Materials and Methods......
3.1 Reagents

Culture media

For all the experiments, cells were cultured in RPMI-1640 (Life Technologies, USA) fortified with 2 mM L-Glutamine (Life Technologies), 10% fetal calf serum (FCS, Biological Industries, Israel), 100 μg/ml streptomycin and 100 U/ml penicillin (Hi Media, India). Supplemented with 5 mM HEPES (Sigma-Aldrich, USA), 50 μM β-mercaptoethanol (Life Technologies), 2 mM L-Glutamine (Life Technologies), 1.35 g/l sodium bicarbonate (Life Technologies), 10% FCS (Biological Industries), 100 μg/ml streptomycin and 100 U/ml penicillin (Hi Media). The OP-9 culture medium was Minimum essential medium (αMEM, 1X with GLUTAMAX and without ribonucleosides and deoxyribonucleosides, (GIBCO) was supplemented with 20% heat inactivated FCS and 1% Pen-Strep for maintenance of OP9-DL1 cells and for OP9DL1-DN1+DN2 cell co-cultures.

Phosphate-buffered saline (PBS) (1X)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>0.2 g/l</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2 g/l</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.0 g/l</td>
</tr>
<tr>
<td>Na₂HPO₄.2H₂O</td>
<td>1.15 g/l</td>
</tr>
</tbody>
</table>

pH adjusted to 7.2-7.4
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**Hanks balanced salt solution (HBSS) (1X)**

A 10X stock of Hank’s balanced salt solution (HBSS) (Biological Industries) was diluted to 1X in 0.035% sodium bicarbonate solution in sterile water, and used for washing the cells.

**Chemicals and inhibitors**

Inhibitors and chemicals used in the study include recombinant human Interleukin-2 (IL-2) (Roche Diagnostics, Mannheim, Germany 10,000 U=5 µg), 5-carboxyfluorescein succinimidyl ester (5-FAM, SE or CFSE) (Molecular Probes, Eugene, OR, USA), H₂O₂ (Sigma), DMNQ (2,3-Dimethoxy-1,4-naphthoquinone) (Enzo lifesciences), staurosporine (Sigma), Etoposide (Sigma), Thapsigargin (Sigma), EUK-134 (Cayman chemicals).

**Reagents used for flow cytometry**

Reagents used for staining mouse specific markers were either unconjugated or conjugated to biotin or fluorescein iso thiocyanate (FITC), phycoerythrin (PE)-, PE-Cy5-, PE-Cy7-, allophycocyanin (APC), Pacific Blue, V₄₄₅₀, PE-Txr, APC-Cy7 (which included antibodies directed against CD44, CD69, CD4, CD8, CD44, CD62L, B220, CD11b (Mac-1), CD11c, NK-1.1, CD69, CD27, CD28, Thy-1.1 (CD90.1), Thy-1.2 (CD90.1), CD45.1, CD45.2, CD11c, Gr1, TCR, Ki67, representative V₅₁ and were procured from BD Biosciences, Invitrogen, eBiosciences, Southern Biotech, Cell Signaling Technologies and Santacruz Biotechnology. Labelled secondary detection reagents used included F(ab')₂ fragments of anti-goat IgG (H+L)-PE, anti-rat IgG (Fc)-
bio, anti-rabbit IgG (H+L)-FITC and streptavidin-PE these and were purchased from Jackson Immunoresearch.

**Reagents Used for ELISA**

For assaying Tnf-α levels in culture supernatants, Mouse Tnf-α ELISA (BD Opt EIA, BD Biosciences Pharmingen) kit was used. It had the following reagents:

Capture antibody: anti-mouse Tnf-α monoclonal antibody, diluted in coating buffer.

Detection antibody: Biotinylated anti-mouse Tnf-α monoclonal antibody.

Enzyme reagent: Avidin-horseradish peroxidase conjugate

Standard: Recombinant

In addition, H₂O₂ (E. Merck) and o-phenylenediamine (Sigma) dissolved in substrate buffer were used for developing Tnf-α ELISA and 2N H₂SO₄ (Qualigens, GSK Pharmaceutical Ltd, Mumbai, India) was used for terminating the reaction.

**Coating buffer (0.1M, pH 9.5):**

0.2 M Na₂CO₃

0.2 M NaHCO₃

Mix 8 ml of 0.2M Na₂CO₃ and 17 ml of 0.2 M NaHCO₃ and make up the volume to 100ml with deionised water.

**Citrate-phosphate buffer (substrate buffer), pH 5.0**

0.1M citric acid

0.1M Na₂HPO₄

Mix 48.5 ml of 0.1M citric acid and 5.15 ml of 0.2 M Na₂HPO₄ and make up the volume to 100ml with deionised water.
3.2 Mice

For all the experiments, 4-8 weeks old C57BL/6 (H-2\(^b\)), B6.Thy-1.1 (H-2\(^b\)), OT-I (H-2\(^b\)), OT-II (H-2\(^b\)), P-14, B6CBACaA\(^w-J/\)A-Pdcd8Hq/J and their WT littermates were obtained from Jackson Laboratories (Bar Harbour, USA) and bred at Small Animal Facility, National Institute of Immunology, New Delhi. All mice were maintained and used according to the guidelines of NII Institutional Animal Ethics Committee.

3.3 Methods

Ex vivo cell preparation:

Spleen, thymus and lymph nodes were dissected out from mice euthanised by cervical dislocation. These tissues were teased between a pair of frosted glass slides to obtain single cell suspensions. Spleen cells were subjected to osmotic shock with distilled water to lyse RBCs, or alternately were treated with Gey’s buffer following which the cells were suspended in complete medium. The RBC lysis was not done for lymph node and thymus cell preparations.

CFSE [carboxyfluorescein, succinimidyl ester (5-FAM, SE)] labelling of cells

CFSE is a dye that binds to the intracellular proteins of a cell and with each cell division the intensity of CFSE labelling gets halved allowing one to estimate number of cell divisions undergone by a cell by flow cytometry. Cells were suspended at a density of 10 \( \times 10^6 \) mL in serum free medium or HBSS. To these cells, CFSE was added at a final concentration of 10 \( \mu \)M followed by incubation at 37\(^{\circ}\)C for 20 mins. After the incubation,
serum containing medium was used to wash the cells. A minimum of two washes was
given, each at 400 x g for 5 mins. Extent of labelling was checked by flow cytometry,
following which the cells were stimulated with different concentrations anti CD3 and anti
CD28 for 48 to 96 hrs. CFSE dilution after indicated time was scored on live gated cells
and analysed using the proliferation platform of Flow Jo software.

3.4 Cell Death Assays

Death in activated T cell blasts
Spleen cells from mice were stimulated with purified anti mouse CD3 mAb (anti-CD3)
for 30-40hrs at a cell density of 3 x 10^6/mL, at the end of which anti-CD3 was washed off
and the cells were cultured at a density of 1 x 10^6/ml with IL-2 (5 IU/mL) and maintained
as such for the next 3 days. The cell density was maintained at 1 x 10^6 /mL by monitoring
cultures and diluting them in IL-2 (5 IU/mL) containing media. On day 5, the cultures
were harvested, washed and dead cells were removed by Lympholyte-Mammal
(Cedarlane, Ontario, Canada) density gradient procedure. The resulting T cell blasts were
analysed by flow cytometry and were found to be 99% pure for T cells.

TSWD: For inducing TSWD, T cell blasts were cultured at 1 x 10^6/mL cell density with­
out IL-2 (-IL2). The T cell blasts cultured with IL-2 (+IL2) served as negative control for
TSWD.

AICD: For inducing AICD, T cell blasts at a cell density of 0.5 x 10^6/mL was stimulated
with plate bound anti-CD3 (+aCD3). T cell blasts cultured without plate bound anti-CD3
stimulus (-aCD3) served as a negative control for AICD. All cultures contained IL-2 (5 IU/L) to rule out death by TSWD.

To study the role of pharmacological modulators in death, 100 μM MnTBAP, 100 μg/mL AG, or 300 μM L-NMMA or 25μM EUK-134 were some of the inhibitors added to T cell blasts at the time of death induction. At different times, cells were harvested and stained either with appropriately labelled anti-CD4 or anti-CD8 mAb’s and Hoechst 33342 (2 μg/mL; Molecular probes Inc., Eugene, Oregon, USA) to score for apoptotic nuclei. In some instances, death was also scored by Trypan blue dye exclusion.

**Death in response to death inducers**

T cell blasts at a cell density of 0.5 x 10^6/mL were plated in Flat bottom 96 well plate, and media containing various death inducers like Etoposide, Staurosporine, Thapsigargin, DMNQ and H_2O_2 added in the medium, at various time points cells were harvested and death was scored by Trypan blue dye Exclusion.

**Death in naive T cells**

Spleen cells from Hq and WT mice were cultured with (+IL2) or without (-IL2) IL-2 to induce TSWD. 24 hrs later cells were harvested and stained either with appropriately labelled anti-CD4 or anti-CD8 mAb’s and Hoechst 33342 (2 μg/mL; Molecular probes Inc., Eugene, Oregon, USA) to score for apoptotic nuclei.
3.5 Purification of cells:

**CD4 and CD8 cells:** Splenocytes (at a cell density of $10^8$/ml) were labelled with biotinylated anti-CD4 or antiCD8 antibody (Miltenyi Biotech) for 30 min on ice followed by 2 washes with MACS buffer (PBS containing 1% FCS), resuspended at $10^8$/ml and incubated on ice for 30 min with magnetic streptavidin microbeads. Labelled cells were washed and separated on LS or MS columns (Miltenyi Biotech) as per manufacturer’s protocol.

**DN1 and DN2 cells:** Thymocytes (at a density of $10^8$/ml) were labelled with a mix of 9 biotinylated antibodies to various receptors (CD4, CD8, CD3, CD11b, CD11c, Gr-1, NK1.1, γδ-TCR, B220, CD19) and incubated for 30 min followed by two washes in MACS buffer and a subsequent incubation with Streptavidin beads. The flowthrough of the first step in this process after passing through column is again set through the same process with antiCD44 antibody and Streptavidin beads and after column purification the cells that are retained in the MS column as per manufacturer’s protocol are the purified population.

**Trypan Blue exclusion**

In order to detect death by Trypan blue exclusion, 0.04% Trypan blue solution made in PBS was added to the cells in a 1:1 ratio. Trypan blue dye enters cells with compromised cell membranes while live cells exclude the dye. Non-viable cells appear blue under a bright field microscope. For each sample, 100 cells were counted to score for % dead
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cells by Trypan blue uptake. Trypan blue exclusion method was used only in experiments involving T cell blasts, which are 99% pure for T cells.

Detection of apoptotic nuclei

In order to detect death by nuclear damage, cells at different times post death induction were harvested and stained with appropriately labelled anti-CD4 or anti-CD8 mAb’s (Pharmingen) and Hoechst 33342 (2 μg/mL; Molecular probes Inc., Eugene, Oregon, USA) to score for death in CD4 and CD8 T cells only. To score for apoptotic nuclei in Hoechst 33342 staining was done. Cells stained for CD4 and CD8 markers were analysed for nuclear morphology, visible due to Hoechst 33342 dye, using a fluorescence microscope (TE2000-U; Nikon Corp., Tokyo, Japan). For each sample 100 to 200 CD4 or CD8 T cells were counted to score for percentage of CD4 or CD8 cells showing apoptotic nuclei.

Primary proliferative responses

Responder spleen cells or purified B and T cell (total and naïve) were plated at a cell density of 3 x 10^5 cells/well, in a 96-well flat bottom plate. To these wells, stimulators were added starting at 3 x 10^5 cells/well and titrated down. The wells having responders alone served as controls for background proliferation. Total culture volume was 200 μL/well. Proliferation was estimated in the last 12 hrs of a total 72-84 hrs culture by [3H] thymidine incorporation.

To score for total T cell and B cell proliferative responses, spleen cells from Hq and their WT counterparts were plated at 3 x 10^5/well of a 96-well flat bottom plate along with
titrating doses of either anti-CD3 for T cell responses or LPS for B cell responses. Total culture volume was 200 μL/well. In some experiments, the total splenic cell numbers were normalised to equal T or B cell numbers/well. Proliferation was estimated in the last 12 hrs of a total 72-84 hrs culture by [3H] thymidine incorporation.

To score for CD4 or CD8 T cell proliferation, spleen cells were labelled with CFSE, and cultured at $1.5 \times 10^5$/ml density with titrating doses of either anti-CD3 for T cell responses or LPS for B cell responses. Frequencies of CD4/CD8 and B cells undergoing proliferation were scored by flow cytometry with CFSE dilution as a read out.

### 3.6 OP9DL1-DN1+DN2 cell co-cultures:

**Maintenance of OP9DL1 cell line**

OP-9 cells were cultured in minimum essential medium (α-MEM) with 20% heat inactivated FCS and split at 80% confluence every 3-4 days using cell dissociation medium. OP-9DL1 cells lose their ability to support T-cell differentiation upon overcrowding or repeated sub-culturing; hence stocks from early passages in 10 cm plates or 6 cm were used for each co-culture. OP9-DL1 and OP-9GFP stocks were made by initial rounds of expansion to 16 to 32 plates and these were frozen in 90% FCS and 10% DMSO as Primary stocks at -70°C.

**OP9DL1-DN1+DN2 cell co-cultures**

The co-cultures were carried out for the in vitro differentiation of CFSE labelled DN1 + DN2 thymocytes as described by Schmitt et al., 2004. DN1 + DN2 cells were labelled
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with CFSE and co-cultured on bone marrow derived stromal cells OP9 expressing the notch ligand (DL1) leading to effective production of mature T-cells following 4 to 6 days of co-culture. The DN1 + DN2 cells were freshly isolated and purified from fresh thymus and dead cells were removed by ficoll hypaque density gradient to ensure that only fresh and healthy living cells were seeded. OP9DL1 feeder cells from primary stocks were expanded on 24 well plates and 6 well plates along with such that on the day of CFSE labelled thymocyte seeding they achieved 75 to 80% confluence on the day of seeding of thymocyte 5-10 ng/ml of Flt-3-Ligand and 5 ng/ml of IL7 was added. The medium (along with Flt-3 ligand and IL-7) was gently changed on day 2 or day 3 as per requirement. On day 3 or day 4 the cells were harvested and the thymocytes were stained for markers CD25, CD44, CD8 and CD4 to see if differentiation and proliferation (based on CFSE dilution) of seeded thymocytes has happened by flow-cytometry.

3.7 Staining for Flowcytometry

Surface staining

For surface staining, 1 x 10⁶ to 3 x 10⁶ cells were incubated with 50 μL of primary staining reagent appropriately diluted in staining buffer which is PBS containing 0.5% BSA (Hi Media) and 0.1% sodium azide (Sigma), on ice for 45 min in 96 well round bottom polystyrene plates (Tarsons, India). Control samples were incubated with appropriate isotype matched control antibody. The cells were washed thrice with cold staining buffer, followed by incubation for 45 mins with 50 μL of appropriate secondary reagent diluted in staining buffer at working concentration. Finally, cells were washed
two to three times with ice cold staining buffer and re-suspended in PBS to be analysed on BD LSR (Becton and Dickinson, San Jose, CA) or BD FACSARia™ I (Becton and Dickinson, San Jose, CA). The flow data was analysed using Flow Jo software (Treestar, San Carlos, CA).

**Para-formaldehyde fixation of cells**

The aim of para-formaldehyde (PFA) fixation was to crosslink to Antibody bound to surface receptors. A stock solution of 1% PFA was prepared in PBS, filtered sterile and stored at -20°C for use. Cells post surface staining were 1% of PFA (tissue culture grade from Sigma) solution made in PBS. Cells were incubated for 15 to 20 min at room temperature (RT) with intermittent shaking followed by centrifugation at 400 X g to remove the PFA solution. The resulting pellet was washed twice with 1ml PBS each time to remove traces of PFA.

**Intracellular staining**

Cells were surface stained for various markers followed by permeabilization (using 0.03% saponin) of the cells. Then the cells were labelled with various intracellular protein, and were washed two to three times with ice cold staining buffer and re-suspended in PBS to be analysed on BD LSR (Becton and Dickinson, San Jose, CA) or BD FACSARia™ I (Becton and Dickinson, San Jose, CA). The flow data was analysed using Flow Jo software (Treestar, San Carlos, CA).
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Staining for microscopy

Cells were fixed with 4% paraformaldehyde for 20 min at 37°C and permeabilized using 0.3% Tween-20 or 0.03% saponin in PBS for 20 min at ambient temperature. Cells were blocked with 2 mg/ml BSA in PBS prior to incubation with antibodies in the same medium. Rabbit and mouse primary antibodies were detected using fluorophore-labeled Fc-specific anti-rabbit IgG or anti-mouse IgG.

Mitotracker red staining for Flowcytometry and Microscopy

Ex vivo cells from spleen and thymus were suspended in complete DMEM at the density 1 x 10^6 adding of MitoTracker Red CMXRos (invitrogen) at final concentration of 1µM. Cells were incubated at 37°C for ½ an hour in dark followed by 2 washes with complete medium. Cells were then resuspended in PBS and staining for surface receptor was carried out. These cells were then analysed on BD LSR (Becton and Dickinson, San Jose, CA) or BD FACSARia™ I (Becton and Dickinson, San Jose, CA). The flow data was analysed using Flow Jo software (Treestar, San Carlos, CA).

MitoSOX red and DCFDA staining

Cells were first stained for surface receptor followed by DCFDA (working concentration 1 and 10 µM) and MitoSOX (reagent working solution 5 µM )staining at room temperature in dark for 10 minutes in case of MitoSOX and 30 min for DCFDA. The reaction is stopped in DCFDA by adding complete medium and in both the cases staining is followed by 2 washes with cold PBS. Cells were analysed on BD LSR (Becton and


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Dickinson, San Jose, CA) or BD FACS Aria™ I (Becton and Dickinson, San Jose, CA). The flow data was analysed using Flow Jo software (Treestar, San Carlos, CA).

Death scoring was done using PI (Sigma), sytox red (Invitrogen) and sytox green (Invitrogen) were used to stain the nuclei of dead cells. For staining dead cells, samples were first stained for different caspases and/or surface markers, washed and re-suspended at a density of 5 x 10^6 cells/ml. To this either PI was added at a final concentration of 1 µg/ml or sytox green at 1 µM or sytox red at a final concentration of 3 nM. For PI staining, samples were analysed immediately by flow-cytometry, whereas, for sytox green and sytox red staining, cells were incubated with the dye for 15 min at RT before they were analyzed on a flow-cytometer.

Caspase Assay staining

To detect caspase activation, 10^6 cells were suspended in 300 µl of complete medium and incubated with 1 µl of the appropriate caspase reagent for 60 min at 37°C and 5% CO2. The cells were then spun at 3000 rpm for 5 min, and were washed thrice with the washing buffer provided by the manufacturer. The cells were then used for surface staining (as described above) followed by staining for dead cells.

The reagents for detecting activated caspases were purchased from Calbiochem, and included FITC-DEVD-FMK (caspase-3), FITC-IETD-FMK (caspase-8), FITC-LEHD-FMK (caspase-9).
3.8 Enzyme linked immunosorbent assay (ELISA)

Amount of cytokine secreted in culture supernatants was assayed by sandwich elisa according to manufacturer’s instruction. 96 well flat bottom (Nunc immunosorp, Roskilde, Denmark) plates were coated with capture antibody which is anti mouse Tnf-α diluted in carbonate-bicarbonate buffer (pH 9.2) and incubated at 4°C for 14-16 hrs. The plates were blocked with blocking buffer (PBS-T containing 1% BSA) for 1 hr at 37°C to prevent non-specific binding of proteins to the plate. After blocking, various dilutions of culture supernatants (from stimulated cultures) or standard were added and incubated for 2 hrs at room temperature (RT). The bound cytokines were detected by adding biotinylated anti-mouse Tnf-α mixed with avidin-horseradish peroxidase (HRP) conjugate, diluted to appropriate concentration in PBS containing 0.5% BSA. Plates were incubated for 1 hr at room temperature. After each incubation, plates were washed thrice with PBS containing 0.5% Tween (Sigma). After the final wash, citrate-phosphate substrate buffer (pH 5.0) containing H₂O₂ (30% w/w; 1 μL/mL of the substrate buffer) and o-phenylenediamine (OPD; 0.5 mg/mL of substrate buffer) was added to each well. H₂O₂ is the substrate for HRP enzyme while OPD is a chromogenic substrate and reacts with radicals released during primary reaction of HRP and H₂O₂ to yield colour. 2N H₂SO₄ (Qualigens) was added to terminate the reaction. Absorbance was measured at a wavelength of 490 nm using a micro-plate reader (Bio-tek instruments) and compared with the Tnf-α standard.
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3.9 SDS-PAGE and Western Blotting

The 1-2 x 10^6 cells treated under various conditions were harvested, washed twice by centrifugation with chilled PBS, lysed by the addition of SDS sample buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.1% bromphenol blue). After addition of the lysis buffer, samples were vortexed to reduce sample viscosity, denatured by boiling, and then either loaded immediately on to the SDS-PAGE gel or stored at –20°C to be used later.

For cytosolic and mitochondrial extracts, 5 µL of 4X SDS sample buffer containing 100 mM of DTT (dithiothreitol) was added to 50 µL of the extracts, followed by boiling and cooling on ice. Samples were microcentrifuged for 5 min before loading on 12% SDS-PAGE gels or were stored at –20°C to be used later.

Proteins were transferred onto Trans-Blot nitrocellulose membrane (Biorad Laboratories, CA, USA). Membranes were blocked for 1 hr in blocking buffer which is 5% Blotto in TBS-T (Tris buffered saline containing 0.1% Tween-20) followed by an overnight incubation with primary antibody diluted in blocking buffer, at 4°C with gentle rocking. Membranes were washed three times for 5 min each, with 10 ml of TBS-T and then incubated for 1 hr at RT with goat anti-rabbit/anti-mouse HRP (CST), diluted at 1/1000 in blocking buffer. After another three washes, proteins were detected by enhanced chemiluminescence, according to the manufacturer’s instructions (Amersham). The light emission can be detected by exposure to blue-light sensitive autoradiography film.
3.10 Analysis of flow cytometry data

Data acquired by flow cytometer was analysed by the analysis software Flowjo (Treestar, San Carlos, CA) and has been shown as two color (parameter) plots. Extent of proliferation in responding cells by CFSE dilution, was analysed by the Flowjo proliferation platform (Treestar, San Carlos, CA) software. The principal of this analysis is that with each cell division the intensity of CFSE labelling gets halved. This software halved allows us to estimate number of cell divisions undergone by a cell as well as the total percentage of cells in each successive division.

3.11 Statistical analysis

For in vivo experiments, data are shown as mean ± SE for three mice per group and are representative of five independent experiments. For in vitro cultures, data are shown as mean ± SE for triplicate samples and is representative of three independent experiments. Wherever mentioned, data have been compared by one way anova student’s t test, where p<0.05 was considered significant.