# Inhibition of Superoxide Generation upon T-Cell Receptor Engagement Rescues Mart-$1_{27-35}$–Reactive T Cells from Activation-Induced Cell Death

Håkan Norell,[1] Telma Martins da Palma,[1] Aaron Lesher,[1] Navtej Kaur,[1] Meenal Mehrotra,[3] Osama S. Naga,[1] Natalie Spivey,[1] Seye Olafimihan,[1] Nitya G. Chakraborty,[4] Christina Voelkel-Johnson,[2] Michael I. Nishimura,[1,2] Bijay Mukherji,[4] and Shikhar Mehrotra[1,4]

Departments of [1]Surgery, [2]Microbiology and Immunology, and [3]Pathology, Medical University of South Carolina, Charleston, South Carolina and [4]Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut

## Abstract

**Cytotoxic T lymphocytes (CTL) may undergo massive expansion upon appropriate antigenic stimulation. Homeostasis is maintained by a subsequent "contraction" of these cells. Activation-induced cell death (AICD) and programmed cell death prevent the untoward side effects, arising from excessive numbers and prolonged persistence of activated CTL, that occur upon uncontrolled and/or continued expansion. However, effector cell persistence has been identified as a hallmark of successful T-cell–mediated adoptive immunotherapy. Thus, prevention of AICD may be critical to achieve more successful clinical results. We have previously shown that treatment with the c-Jun $NH_2$-terminal kinase (JNK) inhibitor SP600125 protects human melanoma epitope Mart-$1_{27-35}$–reactive CTL from apoptotic death upon their reencounter with cognate antigen. However, inhibition of JNK also interferes with the functional ability of the CTL to secrete IFN-γ. Here, we show that reactive oxygen species (ROS) inhibitors, such as the superoxide dismutase mimetic Mn (III) tetrakis (5, 10, 15, 20-benzoic acid) porphyrin (MnTBAP), efficiently protected Mart-$1_{27-35}$–reactive primary CTL from AICD without impairing their functional capability. MnTBAP prevented the increase in intracellular ROS, mitochondrial membrane collapse, and DNA fragmentation observed in control-treated cells upon cognate antigen encounter. Furthermore, the mechanism of AICD prevention in primary CTL included blockade of JNK activation. Finally, tumor-reactive *in vitro* expanded tumor infiltrating lymphocytes, which are used clinically in cancer immunotherapy, also benefit from MnTBAP-mediated antioxidant treatment. Thus, modulation of the redox pathway might improve CTL persistence and lead to better clinical results for T cell–based immunotherapies.** [Cancer Res 2009;69(15):6282–9]

## Introduction

Maintenance of T-cell homeostasis is a complex process controlled by balancing the expansion and contraction of T cells. Programmed cell death and activation-induced cell death (AICD) prevent untoward side effects of an uncontrolled and persistent T-cell effector response and maintain homeostasis (1, 2). However, AICD may be detrimental to immunotherapy, especially if activated cytotoxic T lymphocytes (CTL) undergo apoptosis directly upon reencountering their cognate antigen. Thus, prolonging T-cell survival by inhibiting AICD may enhance the therapeutic benefit of various immunotherapeutic strategies (3, 4).

Several experimental models of tumor immunotherapy show that T-cell persistence is important for therapeutic success. Long-term persistence of adoptively transferred T-cell receptor (TCR) transgenic CTL was associated with tumor regression (5). Overexpression of the antiapoptotic protein Bcl-2 in effectors directly inhibits apoptosis and thereby prolongs T-cell survival. Importantly, Bcl-2 overexpression in TCR transgenic CTL significantly enhanced adoptive immunotherapy of established murine melanomas (6). Cancer regression in patients receiving autologous tumor infiltrating lymphocytes (TIL) was also significantly correlated with their persistence in peripheral blood (7). These studies suggest that AICD may limit T-cell survival *in vivo* and that inadequate T-cell persistence limits current adoptive immunotherapy protocols.

Death receptor ligation and activation of the caspase cascade have been considered the principal triggers for AICD. However, recent findings have established that some death signals originate internally and that not all types of cell death are caspase mediated (8). DNA damage, reactive oxygen species (ROS), nitric oxide, and excess mitochondrial $Ca^{2+}$ may all promote AICD (9). Our previous studies have shown that cognate antigen exposure induces AICD in primary human CTL (10). Furthermore, we found that the c-Jun $NH_2$-terminal kinase (JNK) inhibitor SP600125 rescued $CD8^+$ T cells reactive to either a melanoma-associated epitope (Mart-$1_{27-35}$) or an influenza matrix protein epitope ($MP_{58-66}$) from a caspase-independent AICD (11, 12). However, SP600125 concomitantly interfered with the ability of activated CTL to secrete IFN-γ. A role for ROS in mitochondrial damage and caspase-independent death is documented in diverse models (13, 14). Interestingly, antioxidant Mn (III) tetrakis (5, 10, 15, 20-benzoic acid) porphyrin (MnTBAP) was shown to block death of mouse $CD4^+$ T cells after exposure to strong polyclonal stimuli with the superantigen staphylococcal enterotoxin A (SEA; ref. 15). Protection from cell death was attributed to blockade of ROS production, which is normally initiated upon T-cell activation and sensitizes T cells to apoptosis by decreasing Bcl-2 expression (16).

Here, we evaluated the effect of ROS inhibition on AICD after restimulation with the cognate epitope of Mart-$1_{27-35}$ antigen–reactive primary human CTL. Notably, MnTBAP could protect a large fraction of the activated CTL from undergoing AICD. Importantly, MnTBAP did not interfere with T-cell effector functions, including their ability to secrete cytokines. Furthermore, clinically relevant effectors, such as *in vitro* expanded TIL, were also protected from AICD after MnTBAP pretreatment. Thus,

**Requests for reprints:** Shikhar Mehrotra, Department of Surgery, Hollings Cancer Center, Medical University of South Carolina, 86 Jonathan Lucas Street, Charleston, SC 29425. Phone: 843-792-9195; Fax: 843-792-2556; E-mail: mehrotr@musc.edu.



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strategies to modulate the redox pathway may improve T-cell survival (17, 18) without impairing effector cell functionality, thereby conferring therapeutic benefit to T cell–based immunotherapies for various diseases (19, 20).

## Materials and Methods

**Cells.** Peripheral blood mononuclear cells (PBMC) from HLA-A2–positive healthy donors were obtained with informed consent. TIL1235 (reactive to the human Mart-$1_{27-35}$ antigen), HLA-$A2^+$ human melanoma MEL624, and its HLA-$A2^-$ variant MEL624-28 were obtained from surgical specimens of patients undergoing experimental immunotherapies at the Surgery Branch, National Cancer Institute (21). T2 cells are transporter associated and protein deficient, and its empty surface HLA-A2 molecules were used for direct presentation of epitopes to the antigen-reactive CTL.

**Culture medium and reagents.** Mart-$1_{27-35}$ peptide (AAGIGILTV) and $MP_{58-66}$ peptide (GILGFVFTL) were purchased from MP Systems. Culture medium was Iscove's Modified Dulbecco's Medium (Life Technologies Bethesda Research Laboratories) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Inc.). Media for TIL1235 was supplemented with 6,000 IU/mL interleukin-2 (IL-2; Chiron). Ficoll-Paque was obtained from Amersham Bioscience. Recombinant cytokines were purchased from R&D Systems. MHC class I tetramers and pentamers were purchased from Beckman Coulter and ProImmune, respectively. These reagents bind directly to the TCR of a particular specificity, determined by the MHC allele and peptide combination, and thus can be used to detect and separate antigen-specific $CD8^+$ T-cell populations. Antibody to detect single-stranded DNA (ssDNA) was obtained from Alexis Biochemicals, whereas all other fluorochrome-labeled monoclonal antibodies and Annexin V were purchased from BD Biosciences. 7AAD was purchased from Calbiochem, and hydroethidium was from Sigma Chemicals. Inhibitors as SP600125 for JNK, PD098059 for extracellular signal-regulated kinase (ERK), and antioxidants MnTBAP and L-NAC were purchased from EMD Biosciences.

**Generation of dendritic cells from peripheral blood monocytes.** The procedure for generating dendritic cells (DC) from peripheral blood monocytes has been published (10). Briefly, circulating monocytes were enriched by adherence of Ficoll-Hypaque density gradient–isolated PBMC. The adherent cells were cultured in complete media (CM) containing 1,000 IU/mL granulocyte macrophage colony-stimulating factor and 500 IU/mL IL-4 for 3 to 5 d to obtain a population of immature DCs. Maturation of immature DCs was performed by first adding IFN-γ (1,000 U/mL) for 2 h followed by 100 ng/mL lipopolysaccharide overnight.

**Activation of $CD8^+$ T cells by DC-based presentation of epitopes *in vitro*.** The procedure for peptide-loaded DC-based *in vitro* activation and expansion of epitope-reactive $CD8^+$ T cells has been described (10, 11). Briefly, Ficoll-Hypaque gradient-separated PBMCs were purified for $CD8^+$ T cells (≥90% purity) by Dynal magnetic bead isolation kit (Invitrogen) and cocultured with autologous irradiated (3,000 rad) DCs pulsed with relevant peptides (100 μg/mL) and 5 μg/mL of β2 microglobulin at a $CD8^+$ T cell:DC ratio of 100:1. Activated CTLs were maintained in media containing IL-15 (10 ng/mL).

**AICD induction and evaluation.** CTL were preincubated with inhibitors at predetermined optimal concentration for 30 min at 37°C and then exposed to cognate or noncognate antigens in the form of either MHC class I pentamer reagents or peptide-loaded T2 cells for induction of antigen-specific apoptosis (11, 12). Apoptosis was determined by flow cytometry with three or four color staining (e.g., CD8, HLA-A2 pentamer, 7AAD, and Annexin V) and acquired on a FACSCalibur (Becton Dickinson) or an Accuri C6 (Accuri Cytometers) flow cytometer, and data were analyzed using FlowJo software (Tree Star, Inc.).

**Measurement of mitochondrial membrane potential ($\Delta\psi$).** $\Delta\psi$ was estimated by staining with 20 nmol/L $DiOC_6$ (Molecular Probes), a cationic lipophilic dye, for 15 min at 37°C in the dark before analysis by flow cytometry. Fluorescence of $DiOC_6$ is oxidation independent and correlates with $\Delta\psi$ (22).

**Cytokine release assay.** Cytokine release by the effector cells was determined by coculturing $1 \times 10^4$ to $1 \times 10^5$ effector cells in a 1:1 ratio with melanoma tumor cells or peptide-pulsed T2 cells as described previously (10). After 16 to 24 h, culture supernatants were harvested and cytokine concentrations measured by sandwich ELISA per the manufacturer's protocol (R&D Systems) using a spectrophotometer (BioTek).

**CD107a degranulation assay.** Cell surface expression of CD107a was used as a surrogate marker for degranulation (23). $1 \times 10^5$ CTLs were cocultured with melanoma tumor cells in a 1:1 ratio overnight before staining with an anti-CD107a monoclonal antibody and analyzed by fluorescence-activated cell sorting (FACS).

**Western blot.** Protein extractions were performed in radioimmunoprecipitation assay buffer, and samples were separated on 15% SDS polyacrylamide gels and electrophoretically transferred to polyvinylidene

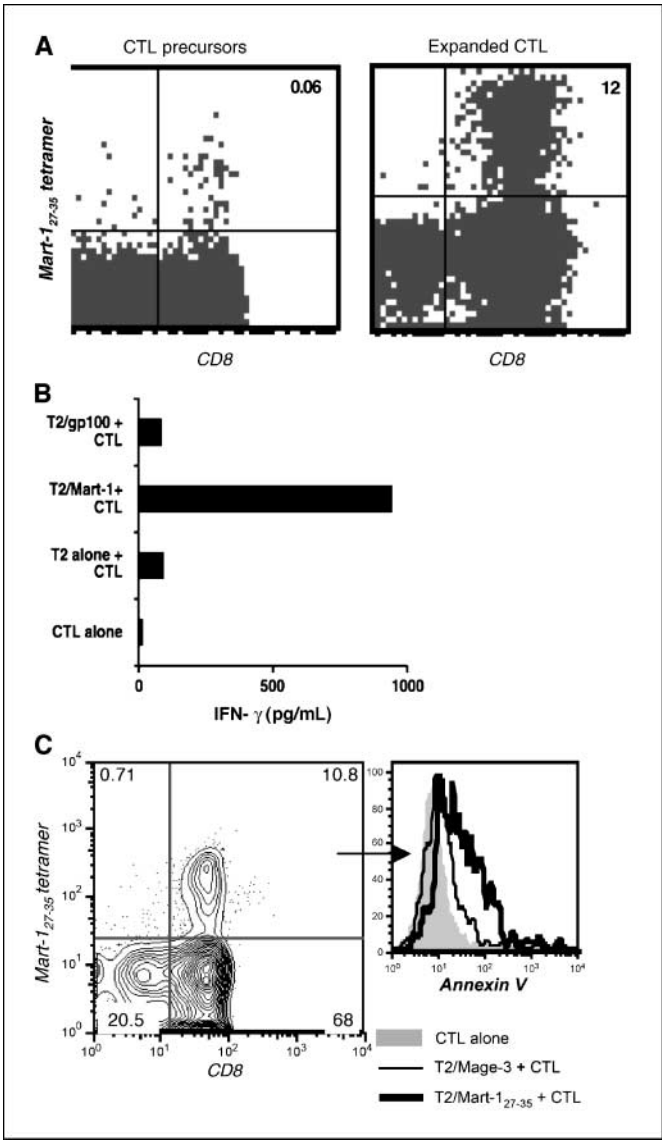


**Figure 1.** Expansion of Mart-$1_{27-35}$–reactive CTL and induction of AICD. *A,* Mart-$1_{27-35}$–reactive CTL precursors (*left*) and CTL expanded with autologous DC pulsed with peptides were tracked by FACS using staining for CD8 and tetramer reagents. *B,* IFN-γ secreted into coculture supernatant collected after overnight incubation of Mart-$1_{27-35}$–reactive CTL stimulated with cognate or control peptide-pulsed T2 cells. *C,* expanded CTLs were restimulated with cognate or control epitope-pulsed T2 cells and stained after 4 h with tetramer, CD8, and Annexin V. Histogram depicts fluorescence intensity for Annexin V in tetramer-gated $CD8^+$ CTL when unstimulated (*gray filled*), stimulated with T2 cells pulsed with control peptide (*thin black line*), and stimulated with T2 cells pulsed with cognate peptide (*thick black line*). All data represent one of at least seven separate experiments with similar results.

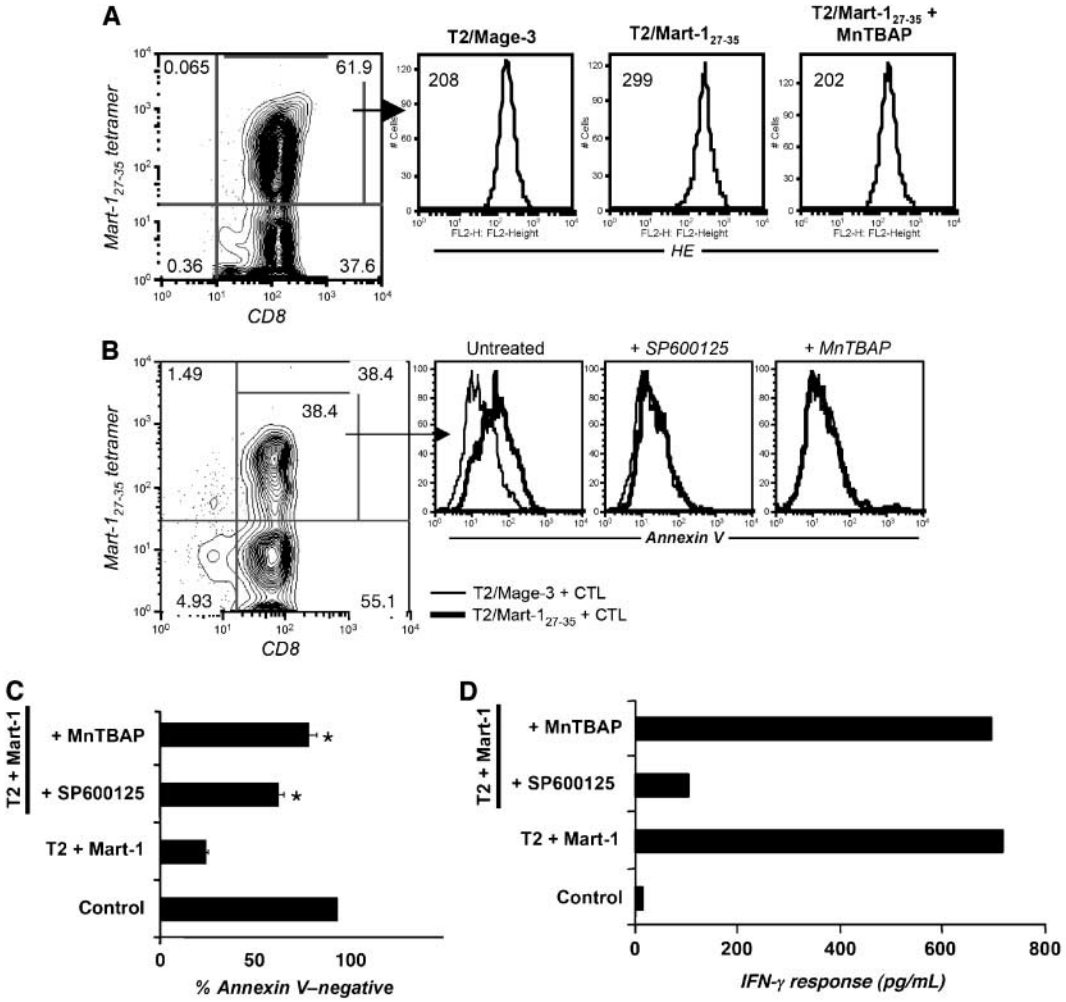


**Figure 2.** Increased endogenous ROS levels after TCR stimulation and rescue of Mart-$1_{27-35}$–reactive CTL from AICD by ROS inhibition. Mart-$1_{27-35}$ epitope–reactive CTLs were stimulated with control or cognate peptide-pulsed T2 cells (1 μg/mL) or first preincubated with 400 μmol/L MnTBAP or 25 μmol/L SP600125 for 30 min and then stimulated with their cognate epitope. *A,* staining with hydroethidium (*HE*) was performed 4 h later. Histogram represents fluorescence intensity of hydroethidium (*HE*) on the antigen-reactive CTL. Numbers represent mean fluorescence intensity. *B,* induction of AICD in CTL was evaluated by staining for tetramer, CD8, and Annexin V 4 h later. Histogram overlays depict Annexin V expression on tetramer-gated CTL stimulated with T2 cells pulsed with control peptide (*thin black line*) and relevant peptide (*thick black line*), respectively. *Mage-3,* control epitope; *Mart-$1_{27-35}$,* cognate epitope. *C,* another representation of how Mart-$1_{27-35}$–reactive CTLs were rescued from death (and thus remained Annexin V–negative) to various degrees in response to pretreatment with SP600125 and MnTBAP before cognate antigen exposure. *, $P < 0.05$ compared with untreated CTL stimulated with T2 cells pulsed with Mart-$1_{27-35}$ peptide. *D,* matched IFN-γ secretion from the setup in *C* measured by ELISA. All data represent one of three separate experiments with similar results.

difluoride membranes (Millipore). The primary antibodies (anti-JNK, anti-pJNK, and anti–β-actin), and secondary antibodies were obtained from Santa Cruz Biotechnologies.

## Results

**Activation and expansion of Mart-$1_{27-35}$–reactive CTL.** Mart-$1_{27-35}$ epitope–reactive CTL were expanded from PBMC of healthy donors using peptide-pulsed autologous mature DCs (Fig. 1*A*). Within 12 days, Mart-$1_{27-35}$ T cells expanded 200-fold (0.06–12%), as measured by staining with HLA-A2 tetramers loaded with Mart-$1_{27-35}$, and secreted significant amounts of IFN-γ when stimulated with their cognate antigen (Fig. 1*B*). Functional Mart-$1_{27-35}$–reactive T cells prepared in this manner were used throughout our studies to understand mechanisms of AICD in primary human antigen-reactive CTL.

**Sensitivity of expanded Mart-$1_{27-35}$–reactive CTL to AICD.** To evaluate the effect on viability of TCR ligation on the Mart-$1_{27-35}$–reactive CTL upon exposure to cognate antigen, cells were stained with the relevant tetramer and Annexin V. Figure 1*C* shows increased Annexin V binding, indicating decreased viability of tetramer-positive cells upon exposure of the TCR to its cognate antigen (thick black overlays) compared with the control peptide (thin black lines) or unstimulated cells (gray-filled overlay). Thus, the cell death observed in this model is antigen specific and consistent with the idea that repeated TCR-mediated activation of CTL results in AICD (11, 12).

**TCR-dependent superoxide generation.** Fluorescence intensity of the intracellular stain hydroethidium, which tracks superoxide levels (15, 22), showed a 35% to 50% increase upon TCR engagement with the cognate peptide compared with a control

peptide in Mart-$1_{27-35}$–reactive CTL (Fig. 2*A*). Preincubation of Mart-$1_{27-35}$–reactive CTL with the antioxidant MnTBAP prevented this increase. These data suggested that superoxide levels increase upon TCR stimulation with the cognate epitope, leading us to further examine the role of ROS in AICD of CTL.

**Inhibition of ROS and AICD.** As shown in Fig. 2*B*, Mart-$1_{27-35}$–reactive CTL preincubated with MnTBAP exhibited less increase in Annexin V staining than untreated CTL upon cognate antigen stimulation compared with control peptide. Protection from AICD by MnTBAP indicates that there is a role of ROS in AICD. Addition of 2-mercaptoethanol alone to the coculture media had no rescuing effect on T cells undergoing AICD (data not shown). The data obtained with MnTBAP were similar to our previous observation with the JNK inhibitor SP600125 (Fig. 2*B* and *C*; refs. 11, 12). One drawback to using SP600125 is that JNK inhibition also inhibits cytokine secretion (11, 12). Therefore, we examined the effect of MnTBAP on IFN-γ production. As shown in Fig. 2*D*, in contrast to SP600125, antioxidant MnTBAP had no effect on the ability of Mart-$1_{27-35}$–reactive T cells to secrete IFN-γ. Moreover, CTL, pretreated with MnTBAP and thus rescued from AICD, accumulated to higher numbers when left in culture for 5 days (data not shown). As a further demonstration that CTL could be rescued from AICD by superoxide quenching, several other commercially available cell-permeable ROS inhibitors (L-NAC, MnTPyP, Tiron, and D1417) were tested (24). All these compounds rescued CTL from AICD (data not shown). These results provide a proof of principle that antioxidants can be used to rescue CTL from TCR-mediated AICD. Furthermore, it indicates that T-cell effector function and AICD signaling pathways are separate. Thus, rescuing T cells from AICD by antioxidants may increase the overall persistence of CTL while preserving effector function.

**Mechanism of ROS inhibition in rescue from AICD.** During apoptosis, mitochondrial membrane permeability changes are accompanied by release of various proteins from the intermembrane space that initiate and maintain a caspase cascade, chromatin condensation, and DNA fragmentation. Release of these proteins results in permeabilization of the inner mitochondrial membrane and dissipation of $\Delta\psi$ (25), which can be assessed through FACS analysis with a fluorescent dye like $DiOC_6$ (26). The mitochondrial membrane integrity of CTL undergoing AICD and the effect of MnTBAP pretreatment on this event was evaluated. Figure 3*A* shows that stimulation of Mart-$1_{27-35}$–reactive CTL with cognate epitope resulted in ~50% decrease in $DiOC_6$ staining compared with controls, indicating an antigen-driven loss of $\Delta\psi$. Pretreatment with the ROS inhibitor MnTBAP or JNK inhibitor SP600125 resulted in less decrease in $\Delta\psi$ (28% and 22%, respectively). Another means of measuring apoptosis is DNA fragmentation caused by frequent single-strand cuts (27). An antibody that recognizes deoxycytidine of ssDNA of at least 25 to 30 bases in length, in the absence of any reactivity to double-stranded DNA, and specifically detects apoptotic, but not necrotic, cells was used (28). As shown in Fig. 3*B*, restimulation of Mart-$1_{27-35}$–reactive CTL with their cognate peptide to induce AICD resulted in around 70% increase of cells with nicked ssDNA compared with controls. In contrast, CTL pretreated with SP600125 or MnTBAP before the exposure to cognate antigen had only marginally higher fractions of cells with ssDNA than CTL restimulated with the control peptide. Thus, pretreatment of epitope-specific CTL with superoxide inhibitor MnTBAP rescued them from AICD by reducing damage to mitochondria and DNA.

**Sensitivity of TIL to AICD and rescue by MnTBAP treatment.** A high proportion of melanoma patients was recently shown to exhibit objective clinical responses when given nonmyeloablative chemotherapy before the transfer of autologous TIL (29). If ROS inhibition could also rescue TIL from AICD, it would have direct clinical application by improving *ex vivo* expansion of TIL for immunotherapy. Importantly, ROS inhibition can reduce telomere shortening, restrict replicative senescence (30), and potentially increase *in vivo* persistence of TIL. Like PBMC-derived CTL, TIL1235 also underwent AICD upon antigen stimulation as

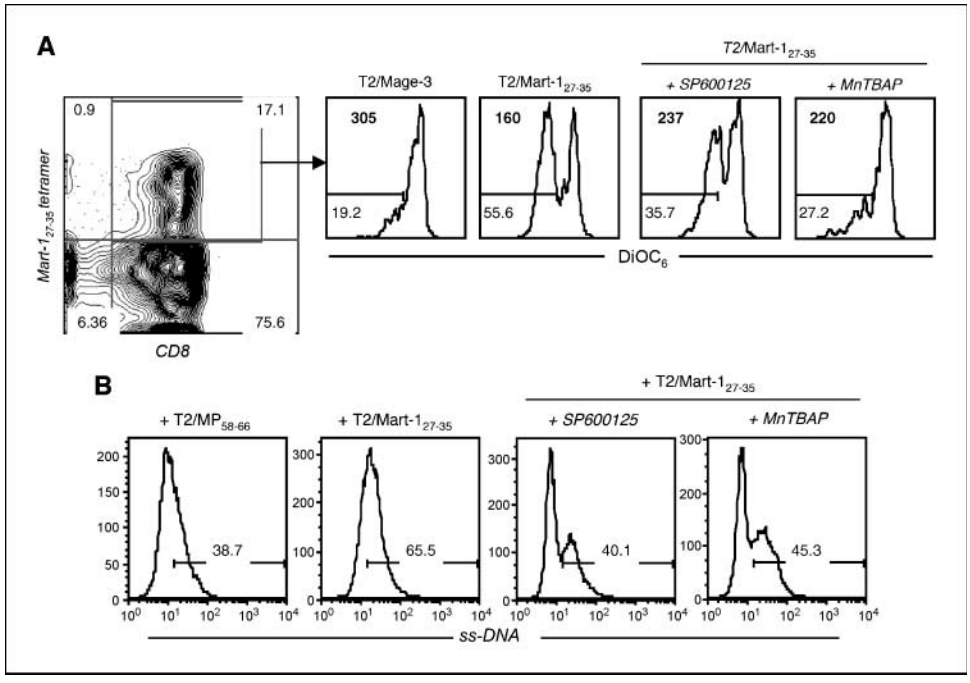


**Figure 3.** Effect of TCR stimulation and ROS inhibition on the membrane potential ($\Delta\psi$) and DNA degradation. Mart-$1_{27-35}$–reactive CTL preincubated with MnTBAP (400 μmol/L) and SP600125 (25 μmol/L) were stimulated with cognate Mart-$1_{27-35}$ and control Mage-3 peptide-pulsed T2 cells for induction of AICD. *A,* histogram represents $DiOC_6$ staining on tetramer-gated CTL. Numbers in upper left corner represent mean fluorescence intensity for $DiOC_6$ staining and numbers in lower left corner represent the percentage of cells that have low membrane potential. *B,* ssDNA levels in tetramer-positive cells were determined with ssDNA-specific antibody. Data presented show one representative experiment of two.

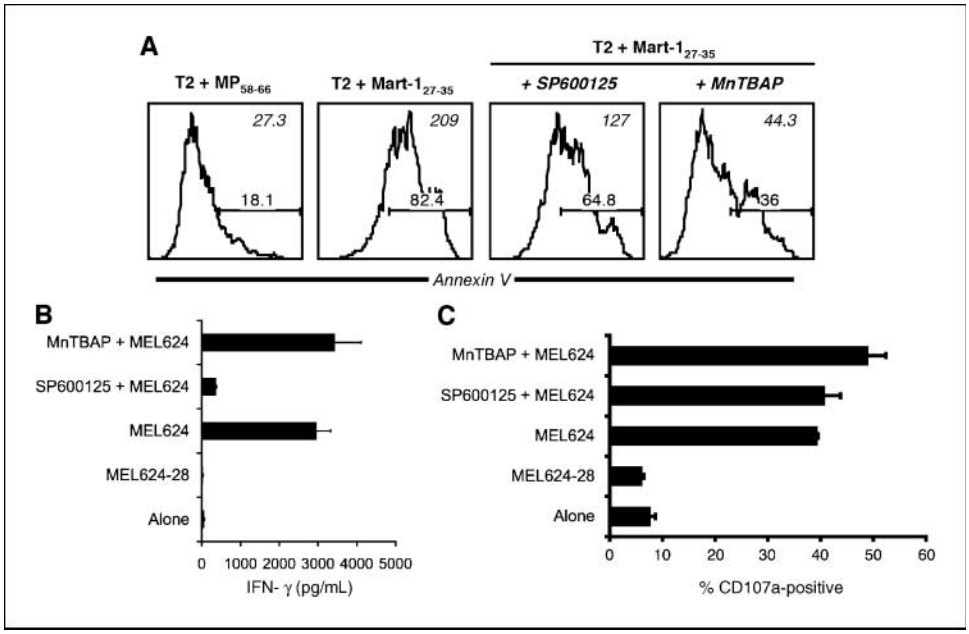


**Figure 4.** Pretreatment with MnTBAP protects TIL from AICD without impairing function. Mart-1–reactive TIL1235 preincubated with media alone, MnTBAP (250-500 μmol/L), and SP600125 (25 μmol/L) were cocultured with target cells. *A,* after 4 h of coculture with T2 cells pulsed with cognate or control peptide (1 μg/mL), cells were stained with Annexin V, 7AAD, and CD8 and analyzed by FACS. Histogram represents the log fluorescence of Annexin V on $CD8^+$ gated TIL. *Top right,* numbers represent mean fluorescence intensity; *bottom right,* numbers represent the percentage of cells that stained positive for Annexin V. *B*, IFN-γ release by, and *C*, CD107a expression on TIL1235 upon coculture with HLA-A2–positive (MEL624) or matched HLA-A2–negative (MEL624-28) melanoma cells. All data are from one representative experiment of at least two.

measured by Annexin V staining (Fig. 4*A*). Importantly, MnTBAP treatment efficiently rescued TIL from AICD without impairing effector functions. Cytokine secretion (Fig. 4*B*), cytolytic activity as measured using degranulation assay (Fig. 4*C*; ref. 23), and the proliferative potential of T cells as measured using Ki-67 staining (ref. 31; data not shown) were all intact in MnTBAP-pretreated cells. As expected, the JNK inhibitor SP600125 impaired the ability of TIL to secrete cytokines (Fig. 4*B*) without affecting their degranulation (ref. 12; Fig. 4*C*). Thus, TIL are also prone to ROS-mediated AICD and benefit from antioxidant treatment.

**Effect of superoxide inhibition on AICD of "nonself" influenza matrix epitope–reactive CTL.** Because tumor-associated human melanoma "self" epitope Mart-$1_{27-35}$–reactive T cells may behave differently than "nonself" epitope–reactive T cells, we evaluated the effect of superoxide inhibition on influenza matrix epitope $MP_{58-66}$–reactive CTL. These nonself-reactive CTL were also expanded from PBMC of healthy donors using peptide-pulsed autologous DC (Fig. 5*A, i*), and secreted significant amounts of IFN-γ when stimulated with their cognate antigen relative to controls (Fig. 5*A, ii*). As with Mart-$1_{27-35}$–reactive CTL, restimulation of $MP_{58-66}$-reactive CTL resulted in an increase in Annexin V staining of tetramer-positive cells upon exposure to the cognate antigen (thick black overlays) compared with the control peptide (thin black overlays) or unstimulated cells (gray-filled overlay; Fig. 5*B*). In addition, fluorescence intensity of hydroethidium, tracking intracellular superoxide levels (22), showed almost 30% increase in $MP_{58-66}$-reactive CTLs after TCR engagement with relevant peptide compared with the control peptide (Fig. 5*C*). This increase was blocked by MnTBAP treatment. An analysis of ssDNA revealed that pretreatment with MnTBAP reduced the extent of DNA fragmentation upon cognate antigen encounter (Fig. 5*D*), as observed with Mart-$1_{27-35}$–reactive CTL. Thus, these observations suggest that *in vitro* expanded tumor epitope Mart-$1_{27-35}$–reactive and viral epitope $MP_{58-66}$–reactive CTLs undergo AICD by similar mechanisms and could be rescued by inhibiting endogenous ROS.

**Effect of superoxide inhibition on JNK phosphorylation.** Our previous data showed that JNK is activated in CTLs undergoing apoptosis (11, 12). Other studies have also suggested that ROS play a role in JNK activation (32, 33). To determine the role of JNK in the MnTBAP-mediated inhibition of AICD in CTL, we examined the level of JNK phosphorylation in untreated or inhibitor-pretreated $MP_{58-66}$-reactive T cells stimulated with cognate and control pentamer reagents. Cell lysates were prepared and analyzed by Western blot using antibodies specific for JNK and phosphorylated JNK. The total level of JNK was relatively constant regardless of pretreatment and antigenic stimulation (Fig. 6*A*). In contrast, the level of phosphorylated JNK increased upon exposure to the cognate antigen compared with the control. This increase was completely blocked by MnTBAP and SP600125 pretreatment (Fig. 6*B*), but not by ERK inhibitor PD098059, which served as specificity control. Because AICD induced in $MP_{58-66}$–reactive T cells using cognate pentamer negates the role of costimulatory signal, the observed phosphorylation of JNK is TCR complex specific. These data suggest that ROS could control CTL viability by regulating the activity of proapoptotic and antiapoptotic proteins through JNK phosphorylation.

## Discussion

Upon antigen stimulus, naive resting T cells go through a process of activation, proliferation, and differentiation. Interestingly, activated T cells may die through AICD after reencountering their cognate antigen (9). It is not clear if AICD contributes to homeostasis, but it plays an important role in preventing untoward side effects of an uncontrolled or persistent effector response (34). However, for the protection of the host against cancer and infection, AICD may be detrimental as disease states might be prolonged if potentially therapeutic CTL are deleted.

Given that the effectiveness of T cells may be significantly improved by protection from AICD, understanding its mechanism in human antigen–reactive CTL is important. We have earlier shown that a fraction of human CTL reactive to Mart-$1_{27-35}$ and $MP_{58-66}$ underwent AICD upon reencountering their respective cognate peptides and that JNK inhibition prevented AICD but also

impaired the ability of the cells to secrete cytokines (11, 12). The current study shows that inhibition of ROS rescues CTL from AICD without impairing their effector functions. In support of this idea, both tumor and viral epitope–reactive primary human CTL activated by their cognate epitope contained more superoxide than their counterparts exposed to a control epitope. Because superoxide is a byproduct of the mitochondrial electron transport chain, elevated levels may reflect increased respiration within activated cells (35). Furthermore, antigen-reactive primary CTL and TIL escaped AICD when treated with MnTBAP. MnTBAP has been shown to protect cells from superoxide-mediated toxicity in neural cells and endothelial cells through its mimicry of superoxide dismutase and/or catalase (36, 37). Loss of $\Delta\psi$ has been shown to be a key event in the apoptosis of mouse thymocytes exposed to various apoptotic stimuli (38) and in peripheral T cells undergoing SEA-induced apoptosis (15). Additionally, death of thymocytes has been accompanied by the production of ROS as $\Delta\psi$ dissipates (39). Our data indicate that similar ROS-dependent mechanisms cause opening of the permeability transition pore and regulate loss of the $\Delta\psi$ and AICD in human CTL. In addition to decreasing superoxide levels, MnTBAP pretreatment rescued CTL from loss of $\Delta\psi$, DNA fragmentation, and death upon recognition of cognate antigens. Moreover, enhanced accumulation of MnTBAP-pretreated, compared with untreated, CTLs, after restimulation with cognate epitope, indicate that they indeed remained viable for a longer time.

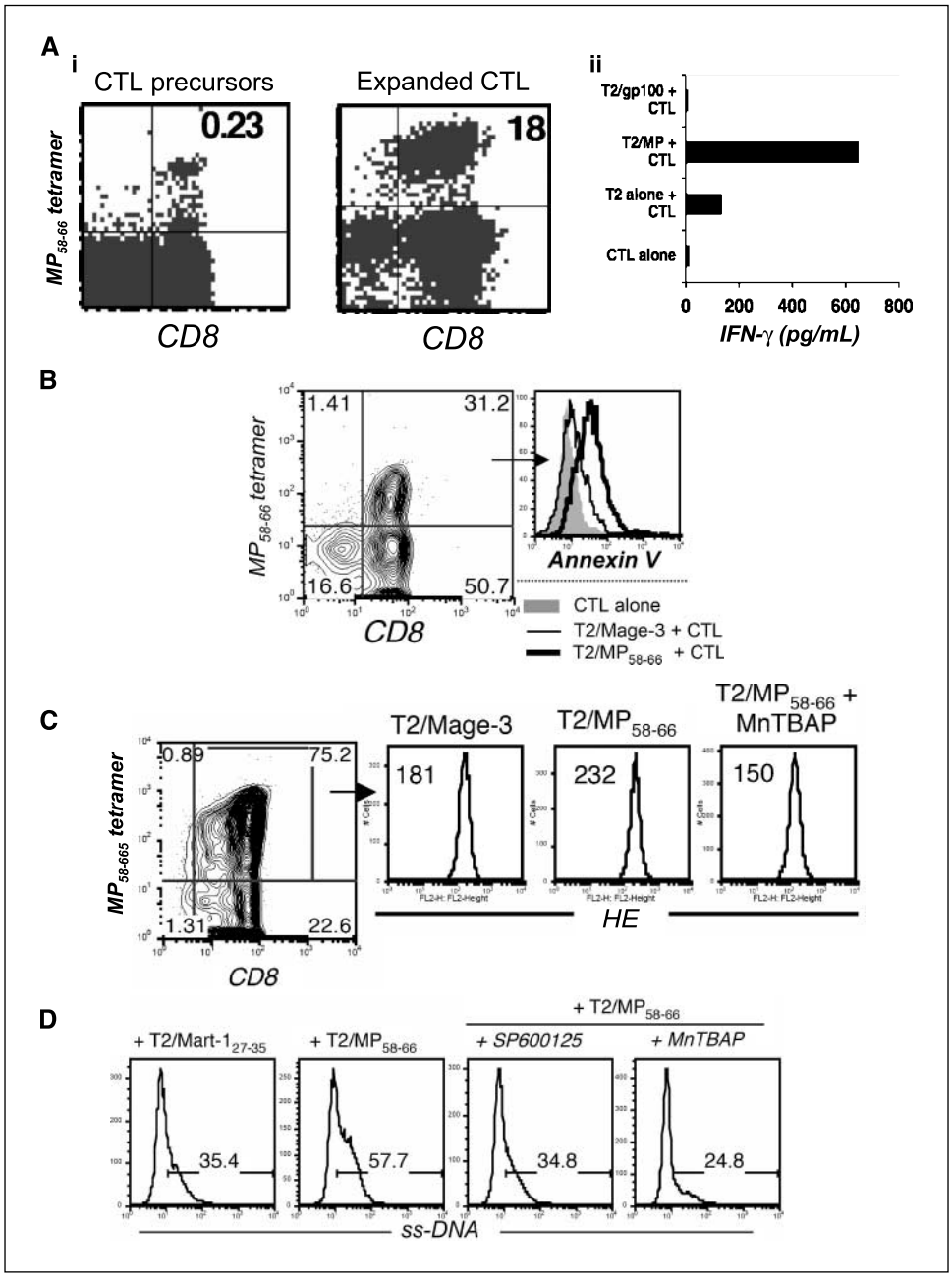


**Figure 5.** Effect of MnTBAP on AICD of influenza epitope $MP_{58-66}$-reactive T cells. *A, i,* tetramer staining to track CTL precursors (*left*) that were expanded using $MP_{58-66}$ peptide-pulsed DC and stained on day 12 (*right*); *ii,* IFN-γ secretion after overnight incubation of the $MP_{58-66}$ CTL stimulated with cognate or control peptide (gp100) pulsed T2 cells. Data represent one of more than five separate experiments with similar results. *B,* histogram overlays depict fluorescence intensity for Annexin V in $MP_{58-66}$ tetramer-gated CTL when unstimulated (*gray filled*), stimulated using T2 cells pulsed with control Mage-3 peptide (*thin black line*), and stimulated using T2 cells pulsed with cognate $MP_{58-66}$ peptide (*thick black line*). *C,* $MP_{58-66}$-reactive CTLs untreated or pretreated with MnTBAP were stimulated with control Mage-3 or cognate $MP_{58-66}$ peptide-pulsed (1 μg/mL) T2 cells. Staining was performed 4 h later. Histogram represents fluorescence intensity of hydroethidium (*HE*) on the antigen-reactive CTLs. Numbers in the upper left corner represent mean fluorescence intensity. Data in *B* and *C* represent one of three separate experiments with similar results. *D,* untreated and MnTBAP- and SP600125-pretreated $MP_{58-66}$ epitope–reactive CTLs were exposed to the cognate ($MP_{58-66}$) and control ($Mart\text{-}1_{27-35}$) peptide-pulsed T2 cells for induction of AICD. Histogram represents ssDNA levels in tetramer-positive cells determined after staining with ssDNA-specific antibody. Data from one representative experiment of two is shown.

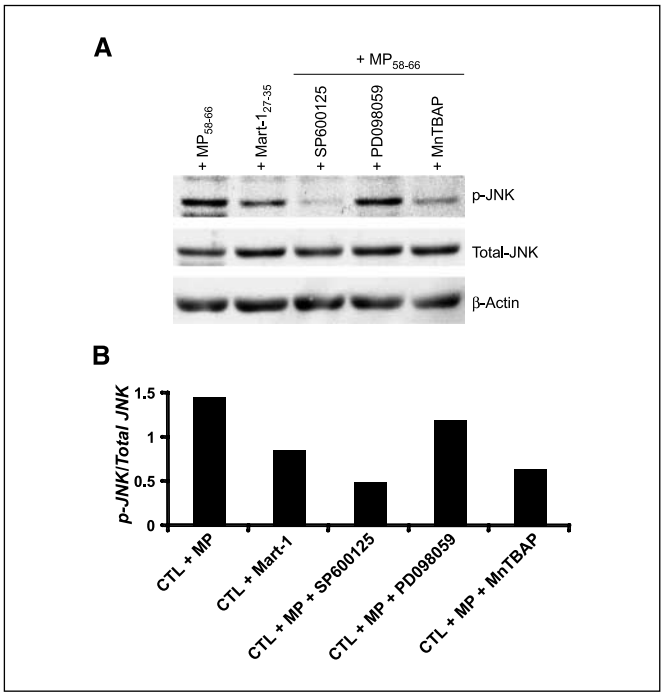


**Figure 6.** Effect of ROS inhibition on JNK phosphorylation. $MP_{58-66}$-reactive SP600125-, MnTBAP-, or PD098059 (ERK inhibitor)-pretreated CTL were exposed to the cognate epitope $MP_{58-66}$, and untreated CTL were exposed to both cognate epitope $MP_{58-66}$ and control epitope Mart-$1_{27-35}$ for induction of AICD. *A,* Western blot depicts total and phosphorylated JNK (*p-JNK*) levels using lysates made from the $MP_{58-66}$-reactive CTLs. *B,* densitometric analysis of the blot in *A*. Bands were scanned into the computer, and band intensity was quantitated using the NIH Image 1.61 software (free software available from the NIH). Values represent the ratio of phosphorylated JNK over total JNK.

TCR activation has been shown to increase ROS within T cells (40), although how excess ROS accumulates is unclear. One explanation is that increased demand for ATP production is imposed on T cells by their conversion from resting precursors to rapidly dividing effectors. In addition to the rate of production, ROS levels depend on the detoxifying activities of antioxidants. For example, superoxide dismutase (SOD) converts superoxide into oxygen and $H_2O_2$ (41). In turn, $H_2O_2$ is detoxified by glutathione peroxidase and/or catalase (42). Thus, the severity of ROS damage is ultimately dependent on both the levels and types of ROS and the levels and activities of antioxidants. Because MnTBAP mimics both SOD and catalase activity, it ensures the complete detoxification of superoxide and its downstream metabolites (15). Hence, MnTBAP may be particularly useful in preventing cellular damage in which superoxide is the initiating ROS.

As such, a role for MnTBAP or other antioxidants in rescuing CTLs from death is understandable. However, the exact pathways that these agents modulate to protect cells from AICD are not clearly understood. Increased intracellular concentration of ROS can lead to selective activation of activator protein-1 (AP-1) transcription factors determining cell fate, i.e., survival versus death (43). Activation of mitogen-activated protein kinases (ERK, p38, and JNK) has also been observed in response to changes in the cellular redox state (44), and the balance between ERK and JNK activation seems to be a key determinant of cell survival. A decrease in ERK and an increase in JNK activity are required for apoptosis (45). Hence, SP600125 and antioxidants (e.g. MnTBAP) might, in fact, rescue CTL from AICD by a common mechanism, i.e., blocking of JNK-driven apoptotic signaling. Our data support the interpretation that AICD observed in *ex vivo* expanded CTL reactive for tumor self antigen or viral nonself antigen is mediated through ROS and JNK activation and involves mitochondrial membrane collapse, which can be prevented by antioxidants such as MnTBAP.

The importance of CTL persistence for successful adoptive T-cell therapy has been highlighted in both experimental models (5) and clinical trials involving cancer patients (7). Preventing AICD of TIL may benefit their *ex vivo* expansion, as well as increasing their persistence and efficacy *in vivo*. Thus, we investigated the sensitivity of TIL to AICD when reencountering cognate antigen and its modulation by ROS inhibition. Pretreatment of TIL1235 with MnTBAP rescued them from AICD upon cognate antigen encounter. Whereas SP600125 concomitantly impaired their ability to secrete cytokines, MnTBAP treatment did not interfere with CTL functionality. A better understanding of how ROS mediate their effects (46) is likely to unveil novel targets that can be exploited to increase persistence of CTL and thereby improve immunotherapies.

An earlier study showed that the natural free radical scavenger vitamin E suppresses the activity of the transcription factors nuclear factor-κB and AP-1, thus blocking expression of CD95L and preventing T-cell AICD (47). Because AICD is a major cause of T-cell depletion in AIDS, a disease associated with high levels of oxidative stress, this study examined 35 HIV-1–positive individuals and found that their T cells were more susceptible to AICD compared with T cells isolated from healthy controls. Administration of vitamin E suppressed CD95L mRNA expression and protected T cells of HIV-1–infected individuals from CD95-mediated apoptosis (47). In addition, a role for inducible nitric oxide synthase in regulating T-cell death and immune memory has been shown (48). These studies taken together with our data suggest that inhibition of free radical generation/oxidative stress can affect T-cell survival and merits further clinical investigation. Since MnTBAP itself has been used *in vivo* in preclinical models (49) and other antioxidants already are approved for clinical use, these findings have direct implications for current immunotherapeutic treatments. Furthermore, engineering T cells to overexpress antioxidant enzymes has recently been shown to protect T cells from exogenous ROS (50). Similar approaches may also protect CTL from AICD and are under way in our laboratory to improve T-cell persistence and the results of immunotherapy.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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