5. TNF-α induces apoptotic cell death in dopaminergic neuronal cells (SH-SY5Y cells): Inflammatory model of Parkinson's disease

5.1 Prolonged exposure of TNF-α induces cell death in SH-SY5Y.

The previously reported clinical studies had provided evidence that TNF-α level is increased in both serum and CSF (cerebral spinal fluid) in PD patients sample (Mogi *et al.*, 1994c; Mogi *et al.*, 2000b). This strongly suggests it has vital role in selective neuronal loss during PD however; its role in path-physiology of PD is not well understood. In the current study, SH-SY5Y cells (*in-vitro* model of dopaminergic neuron) were treated with low concentration of TNF-α (20 ng/ml) for different time period. The soluble TNF-α can bind to TNFR1 and TNFR2 (Caminero *et al.*, 2011), hence the expression pattern of both TNFR1 and TNFR2 in SH-SY5Y was analyzed in the presence of TNF-α using real time PCR. The expression of TNFR1 increased significantly in the presence of TNF-α whereas no change in TNFR2 expression (Fig 5.1A). The binding of TNF-α to TNF-R1 can lead to induction of cell survival as well as death pathway (Christofferson *et al.*, 2014) hence further studies were done to confirm the role of TNF-α in neuronal survival. SH-SY5Y cells were treated with different dose of TNF-α for prolong period and cell survival was monitored using MTT assay (Fig 5.1B). The cell survival significantly decreased after 72 hours of TNF-α treatment as compared to untreated cells (Fig 5.1C). The high level of cell death (>30%) was observed after 96 hours of TNF-α treatment (Fig 5.1C). To determine the type of cell death on prolonged exposure of TNF-α, Acridine orange/Propidium Iodide staining was performed. The cells stained orange (Fig 5.1E & F) after 72 hours of TNF-α treatment, suggesting that the cell death is mediated by apoptosis. The induction of apoptosis was further confirmed by monitoring the PARP cleavage and caspase activation. PARP is a target of executioner caspases and is cleaved during apoptosis (D'Amours *et al.*, 2001; Chaitanya *et al.*, 2010). The western blotting showed 110 kDa corresponding to native form of PARP was observed in control as well as TNF-α treated cell. The band of 89 kDa corresponding to cleaved PARP was observed only after 72 hours of TNF-α treatment (Fig 5.1D). The further experiments were performed to confirm the activation of caspases and its role in TNF-α induced cell death. TNF-α treatment showed the band of 18 kDa corresponding to cleaved subunit of caspase-3 after 72 hours whereas it was not detected till 48 hours of TNF-α treatment and untreated cells (Fig 5.1D).

`

Figure 5.1

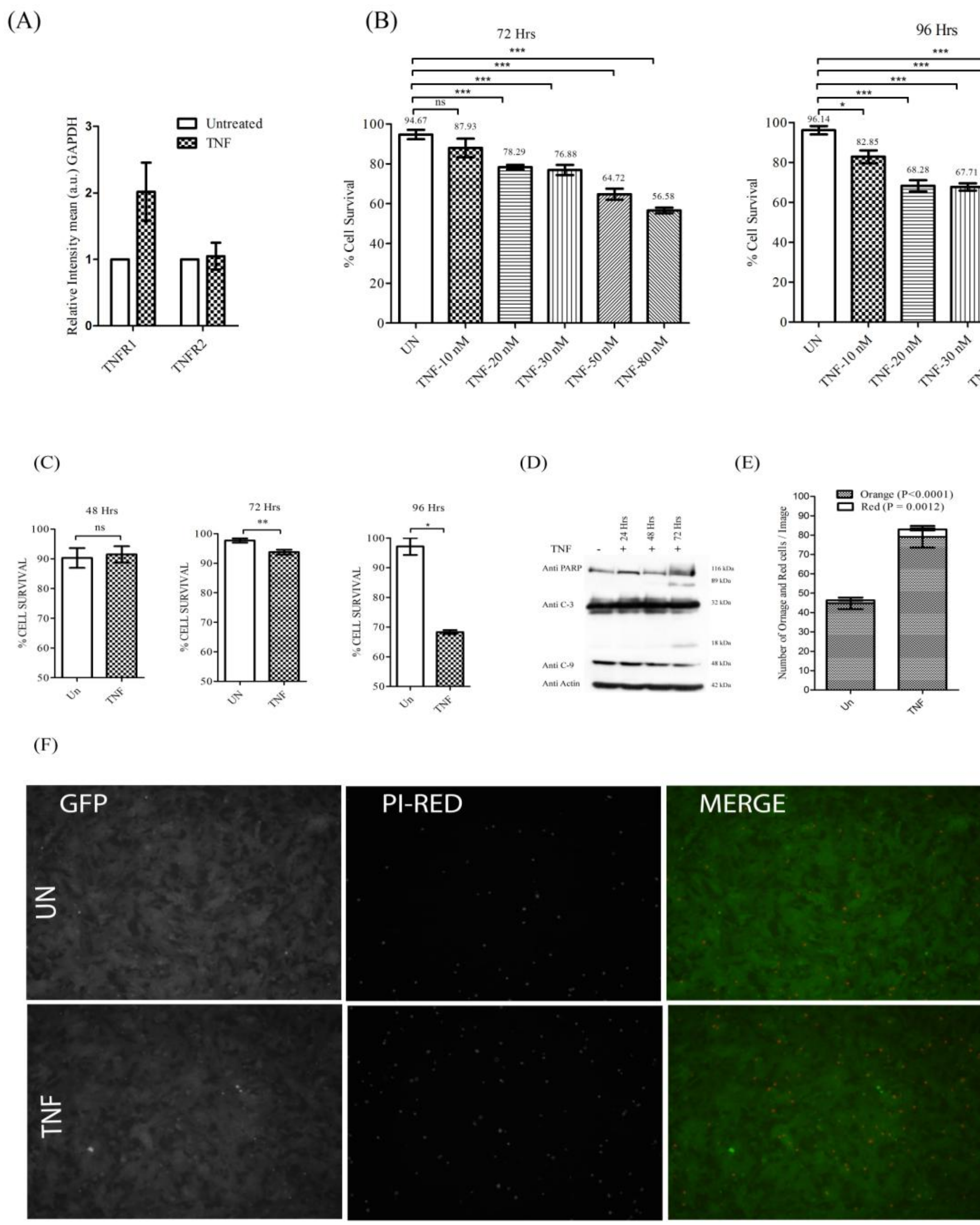

Figure 5.1 TNF-α differentially regulate the expression of TNFR1 and TNFR2 and modulate cell death.

(A) SH-SY5Y cells were treated with TNF-α (20 ng/ml) for 72 hours. Expression pattern

`

of TNFR1 and TNFR2 were determined using GADPH as endogenous control in presence of TNF-α by real time PCR. (B) SH-SY5Y cells were treated with different doses of TNF-α for indicated time period and MTT assay was performed for analysis of cell survival. (C) SH-SY5Y cells were treated with TNF-α (20 ng/ml) for indicated time period and MTT assay was performed for analysis of cell survival. (D) TNF-α activates caspase-3 and 9 in DAergic cells. SH-SY5Y cells were treated with TNF-α (20 ng/ml) for different time period. The activation of caspase-3 and 9 was monitored by western blotting using specific antibodies (E & F) TNF-α induces apoptosis. SH-SY5Y cells were treated with TNF-α (20 ng/ml) for 72 hours. The analysis of the type of cell death was monitored by Acridine orange/Propidium Iodide staining. Number of orange and red cells was plotted per image in presence and absence of TNF-α in Acridine/PI staining. Asterisk (*) indicates that p value <0.05, for SEM

Caspase-9 antibody detects only 48 kDa precursor form of caspases-9. The decreased level of 48 kDa band was observed only after 72 hours of TNF-α treatment (Fig 5.1D). This strongly suggests the activation of caspase-9 in the presence of TNF-α. These observations collectively suggest that prolonged exposure of low doses of TNF-α to dopaminergic neuronal cells, SH-SY5Y induces apoptosis.

5.2 TNF-α sensitizes SH-SY5Y cells to PD stress induced cell death.

Neurotoxicants like Rotenone, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA) induces PD like conditions in experimental animal models (Bove *et al.*, 2005) and induces degeneration of DAergic neurons. SH-SY5Y expresses tyrosine hydroxylase and dopamine transporter and can preferentially uptake 6-OHDA (Luthman *et al.*, 1989). It accumulates in the cytosol and induces cell death by generating ROS. Similarly, Rotenone freely enter into cells and induces neuronal death by inhibiting complex-I activity (Berndt *et al.*, 2013). We further analyzed if TNF-α sensitizes dopmanergic neurons to cell death in PD stress conditions (Rotenone and 6-OHDA). SH-SY5Y cells were co-treated with sub-toxic doses of 6-OHDA and TNF-α. The cell viability was analyzed by MTT assay. The cell viability significantly decreased in the presence of TNF-α and 6-OHDA as compared to 6-OHDA alone and untreated cells (Fig 5.2A). Similar experiments were performed in the presence of rotenone. The presence of TNF-α sensitized rotenone induced cell death as compared to untreated or

Figure 5.2

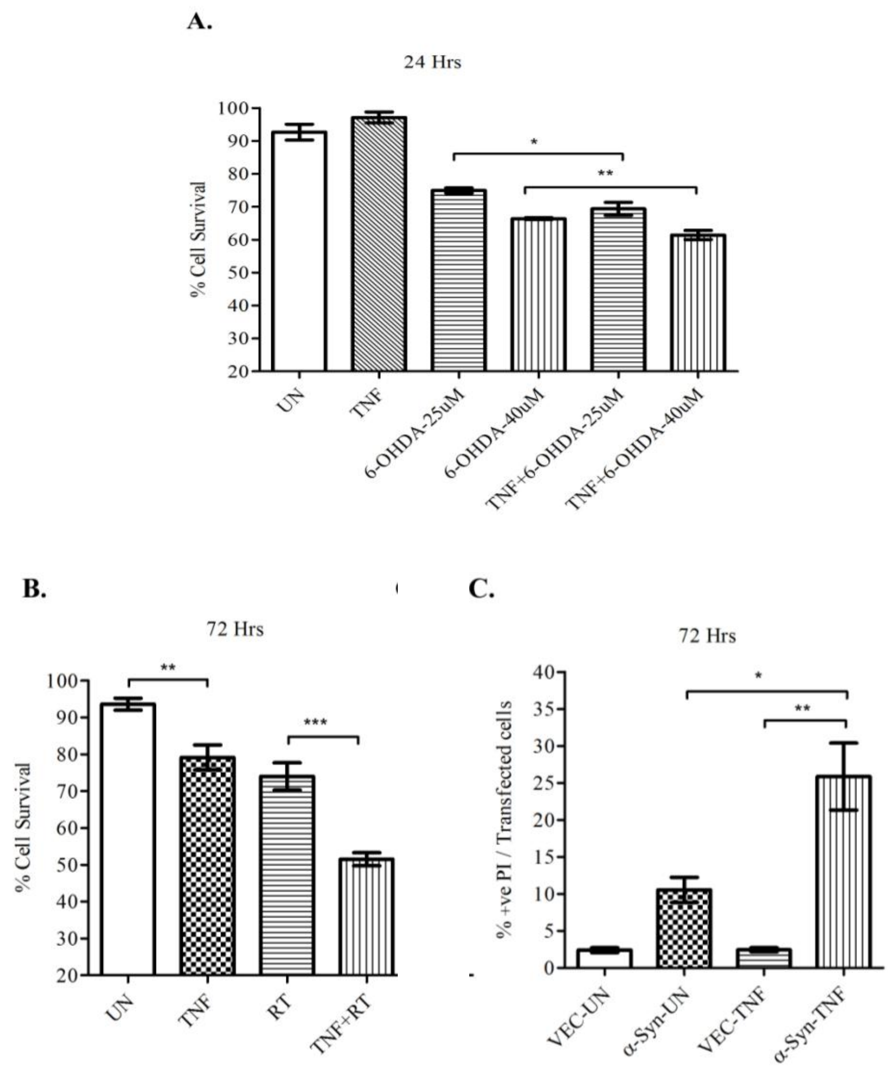

Figure 5.2: TNF-α sensitizes SH-SY5Y to cell death in PD stress conditions.

*(A) SH-SY5Y cells were co-treated with indicated sub-toxic doses of 6-OHDA (25 µM, 40 µM) and TNF-α (20 ng/ml) for 24 hours. Cell viability was analyzed by MTT assay. (B) SH-SY5Ycells were treated with Rotenone (25µM/ml) for 24 hours in the presence of TNF-**α** and MTT assay was performed for cell survival assessment. (C) SH-SY5Y cells were transfected with α-synuclein-GFP and vector control followed by TNF-**α** (20 ng/ml) treatment. After 72 hours of treatment % PI (Propidium Iodide) positive cell/ Transfected was assessed by PI staining. Asterisk (*) indicates that p value <0.05, for SEM.*

treated either of them (Fig 5.2B). The high levels of α-synuclein expression is known to produce PD-like cellular and axonal pathologies in the nigrostriatal region of both rodents and primates brain (Ulusoy *et al.*, 2010). Therefore role of TNF-α was also checked in cell death during α-synuclein mediated in PD stress condition. To confirm this, α-synuclein-GFP was transfected in SH-SY5Y and treated with TNF-α for 72 hours and cell death was monitored by PI Staining. The number of PI positive/α-synuclein-GFP transfected cells significantly increased in the presence of TNF-α (Fig 5.2C). Collectively, these results suggest that TNF-α sensitises SH-SY5Y to cell death in the presence of different PD stress conditions.

5.3 Discussion

The epidemological, GWAS and clinical reports strongly suggest that inflammation is one of the major causes of DAergic neuronal loss and cause of PD. The increased levels of TNF, one of the key pro-inflammatory cytokines have been observed in CSF (Mogi *et al.*, 1994b; Nagatsu *et al.*, 2000) and striatum of the PD brain (Chertoff *et al.*, 2011). The role of TNF in pathogenesis of PD is not well understood. TNF-α is pletotropic cytokine and can act as pro-survival or cell death stimuli depending upon the cell type (Chu, 2013; Juhasz *et al.*, 2013; Shuh *et al.*, 2013; Wallach, 2013). We used DAergc neuronal cell line (SH-SY5Y) to elucidate the molecular mechanisms of TNF-α mediated DAergc loss during PD stress conditions. SH-SY5Y cells were treated with enhanced levels of TNF-α for longer duration mimicking conditions *in vivo*. The presence of TNF-α specifically up-regulates TNF-R1 leading to activation of intracellular cell death pathways. The increased level of TNF-α leads to apoptosis in DAergc neuronal cell line was clearly evident by PARP cleavage, caspase-3 and caspase-9 activation. The increased level of TNF-α showed significantly increased cell death with time. This observation is in consonance with earlier observation of the post mortem brain of PD patients (Nagatsu and Sawada, 2007). This strongly suggests that TNF-α may bind to the cognate receptor and induce the activation of downstream caspase cascade rather than activation of pro-survival pathway of NF-κB activation. TNF-α also sensitized SH-SY5Y to cell death in the presence of rotenone, 6-OHDA and α-synuclein induced PD stress conditions. The activation of caspase-9 in the presence of TNF-α strongly suggests the activation of mitochondrial mediated pathway of apoptosis. In the current study, we first time to report cell based model of inflammatory induced PD model using TNF-α and its mode of cell death in SH-SY5Y cells. TNF-α is a key pro-inflammatory cytokines involved in the pathological mechanisms of PD, targeting TNF-α action seems to be an eye-catching disease-modifying approach.

`