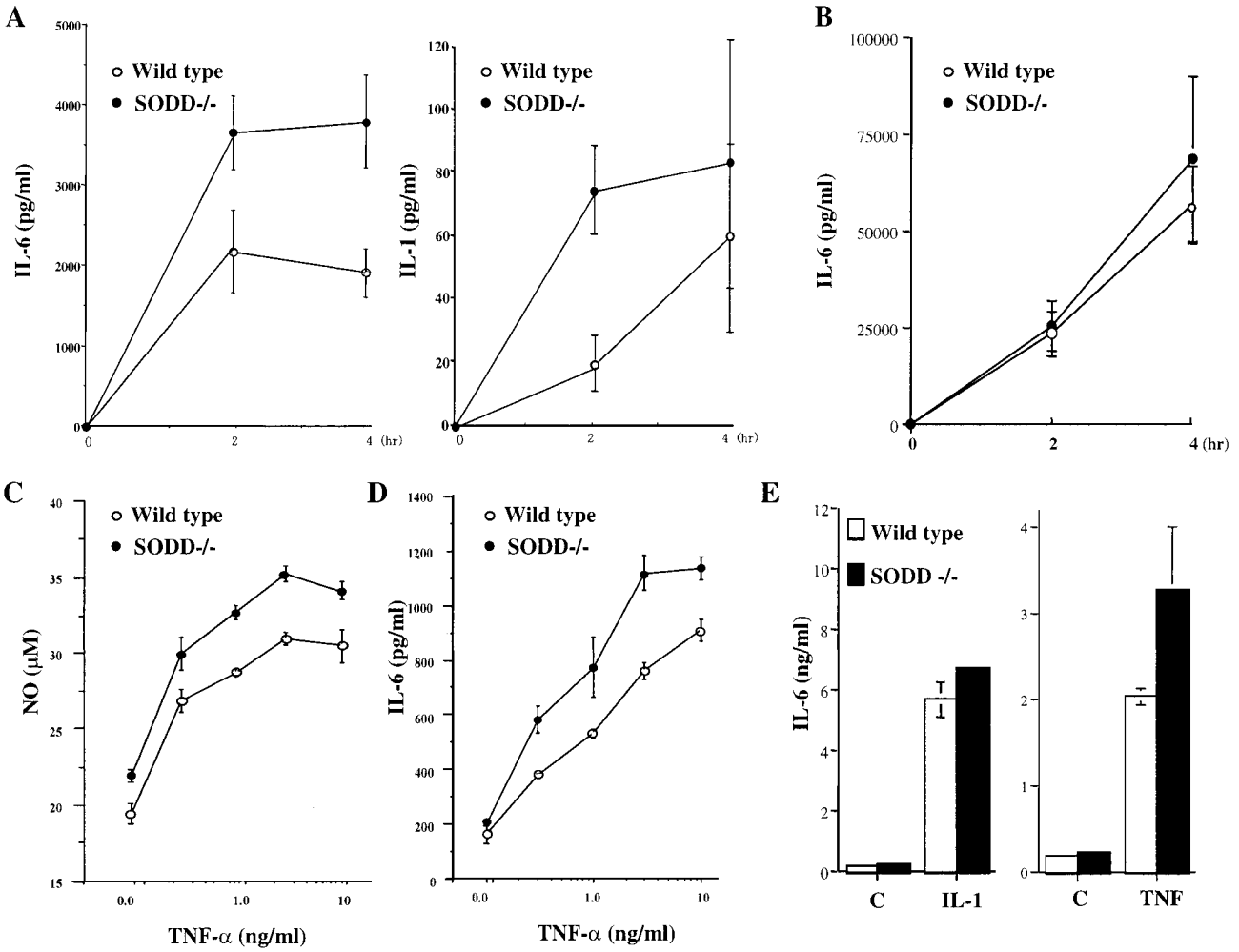

FIG. 2. Increased TNF responses in SODD-deficient mice and cells. (A) In vivo cytokine response to TNF challenge. Concentrations of IL-6 or IL-1 in serum were measured for four wild-type and four $SODD^{-/-}$ mice 0, 2, or 4 h after intraperitoneal TNF injection (10 μg/mouse). (B) In vivo cytokine response to IL-1 injection. Levels of IL-6 in serum were determined for three wild-type and three $SODD^{-/-}$ mice 0, 2, or 4 h after intraperitoneal IL-1 injection (10 μg/mouse). (C) Production of NO from macrophages in response to TNF. Peritoneal resident macrophages isolated from wild-type and $SODD^{-/-}$ mice were stimulated with 100 U of IFN-γ/ml plus increasing concentrations of TNF, and NO production was measured after 24 h. (D) Cytokine responses of primary EF to TNF stimulation. Wild-type and $SODD^{-/-}$ primary EF were stimulated with 100 U of IFN-γ/ml plus various concentrations of TNF for 24 h, and IL-6 production in the culture supernatant was measured. (E) Cytokine response of primary EFs to IL-1 stimulation in comparison to TNF treatment. Wild-type and mutant EFs were stimulated with 10 ng of IL-1 or TNF/ml for 24 h, and IL-6 production was measured. Measurements of NO and cytokines were performed in triplicate for each condition. The error bars indicate standard errors.

RESULTS

Generation of SODD-deficient mice. The *SODD* gene was disrupted in murine embryonic stem (ES) cells by homologous recombination using a targeting vector, as shown in Fig. 1A, which was designed to replace a *SODD* exon (exon 2) with a *PGK-neo* cassette. Southern blot analysis using a flanking probe (probe A [Fig. 1A]) on *Xba*I-digested genomic DNAs of ES cell clones demonstrated a 3.5-kb fragment for the wild-type allele and a 4.5-kb fragment for the targeted allele (Fig. 1B). Heterozygous ES cell lines containing a mutated *SODD* allele were injected into C57BL/6 blastocysts, and chimeras with germ line transmission were used to generate $SODD^{+/-}$ mice by mating with C57BL/6 mice. Heterozygous $SODD^{+/-}$ mice were healthy and fertile, and from heterozygous intercrosses, homozygous $SODD^{-/-}$ mice were born at the expected Mendelian ratio. Southern blot analyses of genomic DNAs from wild-type and heterozygous and homozygous mutant mice are shown in Fig. 1C. To determine whether this was a null mutation, primary EF were derived from wild-type and $SODD^{-/-}$ mice, and SODD protein expression was analyzed by Western blotting using a specific antibody. As shown in Fig. 1D, no protein could be detected in $SODD^{-/-}$ primary EF. $SODD^{-/-}$ mice appeared healthy and showed no obvious abnormalities macroscopically or microscopically in the brain, heart, lung, liver, kidney, and skin (data not shown).

Increased responsiveness of $SODD^{-/-}$ mice and cells to TNF stimulation. Since SODD has been implicated in the regulation of TNF signaling, we first analyzed the in vivo re-

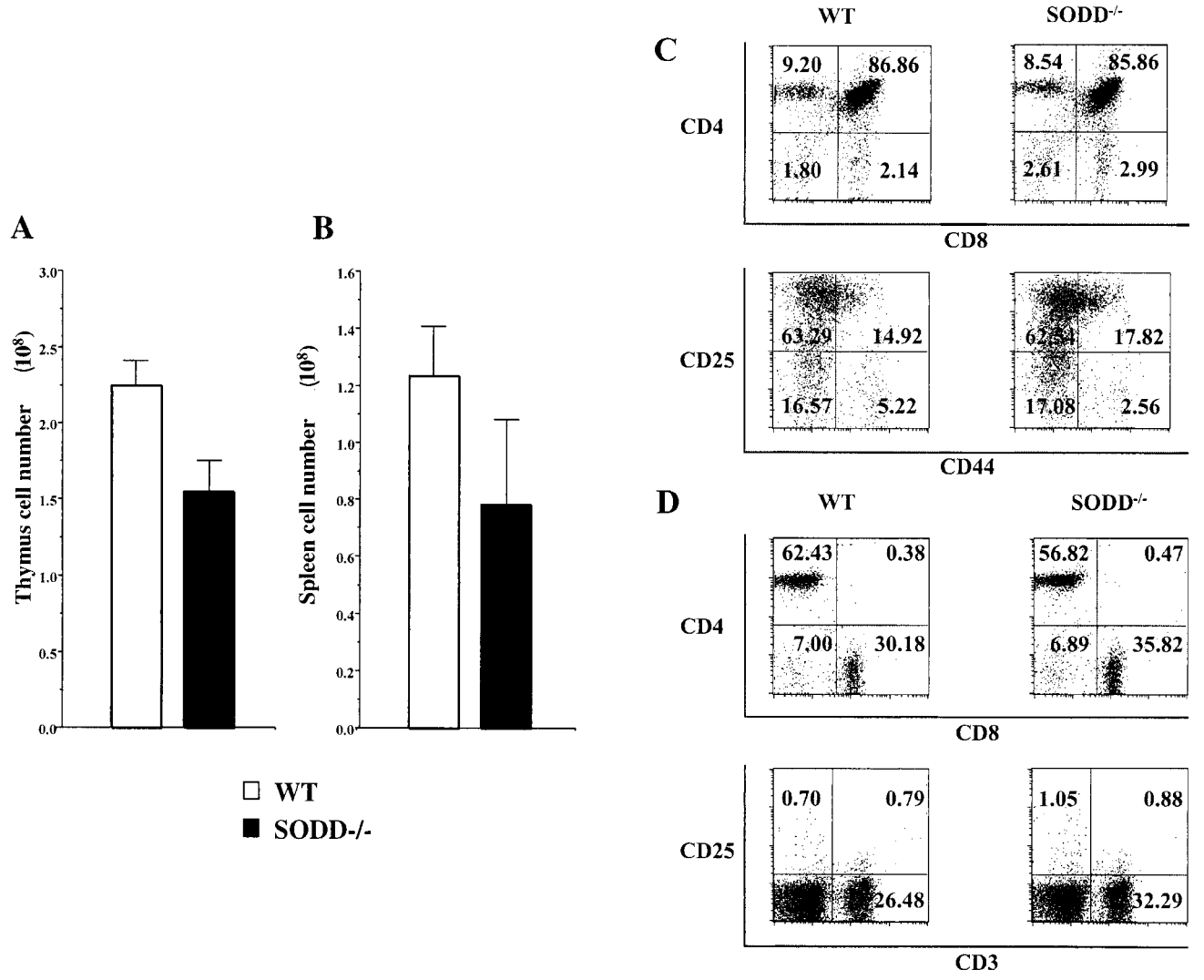

FIG. 3. Analysis of the lymphoid compartment in *SODD*$^{-/-}$ mice. (A) Total thymocyte numbers in wild-type (WT) and *SODD*$^{-/-}$ mice. Thymocyte cell counts were taken from four wild-type and four mutant mice at 6 to 8 weeks of age, and the mean and standard errors are shown. (B) Splenocyte cell counts from wild-type and *SODD*$^{-/-}$ mice. Total splenocytes were counted for four wild-type and four mutant mice, and the mean and standard errors are shown. (C) Thymocyte surface marker expression levels analyzed by flow cytometry. Total thymocytes isolated from wild-type and *SODD*$^{-/-}$ mice were analyzed for the expression of CD4 and CD8 (top). Cells that were double negative for CD4 and CD8 were gated and analyzed for the expression of CD25 and CD44 (bottom). Numbers indicate percentages of total or gated cells. (D) Profiles of surface marker expression in splenocytes. Total splenocytes from wild-type and mutant mice were stained for the expression of CD3, CD25, CD4, and CD8.

sponse of *SODD*$^{-/-}$ mice to TNF challenge. Murine TNF was injected into the peritoneal cavities of wild-type and SODD-deficient animals, and then the levels of IL-6 and IL-1β in serum were measured by enzyme-linked immunosorbent assay. Wild-type mice were the littermate controls for mutant mice. Although the levels of IL-1β 4 h following injection were variable, overall we found that the cytokine responses from *SODD*$^{-/-}$ mice were abnormally elevated both 2 and 4 h after TNF injection (Fig. 2A). In contrast, IL-1β injection into wild-type and SODD-deficient mice induced similar levels of cytokine elevation (Fig. 2B). In addition, no constitutive levels of IL-6, IL-1, or TNF could be detected in sera from unstimulated wild-type or SODD-deficient mice (Fig. 2A and B and data not shown).

To determine whether this defect in the TNF response could be detected at the cellular level, we first examined the responses of wild-type and *SODD*$^{-/-}$ macrophages to TNF. Wild-type and mutant macrophages were derived from mice of the same litter. When peritoneal resident or bone marrow-derived macrophages from wild-type and mutant animals were stimulated with TNF plus 100 U of IFN-γ/ml, we observed a mild increase in nitric oxide (NO) production by SODD-deficient macrophages compared to that by wild-type macrophages (Fig. 2C and data not shown). We also examined the TNF response of *SODD*$^{-/-}$ primary EF. Again, wild-type and mutant EF were derived from embryos of the same litter. Although EF cells do not produce detectable amounts of NO after TNF stimulation (data not shown), they do produce IL-6 in response to TNF. When we assayed IL-6 production, we found that *SODD*$^{-/-}$ primary EF produced an increased

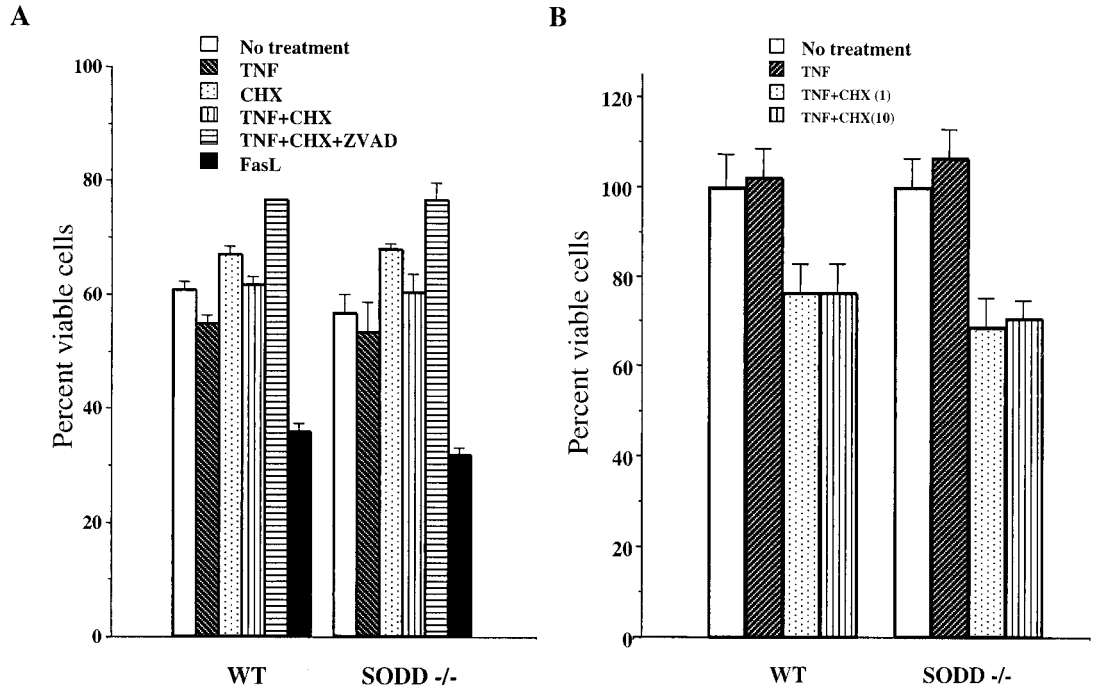

FIG. 4. Normal TNF-induced cell death in SODD-deficient cells. (A) Thymocyte death induced by TNF and Fas ligand (FasL). Wild-type (WT) and *SODD*$^{-/-}$ thymocytes were stimulated with various reagents as indicated, including two death receptor ligands, TNF, and FasL, or were untreated. After 24 h, the cells were stained with CD4, CD8, and 7-AAD. The percentages (plus standard errors) of viable CD4$^+$ CD8$^+$ (7-AAD-negative) cells are shown. CHX, cycloheximide; zVAD, caspase inhibitor. (B) Primary EF cell death induced by TNF plus cycloheximide. Wild-type and *SODD*$^{-/-}$ primary EF were treated with TNF in the absence or presence of cycloheximide (1 or 10 μg/ml) for 24 h. The percentages (plus standard errors) of viable cells capable of excluding trypan blue are shown.

amount of IL-6 compared with wild-type EFs in response to TNF stimulation (Fig. 2D and E). In contrast, levels of IL-6 production induced by IL-1 in wild-type and *SODD*$^{-/-}$ cells were comparable (Fig. 2E). These data suggest that SODD-deficient animals and cells respond to TNF in a deregulated manner.

Since deregulated TNF signaling has been shown to result in reduced lymphoid compartments in various mutant mice (10, 16, 28), we next examined the development and cellularity of lymphocytes in SODD-deficient mice. Interestingly, in *SODD*$^{-/-}$ mice, total thymocyte numbers were significantly reduced compared to those in wild-type mice (Fig. 3A). However, the distribution of thymocyte subpopulations, including CD4$^-$ CD8$^-$ (double-negative), CD4$^+$ CD8$^+$ (double-positive), and CD4$^+$ or CD8$^+$ single-positive cell populations, was not altered in *SODD*$^{-/-}$ mice compared to wild-type mice (Fig. 3C). Also, in the population of CD3$^-$ CD4$^-$ CD8$^-$ triple-negative thymocytes, the proportions of thymocytes in the TN1 to TN4 subpopulations, as delineated by CD25 and CD44 expression, in both wild-type and *SODD*$^{-/-}$ mice were similar (Fig. 3C). In terms of the peripheral lymphocyte compartment, we observed a reduction in total splenocyte numbers in *SODD*$^{-/-}$ mice, although there was variability among individuals (Fig. 3B). Despite this reduction in cellularity, the proportions of CD4$^+$ versus CD8$^+$ T cells (Fig. 3D) and of T cells versus B cells (data not shown) were unaltered in mice lacking SODD. The activation status of T cells was evaluated by expression of the early activation marker CD25, and we found that *SODD*$^{-/-}$ T cells did not exhibit any alterations in activation status compared to wild-type T cells (Fig. 3D).

Enhanced TNF-induced NF-κB activation but unaltered cell death in SODD-deficient cells. To investigate whether SODD deficiency renders cells more sensitive to TNF-induced apoptosis, we examined *SODD*$^{-/-}$ thymocytes and EF along with their wild-type controls. As shown in Fig. 4, SODD-deficient thymocytes and EF showed viability similar to that of their wild-type counterparts in response to TNF stimulation. These results indicated that while SODD deficiency results in increased cytokine production in response to TNF signaling, the TNF-induced cell death pathway either is not subjected to regulation by SODD or is counteracted by other mechanisms in the absence of SODD.

Through TNFR1, TNF is capable of triggering the activation of NF-κB in addition to a cell death pathway. NF-κB is the transcription factor that has been shown to play an important role in cell survival, as well as in the production inflammatory cytokines. We therefore examined the effect of SODD deficiency on TNF-induced NF-κB activation in EF cells. We first performed gel mobility shift assays to evaluate the NF-κB activities by their abilities to bind to DNA but found no significant difference between wild-type and *SODD*$^{-/-}$ EF (data not shown). We then specifically examined an upstream event in the activation of NF-κB, that is, the degradation of IκBα, which allows nuclear translocation of NF-κB. As shown in Fig. 5A and B, the degradation of IκBα appeared to be slightly enhanced in *SODD*$^{-/-}$ EF cells compared to that in wild-type

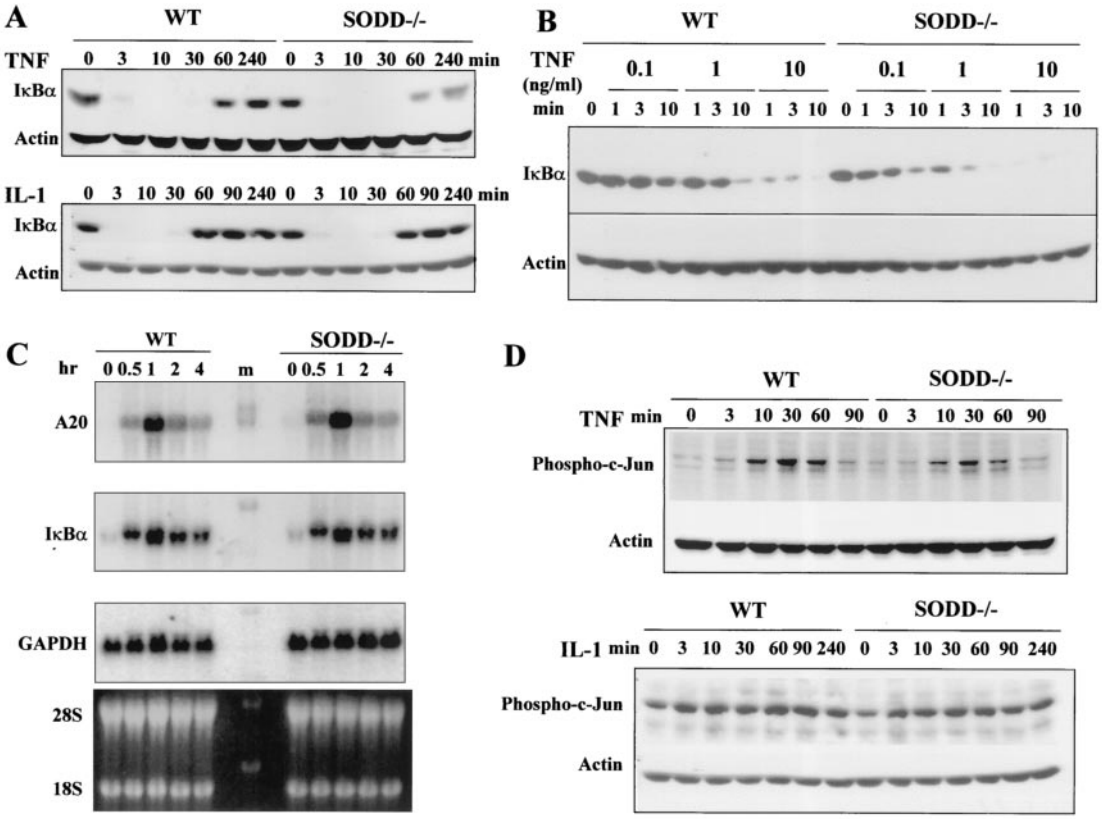

FIG. 5. Effect of SODD deficiency on TNF-mediated downstream signal transduction. (A) Time course of IκB degradation in response to TNF or IL-1 stimulation. Wild-type (WT) and *SODD*$^{-/-}$ primary EF were stimulated with TNF or IL-1 (10 ng/ml), and total cell lysates were harvested at various time points as indicated and analyzed for the expression of total IκBα protein and actin by Western blot analysis. (B) Early time courses of IκB degradation upon stimulation with various concentrations of TNF. Wild-type and *SODD*$^{-/-}$ cells were stimulated with increasing concentrations of TNF, and the extent and kinetics of IκBα protein degradation were determined by Western blot analysis. (C) mRNA induction of A20 and IκBα genes after TNF stimulation. Total RNAs from wild-type or *SODD*$^{-/-}$ primary EF were collected at the indicated time points after TNF stimulation, and Northern blot analysis was performed to determine the kinetics of A20 gene expression. The same blot was stripped and reprobed with IκBα and then GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA. m, marker for RNA size standards on the gel. (D) Kinetics of JNK activation induced by TNF or IL-1. Activation of the JNK pathway was determined by the level of phosphorylated c-Jun in TNF- or IL-1-stimulated wild-type and *SODD*$^{-/-}$ primary EF.

cells. This was particularly evident at the early time points (Fig. 5B). This phenomenon appeared to be specific to TNF stimulation, as IL-1 treatment triggered similar levels of IκBα degradation in wild-type and SODD-deficient EF (Fig. 5A).

The level of IκBα expression at the later time points, which reflected a dynamic balance between the ongoing IκBα degradation that precedes NF-κB activation and new IκBα gene transcription that is dependent on NF-κB activity, was also slightly reduced in SODD-deficient cells compared to that in wild-type cells (Fig. 5A). To further investigate this defect, we examined the kinetics of induced transcription of two NF-κB-dependent genes, IκBα and A20, in SODD-deficient cells. While no difference could be observed in IκBα expression in wild-type and *SODD*$^{-/-}$ EF at various time points, a mild increase of A20 mRNA-induced expression could be detected in *SODD*$^{-/-}$ cells 0.5 and 1 h after TNF stimulation (Fig. 5C).

We noted that the data in Fig. 2C showing increased IL-6 production by SODD-deficient cells indicated that the same level of IL-6 induction by TNF in wild-type cells could be achieved by 1/3 to 1/10 of the TNF concentration in *SODD*$^{-/-}$ cells. To examine whether this phenomenon is correlated with differences in NF-κB activation in SODD-deficient versus wild-type cells, we measured IκB degradation in wild-type and *SODD*$^{-/-}$ EF using serial concentrations of TNF. As shown in Fig. 5B, the extent of IκB degradation was indeed dependent on the concentration of TNF, and *SODD*$^{-/-}$ cells required lower doses of TNF to induce levels of IκB degradation similar to those observed in wild-type cells.

TNF also activates stress kinase pathways, such as JNK and p38 mitogen-activated kinase (MAPK), which play roles in cell death and the regulation of cytokine production (25). We examined activation of these stress kinases in *SODD*$^{-/-}$ EF in response to TNF and found that they were not enhanced. In fact, while the p38 MAPK pathway was induced normally in the mutant cells (data not shown), activation of the JNK pathway, by measuring the level of phospho-c-Jun, was slightly decreased in cells lacking SODD compared to that in wild-type cells (Fig. 5D). However, c-Jun kinase activities as measured by an in vitro kinase assay appeared comparable in SODD-deficient and wild-type cells (data not shown). Taken together, our

results suggested that SODD deficiency causes the enhancement of cytokine production in response to TNF and an alteration of the complicated TNF-induced signaling cascades, including the enhanced NF-κB signaling pathway and slightly repressed JNK activation.

DISCUSSION

The complexity and importance of proper TNF signal regulation can be illustrated by the demonstrated existence of multiple inhibitory feedback mechanisms. For example, IκBα and A20 are both induced by TNF signals and function to shut down further signal transduction by sequestering NF-κB (12) and impinging on the receptor signaling complex (5, 6, 20), respectively. The importance of these molecules in inhibiting TNF signals was further exhibited in the knockout studies (1, 11). Interestingly, TRAF2 and TRAF1 were implicated in the transduction of TNF signals by their recruitment to both TNFR1 and TNFR2 receptor complexes (17, 21). However, gene-targeting studies suggested that TRAF2 and TRAF1 also play roles in shutting down TNF signals (24, 28).

In this study, we examined the physiological function of SODD, a potential signal regulator at the very proximal end of TNFR1 complex assembly. We showed that SODD deficiency results in a mild enhancement of NF-κB activation and cytokine production, but not JNK activation or cell death. However, TNF signaling is not wildly deregulated in the absence of SODD. Elevated expression of A20 plus the presence of IκB proteins and TRAF2 probably participate in keeping TNFR1 signals largely in check in cells lacking SODD. Nonetheless, our results demonstrated that SODD is required for optimal TNF signal regulation and that NF-κB activation, particularly degradation of IκB, is the downstream pathway that is most susceptible to a defect in negative regulation of TNF-mediated events.

There are two major possibilities for the way in which a negative regulator may control TNF signals. It may function as a type of gatekeeper for a TNF receptor(s), and a deficiency of such molecules would result in a lower threshold being required for signal transduction to occur. Alternatively, the negative regulator may shut down TNF signals after the initiation of downstream pathways. Our results seem to support a role for SODD as one of the gatekeepers for the initiation of TNFR1 signaling, and the loss of SODD lowers the amount of TNF required to trigger a certain signaling intensity (Fig. 2C and 5B). This distinguishes SODD from other major negative regulators, such as the A20 and IκB proteins, which function primarily as feedback inhibitors that turn off signals after they have been initiated.

Although we favor a mechanism where there is a direct relationship between the increase in cytokine production and the elevated NF-κB in TNF-stimulated *SODD*$^{-/-}$ cells, we cannot rule out the contribution of alterations in other signaling pathways, such as the JNK pathway, to the abnormal cytokine production. It has been shown that in cells in which NF-κB is repressed or deficient, JNK activity is increased, and this increase mediates an enhancement in cell death (4, 22). Further studies of the role of the JNK pathway alone and the reciprocal control between NF-κB and JNK pathways in TNF-induced cytokine production will be required to address this issue.

SODD was initially proposed to prevent TNFR1 triggering in the absence of ligand stimulation. Although there was no strong evidence from our results to support this hypothesis, we have occasionally observed that *SODD*$^{-/-}$ macrophages and EF cells produced inflammatory cytokines (compared to wild-type cells) in the absence of TNF stimulation. Intriguingly, these SODD-deficient cells displayed a reduced cytokine response to TNF stimulation. The mutant cells may have been preactivated somehow and became refractory to TNF-induced responses. It is also possible that repressed JNK activation in *SODD*$^{-/-}$ cells plays a role in regulating cytokine production in this situation.

The phenotype of reduced thymocyte cellularity was consistently observed in *SODD*$^{-/-}$ mice, but it is not easily explained by the other phenotypes of enhanced cytokine production or NF-κB activation induced by TNF. One possible explanation is that the deficiency of SODD may also enhance the signaling of DR3, which has been shown to play a role in thymocyte negative selection (26). Intriguingly, TL1A, a recently identified DR3 and decoy receptor 3 (DcR3) ligand, has been implicated in T-cell costimulation and in the killing of certain tumor cells, but not primary T cells (14). Future studies investigating the physiological roles of TL1A and DR3 in regulating thymocyte cellularity and the involvement of SODD in this signaling context may clarify whether SODD affects thymocytes through a mechanism involving DR3.

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