MATERIALS AND METHODS
MICE

The mouse strains used for all experimental work were BALB/c [H-2\textsuperscript{d}] and C3H/HeJ [H-2\textsuperscript{k}], obtained from Jackson Laboratories [Bar Harbor, USA] and bred in the Small Animal Facility of the National Institute of Immunology, New Delhi, India by Dr. R. K. Anand and provided by him on request.

CHEMICALS

Culture Medium

Dulbecco's Minimum Essential Medium [DMEM, Gibco, USA] or Click's [Irvine Scientific, USA] medium fortified with L-glutamine [2 mM final concentration] containing $5 \times 10^{-5}$ M \( \beta \)-mercaptoethanol [Sigma, USA], streptomycin [100 \( \mu \)g/ml] and 10% fetal calf serum [FCS, Hyclone, USA] was used for all \textit{in vitro} T-cell assays and maintenance of cultures.

Phosphate-buffered saline [PBS]

PBS contains NaCl-8 g, KCl-200 mg, KH\(_2\)PO\(_4\)-200 mg, Na\(_2\)HPO\(_4\)-7H\(_2\)O-750 mg per liter triple-distilled water at pH 7.2.

Proteins

The foreign and self proteins used for immunizations and as antigens for \textit{in vitro} T-cell proliferation assays were - bovine serum albumin [BSA, Fraction V, Sigma, USA], diphtheria toxoid [DT, National Serum Institute, Pune, India], tetanus toxoid [TT, National Serum Institute,
Materials and Methods

Pune, India], ovalbumin [OVA, Sigma, USA], mouse serum albumin [MSA, Sigma, USA] and mouse hemoglobin [MHb, Sigma, USA]. These proteins were treated with maleic anhydride to obtain maleylated antigens. The non-protein scavenger receptor ligands and non-ligands used for targeting experiments as well as immunizations were poly- guanylic acid [poly-G, Sigma, USA], poly-cytidylic acid [poly-C, Sigma, USA], fucoidin [Sigma, USA] and fetuin [Sigma, USA].

Adjuvant

The adjuvant used for immunizations was Alhydrogel ['alum'; Superfos Biosector a/s, Denmark], containing 2% Al$_2$O$_3$ and 3% Al[OH]$_3$.

COLLECTION OF SERUM

Mice were bled from the retro-orbital venous plexus, under inhalation [diethyl ether] anesthesia and the collected blood was allowed to clot at room temperature for 20 min. The clotted blood was then kept at 4°C for about half an hour and the tubes were spun at 14000 rpm to pellet the clots and separated sera were collected.

PROTEIN ESTIMATION

The concentration of protein in solution was measured by the micro- bicinchoninic acid [BCA] method which detects protein levels of 10-2000 μg/ml in extremely small sample sizes. 50 parts of BCA reagent ‘A’ was mixed with 1 part of reagent ‘B’ to give reagent ‘C’. The BCA reagent ‘A’ was prepared by dissolving BCA [4,4’-dicarboxy-2,2’ biquinolone, \( \text{C}_{20}\text{H}_{12}\text{N}_2\text{O}_4 \)] [Sigma, USA] -0.5 g, Na$_2$CO$_3$ -1 g, sodium tartarate -0.08 g,
Materials and Methods

NaOH -0.2 g, NaHCO₃ -0.475 g [Merck, India] in 50 ml distilled water, at pH 11.25. Reagent ‘B’ was a 4% solution of CuSO₄·5H₂O [Qualigens, India]. To 20 μl of protein sample, 200 μl of BCA reagent ‘C’ was added in a 96-well, non-sterile polystyrene microtiter plate [Tarsons, India]. The plate was incubated at 37°C for 30 minutes.

The absorbance was read at 570 nm in an ELISA reader [Bio-tek™ Instruments, USA]. The assay was blanked on PBS which was used instead of the protein solution. A standard curve of BSA [Sigma, USA] was used for quantitating the amount of protein.

MALEYLATION OF PROTEINS

The proteins [2-3 mg/ml] to be maleylated were dissolved in PBS [pH-7.2]. Prior to maleylation the pH was increased to pH 8. Finely powdered maleic anhydride [C₄H₂O₃] [Sigma, USA] was used at a concentration 2.5 fold higher than the protein concentration. The reaction was carried out at mild alkaline pH [8.5-9.0] with constant stirring. The pH was maintained using 1 N NaOH. After complete addition of the maleic anhydride, the pH was allowed to stabilize to pH 7.4. The proteins were dialyzed against 10 liters of PBS. They were filter-sterilized and the protein concentration was ascertained by the BCA [Sigma, USA] method. Maleylation acylates the α- and ε- amino groups in proteins [Butler and Hartley, 1972].
VALIDATION OF MALEYLATION USING TRINITROBENZENESULFONIC ACID [TNBS] TO DETERMINE FREE ε-AMINO GROUPS

2,4,6-trinitrobenzenesulfonic acid is a reagent that aids in the estimation of free amino groups. It reacts with free amino groups under mild conditions to give rise to trinitrophenyl [TNP] derivatives. To 1 ml of protein solution containing 0.6-1 mg/ml protein, 1 ml of 4% NaHCO₃ [pH 8.5] and 1 ml 0.1% TNBS was added. The reaction was allowed to proceed at 40°C for 2 hours, following which 1 ml of 10% SDS was added to solubilize the protein and its precipitation was prevented by the addition of 0.5 ml 1 N HCl. The absorbance was read at 335 nm in a UV-spectrophotometer [Varian 100-S, Varian Techtron, Australia] against a blank treated exactly as above but containing 1 ml water instead of the protein solution. The difference in absorbance values between the native and maleylated proteins indicated the extent of maleylation, which was complete to more than 90% in all cases.

DEMALEYLATION OF PROTEINS

Demaleylation involves the regeneration of amino groups that have been modified by maleylation. The maleylated protein was treated with 30% glacial acetic acid [CH₃COOH] [Merck, India] in a glass tube till the pH was reduced to 3.5. 5% chloroform [CHCl₃] [Merck, India] was added to prevent contamination and the solution was kept at 37°C in a water-bath for 96 hours. The precipitate was resolubilized in 1 M NaOH. The demaleylated protein was then dialyzed in a buffer containing 0.01 M sodium phosphate [Qualigens, India], 0.15 M NaCl [Qualigens, India] and
0.01% EDTA [Qualigens, India] [pH 7.4] and the available amino groups were estimated by the TNBS method.

PURIFICATION OF MSA FROM NORMAL MOUSE SERUM

Normal mouse serum from BALB/c mice was subjected to precipitation with a final concentration of 33% \([\text{NH}_4\]_2\text{SO}_4\) on ice. On complete dissolution of the \([\text{NH}_4\]_2\text{SO}_4\), the serum was centrifuged at 12000 rpm for 30 min. The supernatant was collected and subjected to further precipitation with a final concentration of 50% \([\text{NH}_4\]_2\text{SO}_4\) on ice. The solution was again centrifuged at 12000 rpm for 30 min. The pellet thus obtained was dissolved in phosphate-buffered saline [PBS] and treated as the albumin-enriched fraction. The protein concentration was determined using the BCA method [Sigma, USA].

IODINATION OF MALEYL-BSA

Radiolabeling of maleyl-BSA for targeting studies was done with radioactive sodium iodide [Na\(_{125}\)I, 10mCi, Amersham, UK]. Two mg of protein [maleyl-BSA] was taken in 250 μl of PBS in a tube along with 250 μl of glycine-NaOH [1 M, pH 10]. In a second tube, 1.25 ml of 2 N NaCl was taken. The entire reaction was carried out on ice. To the second tube, 100 μl of a 33 mM solution of iodine monochloride [ICl] was added. [ICl, prepared by mixing 150 mg sodium iodide in 8 ml of 6 N HCl, and 99 mg sodium iodate (anhydrous) dissolved in 2 ml of water. The iodate solution was injected forcibly into the iodide-HCl solution to prevent precipitation of iodine. The mixture was diluted with water to 40 ml and shaken in a glass container with 5 ml of CCl\(_4\). Residual CCl\(_4\) was removed by reacting the aqueous phase with moist air for 1 h, after which
the volume was adjusted to 45 ml. The solution of NaCl and ICl was mixed thoroughly and 125 µl was taken and added to the first tube containing the protein. The mixture was incubated on ice for 10 minutes. A prewashed Sephadex G-25M column [Pharmacia, Sweden] was loaded with the 'hot' mixture and 1 ml of PBS [pH 7.4]. The entire mixture was eluted with the help of PBS and ten fractions were collected in tubes; 2 µl of solution was taken from each fraction and counted in a gamma-counter [Minigamma, LKB-Pharmacia, Sweden].

The radiolabeled protein was present in the second and third fractions while the free iodine was removed in the last two fractions. The protein-containing fractions were pooled and dialyzed against PBS at 4°C for 24 h with changes every six hours. The protein concentration was estimated by the BCA method. The radiolabeled protein was used in assays after the specific activity had been estimated.

DETERMINATION OF SPECIFIC ACTIVITY OF \(^{125}\)I-maleyl-BSA

The \(^{125}\)I-mBSA was diluted 1:100 in PBS containing 1 mg/ml BSA. From this solution, two aliquots of 10 µl were taken and mixed with 990 µl of PBS-BSA. To one aliquot, 250 µl of 50% trichloroacetic acid [TCA] [SRL, India] was added and kept overnight at 4°C. The tube was spun at 12000 rpm for 15 minutes and the supernatant and pellet were carefully separated and the supernatant was counted in a gamma counter. The second aliquot was counted directly, which gave the total counts present. The counts in the TCA-soluble supernatant was subtracted from the total counts giving the counts associated with the pellet. The specific activity was calculated by dividing the total counts with the protein concentration of the \(^{125}\)I-mBSA [in nanograms] and was expressed as cpm/µg.
Materials and Methods

HARVESTING OF PERITONEAL EXUDATE CELLS [PECs]

BALB/c mice were primed with 4% Brewer’s thioglycollate broth [HiMedia, India] intraperitoneally for 48 hours. Peritoneal lavage was performed by injecting 5-10 ml of chilled PBS into the peritoneum and massaging the abdomen. The fluid was drained by gravity from the peritoneal cavity using an 18-gauge needle [Becton & Dickinson, USA] held over a sterile tube. The cells were spun and washed twice with sterile culture medium before they were counted using a hemocytometer [Neubauer, Germany].

CELL FRACTIONATION

Peritoneal exudate cells [PECs], spleen and thymus cell populations were separated into their component cell types by fractionation. The cells were counted and plated onto sterile, plastic petri-dishes [Corning, USA] in complete culture medium. They were allowed to adhere to the plastic for an hour at 37°C. The non-adherent cells [macrophage-depleted population] were removed by gently swirling and washing the plate with medium. The adherent cells were removed by adding culture medium to the plate and incubating it on ice for 1 h. The cells were vigorously flushed off the plate and collected as the macrophage-enriched fraction.

TARGETING OF MALEYLATED PROTEINS TO SCAVENGER RECEPTORS

The targeting of maleyl-proteins to the macrophage-specific scavenger receptors was assayed by using them to compete with 125I-maleyl-BSA [maleylated bovine serum albumin], a known ligand for the scavenger
receptors. The binding assay estimates the ability of various maleylated proteins to compete with $^{125}\text{I}$-maleyl-BSA for binding to the scavenger receptors while the degradation assay estimates the amount of iodo-tyrosines liberated as a result of proteolytic degradation of maleyl-BSA. Maleyl-BSA is endocytosed through the scavenger receptor and enters the lysosomes where it undergoes proteolytic degradation into short peptides and single amino acids. Among these amino acids are radiolabeled tyrosines, called mono- and di- iodo-tyrosines. These iodo-tyrosines are thrown out into the extracellular tissue culture medium and they serve as a measure of scavenger receptor targeting.

PECs were obtained by peritoneal lavage on mice and the plastic-adherent macrophage-enriched population was isolated as above. Spleen and thymus cell suspensions were also plated onto plastic dishes and the non-adherent fractions were collected as the macrophage-depleted lymphoid populations. Cells were plated [$5 \times 10^5$] in 24-well plates in DMEM containing 2 mg/ml BSA. Radiolabeled maleyl-BSA [$^{125}\text{I}$-maleyl-BSA] [6 µg/ml] was added either by itself or along with 10 µg each of unlabeled competitors or non-competitors [as negative controls], and the cells incubated for 5 hours at $37^\circ\text{C}$. The plates were spun at 1500 rpm for 10 minutes, 500 µl of the culture supernatant was mixed with 200 µl of 50% TCA [SRL, India] and allowed to precipitate overnight at $4^\circ\text{C}$. The tubes were then spun at 12000 rpm and the TCA-soluble supernatant was collected. To 400 µl of the TCA-soluble supernatant, 7 µl of 40% freshly prepared potassium iodide [Merck, India] and 15 µl of $\text{H}_2\text{O}_2$ [Merck, India] were added. After mixing, it was incubated for 10 minutes, followed by the addition of 1 ml of CHCl$_3$ [Merck, India]. The solution was allowed to stand for a further 10 minutes after vigorous mixing. 100 µl of supernatant was taken from the colorless top layer. The amount of
radioactivity was determined with a gamma-counter [Minigamma, LKB-Pharmacia, Sweden]. The cells were washed and the amount of cellular protein in each well was quantitated by lysing the cells with 0.1 N NaOH and estimating the protein content in the supernatant by the BCA method [Sigma, USA]. The degradation of $^{125}$I-maleyl-BSA is expressed as ng $^{125}$I-maleyl-BSA degraded per mg cellular protein.

For the binding assay, PECs were obtained in the same manner. The plastic-adherent or macrophage-enriched population was plated [5 X10$^5$ cells/well] in a 6-well plate and incubated with $^{125}$I-maleyl-BSA [6 µg/ml] in PBS containing 5 mg/ml BSA either by itself, or with 10 µg/well each of unlabeled competitors or non-competitors [as negative controls] for two hours. The cells were washed thrice with PBS containing 5 mg/ml of BSA followed by plain PBS. They were then lysed with 0.1 N NaOH, and 500 µl of the supernatant was collected and the cell-bound radioactivity counted [Minigamma, LKB-Pharmacia, Sweden]. The protein concentrations of the cell lysates were determined by the BCA method [Sigma, USA] and the cell-bound radioactivity was expressed as ng bound protein per mg of cellular protein.

**ADSORPTION OF ANTIGENS ON ADJUVANT-ALUM**

Antigen solutions of appropriate protein concentrations were made in PBS. They were diluted in a 1:1 [vol/vol] ratio with sterile alum [Alhydrogel, Denmark]. After thorough mixing, they were adsorbed overnight at 4°C on a rocking shaker.
IMMUNIZATIONS

BALB/c mice were immunized with native or maleylated antigen intraperitoneally [i.p] either 300 µg on alum as a single dose, or three weekly doses of 100 µg each of antigen in PBS. The B cell responses were assayed either a fortnight after the alum-based immunization or a week after the last soluble dose of antigen. The T cell responses were normally assayed during a period of 2-5 weeks after the last immunizing dose.

For the 'cross-priming' experiments, the first immunizing dose was either three weekly doses of 100µg each of maleylated antigen on alum followed by 100 µg of soluble or alum-adsorbed native antigen, or three weekly doses of 100 µg each of maleyl-antigen in PBS followed by 100 µg of soluble or alum-adsorbed native antigen given intraperitoneally. The B cell responses were assayed before and after the native antigen immunization.

Immunizations were also done intraperitoneally with three weekly doses of either TT, maleyl-TT, MSA or maleyl-MSA in PBS, with or without 1 mg of poly-guanylic acid [poly-G] or poly-cytidylic acid [poly-C] in each dose.

All immunizations were done in groups with five animals each.

ANTIGEN-SPECIFIC ANTIBODY ENZYME-IMMUNOSORBENT ASSAYS [EIAs]

PVC microtiter plates [Falcon, USA] were coated with 10 µg/ml of antigen in coating buffer [0.2 M Na₂CO₃, 0.2 M NaHCO₃, pH 9.6] overnight
at 4°C. The plates were washed with PBS containing 0.05% Tween-20 and blocked with 1% milk powder [Lactogen, Nestle, India] in PBS at 37°C for 1 h. Serum dilutions were made in PBS-Tween buffer containing 0.25% milk powder. Three-fold titrations of the serum samples were done in the plate and incubated for 1 h at 37°C. The specific antibodies in the immune sera were detected with a goat anti-mouse Ig-HRP conjugate [NII, New Delhi, India] followed by the addition of the substrate, o-phenylenediamine tetrahydrochloride [0.5 mg/ml] [Sigma, USA] in substrate buffer [0.1 M citric acid, 0.2 M Na₂HPO₄, 0.1% H₂O₂]. The reaction was stopped using 1 N H₂SO₄. The color was developed for 10-60 minutes at room temperature and the absorbance was read at 490 nm in an ELISA reader [Bio-tek™ Instruments, USA]. The concentrations of specific antibodies were calculated on the basis of affinity-purified mouse Ig standard curves run in parallel assays.

INHIBITION EIAs

Inhibition EIA assays were done in a similar manner with the exception that prior to addition in the antigen-coated plate, graded doses of soluble antigen were mixed with a fixed dilution of the serum sample that yielded half-maximal absorbance in prior experiments. The specific inhibition obtained was expressed as a percent value.

ISOTYPE-SPECIFIC EIAs

EIAs were used to determine the amount of both IgM and IgG antibodies in the immune sera. The relative amounts of IgG1 and IgG2a were also estimated using similar EIAs. IgM levels were determined by using either a monoclonal anti-mouse IgM-biotin [Bet-2, HB88, ATCC] or a polyclonal
rabbit anti-mouse IgM-biotin [Jackson, USA] followed by a HRP-Avidin conjugate [Vector, USA]. The IgG levels were determined with the help of a goat anti-mouse IgG-β-galactosidase conjugate [Fisher Scientific, USA], the enzyme activity of which was revealed by the substrate, CPRG [chlorophenol red β-D-galactopyranoside, Boehringer Mannheim, Germany; 3.29 mM] in the substrate buffer [HEPES-100 mM, NaCl-150 mM, MgCl₂-2 mM, NaN₃-0.1% and BSA-1%, pH-7.0]. The color developed for 10-60 minutes was read at 570 nm in an ELISA-reader.

The antigen-specific IgG1 and IgG2a levels were assayed using goat anti-mouse isotype-specific antibodies for IgG1 and IgG2a [Sigma, USA] followed by rabbit anti-goat Ig-HRP conjugate [NII, New Delhi, India] and o-phenylenediamine tetrahydrochloride in citrate phosphate buffer with H₂O₂ as the substrate. The absorbance was read at 490 nm.

**PREPARATION OF SINGLE CELL SUSPENSIONS FROM SPLEENS**

Mice were killed by cervical dislocation and spleens were dissected aseptically and placed in sterile petri-dishes containing 5 ml complete medium [DMEM or Click’s]. Single cell suspensions of the spleens were prepared by rubbing the spleens gently between two sterile frosted glass slides [Blue Star, India]. The suspension was centrifuged at 1200 rpm for 5 min. The red blood cells [RBCs] were removed from the preparation by a hypotonic shock using distilled water [9 ml] followed by immediate equilibration with 10 X PBS [1 ml]. The cells were washed twice with medium and resuspended in 10 ml of complete medium. The lymphocytes were counted in the WBC counting chamber of a hemocytometer [Neubauer, Germany] and then used in the assay.
ANTIGEN-SPECIFIC T-CELL PROLIFERATION ASSAYS

T-cell stimulation assays were done with mice immunized with either native or maleylated antigen in the presence or absence of adjuvant. Splenic preparations from immune mice were plated into 96-well flat-bottomed microtiter plates [Corning, USA] containing graded doses of antigens [native or maleylated or peptide], at 3-5 × 10^5 cells per well in complete medium in triplicate cultures. The control wells lacked any antigen. The responder T cells were allowed to proliferate and after 96 hours, the plates were pulsed with 0.5 μCi of ^3H-thymidine [Amersham, UK] for 12-16 hours. The cells were harvested onto glass-fiber filter mats and counted by liquid scintillation spectroscopy [Betaplate, LKB-Pharmacia, Sweden].

STATISTICAL ANALYSIS

Statistical analysis was done using the Student's t-test.