Discussion

&

Conclusions

4.1 Discussion

Fas associated death domain (FADD) is a pivotal signaling component of death receptor (DR) mediated apoptosis. FADD plays an important role in determining the fate of cell death or survival (Tourneur et al., 2005). Previous reports have shown that FADD also plays an important role in non-apoptotic functions including embryonic development, cell proliferation, cell cycle progression, inflammation, innate immunity, necrosis, and autophagy (Chinnaiyanetal et al., 1996; Yeh et al., 1998; Zhang et al., 1998; Beisneretal et al., 2003; Osborneta et al., 2010; Tourneur et al., 2005). In contrast, a defect in the expression of FADD contributes to evasion of apoptosis and resistance to cancer therapy (Chinnaiyanetal et al., 1996; Tourneur et al., 2005). Earlier reports suggest that low expression of FADD is corroborated with tumor progression in both mice and humans (Tourneur et al., 2003; Tourneur and Chiocchia, 2010). Interestingly, the dual role of FADD in cell death and survival remains elusive. Hence, it was important to delineate the role of FADD in regulation of cell death and survival signaling. The death receptors (DRs) such as Fas (CD95/ Apo) and tumor necrosis factor receptor 1 (TNFR1) (p55/CD120a), belong to the TNF receptor super family and contain cytoplasmic death domain (DD) that helps to execute downstream signal transduction (Dempsey et al., 2003). Upon binding of ligand to the cognate cell surface receptors, the DD of cell surface receptor homophilically interacts with the DD of FADD and induces oligomerization of DED (death effector domain) of FADD with apical caspases such as, procaspase 8/10 to form a death-inducing signaling complex (DISC) (Scaffidi et al., 1998). The formation of DISC facilitates processing and catalytic activation of procaspases-8 and -10 to transduce downstream signaling of apoptosis (Peter and Krammer, 2003). However, the catalytic activation of procaspase-8 and -10 has been negatively regulated by the anti-apoptotic cellular Flice like inhibitory protein (cFLIP) to abrogate apoptotic instigation (Li et al., 1998; Thornberry and Lazebnik, 1998; Wang et al., 2001). Moreover, cFLIP with the aid of NF-κB activation abrogates death receptor mediated apoptosis to support cell survival (Irmler et al., 1997; Krueger et al., 2001; Piao et al., 2012; Safa et al., 2008). Although, the adaptor protein FADD provides a common platform for binding of procaspase-8 and $cFLIP_L$, but $cFLIP_L$ has a high binding affinity with FADD and tightly regulates the processing and activation of procaspase-8 (Krammer, 2000; Scaffidi et al. 2000; Krueger et al., 2001; Chang et al., 2002; Jonsson et al., 2003). Previous reports suggest that low expression of FADD and elevated expression of cFLIP restricts apoptosis and promotes malignancy (Peter and Krammer, 2003; Tourneur and Chiocchia, 2010). Therefore, it was important to

unravel the cross talk between FADD and cFLIP in the regulation of programmed cell death signaling.

The present study was initiated with the examination of endogenous expression of FADD and $cFLIP_L$ in cancer and transformed cells of different origins. The mRNA and protein expression analyses suggested that, an inadequate expression of FADD and elevated expression $cFLIP_L$ was found in the cancer and transformed cells as compared to non-cancerous NIH 3T3 cells. Most of the cell-surface receptors associated adaptor proteins linked to intracellular signaling are likely to reside in the cytoplasm or the nucleus (Screaton et al., 2003; Bender et al., 2005). Earlier reports have shown that, upon death receptor activation the DED region of FADD recruits the procaspase-8 at the cell membrane to initiate cell death (Scaffidi et al., 2000; Peter and Krammer 2003). An interesting report revealed that FADD also possesses a strong nuclear localization (NLS) and nuclear export signal (NES) (Gomez-Angelats and Cidlowski, 2003). In addition, the DD of FADD phosphorylated at Serine 194 residue within the nucleus and participates in cell cycle regulation (Zhang et al., 2004). Next, the subcellular localization of FADD was examined in HEK 293T cells by immunostaining. Notably, expression of endogenous FADD was detected in the nuclear region, whereas transient expression of the FADD (post 48 h) was observed at the periphery of the nucleus and throughout the cytoplasmic space of cells. In contrast, the overexpression of FADD in HEK 293T cells showed a prominent cytosolic localization and expression from 48 to 96 h of post transfection. Furthermore, it was noticed that induced expression of FADD remarkably attenuates the expression of $cFLIP_L$ in HEK 293T cells. In addition, the over expression of FADD facilitated processing and activation of initiator procaspase-8 and executioner procaspase-3 in HEK 293T for initiation of apoptotic cell death (Ranjan et al., 2012). Indeed, the formation of a death inducing signalling complex (DISC) is important for activation of downstream signalling of extrinsic apoptosis (Peter and Krammer, 2003). This signaling cascade is restricted by $cFLIP_L$, which inhibits the binding of procaspase-8 with FADD to abrogate DISC formation (French and Tschopp, 1999; Golks et al., 2006). Therefore, it was important to investigate the interaction of procaspase-8 and cFLIP with FADD at the DISC. The binding proportion of $cFLIP_L$ and pro-caspase-8 at DISC were examined in wild type (wt.) and mutant (mut.) expressing FADD. It was noticed that added expression of wt. FADD in HEK 293T cells selectively interacts with procaspase-8 rather than $cFLIP_L$ at the DISC. Interestingly, mutant FADD restricts $cFLIP_L$ but allows binding of procaspase-8 at the DISC. Altogether, these findings clearly demonstrate that availability of FADD in sufficient amount initiates

apoptosis by providing more FADD for the processing and activation of procaspase-8 at the DISC by counteracting the expression of $cFLIP_L$. It is widely reckoned that, binding of death ligand CD95 (APO-1/Fas) to their cognate receptor promotes the formation of DISC for the commencement of apoptosis (Chinnaiyan 1996; Krammer and Goldman, 2000). In contrast, the elevated expression of $cFLIP_L$ restricts binding of procaspase-8 with FADD and resist CD95 mediated apoptosis in cancer cells (Scaffidi et al. 1998). In fact, an earlier report suggests that the expression of CD95 was downregulated during cancer progression and adapt cells to resist apoptosis (Debatin and Krammer 2004). Moreover, CD95 also plays an important role in maintaining cellular homeostasis apart from apoptosis (Peter et al., 2005). At lower concentrations, CD95 may trigger cell survival by activation of NF-κB pathways, but at higher concentration it may trigger cell death (Golks et al. 2006; Larvik and Krammer, 2012). On the basis of available report, it could be assumed that unavailability of FADD might be one of the possible rational for the inhibition of death receptor mediated apoptosis. Several lines of evidences demonstrate that CD95L stimulates phosphorylation of FADD to facilitate its translocation from the nucleus to initiate the apoptotic instigation (Scaffidi et al., 2000; Alappat et al., 2005). Now, it was important to investigate the cellular localization and apoptotic signaling of overexpressed FADD upon CD95L stimulation in HEK 293T cells. It was noticed that the CD95L stimulation rapidly localized the overexpressed FADD to the periphery of HEK 293T cell membrane post 48 h of FADD transfection. Furthermore, CD95L treatment to HEK 293T cells shows a moderate activation of caspase-8 and attenuation of $cFLIP_L$ expression. In contrast, the stimulation of CD95L to FADD overexpressed HEK 293T cells drastically reduces the expression of $cFLIP_L$ with concomitant processing and activation of procaspase-8, and capsase-3. The above findings suggest that sufficient availability of FADD in response to CD95L regulates the expression of $cFLIP_L$ to execute death receptor signaling of apoptosis. Altogether, induced expression of FADD has enormous potential to assemble DISC and facilitates activation of caspase-8 by attenuating the expression of $cFLIP_L$, independent of death receptor stimulation. (Ranjan et. al., 2012; Ranjan and Pathak, 2016).

Some earlier reports state that, the death receptor (extrinsic) mediated activation of apoptosis signaling is not obligatory to evoke the mitochondria (intrinsic) mediated apoptosis (Wang, 2001; Tait and Green, 2010). Previous reports demonstrate that Fas/CD95 ligand stimulation challenges the mitochondrial outer membrane (MOM) integrity by caspase-8 mediated processing of Bid protein that translocates (truncated Bid- tBid) to the mitochondrion and dissipates cytochrome c to commence apoptosis. Notably, the activation of extrinsic along with intrinsic signaling relies on the magnitude of

caspase-8 activation and Bid cleavage (Li et al., 1998; Kim et al., 2009). However, the anti-apoptotic protein cFLIP restricts activation of caspases-8 and inhibits Bid cleavage (Irmler et al., 1997; Krueger et al., 2001). In fact, the stimulation of death receptor has limited effect to activate the intrinsic signaling of cell death (Werner et al., 2002)**.** Indeed, the MOM integrity has been tightly regulated by a Bcl-2 family of anti-apoptotic proteins that resist tBid mediated activation of intrinsic signaling (Kim et al., 2009; Madesh et al., 2009; Tait and Green, 2010). As shown above, induced expression of FADD reduces the level of $cFLIP_L$ and commences the activation of procaspase-8 and extrinsic apoptosis signaling, independent of death receptor stimulation. Therefore, it was important to delineate the involvement of FADD and the cFLIP in the regulation of mitochondrial integrity and apoptosis. To achieve this aim, the cells were transiently transfected with FADD and siRNA against $cFLIP_L$ to examine mitochondrial dynamics. The results show that induced expression of FADD and knockdown of $cFLIP_L$ ($cFLIP_L^{KD}$) pulverizes the mitochondrial integrity accompanied by the loss of mitochondrial membrane potential (MMP). In addition, reduced expressions of mitochondrial associated proteins Bcl-2 and cytochrome c was noticed. Moreover, such effects were adverse when cells were transfected together with FADD and siRNA of $cFLIP_L$ in HEK 293T cells. Further, the following outcomes were corroborated by expressing the mutants of the FADD (FADD-SLT2) and a DD of FADD (FADD-DD) in HEK 293T cells. It was noticed that, FADD-SLT2 and FADD-DD has minimal effect on the mitochondrial integrity; suggesting that overexpression of wild type FADD has immense potential to challenge mitochondrial dynamics. Interestingly, transient knockdown of $cFLIP_L$ ($cFLIP_L^{KD}$) facilitates Bid disintegration accompanied by altered expression of Bcl-2 and diffusion of mitochondrial cytochrome c in HEK 293T and MCF-7 cells. In addition, $cFLIP_L^{KD}$ challenges mitochondrial membrane potential (MMP) and downregultes the expression level of mitochondrial cytochrome c that may lead initiation of cell death by mode of apoptosis. Although, this loss of mitochondrial integrity was only noticed in HEK 293T and MCF-7 cells; however, no major changes were observed in non-cancerous NIH 3T3 cells. Furthermore, the induced expression of FADD and $cFLIP_L^{KD}$ initiates processing and activation of caspase-8 and cleavage of Bid to activate the cascade of caspases such as procaspase-9, procaspase-3, procaspase-7 and PARP to execute apoptotic cell death. These results demonstrate that the components of death receptor extrinsic signaling, FADD and $cFLIP_L$ have an imperative role in the intrinsic signaling of apoptosis.

Mechanistically, the stimulation of TNF-α coordinate NF-κB dependent activation of anti-apoptotic genes such as *cFLIP, cIAPs, XIAP* etc. that appears to prevent apoptosis

signaling for cell survival (Micheau et al., 2001). Although, pre-stimulation of TNF-α stabilizes the expression of $cFLIP_L$, but the signaling of TNF-α and $cFLIP_L$ in maintaining mitochondrial integrity are poorly resolved. Therefore, it was important to investigate the cellular fate for death or survival upon silencing the expression $cFLIP_L$ in TNF-α pre-stimulated cells. It was found that, priming of TNF-α failed to accumulate the expression of $cFLIP_L$ in $cFLIP_L$ knockdown HEK 293T cells and concomitantly induces intrinsic signaling of apoptosis similar to $cFLIP_L$ knockdown cells. Altogether, pre-treatment of TNF-α was unable to protect the cellular integrity upon knockdown of $cFLIP_L$ and directs apoptotic demise. Thus, these results indicate that, FADD and $cFLIP_L$ have an imperative role in regulation of extrinsic and intrinsic signaling of apoptosis.(Ranjan and Pathak, Sci. Rep., 2016).

Previous reports highlight that, CD95 and TNF-α are two biologically important molecules that play an important role in cell death and survival signaling. In comparison to Fas/CD95 ligand, the TNF-α also regulates intracellular apoptotic and non-apoptotic signaling in cancer cells (Magnusson and Vaux, 1999). Previous report advocates that, TNF-α may contribute in cell death or survival *via* participating in various signaling, depending upon cellular context and stimulation (Marques-Fernandez et al., 2013). Indeed, the major role ascribed to TNF-α for cell survival has been orchestrated by NF-κB activation and up regulation of anti-apoptotic proteins such as cFLIP, cIAPs, XIAP etc. to prevent apoptosis signaling (Beg and Baltimore, 1996; Micheau and Tschopp, 2003). In fact, the aberrant activation of NF-κB signaling has been associated with stress, infection, immunological response and several human diseases including inflammatory disorders and cancer (Grivennikov et al., 2010). In addition, $cFLIP_L$ advances TNF-α mediated MAPK/ERK1 and NF-κB activation to promote cell survival and malignancy (Micheau and Tschopp, 2003; Golks et al., 2006; Marques-Fernandez et al., 2013). Notably, few previous reports suggest that FADD, casper and caspase-8 can activate NF-κB (Yeh et al., 2000; Chaudhary et al., 2000). On the contrary, it has been also shown that procaspase-8 mediated activation of NF-κB is independent of caspase-8 proapoptotic activity that may only proceed to apoptotic cell death (Hu et al., 2000). Nevertheless, it has also been suggested that low amount of FADD can activate NF-κB, which is mainly relying on concentration and time, but mutation in procaspase-8 neutralizes this activation (Chaudhary et al., 2000). Collectively, the role of FADD in regulation of cell death or survival mediated by TNF-α signaling is still controversial. Therefore, it was important to unravel the cellular dynamics of FADD in regulation of cell death or survival in context of TNF- α stimulation.

In the present study, it was found that TNF-α canonically induces NF-κB activation and associated gene product $cFLIP_L$ in HEK 293T cells, but in the presence of FADD the expression of NF-κB subunit p65 and $cFLIP_L$ was abrogated. Furthermore, ectopic expression of FADD impedes NF-κB activation and restricts cytosolic to nuclear translocation of p65. Moreover, it was found that, FADD ubquitinates IKKβ and stabilizes IκBα to sequester p65 within the cytosol. These results indicate that the stimulation of TNF-α in the presence of FADD was unable to maintain the anti-apoptotic integrity of NF-κB and $cFLIP_L$ for cell survival. Moreover, the sufficient availability and concentration of FADD abrogates NF-κB activation to commence cell death. Next, the constituting structural domains and mutants of FADD and $cFLIP_L$ were expressed in HEK 293T cells to revalidate NF-κB activation and $cFLIP_L$ expression. The transient expression of DD of FADD (FADD-DD) and mutant of the FADD (FADD-SLT2) shows a significant increase in the activity of NF-κB and expression of p65 and $cFLIP_L$. These outcomes suggest that, the DD of FADD binds to DD of death receptors (DRs) and inhibits the downstream apoptosis signaling to favor NF-κB activation and cell proliferation. Moreover, the ectopic expression of mutant $cFLIP_L$ (DM-$cFLIP_L$) has negligible effect on the NF-κB activation, p65 and $cFLIP_L$ expression, as compared to the conventional response generated from the wild type $cFLIP_L$. Altogether, these results suggest that the structural integrity of wild type FADD inhibits NF-κB activation; however expression of $cFLIP_L$ favors NF-κB activation. Next, the potential of FADD on apoptotic commencement was examined in TNF-α stimulated cells. It was noticed that, the cells treated with lower concentrations of TNF-α (5 and 10 ng/ml) had no remarkable alteration in cell viability and cell proliferation. However, the induced expression of FADD activates both extrinsic and intrinsic caspases responsible for apoptotic cell death, even in the presence of TNF-α. Surprisingly, TNF-α could not protect the cell survival during the availability of FADD in HCT 116 and MCF-7 cells. Similarly, pre-treatment of TNF-α followed by knockdown of $cFLIP_L$ was unable to protect NF-κB activation and cell survival. It was important to validate mode of cell death by necrosis. The FADD expressed cells pre-treated by necrostatin-1 followed by TNF- α exposure did not show remarkable release of LDH and inhibition of PARP activation. Therefore, it was confirmed that the FADD mediated cell death was apoptotic rather than necrotic. These results clearly suggest that FADD attenuates activation NF-κB and $cFLIP_L$ expression to restrict cell survival even in the presence of stimulant TNF-α. More interestingly FADD mediated proteolytic cleavage of pro-caspase-8 confers downstream signaling of apoptosis. (Ranjan and Pathak, Sci. Rep., 2016).

Mechanistically, TNF-α triggers the assembly of complex I constituted with RIP1/TRADD/TRAF2/cIAPs to activate NF-κB and downstream anti-apoptotic signaling network for cell survival (Micheau and Tschopp, 2003). In contrast, depletion of cIAP1/2 relieves RIP1 from complex I to form pro-apoptotic complex II along with FADD and procaspase-8 (Bertrand et al., 2010; Feoktistova et al., 2011). An earlier report show that, TNF-α also triggers the formation of complex II, comprising with FADD, caspases-8 and RIP1 to execute apoptosis signaling (Wang et al., 2008). In fact, TNF-α mediated cell death and survival decision is critically regulated by cIAPs dependent ubiquitination of RIP1 as an initial checkpoint and later on sheltered by $cFLIP_L$ (Feoktistova et al., 2011). Consistent with the previous findings, in this study the cellular dynamics of FADD in the regulation of cIAP2 expression and ubiquitination of RIP1 in response to TNF-α were explored. The mRNA and protein expression analysis along with co-immunoprecipitation assay revealed that transient expression of FADD negatively regulates the expression of cIAP2 and favors the interaction of RIP1 with FADD. Moreover, selective knockdown of $cFLIP_L$ in FADD expressed cells greets strong interaction between RIP1 and FADD. Similarly, the mRNA analysis suggests that ectopic expression of FADD upregulate the expression of procaspase-8, however the expression of anti-apoptotic genes such as *$cFLIP_L$* and *cIAP2* were found depleted, independent of TNF-α stimulation. In addition, the *in silico* analysis suggests that the multiple bonding interaction and occupied larger surface area helps to provide a holistic environment for the catalytic interaction of FADD and RIP1 to form complex II. Altogether, these results demonstrate that FADD has fine tuning over regulation of cIAPs, RIP1 and $cFLIP_L$ even in the presence of pro-survival factor TNF-α.

In contrast, the cell death and survival signaling associated with NF-κB and the cFLIP has strong a co-relation with reactive oxygen species (ROS) production (Morgan and Liu, 2011). Previously it has been shown that, the ablation of NF-κB activation induces rapid generation of ROS and JNK activation in response to TNF-α (Tang et al., 2001; Kamata et al., 2005). Indeed, the elevated level of ROS provokes JNK1 activation and leads to Bcl-2 phosphorylation and apoptotic cell death (Nakano, 2004; Shen and Liu, 2006; Wei et al., 2008). JNK activation is a highly regulated process, as short JNK1 activation favors tissue regeneration, whereas prolonged JNK1 activation promotes cell death (Schwabe et al., 2003; Shen et al., 2006). Moreover, it has been earlier shown that activation of JNK orchestrated the E3 ubiquitin ligase ITCH mediated turnover of $cFLIP_L$ expression (Chang et al., 2006). Therefore, further studies were aimed to delineate the cellular signaling of FADD and ROS-JNK1 to regulate the expression of $cFLIP_L$. The results demonstrate that, induced expression of FADD accumulates intracellular ROS and activates JNK1 expression

to trigger the ITCH mediated ubiquitination and degradation of $cFLIP_L$. Moreover, it was noticed that stimulation of TNF-α to FADD expressed cells significantly enhance ROS level and ubiquitination of $cFLIP_L$ in HEK 293T cells. (Ranjan and Pathak, Sci. Rep., 2016).

An earlier report states that, knockdown of the $cFLIP_L$ induces ROS generation and JNK activation in tumor cells (Nakajima et al., 2003). In this study, it was noticed that knockdown of $cFLIP_L$ in TNF-α primed cells progressively enhanced the ROS accumulation and JNK1 activation similar to $cFLIP_L$ knockdown cells. Altogether, these results indicate that ablation of NF-κB activity in FADD expressed and $cFLIP_L$ knockdown cells induces ROS mediated JNK1 activation followed by ITCH activation and Bcl-2 alteration to induce extrinsic and intrinsic signaling of apoptosis. Thus, a balanced expression of FADD and $cFLIP_L$ directs the regulation of NF-κB to determine the fate of cell death or survival.

Indeed, FADD and cFLIP both assume to involve in autophagy signaling (Pyo et al, 2005; Lee et al., 2007). Autophagy and apoptosis are two different programmed physiological processes that required for the maintenance of cellular homeostasis. Autophagy generally referred as "self-eating" process tends to degrade the unwanted cytosolic constituent and target them to lysosome. Although, basal level of autophagy helps to maintain cellular homeostasis in normal physiological condition, however, during stress it supports cell survival (Bergamini et al., 2004; Levine and Klionsky, 2004). Indeed, autophagy plays a cytoprotective role against cell death during cell proliferation in cancer and provides microenvironments for cell survival by availing them nutrition (Kuma et al., 2004). Recent advancement in apoptosis and autophagy signaling provides the close relationship between cell death and survival to determine their cellular fate (Mukhopadhyay et al., 2014). Interestingly, autophagy is a dual sword process which regulates cell death and survival mechanism. Although, during stress condition the process of cell survival is intensified by induction of autophagy, however the irreparable damage induces cell death by the process of apoptosis (Marino et al., 2014). Recent reports revealed that excessive level of autophagy is associated with autophagic cell death (Kroemer and Levine, 2008; Liu and Levine, 2015). The autophagic cell death is characterized by the accumulation of autophagic vacuoles during physiological and pathological conditions. Mechanistically, during cellular stress the DD of FADD interacts with a DD of Atg5 and contributes to autophagic cell death (Pyo et al., 2005). In contrast, cFLIP can abrogate the interaction between Atg3 and LC3 to suppress autophagic induction (Lee et al., 2009).

In this study, the cellular cross talk of FADD and $cFLIP_L$ in the regulation of autophagy was examined. To achieve this aim, the signaling of autophagy was examined during overexpression of FADD and $cFLIP_L$ knowckdown conditions. The results show that, added expression of FADD rescues the autophagosome formation and LC3 conversion as compared to $cFLIP_L$ knockdown cells. Although, the cellular mechanism for cell death by apoptosis or autophagy both are different, but key regulators of apoptosis play an important role in the regulation of autophagy (Thorburn, 2008). Previous report advocates that anti-apoptotic protein Bcl-2 interacts with Beclin-1 and suppresses autophagy (Ciechomska et al., 2009). However, during physiological stress, Bcl-2 dissociates from Beclin-1 and subsequently stimulates autophagy (Pattingre et al., 2005). In fact, it is still debatable to answer at what extent these apoptotic regulatory proteins account for the anti-autophagic activity in the cells at physiological expression levels. Indeed, the metabolic sensor mammalian target of rapamycin complex 1 (mTORC1) regulates autophagy induction during cellular stress (Laplante and Sabatini, 2012; Levine and Klionsky, 2004). However, the implication of mTORC1 inhibitors such as rapamycin induces autophagy and enforces translocation of nuclear binding protein HMGB1 to the cytosol for sustaining pro-autophagic activity, independent of physiological stress (Maiuri et al., 2007; Tang et al., 2010; Li et al., 2014; Park and Lippard, 2011; Galluzzi et al., 2014). In this study, it was found that rapamycin treatment to HEK 293T cells augments autophagic stress with rapid turnover of autophagy marker protein LC3-I to LC3-II, however the ectopic expression of $cFLIP_L$ resists the autophagic stress induced by rapamycin. Moreover, exposure of rapamycin induces oxidative stress and accumulates cytosolic expression of HMGB1, but failed to induce similar effects in $cFLIP_L$ overexpressed cells.(Ranjan and Pathak, J. Cell. Biochem, 2015).

Indeed, HMGB1 is known to regulate autophagy and apoptosis by interfering the conventional interaction of Beclin-1 and Bcl-2 during physiological stress. Endogenous HMGB1 competes with Bcl-2 for interaction with Beclin-1 to sustain autophagy (Pattingre et al., 2005; Tang et al., 2010a). In this context, it was noticed that, overexpression of $cFLIP_L$ maintains the basal interaction of Beclin-1 with Bcl-2, independent of rapamycin treatment. Collectively, these results suggest that $cFLIP_L$ protects the cells against oxidative stress and autophagy induced by mTOR inhibitor rapamycin to provide a line of defense for cell survival in cancer. Earlier, it was observed that knocking down the endogenous expression of $cFLIP_L$ ($cFLIP_L^{KD}$) imparts autophagic stress in cancer cells (Lee et al., 2009). However, the effect of $cFLIP_L^{KD}$ on the downstream expression of pro-autophagic HMGB1 and the fate of Beclin-1 interaction with Bcl-2 remains elusive. Herein,

results suggest that $cFLIP_L^{KD}$ provokes rapid accumulation of autophagosomes with puncta formation and conversion of LC3-I to LC3-II accompanied by loss of cell viability. Astonishingly, re-expressing $cFLIP_L$ in the $cFLIP_L^{KD}$ cells restores the autophagic equilibrium similar to 3-MA (an inhibitor of autophagy) pre-treated cells. Moreover, re-introduction of $cFLIP_L$ in the $cFLIP_L^{KD}$ cells maintains the viability comparable to control cells. Furthermore, it was found that in $cFLIP_L$ knockdown cells, HMGB1 accumulates in cytosolic and expression of Bcl-2 diminishes; however, such effect was inhibited upon re-expression of $cFLIP_L$ in $cFLIP_L^{KD}$ cells to sustain autophagic equilibrium. As reported earlier, the cytosolic translocation of HMGB1 triggers JNK1 mediated phosphorylation of Bcl-2 and disassociates its interaction with Beclin-1 under autophagic stress condition (Tang et al., 2010b; Zhao et al., 2011). Next, the participation of $cFLIP_L$ in maintaining the constitutive interaction of Beclin-1 with Bcl-2 was examined. The results suggest that, knockdown of $cFLIP_L$ rapidly accumulates cytosolic ROS with subsequent activation of kinase protein JNK1. Moreover, selective knockdown of $cFLIP_L$ in HEK 293T cells impinges constitutive ability of Bcl-2 to interact with Beclin-1. On the contrary, the re-expression of $cFLIP_L$ in the $cFLIP_L^{KD}$ cells were capable of restoring this basal interaction to suppress autophagy. In parallel, the ectopic expression of $cFLIP_L$ potentially suppresses H_2O_2 (potent ROS inducer) induced oxidative stress similar to N-acetyl cysteine (NAC- ROS scavenger) and suppresses HMGB1 cytosolic accumulation accompanied by protecting Beclin-1-Bcl-2 interaction. Some earlier finding revealed that tumor suppressor protein p53 may also regulate autophagy by modulating mTOR signaling apart from apoptosis and metabolism (Feng et al., 2005; Rosenfeldt et al., 2013). Mechanistically, p53 regulates the expression of Beclin-1 during cellular stress and promotes autophagy (Green and Kroemer, 2009). Therefore, it was important to dissect the role of $cFLIP_L$ in context of p53 signaling during autophagy. Interestingly, knockdown of $cFLIP_L$ upregulates the expression of p53, which advances the ubiquitination of Beclin-1, but re-expression of $cFLIP_L$ in the $cFLIP_L$ knockdown cells restore the expression of p53 to the basal level to maintain the integrity of Beclin-1. Notably, TNF-α is a potent inducer of $cFLIP_L$, but apparently regulates the cellular mechanism of autophagy (Karin and Lin, 2002; Micheau and Tschopp, 2003; Djavaheri-Mergny et al., 2006). Here, the results suggest that pre-treatment of TNF-α to HEK 293T cells have a moderate effect on the autophagic turnover of LC3 protein; however, knockdown of $cFLIP_L$ triggers autophagic stress. Interestingly, it was found that knockdown of $cFLIP_L$ in TNF-α pre-treated cells failed to restrict the cytosolic accumulation of HMGB1 and transactivation of p53. These results suggest that knockdown of $cFLIP_L$ mitigates TNF-α response for cell survival and promotes cell death

by activating apoptotic and autophagy signaling. Thus, these results indicate that, anti-apoptotic protein $cFLIP_L$ resists drug induced oxidative stress to mitigate autophagy induction. The ectopic expression of $cFLIP_L$ suppresses stress induce activation of JNK1, HMGB1 and p53 to maintain cell survival autophagic equilibrium in HEK 293T cells. Threfore, targeting the expression of $cFLIP_L$ in cancer cells would be a promising approach to breaking the line of defence for cell survival supported by autophagy.

An avoidance of apoptosis having multiple reasons, but among them, inflammation associated tumor progression through production of different inflammatory cytokines and chemokines having strong impact on tumor survival (Aliprantis et al., 1999; Lien et al., 1999; Kawai and Akira, 2007, Kawai and Akira 2010). In response to pro-inflammatory molecule LPS, the cell surface receptor TLR4 recruits adaptor protein MyD88 and IRAK1 to activate NF-κB signaling and synthesis of proinflammatory cytokines IL-1β and IL-18 (Zhande et al., 2007; Ma et al., 2004; Bossaller et al., 2012) Recent reports highlight that the Inflammasome activation together with activated NF-κB, triggers canonical maturation of IL-1β to support tumor progression (Latz et al., 2013; Abderrazak et al., 2015). Therefore, regulation of inflmmasome activation and inflammatory cytokines by targeting NF-κB signaling might be a useful stratagem for cancer prevention and therapy. Some earlier reports state that, FADD interacts with MyD88 and exclude IRAK1 to restrict LPS induced NF-κB activation (Bannerman et al., 2002; Zhande et al., 2007). Moreover**,** FADD has been also reported in the non-canonical activation of IL-1β (Bossaller et al., 2012; Moriwaki et al., 2015). Therefore, it was important to investigate the role of FADD in the regulation of LPS induced activation of NF-κB and proIL-1β. It was noticed that overexpression of FADD suppresses the LPS induced activation of NF-κB and synthesis of proIL-1β. Moreover, the canonical stimulation of LPS and ATP to activate Inflammasome induced IL-1β maturation was reduced in FADD expressed cells. Altogether theses results suggest that induced expression of FADD can negatively act on the activation of NF-κB to suppress the LPS induced synthesis and maturation of proinflammatory cytokines IL-1β.

The above findings emphasize that, FADD is an important functional molecule that can induce apoptosis in cancer cells. In fact, it could be a promising candidate for intervention of cancer therapy. Therefore, further study was intended to explore some novel approach for delivery of FADD protein inside the cell to translate a protein as a therapeutic candidate. In this study, a novel approach was used for delivery of FADD inside the cells with the aid of cell permeable peptides followed by evaluation of anti-proliferative potential in cancer cells. The human *FADD* gene was cloned and successfully expressed in the bacterial expression system *E. coli* BL21 (*DE3*)-pLysS. It was noticed that,

the expressed human FADD (hFADD) mostly resided in the bacterial pellet as inclusion bodies. Therefore, the pellet was denatured and refolded in Gn-HCl re-folding buffer followed by purification by affinity column chromatography and about 28 kDa hFADD protein was obtained and confirmed by Western blotting. Further, hFADD was characterized by wavelength spectrum, and the results suggest that the Trp and Tyr residues were mainly located in a nonpolar environment of a refolded hFADD protein with a characteristic emission spectrum. In addition, the MALDI-TOF analysis confirms that the purified fractions have characteristic properties similar to hFADD. Moreover, the *in vitro* protein-protein interaction analysis shows that elution of cFLIP protein upon binding with hFADD and confirms that purifed hFADD retains full biological activity. Next, the purified hFADD protein was chemically conjugated with a cell permeable (CP) peptide for the intracellular delivery to cancer cells. The FT-IR spectroscopy suggests that, purified hFADD was successfully conjugated with CP peptide (CP-FADD) by forming an acidic amide linkage. Next, the co-immunoprecipitation assay and immunofluoroscence analysis shows that CP-FADD within 3 h of treatment efficiently penetrates across the cell membrane and accumulate inside the cells. Further, anti-proliferative potential of CP-FADD was examined in HEK 293T and HCT 116 cells. The results show that, 5μM of CP-FADD conjugate treatment for 3-12 h effectively suppresses cell viability form 3 -12 h. Next, it was important to validate the apoptotic potential of CP-FADD conjugate in cancer cells. It was found that, CP-FADD conjugate treatment to cells induces apoptotic cell death with subsequent activation of Procaspase-8, -9 and PARP. It is well documented that an aberrant activation of NF-κB inflames secretion of pro-inflammatory cytokines to promote cell proliferation in cancer (Karin and Lin, 2002; Karin and Greten, 2005). Therefore, it was important to validate the effect of CP-FADD on NF-κB activation and pro-inflammatory cytokines. It was noticed that, CP-FADD diminishes the expression of NF-κB subunit p65 and restricts its phosphorylation within 3 h of treatment. In addition, the CP-FADD conjugate treatment to HCT 116 cells negatively act on the mRNA and protein expression of pro-inflammatory cytokine IL-1β as well as diminishes the level IL-1β as compared to LPS treated control cells. Next, the cell death inducing properties of CP-FADD conjugate was compared with conventional apoptotic inducers such as CD95, TNF-α, Cycloheximide, HA14-1 and etoposide. Interestingly, it was noticed that CP-FADD conjugate significantly reduces the cell viability within 3 h of treatment and induces activation of procaspase-7 and PARP within 3 h of treatment as similar to known apoptosis inducers. Thus, these results suggest that FADD is an important functional component in apoptosis signaling and it could be a plausible novel candidate for cancer

therapeutics. Importantly, conjugation of FADD with cell permeable peptide could be used as a tool for delivery of protein inside the cells. Altogether, this study support a considerable and greater emphasis to develop a translational approach to open a new avenue of cancer therapy.