Apoptosis-Inducing Factor Regulates Death in Peripheral T Cells[1]

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Apoptosis-inducing factor (Aif) is a mitochondrial flavoprotein with multiple roles in apoptosis as well as in cellular respiration and redox regulation. The harlequin (Hq) mouse strain carries an *aif* locus modification causing reduced Aif expression. We demonstrate that activated $CD4^+$ and $CD8^+$ peripheral T cells from Hq mice show resistance to neglect-induced death (NID) triggered by growth factor withdrawal, but not to death induced by multiple agents that trigger DNA damage. Aif translocates to the nucleus in cells undergoing NID, and, in Hq T cell blasts, resistance to NID is associated with reduced cytosolic release of mitochondrial cytochrome *c*, implicating Aif in this event. In contrast, Hq T cell blasts express higher levels of CD95L, demonstrating increased susceptibility to activation-induced cell death (AICD) and apoptosis triggered by hydrogen peroxide. Superoxide scavenging protects from AICD in wild-type, but not Hq, T cell blasts, suggesting that Aif plays a crucial superoxide-scavenging role to regulate T cell AICD. Finally, the altered pattern of death susceptibility is reproduced by siRNA-mediated reduction of Aif expression in normal T cells. Thus, Aif serves nonredundant roles, both proapoptotic and antiapoptotic, in activated peripheral T cells. *The Journal of Immunology,* **2007, 179: 797–803.**

Peripheral T lymphocytes undergo extensive proliferation and death upon activation through the TCR. Activated T cells can undergo two distinct forms of cell death following activation. Peripheral tolerance is mediated by repeated activation of T cells leading to activation-induced cell death (AICD)[5] mediated by death receptor members of the TNFR family—CD95/Fas for CD4 T cells and TNFRI/TNFRII for CD8 T cells—a mitochondria-independent pathway in T cells (1). In another pathway critical for control of the magnitude of the surviving pool of Ag-specific memory T cells, a withdrawal of growth factors, perhaps through a lack of triggering of the common γ-chain of the cytokine receptors (reviewed in Ref. 2), results in a death pathway that converges on the mitochondrion.

Mitochondrial damage can occur independent of caspase activation. Three separate categories of mitochondrial proteins are thereupon released (reviewed in Ref. 3). Of these, both cytochrome *c* (cyt c) and the SMAC/DIABLO are caspase dependent in their final death effector function. However, mitochondria also release endonuclease G and apoptosis-inducing factor (Aif), which can mediate large-scale DNA fragmentation, together or separately, in a caspase-independent manner. It is thus possible that mitochondrial damage, followed by Aif/endonuclease G release, could provide a completely caspase-independent pathway of apoptosis, from initiation to death.

Aif is a flavoprotein with flavin-adenine dinucleotide-dependent NADH oxidase activity located in the mitochondrial intermembrane space (reviewed in Ref. 4). In mitochondria, Aif appears to be important in acting as a hydrogen peroxide scavenger, reducing oxidative stress (5). The levels of electron transport complex I in mitochondria are lower in the absence of Aif at least in some cell lineages (6, 7), contributing to reduced oxidative phosphorylation and a tendency to oxidative stress. Aif can leak from mitochondria into the cytosol either early (8) or late (9) in a number of death pathways. Furthermore, Aif release is caspase-independent in some pathways (10), while it is caspase-dependent in others (11). Extramitochondrial Aif appears to be capable of causing further damage to and leakage from mitochondria (reviewed in Ref. 4), as well as localizing to the nucleus and causing nuclear DNA degradation in association with other molecules (4).

Aif is critical for embryonic development and *aif*-null embryos are developmentally nonviable (12). However, the harlequin (Hq) strain of mice has a proviral insertion in the upstream regulatory region of the *Aif* gene leading to an ~80% reduction in levels of Aif expression resulting in increased oxidative stress in cerebellar neurons, which enter an abortive cell cycle and die (5).

There are some indications that Aif may participate in death pathways in T cells (13). Mitochondrial damage is controlled by reactive oxygen species (ROS) in T cells postactivation (reviewed in Ref. 14). However, it is not yet clear whether Aif plays a nonredundant role in the T cell lineage. Using the Hq mouse strain,

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Received for publication October 25, 2006. Accepted for publication May 11, 2007.

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[1] This work was supported in part by grants from the Department of Biotechnology and the Department of Science and Technology, Government of India (to A.G., S.R., and V.B.), the Indian Council of Medical Research, the Government of India (to V.B.), the National Institutes of Health (United States), and the Arkansas Biosciences Institute (to J.M.D.), the Arkansas Biotechnology Institute (to J.M.D.), and the Wellcome Trust (to V.B. and A.S.). National Institute of Immunology is supported by the Department of Biotechnology, Government of India.

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[5] Abbreviations used in this paper: AICD, activation-induced cell death; cyt *c*, cytochrome *c*; Aif, apoptosis-inducing factor; ROS, reactive oxygen species; WT, wild type; AG, aminoguanidine; MnTBAP, Mn-tetrabenzoyl-aminoporphyrin; NID, neglect-induced death; siRNA, small-interfering RNA; SOD, superoxide dismutase; PTP, permeability transition pore; CsA, cyclosporine A; DR, death receptor.

which offers the opportunity to examine potential lineage-specific roles of Aif in an Aif-hypomorphic situation, we now show that Aif plays both pro- and antiapoptotic roles in activated peripheral T cells.

Materials and Methods

Mice

Hq mice (B6CBACa $A^{w\text{-}J}$/A-$Pdcd8^{Hq}$/J; The Jackson Laboratory) are in a mixed B6 × CBA/CaJ background, and therefore all experiments described here used normal littermates as wild-type (WT) controls. Also, because the *aif* gene is on the X chromosome, hemizygous males, either WT or Hq, were used for most experiments. $MnSOD^{+/-}$ mice were obtained from The Jackson Laboratory and bred either in the small animal facility (National Institute of Immunology, New Delhi, India) or at the animal house (National Centre for Biological Sciences, Bangalore, India). All experimental protocols were approved by the Institutional Animal Ethics Committees.

Cell preparations, activation protocols, and death assays

Splenic and lymph node cells from Hq or WT mice were used to generate T cell blasts using anti-CD3 (eBioscience) and IL-2 (Roche) as described (15). Live T cell blasts were isolated on Ficoll-Hypaque gradients (Cedarlane Laboratories) and subjected to further culture with or without supportive IL-2 for inducing NID for 3 more days as described (15). Cell viability in purified T cell blasts was routinely monitored before their use in death assays and was invariably >98%. Death was scored by fluorescent microscopy (TE2000-U; Nikon) in stained CD4 or CD8 cells by the condensed nuclear morphology visible upon staining with the DNA-binding dye Hoechst 33342 (2 μg/ml; Molecular Probes), or by the nonexclusion of the vital dyes trypan blue (Sigma-Aldrich) or Sytox Green (Molecular Probes). Both assays gave similar trends in all experiments. Staurosporine, etoposide (Sigma-Aldrich), cyclosporine A (Sigma-Aldrich), and thapsigargin (Calbiochem) were used in titrating doses as indicated. Cells were gamma irradiated using a ^{60}Co source (BI-2000; BRIT).

To induce AICD, live T cell blasts were cultured in the presence of IL-2 (5 IU/ml) with or without plate-bound anti-CD3 (10 μg/ml) as described (15). At various time points, cells were harvested and stained with fluorochrome-labeled anti-CD4 or anti-CD8 and Hoechst 33342 to score for apoptotic nuclei, and for dye exclusion using trypan blue. Mn-tetrabenzoylaminoporphyrin (MnTBAP; 100 μM; Calbiochem) and aminoguanidine (AG; 100 μg/ml; Sigma-Aldrich) were added to the cultures at the initiation of death where indicated.

Peritoneal exudate cells were induced by i.p. injection of 4% thioglycolate broth (Himedia). At 72 h postinjection, cells were harvested from the peritoneum and macrophages were isolated by plastic adherence as described previously (16). Purified populations were >90% $CD11b^+$. These cells were activated with LPS (30 μg/ml; Sigma-Aldrich) and IFN-γ (50 IU/ml; eBioscience) for 48 h, and analyzed for induction of death, using flow cytometry to follow exclusion of the Sytox Green dye.

Assays for Fas and FasL activity

Splenic cells from WT and Hq mice were used to generate T cell blasts using anti-CD3 and IL-2 as described above. To assay for Fas ligand activity, live T cell blasts isolated on Ficoll-Hypaque gradients were cocultured with CFSE-labeled (Molecular Probes) Jurkat cells at various E:T ratios (1:3, 1:15, 1:25) for 16 h. Cells were stained with Hoechst 33342 (2 μg/ml) and death was scored as apoptotic nuclear morphology in the CFSE-labeled Jurkat cells by fluorescent microscopy (TE2000-U; Nikon).

To assay for Fas activity, live T cell blasts were cultured in the presence of various concentrations of rCD95L-Ig (PeproTech) in 5 IU/ml IL-2 for 16 h. Cells were stained for CD4 and CD8 as well as with Hoechst 33342 and death was scored as frequency of apoptotic nuclei.

TNF-α assays

WT or Hq T cell blasts were maintained in IL-2 (5 IU/ml) in the presence or absence of plate-bound anti-CD3 for 12 h in culture. TNF-α was measured in culture supernatants using a commercial ELISA (BD Biosciences).

Western blot analysis

Splenic cells or T cell blasts were used as appropriate. Whole cell lysates, or cytosolic and subcellular fractions, were prepared as described previously (17, 18). After SDS-PAGE separation, proteins were transferred to nitrocellulose membranes and probed with Abs to Aif (Chemicon International), cyt *c* (BD Biosciences), or p38MAPK (Santa Cruz Biotechnology), followed by appropriate secondary Abs coupled to HRP. Bound HRP was detected by ECL, according to the manufacturer's instructions (Amersham Biosciences). The anti-Aif Ab shows two bands under these conditions in the manufacturer's data as well as in our experiments (see Fig. 1*A*).

Flow cytometry

The Abs used for the stainings were either biotinylated, or directly coupled to fluorescein or PE or CyChrome C. Biotinylated Abs and fluorophore-coupled streptavidin were obtained from BD Biosciences or from eBioscience. Data acquisition was done on a BD-LSR (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

Confocal microscopy

For confocal microscopy, WT T cell blasts undergoing either neglect-induced death (NID) or AICD for various periods of time as indicated were fixed, permeabilized, and stained for Aif using anti-Aif Ab and secondary reagents as indicated earlier, as well as with Hoechst 33342. They were spun onto poly-L-lysine-coated coverslips and confocal images were acquired on a Bio-Rad MRC-1024 confocal microscope (Bio-Rad Microsciences) with factory-set dichroics and a krypton-argon laser, using LaserSharp software (Bio-Rad). Images were processed using MetaMorph (Universal Imaging) and Adobe Photoshop software. Widefield images were used to score the nuclear morphology and the location of Aif in multiple individual cells.

Small-interfering RNA (siRNA)-mediated Aif "knockdown"

Blood was collected from consenting healthy human donors by venipuncture and PBMCs were isolated using a Ficoll-Hypaque gradient. PBMCs were stimulated with plate-coated anti-CD3 (OKT3; 1 μg/ml) for 48 h followed by siRNA transfection, which was done by nucleoporating 2 × 10^6 cells with 2 μg each of a combination of plasmids expressing mouse Thy-1 (gift from Dr. T. Mitchell, University of Kentucky, Louisville, KY) and vectors carrying either *aif* siRNA (pKD-AIF-v3; Upstate Biotech) or control siRNA (pKD-NegCon-v1; Upstate Biotech). Nucleoporation was done using program T-23 according to the manufacturer's instructions (Amaxa Biosciences). Cells were then shifted to medium containing IL-2 (5 U/ml) 6 h later and maintained for a further 48 h to generate T cell blasts. Viable T cell blasts at the end of 96 h were harvested using Ficoll-Hypaque density gradients.

The efficiency of siRNA-mediated Aif knockdown was assessed by either staining cells 48 h posttransfection for surface mouse Thy-1 and intracellular Aif using an anti-Aif Ab, or by MACS-purifying (Miltenyi Biotec) mouse Thy-1-expressing cells and Western blotting their lysates for Aif expression.

Harvested T cell blasts were either cultured in the presence or absence of IL-2 (5 IU/ml) to induce NID, or cultured with IL-2 in the presence or absence of plate-coated anti-CD3 to induce AICD. Cells were stained 24 h later with Hoechst 33342, anti-Thy-1, and anti-CD4 or anti-CD8. Nuclear morphology was visualized by fluorescent microscopy (IX-81; Olympus) in >100 mouse Thy-1-expressing cells in each sample.

Results

Poor NID in peripheral T cell blasts from Aif-hypomorphic Hq mice

The mitochondrial NID pathway in activated T cells controls the magnitude of the CD4 and CD8 memory T cell pools surviving the attrition of the initial immunization-induced expansion (15, 19). The broad spectrum caspase-inhibitor ZVAD-fmk does not appear to enhance the generation of memory T cells (20, 21). Because Aif is one of the few factors released from mitochondria reported to be capable of inducing caspase-independent death, we examined NID in activated Aif-deficient T cells.

Western blot analysis confirmed that Aif levels in Hq splenic cells were substantially lower than in WT splenic cells (Fig. 1*A*). We see the doublet of Aif bands consistently and both bands are equally diminished in Hq cells. Although Aif isoforms have been reported (22), they appear to be shorter than the doublet seen here. When activated T cell blasts were cultured with or without supportive IL-2 for inducing NID as described earlier (15), NID was lower in Hq than in WT CD4 and CD8 T cell blasts, both by nuclear morphology and by vital dye exclusion, at all time points measured (Fig. 1, *B* and *C*). Although the superoxide dismutase (SOD) mimic and peroxynitrite scavenger MnTBAP and the

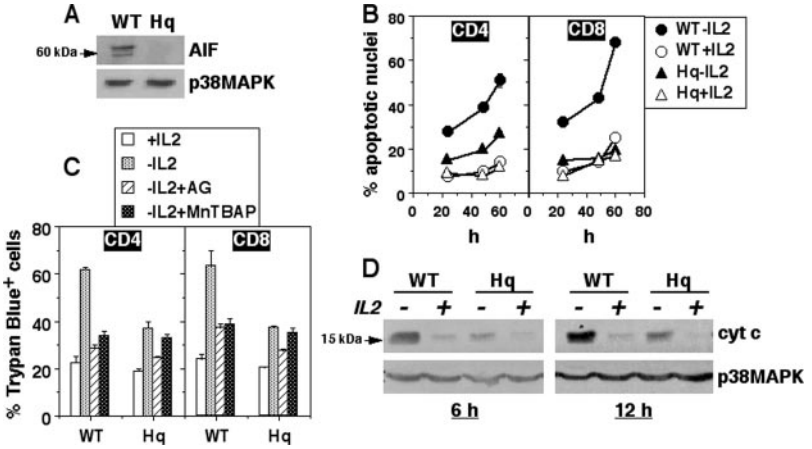

FIGURE 1. Poor death by neglect in peripheral T cell blasts from Aif-hypomorphic Hq mice. *A*, Western blots showing Aif levels in WT and Hq splenic cell lysates upon SDS-PAGE. Levels of p38MAPK are shown as controls. *B*, NID in WT and Hq T cell blasts, measured by the frequency of apoptotic nuclei in CD4 or CD8 cells at various time points, as shown. Control cultures were maintained in IL-2 (5 IU/ml). Data are shown as mean ± SE of triplicate measurements. *C*, NID in WT and Hq T cell blasts, measured by the loss of trypan blue dye exclusion in either CD4 or CD8 cells at 60 h in culture in the presence of absence of MnTBAP (100 μM), AG (100 μg/ml), or IL-2 (5 IU/ml). Data are shown as mean ± SE of triplicate measurements. *D*, Western blots showing cyt *c* levels in cytosolic fractions of WT and Hq T cell blast lysates at 6 or 12 h of culture with or without IL-2 (5 IU/ml) as indicated. Levels of p38MAPK are shown as controls. All data are representative of three to seven independent experiments.

inducible NO synthase inhibitor AG both protected WT T cell blasts against NID as we have shown earlier (15), they provided little protection against the residual death observed in Hq cells (Fig. 1*C*).

Even in Aif deficiency, other mitochondrial mediators of death would be expected to be functional, and in fact mitochondrial death has been reported to be prevented in neurons only by deficiency of both Aif and Apaf-1 (9, 23). It was therefore critical to examine whether other mitochondrial death mediators, notably cyt *c*, were in fact normally released in Hq T cells undergoing NID. The protection of Hq T cell blasts from NID was associated with reduced leakage of the mitochondrial protein cyt *c* into the cytoplasm (Fig. 1*D*).

These data suggested that either all mitochondrial death events were Aif dependent in T cells, or that some death events were Aif dependent but not others. We therefore tested WT- and Hq-activated T cells for susceptibility to death by other known inducers of mitochondrial death, such as etoposide, staurosporine, and gamma irradiation. Hq T cell blasts were as sensitive as WT cells to the induction of death by these agents (Fig. 2*A*). Furthermore, we compared WT and Hq T cell blasts in death induced by endoplasmic reticulum stress triggered by thapsigargin-mediated calcium store release and found that WT and Hq cells did not show any differences in their susceptibility to this form of death (Fig. 2*A*).

We also compared the susceptibility to death of another cell lineage from WT and Hq mice. When death was assayed in thioglycolate-induced peritoneal macrophages triggered with bacterial LPS and IFN-γ, WT and Hq macrophages showed equivalent susceptibility (Fig. 2*B*).

These data suggested that some, but not all, forms of mitochondrial death were Aif dependent. An intriguing possibility was that this could be correlated to the two categories of mitochondrial damage pathways that have been hypothesized: one by extensive damage of the outer mitochondrial membrane and the other by formation of the permeability transition pore (PTP) (24). Aif has been reported to bind to some cyclophilins (25) and cyclophilin family members are essential for PTP formation in mitochondria (24). Thus, the prediction from this possibility was that agents inhibiting PTP formation may protect against an Aif-dependent

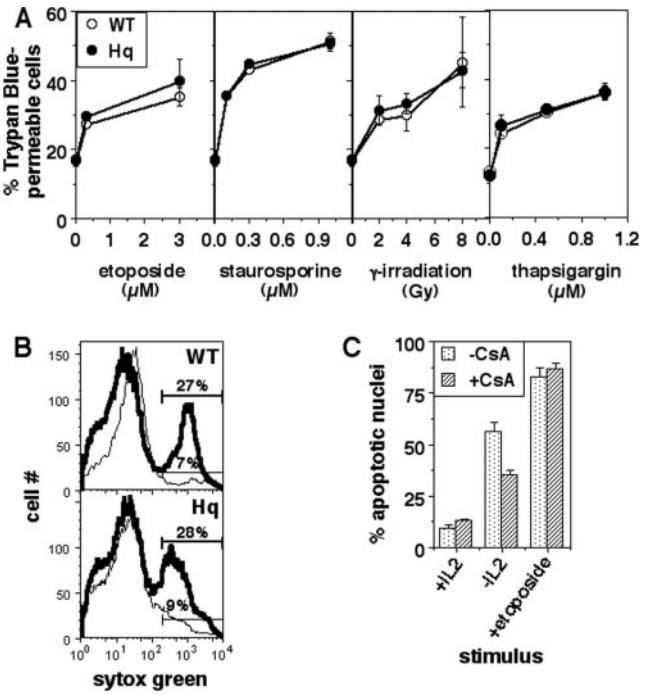

FIGURE 2. Mitochondrial death in all situations is not altered in Aif-hypomorphic Hq peripheral T cell blasts. *A*, Death induced by etoposide, staurosporine, gamma irradiation, or thapsigargin in WT and Hq T cell blasts, measured by the loss of trypan blue dye exclusion in the cells at 16 h in culture in the presence of absence of IL-2 (5 IU/ml) and various concentrations of the stimuli as shown. Data are shown as mean ± SE of triplicate measurements. *B*, Peritoneal macrophages from WT or Hq mice were cultured without (thin lines) or with (thick lines) LPS (30 μg/ml) and IFN-γ (50 U/ml) for 48 h, and death was assayed by exclusion of Sytox Green dye as shown. Dead cell frequencies are indicated. *C*, WT T cell blasts were subjected to NID in the absence of IL-2, or to etoposide-induced death (2 μg/ml etoposide in the presence of 5 U/ml IL-2), in the presence or absence of CsA (10 ng/ml) as indicated. Cells cultured in 5 U/ml IL-2 served as controls. The extent of death was estimated at 16 h by the frequency of apoptotic nuclei. All data are representative of three to six independent experiments.

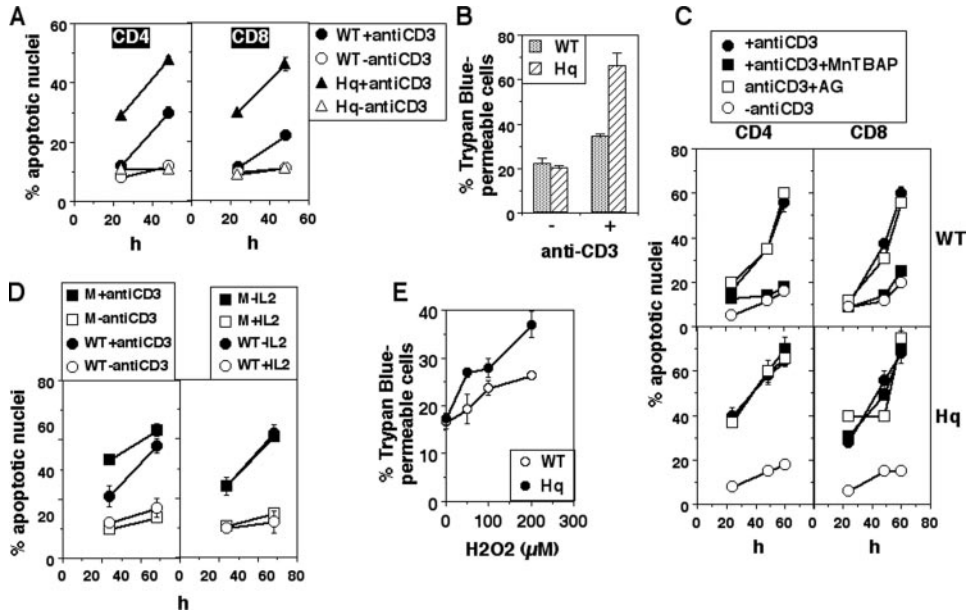

FIGURE 3. Enhanced AICD in peripheral T cell blasts from Aif-hypomorphic Hq mice. *A*, AICD induced by plate-bound anti-CD3 (10 μg/ml) in WT and Hq T cell blasts, measured by the frequency of apoptotic nuclei in CD4 or CD8 cells at various time points as shown. Control cultures (-anti-CD3) were maintained in IL-2 (5 IU/ml). Data are shown as mean ± SE of triplicate measurements. *B*, AICD in WT and Hq T cell blasts, measured by the loss of trypan blue dye exclusion in the cells at 60 h in culture with or without plate-bound anti-CD3 as indicated. Cultures without anti-CD3 were maintained in IL-2 (5 U/ml). Data are shown as mean ± SE of triplicate measurements. *C*, AICD induced by plate-bound anti-CD3 in WT and Hq T cell blasts, measured by the frequency of apoptotic nuclei in CD4 or CD8 cells at various time points as shown. Control cultures (-anti-CD3) were maintained in IL-2 (5 IU/ml). MnTBAP was used at 100 μM and AG at 100 μg/ml. Data are shown as mean ± SE of triplicate measurements. *D*, AICD induced by plate-bound anti-CD3, or NID induced by absence of IL-2 in T cell blasts generated from $MnSOD^{+/+}$ (WT) and $MnSOD^{+/-}$ (M) littermate mice, measured by the frequency of apoptotic nuclei in CD4 or CD8 cells at various time points as shown. Control cultures (-anti-CD3) were maintained in IL-2 (5 U/ml). Data are shown as mean ± SE of triplicate measurements. *E*, Hydrogen peroxide-induced death in WT and Hq T cell blasts, measured by the loss of trypan blue dye exclusion in the cells at 16 h in culture in the presence of absence of IL-2 (5 IU/ml) and various concentrations of H_2O_2 as shown. Data are shown as mean ± SE of triplicate measurements. All data are representative of three to eight independent experiments.

form of mitochondrial death, but not against an Aif-independent form. We tested this using cyclosporine A (CsA), which binds to cyclophilin D to inhibit PTP formation (24). We tested the CsA sensitivity of an Aif-dependent form of mitochondrial death, NID, and of an Aif-independent form of death using etoposide, in T cell blasts. Consistent with the hypothesis, NID in WT T cell blasts was reduced by CsA, while etoposide-induced death was completely unaffected (Fig. 2*C*).

Enhanced AICD in peripheral T cell blasts from Hq mice

NID is mediated by mitochondrial mechanisms and therefore can be conceived to include a role for Aif. AICD in T cell blasts, however, is thought of as an extrinsic death pathway driven by death receptors, with only marginal roles envisaged for mitochondrial mechanisms. We next tested the sensitivity of Hq T cell blasts to AICD, induced by cross-linking CD3 on them (15). Hq T cell blasts began showing significantly greater levels of death, particularly at earlier time points, than WT T cell blasts did (Fig. 3, *A* and *B*), both by nuclear morphology and by vital dye exclusion, indicating their greater sensitivity to AICD.

We further found that MnTBAP could also protect WT T cell blasts against AICD, but inducible NO synthase inhibitors could not do so (Fig. 3*C*). This suggests that the SOD mimetic ability of MnTBAP, rather than any effect on reactive nitrogen intermediates, may be responsible for protection of WT T cell blasts against AICD. In such a scenario, mitochondrial SOD activity would place significant limits on T cell AICD. Consistent with this possibility, we observed that T cell blasts from mice heterozygous for a mitochondrial MnSOD-null mutation also showed enhanced early death during AICD, although NID was unaffected in these cells (Fig. 3*D*). Most notably, MnTBAP was unable to protect Hq T cell blasts from AICD (Fig. 3*C*), suggesting that Aif deficiency limited the protective ability of SOD activity.

Furthermore, if the SOD mimetic ability of MnTBAP contributes to protection against death only in the presence of Aif as a peroxide scavenger acting downstream of SOD activity, Hq T cell blasts would be predicted to show enhanced sensitivity to hydrogen peroxide. Indeed, Hq T cell blasts were more susceptible to death triggered by exogenously added hydrogen peroxide than WT T cell blasts (Fig. 3*E*).

Because ROS can induce expression of the death receptor ligand CD95L (FasL) involved in AICD (14), we examined whether T cell blasts generated from Hq mice showed higher levels of CD95L to begin with than WT T cell blasts did. T cells activated with anti-CD3 and Il-2 would be expected to show significant levels of CD95L, as WT T cell blasts did (Fig. 4*A*). Furthermore, Hq T cell blasts expressed modestly but consistently increased levels of CD95L (but not of CD95) than WT T cell blasts did (Fig. 4*A*). To confirm that this difference in CD95L levels could actually lead to increased cell killing, WT and Hq T cell blasts were next tested for their ability to kill Jurkat cells as targets. T cell blasts were harvested from activation cultures and added to labeled Jurkat cells, which functioned as targets for induction of CD95-CD95L-mediated death as reported earlier (26). Hq T cell blasts showed a consistently better cytotoxic ability (Fig. 4*B*).

The modest increase in CD95L levels observed suggested that Aif deficiency may lead to enhanced AICD of T cell blasts via other pathways as well. To test this possibility, we examined the susceptibility of WT or Hq T cell blasts to death receptor (DR)-mediated death, rather than to activation-induced AICD. For this,

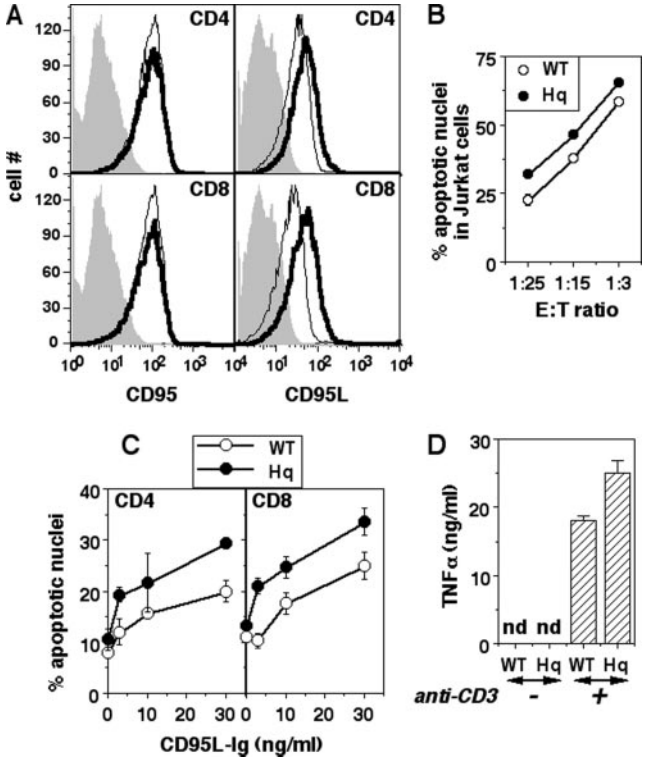

FIGURE 4. Enhanced levels and function of CD95L on Aif-hypomorphic Hq peripheral T cell blasts. *A*, CD95 and CD95L levels on CD4 or CD8 T cell blasts from WT (thin lines) or Hq (thick lines). WT or Hq splenic T cells were activated in the presence of anti-CD3 and IL-2 (see *Materials and Methods*) for 96 h before being stained for CD95 and CD95L. *B*, Death in target Jurkat cells (T) induced by WT and Hq T cell blasts generated as above (E), cocultured at various ratios as shown for 16 h, measured as the frequency of apoptotic nuclei. Data are shown as mean ± SE of triplicate measurements. All data are representative of three to seven independent experiments. *C*, Death induced by graded concentrations of FasL-Ig as shown in WT and Hq T cell blasts, measured by the frequency of apoptotic nuclei in CD4 or CD8 cells after 24 h in culture. Cultures were maintained in IL-2 (5 IU/ml). Data are shown as mean ± SE of triplicate measurements. *D*, TNF-α levels induced by plate-bound anti-CD3 in WT and Hq T cell blasts, measured by ELISA after 12 h in culture. Cultures were maintained in IL-2 (5 IU/ml). Data are shown as mean ± SE of triplicate measurements (nd, not detectable). All data are representative of two to four independent experiments.

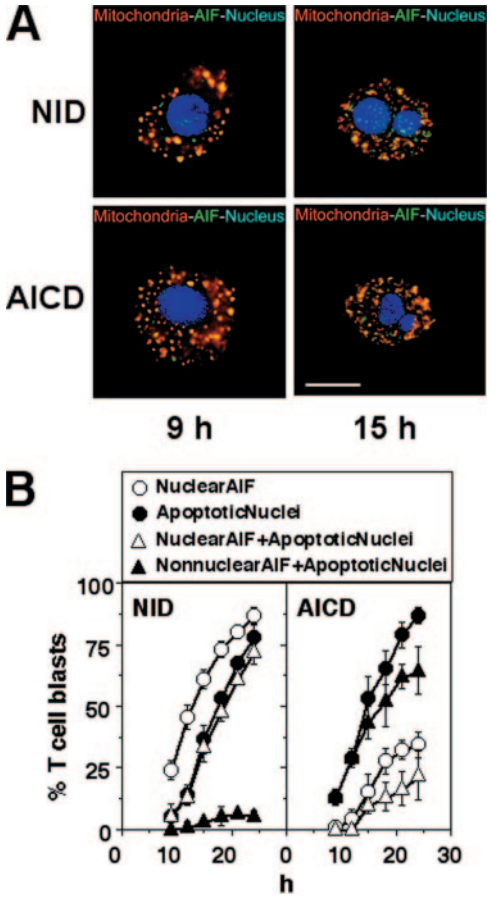

FIGURE 5. Patterns of Aif translocation in WT peripheral T cell blasts undergoing NID or AICD. *A*, Aif localization in WT T cell blasts undergoing NID or AICD. Cells undergoing NID or AICD were stained at 9 or 15 h with anti-Aif (green), Mitotracker Red (red), and Hoechst 33342 (blue). Scale bar, 10 μm. *B*, NID or AICD death pathways were induced in WT T cell blasts and cells were stained as in *A* above at various time points as indicated. Cells showing the various localization patterns indicated in the legend were counted. Data represent ~100 cells each. All data are representative of three to five independent experiments.

purified WT or Hq T cell blasts were cultured with rCD95L-Ig to induce DR-mediated death directly. CD4 as well as CD8 Hq T cell blasts were more susceptible than WT cells to such DR-mediated death (Fig. 4*C*), showing that Aif deficiency modified DR-mediated death pathways in T cell blasts.

Another potential factor in the enhanced death of Hq T cell blasts during AICD could be the levels of TNF-α generated by restimulation by plate-bound anti-CD3. We assayed TNF-α levels in cultures supernatants of WT or Hq T cell blasts in culture with or without plate-bound anti-CD3 for 12 h. Hq T cell blasts secreted higher levels of TNF-α than WT T cell blasts did (Fig. 4*D*).

We also confirmed that, in T cell blasts, Aif translocated to the nucleus during NID but not during AICD. WT T cell blasts subjected to either NID or AICD were stained with an anti-Aif Ab along with Mitotracker Red and Hoechst 33342 to locate the mitochondria and the nucleus. Confocal microscopy showed that Aif could be seen in the nucleus in T cell blasts undergoing NID at both the time points tested, while even at the later time point, it remained non-nuclear in cells undergoing AICD (Fig. 5*A*). When these data were quantified, the trends showed that Aif relocated to the nucleus before apoptosis set in during NID, while the opposite was true of AICD (Fig. 5*B*). The Aif levels expressed in Aif-hypomorphic Hq cells were too low for reliable quantification of Aif localization (data not shown).

Aif knockdown in normal activated T cells alters their death susceptibility

It remained formally possible that these alterations in the death phenotype of Hq peripheral T cell blasts described above could be developmentally programmed in the T cell lineage of Hq mice rather than being due to low levels of Aif in the cells at the time of assay. We therefore tested whether reduction in Aif levels in normal WT T cell blasts led to similar alterations in the death phenotype, using an siRNA-mediated knockdown approach. Because well-validated and controlled *aif*-specific siRNAs are commercially available for human rather than mouse *aif*, we used human T cell blasts for this purpose.

Activated T cell blasts from peripheral blood cells of normal human volunteers were generated by activation with anti-CD3 and IL-2 as described (21). These cells were cotransfected with commercially available plasmid vectors coding for either a control or an *aif*-specific siRNA minigene, and a plasmid vector carrying the

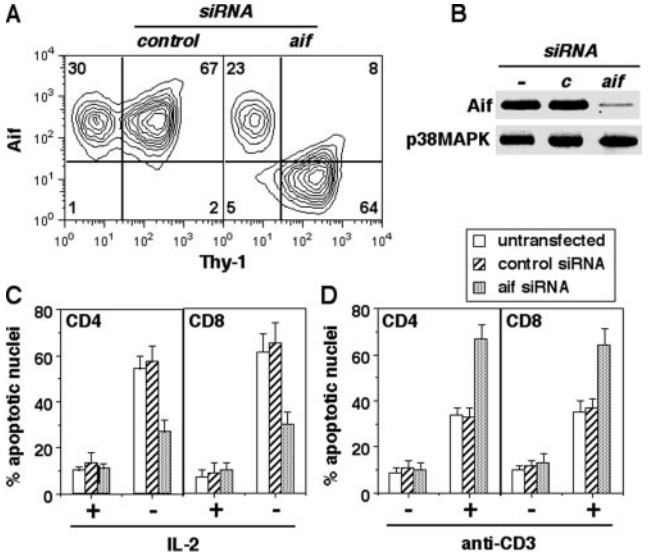

FIGURE 6. Reduction of Aif with siRNA recapitulates the altered death phenotype in human T cell blasts. Human T cell blasts were transfected with control siRNA or aif siRNA vectors along with a marker plasmid carrying the mouse *Thy-1* gene, cultured further in IL-2 for 48 h before being used further. *A*, Two-color plots show the expression profile of mouse Thy-1 and intracellular Aif in human T cell blasts transfected with control or *aif* siRNA. *B*, Western blots showing Aif levels in lysates of human T cell blasts either left untransfected or transfected with control or aif siRNA. Levels of p38MAPK are shown as controls. *C*, NID in human T cell blasts either left untransfected or transfected with control or aif siRNA at 24 h of culture. Control cultures were maintained in IL-2 (5 IU/ml). Death was read by nuclear morphology using Hoechst 33342 in CD4 or CD8 cells along with the expression of mouse Thy-1. Data are shown as mean ± SE of triplicate measurements. *D*, AICD induced by plate-bound anti-CD3 in human T cell blasts either left untransfected, or transfected with control or aif siRNA at 24 h of culture. Control cultures (-anti-CD3) were maintained in IL-2 (5 IU/ml). Death was read by nuclear morphology using Hoechst 33342 in CD4 or CD8 cells along with the expression of mouse Thy-1. Data are shown as mean ± SE of triplicate measurements. Data are representative of two independent experiments.

mouse *thy-1* gene. Two-color flow cytometric analysis of the T cell blasts for cell-surface mouse Thy-1 vs intracellular human Aif showed that, while control siRNA did not lead to any change in Aif expression (Fig. 6*A*), *aif*-specific siRNA led to a >10-fold decrease of cellular Aif levels (Fig. 6*A*). This was also confirmed by MACS-sorting Thy-1-expressing transfected cells followed by Western blot analysis (Fig. 6*B*).

When untransfected, control siRNA-transfected or *aif*-siRNA-transfected T cell blasts were cultured with or without supportive IL-2 for inducing NID; Aif-knockdown CD4 and CD8 T cell blasts showed reduced NID (Fig. 6*C*). In contrast, when these cells were cultured with plate-bound anti-CD3 in the presence of IL-2 for inducing AICD, Aif-knockdown CD4 and CD8 T cell blasts showed enhanced AICD (Fig. 6*D*). These data confirmed that reduction of Aif levels in developmentally normal T cells substantially alters their susceptibility to death.

Discussion

Aif-null mouse embryos are not viable, but the Hq strain is Aif hypomorphic, allowing an investigation of the role of Aif in the T cell lineage. Using this approach, our data provide some interesting insights. Activated peripheral T cells from Aif-hypomorphic mice are less susceptible to NID, while being more susceptible to activation-induced death, indicating that Aif has nonredundant roles in the death of activated peripheral T cells, and that these roles include both pro- and antiapoptotic components.

In peripheral T cells, the magnitude of the CD4 and CD8 memory T cell pool remaining after attrition of primary responses is controlled by NID mediated through ROS/reactive nitrogen intermediate-modulated mitochondrial pathways with a significant caspase-independent component (15, 19). This provides a plausible role for Aif as a caspase-independent mitochondrial mediator of death during postactivation NID in peripheral T cells. However, if Aif-mediated death were to be simply a death mechanism additional to the cyt *c*-mediated caspase-9-dependent pathway, Aif hypomorphism by itself would not be expected to show any modified susceptibility to mitochondrial death. Such a situation has indeed been reported in Hq neurons, which show no enhancement of their susceptibility to mitochondrial cell death unless they have also been rendered Apaf-1 deficient (23). However, we observe that Hq T cell blasts are protected from NID. This suggests that the classical cyt *c*-Apaf-1-mediated pathway of mitochondrial death may be dependent on the presence of Aif in T cells. This explanation is supported by our finding of poor cyt *c* release from Hq T cell blasts undergoing NID, and has also been previously proposed for HIV-1 gp120-mediated death of T cells (13).

However, Hq T cell blasts are not resistant to all mitochondrial death triggers. Neither etoposide, staurosporine, nor gamma irradiation reveal any differences between WT and Hq T cell blasts, just as the endoplasmic reticulum stress inducer, thapsigargin, does not. Furthermore, LPS- and IFN-γ-mediated death of macrophages, which is thought to be mediated by mitochondrial pathways (27, 28), is no different between WT and Hq peritoneal exudate cells. Our data thus suggest that the mechanisms of release of Aif and cyt *c* from mitochondria may differ in different contexts. Some reports have shown that cyt *c* release precedes and is independent of Aif release (11). In other studies, Aif release has been shown to be caspase independent (10). It is also interesting that Aif release is associated with permeability transition in mitochondria in some but not all situations (24). Aif has been shown to bind to cyclophilin A (25), and another member of the cyclophilin family, cyclophilin D, is known to participate in PTP formation, so that agents that bind to cyclophilin D such as CsA inhibit PTP formation and mitochondrial death (24). If this is indeed the case, Aif deficiency may lead to poor PTP formation in at least some cell types such as T cell blasts, which in turn may lead to protection of such cells from PTP-dependent forms of death, but not from other forms of death such as outer mitochondrial membrane damage (24). Our findings, that in T cell blasts, NID is CsA sensitive while etoposide-induced death is not, support such a model.

Furthermore, exogenous Aif has also been shown to be able to damage isolated mitochondria to induce cyt *c* release (10). Thus, in response to some triggers, cyt *c* release from mitochondria may be mediated by release of Aif and subsequent amplification of mitochondrial damage sufficient to allow cyt *c* release. However, other triggers may permit cyt *c* release independent of Aif. Thus, it may be the ability of Aif to cause feedback mitochondrial damage that is critically nonredundant in some situations, rather than its ability to induce DNA fragmentation.

Postactivation NID has been implicated in determining the extent of survival of responding T cells as memory (15, 19). Thus, our data would suggest that Aif-hypomorphic peripheral T cells would survive better than WT T cells as memory upon activation. In support of this possibility, Hq mice show greater frequencies of peripheral $CD44^{high}$ effector/memory T cells (data not shown). Thus, it is attractive to speculate that impaired Aif-dependent NID of activated T cells may contribute to a preferential survival of memory T cells. In contrast, AICD/DR pathways are thought to be

crucial in maintaining peripheral self-tolerance of the T cell repertoire (29). The enhanced AICD in Aif-hypomorphic T cell blasts may thus be related to a more stringent "editing" of the peripheral self-reactive repertoire and the functional consequences of such a resetting of tolerance thresholds would be of considerable interest.

Unlike NID, T cell AICD is a death receptor-mediated pathway. It is thought of as a paracrine event in which the induction of CD95L or TNF-α in T cell blasts allows them to kill bystander cells through the ubiquitously expressed DRs CD95 or TNF-R (1). We find that Aif-hypomorphic T cell blasts show enhanced AICD. This enhanced AICD could involve increased levels of CD95L and/or TNF-α expression, and/or enhanced susceptibility to DR-mediated death signals. Interestingly, we find evidence of both possibilities. Activated Hq T cell blasts do show modest increases in the functional levels of CD95L on them, they secrete greater amounts of TNF-α, and they also show a striking increase in their susceptibility to DR-mediated apoptosis. ROS, possibly mitochondrial in origin, have been thought to play a significant modulatory role in AICD, perhaps through the regulation of CD95L induction (14). Another major link proposed between mitochondria and DR-mediated death is the proapoptotic protein Bid, which is activated via DR signaling and induces mitochondrial damage (30). However, mitochondrial contributions to death are impaired in Hq T cells, suggesting that T lineage cells are likely to have novel connections between DR signals and the mitochondria.

Furthermore, we observe that an SOD mimic, MnTBAP, blocks AICD in WT but not Hq T cells. The absence of MnTBAP-mediated protection in Hq T cell blasts suggests that Aif is required for the protective effects of SOD activity. The conversion of superoxide to peroxide would lead to peroxide accumulation, which would further need to be scavenged. Aif is reported to function as a peroxide scavenger, and the absence of Aif may thus lead to peroxide accumulation in the presence of MnTBAP in Hq T cell blasts, thus helping explain the lack of protection. Further, Aif remains in a mitochondrial location in T cell blasts undergoing AICD, and T cells from mice heterozygous for a null mutation in mitochondrial MnSOD also show enhanced early AICD, indicating that mitochondrially generated superoxide/peroxide species can contribute to AICD. Together, these data suggest that mitochondrial Aif may function as a nonredundant mitochondrial peroxide scavenger (5) to regulate AICD in T cells.

The possible role of Aif as a peroxide scavenger in T cells is also supported by our observation that Hq T cell blasts are more susceptible to the induction of death by hydrogen peroxide than WT T cell blasts. As noted above, the lack of MnTBAP-mediated protection of dying Hq T cell blasts can then be interpreted as an inability to scavenge peroxide successfully in mitochondria, leading to extensive oxidative stress during T cell activation.

Cell death in the T cell lineage occurs as a crucial event at multiple points, and the particular concatenation of molecular death signals which, of the many possibilities available, is essential for mediation of each of these death events is not well-understood despite a great deal of information available on the molecular possibilities involved. Our data show that Aif plays a complex and significant role, sometimes proapoptotic and sometimes antiapoptotic, in controlling the death of activated peripheral T cells.

Disclosures

The authors have no financial conflict of interest.

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