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Copper–zinc superoxide dismutase-deficient mice show increased susceptibility to experimental autoimmune encephalomyelitis induced with myelin oligodendrocyte glycoprotein 35–55

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Abstract

In this report, we have addressed the role of copper–zinc superoxide dismutase (SOD1) deficiency in the mediation of central nervous system autoimmunity. We demonstrate that SOD1-deficient C57Bl/6 mice develop more severe autoimmune encephalomyelitis induced with myelin oligodendrocyte glycoprotein (MOG) 35–55, compared with wild type mice. This alteration in the disease phenotype was not due to aberrant expansion of MOG-specific T cells nor their ability to produce inflammatory cytokines; rather lymphocytes generated in SOD1-deficient mice were more prone to spontaneous cell death when compared with their wild type littermate controls. The data point to a role for SOD1 in the maintenance of self-tolerance leading to the suppression of autoimmune responses.

Keywords

Autoimmunity; Experimental autoimmune encephalomyelitis; Oxidative stress; T cells

1. Introduction

Reactive oxygen species (ROS) are generated by a multitude of biochemical reactions and cellular functions in all mammalian cells. ROS, or pro-oxidants, include superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide, hydroxyl radicals and peroxynitrite (Nordberg and Arner, 2001). In redox homeostasis, the formation of pro-oxidants is balanced by their consumption by anti-oxidants. ROS serve as a “double edged sword,” since low amounts of ROS may act as secondary messengers to physiological processes including cell signaling (Sun et al., 2005; Valko et al., 2007). On the contrary, enhanced production of pro-oxidants in contrast to anti-oxidants can disrupt redox homeostasis,

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causing “oxidative stress.” The excess ROS produced can lead to damage to DNA, proteins, mitochondria and the cell membrane, eventually causing cell death (Nordberg and Arner, 2001).

Production of ROS generally involves three different enzyme systems—nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, mitochondrial enzymes and peroxisomal enzymes—and their levels are regulated by several antioxidant enzymes, such as superoxide dismutases (SODs), catalases, peroxiredoxins and glutathione peroxidases (GPx) (Nordberg and Arner, 2001). SODs are metal-containing enzymes that mediate dismutation of O_2^- into H_2O_2 and molecular oxygen. In humans, the three isoforms of SODs are copper–zinc SOD (SOD1), manganese SOD (SOD2) and extracellular SOD (SOD3). While SOD1 is mostly a cytosolic protein with a minor component located in the mitochondrial inter-membrane space, SOD2 is a mitochondrial protein and SOD3 is present in the extracellular space (Johnson and Giulivi, 2005). Being a cytosolic protein, SOD1 plays a critical role in scavenging ROS. In particular, O_2^- and allelic variations in SOD1 have been implicated in disease conditions such as amyotrophic lateral sclerosis and diabetic nephropathy in type I diabetes (Kunst et al., 2000; Mohammedi et al., 2011). Although mice deficient for SOD1 develop normally, they show degenerative changes in the cochlear ganglion and retinal nerve, as well as progressive motor axonopathy leading to muscular dystrophy as a result of excessive ROS production, and females show reproductive defects (Matzuk et al., 1998; Keithley et al., 2005; Fischer et al., 2012).

The general dogma is that increased ROS production is detrimental and causes tissue injuries. As such, oxidative stress is implicated in the pathogenesis of many inflammatory diseases, including cancer, aging, atherosclerosis, ischemia, and in neurodegenerative and autoimmune disorders such as systemic lupus erythematosus, Alzheimer's disease, multiple sclerosis (MS), diabetes and arthritis (Griffiths, 2005; Valko et al., 2007). At a molecular level, mice deficient for nuclear factor-erythroid 2-related factor 2, the transcription factor that controls the expression of anti-oxidant enzymes, develop multi-organ autoimmune disease due to impaired redox homeostasis (Ma et al., 2006). However, this paradigm has been recently revisited with an observation that oxidative burst in fact is beneficial in controlling autoimmune responses. For example, Dark Agouti rats and B10.Q mice carrying a mutation in Ncf1, the gene encoding for $p47^{phox}$ subunit of NADPH oxidase 2 (Nox 2 alias $gp91^{phox}$), show a reduced oxidative burst and become highly susceptible to T cell mediated autoimmune diseases such as collagen-induced arthritis and experimental autoimmune encephalomyelitis (EAE) (Hultqvist et al., 2004; Gelderman et al., 2006; Holmberg et al., 2006; Gelderman et al., 2007; Sareila et al., 2011). On the contrary, mice deficient for $gp91^{phox}$ are resistant to the development of EAE due to reduced peroxynitrite formation as a result of decreased superoxide production (Li et al., 2011). However, similar to EAE models, MS lesions contain ROS, and show the expression of cyclooxygenase-2, inducible nitric oxide synthase, catalase, heme oxygenase and SOD1; and oligodendrocytes have highest activities of catalase, GPx and SOD2 (Carlson and Rose, 2006). Although a variety of antioxidants have been shown to prevent and/or suppress severity of EAE, the use of dietary antioxidants such as vitamins C and E, and lipoic acid evinced mixed results in MS patients (Gilgun-Sherki et al., 2004; van Meeteren et al., 2004; Schreibelt et al., 2007). Furthermore, in EAE models, therapeutic administration of SOD yielded little or no effects,

but was found to be beneficial in disease models for experimental optic neuritis and uveoretinitis including neuronal injuries (Guy et al., 1986; Yamada et al., 1986; Guy et al., 1989; Barkats et al., 1996; Chan et al., 1998; Qi et al., 2007). These observations raise a question as to the functional role of SODs in various organ-specific autoimmune diseases.

In this study, we have addressed the role of SOD1, an endogenous regulator of ROS, in the mediation of central nervous system (CNS) autoimmunity. We demonstrate that SOD1-deficient C57B1/6 (SOD1-KO) mice develop more severe EAE induced with myelin oligodendrocyte glycoprotein (MOG) 35–55 when compared with their littermate wild type controls. Such an alteration in the disease phenotype is due to neither overt increase in the expansion of MOG-specific T cells, as evaluated by major histocompatibility complex (MHC) class II dextramers staining nor their ability to produce high amounts of proinflammatory cytokines.

2. Materials and methods

2.1. Mice

SOD1-KO mice were procured from the Jackson Laboratory (Bar Harbor, ME), and the animals were maintained in accordance with the animal protocol guidelines of the University of Nebraska-Lincoln, Lincoln, NE. All experiments involved the use of 3-to 5-month-old male SOD1-KO mice and their littermate controls on C57B1/6 genetic background carrying H-2^b haplotype.

2.2. Peptide synthesis and immunization procedures

MOG 35–55 (MEVGWYRSPFSRVVHLYRNGK) and ovalbumin (OVA) 323–339 (ISQAVHAAHAEINEAGR) were synthesized on 9-fluorenylmethyloxycarbonyl chemistry (Neopeptide, Cambridge, MA). All peptides were HPLC-purified (>90%), confirmed by mass spectroscopy and dissolved in sterile 1× PBS prior to use. To measure recall responses, MOG 35–55 emulsified in complete Freund's adjuvant (CFA) was administered s.c., in the inguinal and sternal regions (100 μg per mouse). Disease was induced with peptide emulsified in CFA supplemented with *Mycobacterium tuberculosis* H37RA extract (Difco Laboratories, Detroit, MI) to a final concentration of 5 mg/ml (200 μg/mouse). Additionally, pertussis toxin (PT; List Biological Laboratories, Campbell, CA) was administered i.p., (200 ng/mouse) on day 0 and day 2 postimmunization (Massilamany et al., 2011c).

2.3. Clinical scoring and histopathology

Following EAE induction, the mice were monitored for clinical signs of disease and scored as previously described (Mendel et al., 1995; Massilamany et al., 2011c): 0, no signs of disease; 1, limp tail or hind limb weakness but not both; 2, limp tail and hind limb weakness; 3, partial paralysis of hind limbs; 4, complete paralysis of hind limbs; and 5, moribund. At necropsy, brains and spinal cords were collected in 10% phosphate buffered formalin and analyzed for histological evidence of inflammation. After fixation, brain sections were made through the cerebrum and hippocampus, and the cerebellum and brainstem. Three sections also were made from each segment of the spinal cord including the cervical, thoracic, lumbar and sacral regions. The tissues were stained by hematoxylin and eosin (H and E)

staining. Lesion types were characterized and severity was scored by an observer blinded to treatment. Severity scores were obtained by counting inflammatory foci in both meninges and parenchyma for all sites. Inflammation type was categorized as lymphocytic, suppurative or mixed. Counts were then added across all sections of brain and spinal cord for each mouse (Massilamany et al., 2010, 2011a). Additionally, to verify demyelination, spinal cord sections obtained from wild type and SOD1-KO mice were examined by luxol fast blue staining (Sobel et al., 1990).

2.4. T cell proliferation assay

Groups of wild type and SOD1-KO mice were immunized with MOG 35–55 and euthanized 10 days later; the draining lymph nodes (LN) were harvested to prepare single cell suspensions (Massilamany et al., 2010). Lymph node cells (LNC) were stimulated with MOG 35–55 and OVA 323–339 (0–100 μg/ml) at a density of 5×10^6 cells/ml for two days in growth medium containing 1× RPMI medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 4 mM L-glutamine, 1× each of non-essential amino acids and vitamin mixture, and 100 U/ml penicillin-streptomycin (Lonza, Walkersville, MD). Cultures were then pulsed with 1 μCi of tritiated ^{3}[H] thymidine/well (MP Biomedicals, Santa Ana, CA); 16 h later, the proliferative responses were measured as counts per minute (cpm) using a Wallac liquid scintillation counter (Perkin Elmer, Waltham, MA; Massilamany et al., 2010, 2011b). To determine proliferative responses to polyclonal activators, splenocytes obtained from naive wild type and SOD1-KO mice were stimulated with anti-CD3 (clone, 145-2C11: 0–5 μg/ml; eBioscience, San Diego, CA) or lipopolysaccharide (LPS; 0–1 μg/ml; Sigma-Aldrich, St. Louis, MO) for two days in growth medium, and the proliferative responses were measured by pulsing the cells with ^{3}[H] thymidine as described above.

2.5. Immunophenotyping

Splenocytes from naive wild type and SOD1-KO mice were stained with cocktails of antibodies for various immune cell markers, specifically, T cells (CD3, CD4 and CD8) and non-T cells (B220, CD11b, CD11c and GR1) (all antibodies from eBioscience), and 7-aminoactinomycin-D (7-AAD; Invitrogen, Carlsbad, CA). After cells were acquired by flow cytometry (FACSCalibur, BD Biosciences, San Diego, CA), percentages of cells positive for each marker were determined in the live (7-AAD$^-$) population using Flow Jo software (Tree Star, Ashland, OR).

2.6. Enumeration of the frequencies of MOG 35–55-specific CD4 T cells by MHC class II dextramer staining through flow cytometry

MHC class II dextramers comprising IA^b/MOG 35–55 and IA^s/Theiler's murine encephalomyelitis virus (TMEV) 70–86 were prepared utilizing biotinylated soluble monomers covalently tethered to the respective peptides as we have recently described (Massilamany et al., 2011c). Biotinylated monomers tethered to the respective peptides were incubated with activated dextran molecules to derive dextramers (Massilamany et al., 2011b, 2011c). To enumerate the frequencies of antigen-specific cells, LNC obtained from wild type or SOD1-KO mice immunized with MOG 35–55 were stimulated with MOG 35–55 (20 μg/ml) for two days, and the cultures were maintained in growth medium containing interleukin (IL)-2, hereafter called as IL-2 medium (eBioscience; Advanced

Biotechnologies, Columbia, MD). On day six poststimulation, viable lymphoblasts were stained with IA^b/MOG 35–55 and IA^s/TMEV 70–86 (control) dextramers, anti-CD4 (eBioscience), and 7-AAD (Massilamany et al., 2011c; Gangaplara et al., 2012). Cells were acquired by flow cytometry, and the percentages of dextramer-positive ($dext^+$) cells were then determined in the live (7-AAD^-) $CD4^+$ population.

2.7. Determination of the frequencies of MOG 35–55-specific T cells in CNS infiltrates

Groups of mice were immunized with MOG 35–55 (200 μg), and PT (200 ng) was administered i.p., as above. Mice showing clinical signs of EAE scored 3 to 4 were euthanized, and the hearts were perfused through injection of 10 ml of 1× cold PBS into the left ventricles. Brains were harvested by blunt dissection, and the spinal cords were flushed using 1× cold PBS. The CNS tissues were homogenized and digested with type IV collagenase (400 U/ml; Worthington, Lakewood, NJ) at 37 °C for 1 h, and the mononuclear cells (MNC) were isolated by percoll density gradient centrifugation (70%/30%; Massilamany et al., 2011c). To enumerate the frequencies of MOG 35–55-specific T cells, MNC were stained with IA^b/MOG 35–55 and IA^s/TMEV 70–86 dextramers, anti-CD4 and 7-AAD; the percentages of MOG 35–55 $dext^+$ cells were then enumerated in the live (7-AAD^-) $CD4^+$ subset as above.

2.8. Analysis of cell viability

Splenocytes were prepared from naive wild type and SOD1-KO mice. The cells were cultured in 96-well culture plates at a density of 2×10^6 cells/ml, in triplicates, each containing 200 μl cell suspension in the presence of anti-CD3 (0–1 μg/ml) for 48 h. After the cells were washed with 1× PBS at indicated time points, the cell pellets were suspended in 200 μl of 1× PBS, to which 7-AAD (1:500) was added. Cells were acquired by flow cytometry three times to obtain 10,000 events for each replicate. The percent live (7-AAD^-) and dead (7-AAD^+) cells were determined by plotting the cells that were positive for 7-AAD against forward scatter (FSC) (Reddy et al., 2001; Kruisbeek, 2006). Finally, average values were obtained for all three replicates for each sample, and the mean ± SEM values were then derived for a group of mice.

2.9. Intracellular cytokine staining

Groups of wild type and SOD1-KO mice were immunized with MOG 35–55; after 10 days, the animals were euthanized, and draining LN were harvested to prepare single cell suspensions. Cells were stimulated with MOG 35–55 (20 μg/ml) for two days and maintained in IL-2 medium. On day five poststimulation, viable lymphoblasts were stimulated with phorbol 12-myristate-13 acetate (PMA, 20 ng/ml) and ionomycin (300 ng/ml; Sigma-Aldrich) for 5 h in the presence of 2 mM monensin (GolgiStop, BD Pharmingen, San Diego, CA). After staining with anti-CD4 and 7-AAD, cells, were fixed, permeabilized and stained with anti-cytokine antibodies along with their respective isotype controls (eBioscience) (Massilamany et al., 2010, 2011a). The cells were then acquired by flow cytometry, and the frequencies of cytokine-producing cells were determined in the live (7-AAD^-) $CD4^+$ subset. The clones of cytokine antibodies used were: IL-2 (JES6-5H4), interferon (IFN)-γ (XMG1.2), IL-4 (11B11), IL-10 (JES5-16E3), IL-17A (eBio 17B7), IL-17F (eBio 18F10), granulocyte macrophage-colony stimulating factor (GM-CSF)

(MP1-22E9), tumor necrosis factor (TNF)-α (MP6-XT22) (all from eBioscience), and IL-22 (140301; R&D Systems, Minneapolis, MN).

2.10. Statistics

Differences in T cell proliferative responses, $dext^+$ cells, cell viability and cytokine-producing cells were determined using Student's t-test. Wilcoxon rank sum test was used to compare differences in the severity of clinical and histological EAE induced by immunization with MOG 35–55. The time-course analysis of the clinical disease involved division of the effector phase of EAE into three intervals (days 10–16, days 17–23 and days 24–30); the median values of clinical scores obtained for each period for wild type and SOD1-KO mice were compared (Massilamany et al., 2011b). $p \leq 0.05$ values were considered significant.

3. Results

3.1. SOD1-deficient mice develop severe EAE induced with MOG 35–55

Although oxidative stress has been implicated in the pathogenesis of various degenerative and inflammatory diseases, recent data demonstrating the beneficial effects of ROS in self-tolerance provided a compelling reason to investigate the role of SOD1 in the development of CNS autoimmunity. We hypothesized that mice deficient for SOD1 become more susceptible than wild type mice to autoimmune encephalomyelitis induced with MOG 35–55, and this was the case (Fig. 1a). While the incidence (100%), mean day of EAE onset (12.21 vs. 12.20) and mean maximum scores (3.93 vs. 4.40) were comparable in both wild type and SOD1-KO mice (Table 1), the severity of clinical disease was higher in SOD1-KO than in wild type mice (Fig. 1a). This was ascertained by comparing the mean neurological scores representing three different time periods of clinical EAE: days 10–30 ($p \leq 1.97E{-}05$); days 17–23 ($p \leq 4.69E{-}04$), and days 24–30 ($p \leq 3.86E{-}03$). Evaluation of the CNS tissues for inflammatory infiltrates further verified EAE-severity, revealing that brains and spinal cords of SOD1-KO mice—in particular the meninges—tended to contain an increased number of inflammatory foci, compared to wild type mice (Table 1). We also noted that the spinal cords obtained from both wild type and SOD1-KO mice contained areas of demyelination accompanied with the distended myelin sheaths (Fig. 1b). Likewise, infiltrates predominantly comprised of lymphocytes and macrophages were similar in nature in both groups of mice, raising the question as to the mechanisms underlying their disease phenotypes.

3.2. CNS infiltrates from SOD1-KO mice immunized with MOG 35–55 contain low frequencies of antigen-specific T cells

MOG 35–55-induced EAE is typically mediated by CD4 T cells, although recent data indicate that CD8 T cells responding to MOG 40–54 can induce similar disease (Sun et al., 2001, 2003). As the EAE-severity was greater in SOD1-KO than in wild type mice, we hypothesized that a larger number of MOG-specific T cells could infiltrate into the CNS of SOD1-deficient animals and mediate the disease. To test this hypothesis, we created IA^b/MOG 35–55 dextramers and analyzed by flow cytometry the frequencies of MOG-specific T cells using MNC harvested from brains and spinal cords of wild type and SOD1-

KO mice. We noted that the total number of MNC recovered from wild type and SOD1-KO mice to be comparable ($2.5\pm0.46\times10^6$ vs. $1.7\pm0.23\times10^6$). But, unexpectedly, the CNS infiltrates obtained from SOD1-KO mice contained low numbers of MOG 35–55 $dext^+$ T cells (0.2±0.04%), as compared with wild type mice (0.85±0.02%; Fig. 2). The data suggest either that the antigen-sensitized lymphocytes generated in SOD1-KO mice may have an inherent defect in their expansion or that the cells die rapidly in a SOD1-deficient microenvironment.

3.3. Immune cells generated in SOD1-KO mice respond poorly to antigens

Detection of a low frequency of MOG-specific T cells in SOD1-KO mice prompted us to ask whether they differ from wild type mice in their immune cell subsets or their ability to respond to antigens. First, we verified that the proportions of various immune cells (T cells and non-T cells) were similar in both groups of mice, except that $CD8^+$ T cells would be lower in SOD1-KO than in wild type mice ($p=0.003$; Table 2). Second, we measured antigen-specific proliferative responses by stimulating LNC obtained from wild type and SOD1-KO mice immunized with MOG 35–55. Fig. 3 shows that LNC obtained from both groups of mice exhibited an antigen-specific, dose-dependent response to MOG 35–55, since MOG-sensitized T cells did not respond to a control antigen (OVA 323–339). Although the magnitude of proliferative responses in wild type mice was greater than in SOD1-KO mice ($p<0.05$), the fold induction over background values in medium controls was comparable (~5-fold vs. ~6-fold at high antigenic dose, 100 μg; Fig. 3). One potential possibility for this diminished response in SOD1-KO mice is the apparent presence in their naïve repertoires of low frequencies of CD8 T cells (Table 2: 9.04 ± 0.23% vs. 13.50±0.67%, $p=0.003$), which can respond to MOG 40–54 that lies within MOG 35–55. Therefore, we considered two polyclonal activators, anti-CD3 and LPS, to stimulate splenocytes obtained from wild type and SOD1-KO mice. By measuring the proliferative responses based on ^{3}[H] thymidine-incorporation assay, it was evident that splenocytes from wild type and SOD1-KO mice responded equally to anti-CD3 when compared with their corresponding medium controls, but the proliferative response to LPS in wild type mice tended to be greater than that achieved with cells obtained from SOD1-KO mice (~8-fold vs. 5-fold; Fig. 3b). However, similar to MOG 35–55, the magnitude of responses obtained for anti-CD3 or LPS stimulations was consistently higher in cells generated from wild type than from SOD1-KO mice (anti-CD3, 2-fold; LPS, 2.5-fold; $p=0.014$). As noted above, while low numbers of CD8 T cells can still account for reduced response to anti-CD3 in SOD1-KO mice (Fig. 3b, left panel), the data obtained with LPS stimulation (Fig. 3b, right panel) imply an alternative interpretation. That is, immune cells from SOD1-KO mice may have a higher threshold for antigenic stimuli, or the cells may become vulnerable for activation-induced cell death (AICD).

3.4. T lymphocytes generated in SOD1-KO mice are prone to spontaneous cell death

To address the possibility that the diminished proliferative response of lymphocytes generated in SOD1-KO mice may be due to their susceptibility to AICD, we determined viability of cells using anti-CD3 as a polyclonal activator of T cells. Cell viability was assessed in cultures stimulated with or without anti-CD3 by flow cytometry using 7-AAD as a cell death marker, and the percent of dead cells was derived using the mean values of three

replicates in each treatment (Fig. 4). We noted that when the cells were stimulated with a low dose of anti-CD3, the number of dead cells at both 24 and 48 h was higher in cultures prepared from wild type than from SOD1-KO mice (0.5 μg/ml); at a high dose, the pattern was reversed (1.0 μg/ml) ($p<0.05$). Strikingly, however, in the medium controls containing no antigenic stimulus, a high percent of dead cells was found in cultures derived from SOD1-KO mice, in contrast to wild type mice, at both 24 h ($p=0.058$) and 48 h ($p=0.018$). Furthermore, at 24 h poststimulation with anti-CD3 in the medium controls, cell death in SOD1-KO mice, in contrast to wild type animals, did not increase at a low dose (0.5 μg/ml) and only marginally increased at a high dose (1.0 μg/ml) (Fig. 4, left panel). While such a trend was not apparent at 48 h, the percent of dead cells remained constant regardless of doses of anti-CD3 used for stimulation (Fig. 4, right panel). Nonetheless, the percent of dead cells resulting from AICD in SOD1-KO mice was consistently high in cultures stimulated with a high dose of anti-CD3 (1.0 μg/ml) when compared with wild type mice ($p<0.05$). Taken together, the data suggest that T cells in the absence of SOD1 are prone to spontaneous death, and the threshold for their activation with an antigenic stimulus is higher than in wild type mice, in which a low dose of anti-CD3 is sufficient to achieve the same effect. Finally, by relating the proliferative responses (Fig. 3) and cell viability analysis (Fig. 4), we concluded that the reduced antigen-specific responses observed in SOD1-KO mice are attributable to their susceptibility to spontaneous cell death, making fewer cells available for antigenic stimulation in relation to wild type mice.

3.5. Enhanced susceptibility to MOG 35–55-induced EAE in SOD1-KO mice is not due to their increased ability to produce inflammatory cytokines that favor CNS autoimmunity

It is widely accepted that EAE is typically mediated by cytokines produced by both Th1 and Th17 cells (Bettelli et al., 2007; Stromnes et al., 2008). To determine if the increased EAE-severity in SOD1-KO mice is due to increased production of proinflammatory cytokines, we evaluated the precursor frequencies of cytokine-producing cells by flow cytometry. The cytokine panel included Th1 (IL-2 and IFN-γ), Th2 (IL-4 and IL-10) and Th17 (IL-17A, IL-17F and IL-22), GM-CSF, and TNF-α. As shown in Fig. 5, cells capable of producing all the cytokines tested, except for IL-4, were present in the cultures prepared from both wild type and SOD1-KO mice. However, the frequencies of cells producing each of the individual cytokines, in particular, IL-2 ($p=0.057$) and IL-10 ($p=0.014$), were consistently lower in SOD1-KO than wild type mice. We next derived the frequencies of cytokine-producing cells representing all the three Th subsets, Th1, Th2 and Th17, by pooling the percent of cytokine-producing cells corresponding to each (Th1, IL-2 and IFN-γ; Th2, IL-4 and IL-10; and Th17, IL-17A, IL-17F and IL-22). As expected, frequencies of cells producing these cytokines were found to be lower in SOD1-KO mice than in wild type mice (Th1, 28.17±1.14 % vs. 17.22±4.09 %, $p=0.06$; Th2, 1.34±0.14 % vs. 0.74±0.05%, $p=0.02$; and Th17, 7.36±0.44 % vs. 3.36±0.71 %, $p=0.01$; Fig. 5). Thus, the data do not point to a role for inflammatory cytokines as contributing factors for the increased severity of EAE noted in SOD1-KO mice.

3.6. Antigen-specific T cells derived from SOD1-KO mice have no defect in their ability to respond to an antigen, but their expansion requires supplementation of T cell growth factor, IL-2

To address the possibility that the reduced expansion of MOG-specific T cells in SOD1-KO mice might be due to their inability to produce sufficient amounts of IL-2, we examined antigen-specific T cell expansion by supplementing the cultures with IL-2 as a growth factor. LNC obtained from wild type and SOD1-KO mice immunized with MOG 35–55 were stimulated with peptide, and the cultures were maintained in IL-2 medium in which frequencies of MOG specific T cells were enumerated by IA^b/dextramer staining. The analysis revealed comparable frequencies of MOG 35–55 $dext^+$ CD4 T cells in cultures derived from both wild type and SOD1-KO mice (2.76±0.36% vs. 2.43±0.68%). The dextramer staining was specific since the control IA^s/TMEV 70–86 dextramers did not bind MOG-sensitized cells (Fig. 6). These observations suggest that the antigen-sensitized T cells generated in SOD1-KO mice have no inherent defect in their ability to respond to an antigen, but the limited availability of IL-2 in the microenvironment can negatively affect their expansion.

4. Discussion

Using MOG 35–55-induced EAE as the disease model for MS, we have addressed the role of SOD1 in the development of CNS autoimmunity. Both wild type and SOD1-KO mice developed EAE signs similarly, i.e., progressive paralysis including the nature of CNS infiltrates. While increased disease severity was expected in SOD1-KO mice, owing to their ability to produce high amounts of ROS (Iuchi et al., 2008; Meissner et al., 2008; Fischer et al., 2011; Murakami et al., 2011; Fischer et al., 2012), determination of mechanisms underlying such an altered disease phenotype led to several key findings: a) CNS infiltrates of SOD1-KO mice contained fewer MOG-specific T cells as compared to wild type mice; b) immune cells from SOD1-KO mice responded poorly to antigens; c) MOG 35–55-sensitized T cells generated in SOD1-KO mice contained low frequencies of Th1, Th2 and Th17 cytokine-producing cells; and d) immune cells from SOD1-KO mice were found to be more prone to spontaneous cell death, and their threshold for antigenic stimulation was higher than that of wild type mice. These observations provide new insights into the role of SOD1 in the maintenance of self-tolerance and development of immune-mediated diseases, given that SOD1 is one of the important endogenous regulators of the formation of pro-oxidants.

MOG 35–55-induced EAE is mediated primarily by T cells through the production of Th1 and Th17 cytokines (Langrish et al., 2005; Jager et al., 2009). Numerous studies indicate that SOD1-KO mice produce excessive amounts of ROS (Iuchi et al., 2008; Meissner et al., 2008; Fischer et al., 2011; Murakami et al., 2011; Fischer et al., 2012). We verified that macrophages derived from these animals produce relatively higher amounts of ROS when compared with wild type mice (data not shown). This may be one potential reason for enhanced EAE-severity noted in SOD1-KO mice. But surprisingly, such an alteration in the disease phenotype occurred independent of antigen-specific T cell expansion, as well as cytokines that favor CNS autoimmunity, suggesting that SOD1 can modulate inflammatory processes in multiple ways. It has been shown that mice deficient for SOD1 show reduced

caspase-1 activity and reduced production of the caspase-1-dependent cytokines IL-1β and IL-18 (Meissner et al., 2008). Conversely, lack of or reduced SOD1 activity was associated with increased TNF-α and IL-6 synthesis with a concurrent increase in nuclear factor-κB (NF-κB) activity (Ishigami et al., 2011). In our study, whereas the number of TNF-α-producing cells was unchanged between wild type and SOD1-deficient mice, we noted in the SOD1-KO mice reduced frequencies of cells producing encephalitogenic cytokines, IFN-γ (Th1), IL-17A, IL-22 (Th17) and GM-CSF, including EAE-suppressing cytokines, IL-4 and IL-10 (Th2) (Fig. 5). However, by analyzing the differences at the level of individual cytokines, it was apparent that frequencies of IL-2- and IL-10-producing cells were significantly reduced in SOD1-KO as compared with wild type mice. IL-10 is an anti-inflammatory cytokine, and mice deficient for IL-10 show enhanced levels of O_2^-(Gunnett et al., 2000). Therefore, reduced synthesis of IL-10 in SOD1-KO mice could possibly potentiate their ability to produce high amounts of O_2^-, an environment conducive for predisposition to tissue destruction. IL-2, on the other hand, is a T cell growth factor, and the presence of reduced frequencies of IL-2-producing cells in MOG 35–55-responsive cultures derived from SOD1-KO mice may have contributed to the lower expansion of antigen-specific T cells noted in these mice. These data raise a question as to the relationship between SOD1 activity and IL-2 or IL-10 secretion, given that frequencies of cells producing these cytokines were low in SOD1-deficient mice. Our data point to a role for SOD1 in the synthesis of IL-2 based on an observation that Jurkat cells (human T lymphocyte cell line) cultured in a copper-deficient medium showed reduced synthesis of IL-2, as well as instability of IL-2 transcripts (Hopkins and Failla, 1997). Since IL-2 can mediate the dual functions of cell proliferation and cell death during the course of antigen-specific T cell expansion (Zambricki et al., 2005), underproduction of IL-2 can alter these functions, thereby affecting cell survival.

In evaluating cell viability, we noted that a significantly higher proportion of lymphocytes generated in SOD1-deficient mice were dying spontaneously than those generated in wild type mice (Fig. 4). This was consistent with an observation that SOD1-deficient mice show a higher degree of apoptosis of lymphocytes in the thymus than in spleens (Freeman et al., 2000). Such an effect on apoptosis also was evident with the proliferating lymphocytes in mice overexpressing SOD1 (Pahlavani et al., 2001), implying that too much or too little SOD1 can have similar consequences depending on the nature and activation status of cells. In our studies, we noted by immunophenotyping that only the number of CD8 T cells was lower in SOD1-KO than in wild type mice. Whether SOD1-deficiency can selectively affect T cell maturation processes, particularly of CD8 T cells within the thymus, requires additional studies. Such an imbalance in the proportions of any one specific subset in the peripheral repertoire is unlikely to be due to the inherent ability of SOD1-deficient mice to produce excessive amount of ROS.

Upon cross-linking, T cells can produce H_2O_2 and $O2^-$ and respectively activate FAS ligand (apoptotic) and extracellular-signal-regulated kinase (ERK)1/2 (proliferative) pathways (Devadas et al., 2002); however, ROS can trigger apoptosis of T cells independent of FAS and TNF-pathways (Hildeman et al., 1999). Furthermore, oxidative stress can induce T cell hyporesponsiveness as noted in rheumatoid arthritis, cancer, leprosy and acquired immunodeficiency syndrome (Cemerski et al., 2002, 2003); these hyporesponsive T cells

show an impairment of calcium mobilization and selective downmodulation of T cell signaling molecules, suggesting that the functional consequences of ROS generation can vary from proliferation to cell death. Two observations—that SOD1-KO mice produce more ROS than wild type mice (Iuchi et al., 2008; Meissner et al., 2008; Murakami et al., 2011; Fischer et al., 2012) and that the lymphocytes undergo spontaneous cell death, showing a high threshold for their activation (Fig. 4)—support the notion that lymphocytes become hyporesponsive to antigenic stimulation in the presence of supraphysiologic doses of ROS produced in a SOD1-deficient environment. These events can then possibly trigger apoptotic pathways leading to the death of antigen-activated cells. Such an effect can further be precipitated by other factors like insufficient IL-2 production as noted in our studies (Fig. 5). In support of this notion, we verified that supplementation of IL-2 to MOG 35–55-sensitized T cells derived from SOD1-KO and wild type mice led to their comparable expansion in both groups of mice (Fig. 6).

In summary, we provide evidence that mice deficient for SOD1 become more susceptible to CNS autoimmunity, but our data do not point to a primary role for T cell-derived factors like cytokines as mediators of disease. Instead, the inherent ability of SOD1-KO mice to produce excessive amounts of ROS appears to play a key role in disease induction. However, since EAE-severity was modestly increased in SOD1-KO mice as compared with wild type mice, other anti-oxidant defense systems may compensate for the loss of SOD1 deficiency, but these compensatory mechanisms appear not enough. Previous reports indicate that SOD1 deficiency can augment inflammation, possibly by increasing the expression of adhesion molecules, vascular endothelial growth factor and metalloproteinases, and also by modulating NF-κB-dependent genes and chemokines and their receptors (Siomek et al., 2010; Ishigami et al., 2011). SOD1 deficiency can lead to reduced life span of erythrocytes and autoantibody production, likely as a result of the immune system perceiving oxidized proteins as altered self-proteins (Iuchi et al., 2009). Whether MOG 35–55, which was used as an immunogen to induce EAE in our studies, undergo similar changes leading to an altered recognition by SOD1-deficient, antigen-presenting cells remains to be determined. Alternatively, when O_2^-, the major substrate for SOD1 is increased in a SOD1-deficient environment, it is expected to react with nitric oxide, leading to the formation of peroxynitrite. Being highly toxic, peroxynitrite can potentiate tissue damage and inflammation while making antigen-specific T cells susceptible to cell death due to their continuous exposure to ROS in the absence of endogenous regulators of pro-oxidants like SODs. Taken together, our data provide new insights into the mechanisms by which SOD1 or its mimetics can modulate organ-specific autoimmune and inflammatory diseases.

Acknowledgments

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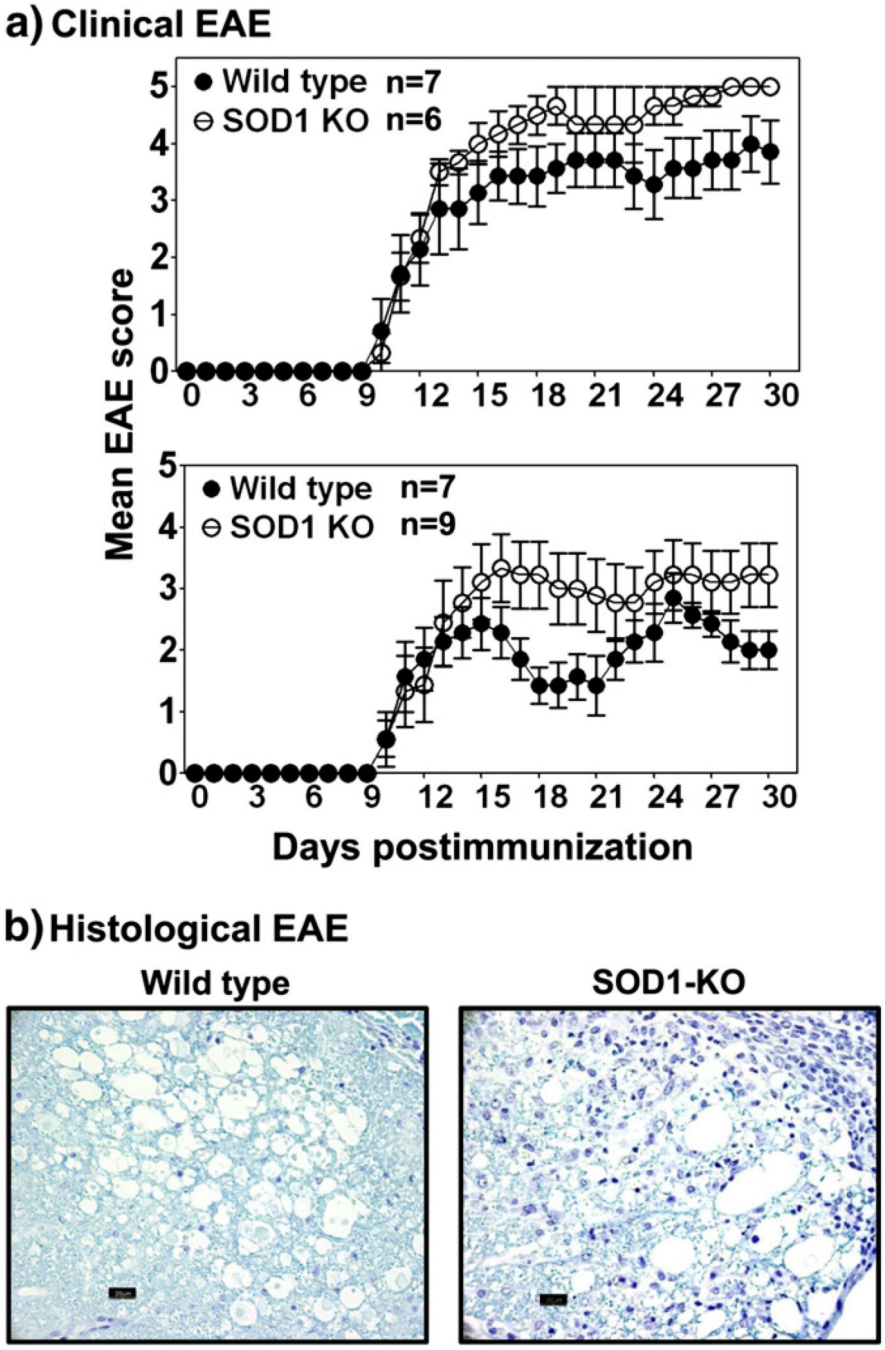

Fig. 1.

SOD1-KO mice show increased susceptibility to MOG 35–55-induced EAE. (a) Clinical EAE. Groups of male wild type and SOD1-KO mice were immunized with 200 μg of MOG 35–55 in CFA s.c., and PT (200 ng) was administered on days 0 and 2 postimmunization. The mice were monitored for clinical signs of EAE and scored. Mean±SEM values obtained for a group of mice from two individual experiments are shown. (b) Histological EAE. Spinal cord sections from the representative animals corresponding to wild type and SOD1-KO mice were examined by luxol fast blue staining, and the sections indicate demyelinated

areas containing swollen myelin sheaths, occasionally, swollen axons. Original magnification, ×400 (bar=20 μm).

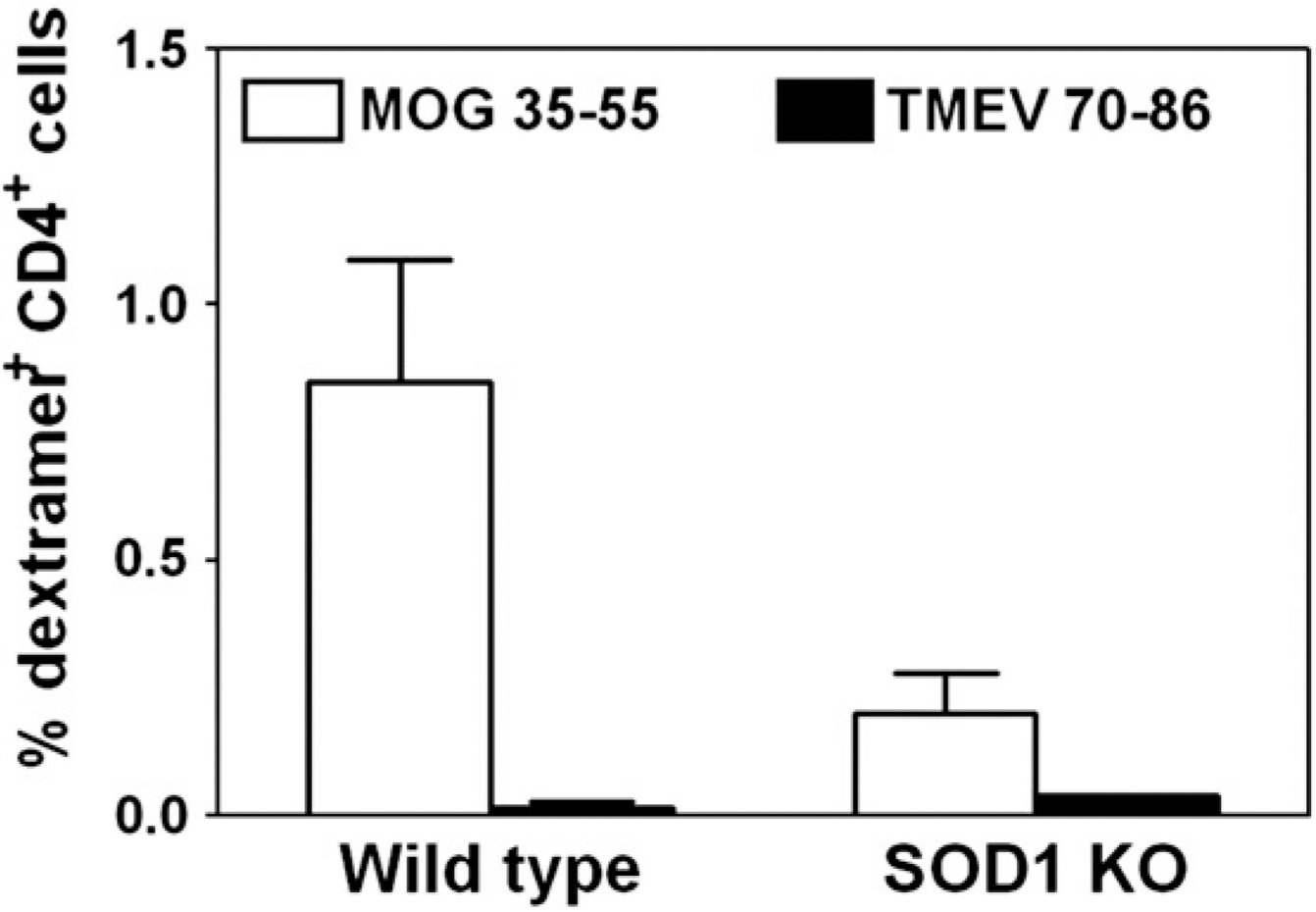

Fig. 2.

CNS infiltrates obtained from MOG 35–55-induced EAE in SOD1-KO mice contain low frequencies of antigen-specific T cells. Groups of wild type and SOD1-KO mice were immunized with MOG 35–55 in CFA s.c., and PT was administered on days 0 and 2 postimmunization. Brains and spinal cords were harvested from EAE mice that scored 3 to 4, and MNC were isolated by percoll density-gradient centrifugation. Cells were stained with MOG 35–55 and TMEV 70–86 dextramers, followed by staining with anti-CD4 and 7-AAD. After the cells were acquired by flow cytometry, percentages of $dext^+$ cells were determined in the live ($7\text{-}AAD^-$) CD4 subset. Mean±SEM values are shown (n=2).

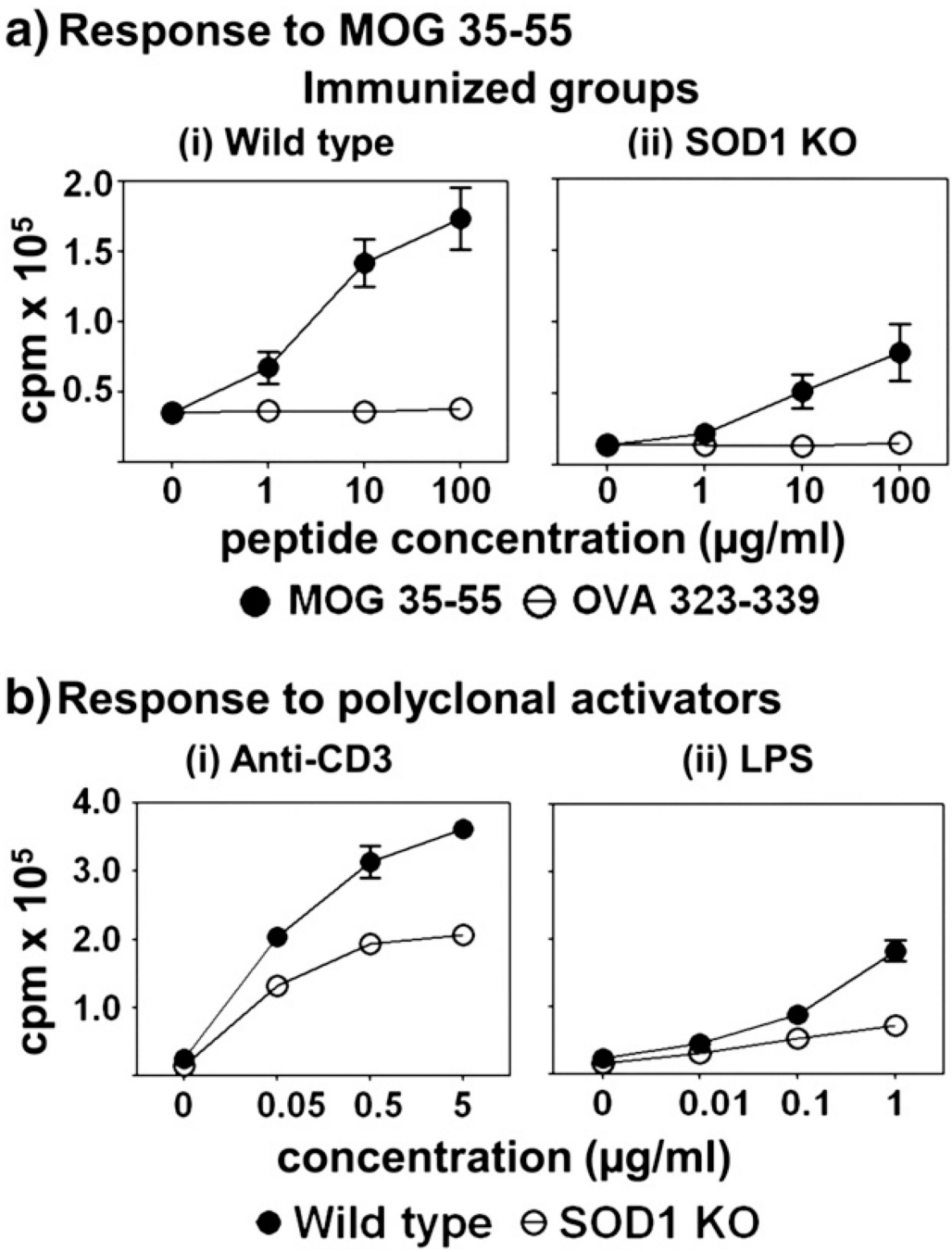

Fig. 3.

Immune cells generated in SOD1-KO mice respond poorly to antigens. (a) Response to MOG 35–55. Groups of wild type and SOD1-KO mice were immunized with MOG 35–55 in CFA. After 10 days, draining LN were harvested and LNC were prepared. Cells were stimulated with MOG 35–55 and OVA 323–339 (control) for two days, followed by pulsing with ^{3}[H]-thymidine. After 16 h, proliferative responses were measured as cpm. (b) Response to polyclonal activators. Splenocytes obtained from naïve wild type and SOD1-KO mice were stimulated with anti-CD3 or LPS at the indicated doses for two days, and the

proliferative responseswere measured as above. Mean±SEM values for a group of mice are shown (n=3).

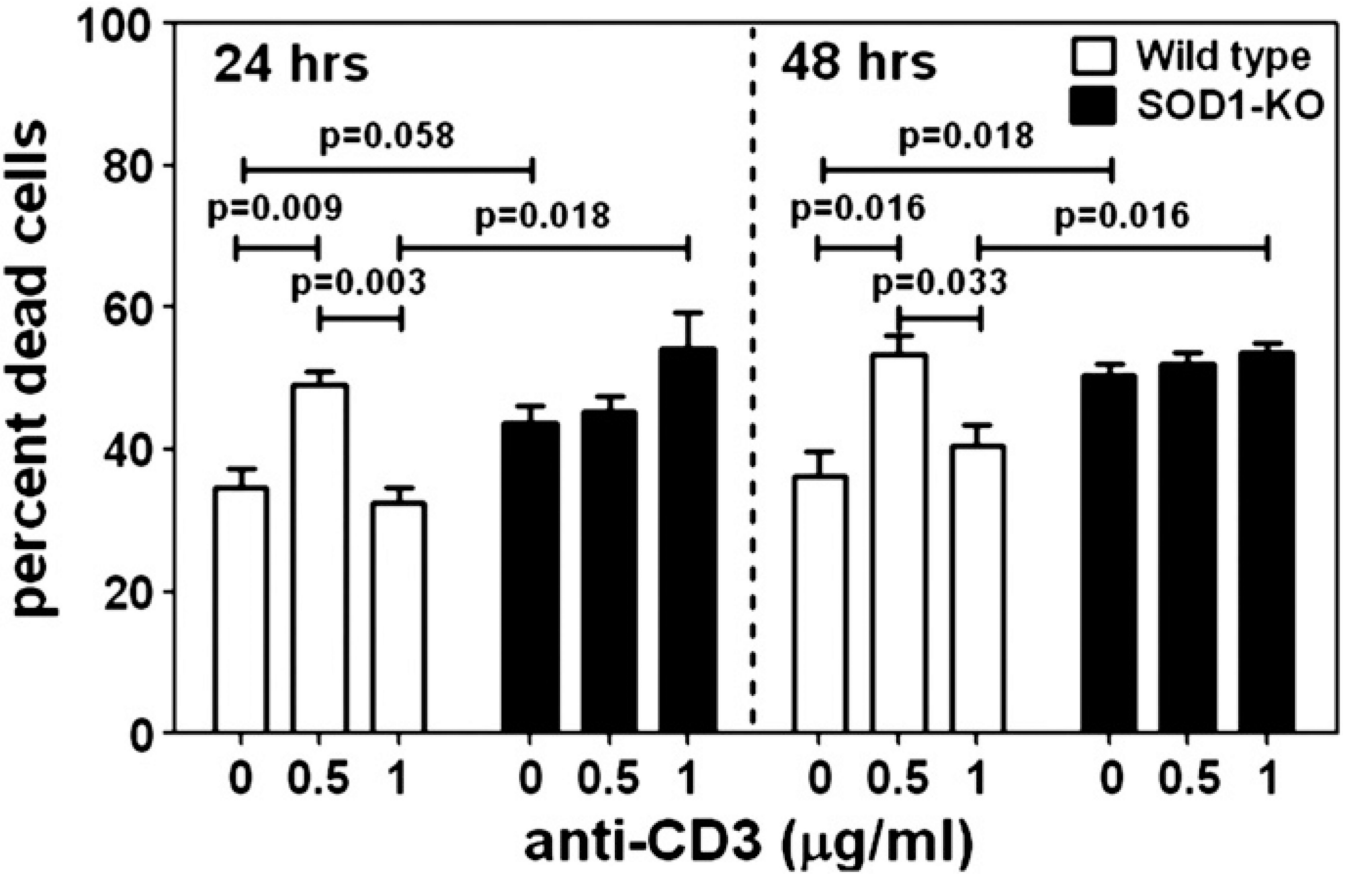

Fig. 4.

T Lymphocytes generated in SOD1-KO mice have a higher threshold for AICD than lymphocytes generated in wild type mice. Splenocytes obtained from naïve wild type and SOD1-KO mice were stimulated with anti-CD3 for 48 h in triplicates, each containing 200 μl cell suspensions at a density of 2×10^6 cells/ml. At indicated time points, cells were washed and stained with 7-AAD, and after the cells were acquired by flow cytometry, percentages of live and dead cells were derived by plotting 7-AAD^+ cells vs. FSC. The average values obtained from a pool of three replicates were then used to determine mean ±SEM values for a group of mice (n=3).

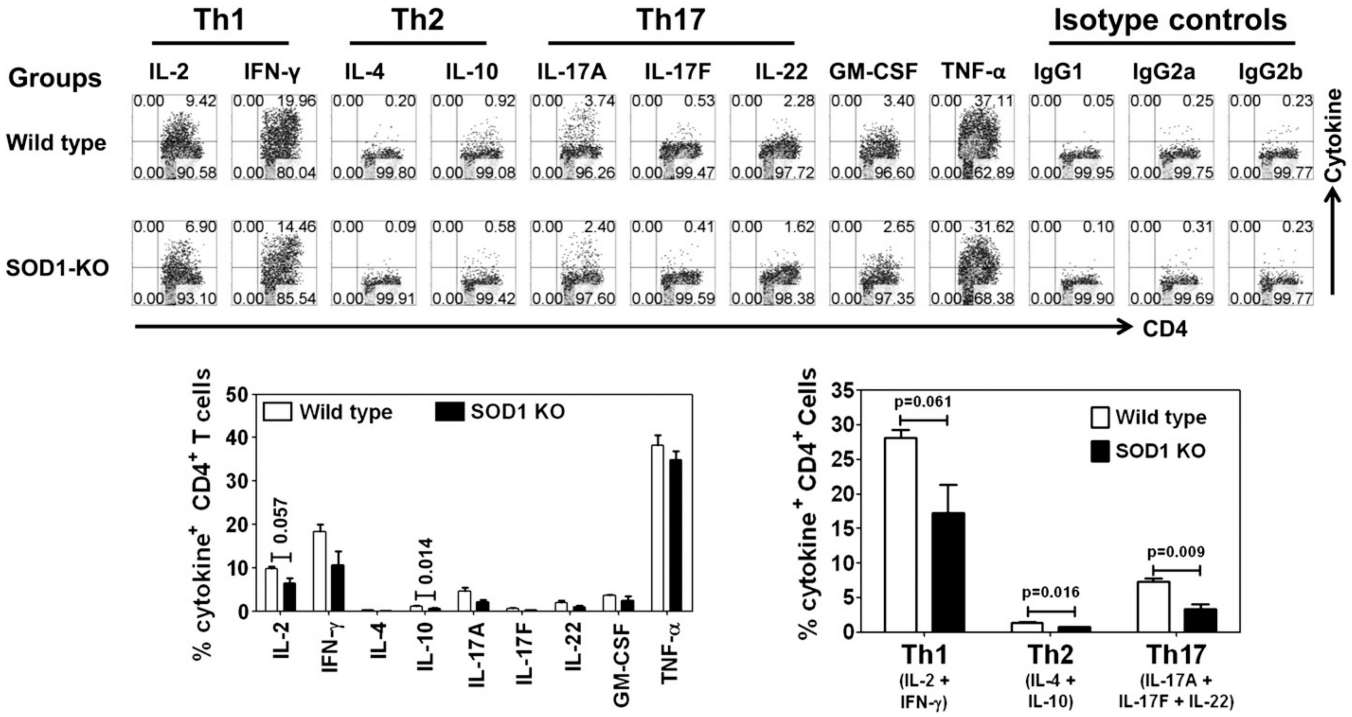

Fig. 5.

Frequencies of Th1- and Th17-cytokine-producing cells were low in SOD1-KO mice. LNC obtained from MOG 35–55-immunized wild type and SOD1-KO mice were stimulated with MOG 35–55 for two days, and the cultures were maintained in IL-2 medium for an additional three days. On day five, viable lymphoblasts were stimulated with PMA and ionomycin for 5 h in the presence of 2 mM monensin, followed by staining with anti-CD4 and 7-AAD. After fixation and permeabilization, cells were stained with cytokine antibodies along with their respective isotype controls and acquired by flow cytometry. Frequencies of cytokine-producing cells were then analyzed in the live (7-AAD$^-$) CD4 subset. Finally, the frequencies of CD4 T cells positive for IL-2 and IFN-γ; IL-4 and IL-10; and IL-17A, IL-17F and IL-22, corresponding to Th1, Th2 and Th17 subsets, were added together to obtain the mean values in each group of wild type and SOD1-KO mice, and compared between the two groups. The top panel represents flow cytometric dot plots obtained for individual cytokines, whereas the bottom panel shows the mean±SEM values obtained for a group of mice (n=3).

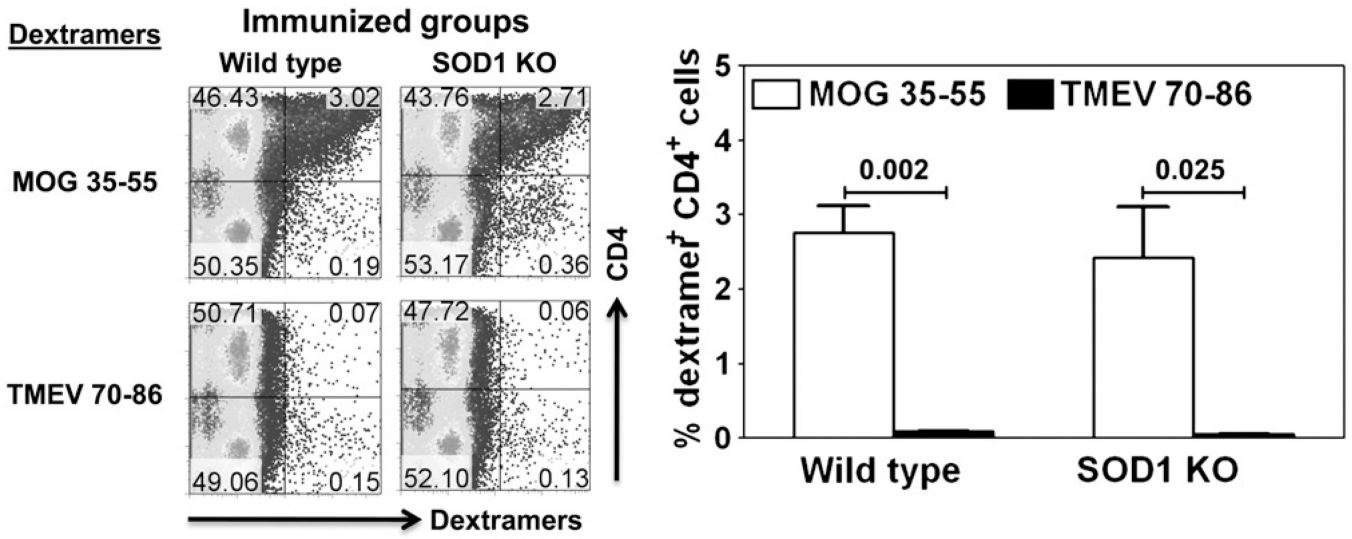

Fig. 6.

MOG-specific T cells generated in wild type and SOD1-KO mice expand comparably in the presence of IL-2. Groups of wild type and SOD1-KO mice were immunized s.c., with MOG 35–55 in CFA. After ten days, the animals were euthanized to harvest LN for preparing single cell suspensions. Cells were stimulated with MOG 35–55 for two days, and the cultures were maintained in growth medium containing IL-2. On day six poststimulation, viable lymphoblasts were stained with MOG 35–55 and TMEV 70–86 (control) dextramers, anti-CD4 and 7-AAD. Cells were acquired by flow cytometry and the percentages of $dext^{+}$ cells were determined in the live ($7\text{-}AAD^{-}$) CD4 subset. Left and right panels, respectively, represent flow cytometric dot plots and mean±SEM values obtained for a group of mice (n=3).

Table 1

Clinical and histologic EAE induced with MOG 35–55 in wild type and SOD1-KO mice.[a]

	Clinical disease			Number of inflammatory foci[c]		
Treatment groups	**Incidence (%)**	**Mean day of onset[b]**	**Mean maximum score[b]**	**Meninges**	**Parenchyma**	**Total**
Wild type	100 (10/10)	12.21±0.87	3.93±0.22	56.10±14.63	24.30±6.38	80.50±20.34
SOD1-KO	100 (15/15)	12.20±0.77	4.40±0.21	81.60±15.80	28.07±5.09	109±20.38

Scoring scale: 0, no signs of disease; 1, limp tail or hind limb weakness but not both; 2, limp tail and hind limb weakness; 3, partial paralysis of hind limbs; 4, complete paralysis of hind limbs; and 5, moribund.

[a] Numbers are mean±SEM values.

[b] Represents only mice that showed clinical disease.

[c] Represents only mice that were euthanized during the observation period.

Table 2

Immunophenotyping of splenocytes obtained from wild type and SOD1-KO mice.[a]

Cell type	Wild type	SOD1-KO
T cells (%)		
$CD3^+$	31.08±3.15	23.62±0.40
$CD3^+$ $CD4^+$	15.70±1.08	13.58±0.21
$CD3^+$ $CD8^+$	13.50±0.67[b]	9.04±0.23[b]
Non T cells (%)		
$B220^+$	47.38±2.38	47.23±2.30
$CD11b^+$	1.57±0.13	1.50±0.16
$CD11c^+$	3.35±0.28	3.30±0.25
$GR1^+$	5.71±0.57	5.89±0.11

[a] Numbers represent mean±SEM values.

[b] p=0.003.