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Manganese superoxide dismutase: A regulator of T cell activation-induced oxidative signaling and cell death

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ABSTRACT

Mitochondrial reactive oxygen species (ROS) are indispensible for T cell activation-induced expression of interleukin 2 (IL-2) and CD95 ligand (CD95L, FasL/Apo-1L) genes, and in turn, for CD95L-mediated activation-induced cell death (AICD). Here, we show that manganese superoxide dismutase (MnSOD/SOD2), a major mitochondrial antioxidative enzyme, constitutes an important control switch in the process of activation-induced oxidative signal generation in T cells. Analysis of the kinetics of T cell receptor (TCR)-triggered ROS production revealed a temporal association between higher MnSOD abundance/activity and a shut-down phase of oxidative signal generation. Transient or inducible MnSOD overexpression abrogated T cell activation-triggered mitochondrial ROS production as well as NF-κB- and AP-1-mediated transcription. Consequently, lowered expression of IL-2 and CD95L genes resulted in decreased IL-2 secretion and CD95L-dependent AICD. Moreover, upregulation of the mitochondrial MnSOD level is dependent on oxidation-sensitive transcription and not on the increase of mitochondrial mass. Thus, MnSOD-mediated negative feedback regulation of activation-induced mitochondrial ROS generation exemplifies a process of retrograde mitochondria-to-nucleus communication. Our finding underlines the critical role for MnSOD and mitochondria in the regulation of human T cell activation.

1. Introduction

T cell receptor (TCR) triggering induces rapid upregulation of a transcription program essential for proliferation, differentiation as well as activation-induced cell death (AICD) of T lymphocytes. The TCR signaling cascade splits downstream of PLCγ1 activation into two arms. On the one hand, inositol 3,4,5-triphosphate (IP_3)-induced rise in intracellular Ca^{2+} concentration activates Ca^{2+}-dependent transcription factors, *e.g.* NF-AT. On the other hand, diacylglycerol (DAG)-mediated activation of PKCθ and RasGRP proteins leads to NF-κB and AP-1 induction. Triggering of these three transcription factors orchestrates a full immediate-early transcriptional response upon T cell activation [1]. Thus, T cell activation can be induced by simultaneous treatment with ionomycin (Iono), a Ca^{2+} ionophore and NF-AT activator, and phorbol 12-myristate 13-acetate (PMA), a DAG mimetic and NF-κB/AP-1 activator [2].

In addition, TCR induction is accompanied by reactive oxygen species (ROS) generation. Although potentially deleterious for a cell, the ROS are kept in check by cellular antioxidative systems which lead to a controlled non-toxic activation-induced rise in the intracellular pro-oxidative status. In particular, hydrogen peroxide (H_2O_2) as a signaling molecule facilitates triggering of the oxidation-dependent transcription factors NF-κB and AP-1 and is therefore indispensible for T cell activation [3–8]. Simultaneous presence of this DAG/PMA-dependent oxidative signal (NF-κB/AP-1 triggering) and the Ca^{2+}-mediated signal (NF-AT triggering) is necessary for T cell activation-induced gene expression (*e.g.* IL-2, IL-4, CD95L). A single signal by itself is insufficient [3,9–12]. Different enzymatic sources, such as the mitochondrial respiratory chain, lipooxygenases and NADPH oxidases (NOX2 and DUOX1) contribute to ROS generation upon TCR triggering [3,10,13–16].

The pivotal role for mitochondria in T cell activation is an emerging field of research. Mitochondria of resting or *in vitro* expanded human T cells localize to the immunological synapse shortly after its formation [17–19]. They modulate amplitude and duration of the Ca^{2+} signal [20]. They are also believed to increase a local ATP gradient necessary for TCR-induced phosphorylation events [19]. Moreover, we and others demonstrated that mitochondria are the main generator of the activation-induced oxidative signal in resting or *in vitro* expanded human T cells [3,9,10,13,21]. We demonstrated

Abbreviations: ROS, Reactive oxygen species; IL-2, interleukin 2; CD95L/FASL/APO-1, CD95 ligand; AICD, activation-induced cell death; MnSOD/SOD2, manganese superoxide dismutase; TCR, T cell receptor

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a crucial role for mitochondria as oxidative signaling organelles in the process of T cell activation-induced transcription as well as CD95L-mediated AICD [3,10]. Our experimental work elucidated a novel signal transduction pathway leading from the TCR signalosome *via* PKCθ activation to superoxide anions ($O_2^{\cdot-}$) release by the mitochondrial respiratory chain complex I. Next, $O_2^{\cdot-}$ leave the mitochondria in a form of H_2O_2 to act as an oxidative signal in a cytoplasm [3,10].

The concept of an oxidative signal implies its tight regulation and transient character. Furthermore, according to the current understanding of the molecular mechanisms of mitochondrial ROS generation, complex I-mediated ROS release poses a potential threat to mitochondrial DNA (mtDNA) integrity [22]. Thus, the oxidative signal generated upon TCR activation is expected to be followed by an antioxidative response eventually leading to the shut-down of ROS production.

Here, we report a regulatory role for manganese superoxide dismutase (MnSOD/SOD2), a major antioxidative enzyme of the mitochondrial matrix, in T cell activation-induced oxidative signal generation. MnSOD, up-regulated upon T cell activation on the transcriptional, translational and enzymatic level, participates in the downregulation of the TCR-induced pro-oxidative intracellular status. Its overexpression inhibits NF-κB- and AP-1-mediated transcription of activation-induced genes, such as IL-2 and CD95L. Consequently, Jurkat T cells which have higher mitochondrial MnSOD content are significantly less susceptible to CD95L-mediated AICD. Moreover, TCR-induced MnSOD expression is dependent on triggering of the oxidation-dependent transcription factor NF-κB. Thus, the negative regulatory function of MnSOD exemplifies a retrograde mechanism of mitochondria-to-nucleus communication.

Decreased MnSOD expression levels, gene silencing, inactivating mutations or gene polymorphisms, all have been associated with tumorigenesis, also in the case of lymphoma and leukemia development [23–33]. Moreover, several reports addressed the importance of MnSOD for T cell differentiation and function [23,32,34,35]. In that respect, our results shed a new light on the understanding of a T cell-specific function of MnSOD, one of the catalytically fastest and thus most intriguing enzymes of the human cell.

2. Materials and methods

2.1. Chemicals

Dichlorodihydrofluorescein diacetate (H_2DCF-DA) was purchased from Invitrogen, Germany. Antibodies were obtained from R&D Systems (murine IgG2a monoclonal, cl. 20102 anti-CD3 antibody isotype control), Millipore, Germany (rabbit polyclonal anti-MnSOD), Santa Cruz, Germany (goat polyclonal anti-CuZnSOD), Cell Signalling, USA (rabbit polyclonal anti-cytochrome *c*), BD, Germany (rabbit polyclonal anti-ERK1), Sigma, Germany (mouse monoclonal anti-γ-tubulin), Merck, Germany (rabbit polyclonal anti-catalase), Thermo Scientific (mouse monoclonal anti-prohibitin 1 antibody, II-14-10) and Abcam (rabbit polyclonal anti-thioredoxin reductase 2). Content of mitochondrial respiratory complexes was analyzed using Total OXPHOS WB Antibody Cocktail from Mito Sciences (Eugene, OR, USA). Monoclonal anti-CD95 (anti-APO-1) and anti-CD3 antibodies (OKT3) were prepared from hybridomas as described previously [9]. All other chemicals, if not noted otherwise, were purchased from Sigma, Roth or Merck.

2.2. Cell culture

The Jurkat T cell line sub-clone J16-145 [9] was cultured in Iscove's Modified Dulbecco's Medium (IMDM), primary human T cells in Roswell Park Memorial Institute Medium (RPMI-1640). Both media were supplemented with L-glutamine and 10% foetal calf serum (FCS).

2.3. Isolation and in vitro expansion of human T cells

Human T cells were isolated *via* Ficoll-Paque density centrifugation followed by rosetting with S-(2-aminoethyl)-isothiuronium bromide hydrobromide-treated sheep erythrocytes as described previously [3,9,10]. T cells were expanded by stimulation with phytohemagglutinin (1 μg/ml) for 16 h and subsequent culture in IL-2-containing (25 U/ml) RPMI-1640 + 10% FCS for 5–6 days ("day 6" T cells).

2.4. Determination of activation-induced ROS generation

Jurkat T cells were stained with 5 μM H_2DCF-DA for 20 min, then stimulated with PMA (10 ng/ml) for 30 min. To determine the kinetics of ROS production, primary T cells were stimulated with plate-bound anti-CD3 antibody (30 μg/ml) for the indicated time periods and stained with 5 μM H_2DCF-DA 20 min before the end of the incubation time. Cells were washed with ice-cold PBS and ROS generation was assayed by FACS (FACS Canto II, BD Germany). ROS generation was quantified as increase in mean fluorescence intensity (MFI), calculated according to the following formula: increase in MFI (%) = $[(MFI_{stimulated} - MFI_{unstimulated}) / MFI_{unstimulated}] \times 100$ [3,10].

2.5. Sub-cellular fractionations

To isolate cytosol, cells were suspended in hypotonic lysis buffer (10 mM Tris, pH 7.4, 1.5 mM $MgCl_2$, 5 mM KCl, "Roche Complete" protease inhibitors cocktail (Roche, Germany)) and passed through a 25G needle 20 times. Cell debris was separated by centrifugation (2 min, 300 *g*). The membrane fraction was then separated from the cytosol by centrifugation at 800 *g* for 45 min. To isolate the mitochondria-enriched fraction, cells were passed through a 25G needle in hypotonic lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 250 mM sucrose, "Roche Complete" protease inhibitors cocktail (Roche, Germany)). The cell debris was pelleted by centrifugation (750 *g*, 10 min) and removed. Thereafter, the supernatant was centrifuged again (11,000 *g*, 30 min) and the pellet lysed in radioimmunoprecipitation assay buffer (RIPA). Protein concentration was determined with the bicinchoninic acid method (BCA, Pierce) and equal amounts of protein were analysed by Western blotting (WB) as described before [10].

2.6. Transfection and MnSOD overexpression

Jurkat T cells were transfected with a CMV-driven expression plasmid carrying human MnSOD cDNA (coding sequence NM_001024465.1, accession number BC012423; pCMV-SPORT6, Open Biosystems) or empty vector (pcDNA3, CMV promoter, Invitrogen; 3 μg/transfection) with or without a RFP expression vector (for measurements of ROS generation; mRFP1-pcDNA3, CMV promoter; 2 μg/transfection) or EGFP expression vector (for cell death assay; pmaxGFP, CMV promoter, Lonza; 2 μg/transfection). Transfections were performed using AMAXA nucleofection technology ("Cell Line Nucleofector® kit V", Lonza) according to the manufacturer's protocol. After over-night recovery cells were subjected to further experiments.

2.7. Luciferase reporter assays

Firefly luciferase reporter constructs containing: (i) the CD95L (−860/+100) promoter, (ii) the IL-2 (−300/+47) promoter, (iii) three copies of the AP-1 binding site from the SV40 enhancer (CGGTTGCTGACTAATTG), (iv) four copies of the NF-κB consensus sequence (GGAAATTCCCC) in the pTATA-Luc vector were kindly provided by Dr. M. Li-Weber (DKFZ, Heidelberg). A *Renilla* luciferase-expression reporter pRL-TK plasmid (Promega) was used as an

internal control for transfection efficiency. For single transfection 1.3×10^6 Jurkat T cells were electroporated with 2 μg of pRL-TK plasmid, 5 μg of the firefly luciferase reporter construct and 20 μg of the MnSOD expression plasmid (or empty vector) as previously described [3,9]. After over-night recovery, cells were divided and treated with PMA (10 ng/ml) and ionomycin (1 μM) for 8 h. Firefly and *Renilla* luciferase activities were measured using a "Dual Luciferase Kit" (Promega, USA) and a 96-well plate luminometer (Berthold, Germany). To normalize obtained results, firefly luciferase activity was divided by the respective *Renilla* luciferase activity. Results presented are normalized relative light units (RLU).

2.8. RNA isolation, reverse transcription and quantitative real-time PCR

RNA was isolated using the "RNeasy mini kit" (QIAGEN, Germany) and reverse-transcribed with "Reverse transcription PCR" kit (Applied Biosystems, Germany). Quantitative real-time PCR was performed using the "Power SYBR Green PCR Master Mix" (Applied Biosystems). The primers used for MnSOD gene expression analysis were: sense 5′-CTGGACAAACCTCAGCCCTA-3′, anti-sense 5′-TGATGGCTTCCAGCA-3′. Primers used for CD95L, IL-2 and GAPDH were reported before [3]. Gene expression was analyzed using the 7500 Real-Time PCR Systems and Sequence Detection Software, Applied Biosystems, v. 2.0.2. Gene expression levels were normalized using GAPDH expression as an endogenous reference as previously described [3].

2.9. Cell death assay

Jurkat T cells were stimulated with PMA (10 ng/ml) and Iono (10 μM) for 24 h. Cell death was analyzed by a drop in the forward-to-side-scatter (FSC/SSC) FACS profile of dead cells as compared to living [3,9,10]. To assess cell death of MnSOD-expressing cells, cells were co-transfected with a GFP expression vector. For FACS analysis GFP-positive (GFP^+), living cells were gated as shown in Suppl. Fig. 3B. The decrease of GFP^+ cells was calculated according to the following formula: decrease in GFP^+ cells (%) $=[(GFP^+_{control}-GFP^+_{stimulated})/GFP^+_{control}]\times100$.

2.10. Determination of mitochondrial mass and mitochondrial membrane potential via FACS

Mitochondrial mass was assessed by fluorescence staining with acridine orange 10-nonyl bromide (NAO, Sigma, a fluorescent dye binding to cardiolipin in the inner-mitochondrial membrane) or MitoFluor Green (MFG, Invitrogen, a fluorescent dye accumulating in mitochondria independently of membrane potential). T cells were stimulated with plate-bound anti-CD3 antibody (30 μg/ml) for indicated time periods. 20 min before the end of stimulation time, cells were stained with NAO (75 nM) or MFG (50 nM). Next, cells were washed twice with PBS and the mean fluorescence intensity was measured by FACS. Rhodamine 123 fluorescent dye (Rh123, Sigma) was used in an unquenched mode to determine changes of mitochondrial membrane potential according to the method of Chen et al. [36]. Briefly, cells were stained with 10 μM Rh123 for 15 min before harvest and directly analyzed by FACS.

2.11. Generation of doxycyclin (DOX)-inducible MnSOD-overexpressing Jurkat T cells

Human MnSOD cDNA was amplified from pCMV-SPORT6-MnSOD (Open Biosystems) with the primer pair 5′-CCGGAATTCATGTTGAGCCGGGCAGTGTGCG-3′ and 5′-CGGGGTACCTTACTTTTTGCAAGCCATGTATC-3′, and cloned into pRev-TRE-Tight (Clonetech, USA). Retroviruses were generated by transfection of Phoenix cells with pRev-TRE-MnSOD. Jurkat M2 cells, harbouring the DOX dependent transactivator (courtesy of Dr. Lars Weingarten and Dr. Tobias P. Dick, DKFZ, Heidelberg) were infected and cultured in medium supplemented with 100 μg/ml hygromycin for 7 days. The resulting cells were sub-cloned twice and screened for DOX-inducible MnSOD expression by WB.

2.12. Western blotting and band intensity determination

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis were performed as described previously [10]. Band intensities were quantified with application of NIH software ImageJA v.1.45b and normalized to the respective loading control.

2.13. Measurement of MnSOD activity

At least 8×10^7 expanded human T cells were stimulated with plate-bound anti-CD3 antibody for 8 h. Thereafter, cells were collected, washed with PBS, snap-frozen in liquid nitrogen and stored overnight at −80 °C. Jurkat T cells (1×10^7 cells) were transfected by nucleofection with MnSOD-encoding plasmid or empty vector. After 24 h of resting period cells were lysed and MnSOD activity was measured. SOD activity was detected using a commercial "SOD Assay Kit-WST" according to manufacturer's protocol (Dojindo Molecular Technologies Inc., Japan). A computer-tuneable spectrophotometer (Spectramax Plus Microplate Reader, Molecular Devices, USA) operating in the dual wavelength mode was applied. Samples were analyzed in temperature-controlled 96-well plates in a final volume of 300 μl. Briefly, cells were disrupted using a 27G needle in ice-cold ETC buffer (20 mM Tris–HCl pH 7.4, 250 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$) and the homogenates were centrifuged at 600 *g*, 4 °C for 10 min. The resulted supernatant was sonicated (Branson Sonifier 450, USA) to disrupt mitochondrial membranes. MnSOD activity was measured in the presence of CuZnSOD inhibitor (3 mM NaCN) and normalized to the protein content. To correct for inter-experimental variation the enzymatic activities obtained from three independent experiments were subjected to z-transformation (see Fig. 1; for measured values, see Suppl. Fig. 1B).

2.14. Determination of IL-2 secretion

IL-2 concentration was measured by enzyme-linked immunosorbent (ELISA) assay (BD OptEIA Set Human IL-2, Becton Dickinson). Jurkat T cells were transfected by nucleofection with vector encoding human MnSOD or control empty vector (as described before). After over-night recovery (18 h) cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μM) for 7 h. Next, the supernatants were cleared by centrifugation and measurements were performed according to the manufacturer's instructions.

2.15. Caspase 8 activity assay

Jurkat T cells were transfected with MnSOD encoding and control empty vector (as described previously). After an over-night resting period (18 h) the cell suspension was cleared from the dead/damaged cells by Ficoll-Paque density centrifugation. Thereafter, cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μM) for 24 h. Caspase 8 activity was measured using a "Caspase-Glo 8®" kit (Promega, USA) and a 96-well plate luminometer (Berthold, Germany) according to manufacturer's instruction.

3. Results

3.1. Rise in MnSOD content and activity inversely correlates with T cell activation-induced oxidative signal generation and gene expression

T cell activation-induced release of H_2O_2 from mitochondria has been described to be crucial for gene expression including CD95L

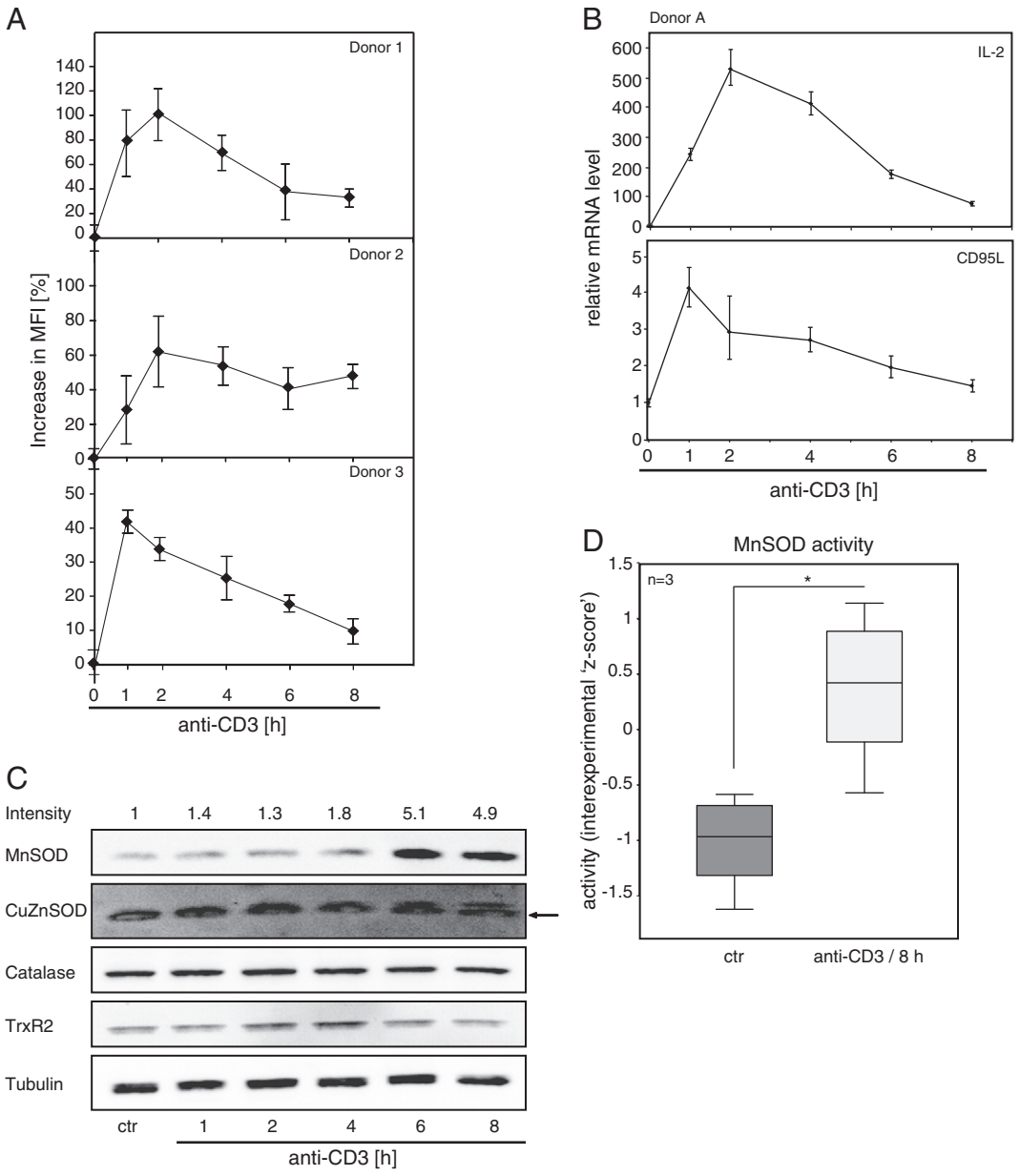

Fig. 1. T cell activation-induced ROS generation and ROS-dependent gene transcription inversely correlate with MnSOD activity and protein content. A, *In vitro* expanded human T cells ("day 6") of three healthy donors were stimulated *via* plate-bound anti-CD3 antibodies (30 μg/ml) for the indicated time periods and stained with H_2DCF-DA. The level of activation-induced ROS was assessed by FACS and calculated as percentage increase in mean fluorescence intensity (MFI) +/− SD of triplicated measurements. B, Expanded human T cells were stimulated by plate-bound anti-CD3 antibodies. Expression of ROS-dependent genes (IL-2, CD95L) was analyzed at the indicated time points using quantitative real-time RT-PCR and normalized to GAPDH expression. Representative results are presented as triplicated measurements +/− SD (for data obtained from cells of other donors, see Suppl. Fig. 1A). C, Expanded human T cells were stimulated *via* plate-bound anti-CD3 antibodies for the indicated time points. Cells were lysed and protein levels were determined by WB analysis (arrow indicates CuZnSOD band). Representative blot for triplicated experiment is presented. The extent of MnSOD upregulation was determined according to signal intensity over loading control (as described in the Materials and methods section). D, T cells from three healthy donors were 8 h stimulated *via* plate-bound anti-CD3 antibodies and MnSOD activity was measured in activated and unstimulated cells. Data are presented as 'z-scores' of three independent experiments +/− SD; Student's *t* test: $p<0.05$ (*). For measured values, see Suppl. Fig. 1D). Cells within the experiments were collected, stained or lysed at the same time, applying the same conditions.

and cytokines, *e.g.* IL-2 and IL-4 [3,9–12]. However, few data are available about the regulation and kinetics of ROS generation in human T cells. In order to determine the profile of oxidative signaling, *in vitro* expanded primary human T cells were re-stimulated by plate-bound agonistic anti-CD3 antibodies and time course of ROS production was determined. T cells of all investigated donors showed a rapid increase in ROS production, with a maximum being reached after 1 to 2 h. Then, a slow decline in ROS generation during the following 6 h was observed (Fig. 1A). Since ROS facilitate NF-κB triggering, we analyzed the expression of NF-κB responsive genes, IL-2 and CD95L. The increase of transcript levels reached maximum after 1 to 2 h which corresponds to the kinetics of ROS production (Fig. 1B and Suppl. Fig. 1A). Of note, stimulation with an isotype-matched control antibody did not lead to an increase in ROS generation or gene expression (Suppl. Fig. 1B). Since ROS are constantly detoxified by dedicated enzymatic systems, protein levels of several antioxidative proteins were analyzed in primary human T cells upon stimulation. Interestingly, the extent of activation-induced ROS production inversely correlated with the MnSOD content (Fig. 1C; Suppl. Fig. 1C). The amounts of other major enzymes of the antioxidative defense, like cytosolic CuZnSOD, mitochondrial thioredoxin reductase (TrxR2) and catalase were not altered. Furthermore, a rise in MnSOD content resulted in a significant enhancement of MnSOD-specific enzymatic activity (Fig. 1D, Suppl. Fig. 1D).

3.2. The oxidative signal generated by mitochondria regulates MnSOD expression

TCR triggering has been associated with increased mitochondrial biogenesis [37]. Thus, we analyzed whether the observed upregulation of MnSOD was due to TCR-induced mitochondrial proliferation, decreased mitochondrial turnover or transcriptional upregulation. To investigate mitochondrial proliferation upon TCR stimulation the mitochondrial mass of primary human T cells was determined by either immunoblotting (Fig. 2A) or the use of the

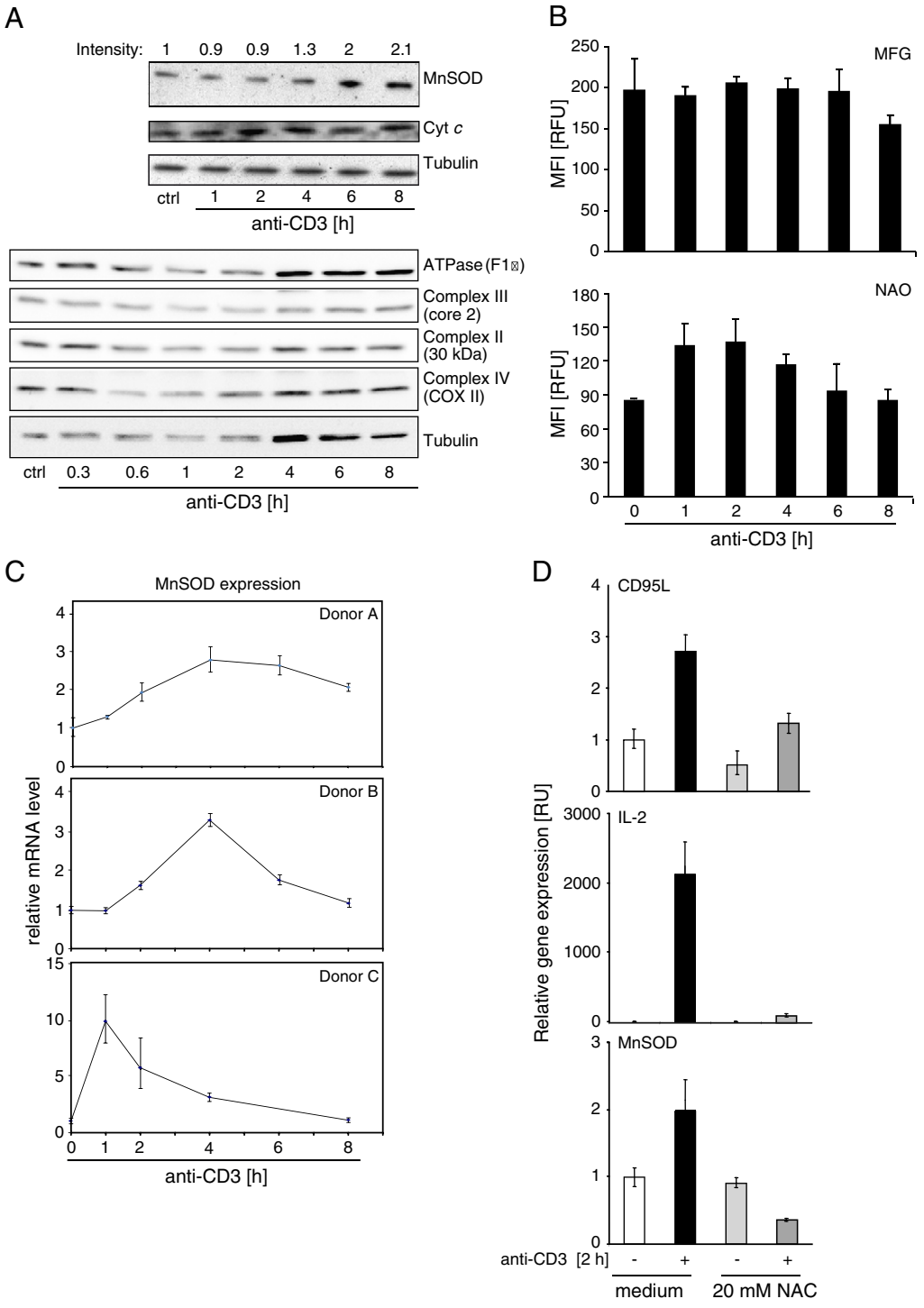

Fig. 2. Upregulation of MnSOD is mediated by oxidation-dependent transcription and not by an increase of mitochondrial mass. A–C, *In vitro* expanded human T cells ("day 6") were stimulated by plate-bound anti-CD3 antibodies (30 μg/ml) for indicated time periods. A, Cells were lysed and protein content was analyzed by WB. Levels of MnSOD upregulation were determined according to signal intensity over loading control. B, Cells were stained with MitoFluor Green (MFG) or 10-nonyl acridine orange (NAO) 20 min before the end of the respective stimulation period. Mitochondrial mass was analyzed by FACS in triplicated samples (+/− SD). A and B, representative results of triplicated experiments (*i.e.* donors) are presented. C, Expression of MnSOD was analyzed at the indicated time points using quantitative real-time RT-PCR and normalized to GAPDH expression. Results of triplicated measurements +/− SD for three independent experiments (*i.e.* donors) are presented. D, Pre-activated T cells were stimulated for 2 h *via* plate-bound anti-CD3 antibodies +/− 30 min pre-incubation with 20 mM N-acetyl-cysteine (NAC). Activation-induced expression of CD95L, IL-2 and MnSOD was analyzed by quantitative real-time PCR and normalized to GAPDH expression. Representative results of triplicated measurements +/− SD for three independent experiments (*i.e.* donors) are presented.

mitochondria-specific fluorescent dyes MFG and NAO (Fig. 2B). The content of mitochondrial proteins remained unchanged when compared with loading control as well as no change in MFG accumulation could be observed. Intriguingly, NAO staining showed a transient increase in fluorescence which paralleled the kinetics of ROS production but not the rise in MnSOD content. Binding of NAO to cardiolipin, a lipid component of the inner-mitochondrial membrane, could be influenced by changes in cardiolipin oxidation or mitochondrial membrane potential [38,39]. Moreover, the transient character of the observed change makes mitochondrial proliferation unlikely. Thus, the increase in MnSOD content does not simply reflect an over-all increase in cellular mass of mitochondria.

The TCR-induced temporal changes of ROS levels paralleled those observed for NF-κB responsive genes, IL2 and CD95L (Fig. 1A and B, Suppl. Fig. 1A). MnSOD transcription has been previously reported to depend on NF-κB induction [40–42]. Analysis of the TCR-induced changes in MnSOD transcript levels revealed positive correlation with intracellular pro-oxidative status (Figs. 2C and 1A). Since NF-κB triggering is oxidation-dependent, we investigated whether the increased MnSOD expression was a result of activation-induced ROS production. To this end, we applied the ROS scavenger, N-acetylcysteine (NAC). We and others previously showed that, addition of NAC efficiently inhibits T cell activation-induced ROS generation and NF-κB triggering [3,9,43] (Suppl. Fig. 2A and B). As presented in Fig. 2D, pre-treatment with NAC abrogated the oxidative signal dependent transcription of IL-2 and CD95L genes. Correspondingly, treatment with NAC (Fig. 2D, Suppl. Fig. 2D) also blocked MnSOD transcription, indicating a dependence of MnSOD upregulation on activation-induced ROS generation. Moreover, Trolox, a water-soluble vitamin E derivative, which blocked activation-induced ROS generation (Suppl. Fig. 2C), also inhibited transcriptional upregulation of MnSOD and IL-2 (Suppl. Fig. 2D). In the applied concentration range both anti-oxidants did not display any toxic effects (Suppl. Fig. 3A and B). Thus, the TCR-triggered upregulation of MnSOD represents a negative feedback loop regulating ROS production.

3.3. Increase in MnSOD content inhibits T cell activation-induced ROS generation

An association between PMA-triggered mitochondrial H_2O_2 generation and increase in MnSOD content and activity was also shown for the Jurkat T cell line [10]. Although, the kinetics of mitochondrial oxidative signal generation differs from that of *in vitro* expanded human T cells (in Jurkat T cells the signal peaks after 30 min–1 h, data not shown), we decided to apply Jurkat T cells as a model system. Moreover, efforts to transiently overexpress MnSOD in expanded human T cells were unsuccessful (data not shown). To analyze the physiological role of T cell activation-induced increase in MnSOD content and activity, MnSOD was transiently overexpressed in Jurkat T cells. MnSOD overexpression neither affected the viability of transfected cells (Suppl. Fig. 4A) nor influenced mitochondrial membrane potential, mitochondrial mass or the content of major subunits of respiratory chain complexes (Suppl. Figs. 4B, C and 5A). As revealed by band intensity analysis, the level of MnSOD protein upon overexpression (1.8–3.1 fold increase in intensity; Figs. 3 and 4A and Suppl. Fig. 5A) resembles the ones reached upon activation of Jurkat T cells (2.1 fold increase in intensity; Suppl. Fig. 5A) as well as activation of expanded human T cells (1.7–5.1 increase in intensity; Figs. 1C, 2A and Suppl. Fig. 1C). Concomitantly, cells

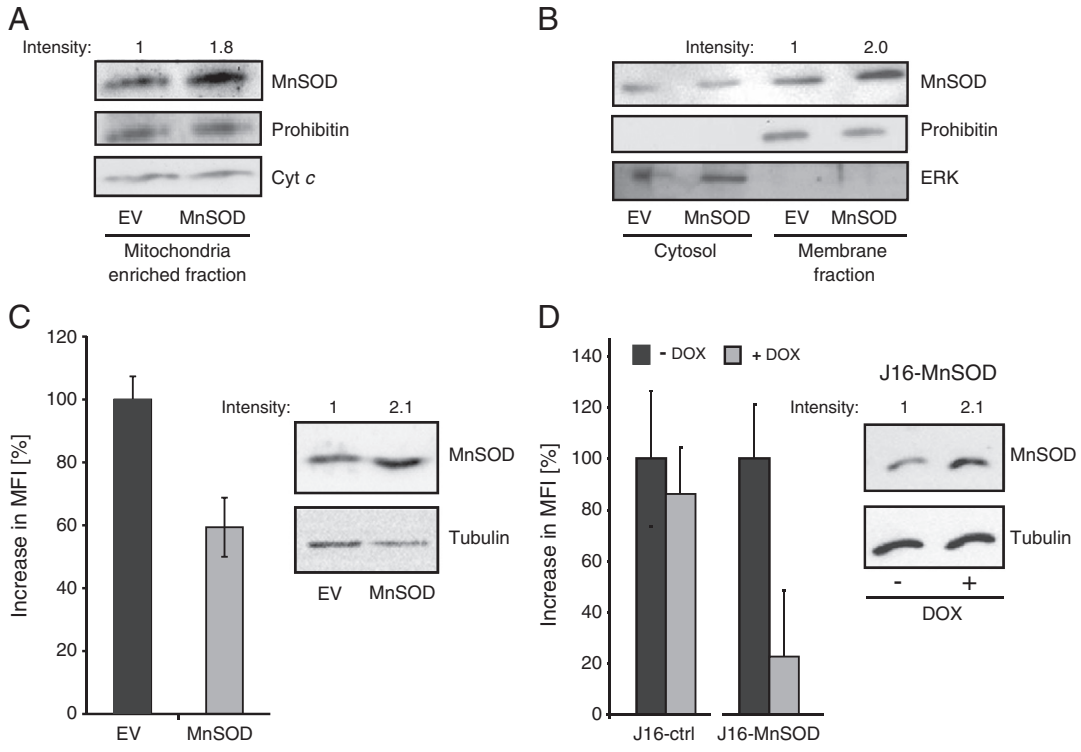

Fig. 3. MnSOD regulates T cell activation-induced ROS production. A and B, Jurkat T cells were transiently transfected by electroporation with MnSOD-expressing or empty (EV) vector. Mitochondria-enriched fractions (A) or cytosol and membrane fractions (B) were isolated by differential centrifugation 24 h after transfection and intracellular localization of MnSOD was analyzed by WB. C, Jurkat T cells transfected with EV or MnSOD-encoding plasmids and RFP reporter plasmid were stained with H_2DCF-DA and stimulated with PMA (10 ng/ml) for 30 min. Left panel, the levels of activation-induced ROS were assessed by FACS in triplicated samples +/− SD (for gating strategy, see Suppl. Fig. 6A) and calculated as percent increase in mean florescence intensity (MFI). Right panel, MnSOD overexpression was verified by WB. D, Jurkat T cells were stably transfected by retroviral transduction using a MnSOD expression construct under control of a doxycycline (DOX)-inducible promoter and sub-cloned in the presence of hygromycin. Thereafter, cells were treated with DOX for 48 h or left untreated. Left panel, cells were stained with H_2DCF-DA and stimulated with PMA (10 ng/ml) for 30 min. The level of activation-induced ROS was analyzed by FACS in triplicated samples +/− SD. Right panel, MnSOD overexpression was verified by WB. A–D, Representative results for triplicated experiments are presented. Levels of MnSOD overexpression were determined by measurements of signal intensity over loading control.

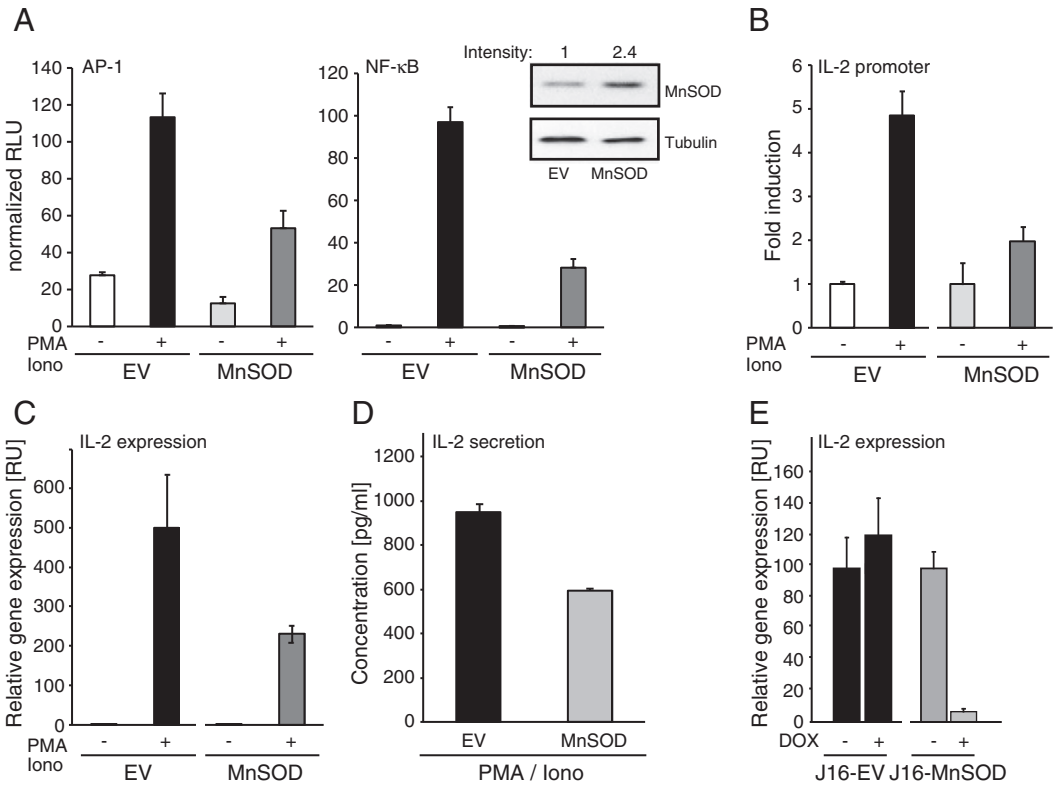

Fig. 4. MnSOD content controls T cell activation-induced gene expression. A-D, Jurkat T cells were transfected with empty vector (EV) or MnSOD expression vector (MnSOD), by electroporation (A and B) or nucleofection (C and D). A and B, Jurkat T cells were additionally co-transfected with firefly luciferase reporter constructs under the control of AP-1- and NF-κB-based promoters (A) or IL-2 promoter (B) and *Renilla* luciferase-harboring pRL-TK plasmid. After over-night recovery (18 h), cells were treated with PMA (10 ng/ml) and ionomycin (Iono, 1 μM) for 8 h and normalized luciferase activity was measured. MnSOD protein levels were analyzed by WB (A, right panel). Extent of MnSOD overexpression was determined by measurement of signal intensity as compared to the loading control. C and D, Transfected cells were stimulated with PMA/Iono for 1 h (C) or 7 h (D) and expression (C) or secretion (D) of IL-2 was determined by quantitative real-time RT-PCR (C) or ELISA (D). E, Jurkat T cells stably transduced with a retroviral construct expressing MnSOD under the control of a doxycycline (DOX)-inducible promoter were treated with DOX for 48 h or left untreated. Thereafter, expression of IL-2 induced by 1 h PMA/Iono treatment was determined by quantitative real-time RT-PCR. Representative results are presented as triplicated measurements +/− SD.

overexpressing MnSOD display an increased MnSOD-specific enzymatic activity (Suppl. Fig. 5B). Since MnSOD activity depends on sub-cellular localization of the enzyme, cells overexpressing MnSOD were fractionated by differential centrifugation into mitochondria-enriched (Fig. 3A) or cytoplasmic and membrane fractions (Fig. 3B). A significant increase in mitochondrial MnSOD content was detected as demonstrated by WB analysis (Fig. 3A and B). Thus, overexpression of MnSOD in Jurkat T cells seems to be a suitable model system to mimic T cell activation-induced MnSOD upregulation.

To directly measure the influence of increased MnSOD levels on activation-induced ROS production, MnSOD was overexpressed together with the red fluorescent protein (RFP) to control for transfection efficiency (for gating strategy, see Suppl. Fig. 6A). As shown by FACS analysis, the release of H_2O_2 from mitochondria was significantly decreased in MnSOD-overexpressing RFP-positive cells (Fig. 3C). To further investigate this finding, a Jurkat T cell line stably transfected with MnSOD under control of a doxycycline (DOX) dependent transactivator/promoter system (J16-MnSOD) was generated. Pre-treatment with DOX for 48 h resulted in enhanced MnSOD protein levels (2.1 fold increase in band intensity). In consequence, activation-induced ROS generation was decreased (J16-MnSOD, Fig. 3D). In contrast, in the control cell line containing the transactivator/promoter system alone no changes in the activation-induced ROS production could be observed upon DOX pre-treatment (J16-ctrl, Fig. 3D). In conclusion, elevated MnSOD levels lead to a decrease in stimulation-induced ROS production.

3.4. Higher mitochondrial abundance of MnSOD inhibits T cell activation-induced gene expression

The activation of AP-1 and NF-κB transcription factors is highly dependent on the increased intracellular pro-oxidative status. Thus, we analyzed the effect of higher mitochondrial MnSOD content on T cell activation-mediated AP-1 and NF-κB triggering using luciferase reporter constructs. Upon MnSOD overexpression, AP-1 and NF-κB triggering was decreased which coincided with the reduced ROS release from mitochondria (Fig. 4A).

Next, we investigated NF-κB and AP-1 dependent induction of the IL-2 promoter. Cells with higher amounts of MnSOD protein showed a lowered IL-2 driven luciferase signal (Fig. 4B). Activation-induced IL-2 transcription was also clearly decreased in Jurkat T cells overexpressing MnSOD in a transient (Fig. 4C) and DOX-inducible manner (Fig. 4E). Furthermore, increased MnSOD content resulted in a decreased ability to secrete IL-2 upon activation (Fig. 4D). Therefore, the MnSOD level critically regulates the T cell activation-triggered transcriptional response.

3.5. Increase in MnSOD content negatively regulates T cell activation-induced CD95L-mediated AICD

Expanded T cells as well as Jurkat T cells undergo CD95L-mediated AICD upon TCR triggering or PMA/Iono stimulation. T cell activation-induced upregulation of CD95L transcription strictly depends on oxidative signal-mediated NF-κB and AP-1 triggering [9–12]. Therefore,

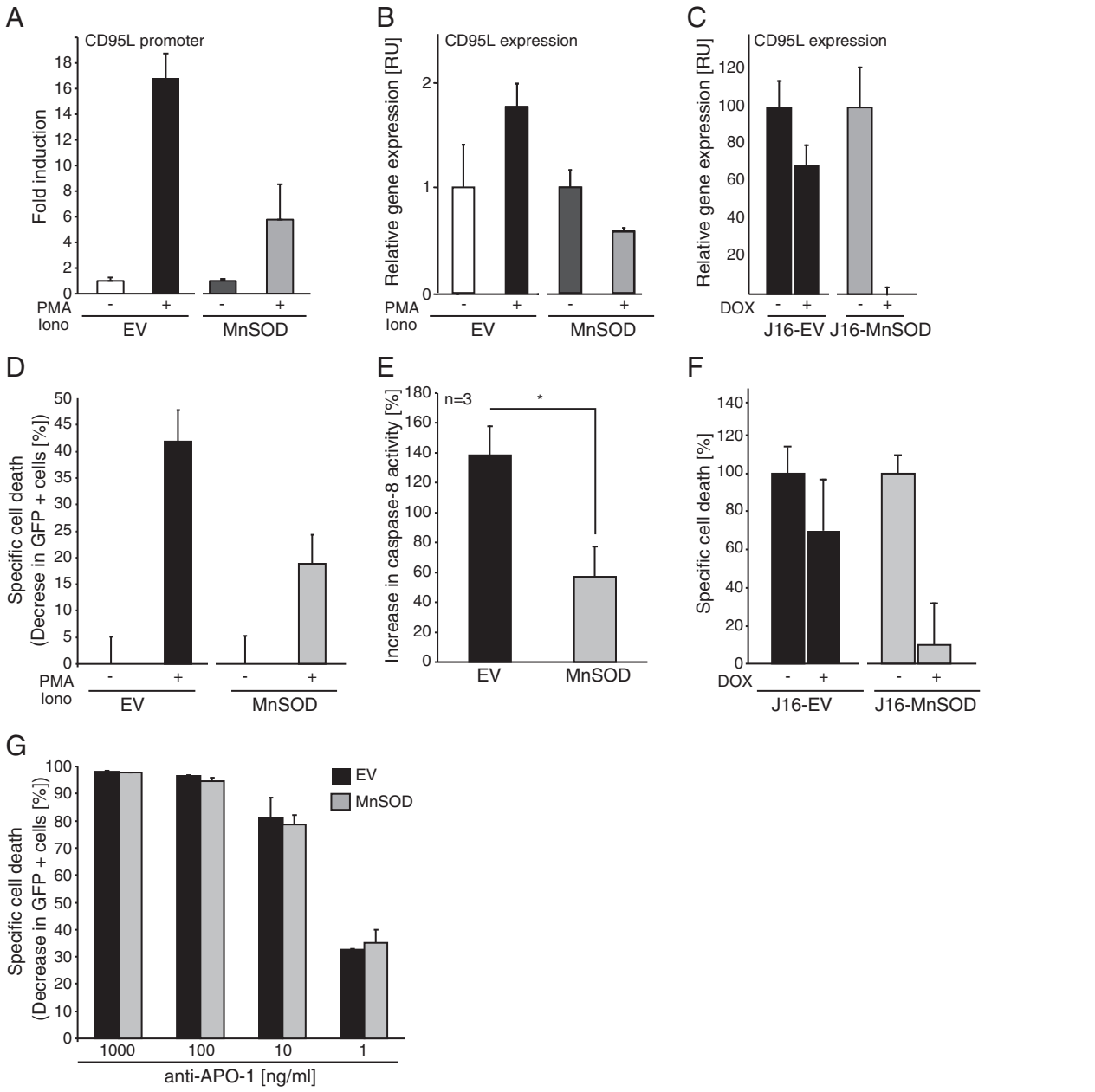

Fig. 5. MnSOD is a negative regulator of activation-induced CD95L-mediated cell death. A, B and D–G, Jurkat T cells were transiently transfected with empty vector (EV) or MnSOD expression vector (MnSOD), by electroporation (A) or nucleofection (B, D, E and G). A, Cells were additionally co-transfected with firefly luciferase reporter construct under the control of CD95L promoter and *Renilla* luciferase-harboring pRL-TK plasmid. After over-night recovery (18 h), cells were treated with PMA (10 ng/ml) and ionomycin (Iono, 1 μM) for 8 h and normalized luciferase activity was assayed. C, D, E and G, Transfected cells were stimulated with PMA/Iono for 1 h (B) or 24 h (D and E) and CD95L expression (B), activation-induced cell death (C) or caspase 8 activity (E) were determined. In D, cells were additionally co-transfected with a GFP expression vector. Cell death was measured by assessing the decrease in number of living GFP^+ cells (see Suppl. Fig. 6B for details). C and F, Jurkat T cells stably expressing MnSOD under control of DOX were stimulated with PMA/Iono for 48 h and cell death was determined by a drop in the FSC/SSC profile. G, Transiently co-transfected cells, as in (D), were treated with different concentrations of agonistic anti-CD95 antibody (anti-APO-1) for 24 h. Next, cell death was determined by assessing the decrease in the number of living GFP^+ cells. A–G, Representative results are presented as triplicated measurements +/− SD.

we investigated whether reduced levels of the oxidative signal in cells overexpressing MnSOD affect CD95L expression and AICD. To this end, we transiently co-expressed a CD95L promoter luciferase reporter construct together with MnSOD. MnSOD overexpression abrogated activation-induced CD95L promoter activity (Fig. 5A). Moreover, MnSOD overexpression led to reduced CD95L transcript levels (Fig. 5B). In addition, DOX-treated J16-MnSOD cells showed lower CD95L transcript levels upon stimulation as compared to control cells (Fig. 5C).

To test whether reduced CD95L transcription results in reduced cell death, Jurkat T cells were transiently co-transfected with MnSOD and green fluorescent protein (GFP). Next, apoptosis was induced by stimulation with PMA/Iono and the decrease in the number of living GFP-positive cells (GFP^+) was determined by FACS measurement (Suppl. Fig. 6B). Overexpression of MnSOD resulted in approximately 50% decrease in AICD (Fig. 5D). Moreover, increased abundance of MnSOD protein led to significantly lower caspase 8 activity measured 24 h after AICD induction (Fig. 5E). AICD was also decreased in DOX-treated J16-MnSOD cells (Fig. 5F). However, in the latter case an additional effect of DOX cannot be ruled out—the control cell line showed a slight decrease in cell death after DOX treatment (Fig. 5F).

Due to its protective action on mitochondria MnSOD inhibits apoptosis triggered by TNF-α, irradiation or anti-cancer drugs [44–48]. Particularly, in the case of cell death induced by TNF-α receptor engagement MnSOD was suggested to provide protection against toxic effects of mitochondrial $O_2^{\cdot-}$ [45,48]. Thus, we analyzed whether observed reduction in AICD of MnSOD-overexpressing Jurkat T cells is a consequence of general disturbance in downstream mitochondrial apoptotic events or reduced CD95L expression. To this end, MnSOD-overexpressing cells were treated with the agonistic CD95 antibody (anti-APO-1). Thereafter, the decrease in GFP^+ cells was measured by FACS. No difference in apoptosis induction could be observed for all antibody concentrations tested (Fig. 5G) showing that the reduction of AICD does not relate to reduced sensitivity towards CD95-induced apoptosis but is indeed an effect of decreased CD95L expression.

In conclusion, we showed that increased MnSOD levels in stimulated T cells negatively correlate with ROS production. Furthermore, we demonstrated that increased MnSOD levels down-regulate T cell activation-induced ROS production, AP-1 and NF-κB activation, ROS-dependent gene transcription as well as CD95L-mediated AICD. Thus, the activation dependent upregulation of MnSOD comprises a new regulatory negative feedback loop in TCR signaling (Fig. 6).

4. Discussion

TCR-mediated gene expression strictly depends on the simultaneous presence of two signals, a DAG/PMA-dependent H_2O_2 signal and an IP_3/Iono-dependent Ca^{2+} signal, and consequently on simultaneous activation of oxidation-dependent (*e.g.* NF-κB, AP-1) and Ca^{2+}-dependent (*e.g.* NF-AT) transcription factors [1–3,7,10]. Neither signal is sufficient by itself for induction of IL-2/CD95L expression or CD95L-dependent AICD.

The activation-induced oxidative signal is generated by different enzymatic sources including the phagocytic NADPH oxidase NOX2 [14] and the non-phagocytic NADPH oxidase DUOX1 [16]. We and others have previously identified mitochondrial respiratory complex I as an essential source of ROS generation upon TCR stimulation [3,10]. Treatment with complex I inhibitors or siRNA mediated down-regulation of NDUFAF1, a crucial complex I assembly factor, efficiently block TCR triggering-induced ROS production and gene expression [3,10]. Thus, mitochondria function as oxidative signaling organelles during T cell activation

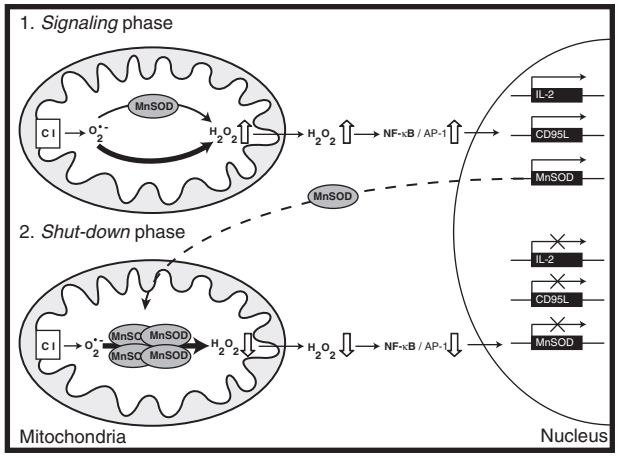

Fig. 6. Schematic representation of the MnSOD-mediated regulation of T cell activation-induced oxidative signaling. *Signaling* phase (1), upon T cell activation mitochondrial complex I-mediated $O_2^{\cdot-}$ release is up-regulated. MnSOD participates in generation of H_2O_2 by mitochondria. Due to low MnSOD levels, H_2O_2 could also be generated by reactions other than $O_2^{\cdot-}$ dismutation, which have product/substrate ratio higher than that of the dismutation reaction. A rise in the cytoplasmic H_2O_2 concentration leads to activation of the redox-dependent transcription factors, NF-κB and AP-1, and therefore facilitates expression of immediate-early CD95L/IL-2 genes as well as MnSOD. *Signal shut-down* phase (2), transcriptional upregulation of MnSOD results in its higher mitochondrial content and activity. Mitochondrial H_2O_2 release is inhibited, presumably due to the out-titration effect of higher intra-mitochondrial MnSOD abundance. Lowering of cytoplasmic H_2O_2 levels leads to a less pro-oxidative environment and blunts NF-κB and AP-1 activation.

According to experiments on isolated mitochondria, the first step of complex I-mediated H_2O_2 generation is formation of $O_2^{\cdot-}$ and its release into the mitochondrial matrix [22]. Next, $O_2^{\cdot-}$ has to be transformed into H_2O_2, which acts as an oxidative signaling molecule. As electrically neutral and relatively stable compound, H_2O_2 crosses mitochondrial membranes, diffuses into the cytosol and potentiates TCR signaling *via* reversible inactivation of negative regulatory phosphatases or direct influence on NF-κB/AP-1 activity or DNA binding [7,8,49].

In order to avoid potentially deleterious ROS effects as well as to allow for spatiotemporal signaling integration, the extent and duration of the oxidative signal have to be tightly regulated. Therefore, the TCR-induced generation of the mitochondrial oxidative signal is expected to be limited by a subsequent antioxidative response.

MnSOD is a major antioxidative enzyme, localized exclusively in the lumen of the mitochondrial matrix. It provides a first line of defense against $O_2^{\cdot-}$ released by the electron chain as a byproduct of respiration [50]. MnSOD protects mitochondrial proteins, lipids and mtDNA from $O_2^{\cdot-}$ by efficiently dismutating it to H_2O_2 and O_2. Importantly, the enzyme operates with one of the fastest catalytical rates known (reaction rate constant at pH 7.8, $k = \text{app. } 2\times10^9\ M^{-1}\ s^{-1}$ [50]). Moreover, in activated T cells residual MnSOD activity fulfills an additional role. It mediates the first step in the process of mitochondrial oxidative signal generation by facilitating $O_2^{\cdot-}$ to H_2O_2 conversion.

Interestingly, we have observed that the kinetics of the TCR-induced oxidative signal generation as well as NF-κB/AP-1-dependent IL-2 and CD95L gene expression inversely correlates with the protein content of MnSOD (Figs. 1A–C, 2A, Suppl. Fig. 1A, C). Strikingly, the amounts of other major antioxidative enzymes, such as CuZnSOD (SOD1, located in the cytoplasm and mitochondrial inter-membrane space) as well as H_2O_2-removing proteins, catalase (located in peroxisomes, cytoplasm, mitochondrial inter-membrane space) and thioredoxin reductase 2 (TrxR2, located in the mitochondrial matrix) remained unaltered. In addition, rise in the MnSOD protein level corresponded with a significant increase in MnSOD-specific activity (Fig. 1D, Suppl. Fig. 1D).

The influence of enhanced MnSOD activity and content on mitochondrial H_2O_2 generation is a matter of debate. Nevertheless, theoretical models underline that in the case of steady-state $O_2^{\cdot-}$ generation increased MnSOD activity potentially results in over-all decreased H_2O_2 release from mitochondria [51,52]. Moreover, in different experimental systems where MnSOD was overexpressed or down-regulated respective decrease or increase of cellular oxidative stress was observed [32,53,54]. Increased amounts of MnSOD more efficiently remove $O_2^{\cdot-}$ due to the out-titration effect. Thus, there is a reduced probability of $O_2^{\cdot-}$ to participate in other H_2O_2-generating reactions (*e.g.* reduction of aconitase [4Fe–4S] cluster), that yield more H_2O_2 per single $O_2^{\cdot-}$ molecule than the dismutation reaction itself [52]. Consequently, TCR-induced $O_2^{\cdot-}$ release by complex I, which during the *signaling phase* manifests itself in H_2O_2 generation ($O_2^{\cdot-}$ generation increased, MnSOD "low", H_2O_2 release increased) is followed by a signal *shut-down phase* ($O_2^{\cdot-}$ generation increased, MnSOD "high", H_2O_2 release decreased, see Fig. 6) [51,52].

Results obtained with Jurkat T cells overexpressing MnSOD in a transient or DOX-inducible fashion support this assumption. In both experimental systems used, enforced upregulation of MnSOD content significantly blocked PMA-induced mitochondrial oxidative signal

generation already during the *signaling phase* (Fig. 3C, D). Interestingly, as reported before [10], MnSOD activity in non-transfected Jurkat cells initially rises upon PMA or TCR stimulation independently of subsequent activation-induced upregulation of MnSOD content, thus facilitating at first the oxidative signal generation. In addition, as we reported in a recent study [55], when the T cell activation-induced rise in MnSOD content was abolished, due to NF-κB pathway inhibition by constitutive IκBα expression, oxidative signal generation was enhanced.

It is therefore apparent that MnSOD serves as a regulatory and limiting switch in the process of oxidative signal generation of activated T cells. However, since the mitochondrial redox equilibrium depends on a confined glutathione pool [56] and separate thioredoxin system [57] analysis of secondary steps governing TCR-induced mitochondrial H_2O_2 generation and scavenging is necessary to fully elucidate the mechanisms of the oxidative signal generation.

Concomitantly with an inhibition of the mitochondrial oxidative signal, MnSOD overexpression leads to the abrogation of T cell activation-induced NF-κB and AP-1 triggering (Fig. 4A). Similar effects were reported for other experimental systems [58–61]. Consequently, IL-2/CD95L promoter activities, IL-2/CD95L gene expression as well as IL-2 secretion were inhibited (Figs. 4B–E and 5A–C). Since CD95L is the major mediator of AICD in Jurkat T cells [9–11], transient or DOX-inducible MnSOD overexpression resulted in diminished cell death upon T cell activation (Fig. 5D, F). Extent of activation-induced caspase 8 processing was also decreased (Fig. 5E). Previously, it has been shown that high levels of MnSOD could protect malignant cells against cell death induced by TNF-α, anti-cancer drugs or irradiation [44–48]. Moreover, cleavage and inactivation of MnSOD is a necessary step during CD95 receptor-induced apoptosis of Jurkat T cells [62]. However, MnSOD-overexpressing Jurkat T cells were equally prone to CD95 receptor-triggered apoptosis as control cells (Fig. 5G). Thus, observed lower AICD could be attributed to the decreased activation-induced CD95L level but not to anti-apoptotic effects of MnSOD at the stage of mitochondrial rupture.

A rise in MnSOD content could not be associated with increased mitochondrial mass or decreased mitochondrial turnover (Fig. 2A, B). However, a number of reports demonstrate a positive regulatory role for NF-κB in MnSOD transcription [40–42]. Indeed, MnSOD expression is induced upon activation of *in vitro* expanded human T cells and the transcript levels temporarily coincide with the extent of the oxidative signal (Fig. 2C). Of note, as compared to immediate-early genes, such as IL-2 and CD95L, MnSOD transcript levels decrease less abruptly, which could be indicative for higher mRNA stability (Fig. 1B).

Treatment with NAC, an antioxidant capable of inhibiting activation-induced oxidative signal generation, NF-κB activation as well as NF-κB-dependent gene expression [3,9,10] (Suppl. Fig. 2A, B), efficiently blocked MnSOD transcription (Fig. 2D, Suppl. Fig. 2D). Moreover, Trolox, a compound with antioxidative properties different from those of NAC, did not only efficiently inhibit activation-induced ROS generation but also MnSOD expression (Suppl. Fig. 2C and D). In addition, in a recent study by Kiessling et al. we showed that overexpression of IκBα, a major inhibitor of the NF-κB pathway, abrogates upregulation of MnSOD upon T cell activation [55]. Therefore, a negative feedback loop could be postulated between ROS/NF-κB-dependent MnSOD expression and MnSOD-dependent control of ROS generation. Moreover, a signaling process involving T cell activation-induced mitochondrial oxidative signal and a regulatory role of MnSOD exemplifies a mechanism of mitochondria-to-nucleus retrograde communication [63] (Fig. 6). A similar mechanism for regulation of MnSOD expression by mitochondria-generated ROS (H_2O_2) and NF-κB activation has been also proposed by others [64,65].

The MnSOD$^{-/-}$ mice, constitutively lacking protein expression, die shortly after birth [66]. Nevertheless, application of other genetic mouse models demonstrates a regulatory role for MnSOD in T cell development and function. Using Lck promoter-controlled T cell-confined MnSOD knock-out mice, Case et al. showed that MnSOD is crucial for proper thymocyte differentiation, homeostatic survival of peripheral T cells as well as for T cell-mediated immune responses [34]. Conditional loss of MnSOD led to increased mitochondrial $O_2^{\cdot-}$ levels, increased thymocyte apoptosis, decreased number of peripheral T cells and impaired clearance of the influenza A H1N1 virus. Interestingly, peripheral T cells and thymocytes were in a more activated state as judged by a greatly increased proportion of Mel14^{-}/CD44^{+} cells. In addition, Maric et al. reported that a lowered content and activity of MnSOD in peripheral T cells of INFγ-inducible lysosomal thiol reductase (GILT) knock-out mice manifests itself in increased sensitivity to TCR ligation and accelerated development of T cell-dependent diabetes [35]. CD3 triggering of GILT$^{-/-}$ T cells resulted in higher levels of $O_2^{\cdot-}$, both before and after stimulation, as well as increased and more persistent ERK1/2 activation.

Multiple studies support tumor suppressor function of MnSOD [28]. Reduced protein level, inactivating mutations, genetic polymorphisms or gene silencing of MnSOD have been associated with increased intracellular oxidative stress, resulting in higher susceptibility to tumor development or enhanced tumorigenic potential [24,26,27,31]. On the contrary, increased MnSOD expression exerts an anti-tumor effect, *i.e.* reduces proliferation of cancer cells *in vitro* or tumor formation *in vivo* [29,58,67]. To this end, MnSOD$^{-/+}$ mice are prone to spontaneous lymphoma development [32]. Moreover, studies using the Lck-Bax 38/1 mouse model of T cell lymphoma demonstrated that crossing with Lck-MnSOD transgenics abrogates lymphoma development [23]. Increased gene dosage of MnSOD significantly improved chromosomal stability of the Lck-Bax 38/1T cells, and in turn decreased aneuploidy of thymocytes and delayed the onset of lymphoma.

In conclusion, the existence of a MnSOD-mediated switch in the process of T cell activation-induced gene expression and CD95L-dependent AICD provides yet another argument for a crucial importance of mitochondria, and MnSOD in particular, for the regulation of T cell function.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2012.03.003.

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