MATERIALS AND METHODS
MICE

The mice strains used for all experimental work were C57BL/6 [H-2^b], obtained from Jackson Laboratories [Bar Harbor, USA], IgH transgenic [H-2^b] [gift of Dr. J. M. Durdik, USA], TAP-1 deficient [H-2^b] [gift of Dr. L. Van Kaer, USA] and bred in the Small Animal Facility of the National Institute of Immunology, New Delhi by Dr. R. K. Anand and provided by him on request.

CULTURE MEDIA

Dulbecco's Minimum Essential Medium [DMEM, Gibco, USA] or Click's [Irvine Scientific, USA] medium fortified with L-glutamine [2 mM], 5X10^{-5} M β-mercaptoethanol [Sigma, USA], streptomycin [100 μg/ml] and 10% fetal calf serum [FCS, Hyclone, USA] was used for maintenance of all cell lines and experiments. Selection medium for B and T cell fusions was made by adding Hypoxanthine [0.1 mM], Aminopterin [0.4 μM] Thymidine [16 μM] mixture [HAT, Boehringer Mannheim, Germany] to culture medium containing 20% FCS. T and B cell hybridomas were weaned on medium containing Hypoxanthine [0.1 mM] and Thymidine [16 μM] mixture [HT, Boehringer Mannheim, Germany] and 20% FCS.

PHOSPHATE-BUFFERED SALINE [PBS]

PBS contains 8 g of NaCl [Qualigens, India], 200 mg of KCl [Qualigens, India], 200 mg of KH₂PO₄ and 750 mg of Na₂HPO₄·7H₂O [Qualigens, India] in 1 liter of triple-distilled water.
PROTEINS

Proteins used for T cell hybridoma assays was ovalbumin [OA, Sigma, USA]. These protein was treated with maleic anhydride to obtain maleylated protein. Both native and maleyl proteins were adsorbed on Alum [Alhydrogel, Superfos Biosector a/s, Denmark] at 4°C to get particulate preparations of proteins.

PEPTIDES

The H-2K^b-binding ovalbumin peptide 257-264 [SIINFEKL], its lysine-substituted form [SIINFEEL] and another H-2K^b-binding peptide from VSV [RGYVYWL] were the gift of Dr. D. M. Salunke, NII [New Delhi, India].

CELL LINES

T CELL LINES

MHC class I [H-2^b]-restricted, ovalbumin-specific T cell transfectant [B3.4.5] was a gift from Drs. C. Hogquist and M. Bevan, University of Washington, Seattle, USA. T cell lines RMA [H-2^b] and its TAP-1/TAP-2 deficient mutant cell line RMA-S [H-2^b] are from American Type Culture Collection [ATCC, USA].

B CELL LINES

LB27.4 [H-2^b,d, HB99] is an ATCC [USA] cell line. The 3F1 hybridoma was generated from fusion of transgenic spleen cells with the plasma cell fusion partner, Sp2/0 and checked for surface expression of transgenic Ig by flowcytometric analysis.
MACROPHAGE CELL LINES
IC-21 [H-2<sup>b</sup>] is an ATCC cell line. The bone marrow derived macrophage cell line BMC2 [H-2<sup>b</sup>] was a gift of Dr. A. Rudensky [University of Washington, Seattle, USA].

MONOCLONAL ANTIBODIES
Anti-MHC class I antibody [HB176] is an ATCC cell line. Anti-B220 [6B2], anti-mouse IgM<sup>b</sup> [RS3.1] and anti-arsonate [R16.7] antibodies are gifts from Dr. J. M. Durdik [Denver, USA].

B CELL AND T CELL FUSION PARTNERS
Sp2/0 [H-2<sup>d</sup>, CRL 1581], a mouse myeloma line, used as plasma cell fusion partner and BW5147.3 [H-2<sup>k</sup>], a mouse T cell lymphoma line, used as T cell fusion partner, are cell lines from ATCC [USA]. BWCD8.7 is T cell receptor deficient derivative of BW5147.3 transfected with CD8 and was provided by Prof. C. A. Janeway [Yale University, New Haven, USA].

IL-2 DEPENDANT CELL LINE
CTLL-2 [H-2<sup>b</sup>, TIB214, ATCC, USA] is a mouse cell line that responds to IL-2 and IL-4 by proliferation.

METHODS

PROTEIN ESTIMATION

The concentration of protein in solution was estimated by micro-bicinchoninic acid [BCA] method. BCA reagent 'A' was prepared by dissolving 0.5 g BCA [4,4' -dicarboxy- 2,2' biquinolone, C<sub>20</sub>H<sub>12</sub>N<sub>2</sub>O<sub>4</sub>] [Sigma,
USA], 1 g sodium carbonate [Na$_2$CO$_3$], 0.08 g sodium tartarate, 0.2 g sodium hydroxide and 0.475 g sodium bicarbonate [Merck, India] in 50 ml of distilled water at pH 11.25. Reagent ‘B’ was 4% cupric sulphate [CuSO$_4$.5H$_2$O, Qualigen, India]. 50 parts of reagent ‘A’ was mixed with 1 part of reagent ‘B’ to prepare reagent ‘C’. To 20 µl of protein sample, 200 µl of reagent ‘C’ was added in 96-well, non-sterile polystyrene microtiter plates [Nunclon, USA]. The plate was incubated at 37°C for 30 minutes. Absorbance was read at 570 nm in an ELISA reader [Bio-tektm Instruments, USA]. The assay was blanked on 20 µl of water with 200 µl of reagent ‘C’. A standard curve of BSA was used from 1000 µg to 100 µg to quantitate the amount of protein.

**MALEYLATION OF PROTEINS**

Maleylation acylates α- and ε- amino groups in protein [Butler, P. J. G. and Hartley, B. S. 1972. Maleylation of amino groups. Method in Enzymology, 14:191]. The proteins [10-25 mg/ml] were dissolved in PBS [pH 7.2]. Before maleylation, the pH of protein solution was increased to 8.0 using 1N NaOH. Finely powdered maleic anhydride [C$_4$H$_2$O$_3$, Sigma USA] was used at a concentration 2.5 fold higher than protein concentration. The pH was maintained between 8.5 to 9.0 throughout the addition of maleic anhydride, using 1N NaOH with constant stirring. The pH was allowed to stabilise to 7.4 after complete addition of maleic anhydride. The proteins were than dialysed against 10 liters of PBS at 4°C. The concentrations of proteins were estimated using BCA method and maleyl proteins were filter-sterilised before use.
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MALEYLATION OF PEPTIDES

Peptides were dissolved in PBS [5 mg/ml] and maleylated following the protocol mentioned above. After maleylation, peptides were passed through Sephadex G-10 [Pharmacia LKB, Sweden] which was swollen in PBS and packed in a 5 ml syringe. This step was carried out to remove excess maleic anhydride. Peptide concentrations were quantitated by BCA method. The maleyl peptides were filter-sterilised before use.

VALIDATION OF MALEYLATION USING TRINITROBENEZENE SULFONIC ACID [TNBS] METHOD

2,4,6-trinitrobenzenesulfonic acid reacts with free amino groups under mild basic conditions to form trinitrophenyl [TNP] derivatives. To 1 ml of solution containing 1 mg of protein or peptide, 1 ml of 4% sodium bicarbonate [Qualigens, India] [pH 8.5] and 1 ml of 0.1% TNBS [Pierce, USA] solution was added and the reaction was allowed to proceed at 40°C for 2 hours. After this time, 1 ml of 10% SDS [Sigma, USA] was added to solubilise the protein and its precipitation was further prevented by addition of 0.5 ml 1N HCl. The absorbance was read at 335 nm in a UV-spectrophotometer [Varian 100-S, Varian Techtron, Australia] against a blank containing 1 ml of distilled water and treated similar to the protein solutions. The difference between native and maleylated protein indicated the extent of blockade of free amino groups by maleyl residues, which was more than 90% in all cases.
BIOTIN LABELING OF PEPTIDES

Peptides [SIINFEKL and SIINFEEL] were labeled with biotin [NHS-biotin, Pierce, USA]. 1 mg of biotin was dissolved in 1 ml of water. 2 mg of peptides were dissolved in 0.1M NaHCO₃ [Qualigens, India]. 60 μl of biotin solution was added for each ml of peptide solution and mixed properly. The solution was allowed to stand at room temperature for 4 hours. Peptide solution was passed through G-10 sephadex [Pharmacia, Sweden] column to remove unlabeled biotin molecules.

To check for presence of biotin molecules in the peptides, 20 μg of native or labeled peptides were spotted on nitrocellulose membrane [Hibond NC; Amersham, UK] and air dried for 1 hour. Nitrocellulose membrane was then blocked with 2% BSA [HiMedia, India] made in PBS, for 1 hour. Membrane was then washed three times with PBS containing 0.05% Tween-20 [CDH, India], followed by incubation with avidin-HRP conjugate [Vector, USA] for 1 hour. Membrane was washed again three time with PBS containing 0.05% Tween-20. Colour was developed using 3,3',5,5'-tetramethyl benzidine substrate according to the instructions of the kit [TMB substrate systems, Vector, USA].

DEMALEYLATION OF PROTEINS

Demaleylation involves the regeneration of amino groups that have been modified by maleylation. The pH of maleylated protein was brought down to 3.5 with 30% glacial acetic acid [Qualigens, India]. 5% chloroform [Qualigens, India] was added to prevent contamination and the solution was kept at 37°C in a water bath for 96 hours. The precipitate was resolubilised in 1 M NaOH.
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The demaleylated protein was dialysed against buffer containing 0.01 M sodium phosphate [Qualigens, India], 0.15 M sodium chloride [Qualigens, India] and 0.01% EDTA [Qualigens, India]. The available free amino groups were estimated using TNBS method. Extent of demaleylation was about 65-70%.

HARVESTING OF PERITONEAL EXUDATE CELLS AND PURIFICATION OF MACROPHAGES

TAP-deficient or normal mice were primed with 1 ml of 4% Brewer’s thioglycollate broth [HiMedia, India] intraperitoneally for 72 hours. Peritoneal lavage was performed by injecting 10 ml of chilled PBS into the peritoneum. The fluid containing cells was drained by gravity from the peritoneal cavity using an 18-gauge needle [Becton & Dickinson, USA] held over a sterile tube. Cells were washed twice with sterile culture medium and counted using a haemocytometer [Neubauer, Germany]. $30 \times 10^6$ cells in 5 ml of medium were plated on 60 mm petri plates [Nunclon, Denmark] and incubated at $37^\circ$C for 30 minutes. Non-adherent cells were removed and plates were washed twice with medium. Adherent macrophages were removed by flushing the plates with 5 ml of medium.

CELL PREPARATIONS FROM SPLEEN AND THYMUS

Mice were killed by cervical dislocation and spleen and thymus were dissected aseptically and placed in a sterile petri plate containing 5 ml culture medium. Single cell suspensions from spleen were prepared by rubbing spleen or thymus gently between two sterile frosted glass slides [Blue Star, India]. Cell suspensions from thymus were used immediately after two
washes in sterile medium. Cell suspensions from spleen were cleared of red blood cells [RBCs] by hypotonic shock using distilled water [9 ml] followed by immediate equilibration with 10X PBS [1 ml]. The cells were washed twice with medium and counted before use.

MITOGENIC ACTIVATION OF SPLEEN CELLS

Splenic cells were incubated with 20 μg/ml of bacterial lipopolysaccharide [LPS from Salmonella typhosa, Difco, USA] or 1 μg/ml of Concanavalin A [Con A] [Boehringer Mannheim, Germany] in culture medium containing 10% FCS for 48 hours. Cells were then layered on a density cushion [Lympholyte M, density= 1.071, Sigma, USA] and centrifuged at 1800 rpm for 20 minutes. Live cells, which formed a layer at the interface, were collected and washed twice with medium before using in T cell stimulation assays.

PURIFICATION OF T AND B CELLS FROM SPLEENS

Spleen cell preparations from C57BL/6 mice were prepared as described above. Macrophages were removed by plastic adherence at 37°C for 30 minutes followed by panning on anti-mouse-Ig coated plates at 37°C for 1 hour to deplete B cells. Non-adherent cells were T-enriched population. B cells were removed from anti-mouse Ig coated plates by flushing with medium.

LOADING OF ANTIGENS IN THE CYTOSOL OF APC BY OSMOTIC SHOCK

1-3x10^6 APCs were incubated with 0.5 ml of prewarmed hypertonic solution containing 0.5 M sucrose [Sigma, USA], 10% PEG 800 [Boehringer Mannheim,
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Germany], 10 mM HEPES [Sigma, USA] and 10 mg/ml of antigen in FCS-free DMEM, at 37°C for 10 minutes. This leads to the formation of pinosomes in the cells, through which the antigen is taken inside the cell. This was followed by the addition of 10 ml of isotonic solution of FCS-free DMEM and incubation at 37°C for 3-4 minutes. This swells and ruptures the pinosomes releasing the antigen in the cytosol of the cell. Cells were washed three times before using them as stimulators in T cell stimulation assay.

ANTIGEN-SPECIFIC T CELL STIMULATION ASSAYS

1X10^5 per well of OA-specific T cell line [B3.4.5] was stimulated with varying concentrations of antigens [peptides, maleylated peptides, OA, maleyl-OA] in the presence of macrophages purified from peritoneal exudate cells extracted from either C57BL/6 or TAP-deficient mice. For stimulation with endogenously available antigen, APCs were loaded with antigens cytosolically, as described above. Varying numbers of these loaded cells were used as stimulators for the T cell lines.

100 μl of culture supernatant was harvested from each well after 20 hours and subjected to freeze-thaw procedure to kill any live cell present. Amount of IL-2 secreted by the T cell line after stimulation was measured in an IL-2 bioassay. All the assays were done in triplicates and mean ± standard error was reported.

DRUG INHIBITION ASSAYS

Peritoneal macrophages were either not treated or pretreated with different concentrations of ammonium chloride [Qualigens, India] or 5 μg/ml of brefeldin A [Sigma, USA] for 30 minutes. After this, 3 μM of peptide, or 1
mg/ml of OA or maleyl-OA was added to the cells, or the cells were pelleted and loaded with OA by osmotic lysis of pinosomes, as described earlier. Cells were allowed process the antigens in the presence of absence of ammonium chloride or brefeldin A for 4 hours. Cells were washed three times with FCS-free medium and fixed with 0.01% paraformaldehyde [Sigma, USA] for 20 minutes. Cells were washed three times with FCS-containing medium. Varying numbers of these cells were used as stimulators for the T cell line, B3.4.5. Activation of the T cell line was measured by assaying IL-2 production.

IL-2 BIOASSAY

IL-2 dependent cell line, CTLL-2 was used to measure IL-2 in the culture supernatants harvested from the T cell stimulation assay. CTLL-2 was maintained on a two day cycle of medium change containing 5 Units/ml of human recombinant IL-2 [Genzyme, USA]. To 100 μl of supernatant, CTLL-2 cells were added at 1X10⁴ per well. The plates were pulsed with 0.5μCi of ³H-thymidine [Amersham, UK] per well after 24 hours. 12-16 hours later, the cells were harvested onto glass-fiber mats [Pharmacia, Sweden] and counted by liquid scintillation spectroscopy [Betaplate, LKB-Pharmacia, Sweden].

MHC CLASS I-STABILISATION ASSAY

To test whether different modified peptides bind to MHC class I [H-2b], TAP-1 and TAP-2 deficient cell line RMA-S was used. These cells do not express stable MHC class I on their surface. However, if synthetic peptide, given from outside, binds to MHC class I there is accumulation of stable MHC-peptide complex on the surface of RMA-S. 1X10⁵ RMA-S cells were incubated with varying concentrations of different peptides in a 96-well round bottom plate
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[Nunclon, Denmark] at 37°C for 18-20 hours. Cells were then incubated with anti-MHC class I antibody [Y3] and incubated on ice for 60 minutes. Control samples were incubated with washing buffer made of 1X PBS containing 0.5% BSA [HiMedia, India] and 0.1% sodium azide [Loba Chem, India]. This was followed by 3 washes using the washing buffer. Cells were then incubated with anti-mouse Ig b-galactosidase conjugate [1:1000 dilution, Southern Biotechnologies, USA] for 60 minutes on ice. Cells were washed three to four times with washing buffer. Colour was developed using 1 mg/ml chlorophenolred β-D-galactopyranoside [CPRG, Boehringer Mannheim, Germany] dissolved in substrate buffer containing 100 mM HEPES [Gibco, USA], 150 mM NaCl [Qualigens, India], 2 mM MgCl₂ [Qualigens, India], 0.1% sodium azide [Loba Chemie, India] and 1% BSA [HiMedia, India]. Colour was allowed to develop for 10-30 minutes and 100 µl of supernatant was transferred to another 96-well plate [Nunclon, Denmark] and absorbance measured at 570 nm in a spectrophotometer [Bio-Tek™ Instruments, USA].

IMMUNISATIONS AND FUSION FOR B CELLS

C57BL/6 mice were immunised with 500 µg of purified immunoglobulin in PBS, which shares VDJ region with transgenic Ig heavy chain, intraperitoneally. Fifteen days later these animals were given a booster of 500 µg of purified antibody in PBS. One week later, mice were sacrificed and spleen cells harvested. Spleen cells were mixed with B cell fusion partner, Sp2/0 in a 1:1 ratio in the presence of polyethylene glycol [PEG 1500, Boehringer Mannheim, Germany] [Yokoyama, W. M. , Current Protocols in Immunology, Greene Publishing Associates and Wiley-Interscience. Volume 1, Chapter 2.5]. Briefly, 1 ml of PEG was added drop by drop to the cell mixture
over a period of 1 minute followed by gradual addition of FCS-free DMEM to make total volume 10 ml. Fused cell mixture was centrifuged and resuspended in DMEM containing 20% FCS and HAT [Boehringer Mannheim, Germany] at a density of 1X10^6 per ml. Cells were plated at 1X10^5 per well in 96-well flat bottom plates [Corning, UK]. Plates were incubated at 37°C in 7.5% CO_2. Culture supernatants from growing wells were collected and checked for anti-transgene antibody by staining a transgenic B cell hybridoma [3F1] that expresses the transgenic protein on the surface followed by flowcytometric analysis. Specific B cell hybridomas were weaned gradually on DMEM containing 20% FCS and HT [Boehringer Mannheim, Germany], DMEM with 20% FCS and finally on DMEM containing 10% FCS. Culture supernatants from the B cell hybrids found positive on the B cell line, 3F1, were used to stain spleen cells from the transgenic animals. Positive cells were subcloned repeatedly to obtain stable B cell hybridomas.

**IMMUNISATIONS AND FUSION FOR T CELLS**

C57BL/6 mice were immunised intraperitoneally with 5X10^7 γ-irradiated [10,000 rads] 3F1 cells. Seven to ten days later, mice were sacrificed and spleen cells were harvested. These cells were mixed with T cell fusion partner, BWCD8.7, in 1:1 ratio in presence of PEG 1500 [Kruisbeek, A. M., Current Protocols in Immunology, Greene Publishing Associates and Wiley-Interscience. Volume 1, Chapter 3.14]. Fusion was carried out as described above. Fused cells were plated at 1X10^5 per well in selection medium containing 20% FCS and HAT. Growing cells were screened for reactivity against the transgenic cells, using T cell stimulation assay described above. T cell hybrids which showed responses against the transgenic cells were
weaned into culture medium with 10% FCS and subcloned to get stable hybrids.

FLOW CYTOMETERIC ANALYSIS

B cell lines or spleen cells [1x10^6 per well] were taken in a 96-well round bottom polystyrene plates [Tarsons, India] and incubated with monoclonal antibody culture supernatants for 60 minutes on ice. Control samples were incubated with washing buffer [1XPBS containing 0.5% BSA and 0.1% sodium azide]. The cells were then washed thrice with washing buffer and incubated with fluorescein labelled appropriate secondary reagent for 60 minutes on ice. After washing thrice with washing buffer, cells were analysed on either EPICS 751 [Coulter, USA] or BRYTE [Biorad, UK]. Flow cytometric data was analysed using appropriate software.

STAINING FOR TOTAL IgM

Transgenic spleen cells or peripheral blood lymphocytes were stained for total IgM by making both the surface and intracellular IgM available for staining with appropriate antibody. For this, 100 µl of 1% paraformaldehyde [Loba Chemie, India] was added to the cells and incubated on ice for 30 minutes. Cells were washed thrice with 1X PBS and 50 µl of 0.2% Triton X-100 [Sigma, USA] was added and incubated on ice for 10 minutes. Flourescein labelled antibody dilution was made in washing buffer containing 0.2% Triton X-100. After incubation for 60 minutes on ice, cells were washed with washing buffer three times before analysis on the flow cytometer.