Cytokine-dependent regulation of NADPH oxidase activity and the consequences for activated T cell homeostasis

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Cellular dependence on growth factors for survival is developmentally programmed and continues in adult metazoans. Antigen-activated T cell apoptosis in the waning phase of the immune response is thought to be triggered by depletion of cytokines from the microenvironment. T cell apoptosis resulting from cytokine deprivation is mediated by reactive oxygen species (ROS), but their source and position in the apoptotic cascade is poorly understood. RNA interference approaches implicated the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in neglect-induced apoptosis in T cells. Using mice deficient for the catalytic subunit gp91<sub>phox</sub> to characterize the molecular link to activated T cell apoptosis, we show that gp91<sub>phox</sub>-deficient T (T<sup>−/−</sup>) cells generated mitochondrial superoxide but had diminished hydrogen peroxide production in response to neglect, which, in turn, regulated Jun N-terminal kinase–dependent Bax activation and apoptosis. Activated T<sup>−/−</sup> cells were distinguished by improved survival after activation by superantigen in vivo, adoptive transfers into congenic hosts, and higher recall responses after immunization. Thus, the NADPH oxidase may regulate adaptive immunity in addition to its previously well-characterized role in the innate response.

The regulated deletion of T cells is critical for the maintenance of homeostasis in the mammalian immune system (Goldrath and Bevan, 1999; Plas et al., 2002). Two pathways of cell death have been well characterized in mammalian cells (Hengartner, 2000): the extrinsic pathway, which is triggered by death receptors of the tumor necrosis factor receptor superfamily (Ashkenazi and Dixit, 1998), and the intrinsic pathway, which culminates in the release of apoptotic intermediates from mitochondria (Wang, 2001). The Bcl-2 family proteins are key intermediates in the latter because their activities principally converge on the regulation of mitochondrial integrity (Cheng et al., 2001; Youle and Strasser, 2008).

T cell apoptosis not only shapes the immune repertoire but is essential for immune responses to new and repeated antigenic challenges (Goldrath and Bevan, 1999; Plas et al., 2002). Apoptosis of excess T cells after antigen clearance provides the immune system functionality to manage multiple encounters with infectious organisms (Goldrath and Bevan, 1999). The undesired activation of autoreactive T cells accompanying this event is curtailed by Fas–Fas ligand–mediated activation-induced cell death (AICD; Lenardo et al., 1999). Although emerging evidence suggests that aspects of initial antigenic encounter may regulate the timing of T cell contraction, activated T cell death in many systems correlates with the depletion of cytokines after antigenic clearance (Vella et al., 1998; Strasser and Pellegrini, 2004). Activated T cell apoptosis during contraction is independent of both caspases and death receptor signaling and is rescued by exogenous cytokines (Vella et al., 1998; Nussbaum and Whitton, 2004; Yajima et al., 2006). However, only some effectors of T cell apoptosis are well characterized, and molecular interactions linking cytokine signaling to the regulation of Bax/Bak–BIM–mediated mitochondrial damage remain unresolved. In this context, the identification of reactive oxygen species (ROS) as the effectors of activated T cell apoptosis acquired considerable

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RESULTS AND DISCUSSION

Activated T cell neglect−induced death in vitro

T cells activated in vitro using antibodies to CD3-CD28 as surrogate antigen were maintained in culture with the cytokine IL−2. IL−2 withdrawal (neglect) triggered cell death, which was characterized by apoptotic nuclear damage (Fig. 1 A), was inhibited by the broad-spectrum antioxidant Mn(III)tetrakis (4−benzoic acid)porphyrin chloride (MnTBAP), and was FasL independent (Fig. S1, A and B). Several derivatives of superoxide accumulate in T cells undergoing neglect-induced death (Hildeman, 2004). The H2O2-sensitive reagent CM-H2DCFDA (referred to as DCFDA) revealed a temporally regulated increase in levels of H2O2 in T cells cultured without IL−2 (Fig. 1 B). ROS accumulation peaked between 6 and 8 h (with some variation between mice), before eventual decline by 10 h, preceding nuclear damage by several hours (unpublished data). The surge was suppressed by MnTBAP or diphenyliodonium, a noncompetitive inhibitor of NADPH oxidase (Fig. 1 C). NADPH oxidase is a multisubunit complex that includes catalytic and regulatory subunits forming core elements of a complex that also includes adaptor proteins (Lambeth, 2004). Confocal imaging verified that the catalytic (gp91phox) and regulatory (p67phox) subunits are expressed (Fig. 1 D) and can be immunoprecipitated.

Figure 1. ROS regulate activated T cell neglect−induced death. (A) Activated T cells were cultured with or without (deprivation) IL−2 for 18 h before analysis of nuclear damage (mean ± SD of five experiments; P < 0.001). (B) T cells were cultured as in A and change in DCFDA fluorescence relative to T0 (onset of assay) assessed at the time points indicated. (C) Change in DCFDA fluorescence (relative to T0) in cells cultured with MnTBAP (P < 0.005) or diphenyliodonium (P < 0.05) or the vehicle control (veh), without IL−2 for 6 h (neglect). B and C show mean ± SD of three experiments. (D) Merged confocal image of an activated T cell (representative of 60 cells) stained for p67phox (green) and gp91phox (red). Bar, 2 µm. (E) Representative immunoblot of two immunoprecipitation analyses of gp91phox or p67phox in T cells. WCL, whole cell lysate. p38MAPK and tubulin established specificity. Black lines indicate that intervening lanes were spliced out.
apoptosis (Fig. 2 D), the depletion of p40phox protein blunted cell death (Fig. 2 E). Furthermore, p40phox and gp91phox immunoprecipitated in a complex (Fig. 2 F), which also included the p67phox protein from T cells (Fig. 1 E).

Thus, perturbations of gp91phox, p67phox, or p40phox inhibited apoptosis, implicating this complex in the apoptotic response to cytokine deprivation. Consequently, events regulated by NADPH oxidase in activated T cell apoptosis were characterized in mice with a targeted deletion of the catalytic subunit gp91phox.

gp91phox regulates premitochondrial events in the apoptotic cascade

In T cells derived from mice with a null allele for gp91phox (T−/− cells), apoptotic nuclear damage and cell lysis were substantially reduced compared with WT T cells (T+/+) from age-matched controls (Fig. 3, A and B). Both CD4+ and CD8+ gp91phox−/− subsets were protected from apoptosis (Fig. 1 E) from activated T cells, confirming a recent result showing this complex in T cells (Jackson et al., 2004). Subsequently, NADPH oxidase function in T cell apoptosis was probed using RNA interference approaches.

Retroviral infection with Cybb2 short hairpin RNA (shRNA), to deplete gp91phox protein in activated T cells, inhibited neglect-induced death (Fig. 2, A and C). Comparable inhibition was obtained with one other shRNA construct, Cybb3 (unpublished data). Depletion of p67phox by the shRNA Ncf2-2 also attenuated the apoptotic response (Fig. 2, B and C). shRNA Cybb1 or Ncf2-1 to gp91phox or p67phox, respectively, did not deplete protein (Fig. 2 C) or protect from apoptosis (Fig. 2, A and B). p47phox/Ncf1 and p40phox/Ncf4 are adaptor proteins that can translocate p67phox to the membrane for a functional NADPH oxidase complex (Heyworth et al., 1991; Suh et al., 2006). Although shRNA to p47phox did not inhibit apoptosis (Fig. 2 D), the depletion of p40phox protein blunted cell death (Fig. 2 E). Furthermore, p40phox and gp91phox immunoprecipitated in a complex (Fig. 2 F), which also included the p67phox protein from T cells (Fig. 1 E).

Figure 2. Apoptosis after shRNA-mediated disruption of NADPH oxidase (A and B) Apoptotic damage in T cells infected with shRNA to gp91phox/Cybb (A) or p67phox/Ncf2 (B), or a scrambled control. (C) Immunoblots in T cells infected with the shRNA shown. (D and E) Apoptotic damage in cells infected with shRNA to p47phox (Ncf1) or scrambled control (D) or p40phox (Ncf4) and a scrambled control (E). Immunoblots are shown of T cells infected with the different shRNA. (F) In activated T cells, p40phox immunoprecipitated gp91phox. p38MAPK is the specificity control. White lines indicate that intervening lanes were spliced out. Data in all graphs are normalized to nuclear damage at T0 and are the mean ± SD of three independent experiments. P-values above the bars were calculated relative to the scrambled group.
(Fig. S2, A and B). Immunoblot analysis confirmed that the gp91phox protein was not detected in mutant T cells (Fig. 3 A, inset). To assess if protection was restricted to cells activated in vitro, the apoptotic responses of T cells activated in response to staphylococcal enterotoxin B (SEB) in vivo was tested, as apoptosis of superantigen-activated T cells is ROS dependent (Hildeman et al., 1999). 2 d after intravenous injection of SEB, LN cells were harvested and cultured without cytokine. Apoptosis in the Vβ8+ T cells was substantially lower in cells derived from gp91phox−/− as compared with WT mice (Fig. 3 C). There were no differences in the nonreactive Vβ6+ subset (Fig. 3 C).

DCFDA oxidation was minimal in T−/− cells even after prolonged culture without cytokine (Fig. 3 D). However, gp91phox−/−-independent DCFDA oxidation in response to CD3-CD28 stimulation was comparable in primary T+/+ and T−/− cells (Fig. 3 D, inset). The dye MitoSOX red was used to detect mitochondrial superoxide. Oxidation of MitoSOX red in freshly activated T cells (0 h) or after neglect (6 h) was comparable in both genotypes (Fig. 3 E). Similarly, MitoSOX red oxidation was independent of gp91phox−/− in response to CD3-CD28 restimulation of activated T cells (Fig. 3 F). Thus, T−/− cells present a specific defect in neglect-induced NADPH oxidase-dependent ROS.

Loss of mitochondrial outer membrane integrity, an irreversible event in dying cells is characterized by a drop in mitochondrial transmembrane potential (MTP). Partitioning of the potentiometric dye TMRM into mitochondria is dependent on MTP, with reduced dye uptake indicating compromised organelle integrity. Both TMRM (Fig. 3 G) and Mitotracker red (Fig. S3 A) show that T+/+ cells, but not T−/− cells, undergo a substantial loss in MTP after cytokine deprivation.

Mitochondrial damage is regulated by proteins of the Bcl-2 family (Rathmell et al., 2002; Pellegrini et al., 2003). After receipt of an apoptotic stimulus, proapoptotic Bax undergoes a change in conformation, which can be monitored by an epitope-specific antibody (clone 6A7; Hsu and Youle, 1997). In live activated T cells from either background, reactivity to clone 6A7 was undetectable (not depicted), but 6A7 reactivity was revealed in T+/+ cells, but not T−/− cells, cultured without IL-2 (Fig. 3 H, top). An antibody that is not conformation specific (p19) gave comparable staining in both T+/+ and T−/− cells (Fig. 3 H, bottom), indicating that gp91phox did not suppress Bax expression.

Thus, neglect-induced Bax activation, loss of mitochondrial integrity, and caspase-3 processing (Fig. S3 B) were inhibited in activated T−/− cells, positioning gp91phox at an early step of the apoptotic cascade. Subsequent experiments investigated the mechanism by which gp91phox activity impinged on Bax activation.

![Figure 3](image-url)
Sustained Jun N-terminal kinase (JNK) activation is ROS dependent in T cells

JNK can be activated by the inactivating oxidation of thiol protein tyrosine phosphatases (Kamata et al., 2005). T cell death was substantially blocked by the JNK inhibitor SP600125 (Fig. 4 A), which is indicative of a role for this kinase. JNK is reported to regulate Bcl-2 family proteins (Lei et al., 2002). Consistently, Bax activation (6A7 reactivity) was suppressed by SP600125 at drug concentrations that inhibited apoptosis (Fig. 4 B). To ascertain modulation of JNK activity, its phosphorylation was assessed in activated T cells undergoing neglect. An antibody recognizing JNK phosphorylated on Thr183/Tyr185 showed that JNK was phosphorylated for an extended duration in T+/+ cells but not in T−/− cells (Fig. 4 C). Furthermore, MnTBAP prevented sustained JNK phosphorylation (Fig. 4 D). High levels of JNK phosphorylation at T0 in freshly activated cells may reflect their recent experience of cytokine-enriched environments. However, in the absence of exogenous cytokine, reduced JNK phosphorylation correlated with survival. These findings are consistent with ROS modulation of Bax function via the regulation of JNK activation and position gp91phox as a key source of ROS in this context.

Activated T−/− cells were susceptible to AICD, dexamethasone, hydrogen peroxide, or etoposide (Fig S4), indicating that gp91phox specifically regulated the apoptotic response triggered by cytokine deprivation. To confirm these results, subsequent experiments assessed activated T cell survival in vivo.

T−/− cells display improved survival in vivo

We assessed the recovery of in vitro activated T cells after adoptive transfer into congenic hosts. Expression of the homing antigens CD62L (not depicted) or CCR7, which regulate migration in vivo, was comparable (Fig. 5 A). T+/+ or T−/− cells activated in vitro (donors) were injected into the tail veins of immune-competent WT hosts and recoveries assessed after an interval of 7 d. Donors were distinguished from host lymphocytes (isolated from LNs or spleens) by the expression of a congenic (CD45.2+) cell surface marker. Compared with adoptively transferred T+/+ cells, the number of T−/− cells retrieved was significantly higher from both host spleen (Fig. 5 B) and LN (Fig. 5 C), indicating improved survival. The proliferative responses of WT and mutant T cells were comparable (not depicted).

Apopoptosis of T cells activated in response to bacterial superantigens in vivo was assessed subsequently. Both WT and null mice injected with SEA on day 2 after injection present a comparable increase in Vβ3+ T cell number and low annexin V reactivity (unpublished data). Responses diverge on day 3, with increased annexin V+ Vβ3+ T cells detected in WT LNs as compared with mutant mice (Fig. 5 D). In both backgrounds,
apoptosis was low in the Vβ6+ subset (Fig. 5 D). Consistently, apoptosis of Vβ3+ was inhibited in WT mice injected with SEA and MnTBAP (Fig. 5 E). Similarly, in both genotypes, CD4+Vβ8+ cells showed a comparable increase in response to SEB injection (Fig. 5, F and G). However, this subset persisted in greater numbers in gp91phox−/− mice on day 4 at a time when numbers had begun to show a decline in WT mice (Fig. 5, F and G). Again, there were no accompanying changes in the Vβ6+ subset in either genotype (unpublished data).

**Figure 5.** T−/− cells show improved survival in vivo and elevated recall responses to antigen. (A) Cell surface CCR7 on day 2 activated T+/+ and T−/− cells. (B and C) Total numbers of WT or gp91phox−/− T cells recovered, from host spleen or LN, 7 d after adoptive transfer. (D) LN from mice injected with SEA 48 h before harvest were stained with annexin V and antibodies to CD4, CD8, Vβ3, and Vβ6. (E) annexin V staining in LN T cells from mice injected with SEA under the cover of MnTBAP. (F and G) CD4+Vβ8+ subsets in LN of SEB-injected WT and null mice. P-values shown above the bars were calculated relative to day 2 and are derived from a minimum of three independent experiments. Error bars in B–G indicate the mean ± SD of three experiments. (H–J) Responses to mOVA in LN cells from WT or gp91phox null mice injected with mOVA or PBS (control) in vivo using WST-1 (H), BrdU uptake (I), or CFSE dye dilution (J). In H, p-values comparing responses in WT and null mice (four mice in each group) were the following: 5 µg/ml, P < 0.01; 10 µg/ml, P < 0.001; 20 µg/ml, P < 0.005. In I and J, the profiles represent three experiments. Error bars in H and I indicate the mean ± SD of replicates (triplicate wells).
cells, as T<sup>−/−</sup> cells would progress in greater numbers to making memory. To test this, T cell recall responses were assessed after immunization.

gp91<sup>pox</sup>−/− and WT mice were immunized with maleylated ovalbumin (mOVA) and recall responses assessed 12–15 d later using different assays of proliferation. Basal response to antigen was negligible in unimmunized animals of either genotype (Fig. 5 H, triangles). Antigen-specific proliferative responses, using WST-1 or BrdU uptake, were consistently elevated in T cells from draining LN of gp91<sup>pox</sup>−/− mice compared with WT animals (Fig. 5, H and I). Tracking dilution of the CFSE label in T cells loaded with dye before culture with antigen revealed higher numbers of CFSE<sup>+</sup> T<sup>−/−</sup> cells in every division cycle. We also noted a small but consistent increase in the number of T cells entering division in null mice (Fig. 5 J). These data are in concordance with the survival of a higher number of mOVA responder T cells in mutant mice. The initial representation of effector-memory cells (CD44<sup>high</sup>:CD62L<sup>low</sup>:CD25<sup>neg</sup>) in input populations was comparable in WT and mutant mice (unpublished data).

NADPH oxidase is a central well-characterized component of the innate immune response against fungal and bacterial infections (Bjorgvinsdottir et al., 1997; Blanchard et al., 2003). In this paper, we characterize an apoptotic cascade triggered by NADPH oxidase in activated T cells. Cytokine deprivation–induced activated T cell apoptosis in vitro mimics several features of activated T cell death in vivo, permitting analysis of the molecular mechanisms underlying this process (Hildeman et al., 1999; Vig et al., 2004). Using this approach, we describe a mechanism linking ROS and Bel-2 family proteins. We propose that NADPH oxidase is the interacting redox partner responsive to cytokine–dependent survival cues in activated T cells because cytokine withdrawal triggered a gp91<sup>pox</sup>-associated ROS surge. Investigations into the spatio-temporal organization of gp91<sup>pox</sup> and its regulation by cytokines in T cells are ongoing. We report a specific defect in the temporal organization of gp91<sup>pox</sup> and its regulation by cytokines. We propose that NADPH oxidase is the interacting redox partner responsive to cytokine-dependent survival cues and recruitment for activated T cell attrition at the end of the immune response.

MATERIALS AND METHODS

Animals. C57BL/6J, B6/SJL, gp91<sup>pox</sup>−/− (B6.129S6-Cybb<sup>tm1Din</sup>/J), p40<sup>pox</sup>−/− (B6(Cg)-<sup>N47</sup>/J<sup>9</sup>/J), and B6Smn.C3-Fas<sup>b6J</sup> mice strains were obtained from The Jackson Laboratory. Experiments were approved by the Institutional Animal Ethics Committees of the National Center for Biological Sciences (Bangalore, India).

Reagents. Antibodies were procured from the following sources: gp91<sup>pox</sup>, BD; gp91<sup>pox</sup>−/−, p40<sup>pox</sup>−/−, p67<sup>pox</sup>, p38MAPK, and p19-Bax, Santa Cruz Biotechnology, Inc.; iapase-3 and phosopho-JNK, Cell Signaling Technology; and 6A7, tubulin (NeoMarkers), and CCR7, CD25, and CD48.2 (eBioscience). All shRNA sets were obtained from OriGene Technologies. All other reagents were obtained from Sigma-Aldrich, EMD, or Invitrogen.

T cell activation. T cell subsets purified from mouse spleens using Mag Cellect isolation kits (R&D Systems) were stimulated with α-CD3–α-CD28–coated beads (Invitrogen). Cells were separated from beads after 48 h and either continued in culture with 1 µg/ml IL-2 (R & D Systems) or used in assays of neglect wherein cells were cultured (0.3 × 10<sup>6</sup>/ml) with or without IL-2 for 16–18 h.

Retroviral delivery of shRNA. Retroviruses were generated and cells infected according to standard procedures. Infected T cells were switched to media supplemented with 1 µg/ml IL-2, 2 ng/ml IL-7, and 1 µg/ml puromycin for 3 d (day 6 from onset of culture). Live cells were selected on day 7, continued in culture with IL-2 for another 18–24 h, and were then used in assays of neglect. Loss of protein expression was assessed on day 7 by Western blot analysis of cell lysates.

Assays of apoptotic damage and intracellular ROS. Apoptotic damage was assessed after 18–24 h using 1 µg/ml Hoechst 33342, annexin V–FITC, or the uptake of propidium iodide. Cells were stained with annexin V–FITC for 15 min at room temperature, washed in excess of buffer, and analyzed. MTP was measured...
by staining cells with 150 nM TMRE in medium for 30 min at 37°C. Cells were washed once in excess medium and analyzed by flow cytometry.

T cells were incubated with 2 μM CM-H2DCFDA in RPMI at 37°C for 30 min or 5 μM MitoSOX red for 10 min at 37°C. At the end of incubation, cells were washed twice and immediately analyzed by flow cytometry. Data are presented as the percentage of change in mean fluorescence intensity for each time point with respect to the initial fluorescence intensity (time = 0 h).

Immunoprecipitation. 6–8 × 10^6 cells were lysed for 30 min at 4°C in 1% CHAPS buffer supplemented with protease inhibitors (Sigma-Aldrich). gp91^phox was immunoprecipitated using 1% NP-40. Immune complexes were precipitated for 2 h at 4°C using Sepharose G plus beads on a rotational cell mixer. Beads bound to complexes were washed five times with ice-cold PBS at 1,700 rpm. Finally, beads were boiled in SDS lysis buffer for 10 min before Western blot analysis.

Confocal imaging. T cells were fixed with 1% paraformaldehyde and permeabilized with 0.2% NP-40 for 5 min at room temperature. 5% BSA for 1 h was used for blocking and antibody incubations were performed for 1 h on ice. For a7 staining, 0.2% CHAPS for 15 min on ice was used for permeabilization, followed by 5% BSA for 1 h at room temperature. Cells were imaged on an FV1000 (Olympus) at 60x with a 1.4 NA objective lens and Z-stacks were acquired at a step size of 0.8 μm.

In vivo responses. WT (C57BL/6) and gp91^phox−/− mice were immunized subcutaneously with 100 μg mOVA (Abraham et al., 1995) in CFA. 10–12 d later, 10^5 cells/ml in starvation LNs were cultured in 0.2 ml in 96-well plates with or without mOVA for 72 h. Mice were injected with 40 ng SEA intravenously. Cells from draining LNs were harvested on day 3 and stained for Vβ subsets, CD4 and CD8 subsets, and annexin V–FITC. In other assays, LN were harvested on day 2 from mice injected with 100 μg SEB, cultured overnight in complete medium without cytokine, and apoptosis was assessed using annexin V–FITC in the Vβ subsets of CD4^+ and CD8^+ T cells. Proliferation was measured using WST-1 cell proliferation kit or the BrdU colorimetric kit (Roche) as per the manufacturer’s instructions. Cells were loaded with 0.5 μM CFSE in PBS at ambient temperature for 10 min, followed by three washes in chilled medium to remove excess dye. Dilution of dye after culture in vitro was assessed by flow cytometry.

For adoptive transfers, 10 × 10^6 activated T cells (CD45.2^+) were injected into the tail vein of congenic strains (CD45.1^+) of mice. 7 d after injection, spleen and LNs of host were analyzed for the donor and host cell populations. The number of T cells in each spleen and LN preparation was estimated using an f-test and a two-population Student’s t test.

Statistical analysis and data presentation. In immunoblots, the spacer indicates a splice line between separate sections of the same membrane. All data are presented as mean ± SD derived from a minimum of three to five independent experiments unless stated otherwise. Statistical significance was calculated using an f-test and a two-population Student’s t test.

REFERENCES


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SUPPLEMENTAL MATERIAL

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Figure S1. Activated T cell neglect-induced death is ROS dependent but independent of Fas–Fasl signaling. (A) Activated T cell neglect-induced death is inhibited by the synthetic antioxidant MnTBAP. Apoptotic nuclear damage in WT day-2 activated T cells, cultured in the absence of cytokine (neglect) and in the presence of MnTBAP. (B) Day-2 activated T cells derived from mice with a genetic defect in Fas ligand were cultured without cytokine for 18 h and apoptotic nuclear damage was assessed. Data are the mean ± SD of five experiments in A and B.

Figure S2. Both CD4+ and CD8+ T cells from gp91phox−/− mice are protected from neglect-induced death. WT and gp91phox−/− freshly activated (day 2) cells derived from either CD4+ (A) or CD8+ (B) subsets were cultured without cytokine for 18 h and assayed for apoptotic nuclear damage. T cell subsets were isolated from mouse splenocytes using commercial reagents (R&D systems). In A and B, data represent the mean ± SD of three experiments.
Figure S3. Measures of apoptotic damage in WT and mutant T cells. (A) Field views of confocal images of WT (top left) and gp91phox−/− cells (bottom left) cultured without cytokine for 24 h. Cells were stained with Mitotracker red and Hoechst 33342 to mark mitochondria (red) and nuclei (blue), respectively. The number of cells positive for Mitotracker (red) counterstained with Hoechst are higher in the gp91phox−/− set. The single cells on the right imaged at the onset of the assay (T0) show that Mitotracker loading was comparable in live cells. The field views shown are representative of 75 cells imaged in three independent experiments. Bars, 2 µm. (B) Processing of caspase 3 (cleaved) in whole cell lysates of WT or gp91phox−/− T cells cultured with (IL-2) or without (NID) IL-2 for 16 h. Parity of loading was established by probing the same membrane for levels of p38MAPK. The immunoblot is representative of three separate experiments.

Figure S4. Activated T cells from gp91phox-null mice are susceptible to apoptosis triggered by a variety of stimuli. 0.3 × 10⁶ activated T cells/ml, derived from WT or gp91phox−/null mice, were analyzed for the induction of apoptosis in response to AICD/CD3-restimulation after 24 h (A), 100 nM dexamethasone for 9 h (B), 50 µM of exogenous hydrogen peroxide after 9 h (C), or 5 µg/ml etoposide for 14–16 h (D). In A, C, and D, gray bars indicate cells continued in culture in IL-2 in the absence of the apoptotic stimulus. In B, the gray bars represent cells treated with dexamethasone in the presence of IL-2. In all panels, the data are shown as the mean ± SD from a minimum of three separate experiments.