Materials

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Methods

2.0 Materials & Methods

2.1 Chemicals

Molecular biology grade reagents were purchased commercially. Poly-L lysine, protease inhibitor cocktail, H_2DCFDA, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride, 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), Thiazolyl Blue Tetrazolium Blue (MTT), Fluoromount, BCA protein estimation kit, Cyclodextrin, Etoposide, LPS, IPTG, Kanamycin, Ampicillin, DMSO, Sodium vandate, PMSF and anti-ITCH antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Annexin V-FITC Apoptosis detection kit, Caspase-8/FLICE fluorometric assay kit, Necrostatin-1, G418, Imidazole, Guanidium chloride, Sepharose-A beads, MG 132, Protein A sepharose beads, Caspase-8/FLICE fluorometric assay kit, 3-Methyl Adenine (3-MA) and JC-1 dye were purchased from BioVision, (Mountain View, California). Dulbecco's Modified Eagle's Medium (DMEM), RPMI 1640, Dulbecco's Phosphate buffer saline (DPBS), Fetal bovine serum (FBS), 0.25% Trypsin EDTA, PSN antibiotic solution, ER tracker Green, Lysotracker DND red, Ni-NTA resins, Caspase-8/FLICE fluorometric assay kit, EnzChek Caspase-3 assay kit, MitoTracker Red CMXRos dye, Tali Apoptosis detection kit, Prestoblue viability assay kit, Lipofectamine, RNAi-MAX transfection reagent, Miniprep Plasmid isolation kit, FG Power SYBR Green MasterMix, MitoTracker Red CMXRos dye, Caspase-3 assay kit, primary antibody for JNK1, Bcl2 and anti-mouse HRP linked secondary antibody and Alexa fluor 635 were purchased from Invitrogen (Life Technologies, USA). The iScript cDNA synthesis kit, IQ Tm SYBR Green RT PCR kit, Clarity Western ECL Substrate, Unstained SDS-PAGE Standards marker (161-0304) and PVDF membrane were purchased from BioRad (USA). The O'GeneRuler 1 kb DNA Ladder (SM1163) was purchased from ThermoFisher (USA). The IL-1β ELISA kit was purchased from eBiosciences (USA). Dual glow NF-κB luciferase assay kit was purchased from Promega (Madison, WI, USA). Primary antibodies against p65, Procaspase-9, Procaspase-3, Procaspase-7, Cytochrome c, PARP, RIP1, NLRP3, Procaspase-1, β-actin, LC3, Bid, COX IV, GAPDH, p53, Beclin-1, His, HMGB1 and anti-rabbit HRP linked secondary antibody were purchased from Cell Signaling Technology (Danvers, MA). Primary antibodies against $cFLIP_L$, IL-1β and FADD were purchased from Novus Biologicals (USA). Rapamycin was purchased from ApexBio (USA). LDH Cytotoxicity Detection Kit and In-Fusion cloning Kit were purchased from TaKaRa-Clontech (USA). HA14-1 was purchased from Thermo Fisher (Maybridge, UK). Annexin V-FITC Apoptosis detection kit was purchased from BD Biosciences (USA). CD95L and TNF-α was purchased from ProSpec (Israel). Anti-Ubiquitin primary antibody and Cyto ID Autophagy detection

kit were purchased from Enzo life sciences (USA). Anti-cIAP2 and anti-caspase-8 were purchased from BD Pharmigen (USA). Anti-IKKβ was obtained from Abcam (USA). Luria agar, Luria broth and EZ Blue™ cell assay kit were purchased from HiMedia (Mumbai, India). All Restriction enzymes and T4 DNA ligase kit were purchased from New England Biolabs (USA). All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany).

2.2 *In vitro* cell culture

The HEK 293T, NIH 3T3, RAW 264.7, HCT 116 and HaCaT cell lines were obtained from ATCC (Manassas, VA, USA). MCF-7, HT-29, HeLa and A549 were obtained from NCCS, pune India. HEK 293T, MCF-7, HeLa, HaCaT and NIH 3T3 cells were grown in DMEM culture media containing L-glutamine (2 mmol/l). HT-29, RAW 264.7, HCT 116, and A549 cells were grown in RPMI-1640 culture media containing L-glutamine (2 mmol/l). All the media were supplemented with 10% (v/v) heat inactivated FBS and 1% (v/v) penicillin-streptomycin-Neomycin. The cells were kept in a humidified atmosphere of 95% O_2 and 5% CO_2 in a CO_2 incubator at 37^0C. Exponentially growing cultured cells were used for all the experiments. The adherent cells were routinely passaged by trypsinization protocol. In brief, the culture medium form the cell culture flask was aspirated and the cells were washed once with pre-warmed DPBS (pH-7.0). Further, cells were incubated with 0.25% trypsin/EDTA solution and incubated at 37°C for 3 min, the detached cells were re-suspended in pre-warm culture medium and centrifuged at 1100 rpm for 2 min. The cell pellet was re-suspended in fresh pre-warmed culture medium and split into the new flasks in 1:4 ratio of cell suspension to culture medium. The remaining cell suspension was used for plating and stock preparation.

2.3 Plating and Stock preparation

The freshly splitted cells were counted with the aid of haemocytometer. The number of cells were calculated as: Average no of cells counted in haemocytometer X dilution factor X 10^4. The remaining cells were spun down and the pellets were re-suspended in 90% heat inactivated FBS and 10% DMSO followed by storage in a cryocooler at -80^0C.

2.4 *In Vitro* transfection and expression of proteins

2.4.1 Lipofectamine method

The cells were plated in the respective culture plate/ dish and incubated at 37^0C for 16-24 h in a CO_2 incubator to reach the confluency of about 60-70%. One hour prior to transfection, the culture medium of the plate/ dish was replaced with fresh medium. The transfection of plasmids was carried by using Lipofectamine LTX Plus reagent (Invitrogen, USA) as per manufacturer's instructions and the cells were incubated for 24 h. The transfection efficiency was monitored by fluorescent microscopy and Western blotting. Following mentioned plasmids were used in this study: pEGFP- Bcl-2, pmCherry-Bid, pLXSN cFLIP$_{L,}$ pEGFP-Cytochrome c, pmCherry-Bid, pCR3-DM-FLIP, pEYFP-FADD, pEYFP-FADD-DD, pcDNA-FADD, pcDNA-FADD-SLT2, pcDNA-FADD-SLT4, pECFP-IκBα, pEGFP-LC3, pCR3-MC 159 (cFLIP$_L$), pGL3b-NFκB-Luc, pRL-TK, pEGFP-p65 as mentioned in our published research article by Ranjan and Pathak 2015; Ranjan & Pathak, 2016.

2.4.2 Calcium Phosphate method

Cells were also transfected by calcium phosphate transfection method (Song and Lahiri, 1995). In brief, $3.5x10^5$ cells were seeded in 6-well plate and incubated for 16-24 h. Media were changed 1 h prior to transfection and fresh media added to each well. The reaction mixture containing 2 µg of pEYFP-FADD, 15 µl of Calcium Chloride, 150 µl of 2X HEPES buffer in a final volume of 300 µl was added to each well and incubated for 8 h in a CO_2 incubator (NuAire, Plymouth, USA) supplied with 5% CO_2 and 95% O_2 at 37^0C. Culture medium was aspirated from the wells after every 24 h and replaced with fresh medium. Transfection efficiency was examined under a fluorescence microscope (DP71, Olympus, Japan) at different time intervals. (Ranjan et al., 2012).

2.4.3 Evaluation of plasmid transfection

To confirm the expression of transfected plasmid constructs, post 48 h of transfection the total protein was extracted from the cells and subjected to Western blot analysis.

2.5 Knockdown of cFLIP$_L$ by siRNA

The siRNA oligoncleotides targeted against cFLIP$_L$ were custom synthesized from Invitrogen (Life Technologies, USA). In brief, HEK 293T cells were seeded at a density of $5x10^5$ cells in a 6 well plate ($1x10^6$ cells in a 60 mm dish) and incubated for 16 h followed by transfectetion of siRNA-cFLIP$_L$ (75 nM) along with 7.5 µl of RNAiMAX™ (Life Technologies, USA) in a total volume of 200 µl per well in serum free OptiMEM culture

media (Life Technologies, USA). Cells were incubated with complex of siRNA-cFLIP$_L$-RNAiMAX™ for further 48 h (in this study the knockdown of cFLIP$_L$ were carried out for 48 h in the mention experiments). The non-targeting siRNA was taken as a negative control as mentioned in our published research article by Ranjan and Pathak 2016; Ranjan & Pathak, 2016.

2.6 Cell Viability assay

The cell viability assay was performed by trypan blue exclusion assay. In brief, treated cells at various time intervals were harvested, washed once with Dulbecco's Phosphate Buffered Saline (DPBS) and resuspended in 100 µl of DPBS containing 0.4% trypan blue and incubated for 15 min in the dark at 37^{0}C. The cell suspension (10µl) was placed on Neubauer's slide of hemocytometer and live and dead cells were counted under a microscope (Olympus, Japan). The percentage of cell death was calculated (% cell death = Number of dead cells/Total number of cells x100). In addition cell viability was also assessed in certain experiments using a Prestoblue cell viability kit (Life Technology, USA) according to the manufacturer's instructions. In brief, 2 × 10^4 cells were seeded in a 96 well plate and incubated for 24 h, further cells were treated as mentioned in the figure legends. At the end of incubation, 10% prestoblue reagent was added to the total volume of culture medium and allowed to incubate for 2 h in a CO_2 incubator at 37^{0}C. Thereafter, the absorbance of each well was recorded at 570 nm using a Multimode micro plate reader (Molecular Devices, USA). The results were represented in terms of percentage of cell viability (Ranjan et al., 2012; Ranjan & Pathak, 2016).

2.7 Cell Proliferation Assay

The proliferative capacity of cells was examined by MTT assay. In brief, 2x10^4 cells were grown in a 96-well plate for 24 h and cells were treated as mentioned in the figure legends. At the end of incubation, 10% MTT solution was added to the total volume of culture medium and allowed to incubate for 4 h in a CO_2 incubator at 37^{0}C in the dark. Thereafter, 0.5 ml of SDS-HCl was added to the each well, mixed thoroughly and incubated for 4 h at 37^{0}C in the dark. The absorbance of each well was recorded at 570 nm using a Multimode micro plate reader (Molecular Devices, USA). The results were represented in terms of percentage of cell proliferation (Ranjan & Pathak, 2016).

2.8 Apoptotic Cell Death Analysis

2.8.1 Propidium iodide (PI) staining

Apoptotic cell death was monitored by AnnexinV-FITC/PI staining using an apoptosis detection kit (BioVision, USA). In brief, $5x10^5$ cells were grown on poly L-lysine coated coverslips kept in six-well plates for 24 h and then cells were treated as mentioned in the figure legends. Post incubation, coverslips were removed and washed once with DPBS. Thereafter, a mixture of 100 µL binding buffer, 1 µL of PI (20 µg/ml) and 1 µL of Annexin-V FITC was added on a coverslip and incubated for 15 min in dark at room temperature. Annexin/PI stained cells were detected under a fluorescent microscope (DP71, Olympus, Japan). More than 150 cells from three random fields were taken to examine the percentage of cell death. All the images were analyzed by image analysis software (Image-Pro MC 6.1, Bethesda, MD, USA) (Ranjan et al., 2012).

2.8.2 Evaluation of apoptotic cell death by image based cytometer

Apoptotic cell death was validated by using Tali™ image based cytometer (Life Technologies, USA). For analysis of cell death $1x10^6$ cells were grown in six-well plate for 24 h and treated as mentioned in the figure legends. After completion of incubation apoptotic cell death was analyzed using the apoptosis detection kit (Life Technologies, USA) under the image based cytometer as per manufacturer's instructions. The observation was taken from 20 random fields to examine the apoptotic cell death (Ranjan et al., 2014).

2.8.3 Flow Cytometry

The apoptotic cell death was also confirmed by flow cytometry (FACSAria 3, BD Biosciences, San Jose, CA, USA). In brief, cells were seeded at a density of 2×10^6 cells in a 60mm dish and incubated for 24 h. Cells were subjected to treatments as mentioned in the figure legends. Thereafter, cells were collected, washed and re-suspended in 1X Annexin binding buffer followed by the addition of Annexin-V-FITC and Propidium Iodide solution (BD Biosciences, New Jersey, USA). Cells were incubated in the dark for 15 min at room temperature and thereafter subjected to flow cytometric analysis (BD FACSAria, BD Biosciences, San Jose, CA, USA). Data were acquired by BD FACSDiva software (BD Biosciences, San Jose, CA, USA) using standard fluidics, optical and electronic configuration. The light source used was blue laser 488nm with filters, FITC (530/30) and PI (585/42). The FITC and PI channels were compensated with appropriate controls. The

Gating on the cell population was set up by FSC/SSC scatter plot. 10,000 events were recorded and analysed for Annexin-V/Propidium Iodide stains. The results represented in contour plots with quadrant gates showing early apoptosis in quadrant 4 (Q4) and late apoptosis in quadrant 2 (Q2). (Ranjan & Pathak, 2015).

2.9 Colony formation assay

The colony formation assay was performed by crystal violet staining method. In brief, 1x 10^5 cells were seeded in a 24 well plate and incubated for 18 h. cell were treated as described in the figure legends. Post incubations, cells were trypsinized and 1000 cells were seeded in a 6 well plate containing culture medium. Cells were allowed to grow for 10 days, until small colonies were visible. The colonies were fixed with methanol and stained with 0.2% crystal violet stain. The ability of a single cell to survive and to grow in a form of colony was considered as plating efficiency. Plating efficiency was defined by the following formula: Percentage plating efficiency (PE) = (Number of colonies formed/Number of cells seeded) x100 (Waghela et al., 2015; Ranjan & Pathak., 2016).

2.10 Establishment of GFP-LC3 stable cell line and autophagy assay

The GFP-LC3 stable cell line was prepared for autophagy assay. In brief, HEK 293T cells were transfected with 2µg of purified plasmid, pEGFP-Light Chain 3 (LC3) (kindly gifted by Dr. Tamotsu Yoshimori, Osaka University, Japan), using X-treameGENE 9 transfection reagent (Roche, Basel Switzerland) according to manufacturer's instructions. The stable clones expressing GFP-LC3 were selected by addition of G418 (400 µg/mL) in the culture medium. The cells were incubated for 24 h and the medium was changed after every three days. After three weeks, GFP-LC3 expressing stable cells were validated under the fluorescent microscope and used for further assays. Autophagosome formation for induction of autophagy was detected by the formation of GFP-LC3 puncta within autophagic vacuoles of cells. Cells stably expressing GFP-LC3 were plated at a density of 1x 10^4 on poly L-lysine treated glass coverslip and exposed to the treatment as mentioned in the figure legends. GFP-LC3 puncta were examined under an inverted fluorescence microscope. A minimum of 150 cells from random fields were analysed and the number of puncta present per cells was counted with the aid of NIH image software (ImageJ, NIH, USA) (Ranjan et al., 2014; Ranjan & Pathak, 2016).

2.11 Detection of autophagic vacuoles

The formation of autophagic vacuoles was detected by Cyto-ID® Autophagy Detection Kit (Enzo Life Sciences, USA). In brief, HEK 293T cells were seeded at a density of $1x10^5$ cells per well in a 24 well plate and incubated for 24 h. Cells were subjected to siRNA directed against $cFLIP_L$ (48 h) and Rapamycin (100 nM for 3 h) as mentioned in the figure legends. At the end of incubation, cells were washed once with DPBS followed by addition of Cyto-ID® Autophagy Detection reagent diluted in the culture medium and incubated for 30 min in the dark. The cells were counter stained with nuclear staining dye Hoechst 33342 and observed under a fluorescent microscope (Olympus, DP 71, Japan), more than 150 cells from three different fields were analyzed. The percent change in the intensity was evaluated by counting the cells with the aid of NIH image software (ImageJ, NIH, USA) (Ranjan & Pathak, 2016).

2.12 Mitochondria staining

To examine the mitochondrial mass, the mitochondrial staining was performed using MitoTracker® Red CMXRos dye (Invitrogen, USA) according to manufacturer's instructions. In brief, $1x\ 10^5$ cells were grown on poly L-lysine coated coverslip kept in a 24 well plate and incubated for 18 h, further cell were treated as described in the figure legends. The treated cells were stained with MitoTracker dye for 30 min in the dark at 37^oC followed by counterstaining with DAPI for 5 min in the dark and then covered with fluoromount mounting medium (Sigma-Aldrich, USA). The images were visualized under the laser scanning confocal microscope (Leica TCS SP5 II, Germany) (Ranjan & Pathak, 2016).

2.13 p65 translocation assay

The cells were seeded at a density of 1×10^5 on a poly L-lysine coated coverslip kept in a 24 well plate and incubated for 24 h. Thereafter cells were transfected with pEGFP-p65 using lipofectamine LTX-plus reagent (Invitrogen, USA) and expressed for an additional 24 h. Cells were further subjected to treatments as mentioned in the figure legends. The cytosol to nuclear translocation of GFP tagged p65 was monitored under a fluorescent microscope (DP71, Olympus, Japan). More than 150 cells from three random fields were analyzed (Ranjan & Pathak, 2016).

2.14 Luciferase reporter assay for determination of NF-κB activation

The cells were seeded at a density of 1 × 10^5 cells in a 24 well plate and incubated for 24 h. Thereafter, cells were co-transfected with pNFκB-Luc, a luciferase reporter plasmid and pRL-TK, a thymidine kinase promoter-Renilla luciferase reporter plasmid using lipofectamine LTX plus transfection reagent (Invitrogen, Life Technologies, USA) according to manufacturer's instructions. Cells were subjected to treatments as mentioned in the figure legends. The NF-κB luciferase reporter assay was performed with Luciferase Assay Kit (Promega, USA) as per manufacturer's instructions. At the end of incubation, cells were lysed in 100 µl of lysis buffer and cell lysate was mixed with 1:5 ratio of luciferase assay reagent (LARII). The values of firefly and renilla were recorded in a Luminometer (Centro LB 960, Berthold, USA). The results were expressed as the ratio of firefly luciferase activity to that of renilla and normalized to protein concentration (Ranjan & Pathak, 2016).

2.15 Measurement of mitochondrial membrane potential (ΔΨm)

In order to quantify the change in mitochondrial membrane potential (ΔΨm), a lipophilic fluorescent probe JC-1 (Biovision, USA) was used for analysis. JC-1 probe in healthy cells exists as J-aggregates and gives red fluorescence with high mitochondrial membrane potential, but as the mitochondrial membrane loses the integrity during apoptotic conditions, aggregates disperse into monomers and give a green fluorescence. Therefore, a ratio of red/green fluorescence intensity has been taken as an indicator of the relative mitochondrial membrane potential. In brief, 1x10^5 cells were grown on a poly L-lysine coated coverslip kept in a 6-well plate and incubated for 24 h. Cells were further treated as mentioned in the figure legends. Subsequently, medium was changed and co-incubated with JC-1 dye (5 µg/ml) for 20 min followed by counterstained with DAPI (1 µg/ml for 5 min) in the dark. The cells were examined under laser scanning confocal microscope (Leica, Germany). In addition, 2x10^4 cells were grown in a 96-well plate and incubated for 24 h. Cells were further treated as mentioned in the figure legends. Subsequently, medium was changed and co-incubated with JC-1 dye (5 µg/ml) for 20 min in the dark. The fluorescence was quantified at excitation and emission wavelengths of 485 nm and 525 nm, respectively, using a Multimode microplate reader (Molecular Devices, USA) (Ranjan et al., 2014, Ranjan & Pathak, 2016).

2.16 Confocal microscopy

The confocal microscopy was carried out for Immunofluorescence analysis, MitoTracker staining and JC-1 staining. In brief, $1x10^5$ cells were grown on poly-L lysine coated glass coverslips kept in a 24-well plate and allowed to incubate for 24 h. The adherent cells were subjected to treatments as mentioned in the figure legends. Post incubation, cells were washed with DPBS (pH 7.4) and fixed with 4% paraformaldehyde at room temperature for 5 min. Cells were stained as mentioned in figure legends and washed with DPBS and counterstained with DAPI (1 µg/ml) or propidium iodide (0.5 µg/ml) for 5 min in the dark and covered with fluoromount mounting medium (Sigma-Aldrich, USA). The images were captured with a laser scanning confocal microscope (Leica SP5, Germany). All the images were further analysed and processed with Leica SP5 II software (Leica TCS SP5 II, Germany) (Ranjan et al., 2014; Ranjan & Pathak, 2016).

2.17 Real Time-qPCR (RT-qPCR)

A Real-time PCR was performed using the Step One plus real-time PCR detection system (Applied Biosystems, Carlsbad, California). The reaction was conducted in 20 µl final reaction volume containing 4 µl of cDNA (230 pmolar), 10µl iQ™SYBR® GreenSupermix (Bio-Rad, USA) and 2µl of each primer (1 µM) and remaining 2 µl of nuclease free water. All reactions were performed in MicroAmp fast optical 96-well PCR plates (Applied Biosystem) and sealed with optical adhesive covers (Applied Biosystem). 18S rRNA was used as the endogenous control (Ranjan et al., 2012; Ranjan & Pathak, 2016).

Thermal cycler conditions were as follows: Initial denaturation- 95 °C for 10 min; Denaturation- 95 °C for 15 s; Annealing/extending- as per primer sequences for 1 min, Total no. of cycles- 40. Afterwards, final PCR products were heated to 72 °C for 30 s and the products of the expected size were confirmed by melting curve analysis. Each assay was normalized by using the difference in critical thresholds (CT) between target genes and 18S rRNA. The expression of mRNA of respective genes was compared with control using the values of $2^{-\Delta\Delta CT}$. The following sets of primers were used in this study:

Gene	**Sequences**
	Real Time quantitative PCR (RT-qPCR) primers
RIPK1	F-AATGGCGGCACCCTCTACTA R-TCCGACTTCTCTGTGGGCTTT

cIAP2	F-TCCAAGGTGTGAGTACTTGATAAGAATT R-CTGATGTGGATAGCAGCTGTTCA
IL-1β	F-GACAACGAGGCGTACGTTCA R-CGATTTCTGTTGACTATCCCGTAA
p65	F-GCCGGGATGGCTTCTATGA R-TGGATTCCCAGGTTCTGGAA
18SrRNA	F-AGAAACGGCTACCACATCCAA R-TGTCACTACCTCCCCGTGTCA
cFLIP$_L$	F-TGGCCTCCCTCAAGTTCCT R-TGGAATAACATCAAGGCATCCTT
FADD	F-GTGTGCGGGAGTCACTGAGA R-GGGCCACTGTTGCGTTCT
Pro-CASPASE-8	F-TGGCCTCCCTCAAGTTCCT R-TGGGTTCTTGCTTCCTTTGC

Table 1. RT-qPCR primers. Following primers were used in this study.

2.18 Sucellular fractions, SDS-PAGE and Western blotting

In brief, 3.5x 10^5 cells per well in a 6-well plate (1x10^6 cells in a 60 mm dish) were seeded and incubated for 24 h. Cells were further treated as mentioned in the figure legends and harvested at the mentioned time point followed by washing in DPBS (pH 7.4). Cells were resuspended in lysis buffer (50 mM Tris Cl, pH 8; 150 mM NaCl, 1 mM MgCl2, 150 mM CaCl2, 1 mM PMSF, 1 mM Na-Vanadate) containing protease inhibitor cocktail and kept on ice for 30 min followed by disruption and subsequent centrifugation at 8,000 rpm (5415R, Eppendorf, Germany) for 10 min at 4^{o}C. The supernatant was collected and labeled as cytosolic fraction. Further, the cell pellet obtained from cytosolic fraction was resuspended in mitochondrial extraction buffer (50 mM TrisHCl pH 6.8, 250 mM sucrose, 1 mM EDTA, 0.5% Triton-X-100, protease and phosphatase inhibitors) and spun down at 11,000 g for 10 min at 4^{o}C and supernatant was collected and labeled mitochondrial fraction. The resulting supernatant was collected and protein concentration was determined using a BCA protein estimation kit (Sigma Aldrich, USA) according to manufacturer's protocol. The equal amount of protein (30 µg) from each sample was fractionated on 12 % SDS-PAGE as described in Laemmli, 1970. The fractionated proteins were transferred to PVDF membrane by wet electroblotting method at 4 °C. The membrane was blocked with 5% nonfat milk in Tris buffered saline for 3 h at room temperature followed by overnight incubation with primary antibodies as per

recommended dilutions at 4^0C. After washing with TBST (0.01% Tween-20), the membrane was probed with horseradish peroxidase conjugated secondary antibodies (1:10,000). Expression of immune reactive protein was detected by using Novex® ECL HRP linked chemiluminiscent substrate kit (Invitrogen, USA) according to the instruction manual and developed in Kodak X-Omat blue film (NEN Life Sciences, Inc., Boston, MA) in the dark.

2.19 Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA assay of human cytokine IL-1β was performed by commercially available kits for human IL-Iβ (eBioscience, USA) as per manufacturer's instructions. In brief, $2x10^4$ cells were seeded in a 96-well plate and cultured for 24 h in a CO_2 incubator. Cells were treated as described in the figure legends. Post incubation, the supernatant was collected and centrifuged (850 x g for 10 min). Further, the supernatant was incubated in antibody coated micro plate for 16 h at 4^0 C. After completion of incubation the wells were washed three times (1 min each). Further, the diluted detection antibody was added to each wells and incubated for an additional 1 h at room temperature. Post incubation, the avidin-HRP was added to each well and incubated for an additional 30 min at room temperature followed by three washes. Finally, the substrate solution was mixed to each well and incubated for 15 min at room temperature followed by addition of stop solution. The absorbance of each well was recorded at 450 nm with background subtraction at 570 nm using a Multimode micro plate reader (Molecular Devices, USA). The results were represented in terms of picogram (pg) release of IL-1β as compared to control.

2.20 Immunostaining

In brief, $1x10^5$ cells were seeded on poly L-lysine coated coverslips kept in a 24 well plate and incubated for 24 h. Cells were further subjected to treatments as mentioned in the figure legends. Further cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS at room temperature for 30 min. Coverslips were then washed three times for 5 min each with DPBS (pH 7.4) and then blocked for 1 h in 1% BSA at room temperature. Cells were incubated with Primary antibodies (anti-FADD, anti-$cFLIP_L$, anti-His) at a dilution of 1:50 for 16 h at 4°C. Coverslips were washed three times for 5 min each with TBS and incubated at room temperature for 1 h with the Alexa conjugated secondary antibody at a dilution of 1:100. The nucleus was counterstained with DAPI (1 μg/ml) or propidium iodide (0.5 μg/ml) for 5 min at room temperature. Cover-slip were then washed two times with TBS and mounted with Fluoromount

mounting media. The images were visualized under the laser scanning confocal microscope (Leica TCS SP5 II, Germany) (Ranjan & Pathak, 2016).

2.21 Co-immunoprecipitation analysis

2 x10^6 cells were seeded in a 60 mm dish followed by treatments as mentioned in the figure legends. The cells were lysed in 1 ml of Co-IP lysis buffer for 30 min on ice. The total cell lysate was centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was collected and protein concentration was determined by the BCA protein estimation kit (Sigma, USA). Next, 300 µg of protein was preincubated with 20 µl of Sepharose A beads (BioVision, USA) for 1 h at 4°C with gentle shaking. The mixture was spun down at 3000 rpm for 5 min at 4°C and the collected supernatant was incubated with 1µg of respective antibodies and 20 µl of Sepharose A beads for overnight at 4°C with gentle shaking. The beads were washed three times with 1 ml of RIPA buffer and finally resuspended in 6X Laemmli sample buffer. The protein samples were fractionated on 12% SDS-PAGE followed by Western blot as described above (Ranjan & Pathak, 2016).

2.22 Caspases 8 and Caspase 3 activity assay

The assay was performed using the Caspase-8/FLICE fluorometric assay kit according to the manufacturer's instructions (Biovision, U.S.A.). Post incubations, the treated cells were washed twice with the DPBS and spun down at 6000 rpm (5415R, Eppendorf, Germany) for 10 min. The pellet was resuspended in 50 µl of chilled lysis buffer and incubated on ice for 10 min. 50 µl of lysate was mixed with equal volume of reaction buffer and thereafter substrate IETD-AFC was added into the reaction mixture. The incubation was done at 37 °C for 30 min. At the end of incubation, the liberated fluorescence (Ex-400 nm; Em-505 nm) was estimated using a Multimode microplate reader (Molecular Devices, USA). The result represented fold activity of caspases-8 with respect to control cells. Next, caspase-3 activity was determined by EnzChek VR Caspase-3 Assay Kit (Life Technologies, USA) as per manufacturer's instructions. The result represented fold activity of caspases-3 with respect to control cells (Ranjan et al., 2012; Ranjan et al., 2014).

2.23 ROS measurement

To determine the level of ROS in culture media and inside the cells, a fluorescent dye H_2DCF-DA was used. In detail, 2 x10^4 cells were plated per well in a 96-well plate and incubated in a CO_2 incubator for 24 h to reach the confluency. Cells were subjected to treatment as mentioned in the figure legends. Thereafter, cells were coincubated with

H_2DCF-DA (25 µM) for 30 min at 37^{0}C in the dark and washed with ice cold PBS (pH-7.4). The cells were lysed in DPBS (pH 7.4) containing 0.1% Triton-X 100 with vigorous vortexing for 5 min and finally spun down at 10,000 rpm (Eppendorf, 5241R) for 5 min at 4^{0}C. The supernatant was collected and subjected to monitoring the fluorescence value of DCF molecules at the excitation of 485 nm and emission of 525 nm using a Multimode micro plate reader (Molecular Devices, USA) (Ranjan et al., 2014; Ranjan & Pathak, 2016).

2.24 Cytotoxicity assay

The necrotic extracellular release of lactate dehydrogenase (LDH) was analysed by LDH cytotoxicity detection kit (Takara-Clontech, USA). Cells were seeded at a density of 2 x 10^4 cells in a 96 well plate and incubated for 24 h. Cells were subjected to treatments as mentioned in the figure legends. Post incubation, the culture media were equally mixed with the reaction buffer and incubated for 30 min at room temperature in dark. The absorbance was recorded at 490 nm with reference at 650 nm using a Multimode microplate reader (Molecular Devices, USA). The percentage LDH release was calculated relative to that of control (Ranjan & Pathak, 2016).

2.25 Purification, Characterization and conjugation of hFADD

2.25.1 Isolation of total RNA from mammalian cells

Total RNA was isolated with Trizol reagent (Invitrogen, USA) according to manufacturer's protocol. To prevent contamination with RNases, the gloves were worn and working table was wiped with RNAZap solution, RNase-free tubes, filter pipette tips, glassware and solutions were used. The concentration of total RNA was calculated by taking the OD at 260 nm/ 280 nm under UV–Vis spectrophotometer (Molecular Devices, USA). The concentration of RNA was calculated with the following formula:

RNA concentration (µg/ml) = $OD_{260\ nm}$ × 40 × dilution factor.

Furthermore, the quality of the isolated RNA was examined by running the samples on a 1.5% agarose gel and visualization under Gel doc system (Vilber Lourmat).

2.25.2 Synthesis of cDNA and amplification of FADD

The principle of reverse transcription was employed to convert mRNAs into cDNAs by using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). In brief, 1 µg of total mRNA was mixed with 1X assay buffer containing 10mM dNTPs, primers of FADD (forward and reversed- 1 µM of each) FADD-Forward primer-

CGCGGATCCATGGACCCGTTCCTGGTGCTGCT; Reverse primer-CCGGAATTCTTAAGACGCTTCGGAGGTAGATGCGT and 1X reverse transcriptase enzyme and DNAse/ RNAse free H_2O to make the final volume to 20 µl. This reaction mixture was kept on thermo cycler (BioRad, USA) and incubated as per manufacturer's instructions. The final product of PCR amplicon were quantified as follows:

cDNA concentration (µg/ml) = $OD_{260\ nm}$ × 50 × dilution factor.

The cDNA amplicons was subjected to gradient PCR. A typical 20 µl PCR reaction mixture consists of: Template DNA-25 ng, forward primer-20 pmol, reverse primer-20 pmol, 10 mM dNTP mix-1 µl, 10 x PCR buffer-5 µl, DNA Taq polymerase-1.0 U and Dnase free H_2O to 20 µl. The reaction mixture was kept in a thermocycler (BioRad, USA) with the following program of a preheating step of 5 min at 95°C, followed by 30-35 cycles of denaturation for 30-60 sec at 95°C, annealing temperature was from the gradient of 60-70°C for 45 sec. The extension was fixed for 1:30 min/kb at 72°C followed by final hold at 4°C for 30 min. The obtained product was examined by running on a 1.2% agarose gel and visualized under Gel doc system (Vilber Lourmat, Germany).

2.25.3 Cloning of the human FADD (hFADD)

Furthermore, the cDNA amplicon of human *FADD* was purified from agarose gel and were double digested with the high fidelity restriction endonucleases (REs) *Eco*R1 and *Bam*H1. Similarly, the bacterial expression vector pET28a (+) was double digested using restriction enzymes *Eco*R1 and *Bam*H1 according to the manufacturer's instructions (NEB, USA). The REs digested products of the inserts and vectors were extracted from 1.2% agarose gel and further quantified by UV-spectrophotometer. Ligation of the inserts and the vector were carried out by T4 DNA ligase (NEB, USA) under optimum condition at 25^0 C for 16 h by taking the 3 (insert): 1 (vector) ratio of oligonucleotides and according to the manufacturer's instructions (NEB, USA). The ligation mixture was transformed into competent cells of *E. coli* DH5α strain and selected with a kanamycin antibiotic selection marker. The recombinant clones of pET28a–FADD (pET-FADD) were confirmed by PCR amplification and the restriction digestion analysis.

2.25.4 Transformation and expression of pET-FADD

Preparation of competent cells:

Competent E.coli cells were prepared by the CaCl2 method. A single colony of E. coli BL21 (DE3)-pLys were grown in 20 ml of antibiotic-free LB medium at 37°C overnight. This 1 % of bacterial culture was then diluted in 100 ml of pre-warmed LB medium and cultured

until an OD600nm of 0.4-0.6 was achieved. Further cells were cooled on ice for 20 min and then centrifuged 3000 g for 15 min. The cell pellet was resuspended in 50 ml of sterilized pre-cold 0.1 M CaCl2 and incubated for 30 min at 4°C. The suspension was centrifuged at 3000 g for 15 min, and the pellet was gently resuspended in 5 ml 0.1 M CaCl2 solution containing 10% glycerol. The 50 µl aliquots of the competent bacteria suspension were transferred to pre-chilled tubes and stored at -80°C for future use.

Expression of pET-FADD:

For the stable expression of human His tagged FADD, the recombinant clones of pET-FADD were transformed into the competent cells of *E. coli* BL21 (*DE3*)-pLys in the presence of kanamycin (30 µg/ml) and incubated at 37^0C for 18 h in an incubator. Next day, a single colony from the grown plate was inoculated in a 10 ml of fresh LB medium containing kanamycin and incubated over night at 37^0C on a rotating shaker. Post incubation, the 1% of grown culture was further inoculated into 200 ml of fresh LB culture medium containing kanamycin and incubated at 37^0C till the OD at 600 nm to reach 0.2-0.25. The expression of His-FADD was induced with 0.5 mM IPTG and incubated at 25^0C for an additional 4 h on a rotating shaker. Post incubation, 1 ml induced culture was spun down at 4000 g for 5 min and the cell pellet was mixed with 6X loading dye (4X Tris buffer (pH 6.8)-7 ml, Glycerol- 3 ml, SDS- 1g, β-ME-0.6 ml, Bromophenol blue-0.15 mg) and boiled for 10 min. The induced band of His-FADD was analyzed by SDS-PAGE analysis followed by Western blotting. The data were compared with vector transformed cells and recombinant clone transformed cells without IPTG induction.

2.25.5 Purification of human FADD (hFADD)

The culture of *E. coli* BL21 (*DE3*)-pLys containing recombinant clones of His-FADD was spun down at 8000 rpm for 15 min and the pellet was suspended lysis bufer (50 mM sodium phosphate buffer (pH 8.0), 150 mM NaCl, 1 mM PMSF and 0.1% Triton-X 100) followed by ultrasonication for 15 min on ice. The sonicated fraction was spun down at 8000 rpm for 20 min and the pellet (insoluble fraction) was resuspended in refolding buffer (20 mM TrisHCl (pH 8.0), 100 mM NaCl, 6 M Gn-HCl, 10 mM β-mecaptoethanol, 20 mM Imidazole and dH_2O) followed by overnight agitation at 4^0C. The fraction was centrifuged at 12,000 rpm for 20 min at 4^0C. The supernatant was added to the Ni^{2+}-NTA affinity column pre-equilibrated with equilibration buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Triton-X 100 and 10mM βME). The Ni^{2+} NTA column was washed with wash buffer (50 mM sodium phosphate (pH 8.0), 20 mM imidazole, 150 mM NaCl) to remove nonspecific bound proteins. The His tag hFADD (His-hFADD) was eluted with elution

buffer (50 mM sodium phosphate (pH 8.0), 300 mM imidazole, 150 mM NaCl). Different eluted fractions were collected and analyzed by SDS-PAGE. Furthermore, the eluted fractions containing human His-FADD protein was quantified by UV-Vis spectrophotometer and BCA protein estimation kit (Sigma Aldrich, USA). The degree of purity was confirmed by MALDI-TOF analysis.

2.25.6 *In vitro* protein-protein interaction

To examine the biological activity of purified hFADD, the total cell lysate of HCT 116 cells were equally mixed with purified hFADD. The mixture was incubated at 23^0C for 18 h with gentle shaking. Furthermore, the mixture was co-immunoprecipitated with anti-$cFLIP_L$ antibody and incubated for an additional 4^0C for 18 h with gentle shaking. The co-immunoprecipitated product of hFADD-$cFLIP_L$ was washed with 1 ml of RIPA buffer ((50 mM Tris Cl, pH 8; 150 mM NaCl, 1 mM MgCl2, 150 mM CaCl2, 1 mM PMSF, 1 mM Na-Vanadate, 0.1 % Triton X-100) and resolved on 12% SDS-PAGE followed by Western blotting using anti-His antibody.

2.25.7 Conjugation of purified FADD with a cell permeable (CP) peptide

The conjugation of purified hFADD with CP peptide was carried out by chemical conjugation method. (The protocol is under the process of patent).

2.25.8 Fluorescence spectroscopy

Fluorescence emission spectra of purified hFADD, CP peptide and conjugated CP-FADD were recorded by a wavelength scanner (Molecular Devises, USA) using a protein concentration of 50 mg/ml in 20 mM Tris-HCl (pH 8.0) taking elution buffer (50 mM sodium phosphate (pH 8.0), 300 mM imidazole, 150 mM NaCl) as a reference. Fractions were kept in a semimicro quartz cuvette of 1 cm light path length and excited at 280 nm followed by recording of emission spectra from 300 to 400 nm. The excitation and emission spectral bandwidths were both 5 nm.

2.25.9 MALDI-TOF mass spectrometry

Protein spots were excised from the gel and a “control” piece of gel was cut from a blank region of the gel and processed in parallel with the sample. The gel pieces were incubated with 10 mM dithiotreitol (DTT) in 100 mM NH_4HCO_3 for 1h at 56^0C followed by trypsinization. The 0.5 µl aliquots of analyte solution were deposited onto these matrix surfaces. All mass spectra were obtained on a modified Bruker REFLEX mass spectrometer (BrukerFranzen Analytik, Bremen, Germany).

2.25.10 FT-IR spectral analysis

FT-IR spectra were recorded in the wavelength range of 4000–700 cm−1 at 4 cm−1 resolution and an interferogram of 64 scans was co-added to each sample. Spectral data were displayed in terms of transmission and viewed using Win-IR Pro Software (Agilent Technologies Inc., Santa Clara, CA, USA).

2.26 *In silico* protein-protein interaction

Unavailability of suitable crystallographically resolved structure of death domain of RIP1 (RIP1-DD; 583-669) and FADD (FADD-DD; 97-181) in the available structural databases, forced us to construct a molecular model of both by computational techniques. The 3D models of death domain (DD) of FADD (97-181; PDB id: 2GF5) with 100% sequence identity and more than 99% confidence and DD of RIP1 (583-669; PDB ids: 2YQF, 2OF5, 1FAD, 1DDF, 4O6X, 1WXP) with a sequence identity ranging from 24-33% and more than 99% confidence level, were generated by Phyre2 protein modelling tool. The generated models of both death domain sequences were inspected with the help of CHIMERA and PyMol (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC) structural tools. The best model was selected for each; based on the sequence identity, query coverage and confidence level of model building. The molecular docking between DD models of FADD and RIP1 was performed by program GRAMMX for unraveling protein-protein interaction (PPI) (Tovchigrechko and Vakser, 2006) (Ranjan et al., 2016).

2.27 Statistical analysis

Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Duncan's multiple range t-test using SigmaStat statistical analysis software. Values were expressed as mean ±S. E. M. from three independent experiments. Differences were considered statistically significant at *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.001.