2.1 Materials:

2.1.1 Chemicals and Reagents

3, 3'-diaminobenzidine tetrahydrochloride (DAB), 3, 3’, 5, 5’-Tetramethyl benzidine (TMB), phorbol 12-myristate 13-acetate (PMA), propidium iodide (PI), bovine serum albumin (BSA), and proteinase K were procured from Sigma Chemicals Co. (St. Louis, MO). Ficoll-Paque Plus™ reagents were purchased from Amersham Biosciences (Amersham, Piscataway, NJ). Src-kinase inhibitor Genistein, MAPK (MEK-1) inhibitor PD98059, p38 inhibitor SB202190, PI3 kinase inhibitor Ly294002, JNK inhibitor JNKII, NF-κB inhibitors PDTC, Whortmanin were obtained from Calbiochem (Darmstadt, Germany) and/or Cell Signaling Technology Inc. (Danvers, MA). EGFR (tyrosine kinase) inhibitor Tyrphostin AG1478, and Farnesyl Thiosalicylic acid (FTS) was procured from Caymen Chemicals Inc (USA). RPMI-1640, DMEM and fetal calf serum (FCS) were obtained from Biological Industries (Kibbutz, Israel). BMEM was obtained from Lonza Inc. (USA). Mouse TNF, Mouse IL-6, Human TNF-α, Human IL-6, Human IL-10, Human IL-12p40, Human IL-12p70, Human IL-1β detection kits were obtained from BD Biosciences (San Jose, CA) and eBiosciences Ltd. (San Diego, CA). Micro BCA™ (bicinchoninic acid) protein assay kit was procured from Pierce (Rockford, IL). Nitrocellulose membranes for immuno-blotting were obtained from Advanced Microdevices Pvt. Ltd. (Ambala, India). Collagen and Fibronectin were procured from Sigma Chemical Co. (USA). The reagents used in electrophoresis and immuno-blotting were purchased from Sigma Chemical Co. (St. Louis, MO). Chemicals used in the preparation of buffers and other solutions were of analytical grade, and unless otherwise stated were obtained from E. Merck Ltd. (Mumbai, India).

2.1.2 Cell lines

The human leukemic T cell line Jurkat, human monocytic cell line THP-1 were all obtained from American Type Culture Collection (ATCC) (Manassas, VA). The Human Adenocarcinoma Cell Line, A549, was a kind gift from Dr. Devinder Sehgal, National Institute of Immunology, New Delhi, India. Human undifferentiated cell line, ChaGoK-1, Human Colon Adenocarcinoma cell line, Caco-2, Human cervical cancer cell line, HeLa, Human T-cell line, Jurkat and Mouse macrophage cell line, RAW264.7, were kindly
provided by Dr. Ayub Qadri, National Institute of Immunology, New Delhi. Mouse Lung carcinoma cell line, LL/2 (Lewis Lung carcinoma) was a kind gift from Dr. Rahul Pal, National Institute of Immunology. Human lung SV-40 transformed cell line, BEAS-2B was kindly provided by Dr. Balaram Ghosh Laboratory, Institute of Genomics and Integrative Biology, New Delhi, India.

2.1.3 Antibodies

TLR-2 antibody Monoclonal Anti-Human/Mouse CD282 TLR-2 purified Ab (T2.5 clone) was obtained from eBioscience (San Diego, CA). Human anti-CD3 antibody OKT3 were purified from culture supernatants (Hybridoma Laboratory, NII, New Delhi). Human anti-CD28 antibodies were obtained from eBiosciences Ltd. (San Diego, CA).

2.2 Preparation of buffers and other reagents

2.2.1 Phosphate-buffered saline (50mM phosphate, 150mM NaCl, pH 7.4)

<table>
<thead>
<tr>
<th>Na₂HPO₄</th>
<th>40.5mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH₂PO₄·2H₂O</td>
<td>9.49mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>150mM</td>
</tr>
</tbody>
</table>

2.2.2 PBS-Tween: Tween-20 was added to PBS to a final concentration of 0.05%.

2.2.3 Tris-buffered saline (Tris base, NaCl, pH 7.6)

For 10X

C₄H₁₁NO₃ (Trizma Base) 24.2 g

NaCl 80 g

Adjust pH to 7.6 with conc. HCl.
2.2.4 TBS-Tween: Tween 20 was added to TBS to a final concentration of 0.05%.

2.2.5 Protein Extraction buffer (SDS lysis buffer, pH 7.5)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>20mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>1mM</td>
</tr>
<tr>
<td>SDS</td>
<td>2%</td>
</tr>
</tbody>
</table>

2.2.6 Acetic acid-NaCl solution, pH 3.0

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>0.1M</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.15M</td>
</tr>
</tbody>
</table>

2.2.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The solutions were prepared according to the following recipe:

2.2.7.1 Resolving gel (for 10ml) 12.0%

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide 30%, bis-acrylamide 0.8%</td>
<td>4.0ml (3.35ml for 10%)</td>
</tr>
<tr>
<td>Tris HCl buffer (1.5M Trizma base), pH 8.9</td>
<td>2.5ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>3.35ml (4.0 ml for 10%)</td>
</tr>
<tr>
<td>SDS 10%</td>
<td>100µl</td>
</tr>
<tr>
<td>APS 10%</td>
<td>50µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>8µl</td>
</tr>
</tbody>
</table>

2.2.7.2 Stacking gel (for 5 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide 30%, bis-acrylamide 0.8%</td>
<td>0.65ml</td>
</tr>
<tr>
<td>Tris-HCl buffer (1M Trizma base), pH 6.8</td>
<td>0.65ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>3.65ml</td>
</tr>
</tbody>
</table>
Materials and Methods

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS 10%</td>
<td>50μl</td>
</tr>
<tr>
<td>APS 10%</td>
<td>25μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>6μl</td>
</tr>
</tbody>
</table>

SDS-Sodium dodecyl sulphate; APS-Ammonium persulphate; TEMED-N,N,N’N’-Tetramethylethylenediamine.

2.2.7.3 Laemmli sample buffer (non-reducing)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl buffer, pH 6.8</td>
<td>0.16M</td>
</tr>
<tr>
<td>SDS</td>
<td>2.3%</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10%</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

2.2.7.4 Electrode buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>192mM</td>
</tr>
<tr>
<td>Trizma base</td>
<td>25mM</td>
</tr>
<tr>
<td>SDS</td>
<td>3.5mM</td>
</tr>
</tbody>
</table>

2.2.7.5 Staining solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie brilliant blue</td>
<td>0.25%</td>
</tr>
<tr>
<td>Methanol</td>
<td>40%</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>10%</td>
</tr>
</tbody>
</table>

2.2.7.6 Destaining solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>40%</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>10%</td>
</tr>
</tbody>
</table>
2.2.8 Western Blot

The reagents used in Western blotting were as follows:

2.2.8.1 Transfer buffer

Glycine 192mM
Trizma base 25mM
Methanol 20%

2.2.8.2 Ponceau-S (10X)

Ponceau S 26.3mM
Sulphosalicyclic acid 1.18M
Trichloroacetic acid 1.84M

2.2.8.3 Substrate for Western blot

0.05mg 3, 3’-diaminobenzidine was dissolved in 1ml PBS and 1µl of 30% H₂O₂ was added. Substrate was prepared fresh.

2.2.9 Buffers for ELISA:

2.2.9.1 Carbonate buffer, pH 9.5

Na₂CO₃ 32 mM
NaHCO₃ 74 mM

2.2.9.2 Citrate phosphate buffer, pH 5.6

Citric acid 22.1 mM
Na₂HPO₄ 51.4 mM
2.2.10 Substrates for ELISA

2.2.10.1 TMB-TBABH solution

3, 3', 5, 5'-Tetramethyl benzidine (TMB) 41mM
Tetramethylammonium borohydride (TBABH) 8.2mM
N,N-Dimethylacetamide (DMA) 10ml

The solution was stored in an airtight dark glass container at 4°C.

200µl of TMB-TBABH solution and 3µl of 30% hydrogen peroxide (H₂O₂) were added to 8ml citrate phosphate buffer. The substrate was prepared immediately before use.

2.2.10.2 Ortho-phenylene diamine (OPD)

0.5mg OPD was dissolved in 1ml citrate phosphate buffer and 1µl H₂O₂ was added to it. The substrate was prepared fresh before use.

2.3 Methods

2.3.1 Maintenance of cell lines

Cells were maintained in RPMI-1640 (for THP-1, PBMC, Caco-2, ChaGoK-1, RAW264.7) or DMEM (for A549, HeLa, LL/2) or BEBM (along with additives i.e. fibronectin, collagen for BEAS-2B) supplemented with 10% heat inactivated fetal calf serum (RPMI-10) at 37°C in a humidified CO₂ (5%) incubator. Cells were centrifuged at 315 \times g for 5 min, washed twice in serum-free RPMI-1640, resuspended in RPMI-10 and grown in 75cm² tissue culture flasks. The cells were subcultured as per ATCC recommended guidelines.

2.3.2 Co-culture of Carcinoma (tumor) cells and immune cells

Representative Human Lung Carcinoma cells, A549 (Well differentiated Adenocarcinoma) & ChaGoK-1 (Undifferentiated Squamous Cell Carcinoma) were co-cultured with Human Monocytic cell line, THP-1 (in-vitro) and Peripheral Blood Mononuclear Cells, PBMCs (ex-vivo) in standardized ratio of ~1 : 10 for respective cell
types. Similar, ratios were also used for co-cultures of Human Lung Epithelial cells, **BEAS-2B** (Transformed only), Human Colon Adenocarcinoma, **Caco-2** and Human Cervical Adenocarcinoma, **Hela**, if not stated otherwise. Mouse co-culture systems between LL/2 and Raw264.7 or mixed co-cultures between Human tumor cells and Mouse macrophages or vice versa were also set accordingly. Tumor and Jurkat cells were used in ~1: 100 ratio.

### 2.3.3 Culture supernatants

Briefly, tumors cells were plated first and allowed to grow and adhere for about 24 hours. Then, the culture media was taken out, the cells washed and added with monocytes along with fresh culture medium. Culture supernatants were collected at various time points and assayed for various cytokines. Also, conditioned media from tumor cells was collected at various time points, added to monocytes and culture supernatants were assayed for cytokines.

### 2.3.4 Protein estimation

Protein concentrations were determined using the Micro BCA™ (bicinchoninic acid) protein assay kit (Pierce, USA). The assay was performed according to the instructions provided by the manufacturer. The dilutions of the sample were made in PBS and mixed with equal volume of reagent mix (B: C: A :: 24 : 1: 25). The plate was incubated at 37°C for 1 h and absorbance was measured at 540 nm. BSA of known concentration provided with the kit was used as a standard.

### 2.3.4 SDS-PAGE

SDS-PAGE was carried out using the Laemmli buffer system (Laemmli, 1970). The resolving gel was polymerized in a Hoefer or BioRad Protean-3 mini gel apparatus for 30-45 min. The thickness of the gel was 1.5mm. The stacking gel prepared afresh was layered on top of the resolving gel and allowed to polymerize for 15-20 min. Samples to be analyzed were mixed with Laemmli sample buffer and placed in a heating block at 100°C for 5 min before loading into wells. Electrophoresis was carried out at a constant current of 30mA.
2.3.5 Western Blot

Western blot was carried out by the method described by Towbin et al. (1979). The sample to be analyzed separated in a 12% SDS-PAG and transferred to a nitrocellulose (NC) membrane (MDI, India) at a constant current of 300mA for 2 h using a Bio-Rad transfer apparatus (BioRad, USA). The transfer of proteins was ascertained by staining the NC membrane with Ponceau-S (1X). The membrane was blocked for 1 h at room temperature with 1% non-fat milk protein prepared in PBS and subsequently probed with the appropriate primary antibody, followed by HRP-labeled secondary antibody and developed using Enhanced Chemiluminescence reagents.

2.3.6 Human TNF-α, Human IL-6, Human IL-10, Human IL-12p40, Human IL-12p70, Human IL-1β, Human IL-8, Human IFN-γ, Mouse TNF, Mouse IL-6 ELISA

The assay was carried out according to the instructions provided by the manufacturer with slight modifications. Briefly, a 96-well microplate (Maxisorp, Nunc) was coated overnight at 4°C with 50µl capture antibody (diluted 1: 250 in 100mM carbonate buffer, pH 9.5 or as provided in manufacturers-BD Biosciences instructions). The plate was washed 3 times with PBS-Tween (PBST) and blocked with PBS-BSA-1% (200µl/well) for 1 h at 37°C. After washing, samples were added to each well and the plate was incubated for 1 h at 37°C. Subsequently, the plate was washed and incubated with detection reagent mix (detection antibody + avidin-HRP) diluted 1: 250 in PBS-BSA 1%. After 1 h incubation, the plate was washed and the enzyme activity determined by adding freshly prepared substrate solution containing TMB/TBABH/H₂O₂ (75µl/well). The reaction was stopped with 125µl of 2N H₂SO₄ and the absorbance was read at 450nm (or as advised in manufacturer’s instructions).

2.3.7 Generation of Macrophage precursors and T cell blasts from human PBMCs

Blood was collected in heparin-coated vacutainers, by venipuncture from healthy human volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation. Briefly, fresh heparinized blood was diluted with an equal volume of PBS and slowly layered over Ficoll-Paque solution in 15ml poly-propylene tubes. 3ml of Ficoll-Hypaque was used per 10ml of blood/PBS mixture. The tubes were centrifuged at 2000 × g for 30 min at 20°C. The upper layer containing the
plasma and most platelets was removed and the mononuclear cell layer at the plasma/Ficoll-Paque interface was collected in a separate tube (Strober W, 2001). For Macrophage precursors, these cells were plated (24 well or 6 well or 25-cm² flask) for 12 hours. The adhering cells were analysed as macrophage precursors. The non-adherent cell were further analysed for cell blast generation. For T-cell blast generation, these non-adherent cells were washed with RPMI-1640, resuspended in RPMI-10 containing 2μg/ml of anti-CD28 antibody. Cells were then transferred to a 25-cm² flask (or in plates) coated with 10μg/ml of anti-CD3 antibody, and incubated for 48 h at 37°C. Dead cells were removed by Ficoll-Paque density gradient centrifugation and live cells were used to study effect of cell-free extracts.

2.3.8 Wound Healing Assay

Wound Healing Assay is used to study the effects of a variety of experimental conditions on cell migration and proliferation. Briefly, cells were grown in DMEM/RPMI supplemented with 10% FBS. Cells were seeded into 24-well or 6 well tissue culture plates in a density that, after 24 hours of growth, they should reach ~70-80% confluence as a monolayer. Gently and slowly a scratch (wound) was made on the monolayer with a new 1 ml pipette tip across the centre of the well. While scratching across the surface of the well, the long-axial of the tip was kept perpendicular to the bottom of the well. Scratch a straight line in one direction. After scratching, the wells were gently washed twice with medium to remove the detached cells. The well/s was replenished with fresh medium. (Medium may contain ingredients of interest, e.g., components that inhibit/promote cell motility and/or proliferation.) Cells were grown for additional 48 hours (or the time required). Cells were washed twice with 1x PBS, then fixed with 3.7% paraformaldehyde for 30 minutes/ or directly visualised and photographed. Same configurations of the microscope were maintained while taking pictures for different views of the stained monolayer or monolayer of comparing wells.

2.3.9 Propidium Iodide (PI) Staining

Propidium iodide (PI) is a membrane impermeant dye that is generally excluded from viable cells. It binds to double stranded DNA by intercalating between base pairs. PI is excited at 488 nm and, with a relatively large Stokes shift, emits at a maximum wavelength of 617 nm. Because of these spectral characteristics, PI can be used in
combination with other fluorochromes excited at 488 nm such as fluorescein isothiocyanate (FITC) and phycoerythrin (PE). Cells were harvested and aliquoted up to $1 \times 10^6$ cells/100 μL into FACS tubes. Cells washed 2 times by adding 2 mL of PBS, centrifuged at 300 x g for 5 minutes, and then decanted for buffer from the pelleted cells. Cells were re-suspended in 100 μL of Flow Cytometry Staining Buffer. To adjust flow cytometer settings for PI, 5 - 10 μL of PI staining solution was added to a control tube of otherwise unstained cells followed by gentle mixing and incubation for 1 minute in the dark. PI fluorescence (using the FL-2) was determined with a BD FACS caliber™ instrument. Data was acquired for unstained cells and single-color positive controls. 5 - 10 μL of PI staining solution was added to each sample just prior to analysis. The stop count was set on the viable cells from a dot-plot of forward scatter versus PI.

2.3.10 Inhibitor Assay

Tumor cells were treated with various signaling inhibitors for two (2) hours after overnight plating. Cells were washed 3-5 times with serum free culture medium to wash off the inhibitors. Subsequently, tumor cells were co cultured (as shown in section 2.3.2) and assayed for cytokines in the culture supernatants.

2.3.11 Concentration of Inhibitors

The viable inhibitor concentrations were first standardized for each tumor cell type based on their IC$_{50}$ scores. Subsequently, the concentrations used were

MAPK (MEK-1) inhibitor, PD98059  $\quad 200$μM

p38 inhibitor, SB202190  $\quad 50$μM

PI3 kinase inhibitor, Ly294002  $\quad 10$μM

JNK inhibitor, JNKII  $\quad 50$μM

NF-κB inhibitor, PDTC  $\quad 200$μM

Whortmanin  $\quad 10$nM

DMSO was the vehicle (solvent) for inhibitors.
2.3.12 TLR-2 Blocking Assay

Tumor cells were incubated with TLR-2 blocking antibody (Monoclonal Anti-Human/Mouse CD282 TLR-2 purified Ab) along with its Isotype (IgG1) control for two (2) hours at room temperature after overnight plating. Cells were washed 3-5 times with serum free culture medium to wash off any free antibody. Subsequently, tumor cells were co-cultured (as shown in section 2.3.2) and immune-assayed for cytokines in the co-culture supernatants.

2.3.13 Statistical Analysis

Statistical analysis was done using Graph Pad Prism (Ver. 5.0) software. For comparative studies, data were analyzed by One Way ANOVA with multiple comparisons using Dunnett’s test. Data were represented as Mean ± SD and values were considered statistical significant for $p < 0.05$ (CI = 95%) from at least three (or five) independent experiments or otherwise mentioned.