Materials and Methods
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Animals
BALB/c mice and golden hamsters (*Mesocricetus auratus*) were maintained and bred under pathogen-free conditions. C57BL/6, TLR2\(^{-/-}\) and C3He/J (TLR4\(^{-/-}\)) mice were obtained from IISc, Bangalore. MyD88\(^{-/-}\) mice were a kind gift from Dr. Gobardhan Das, ICGEB, New Delhi. Use of both mice and hamsters was approved by the Institutional Animal Ethics Committees of Indian Institute of Chemical Biology, India. All animal experiments were performed according to the National Regulatory Guidelines issued by CPSEA (Committee for the Purpose of Supervision of Experiments on Animals), Ministry of Environment and Forest, Govt. of India.

Parasite cultures and maintenance
Antimony resistant (Sb\(^{R}\)LD; MHOM/IN/2009/BHU575/0 and MHOM/IN/2005/BHU138) and sensitive *Leishmania donovani* (Sb\(^{S}\)LD; MHOM/IN/83/AG83 and MHOM/NP/03/BPK206/0) (Mukhopadhyay et al., 2011) maintained in golden hamsters (Mukhopadhyay et al., 1999) were used for this study. Amastigotes were obtained from the spleen of infected hamsters (Hart et al., 1981) and subsequently transformed into promastigotes and maintained (Chakraborty et al., 2005).

Cell culture and infection
Peritoneal exudate cells, conveniently named macrophages (Mφs) were harvested from BALB/c, C57BL/6, TLR2\(^{-/-}\), TLR4\(^{-/-}\) and MyD88\(^{-/-}\) mice by lavage, 48 h after intraperitoneal injection of 2% (w/v) soluble starch (SIGMA) (Chakraborty et al., 2005). Mφs were plated on 90 mm tissue culture petridishes or 24 well plates (Nunc), at a density of 1×10\(^7\) or 1.5×10\(^6\) respectively, and also on sterile 22 mm square coverslips in 35 mm disposable petriplates at a density of 1.5×10\(^5\)/coverslip in RPMI 1640 medium (SIGMA) supplemented with 10% heat inactivated FBS, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin (GIBCO), i.e. RPMI complete medium. The cells were left to adhere for 48 h at 37°C under 5% CO\(_2\) before infection. Mφs were plated, maintained and infected (MOI of 1:10) with stationary phase LD promastigotes. Mφs were infected with stationary phase LD promastigotes at a ratio of 1:10 for 6 h, then washed to remove free parasites, and incubated for another 24 h or 48 h. Supernatants obtained from Mφs infected with stationary phase LD promastigotes at a ratio of 1:10 for 48 h were subsequently used for ELISA experiments unless otherwise mentioned. Parasite SLA was prepared as described (Chakraborty et al., 2005). Parasite lysates were prepared as described in (Kapleret al., 1990) with slight modifications. The protein content of the lysate was estimated and equal concentration of the lysate protein was directly used for the experiment without further centrifugation step.

Infection and purification of amastigotes
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Hamsters and mice (6 weeks old), were infected with either Sb⁸LD (BHU575/BHU138) or with Sb³LD (AG83) in an inoculum of \(10^7\) parasites in 200 mL via intracardiac routes as described previously (Mukherjee et al., 2012). Soluble Leishmanial Antigen (SLA) was prepared from stationary phase LD promastigotes of LD Parasite as described previously (Mukherjee et al., 2012).

**In vivo infection**

For experimental visceral infections, female BALB/c mice (4–6 week old and 20–25 g each) were injected via intracardiac route with 2 × \(10^7\) hamster spleen-transformed *L. donovani* stationary promastigotes (suspended in 200 µl of 0.02 M PBS per mouse). Infected mice were grouped with 3 to 5 mice in each experimental group. Each group was sacrificed 14, 28 days post infection.

**Treatment**

Mφs were treated with various pharmacological inhibitors prior to infection in serum free medium. Mφs were treated with 10 µM of ERK1/2 inhibitor U0126 (Yanagawa et al., 2007), 20µM of PD 98059 (Feng et al., 1999), 1µM of PI3K inhibitor wortmannin (Hirji et al., 1998), 20µM of IKK inhibitor BAY 11-7082 (Choi et al., 2006), 25µM of JNK inhibitor SP 600125(Mingo-Sionet et al., 2004), or 10µM of p38 inhibitor SB 203580 (Lee et al., 2004) for 45 min prior to infection in serum free medium. In some experiments Mφs were stimulated with Cyclohexamide (CalBiochem) at concentration of 1µg/ml for 1h before infecting the Mφs. In some experiments, Mφs were stimulated with LPS (1µg/ml) and used as a positive control. Stationary phase LD promastigotes were centrifuged at 3000 rpm for 10 min. The supernatant was discarded; the pellet was re-suspended in a minimum volume of PBS and treated with galactosidase (0.2 U; SIGMA) for 1 h at 37°C to remove surface sugar. The parasites were washed and fixed with 1% para-formaldehyde for 15 min, and washed five times with cold PBS (Chandra et al., 2008). These para-formaldehyde treated parasites were then re-suspended in complete medium before infecting the Mφs. Parasite lysates were also used to treat Mφs. In some experiments, Mφs were incubated with parasite free culture supernatant of stationary phase LD promastigotes for 48 h in varying concentration of 1:1 or1:2 or 1:10 with RPMI medium after which centrifuge and supernatants were collected for performing ELISA.

In some experiments, TLR2 Ab (10ng/ml) or TLR1 Ab (10ng/ml) or TLR6 Ab (10ng/ml) were pre-incubated with Mφs for 2h in a serum free medium before infection. In some experiments Mφs were stimulated either with FSL-1 (Invivogen) or with Pam3CSK4 (Invivogen) both at concentration of 10ng/ml for 24 h.

Mφs were treated with murine rIL-10 or rIL-12 (10 pg/ml, 20 pg/ml, 200 pg/ml) (BD Pharmingen) or with αIL-10Ab (BD Pharmingen), which was pre-incubated before infection. Mφs were treated with siRNA for MyD88 (1µg) or control siRNA (1µg) (Santa Cruz) for 2h before infecting them with Sb⁸LD or Sb³LD. In some experiments Mφs were pre-treated with 100µM of TRIF inhibitor peptide (Invivogen) 30mins before infection. In some experiments, Mφs were stimulated with LPS (1µg/ml) and used as a positive control.
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Plasmids
EGFP-p65 plasmid was used (Halder et al., 2010). The murine IL-10 promoter -17/-1576 (1.57 kb), -17/-292 (275 bp) and -864/-1138 (274bp) were cloned into a pGL3-Basic vector. The Mut IL-10 (with deletion at NF-κB binding site -583/-593) promoter construct was generated from the IL-10 (1.57 kb) promoter construct by QuickChangeII PCR-based site-directed mutagenesis kit (Stratagene) according to manufacturer’s protocol. The galactosyltransferase (GalT) gene construct was generated and cloned in an antisense orientation in the pXG-B2863 vector (a kind gift from Dr. S.M. Beverley, University of Washington) and termed anti-LdGalT

Transfection
RAW264.7 cells were grown and transiently transfected with complete IL-10 (1.57kb) or truncated IL-10 (275, 274 bp) or Mut IL-10 (NF-κB binding site -583/-593) promoter constructs using lipofectamin 2000 (Invitrogen). Anti-LdGalT constructs and corresponding empty vector pXG-B2863 was transfected into L. donovani promastigotes by electroporation as described (Kapler et al., 1990) and Gal T Knock Down parasites (KD Sb8LD) were generated. The antisense construct of the galactosyltransferase (GalT) gene was generated by PCR amplification of the 1414-1653 nucleotide region of the phosphoglycan beta 1,3 galactosyltransferase 3 gene (LdGalT3) from the genomic DNA of LD parasites using the sense primer 5′-CGGGATCCCTGTCAGGAGGCTCGACCACCGCAA-3′ and antisense primer 5′-TCCCCGGGTGCTCAGTACTGTGGGCACCTCCAGGTA-3′, and cloned in an antisense orientation in the SmaI/BamHI sites of the pXG-B2863 vector (a kind gift from Dr. S.M. Beverley, University of Washington); this was termed as anti-LdGalT. The construct along with the empty vector pXG-B2863 was transfected into L. donovani promastigotes by electroporation as described earlier (Kapler et al., 1990). Briefly, late log phase promastigotes were harvested and washed twice in OPTI-MEM (GIBCO). Cells were then suspended at a density of 1×10⁸/ml and 0.4 ml of this was taken into a 0.2 mm ice-chilled electroporation cuvette. Thirty microgram of plasmid DNA was taken in 100 µl of electroporation buffer and added to the cells. After 10 min on ice, the cells were electroporated with a single pulse by Bio-Rad Gene Pulsar apparatus using 450 V and 550 µF capacitance. The cells were incubated on ice for a further 5 min and then added to 10 ml of drug free growth medium. After 24 h of survival, 10 µg/ml G418 was added and kept at 22° C. The transfected cells were monitored visually by microscope and the drug concentration was increased gradually. Finally the transfected cells were routinely maintained in medium containing 200 µg/ml G418.

RT-PCR analysis
RT PCR of IL-10 mRNA in infected Mφs were performed using IL-10 specific primer 5′-TCTCTAATGCAGGACTTAAAGGGTTACTTG-3′ and 5′-GACACCTTGGTCTTTGAGCTATTAAAATC-3′ as mentioned previously (Mukhopadhyay et al., 2011). Total RNA was isolated from GalT KD or WT or vector control isolates using the Total RNA
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isolation kit (Roche Biochemicals) according to the manufacturer’s protocol. RNA was stored at -70 °C until further use. RT-PCR was performed by using specific primers for LdGalT3 and GAPDH following the standard protocol.

**RT-qPCR to estimate expression of autophagy genes**

The expression of MyD88 mRNA in Sb^5LD or Sb^3LD or LPS treated Mφs was analysed. The cDNA synthesis and RT-qPCR were done as described elsewhere (Decuypere et al., 2005). Briefly, the resulting cDNA from the Mφs was diluted 7.5 times and subjected to qPCRs for expression profiling referred to as target genes and one gene β-actin referred to as the control gene. The qPCR contained 2 X Sybr Green Supermix (Applied Biosystems, CA, USA) diluted twice, and forward and reverse primers as specified (Decuypere et al., 2005). The sequences of the forward and reverse primer are presented in Table 1. Reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR system using the following thermal profile: initial denaturation at 95°C for 5 min followed by 30 cycles with denaturation at 95°C for 30 s, annealing at 60°C for 15 s and extension at 72°C for 15 s. The PCR was immediately followed by a melt curve analysis using temperature increments of 0.5°C every 30 s to ascertain whether the expected product was amplified and to ensure that no non-specific products or primer dimmers (which could bias the quantification) were formed. Experiments on negative controls of cDNA synthesis (i.e., without reverse transcriptase) and no-template controls (i.e., without cDNA template) were also done for each gene. All reactions were done in duplicate, and their arithmetic average threshold cycle (Ct) was used for data analysis. The fold of gene expression compared with the control was calculated using the formula: Fold of expression= 2^{(-ΔΔCt)} by 7500 Fast System SDS software, version 1.4 (Applied Biosystems, CA, USA).

**Short hairpin RNA construct**

The MyD88-specific short hairpin RNA (shRNA) cassette, driven by the promoter of the small nuclear RNA U6, was generated by PCR-mediated amplification of positions 915–934 of the MyD88 gene (GenBank accession number NM_010851.2), and the selection of shRNA target sequences was based on published guidelines (Pei and Tuschl, 2006). The shRNA cassette was subsequently cloned into a self-inactivating lentiviral vector pCRI.LVand virus produced and concentrated by ultracentrifugation as described previously (Santhosh et al., 2008). Virus titer was measured at 2 3 106 infectious units/ml. shRNA target sequence selection and nonrelevant control vector use was the same as reported elsewhere (Basu Ball et al., 2011). For in vivo knockdown of MyD88, 50 ml of the 1000× vector concentrate of MyD88- specific shRNA construct was injected into spleen tissue in anesthetized BALB/c mice 3 d prior to infection.

**Cytokine measurement**
IL-10 and IL-12 levels were measured in the supernatant by ELISA (BD Bioscience) following the manufacturer’s manual.

**Determination of parasite burden and Histologic responses**

Parasite burdens are expressed as Leishman-Donovan units (where LDU represents the number of parasites per 1000 host cell nuclei multiplied by the organ weight) and were determined from Giemsa-stained tissue impression smears, as previously described (Mukherjee et al., 2012). Liver sections from infected mice were stained with hematoxylin and eosin and the degree of the granulomatous responses assessed in two ways: 1) granuloma density was determined from; 50 fields of view per mouse liver (X40 magnification) the degree of maturation, of granulomas was scored around infected Kupffer cells (KC), as described elsewhere (Murphy et al., 1998). Photomicrographs were taken with a Nikon Eclipse E200 microscope.

**MACS cell preparation**

Cells were MACS purified from whole splenocytes of mice using either CD11b or CD4 micro beads or CD4+CD25+ Regulatory T Cell isolation kit, (MiltenyiBiotec) according to manufacturer’s protocol.

**Re-stimulation of T cells for cytokine analysis.**

For in vitro restimulation, MACS purified CD4+CD25+ and CD4+CD25- cells were incubated separately for 72 h at 37°C, 5% CO2 at a concentration of 2 × 10^6 cells in 1ml of complete RPMI medium (Sigma), in round-bottom 24 well plates (BD Bioscience) in the presence of 2 × 10^5 CD4- cell population in presence of 5 μg/ml of specific *Leishmania* antigen prepared either from AG83 or BHU575 or BHU138 stationary phase promastigotes. Supernatant was collected after 24h and ELISA was done using IL-10 ELISA kit (BD Biosciences) following manufacturer’s protocol. For measurement of intracellular IL-10, Mφ or T cell population were incubated with 10 ng/ml PMA and 500 ng/ml ionomycin for 4 h. The cells were analyzed for surface markers and intracytoplasmic staining for cytokines.

**Immunostaining and confocal microscopy**

For nuclear staining, Mφs were cultured on Lab-TEK chamber slides (Nunc International Corp, Rochester, NY) and infected with Sb^R^LD or Sb^S^LD for 6 h. The standard immunocytochemical method was used as described previously (Nancy et al., 1999). For immunostaining, the cells were fixed in 100% methanol for 30 min and washed three times with PBS. After blocking in 5% bovine serum albumin in PBS for 1 h at room temperature or overnight at 4°C, the cells were incubated for 1 h with polyclonal rabbit anti-p50 antibody (1:100) in PBS containing 0.5% bovine serum albumin, washed and re incubated with rabbit anti-c-Rel antibody (1:100) as mentioned previously. The cells were incubated with conjugated goat anti-rabbit IgG antibody Alexa488 and Alexa-535 (1:100) after serial washings with PBS. Counter-staining with DAPI in the mounting medium verified the location
and integrity of nuclei. Stained cells were washed and examined using a laser scanning confocal microscope (LSM 510; Carl Zeiss, Jena, Germany).

**Immunolabelling and Flow cytometry**

The expression levels of N-acetylgalactosamine residue in WT as well as KD LD was determined flow cytometrically by FITC labelled *Dolichos biflorus* agglutinin and compared to their mannose level (specific to Con A lectin) (Mukhopadhyay et al., 2011). Stationary phase LD parasites, (2 x10$^6$) each, were stained with FITC labelled *Dolichos biflorus* agglutinin and compared to their mannose level specific to Con A lectin. Samples were subjected to flow cytometry analysis in a BD FACS Aria II cell sorter. The mean fluorescence intensity was analysed by FACS DIVA software and accordingly the relative expression of surface sugar was determined. The following antibodies used for immunophenotyping were purchased from BD Biosciences, APC anti–mouse CD25, and FITC anti–mouse IL-10. The isotype controls used (all obtained from BD Biosciences) were rat IgG2b, rat IgG1γ. Before staining, MACS enriched CD4+ population were incubated with FcR Blocking Reagent (MiltenyiBiotec) for 15 mins at room temperature. The staining of surface and intracytoplasmic markers was performed sequentially: the cells were stained first for their surface markers, followed by a permeabilization step and staining for IL-10. The data were collected and analyzed using FACS DIVA software (BD Biosciences) and a flow cytometer (BD FACS Aria II; BD Biosciences).

**EMSA**

Nuclear extracts were prepared from Mφs as described (Haldar et al., 2010). These extracts were incubated with radio-labeled double stranded oligonucleotide probes for either murine IL-10 promoter containing WT or mutant NF-κB binding sites or MDR1 promoter containing WT or mutant AP1 binding sites respectively. Protein-DNA complexes were separated on acrylamide gels. EMSA was performed using Gel shift assay system (Promega) according to the manufacturer’s protocol. DNA probes specific for murine IL-10 promoter containing wild type or mutant NF-κB binding sites, represented by the WT-mIL-10 probe, 5’-GCCCTCTCGGGGTTTCTTGG-3’ and the Mut-mIL-10 probe, 5’-GCCCTCTCCCTTTTATAATTGGG-3’ (NF-κB binding site is underlined and mutated bases are in italics) respectively, were used. Supershift EMSA was carried out using the following antibodies: αp50, αc-Rel (Santa Cruz Biotechnology) and αp65 (Cell Signaling Technology). Bands were visualized using a phosphoimager.

**Western blot and Co-Immunoprecipitation**

Cytoplasmic and nuclear protein was prepared and Western blotting was performed for p50, phospho p50 and c-Rel and MyD88 (Santa Cruz Biotechnology); pIκκα/β, pIκBα, IκBα, IκBβ, IκBε, pERK1/pERK2 (Thr202/Tyr204), ERK1/ERK2, p65, β-actin and histone (Cell SignalingTechnology). Blots were probed with specific Abs. Binding of secondary HRP-labeled goat-anti rabbit or goat-anti mouse Abs (Cell Signaling Technology) was analyzed using SuperSignalR West Pico or West Dura.
Chemiluminescent Substrate (Pierce, Rockland, IL). Co-IP was performed as described previously (Sen et al., 2011) using specific TLR2 (Santa Cruz Biotechnology) Ab or unrelated whole rabbit IgG (Cell Signaling Technology), and blots were probed either with Abs against TLR1 (Santa Cruz Biotechnology) or TLR6 (Santa Cruz Biotechnology).

**Reporter assay**

Luciferase activity in cell extracts from infected or treated Mφs was measured using the Dual Luciferase Reporter kit (Promega) according to the manufacturer’s protocols and the luciferase activity was normalized to the level of the protein content. The murine IL-10 promoter -17/-1576 (1.57 kb; 5′-GCTGGGTCTTGGAGCCTCTTCTGG-3′ and 5′-CTGCAAGGCTGCGCTTGGGCTTTG-3′), -17/-292 (275 bp; 5′-GAGGTAGCCCATAACTAAAAATAGC-3′ and 5′-CTGCAAGGCTGCGCTTGGGCTTTG-3′) and -864/-1138 (274 bp; 5′-GGAAGGACAGCCCCGGAGTACC-3′ and 5′-CCTGCGAGATCTCTGCTGCTCC-3′) were PCR-amplified and cloned into a pGL3-Basic vector (Promega). Using the IL-10 promoter construct (1.57 kb) and a QuickChangeII PCR-based site-directed mutagenesis kit (Stratagene), the Mut IL-10 promoter construct, containing a deletion at NF-κB binding site -583/-593 of IL-10 promoter, was generated. All the inserts were confirmed by sequencing. RAW264.7 cells were transiently transfected with these IL-10 (2 μg) using lipofectamine 2000 (Invitrogen), rested for 12 h, and infected with Sb8LD, Sb5LD for 48 h. Luciferase activity in cell extracts was measured using the Dual Luciferase Reporter kit (Promega) according to the manufacturer’s protocols and the luciferase activity was normalized to the level of the protein content.

The murine IL-10 promoter -17/-1576 (1.57 kb), IL-12 p35 promoters -6/-1411 (1.4 kb), and p40 promoter -103/-1165 (1.06 kb), MyD88 promoter1087/+207 (1.2 kb) were PCR-amplified and cloned into a pGL3-Basic vector (Promega). -6009/-5008 (1 KB) region of miR-466i were cloned into pGL3-Basic vector. +12/+802 region of murine MyD88 3′UTR was cloned in psiCHECK™ vector. miR-466i promoter sequence (with deletion at the NF-κB binding sites -6009/-5008, -5862/-5852, -5573/-5558 and psiCHECK™-Mut3′UTR (with deletion at miR-466i binding site Δ751-757 and Δ768-775) construct was generated from the miR-466i whole promoter construct (1 KB), and MyD88-3′UTR-psiCHECK™ construct respectively by QuickChangeII PCR-based site-directed mutagenesis kit (Stratagene) according to manufacturer’s protocol. The primer sequences are given in Table 1. All the inserts were confirmed by sequencing. The Mφs were transfected with miR-466imimics (SIGMA) at different concentration ranging from 10 to 200 nM or with 25 nM of synthetic miR-30a inhibitor (SIGMA). Mφs were transfected with pUNO-MyD88 vector (Invivogen) in some case. In some experiments RAW264.7 cells were transiently transfected with these constructs (2 μg) using lipofectamin 2000 (Invitrogen), rested for 12 h, and either infected with Sb8LD or Sb5LD for 24h or co-transfected with miR-466i mimic or treated with r-IL-10 or r-IL-12. Luciferase activity
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in cell extracts was measured using the Dual Luciferase Reporter kit (Promega) according to the manufacturer’s protocols and the luciferase activity was normalized to the level of the protein content.

**Pri-miR-466i quantification**

Real-time PCR using miRNA-specific stem-loop primers for reverse transcription and TaqMan probes for mature murine miRNA was performed in accordance with manufacturers’ protocols using an Applied Biosystems 7500 Fast Real-Time PCR. RNA was extracted from an equal amount of cells per time point using the RNeasy Mini Kit (Qiagen). cDNA synthesis was performed using SuperScript III (Invitrogen). pri-miR-466i levels were determined using the TaqManPri-miRNA assays developed by Applied Biosystems. Data analysis was performed using the Fast System SDS software, version 1.4 (Applied Biosystems, CA, USA).

**Northern blotting**

Total RNA (10 μg/lane) was resolved on 15% polyacrylamide-urea gels and electroblotted onto HyBond N+ membranes (GE Healthcare, Little Chalfont, UK). Membranes were hybridized overnight with radiolabeled antisense miRNAs in Express Hyb solution (Clontech, Mountain View, CA, USA). After hybridization, membranes were washed 3 times with 2× SSC and 0.05% SDS, and twice with 0.1× SSC and 0.1% SDS, exposed overnight to imaging screens, and analyzed using a Storm 860 PhosphorImager (GE Healthcare). The same blot was hybridized (on stripping in boiling 0.1% SDS) with control U6 RNA. The probe sequence for miR-466i is 5’-CACACACACACACACACACACATAC-3’.

**ChIP Analysis**

ChIP assays were performed on infected Mφs using ChIP assay kit (Upstate) following the manufacturer’s instructions. Immunoprecipitation was done using rabbit IgG or NF-κB Abs such as αp50, αp65 and c-Rel overnight at 4°C followed by DNA extraction. PCR was performed to amplify the -482/-645 region of IL-10 promoter using primer 5′-GCCCCACAGCACATATCC-3’ and 5′-CCTGGGTTGAACGTCCG-3’ or specific region of miR-466i promoter using primer 5′-CTCAGGTATACCGATAGG-3’ and 5′-GAC CTG GAG TTT TTA GCC TGC C-3’ or 5′-CTCAGCTTACAATAAGC-3’ and 5′-CAG TAG GAG AAG ATT AGT TG-3’ or 5′-CAACTAATCTTCTCCTACTG-3’ and 5′-GAT AGG ACA GTT AGG ATA GC-3’.

**Partitioning of plasma with the IgY-R7-LC2 affinity column**

Briefly 50 μl plasma were diluted with 200μl Tris-buffered saline (TBS, 10mM Tris/HCL, pH 7.4, 150mM NaCl) and filtered through a 0.45 μm membrane filter. Two hundred μl of diluted plasma sample were loaded onto the affinity column at 0.1 ml/min. Depleted plasma samples were initially collected at 0.1ml/min for 10 min and then for an additional 7 min at 0.2 ml/min. The column was washed for 5 min at 1 ml/min. Bound plasma proteins were eluted with 14 ml of 0.1 M glycine solution (pH 2.5). Elution was monitored at 280 nm. Plasma fraction were pooled and concentrated.
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**SDS-PAGE**

SDS-PAGE analysis was carried out with the Tris/Tricine buffer system according to Schagger and Von Jagow. Protein were separated under reducing condition on 10% SDS-PAGE mini gelss (10 cm x 7 cm) and visualized by colloidal Coomassie Blue G-250 or silver staining.

**Two-dimensional gel electrophoresis**

Two hundred μg of precipitated samples were diluted in 185 μl sample rehydration buffer (8 M urea, 2 % CHAPS, 50 mM DTT, 0.2 % (w/v) 3/10 ampholytess, 0.002% Bromophenol Blue) and incubated for 16 h with an IPG strip (ReadyStrip™, pH 3-10, 11cm). Following rehydration, IPG strips were focused for a total of 35,000 V-hr at 20°C in a PROTEAN IEF cell (Biorad). Prior to 2nd-dimension SDS-PAGE, focused IPG strips were first reduced with DTT in the equilibration buffer (6 M urea, 0.375 M Tris/HCl, pH 8.8, 2 % SDS, 20 % glycerol and 2 % (w/v) DTT) and then alkylated with iodoacetamide (6 M urea, 0.375 M Tris/HCl, pH 8.8, 2% SDS, 20 % glycerol, 2.5% (w/v) iodoacetamide) for 10 min each at room temperature. Second-dimension separations were carried out on 4-12 % Bis-Tris gradient gels in a MES buffer system. Two-dimensional gels were stained with colloidal Coomassie Blue, or silver stain, scanned and analyzed with the Imagemaster 2D Platinum 6.0 software package.

**Image analysis**

ImageMaster 2D Elite 4.01 analysis software (Amersham Bioscience) was used for spot-intensity calibration, spot detection, background abstraction, matching, 1-D calibration, and the establishment of average-gel. Intensity of each spot was quantified by calculation of spot volume normalization method multiplied by the total area of all the spots. The reproducibility of spot position was calculated with Corbett’s method (Gharahdaghi et al., 1999).

**In-gel digestion of protein**

Differential spots were excised from preparative gels using spot cutter or manually excised. The excised gels were transferred to Eppendorf tube and destained according to (Gharadaghi et al, 1999) with 50 μl of Farmers’ reducing solution (15 mM potassium ferricyanide and 50mM sodium thiosulfate) and then washed three times for 5-10 min with 150 μl water. Afterward, the gel spots were soaked in acetonitrile and dried under vacuum. The gel pieces were re-swollen in 7.5 μl of 5 mM ammonium bicarbonate with 75 ng of modified porcine trypsin (sequencing grade) to fragment the protein. After 10 min, 7.5 μl of 5 mM ammonium bicarbonate was added and the solution with the gel pieces was incubated for 4 hrs at 37°C. For MS analysis, 1.5 μl of the aqueous supernatant
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was mixed with 1 μl of 2, 5-dihydroxybenzoic acid (5 mg/ml in water) directly on MALDI target and dried.

Mass spectrometry

Individual spots are cut out of the gel and cleaved into peptides with proteolytic enzymes. MS was done with a Reflex IV MALDI-TOF mass spectrometer in reflection mode at an acceleration voltage of 20 kV. The MS was calibrated either with angiotensin II (av. m/z: 1046.5 Da), angiotensin I (av. m/z: 1296.6 Da), bombesin (av. m/z: 1619.8 Da). Substance P (av. m/z: 1347.7 Da), ACTH standards and with the autolytic trypsin fragments- av. m/z: 842.50 Da and 2211.10 Da respectively, as internal standards. The spectra were processed by the “Xmass” software (BrukerDaltonik, Bremen) and the peaks annotated manually. Post-source decay (PSD) analysis was done with up to 300 shots per section.

Data analysis

The peak lists of the mass spectra were used for peptide mass fingerprint analysis with the Mascot software (Matrix Science; http://www.matrixscience.com/search_form_select.html) and profound (prowl; http://prowl.rockefeller.edu/profound_bin/Web-ProFound.exe) together with the NCBI sequence database. Most proteins were identified using the following parameter- database, enzyme, variable modification, mass value, protein mass, peptide mass tolerance, peptide charge state and maximum missed cleavage. The analysis of PSD database was done either by peptide mass fingerprint or peptide fragmentation fingerprint analysis with Mascot.

Statistical variation and presentation

Each experiment was performed thrice and representative data from one set are presented. Inter assay variation was within 10%. Statistical significance between means of various groups was determined using a two-tailed Student’s t test. Only P-values below 0.05 were considered to be statistically significant. P value<0.001 were considered extremely significant (***) , P value ranging between 0.001 to 0.01 were very significant (**), P value 0.01 to 0.05 as significant (*) and P value >0.05 were not significant (ns). Error bars indicate the mean ± standard deviation. Data was analyzed using Prism 5.0 (GraphPad, San Diego, CA).
Table 1: Primer sequences used in the study

<table>
<thead>
<tr>
<th>SL No</th>
<th>Primer</th>
<th>Symbol</th>
<th>Forward /Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Myeloid differentiation primary response gene 88</td>
<td>MyD88</td>
<td>5'-TCGAGTTTGGCAGGAGATG-3' 5'-AGGCTGAGTGCAAACCTTGGT-3'</td>
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<tr>
<td>2.</td>
<td>Myd88 whole promoter</td>
<td>MyD88_P (-1087/+207)</td>
<td>5'-CGCCCACTCACCCTTTGCCCA-3' 5'-GGCCCGCGTGCGCCGTCAGTCG-3'</td>
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<tr>
<td>3.</td>
<td>MyD88_3'UTR</td>
<td>WT</td>
<td>5'-GGAGGGCCCTAGGGCAGA-3' 5'-CCGCTGCAAGAGGCAC-3'</td>
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<tr>
<td>4.</td>
<td>MyD88_3'UTR Δ751-757</td>
<td>MUTI</td>
<td>5'-CCTGAGTCCCCAAGGTCTCCCCTCAGTG-3' 5'-CACTGAGGGAGACCTTGGGGAATCAGG-3'</td>
</tr>
<tr>
<td>5.</td>
<td>MyD88_3'UTR Δ768-775</td>
<td>MUTII</td>
<td>5'-CCTCATTCTCCCCGAGGGAGACTAC-3' 5'-GTGAGTCTCCCCCTGAGGAATGAGG-3'</td>
</tr>
<tr>
<td>6.</td>
<td>Myd88_3'UTR ΔΔ751-757/768-775</td>
<td>MUTΔΔ</td>
<td>5'-CCTCATTCTCCCCGAGGGAGACTAC-3' 5'-GTGAGTCTCCCCCTGAGGAATGAGG-3'</td>
</tr>
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<td>7.</td>
<td>miR-466i murine</td>
<td></td>
<td>5'-AGAGCAGCTGCCCCAACCTAA-3' 5'-TCAGATGGGACTGTCATGT-3'</td>
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<tr>
<td>8.</td>
<td>miR-466i whole promoter</td>
<td>miR-466i_P (-6009/-5008)</td>
<td>5'-CTCAGGTATACGGAATAGG-3' 5'-CATTACAGTGGAGATGAC-3'</td>
</tr>
<tr>
<td>9.</td>
<td>miR-466i ΔNF-κB_1</td>
<td>miR-466i_P(Δ-5862/-5852)</td>
<td>5'-TGTTGGTGTGGTGGTGTGGTGGTTATAC-3' 5'-GGCCAGCTAAAAACTC-3' 5'-GAGTTTCTGATCGTATAG-3' 5'-TATAAACACACACAAACACACACA-3'</td>
</tr>
</tbody>
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