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 gp91phox to characterize the molecular link to activated T cell apoptosis, we show that gp91phox-deficient T (T−/−) 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−/− cells were distinguished by improved survival after activation by superantigens 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.
independent (Fig. S1, A and B). Several derivatives of superoxide accumulate in T cells undergoing neglect-induced death (Hildeman, 2004). The H$_2$O$_2$-sensitive reagent CM-H$_2$DCFDA (referred to as DCFDA) revealed a temporally regulated increase in levels of H$_2$O$_2$ 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and 8 h (with some variation between mice), before eventual decline by 10 h, preceding nuclear damage by several hours (unpublished data).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 < 0.0001). (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.
(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.

**NADPH oxidase regulates activated T cell death**

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).

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). Consequently, events regulated by NADPH oxidase in activated T cell apoptosis were characterized in mice with a targeted deletion of the catalytic subunit gp91phox.
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 impacted on Bax activation.

Loss of mitochondrial outer membrane integrity, an irreversible event in dying cells is characterized by a drop in mitochondrial transmembrane potential (MTP). T+/+ and T−/− day-2 activated cells were analyzed for nuclear damage (A) or lysis (B) after culture without IL-2 for 18 h. Data are normalized to values at T0 and mean ± SD of five experiments is shown. Inset shows immunoblot for gp91phox in lysates of WT and mutant (gp91−/−) T cells. White lines indicate that intervening lanes were spliced out. (C) T cells were harvested from mice injected 48 h before with SEB, cultured overnight in vitro, and stained with annexin V and antibodies to CD4, Vβ8, and Vβ6. Data are from four mice tested in two separate experiments. Error bars indicate the mean ± SD of four mice tested separately. (D) DCFDA oxidation in T−/− cells cultured without IL-2. Inset shows DCFDA oxidation in CD3-CD28–stimulated primary T cells. Data shown are representative of three mice. Error bars indicate the mean ± SD of replicates (triplicate wells). (E) MitoSOX red oxidation in cells cultured without IL-2 plotted as mean ± SD of three experiments. (F) Representative (n = 3) flow cytometry profile of MitoSOX red oxidation in CD3-CD28-stimulated activated T cells. (G) MTP loss assessed by TMRM in activated T cells cultured as in (A). MTP loss in cells cultured without IL-2 relative to cells cultured with cytokine is shown as the mean ± SD of four experiments (P < 0.0005). (H) Confocal images (central plane) of activated T cells (representative of 50 cells from three independent experiments) from WT or mutant mice cultured without IL-2 for 6–8 h and stained with clone 6A7 (top) or clone p19 (bottom). Bars, 2 µm.

Figure 3. Activated T−/− cells are protected from neglect-induced death. (A and B) T+/+ and T−/− day-2 activated cells were analyzed for nuclear damage (A) or lysis (B) after culture without IL-2 for 18 h. Data are normalized to values at T0 and mean ± SD of five experiments is shown. Inset shows immunoblot for gp91phox in lysates of WT and mutant (gp91−/−) T cells. White lines indicate that intervening lanes were spliced out. (C) T cells were harvested from mice injected 48 h before with SEB, cultured overnight in vitro, and stained with annexin V and antibodies to CD4, Vβ8, and Vβ6. Data are from four mice tested in two separate experiments. Error bars indicate the mean ± SD of four mice tested separately. (D) DCFDA oxidation in T−/− cells cultured without IL-2. Inset shows DCFDA oxidation in CD3-CD28–stimulated primary T cells. Data shown are representative of three mice. Error bars indicate the mean ± SD of replicates (triplicate wells). (E) MitoSOX red oxidation in cells cultured without IL-2 plotted as mean ± SD of three experiments. (F) Representative (n = 3) flow cytometry profile of MitoSOX red oxidation in CD3-CD28-stimulated activated T cells. (G) MTP loss assessed by TMRM in activated T cells cultured as in (A). MTP loss in cells cultured without IL-2 relative to cells cultured with cytokine is shown as the mean ± SD of four experiments (P < 0.0005). (H) Confocal images (central plane) of activated T cells (representative of 50 cells from three independent experiments) from WT or mutant mice cultured without IL-2 for 6–8 h and stained with clone 6A7 (top) or clone p19 (bottom). Bars, 2 µm.
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; Putcha et al., 2003). 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).

Apoptosis of T cells activated in response to bacterial super-antigens in vivo was assessed subsequently. Both WT and null mice injected with SEA on day 2 after injection present a comparable increase in Vß3+ T cell number and low annexin V reactivity (unpublished data). Responses diverge on day 3, with increased annexin V+ Vß3+ 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).

T−/− cells have improved recall response to antigen

After antigen clearance, the majority of antigen-reactive T cells are deleted with a small proportion set aside to form T cell memory. Although challenged by recent studies (Kaech et al., 2003; Badovinac et al., 2004), contraction of activated T cells is thought to be triggered by competition for limiting amounts of cytokine in the waning phase of the immune response. Because gp91phox regulated activated T cell apoptosis, its absence should manifest in the persistence of antigen-reactive

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^-/- cells would progress in greater numbers to making memory. To test this, T cell recall responses were assessed after immunization.

gp91^phox^-/- 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^phox^-/- mice compared with WT animals (Fig. 5 H, triangles). Tracking dilution of the CFSE label in T cells loaded with dye before culture with antigen revealed higher numbers of CFSE^+ T^-/- 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^high CD62L^low CD25^neg) 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^phox-associated ROS surge. Investigations into the spatiotemporal organization of gp91^phox and its regulation by cytokines in T cells are ongoing. We report a specific defect in the regulation of the innate immune response is well characterized, these data offer new insight into a novel role for this oxidase in the coordination of the adaptive immune response. A more comprehensive analysis of the organization of this complex in T cells is needed to understand its response to cytokine–dependent cues and recruitment for activated T cell attrition at the end of the immune response.

MATERIALS AND METHODS

Animals. C57BL/6j, B6/SJL, gp91^phox^-/- (B6.129S6-Cyberv^tb^J/j), p47^phox^-/- (B6.Cg-Ncf1^j1/J), and B6Smm.C3-Fab2^J/j mice strains were obtained from The Jackson Laboratory. Experiments were approved by the Institutional Animal Ethics Committees of the National Centre for Biological Sciences (Bangalore, India).

Reagents. Antibodies were procured from the following sources: gp91^phox, BD; gp91^phox, p40^phox, p67^phox, p38MAPK, and p19-Bax, Santa Cruz Biotechnology, Inc.; cdk-3 and phospho-JNK, Cell Signaling Technology; and 6A7, tubulin (NeoMarkers), and CCR7, CD25, and CD45.2 (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 Cellct 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 × 10^6/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. Apopotic damage was assessed after 18–24 h using 1 μg/ml Hoechst 33342, annexin V–FITC, or the uptake of propidium iodide. Stained 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<sup>phox</sup> 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. Bead 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 6A7 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 60× 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/6j) and gp91<sup>phox</sup>−/− mice were immunized subcutaneously with 100 µg mOVA (Abraham et al., 1995) in CFA. 10–12 d later, 10<sup>5</sup> cells/mL from draining LNs were cultured in 0.2 µL in 96-well plates with or without mOVA for 72 h. Mice were injected with 40 ng SEA intra-nasally. Cells from draining LNs were harvested on day 3 and stained for V<sup>B</sup> subsets, CD4<sup>+</sup> and CD8<sup>+</sup> 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 V<sup>B</sup> subsets of CD4<sup>+</sup> and CD8<sup>+</sup> 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<sup>5</sup>–10<sup>6</sup> activated T cells (CD45.2<sup>+</sup>) were injected into WT (C57BL/6J) and gp91<sup>phox</sup>−/− mice. T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. Fig. S1 shows that activated T cell neglect-induced death is dependent on ROS but independent of Fas-FasL. Fig. S2 shows that CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets from gp91<sup>phox</sup>−/− mice are protected from apoptosis. Fig. S3 shows that mitochondrial integrity is maintained in gp91<sup>phox</sup>−/− T cells as compared with WT T cells and that processing of caspase3 is diminished in T−/− cells. Fig. S4 shows the susceptibility of T−/− cells to apoptosis triggered by a variety of stimuli. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082851/DC1.

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