Supplementary text

T cell death following immune activation is mediated by mitochondria-localized SARM

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Plasmids and reagents

Full length SARM (SARM FL) and domains ARM-SAM-TIR, SAM-TIR, TIR and SAM, were cloned in pEGFPN1. The constructs expressing ARM-SAM, ARM-TIR, N124, N-ARM, N-ARM-SAM, were subcloned from pUNO SARM cDNA (Invivogen Inc., USA) into pEGFPN3 vector (Supplementary Table 1). Bcl-xL expression plasmid in pCMV construct was obtained from Clontech. SARM specific and non-targeting control shRNA sequences (Supplementary Table 1) were cloned in pMKO.1 GFP plasmid. SIINFEKL peptide was synthesized and purified to >95% purity by Genemed Synthesis Inc. (SF, USA). Mitochondrial membrane potential-sensitive dye (tetramethylrhodamine ester perchlorate, TMRE-87917), mitochondrial charge uncoupler (carbonyl cyanide 3-chlorophenylhydrazone, CCCP), Propidium Iodide, Hoechst 33342, N-acetyl cysteine and Complete Freund's Adjuvant were from Sigma. ROS indicator (CMH_2DCFDA), Lipofectamine^{TM} LTX, Mitotracker, far-red labeled transferrin, CFSE and recombinant mouse IL-2 were from Invitrogen, USA. Recombinant mouse IL-7 was from R & D systems. ERK inhibitors- PD98059 and U0126 were from Cell Signaling Technology. Fluorogenic apoptosis assay substrate, caspalux and phiphilux, were from OncoImmunin Inc., USA. Anti-CD3 was from Ebioscience. Rabbit polyclonal anti-SARM and mouse monoclonal anti-GAPDH was from Santa Cruz. Rabbit polyclonal anti-actin was from Sigma. Rabbit polyclonal anti-VDAC and rabbit monoclonal anti-pERK were from Cell Signaling Technology. Rabbit polyclonal anti-GFP was from Clontech. Dead cell dye – 7-AAD, Fluorochrome-conjugated anti-CD44-FITC, anti-CD69–APC, anti-CD4-PE, anti-CD8-PE, anti-CD3-APC, anti-CD95-

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PE, anti-CD95L-PE, anti-Thy1.2- PE Cy7, anti-CD25 APC, anti-CCR7 APC, anti-CD62L APC and Annexin V- FITC were from Ebioscience.

Cell lines

T cells were activated using plate-bound anti-CD3 and maintained in RPMI containing 10% (v/v) fetal bovine serum, 50 μM 2-mercaptoethanol, 100 Units/ml penicillin, 100 μg/ml streptomycin and 100 Units/ml IL-2. HEK 293T, HEK 293 Bcl-xL (Invivogen), NIH3T3, Wild type and $BAX^{-/-}$ $BAK^{-/-}$ MEF cell lines were maintained in DMEM containing 10% (v/v) fetal bovine serum, 100 Units/ml penicillin and 100 μg/ml streptomycin. HEK 293 Bcl-xL cell line was continuously selected in the medium containing 10 μg/ml Blasticidin. NK-YS (NK/T lymphoma cell line) was maintained in RPMI containing 10% (v/v) fetal bovine serum, 100 Units/ml penicillin, 100 μg/ml streptomycin and 100 Units/ml recombinant human IL-2. Raji B (Burkitt's lymphoma cell line) was cultured in RPMI containing 10% (v/v) fetal bovine serum, 100 Units/ml penicillin and 100 μg/ml streptomycin. Neuro-2a (Neuroblastoma cell line) was grown in MEM containing 10% (v/v) fetal bovine serum, 100 Units/ml penicillin and 100 μg/ml streptomycin.

Transfection

HEK 293T cells were seeded in 12-well plates (Nunc) at a density of 2.5×10^5 cells/well in 1 ml DMEM, and grown overnight before transfection. Each transfection mixture containing 800 ng of plasmid in 200 μl of incomplete medium and 2 μl of Lipofectamine™ LTX (Invitrogen) was incubated at room temperature for 30 min. The transfection mixture was then added to the cells.

6 x 10^4 NIH 3T3 cells/well seeded in 12-well plates were transfected as described for HEK 293T cells. 8 x 10^4 MEF cells/well seeded in 12-well plates were transfected with Lipofectamine LTX following the manufacturer's instructions. For gene delivery into primary T cells, 1-2×10^6 activated cells were resuspended in 100 µl Nucleofector solution (Amaxa GmbH, Köln, Germany) and electroporated using program X-100 in the Nucleofector device. Following nucleofection, the cells were immediately transferred into pre-warmed RPMI 1640 medium in 12-well plates and incubated at 37^{o}C with 5% CO_2. Similarly, NK-YS, Neuro-2a and Raji B cells were electroporated using A-024, T-024 and M-013 nucleofector programs, respectively.

Fluorescence imaging

1 × 10^6 activated primary T cells were nucleofected with varying truncated constructs of SARM. Cells were collected 6 h post-nucleofection. 2 x 10^5 HEK 293T cells were seeded overnight in a 12-well plate before transfection for 12 h with different truncated constructs of SARM. Following transfection or nucleofection, cells were incubated with 10 nM mitotracker or 10 µg/ml labeled transferrin for 20 or 35 min, respectively. The cells were washed and fixed in 4% formaldehyde for 15 min at room temperature, washed thrice with PBST, and mounted on the glass slide with mounting medium containing DAPI (Invitrogen). The images were obtained using LSM META 510 confocal laser scanning microscope under 100X / 1.3 oil (DIC) objective and numerical aperture of 0.55 (Carl Zeiss). Images were acquired using Carl Zeiss LSM 5 software, version 4.0 SP2.

Immunoblot analysis

Primary CD8 T cells were stimulated with plate-coated anti-CD3 and cells were collected daily for 8 days post-activation. Mitochondria were isolated from primary T cells using mitochondria isolation kit by following the manufacturer's protocol (Pierce Biotechnology). 2.5 x 10^5 HEK 293T cells or 6 x 10^4 NIH 3T3 cells were seeded in a 12-well plate. HEK 293T cells were transfected for 24 h with varying truncated constructs of SARM. NIH 3T3 cells were transfected for 36 h either with SARM-specific shRNA constructs or SARM-specific siRNA. T cells were transduced with SARM-specific shRNA and non-targeting control shRNA and GFP. GFP-positive transduced cells were sorted by flow cytometry. Whole cell lysates were prepared using 1x RIPA buffer (Cell Signaling Inc.). Lysates were resolved in 12 % SDS-PAGE gel and transferred on to PVDF membrane. The membrane was blocked with 5 % skimmed milk in PBST (PBS containing 0.05% v/v of Tween 20) for 1 h and incubated overnight at 4°C with the primary antibody against SARM/ GFP/ VDAC/ GAPDH/ pERK in the blocking buffer. The membrane was washed three times and incubated with the corresponding secondary antibody coupled to HRP in blocking buffer, followed by another three washes before detection of the bound HRP using Supersignal® West Pico (Pierce) chemiluminescent substrate according to the manufacturer's protocol. To ensure equal loading, the same membrane was stripped using Thermo scientific restore Western blot stripping buffer according to the manufacturer's protocol. The membrane was then reprobed with β-actin as described above.

Reverse transcriptase PCR and quantitative real time PCR

Primary CD8 T cells were stimulated with plate-coated anti-CD3 and cells were collected on indicated days post-activation. 6 x 10^4 NIH 3T3 cells were seeded in 12-well plates. NIH 3T3 cells were transfected for 36 h either with SARM-specific shRNA constructs or SARM-specific siRNA. RNA was also prepared from NK-YS, Neuro-2a and Raji B cells. Total RNA from cells was isolated with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Oligo-dT primed RNA was reverse-transcribed with Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA obtained was used in PCR with Taq DNA polymerase (New England Biolabs) or by real time PCR with SYBR Green I Master mix (Roche) using the *LightCycler*® 480 II cycler detection system to determine the relative amount of SARM mRNA (see Supplementary Table 1). Tubulin was used as the internal control in both cases.

Flow cytometry

Primary CD8 T cells were stimulated with anti-CD3 coated on the plate, and cells were collected on indicated days post-activation. To the activated T cells resuspended in FACS buffer (PBS with 0.5% BSA and 0.1% sodium azide), specific fluorochrome conjugated antibodies were added at the dilution of 1:100. The cells were incubated on ice for 10 min and washed with FACS buffer. Cells were pelleted at 1000 g for 5 min at 4°C, resuspended in FACS buffer and analyzed using either LSR II or ARIA (BD Bioscience) depending on the $\lambda_{ex}/\lambda_{em}$ of the conjugated fluorochrome. The data files were analyzed using Flow Jo software.

Supplementary Figure Legend

Supplementary Figure S1: Evolutionary conservation and isoforms of SARM. (a) Primary T cells were activated with plate-bound anti-CD3. Cells were collected on days 1, 2 and 3 post-activation. RNA was prepared by Trizol method. cDNA, reverse-transcribed from RNA was used as template for quantitative real time PCR of SARM, MyD88 and TRIF. Data were normalized to mouse tubulin, GAPDH and actin. **(b)** Domain architecture of human and mouse SARM, showing the location of two ARM, two SAM and TIR domains. The difference in the two splice variant isoforms of mouse SARM is underlined, indicating the 40 amino acid insertion in isoform 764. The human and mouse SARM 724 share 93% sequence homology**. (c)** SARM is evolutionarily conserved as shown by multiple sequence alignments using ClustalX. A rooted phylogram was drawn for various SARM homologues by neighbor-joining algorithm and visualized by Njplot with >10000 bootstrap. Branch lengths are proportional to sequence divergence and can be measured relative to the scale bar shown (0.05 nt-change per site). **(d)** PCR reaction was carried out using cDNA prepared from RNA isolated from primary CD8 T cells. Blue and red arrows indicate forward and reverse primers, respectively. Lanes L: 100 bp DNA ladder, 1 and 2 are amplification with SARM-specific primers without/with cDNA, respectively, and 3 and 4 are amplification with tubulin-specific primers without/with cDNA, respectively. RT PCR amplified only the 367 bp product corresponding to SARM 724, and not the 487 bp product, which would have corresponded to SARM 764. **(e)** Purity of the lymph node cells isolated from OTI^{+} $RAG^{-/-}$ mice: Lymph node cells from OTI^{+} $RAG^{-/-}$ were stained with anti-CD8 APC and anti-CD3 PE to

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examine the purity of the cells, which reflects the integrity of the isolation procedure. Post-staining, the cells were washed and resuspended in FACS buffer and analyzed by flow cytometry. (f) HEK 293T cells in 12-well plate were transfected with 800 ng of the indicated truncated constructs of SARM. 24 h post transfection, the cells were stained with 7-AAD and cell death was quantified by flow cytometry. Results are means ± S.D. of triplicate measurements.

Supplementary Figure S2: GFP fusion does not affect the function of SARM. HEK 293T cells were cotransfected with pCDNA/ SAM-TIR/ TIR and GFP in the molar ratio of 5:1 each. **(a)** At 24 h post-transfection, GFP-positive cells were analyzed by flow cytometry. Flow cytometry indicates the altered GFP expression in SAM TIR transfected cells. **(b)** Percentage of GFP-positive cells (GFP+) and **(c)** Mean GFP fluorescence intensity was measured by flow cytometry at 12, 24, 36 and 48 h post-transfection for each of the SARM constructs. **(d)** Dead cells were stained with trypan blue dye and enumerated using a Neubauer hemocytometer chamber. **(e)** HEK 293T cells were transfected with varying ratios of GFP: SAM-TIR (1:1, 1:2, 1:3, 1:4, 1:5) and at 24 h post-transfection, the mean fluorescence intensity was measured by flow cytometry. The total amounts of the recombinant plasmid DNA were balanced with pCDNA vector.

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Supplementary Figure S3: The proapoptotic role of SARM is conserved in different cell types. PCR was carried out using cDNA prepared from RNA isolated from **(a)** mouse Neuro-2a cells. RT PCR specifically amplified a 125-

bp product corresponding to endogenous SARM. **(b)** Neuro-2a cells were nucleofected with GFP (control) or SARM-FL- or SAM-TIR- GFP. Cell death was quantified at 12 h post-nucleofection by staining the cells with the dead cell stain, 7-AAD. **(c)** RT PCR specifically amplified a 465-bp product corresponding to endogenous SARM in the cDNA prepared from NK-YS and Raji B cells. **(d)** Raji B and **(e)** NK-YS cells were nucleofected with GFP or SARM-FL- or SAM-TIR- GFP. At 12 h post-nucleofection, the cells were stained with 7-AAD and cell death was quantified by flow cytometry.

Supplementary Figure S4: Caspases and MPTP are induced by SARM expression. Activated primary cytotoxic T cells were nucleofected with SAM-TIR- GFP or GFP alone and assayed for **(a)** caspase-3, **(b)** caspase-9 and **(c)** caspase-8 activities at 3, 6, 12, 24 h post-nucleofection. Representative flow cytometry data depicts the overlay of caspase activity histogram of SAM-TIR and GFP- transfected cells at indicated time points. **(d)** HEK 293T cells transfected with equal amounts of SARM-FL/ ARM-SAM-TIR / SAM-TIR /GFP alone, were loaded with 20 nM of TMRE dye at 12, 18 and 24 h post-transfection. $\Delta\Psi m$ (a measurement of mitochondrial membrane potential) was quantified based on TMRE retention. Cells treated with CCCP (mitochondrial decoupler) served as positive control. Representative flow cytometry data depicts the overlay of TMRE intensity histogram of various truncated constructs of SARM at indicated time points. **(e)** Intracellular ROS was detected in SAM-TIR-transfected HEK 293T cells, using CMH_2DCFDA at 12, 18 and 24 h post-transfection. Cells treated with H_2O_2, a strong oxidizing reagent to generate ROS, served as the positive control. Representative flow

cytometry data depicts the overlay of CMH_2DCFDA intensity histogram of SAM-TIR at indicated time points.

Supplementary Figure S5: Effect of antioxidant and ERK inhibitors on SARM induced cell death. (a) HEK 293T cells were pretreated with the varying concentrations (100 μM, 250 μM and 500 μM) of the antioxidant – N-acetyl cysteine (NAC) for 1 h and then transfected with SARM-FL GFP fusion construct. At 12 h post-transfection, the cells were stained with 7-AAD and cell death was quantified by flow cytometry. (b) At 12 h post-transfection, NAC treated SARM-FL transfected HEK 293T cells were loaded with 20 nM of TMRE. The drop in mitochondrial membrane potential was assessed by flow cytometry. (c) HEK 293T cells were pretreated with varying concentrations of ERK inhibitors, PD98059 (25 μM and 50 μM, Panel I) and U0126 (30 μM and 50 μM, Panel II) for 1 h and then transfected with SARM-FL GFP fusion construct. At 12 h post-transfection, cell death was quantified using dead cell dye, 7-AAD. (d) Similarly, PD98059 pretreated cells were transfected with SARM-FL. Intracellular ROS was detected using CMH_2DCFDA, at 12 h post-transfection. The proportion of cells with increased intracellular ROS were calculated and plotted. Results in a, b, c and d are means ± S.D. of triplicate measurements. (e) HEK 293T cells were pretreated with indicated concentrations of NAC for 1 h. The cells were then transfected with SARM-FL and cells were lysed 24 h post-transfection and immunoblotted with anti-pERK antibody. The membrane was stripped and reprobed with anti-ERK antibody. Activated primary cytotoxic T cells were pretreated with indicated concentrations of (f) ERK inhibitors PD98059 and U0126 and (g) N-acetyl

cysteine for 1 h. The cells were then nucleofected with SARM-FL and lysates were analyzed by Western blot using anti- Bcl-xL antibody. Actin was used as a loading control. * p<0.05, ** p<0.005, *** p<0.0005.

Supplementary Figure S6: SARM is localized in the mitochondria. (a) Control untransfected HEK 293T cells were embedded in LR white resin and cut into ultrathin sections. The sections were then stained with anti-GFP followed with 10 nm gold-conjugated anti-rabbit secondary antibody. Under transmission electron microscope JEM1010, the control untransfected cell sections showed no gold particles in the mitochondria. Scale bar is 100 nm. **(b)** HEK 293T cells were transfected with N-ARM or N124. The cells were lysed 24 h after transfection. Lysates were size-fractionated by SDS PAGE and then immunoblotted with anti-GFP. The membrane was stripped and reprobed with anti-actin for the loading control. Primary cytotoxic CD8 T cells were activated using the plate-bound anti-CD3. The activated cells were nucleofected with **(c)** N-ARM or **(d)** N124. At 6 h, the cells were stained with mitotracker (Mito), fixed and observed under fluorescence microscope LSM 510 Meta. Scale bar is 5 μm. **(e)** Summary of the apoptotic potential of various truncated constructs of SARM.

Supplementary Figure S7: Phenotypic changes in primary T cells following *in vitro* activation: Activated primary CD8 T cells were collected at 24 and 48 h post activation, and stained with activation markers: **(a)** CD44 and **(b)** CD69. Post-staining, the cells were washed and resuspended in FACS buffer and analyzed by flow cytometry. Upregulation of CD44 and

CD69 indicates successful activation of the primary T cells. **(c)** CFSE-labeled naïve T cells were activated with plate-bound anti-CD3 and cell proliferation was studied by flow cytometry on days 1, 2, 3 and 4 post-activation. **(d)** Primary T cells were pretreated with either 1 μM MG132 or DMSO (solvent diluent for MG132, used as basal control) for 1 h and activated for 12 h in plate-bound anti-CD3. The cells were collected before and after T cell activation and immunoblotted with anti-SARM. Actin was analyzed as a loading control. **(e)** Activated primary T cells were collected at indicated days and stained with PE-conjugated CD95 and CD95L, which are death receptor and death receptor ligand, respectively. Post-staining, the cells were washed and resuspended in FACS buffer and analyzed by flow cytometry. **(f)** Activated primary T cells were photographed daily for 8 days post-activation to follow the morphological change. **(g)** Popliteal and inguinal lymph nodes were isolated from OTI^{+} $RAG^{-/-}$ mice injected with SIINFEKL peptide in Complete Freund's Adjuvant. The cells were stained with CD69. Post-staining, the cells were washed and resuspended in FACS buffer and analyzed by flow cytometry. Grey and black lines indicate cells from PBS injected control mice and SIINFEKL-injected mice, respectively. **(h)** Popliteal and inguinal lympnode cells isolated from control/ SIINFEKL injected mice were pooled and stained with trypan blue. The live cells are then counted using a Neubauer hemocytometer chamber. Data represent means of four mice per group. **Knock down of SARM by siRNA and shRNA:** NIH 3T3 cells were transfected with **(i)** either non-targeting control or SARM shRNA constructs (N1 and N3) and **(j)** SARM-specific siRNA or control siRNA. By 36 h, the cells were collected and RNA was prepared using Trizol. cDNA,

reverse-transcribed from RNA, was used as template for quantitative realtime PCR and data were normalized to mouse tubulin. NIH 3T3 cells were transfected with **(k)** non-targeting control or SARM shRNA constructs (N1 and N3) and **(l)** SARM specific siRNA or control siRNA**.** By 36 h, the cells were lysed and lysates were size-fractionated by SDS PAGE, immunoblotted and probed with anti-SARM. The membrane was stripped and reprobed with anti-actin for the loading control. (m) Activated primary cytotoxic T cells were transduced with retrovirus containing either SARM-specific or non-targeting control shRNA and GFP reporter. Lysates were prepared from (non-targeting control and SARM-specific shRNA) T cells on days 3, 6 and 9 post-transduction and immunoblotted with anti-SARM antibody. Actin was used as a loading control. * p<0.0005.

Supplementary Figure S8: The conceptual link between SARM and Bcl-2 family of proteins. Panel 1: In healthy cells, Bcl-xL is known to retrotranslocate BAX from mitochondria outer membrane (MOM) to cytosol, thus preventing mitochondrial outer membrane permeabilization (MOMP) formation (Edlich, F. *et al. Cell* **145**, 104-116, 2011). Panel 2: Bcl-xL levels are reduced during SARM-induced apoptosis and thus BAX is free to homo-oligomerize to form MOMP (based on figure 3a). Panel 3: Bcl-xL overexpression prevents SARM-induced apoptosis (based on figure 3b and d) and BAX, BAK double knockout abrogates MPTP-induced by SARM (based on figure 3e). Both these data support that SARM mediates intrinsic apoptosis via Bcl-2 family members.

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Supplementary Table S1. Oligonucleotides used in the study

SARM primers (to differentiate the 724 and 764 isoforms)	Forward	5′- ACACCTATGGCCTGGTCAGC -3′
	Reverse	5′- GCAGACAGCACCAGGACAAA -3′
SARM primers (RT PCR)	Forward	5′- CATCACCCGCAAGAGGTTC -3′
	Reverse	5′- CCATAGGTGTACTGGCGGAA -3′
Tubulin primers (RT PCR)	Forward	5′- CAGCAGGTCTTCGATGCCAA -3′
	Reverse	5′- CCTCCTTCATGGACATCCGTC -3′
SARM siRNA (Invitrogen)	Primer1	5′- UCCGGUAACUGAUAAAGACAUCUGG -3′
	Primer2	5′- CCAGAUGUCUUUAUCAGUUACCGGA -3′
Non-targeting control shRNA	Plasmid	5′- CAACAAGATGAAGAGCACCAA -3′
SARM shRNA (N3)	Plasmid	5′- CAGCCAGAGAAATGCTACATT -3′
SARM shRNA (N1)	Plasmid	5′- CCACATTATCAGAGAGCCAAA -3′
N-ARM cloning primer	Forward	5′- GGAATTCGCCACCATGGTCCTGACGCT -3′
	Reverse	5′- ATGGATCCAGCGCGCAGTTGCCCA -3′
N124 cloning primer	Reverse	5′- ATGGATCCGCCATCGAGGCGGAT -3′
N-ARM-SAM cloning primer	Forward	5′-CCGCTCGAGCCACCATGGTCCTGACGCTGCTCTTC- 3′
	Reverse	5′-GGAATTCGAGGTGTCCCCACTGAGCTTGCC-3′
ARM-SAM cloning primers	Forward	5′- CCGCTCGAGGCCACCATGGGTGGCCTCGACTTG-3′
	Reverse	5′-CGAATTCGATTCTCTGGCTGCAGAGAG-3′
ARM cloning primers	Forward	5′-CCGCTCGAGGCCACCATGGGTGGCCTCGACTTG-3′
	Reverse	5′-CGAATTCGACGCGCAGTTCGCCAGCGC-3′
TIR cloning primers	Forward	5′-CGAATTCGATGCTACATTCCCCGCTG-3′
	Reverse	5′-TCGGTACCCTGTAGGAAGCGGATGAT-3′