MATERIALS & METHODS
STOCK 2:

- CaCl₂·2H₂O 1.86 g
- KCl 4.00 g
- NaCl 80.00 g
- MgCl₂ (anhydrous) 1.04 g
- MgSO₄·7H₂O 2.0 g

Dissolve and made the volume up to 1000 ml.

Both the stocks were stored at 4°C. For making one liter of working solution of BSS, both the stock 1 & 2 were mixed in 100 ml each and the volume was made up to 1000 ml with double distilled water.

2) Membrane preparation: The macrophages were thawed and suspended in 20 mM Tris-HCl pH = 7.4 containing 0.25 M sucrose, 1 mM EDTA, proteolytic inhibitor cocktail (leupeptin, Aprotinin, Antipain, pepstatin, iodoacetamide, PMSF) was added. A small amount of glass beads was added. This was followed by homogenization in a hand driven homoginiser. Before homogenization a small amount of glass beads (Type 1 75-150 microns, Sigma). The cell debris were removed by spinning at 600 x g for 15 min at 4°C. The supernatant thus obtained was centrifuged at 110,000 x g for 2 hrs. The pellet obtained was solublised in 20 mM Tris HCl (pH = 7.5) containing 1 % Triton X-100, 20 % glycerol and proteolytic inhibitor cocktail overnight at 4°C. The insoluble materials were removed by centrifugation at 100,000 x g for 1 hr at 4°C.

The membrane proteins present in the supernatant were seperated by SDS PAGE using 10 % polyacrylamide gel (Laemmli, 1970).
After electrophoresis M150 was located by staining a strip of gel with coomassie blue. The relevant region was cut, crushed and eluted in elution buffer, at 37°C for 24 hrs. The supernatant was recovered from polyacrylamide gel particles.

**REAGENTS USED:**

*Homogenisation Buffer:*
- Sucrose: 250 mM
- Tris HCl: 20 mM
- EDTA: 1mM
- pH was adjusted to 7.0.

**PROTEASE INHIBITOR COCKTAIL:**
- Leupeptin: 5 mg
- Aprotinin: 5 mg
- Antipain: 5 mg
- Pepstatin: 5 mg
- Iodoacetamide: 5 mM.

All components were resuspended in 5 ml of Dounce Buffer: Tris Cl (10 mM), MgCl2 (0.5mM) pH was adjusted to 7.0. Working concentration of protease inhibitor was 10 μg/ml. PMSF was made fresh by dissolving 0.00174 g of PMSF in 100 μl ethanol and adding to 10 ml of Dounce buffer containing protease inhibitor cocktail.

**SOLUBLISATION BUFFER:**
- Triton X 100: 1 %
- Glycerol: 20 %
- Tris Hcl: 20
- Adjust the pH to 7.5.

**ELUTION BUFFER:**
- Tris base: 50 mM
- SDS: 0.1 %
- NH₄HCO₃: 100 mM
- EDTA: 0.1 mM
- NaCl: 50 Mm

Make up the volume to 100 ml after adjusting the pH to 8.0.
REMOVAL OF SDS:

The SDS in supernatant containing protein was removed by Extracti-D gel (PIERCE). The gel was equilibrated with 0.05 M Tris HCl buffer pH=9.0. Prior to loading of supernatant 1mg / ml BSA was loaded to minimize loss of M150 due to non specific binding. The column was washed thoroughly and the supernatant was loaded. The SDS bound to the column while the protein (M150) eluted out. M150 was dialysed against PBS.

RECONSTITUTION OF M150 INTO LIPID VESICLES:

L-_-phosphotidyl choline (PC: Sigma) was dissolved in a 1:1 ratio of chloroform and methanol and evaporated under N₂-gas using a rotavapour to make a deposit of thin film. The lipid was dried under vacuum for 2 hrs and dissolved in bath type sonicator of 30 minutes. M150 from which SDS had been removed was added and mixed and vortex properly .The mixture was then dialysed at 4°C against 10 mM Tris HCl ( pH= 8.0 ) 0.01 mM EDTA & 50 mM NaCl for 24 hrs with changes in the buffer. It was then centrifuged for 2 hrs. at 4°C at 178,000 x g. The supernatant was discarded and the pellet was dissolved in 0.9 % NaCl. The unbound protein was eliminated by passing the solution through Sephadex G50 mini column (Fry et al. 1987). A 2 ml sample was layered on top of a discontinuous density gradient of 5-40 % sucrose in 10 mM Tris HCl pH=6.8, 0.15 M NaCl and 1 mM EDTA. The sample was centrifuged overnight at 98,000 x g overnight at 4°C. The 2 ml sample was collected from the 10 % interface layer and washed in 0.9 % NaCl and passed initially through 0.45 μm and 0.22 μm sterile filter and stored at -20°C until further use.
PREPARATION OF CD8+ T CELLS:

The mice were sacrificed by cervical dislocation and spleen was removed under aseptic conditions in the Bio safety hood. The spleen were crushed using frosted end slides in cold HBSS in a petridish (Tarsons, India). The cells were transferred in a 50 ml tube, which was kept on ice for 5 minutes to remove debris. The cells were then transferred to a fresh 50 ml tube and centrifuged at 1000 rpm for 10 minutes at 4°C. The pellet was resuspended in 1 ml HBSS containing 0.1 % FCS and 10 ml Gey's solution was added and held on ice for six minutes (B.B Mishell & S.M Shiigi 1979). 5 ml FCS was layered gently and the tube was centrifuged at 1000 rpm at 4°C for 10 minutes. The cells were washed once with cold HBSS and the pellet was resuspended in RPMI 10 % FCS and added in a plastic petridish and panning was done for 1 hr at 37°C, 7 % CO₂. The panning was repeated once more. When the second panning was going on nylon wool column was equilibrated with RPMI 10 % FCS. The cells from second panning was centrifuged and the pellet resuspended in RPMI 10 % FCS and passed through nylon wool column. Once the cells entered in the nylon wool the column was closed and cells were maintained in it for 1 hr at 37°C. The cells were eluted with RPMI 10 % FCS and centrifuged at 1000 rpm. The pellet was resuspended in a cocktail of antibodies against different surface molecules of CD4+ T cells (anti-L₃T₄), B cells (anti-IgM & anti-IAᵈ), macrophages (anti-Mac2, anti-Mac3, anti-IAᵇ) and Dendritic cells (anti-33D1) and incubated on ice for 1 h. The cells were washed once and baby rabbit complement (BRC) was added and incubated at 37°C, 7 % CO₂ for 30 minutes. The cells were then washed twice and resuspended in RPMI 10 % FCS. The cells were then on anti-L₃T₄, anti-IAᵈ, anti-mouse-IgM coated petridish twice for each of them (Antibodies were coated on petridishes CO₂/HCO₃ buffer pH = 9.6). This
preparation was subsequently followed for CD8⁺ T cell preparation and gave a purity of > 98 % as analysed by FACS.

**REAGENTS USED:**

**GEY'S SOLUTION:**

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Dissolve and make volume to 1000 ml, and autoclave.

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Dissolve and make the volume to 1000 ml, and autoclave.

<table>
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<th>STOCK C- Column</th>
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</thead>
<tbody>
<tr>
<td>NaHCO₃</td>
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</table>

Dissolve and make the volume to 1000 ml and autoclave.

To make 1X Gey's solution mix,

20 parts of stock A
5 parts of stock B
5 parts of stock C, and
70 parts of distilled water (sterile).

**PROCEDURE:** Resuspend approximately 10⁶ pelleted spleen cells in 1 ml of BSS, 5 %FCS and add 5 ml of 1 x Gey's solution and hold on ice for 6 minutes. This is followed by underlaying the cells with 100 % fetal calf serum and centrifuging at 1000
rpm for 10 minutes. The cells are washed once in BSS 5% FCS prior to proceeding to 
next step.

For separation of live cells from dead cells after the cells have been prepared on 
Ficoll- Hypaque : the cells and Ficoll- Hypaque (Sigma) was prewarmed to 20°C in a 
water bath and the centrifuge maintained at 20°C. 4 ml of Ficoll- Hypaque was added 
to polycarbonate tube followed by gentle layering of 2-4 ml of cell suspension on it. 
This was followed by centrifugation at 2000 x g for 20 minutes. The live cells were 
collected from the media-ficoll interface and washed once in RPMI, 10% FCS at 300 x 
g for 15 minutes.

PREPARATION OF B CELLS:

A single cell suspension of mice spleen was prepared in balanced salt solution. The 
red blood cells were depleted by treatment with haemolytic Gey's solution. The cells 
were then plated on petridish for 1 hr at 37°C and 7% CO₂ twice. The non adherent 
cells were treated with a mixture of anti-Mac2 and anti-Mac3 and a mixture containing 
anti-Thy1.2, and anti-L3T4 and antibodies followed by complement mediated killing. 
The cells were then incubated at a concentration of 4x10⁶/ml/ petridish with 10 µg/ml 
LPS (from Salmonella typhosa, Sigma) for 48 hrs at 37°C and 7% CO₂. The purity of 
such cells was over 85% as analysed by FACS (Becton Dickinson).

RAISING OF POLYCLONAL ANTIBODY AGAINST M150 IN 
HAMSTER :- Syrian Golden hamster was immunised subcutaneously with 100 µg of 
M150 in complete Friend's adjuvant (CFA) at four sites. After 21 days booster was 
given with 50 µg M150 in incomplete Freund's adjuvant subcutaneously. Titre was 
checked by Dot blot and ELISA and surface expression on macrophages and B-cells
ELISA FOR DETECTION OF M150 WITH ANTI-M150:

96 well plate was coated with M150 in 10 mM Tris containing 0.02 % sodium deoxycholate, NaCl overnight at 4°C. This was followed by three washes with PBS Tween 20. preimmune serum and immune serum was added at different dilutions. Dilutions were made in PBS Tween 20. Incubation was done for 2 hrs. at room temperature. The wells were subsequently washed 3 times in PBS Tween 20. This was followed by incubation with rabbit-anti-hamster-IgG for 1 hr followed by three washes with PBS Tween 20. Next anti-rabbit-HRP was added and incubated for 1 hr. Three washes were given by PBS Tween 20 followed by a single wash by wash buffer containing 0.2 % NP40. HRP substrate as added. OPD was added and reaction was terminated by 7 % H2SO4 1:1 volume and OD was read at 490 nm.

REAGENTS USED:

Coating buffer containing deoxycholate:

- Sodium deoxycholate 0.02%
- Tris 10 mM
- Sodium Chloride 150 mM
- Adjust the pH to 8.0.

DOT BLOT ANALYSIS:

M150 was applied on nitrocellulose aligned in a dot blot apparatus and dried with warm air blower. Preimmune serum and anti-M150 serum was added at different dilutions and incubated for 2 hrs. The dot blot was thoroughly washed with PBS Tween 20 and anti-hamster-antibody was added diluted in PBS Tween 20 and incubated for 1 hr. This was followed three washes in PBS Tween 20. Next anti-rabbit-
Materials & Methods

HRP was added diluted in PBS Tween 20 and incubated for 1 hr. The membrane was washed three times with PBS Tween 20 and color was developed with DAB.

SURFACE STAINING FOR PRESENCE OF M150 BY FACS:

ISOLATION OF MACROPHAGES

Mice were injected with 2 ml of 4% thioglycollate intraperitoneally. Peritoneal macrophages were isolated by peritoneal lavage after four days. The cells were panned and adherent population was taken. This isolation method has given > 95 % purity.

ISOLATION OF B CELLS (as mentioned earlier)

SURFACE STAINING: Macrophages and B cells were incubated with 3 % rabbit serum in PBS (decomplemented by incubating for 45' at 56°C) for 1 hr. Anti-hamster serum and preimmune serum were added at 1:100 dilution in 3 % rabbit serum and incubated for 2 hrs on ice. The cells were washed three times with 3 % rabbit serum in PBS. Rabbit-anti-hamster-FITC was added (diluted in 3 % rabbit serum) and incubated for 1 hr. The cells were washed in 3 % rabbit serum. Next the cells were fixed in 1 % paraformaldehyde and analyzed on FACS.

ISOLATION OF CD8+ T CELLS (AS MENTIONED EARLIER),

STUDY OF PROLIFERATION OF CD8+ T CELLS IN RESPONSE TO ANTI-CD3 AND M150.

The purified CD8+ T cells were added to 96 well plate at a concentration of 10^5 cells per well. The wells had been previously coated with anti-CD3 at a concentration of 10 μg/ml in carbonate/bicarbonate buffer overnight at 4°C. The wells were washed
prior to addition of cells with BSS. M150 was added at different concentrations starting from 10 μg/ml. As control PMA + anti-CD3 and anti-CD3 + ionomycin was used.

BLOCKING OF PROLIFERATIVE RESPONSE OF CD8+ T CELLS IN ALLOGENIC RESPONSE:-

CD8+ T cells were isolated from Balb/c while macrophages and B cells were isolated from C57B1/6 as mentioned earlier. The CD8+ T cells were cultured with either Macrophage or with B cells (LPS activated) in presence of anti-M150 serum at 1:500 dilution. The proper controls were taken care of.

ANALYSIS OF EXPRESSION OF CD45 AFTER COSTIMULATION:
Antibodies against CD45 were biotinylated before use for FACS analysis. The cells were incubated with these antibodies for 1 hr in 2 % PBS containing 0.02 % azide on ice. The cells were washed thrice with PBS 2% BSA. The cells were resuspended in PBS 2% BSA and avidin-FITC was added. The cells

EXPRESSION OF Fas AND Bcl-2:
The cells were cultured for 96 hrs in following experimental conditions: (i) Cells + medium (ii) Cells + anti-CD3 (iii) Cells + anti-CD3 + M150 and cells + M150 alone in 24 well plate. The cells were taken out by gentle pipetting and washed in PBS 2% BSA. Anti-Fas and anti-Bcl-2 were added and incubated for 1 hr on ice. The cells were washed in PBS 2% BSA and anti-rabbit-FITC was added and incubated for 1 hr followed by three washings in PBS 2% BSA. The cells were then fixed in 1% paraformaldehyde and analyzed on FACS.
DETECTION AND ANALYSIS OF APOPTOTIC CELLS: Apoptotic cells were detected by staining with propidium iodide according to (Reep et al. 1995). The cells were washed thrice with PBS containing 5% FCS. 1ml of 70% cold ethanol was added, vortexed and kept at 4°C overnight. The cells were washed thrice and suspended in 0.1ml PBS containing 1mg/ml of RNase (Sigma Chem. Co., St. Louis, MO) followed by 0.2ml of 100μg/ml Propidium iodide and left in dark for 30min. Induced apoptosis was analyzed on FACScan (BD with cytomation, data acquisition and software Lysis II) for red fluorescence. Initial identification of cells was made with the help of FSC/SSC plots. The apoptic cells were selected on the basis of showing to higher SSC, due to condensation of nuclear chromatin. The debris were excluded based on their very low SSC and FSC signals and there were no overlaps between these two populations. The apoptic and normal cells were gated out and subsequently analyzed.

ANALYSIS OF DIFFERENT EFFECTOR MOLECULES IN CD8+ T CELLS AFTER COSTIMULATION:

GRANAZYME A: Assay for granazyme A was done by its enzymatic action on azocaesin (Sigma). The cells were recovered by gentle pipetting and washed twice in 10mM Tris HCl buffer pH 8.0 and resuspended in 100μl Tris HCl buffer. 5μl of 5% NP40 was added. This was followed by addition of 2μl of 5% azocaesin (dissolved in 10mM Tris HCl buffer pH 8.0). The mixture was incubated in dark for 2 hours and OD was taken at 450 nm (Jenne and Tschopp 1988).

PERFORIN: The cells were collected from culture wells (96 well plate) and pelleted in microcentrifuge tubes by spinning at 1000 rpm. The cells were resuspended
Materials & Methods

in 100 μl of TBS containing 1 %BSA and 1 mM EDTA. The cells were lysed by rapid freeze and thaw and 300 μl of sheep RBC(10^7 cells/ ml) in Veronal buffer was added and incubated for 15 minutes. The tubes were spun for 5 minutes to remove intact RBC. Hemoglobin release was quantitated at 412 nm by means of ELISA reader.

SIGNAL TRANSDUCTION STUDIES:

Calcium: 10^7 CD8+ T-cells were resuspended in 1 ml of loading buffer containing 3.4μg /ml Fluro3 and incubated for 30 minutes at 30°C. Simultaneously 5 μg/ml of pluronic F127 was added to improve loading.

The cells were diluted to a concentration of 10^6 cells/ ml using loading buffer. Different required signals were added and analyzed for mobilization of intracellular calcium by FACS (Vandenberghe et. al.).

REAGENTS USED:

Cell loading medium: Hanks balanced salt solution containing:

1 mM Calcium
1 mM Magnesium
1 % FCS heat inactivated for 1 hr at 56°C

1 mg/ ml Ionomycin dissolved in DMSO

Tyrosine phosphorylation:

The CD8+ T cells were isolated as described earlier. They were stimulated with anti-CD3 for 3 minutes at 37°C water bath and M150 was added (0.01 μg/ ml). The incubation was stopped at different time intervals i.e. 1, 3, 5, 7 & 10 minutes by centrifuging briefly at high speed and supernatant was discarded. Immediately 0.5 ml of 1x ice cold lysis buffer was added and vortexed immediately and put on ice for 20 minutes.
The cell lysate was microcentrifuged for 15 min at 16,000 rpm at 4°C. Prior to stimulation of CD8+ T cells with appropriate signals, the anti-phosphotyrosine antibody was incubated and 2µl of it was added to protein A sepharose suspended in 0.5 ml PBS and placed on eppendorf shaker at 4°C.

While the cell lysate was undergoing centrifugation, the anti-phosphotyrosine antibody coated protein sepharose beads was washed twice with cold PBS, followed by one wash in 1 x lysis buffer. The supernatant was taken out gently and discarded.

The cell lysate was added to antibody coated beads and the microcentrifuge tubes were put on eppendorf shaker for 6 hrs at 4°C.

The antibody coated beads were microcentrifuged briefly at high speed and washed three times with 1x lysis buffer followed by one wash with 1 mM sodium orthovanadate (in 0.04 M tris HCl buffer).

50 µl 1x SDS sample buffer was added and heated for 5 min at 100°C. It was centrifuged briefly at high speed and the supernatant was loaded on 10% SDS PAGE and electrophoresed.

This was followed by electrotransfer of proteins on nitrocellulose at 150 mA for 30 minutes. The transblot was placed in blocking solution for 1 h at room temperature on rocker platform with constant shaking. The blocking solution was poured off and anti-phosphotyrosine antibody was added at 1:1000 dilution in TBST and incubated overnight at 4°C on orbital shaker. The antibody was removed and the blot was washed three times in 50 ml TBST for 5 minutes each time at room temperature. Since anti-phosphotyrosine is mouse IgG, hence anti-mouse IgG, Biotin (Binding site) was
added in TBST and incubated for 2 hrs. The blot was then washed with 50 ml TBST for 5 min each and streptavidin peroxidase was added in TBST and incubated for 1 hr. The conjugate was poured off and the blot was washed three times in TBST followed by three washes in wash buffer. The membrane was washed thrice and developing solution was added. The reaction was stopped by washing with water.

**REAGENTS FOR TYROSINE PHOSPHORYLATION:**

Anti-Phosphotyrosine was a kind gift from Dr. K.V.S Rao, ICGEB, New Delhi.

**2 X LYSIS BUFFER**

1 ml, 1M Tris Cl pH 8.0 (Final conc. 0.04 M)
2.3 ml, 3M NaCl (Final conc. 0.276 M)
10 ml 50 % v/v Glycerol
5.0 ml 10 % v/v NP40
25 μl of 50 mM p-nitrophenylguanidine benzoate (final conc. 0.5 mM)
0.5 ml of 0.1 M Sodium orthovanadate (0.002 M)
0.2 ml of 0.5 M EDTA pH 8.0 (0.004 M)
1 ml of 0.5 M NaF (0.02 M)

The volume was made up to 25 ml. 10 ml was taken from here and 100 μl of protease inhibitor cocktail was added before use.

Tris buffered saline containing Tween 20 (TBST)

10 mM TrisCl pH 8.0
150 mM NaCl
0.5 % v/v Tween 20
Store at room temperature.

Tris /saline/azide (TSA) solution :

50 mM Tris Cl pH 7.6
150 mM Na Cl.
0.2% v/v sodium azide at room temperature

DAB :- 60.0 mg of DAB in 100 ml of 0.05 M Tris Cl pH 7.6, NiCl2 and 100 µl hydrogen per oxide.

**CHEMICALS**

**SIGMA:** Ammonium persulfate, Antipain di hydrochloride, aprotinin, Avidin ,ß-mercaptoethanol, Bovine Serum Albumin, Bromophenolblue, Chymostatin, Concanavalin A, Coomassie blue R, DMSO, FITC, HEPES, Leupeptin, Nonidet 40, Pepstatin, Phosphatidyl Choline, Sodium Azide, TEMED, Triton X 100, Tween 20, Glutarylaldehyde,Paraformaldehyde, Acrylamide, Bis-acrylamide, OPD,DAB, Azocaein, glass beads, P-nitrophenylguanidinobenzoate, Sodium orthovanadate, Sodium Fluoride.

**GIBCO:** DMEM, HBSS, RPMI 1640, L-Glutamine, L-pyruvate, penicillin, Streptomycin.

**SERA LAB:** Fetal Calf Serum

**BDH:** Ammonium sulphate, Calcium chloride, Ferrous sulphate, Formaldehyde, Glycerol, Glycine, Potassium Chloride, Potassium dihydrogen orthophosphate, Sodium bicarbonate, Sodium carbonate, Sodium hydroxide.

**Genzyme:** Recombinant IL-2, IL-4, and IFN-γ.

**Robbins Scientific:** Nylon wool
**Materials & Methods**

DIFCO: *Freund's adjuvant (Complete & Incomplete), Thioglycollate Medium,*

RANBAXY: *Acetone & methanol.*

CALBIOCHEM: *Ionomycin.*

PIERCE: *BCA Protein Estimation Kit. PMSF, Extracti D Gel, NHS- Biotin, DSS.*

PHARMACIA: *Cyanobromide activated Sepharose Gel.*

QUALIGEN: *Toulene.*

**HYBRIDOMAS AND ANTIBODIES:**

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<th>No.</th>
<th>NAME</th>
<th>ANTIBODY</th>
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