Chapter 3

Materials and methods

3.1 Reagents

All tissue culture materials were obtained from Gibco, Thermo-Scientific, Grand Island, NY. Phospho-Akt (Thr-308), phospho-Akt (Ser-473), Akt , IRS-1, Glut-4 antibodies from Santa Cruz Biotechnology, Santa Cruz, CA. Phospho-IκBα (Ser-32), phospho-CREB (Ser-133), phospho-p44/42 MAPK (Erk1/2) (Thr-202/Tyr-204; D13.14.4E), p44/42 MAPK (Erk1/2) (137F5), phospho-p38 MAPK (Thr-180/Tyr-182; D3F9) and p38 MAPK (D13E1), Phospho-PKC (pan, βII Ser660), Phospho-c-Jun (Ser73) (D47G9) XP® Rabbit mAb, Phospho-c-Fos (Ser32) (D82C12) XP® Rabbit mAb, Phospho-IRF-3 (Ser386) (E7J8G) XP® Rabbit mAb, Phospho-SAPK/JNK (Thr183/Tyr185) (G9) Mouse mAb, Phospho-TAK1 (Ser412) antibody, IRAK4 antibody, IRAK1 (D51G7) Rabbit mAb, IRAK2 antibody, IRAK-M antibodies were procured from Cell Signaling Technology, Danvers, MA. TNF-α Rabbit pAb, IFNγ Rabbit pAb, IL-10 Rabbit pAb and TGFβ-1 Rabbit pAb were purchased from ABclonal,Woburn,MA. Phospho-NF-κBp65 (Ser-281), Anti-pIKKα/β (pSer-176), Phosphotyrosine, Monoclonal Anti-Phosphoserine Clone PSR-45, Horseradish peroxidase-conjugated anti-rabbit and anti mouse secondary antibodies were purchased from Sigma–Aldrich, St. Louis, MO. Anti-GFP, anti-HA, anti-FLAG and anti-MYC antibodies were purchased from BioBharati LifeScience, Kolkata, India. β-actin monoclonal antibody (AC-15), Alexa Fluor 488 conjugated goat anti-rabbit IgG (H + L) secondary antibody, Recombinant Protein G Agarose, Lipofectamine 2000 Transfection Reagent and NP40 lysis buffer from Invitrogen, Thermo-Scientific, Grand Island, NY. Millicell EZ SLIDES was obtained from EMD-Millipore, Darmstadt, Germany. Halt Protease and Phosphatase Inhibitor Cocktail was purchased from Thermo-Scientific, Grand Island, NY. Clarity™ Western enhanced chemiluminescence (ECL) Substrate, Precision Plus Western C Pack, iScript Reverse Transcription Supermix and iTaq™ Universal SYBR® Green Supermix were obtained from Bio-Rad Laboratories, Hercules, CA. Indirubin-3'-monoxime, vanillin, Cytisine, 8-Cyclopentyl-1,3-dipropylxanthine, SCH 58261, Alloxazine, VUF 5574 and Adenosine deaminase, Lipopolysaccharide (LPS), phorbol-12-myristate-13-acetate (PMA), TRI Reagent, were procured from Sigma–Aldrich, St. Louis, MO. MTT was procured from HiMedia, India, Glucose Uptake Cell-Based Assay Kit and Glycerol Cell-Based Assay Kit were procured from Cayman, Ann Arbor, MI. Cyclic AMP XP Assay Kit was purchased from Cell Signaling Technology, Danvers, MA. Legend Max Mouse IL-6 and IL-10 ELISA Kit from BioLegend, San Diego, CA. Steady-Glow

Luciferase Assay System from Promega, Madison WI; RNeasy Lipid Tissue Mini Kit from Qiagen, Hilden, Germany; Vectashield anti-fade mounting medium containing DAPI from Vector Laboratories, Burlingame, CA; κB luciferase plasmid from BioBharati Life Science, Kolkata, India; and EZcountTM LDH Cell Assay Kit, Homogeneous from HiMedia Laboratories, Mumbai, India. pGL2B −1538/+64 and CMV500 A-CREB were a kind gift from Stephen Smale (Addgene plasmid #24942) and Charles Vinson (Addgene plasmid # 33371), respectively. Control siRNA, Adenosine A_{2A}AR siRNA and CGS 21680 Hydrochloride were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. A_{2A}AR cDNA ORF Clone was purchased from Sino Biological Inc., Beijing, China. Lipofectamine 3000 Transfection Reagent and Pierce BCA Protein Assay Kit were purchased from Invitrogen, Thermo-Scientific, Grand Island, New York. Human interleukin 1beta (IL-1β), Taq DNA Polymerase Kit, Super Reverse Transcriptase Kit, pBBL-kBLuc and pBBL-AP1- Luciferase were purchased from BioBharati LifeScience, Kolkata, India, EZ-10 Spin Column Plasmid DNA Minipreps Kit was purchased from BIO BASIC, Human TLR1-9 Agonist Kit was purchased from Invivogen, San Diego, CA, USA, QuikChange Lightning Multi Site-Directed Mutagenesis Kit was purchased from Agilent Technologies, Santa Clara, California, United States. Different gene-specific primers were procured from Integrated DNA Technologies, and Imperial Life Science (P) Limited, India. All other chemicals and reagents used were purchased from Sigma Chemical Co., St. Louis MO, U.S.A.

3.2 Cell culture and treatments

Mouse 3T3-L1 preadipocytes (Cat. No. #SP-L1-F) were procured from the ZenBio, NC, U.S.A. and cultured in Preadipocyte Medium (Cat. No. #PM-1-L1, ZenBio, NC, U.S.A.) supplemented with 1% Penicillin–Streptomycin solution (100 mg/ml) in a humidified 5% CO_2 environment at 37°C. Confluent 3T3-L1 preadipocytes were differentiated using Differentiation Medium (Cat. No. #DM-2-L1, ZenBio, NC, U.S.A). Rat L6 myoblasts were obtained from the National Center for Cell Science (NCCS), Pune, India and cultured in a similar manner as described by us previously [1]. Human THP-1 monocytes were a kind gift from Dr. Rupak Mukhopadhyay's laboratory, Department of Molecular Biology and Biotechnology, Tezpur University cultured in RPMI-1640 Medium (Cat.No.) supplemented with 1% Penicillin–Streptomycin solution

(100 mg/ml) in a humidified 5% CO_2 environment at 37°C. THP-1 monocytic cells were differentiated using PMA treatment at a concentration of 5 ng/ml for 48h in complete RPMI 1640 medium. After 48 h, PMA-containing media was discarded and cells were washed twice with DPBS (Dulbecco's Phosphate Buffered Saline), replenished with complete RPMI 1640 medium and allowed to rest for another 24h. HEK243 cells a kind gift from Dr. Rupak Mukhopadhyay's laboratory, Department of Molecular Biology and Biotechnology, Tezpur University, PANC-1 and MIA PaCa-2 were procured from Dr. K.B Harikumar, Rajiv Gandhi Centre for Biotechnology, Kerala and cultured in a similar manner as described by us previously [1]. Upon termination of incubations, cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline (DPBS) and harvested with trypsin–EDTA solution. Harvested cell pellets were resuspended in NP40 lysis buffer supplemented with Halt protease and phosphatise inhibitor cocktail, vigorously vortexed in every 10 min for 30 min, centrifuged for 10 min at 13 000 rpm at 4°C and the supernatant was collected. The protein concentration of the supernatant was determined by following the method of Lowry et.al. [2].

3.3 Development of A_{2A}AR stable clone

For the establishment of Chinese hamster ovary (CHO) cell line stably expressing human A_{2A}AR, CHO cells were transfected with pCMV3-His-ADORA2A using Lipofectamine 2000 according to the manufacturer's instructions. After hygromycin B selection at 500 mg/ml for 3 weeks, stable transfectants were obtained and single clonal cell line (CHO/ADORA2A) was isolated by limiting dilution. Expression of the A_{2A}AR receptor was verified in RT-qPCR analysis. A_{2A}AR over expressing clonal cells were used for the determination of EC_{50} value (the concentration that produces a half-maximum response) of indirubin-3'-monoxime by performing a cAMP assay.

3.4 Radioligand-binding assay

Radioligand binding experiments were performed following the previously described procedures [3, 4]. The human A_{2A}AR or A_{2B}AR were individually transfected into CHO cells and studied in membranes prepared from these cells [3]. The radioligand [^{3}H] NECA (10 nM) was used for A_{2A}AR. Due to the lack of a useful A_{2B}AR radioligand,

the relative affinity for this receptor subtype was determined in adenylyl cyclase experiments as described previously [3].

3.5 Glucose uptake assay

Glucose uptake assay was performed using a glucose uptake cell-based assay kit (Cayman, U.S.A.) following manufacturer's instruction. Briefly, 3T3-L1 adipocytes were serum-starved overnight in Kreb's Ringer Phosphate (KRP) buffer supplemented with 0.2% bovine serum albumin (BSA). Cells were pretreated with different compounds for 1h followed by palmitate (0.75 mM) incubation for 6 h and 30 min before the termination of incubations; cells were treated with insulin (100 nM). Fluorescent labeled glucose analog 2-NBDG was added to each of the incubations for 5 min before termination of the experiment. Cells were then lysed and fluorescent intensity was measured by Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Finland).

3.6 Cell viability assay

Cell viability was assessed using the lactate dehydrogenase (LDH) release and MTT assays. LDH release was measured using EZcountTM LDH Cell Assay Kit following the manufacturer's instructions. Briefly, confluent 3T3-L1 adipocytes were either subjected to lysis to measure the maximum LDH release or treated without or with different concentrations of I3M (0, 5, 10, 50 mM) for 6h followed by the addition of LDH reagent to each well and incubated for 10 min at room temperature. Stop solution then added to terminate the incubations and fluorescent intensity (ex/em 560/590 nm) was measured by Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Finland). LDH release was calculated as Experimental-Background control/ Max.LDH control-Background control × 100.

MTT assay was performed following the method described previously [5]. Briefly, 3T3-L1 adipocytes were incubated with varied concentration of I3M or CYT for 24 h followed by the addition of MTT and incubated for 4 h. On termination of incubations, formazan crystals formed in cells were dissolved in acidic isopropanol and incubated further for 30 min at 37°C. Cytotoxicity was measured spectrophotometrically at 570 nm with Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Finland). Absorbance values were blanked against acidic isopropanol and the absorbance of cells

exposed to medium only (without any treatment) were taken as 100% cell viability (control).

3.7 Immunoblotting

Immunoblot analysis was performed following our previously described method [6]. Briefly, cell lysates (40 mg of protein) were subjected to either 10% or 12.5% SDS–PAGE and transferred on to ImmbilonP PVDF membranes (Millipore, Bedford, MA) with the help of Wet/Tank Blotting System (Bio-Rad Laboratories, Hercules, CA). Membranes were first blocked with 5% BSA in TBS (Tris-buffered saline) buffer for 1h followed by the overnight incubation with primary antibodies (1:500 or 1:1000 dilutions) in a rotating shaker at 4°C. The membranes were then washed three times with TBST (TBS containing 0.1% Tween 20) buffer for 10 min interval and incubated with peroxidise conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (1: 20000 dilution) for 2h at room temperature. Membranes were then washed three times with TBST for 10min interval and subjected to ClarityTM Western ECL Substrate incubation for 5 min at room temperature. Protein bands were visualized and quantified in Chemidoc XRS+ System (Bio-Rad Laboratories, U.S.A.) using Image Lab Software.

3.8 Coimmunoprecipitation

Coimmunoprecipitation study was performed according to our earlier published method [6]. Briefly, 200 mg of protein from cell lysate was incubated with 2 mg of the anti-IRS antibody for overnight at 4°C in a shaking platform followed by the incubation with 50 ml of Protein-G Agarose for 1h under rotation at 4°C. The samples were then centrifuged at 5000 rpm at 4°C for immune-complex precipitation. Pelleted immune-complex was washed thoroughly, boiled in 4× SDS sample buffer, vortexed and then centrifuged at 13 000 rpm for 10 min. The supernatant was isolated, run on 10% SDS–PAGE gel and transferred on to PVDF membrane. This was followed by immunoblotting with anti-phosphotyrosine or anti-IRS1 antibodies (1:1000 dilution). The blots were subjected to ClarityTM Western ECL Substrate and protein bands were observed and quantified in Chemidoc XRS+ System (Bio-Rad Laboratories, U.S.A.) using Image Lab Software.

3.9 Immunofluorescence analysis

L6 cells or THP1 macrophages were cultured and differentiated on sterile Millicell® EZ-Slide (Cat. No. #PEZGS0896, EMD-Millipore, Darmstadt, Germany), and treated under different conditions. On termination of incubations, cells were fixed with 4% paraformaldehyde for 10 min followed by blocking with 1% BSA in PBS for 1 h at room temperature. Cells were then incubated with anti-Glut4 antibody (1:50 dilution) or anti-pNF-κBp65 (S536) antibody at 1:50 dilution overnight at 4°C with gentle shaking overnight at 4°C in a rotating platform. After washing with ice-cold PBS, cells were incubated with AlexaFluor 488-conjugated goat anti-rabbit secondary antibody (1:200 dilution) or Alexa Fluor 594 conjugated anti-mouse IgG antibody at 1:200 dilution for 2h at room temperature. Cells were then washed thrice with ice-cold PBS and mounted in Vectashield™ anti-fade mounting medium with DAPI for nuclear counterstaining. Cellular images were taken using inverted fluorescent microscope (Leica DMi8, Germany) using LAS X software.

3.10 Semi-quantitative RT-PCR and real-time quantitative PCR

Total RNA was extracted from the cells of different incubations using RNeasy Lipid Tissue Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. RNA was treated with DNase I and reverse transcribed using the iScript Reverse Transcription Supermix. We used 2X PCR Master Mix for semi-quantitative RT-PCR in BioRad C-1000 Thermal Cycler and iTaq™ Universal SYBR® Green Supermix to perform real-time quantitative PCR in ABI-7500 system using gene-specific primers. The following cycling conditions were used for realtime qPCR: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. After the final extension, a melting curve analysis was performed to ensure the specificity of the products. The fold changes in expression were determined using $2^{\Delta\Delta Ct}$ and the expression of target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The oligonucleotides used in our study are listed in **Table 3.1.**

Table 3.1: List of primers and oligos used in this study

Oligo name	Sequence (5` -3`)
mMCP-1	F: 5′- GATGCAGTTAACGCCCCACT -3′ R: 5′- AGCTTCTTTGGGACACCTGC -3′
mTNF-α	F: 5′- CGCTGAGGTCAATCTGCCCAAG -3′ R: 5′- GGTCAGAGTAAAGGGGTCAGAGTGG -3′
mIL-1β	F: 5′-GCCACCTTTTGACAGTGATGAG -3′ R: 5′-GACAGCCCAGGTCAAAGGTT -3′
mIL-6	F: 5′- GCTGGAGTCACAGAAGGAGTGGC -3′ R: 5′-CTGACCACAGTGAGGAATGTCCAC -3′
mIL-10	F: 5′-GCATGGCCCAGAAATCAAGG -3′ R: 5′-GAGAAATCGATGACAGCGCC -3′
mIL-13	F: 5′-TGATGCTCCATACCATGCTG -3′ R: 5′-GTCATTGCTCTCACTTGCCT -3′
mIL-4	F: 5′-CTTTGCTGCCTCCAAGAACAC -3′ R: 5′-GCGAGTGTCCTTCTCATGGT -3′
mTGF-β	F: 5′-CACCCGCGTGCTAATGG -3′ R: 5′-ATGCTGTGTGTACTCTGCTTGAACT -3′
mIFNα	F: 5`-GGA CTT TGG ATT CCC GCA GGA GAA G-3` R: 5`-GCT GCA TCA GAC AGC CTT GCA GGT C-3`
mIFNβ	F:5`-AAC CTC ACC TAC AGG GCG GAC TTC A-3` R:5`-TCC CAC GTC AAT CTT TCC TCT TGC TTT-3`
mIFNγ	F: 5`-GCT ACA CAC TGC ATC TTG GCT TTG-3` R: 5`-GTG GGT TGT TGA CCT CAA ACT TGG-3`
mA_{2A}AR	F: 5′- TGAAGGCGAAGGGCATCA -3′ R: 5′- GGGTCAGGCCGATGGC -3′
GAPDH	F: 5′-CGACTTCAACAGCAACTCCCACTCTTCC -3′ R: 5′-TGGGTGGTCCAGGGTTTCTTACTCCTT -3′
β-actin	F: 5'-AGGCATCCTCACCCTGAAGTA-3' R: 5'-CACACGCAGCTCATTGTAGA-3'
hTGF-β	F: 5'-CACCCGCGTGCTAATGG-3' R: 5'-ATGCTGTGTGTACTCTGCTTGAACT-3'
hTNF-α	F: 5'-CCAGGGACCTCTCTCTAATCA-3' R: 5'-TCAGCTTGAGGGTTTGCTAC-3'
hIL-1β	F: 5'-GGTGTTCTCCATGTCCTTTGTA-3' R: 5'-GCTGTAGAGTGGGCTTATCATC-3'
hMCP-1	F: 5'-TCATAGCAGCCACCTTCATTC-3' R: 5'-CTCTGCACTGAGATCTTCCTATTG-3'
hIL-6	F: 5'-AATTCGGTACATCCTCGACGG-3'

	R: 5'-GGTTGTTTTCTGCCAGTGCC-3'
h IFNG	F: 5`-CGA CAG TTC AGC CAT CAC TT-3` R: 5`-GCA ACA AAA AGA AAC GAG ATG AC-3`
hIL10	Cat. No. 330001 PPH00572B Qiagen
hIL1N	Cat. No. 330001 PPH00555E Qiagen
h18srRNA	F: 5'-GTCTGTGATGCCCTTAGATG-3' R: 5'-AGCTTATGACCCGCACTTC-3'
hMyD88 _L265P	F: 5'-GGCACCTCTTTCGATGGGCTCACTAGCAATAGAC-3' R: 5'-GTCTATTGCTAGTGAGCCCATCGAAAAGAGGTGCC-3'
hIRAK1 _F196S	F: 5'-CCA GCA AAA CGG ACT GGG GCG GGC TCC C-3' R: 5'-GGG AGC CCG CCC CAG TCC GTT TTG CTG G-3'
hIRAK4 _Y262A	F: 5'ACCATTAGGCATGTAAACAGCTACTAAGCAGAGGTCATCTCCATC3' R: 5'GATGGAGATGACCTCTGCTTAGTAGCTGTTTACATGCCTAATGGT3'
h IRAK4_V263F	F: 5'TGAACCATTAGGCATGTAAAAATATACTAAGCAGAGGTCATCTC3' R: 5: 5'GAGATGACCTCTGCTTAGTATATTTTTACATGCCTAATGGTTCA3'
hIRAK4 _D329A	F: 5'-CCCGTGCAAGGCCAAAGGCAGATATTTTAGCAGTAAAAGC-3' R: 5'-GCTTTTACTGCTAAAATATCTGCCTTTGGCCTTGCACGGG-3'
hIL6 promoter	F: 5′-CAGAGCACCTGGTTGGT-3′ R: 5′-GCCCCAGAGCTGAGCAA-3′
IL-10 promoter	F: 5′-CGCCTGTACTGTAGGAAGCC-3′; R: 5′-GCCACAATCAAGGTTTCCCG-3′
hIRAK4-L	F: 5'-ATATGTGCGCTGCCTCAATG-3' R: 5'-GGTAGTGTATTAGCAGTTTTGGG-3'
hIRAK4-S	F: 5'-GCTGCCTCAATGTTGGACTA-3' R: 5'-TCTGGACTTGAGGAGTCAGG-3'

F: Forward primer; R: Reverse primer.

3.11 Enzyme-linked immunosorbent assay (ELISA)

To assess the protein levels of IL-6 and IL-10 in the control and treated cell culture supernatants, ELISA were performed using LEGEND MAX™ mouse IL-6 and IL-10 ELISA kits according to the manufacturer's instructions. We measured TNF-α and IL-6 cytokine levels in the serum samples of control and treated mice using mouse TNF-α (cat. no. #430907) and mouse IL-6 (cat. no. #431307) ELISA kits (BioLegend, San Diego, CA) following manufacturer's instructions.

3.12 Chromatin immunoprecipitation (ChIP) assay.

ChIP assay was performed using a ChIP assay kit (Upstate) following manufacturer instructions using 2 µg of anti-pNF-κBp65 or anti-IgG antibodies. Primers used for the amplification of the mouse *IL-10* promoter and human *IL6* promoter sequence are listed in **Table 3.1**. PCR products were run on ethidium bromide-stained 1.5% agarose gel, and the image was captured by the Chemidoc XRS+ System (Bio-Rad Laboratories, USA) using Image Lab Software.

3.13 Cyclic AMP assay

The cytosolic cAMP level was measured using the Cyclic AMP XP Assay Kit (Cell Signaling Technology, U.S.A.) in accordance with the manufacturer's protocol. Briefly, CHO cells stably expressing A_{2A}AR were treated without or with IBMX (0.5 mM) for 30 min followed by the 15 min incubation of varied concentrations of indirubin-3'-monoxime. On termination of incubations, cells were lysed with 100 ml of lysis buffer and 50 ml of cell lysate was incubated with 50 ml of HRP-linked cAMP solution in 1:1 ratio for 3h at room temperature on a horizontal orbital plate shaker. After incubation, the plate content was discarded and wells were properly washed with 1× wash buffer. An amount of 100 ml of TMB substrate was added to the wells and incubated until color develops. Upon color development, 100 ml stop solution was added and absorbance is measured at 450 nm in Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Finland). The percentage of activity was calculated. % activity = $100 \times [(A - Abasal)/(Amax - Abasal)]$, where A is the sample absorbance, Amax is the absorbance at maximum stimulation, and Abasal is the absorbance at basal level (without indirubin-3'-monoxime). The % activity was plotted versus the concentrations of indirubin-3'-monoxime and the dose-response curve fitted to a non-linear regression model using GraphPad Prism 5 software (San Diego,CA) for the determination of EC_{50} value of indirubin-3'-monoxime.

3.14 Glycerol release assay

Lipolysis was determined in 3T3-L1 adipocytes by measuring free glycerol level in the control and treated cell media using Glycerol Cell-Based Assay Kit following the manufacturer's instructions.

3.15 Luciferase reporter assay

3T3-L1 adipocytes and THP1 macrophages (2 × 10^5 cells/well) were transfected with either κB-luciferase or IL-10 promoter-luciferase expression plasmid (0.25 mg/well) using Lipofectamine 2000 Transfection Reagent (Invitrogen, U.S.A.) following manufacturer's protocol. Briefly, 7.5 ml of Lipofectamine 2000 reagent and 6 ml of 0.25 mg κB-luciferase or IL-10 promoter-luciferase plasmid were added separately into 100 ml of Opti-MEM medium. After 5 min incubation, both solutions were mixed and incubated for 30 min. The transfection mixture was added to the cells containing 0.8 ml of 2% FBS containing Dulbecco's modified Eagle's medium (DMEM) without antibiotics. After incubation at 37°C for 6 h, the culture medium was changed to DMEM containing 10% FBS. After 48 h of transfection, cells were washed with DMEM and used for different incubations. On termination of incubations, cells were lysed and luciferase activity was measured using Steady-Glo Luciferase Assay System (Promega, U.S.A.) with the help of Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Finland). The THP-1 macrophages (2 × 105 cells/well) were transfected with κB-luciferase or AP1-luciferase or IRF3-luciferase plasmids (0.1 μg/well) using Lipofectamine 3000 Transfection Reagent following manufacturer's protocol. Briefly, 0.3 μl of Lipofectamine 3000 Transfection Reagent and 0.1 μg of respective plasmids were added separately into 10 μl of Opti-MEM media, both of these solutions were mixed and incubated for 10 min. The transfection mixture was added to the cells containing complete growth media without antibiotics. After incubation at 37°C for 6 h, the culture medium was changed to growth media containing 20% FBS. After 48 h of transfection, cells were washed and used for different incubations. On termination of incubations, THP-1 cells were lysed and luciferase activity was measured using Steady-Glo Luciferase Assay System with the help of Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Finland).

3.16 RNA interference study

3T3-L1 adipocytes were transfected with Control siRNA or $A_{2A}AR$ siRNA using Lipofectamine 2000 Transfection Reagent (Invitrogen, U.S.A.) following the manufacturer's protocol. The transfection mixture prepared in Opti-MEM was added to the cells and incubated for 6 h at 37°C. After the addition of 20% FBS in culture media,

cells were kept for an additional 18h. Media replaced with fresh culture medium containing 10% FBS and incubated for 48h. After 48h of transfection, knockdown efficiency was analyzed by RT-qPCR. Control or A_{2A}AR siRNA transfected cells were incubated without or with indirubin-3'-monoxime (10 μM).

3.17 Surface plasmon resonance (SPR) analysis:

The surface plasmon resonance (SPR) analysis was performed to confirm the binding of vanillin with IRAK4. The measurements were performed at 25°C using the Biacore X100 instrument using a CM5 sensor chip (GE Healthcare). PBS buffer at pH 7.2 was used as a running buffer. The surface of the CM5 sensor chip was modified with an anti-GST antibody using EDC-NHS coupling at a flow rate of 10 μl/min in both flow cell 1 and 2 (FC-1 and FC-2). Then GST-tagged IRAK4 protein was coated on the same surface in flow cell 2 (FC-2) only. The binding efficacy of vanillin was then determined by measuring the change in response unit (RU) of the SPR sensorgrams in FC-2 and FC-1 and taking the difference i.e. (FC-2) – (FC-1). This was done by an inbuilt kinetics/affinity experiment in Biacore X100 control software with vanillin concentrations varying from 0 to 1000 μM with a flow rate of 30 μl/min. The equilibrium dissociation constant (KD) value of vanillin to IRAK4 was calculated using the inbuilt Biacore Insight Evaluation software.

3.18 In-vitro IRAK4 kinase assay:

IRAK4 kinase assay was performed using a Universal Fluorometric Kinase Assay Kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. Briefly, in a 96-well black plate, 20 μl of ADP sensor buffer, and 10 μl of ADP sensor were added with 20 μl of IRAK4 protein (Promega, 1 μg/ml) or immunocomplex IRAK4 (1 μg/ml) with or without different concentrations of vanillin (10, 50, 100 and 1000 nM) diluted in kinase reaction solution to prepare a final ADP assay volume of 50 μl/well. The reactions were carried out in the presence of ATP (1 mM) and myelin basic protein as substrate (1 mg/ml, 54 μM). The reaction mixture was incubated at room temperature for 15 minutes to 1 hour. On termination of incubations, the kinase activity was measured spectrophotometrically at Ex/Em=540/590 nm with Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Finland).

3.19 Flow cytometric analysis:

Control and treated RAW264.7 macrophages and the peritoneal cells isolated from the mice were harvested, centrifuged at 350 g for 5 min, and washed with ice-cold PBS. The cell pellets were re-suspended in cell staining buffer (PBS containing 0.2% FBS and 0.09% $NaNO_3$) and blocked with TruStain FcX™ (Fcγ blocker, mouse anti-CD16/32 antibody, BioLegend, USA) for 15 min at 4°C. RAW264.7 cells were stained with fluorochrome-conjugated primary antibodies against mouse CD80 and CD206 for 1 h in ice followed by two steps of washing. Cells isolated from the mice peritoneal fluid was washed thoroughly with ice-cold PBS and the cells were incubated with fluorochrome tagged F4/80 along with CD80 and CD206 antibodies for the staining of peritoneal macrophages for 1 h. Cells were then washed twice with chilled PBS, re-suspended in FACS staining buffer, and analyzed in a flow cytometer (BD Accuri C6+, BD Biosciences, San Jose, CA) using FlowJo™ v10.6.1 software.

3.20 Site-directed mutagenesis

Human IRAK4-FLAG, IRAK1-FLAG, and MyD88-YFP plasmids were used as templates for the generation of their mutants ($IRAK4_{Y262A}$, $IRAK4_{V263A}$, $IRAK4_{D329A}$, $IRAK1_{F196S}$, $MyD88_{L265P}$) by using QuickChange Lightning Multi Site-Directed Mutagenesis Kit following manufacturer's protocol. Mutated IRAK4, IRAK1, and MyD88 primers were designed with the help of the Agilent QuikChange Primer Design Program available online at www.agilent.com/genomics/qcpd. Forward and reverse primer sequences used for mutated IRAK4, IRAK1, and MyD88 plasmids constructions are listed in **Table 3.1.**

3.21 Animals and treatments

Wild-type C57BL/6 J male mice aged 5–6 weeks and weighed 20–24 g were procured from the IISER Mohali animal facility and kept in the National Toxicological Center at NIPER S.A.S. Nagar animal house for 7 days in 12-h light/dark cycle at 23 ± 2°C with relative humidity 55 ± 5% and fed with normal rodent pellet diet and water ad libitum. In the present study, vehicle control or vanillin (1.5 mM/g bw) was administered i.p. twice in 1 h intervals, then challenged i.p. with saline or LPS (100 μg) for 4 h. Mice

were then sacrificed, serum and peritoneal fluid were isolated. Peritoneal macrophages were isolated from these mice and used for RT-qPCR analysis and immunoblotting. Serum samples were used for the analysis of TNF-α and IL-6 pro-inflammatory cytokines levels by ELISA. All mouse experiments were performed following the guidelines prescribed by and with the approval of the Animal Ethics Committees of NIPER, S.A.S. Nagar, Punjab (Protocol No.: IAEC/21/77-ext1&M).

3.22 Molecular docking studies:

Molecular docking investigation of IRAK4 with vanillin has been carried out with the AutoDock 4.2 program utilizing the Lamarckian genetic algorithm [7]. The 3D coordinates of IRAK4 have been taken from Protein Data Bank (PDB ID: 2NRU) while the 3D conformer of vanillin has been considered from the PubChem database (https://pubchem.ncbi.nlm.nih.gov). Both the protein and the small molecule structures have been prepared for the docking with the aid of AutoDock Tools. Polar hydrogens and Kollman charges were assigned to the protein. The torsional bonds of vanillin were made detectable with the detect root option along with checking the Gasteiger charges. To decipher the binding mode of vanillin within IRAK4, the search parameters were set as: grid sizes along the X, Y, and Z axes to 36 × 46 × 40 with a grid spacing of 0.375Å, and the box was centered at x=31.022, y=8.659, and z=-10.22 of IRAK4 which comprises the major binding site residues of IRAK4 (Lys213, Tyr262, Tyr264, and Asp329) [8,9]. The docking parameters conforming to population size were fixed at 150; the maximum number of energy evaluations was fixed at 2500000 and GA runs were set to 50. The resultant poses were analyzed based on the binding energy and 3D visualization of the interactions.

3.23 Development of vanillin analogs:

All the reagents and solvents utilized for the reactions were employed without further purification unless otherwise mentioned. Reactions and fractions from column chromatography were monitored by thin layer chromatography (TLC). Commercial aluminum sheets precoated (0.2 mm layer thickness) with silica gel 60 F254 were used for this purpose. Visualization of TLC plates was performed by UV fluorescence at 254 nm and/or by immersion in an ethanolic vanillin solution or by immersion in $KMnO_4$

solution followed by heating. Product purification by column chromatography was executed using silica gel (100–200 mesh) procured from Merck. ^{1}H NMR and ^{13}C NMR spectra were run on JEOL 400 MHz and Bruker Avance III 400 MHz spectrometers in $CDCl_3$ as solvent. Chemical shifts (δ) are reported in ppm. For ^{1}H NMR spectra, δ values were referenced to residual $CHCl_3$ (7.27 ppm) or DMSO (2.50 ppm); and for ^{13}C NMR spectra, δ values were referenced to deuterated solvent $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.51 ppm). Multiplicities in ^{1}H NMR are abbreviated as follows: singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), doublet of doublet of doublets (ddd), doublet of triplets (dt), triplet of doublets (td), multiplet (m), and broad (br). All spectra were recorded at 25 °C. Coupling constants (*J* values) are given in hertz (Hz).

Statistical analysis:

All data were derived from at least three independent experiments and statistical analyses were conducted using Sigma Plot 10.0 software. Densitometric analysis of RT-PCR and Western blot data was performed using ImageJ software (1.48v, NIH, USA). Data were analyzed by unpaired Student's t-test, where the p-value indicated significance. All values were means ± SEM. A level of $p < 0.05$ was considered significant.

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