Dexmedetomidine protects astrocytes from TNFα-induced cell cycle arrest and mitochondrial dysfunction in vitro

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Abstract

TNFα-induced systemic inflammation and cell injury play pivotal roles in many chronic nervous system diseases. This study assessed the protective effect of dexmedetomidine (Dex), an agonist of the adenosine α2 receptor, on TNFα-induced astrocyte injury and the underlying mechanism. The 1321N1 astrocyte cell line was pretreated with or without with 1 nM Dex and then cultured with 20 μM TNFα for 24 hours. The effects of Dex against TNFα-induced cell injury were examined by flow cytometry and immunocytochemistry. Our results indicated that Dex attenuated the TNFα-induced arrest of the cell cycle and upregulated the expression of cell cycle-related regulatory proteins, including Ki67 and cyclins A, B, D, and E. Furthermore, Dex significantly suppressed the TNFα-induced accumulation of reactive oxygen species (ROS), prevented the decrease in mitochondrial membrane potential (Δψm), and inhibited the upregulation of cleaved caspase 9 but not cleaved caspase 8. Our data suggested that Dex attenuated TNFα-induced cell cycle arrest and the early phase of apoptosis, possibly by improving the function of mitochondria and inhibiting the intrinsic caspase pathway in the early stage of apoptosis through the activation of the α2 receptor.

Introduction

TNFα is an important inflammatory factor inthecentral nervous systemthat inducesmany nervous system injuries and chronic diseases[1]. Inflammation and/or mechanical injuries contribute to increasing the concentrations of TNFα to noxious levels intheintracellular space.Some studies haveindicated that lowlevelsof TNFαstimulatethe proliferation of glial cells andprotectneurons from injury[2].However,in most serious nervous systemdiseases, such as brain trauma orsystemicinflammatory reaction syndrome(SIRS), TNFα is released from inflammatory cells into the extracellular space inthebrain or spinal cord andisnoxious to nervous system cells[3,4]. A pilot study showed that injuries to peripheral nerves, such as compression of the sciatic nerve, increased the concentration of TNFα in cerebrospinal fluid(CSF) and injured of nervous system cells, although the underlying mechanism is not clear[5].

As an adenosine α2 receptor agonist, Dexisusually used as a sedative and anesthetic in surgicalproceduresor in intensive caresettings. A growing bodyof evidence suggeststhat Dex significantlyimprovesthe quality of patient-controlled analgesia[6,7]. In a series of preliminary studies, the anti-inflammatory and antiapoptotic effects of Dexwereobserved on lung, renal and intestinal cells[8-11]. Dex binds to the α2 receptor, which is expressed on nervous system cells, and investigating its effect on neurons, astrocytes and microglia will help us to better understand its underlying mechanism beyond sedation and analgesia.

Materials And Methods

1. Cell culture and treatments

The1321N1 human astrocyte cell line (American Type Culture Collection, USA)wascultured in DMEM-F12 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Beyotime Institute of Biotechnology, Shanghai, China) at37 °Cin 5% CO_2. After 2 daysofincubation,thecells were incubated with serum-free DMEM-F12 medium for 24hours and thenchallenged with20 μMTNFα (Sigma–Aldrich, St. Louis, MO, USA) for 24 hours after being preincubated with phosphate-buffered saline (PBS, naïve control),1nMDex (Sigma–Aldrich, St. Louis, MO, USA) or10 nMAtipamezole (α2 receptor antagonist) (Ati, MedChem Express, Monmouth Junction, NJ,USA) mixedwith1 nMDex for 15 minutes. TNFα and Dex concentrations were chosen based on our previous study[5], and the dose of Ati was based on an agonist-antagonist binding affinity ratioof10:1.

2. Cell cycle analysis by flow cytometry

The cell cyclewasanalyzed by flow cytometry (ACEA Biosciences Inc, San Diego, California, USA)[9]. In brief, the cells were detached fromtheflask after trypsinization and then fixed with 70% ethanol at4 °Cfor 12 hours.After being centrifuged at2500 rpm,thecells were suspendedin0.5 ml of PBS containing 500 ng/L RNase and 10μL of40μg/L propidium iodide(PI) and incubated for 10 minutes inadark box at room temperature. Fluorescence was measured by flow cytometry in at least 10,000 gated events andwasanalyzed by FlowJo 7.6.1 software.

3. Immunocytochemistry

Cells were incubated on cover slips and washed with 0.01% PBS3 timesfor 5minutes each,followed bytreatmentas previously described. Then, thecells were fixed with 4% paraformaldehyde (PFA) for 10 minutes and washed again. After being blocked with donkey serum for 10 minutes,thecells were incubated with monoclonalantibodies,including GFAP and α2antibody mixtures(for double labeling), cyclins A, B, D and E, Ki67,andcleaved caspase 8 and 9(1:200, Abcam, Cambridge, MA, USA),overnight at4 °C. Afterthe primaryantibody was removed and the cells were washed again,afluorochrome-conjugated secondary antibody (Abcam, Cambridge, MA, USA) was added and incubated for 1 hour. Then, the cellnuclei werecounterstained with DAPI,andthe slides were examined using an Olympus IX71 microscope underaconstant exposure level. Each experimentwasrepeated 4 times,and 10 areas were chosen randomly on each slide. The number of positive cells relative to the number of DAPI-positive cells and immunofluorescence levels were quantified using ImageJ (National Institutes of Health, Bethesda, MD).

4. Detection of △ψm

Astrocytes were labeled withthelipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanineiodide (JC-1) and analyzed byfluorescence microscopyor flow cytometry previously described[2].To investigatethe direct visual changesinfluorescence after JC-1 staining, cells were incubated on cover slips, treated asadivided group and visualized underamicroscope. In brief,the coverslipswerecarefullywashed with warm PBS twice and then incubated with 0.2 μM JC-1 for 30 minutes at 37 °C inadark box. Subsequently, thecoverslipswere washed again and moved to glass slides,and then immunofluorescence imageswereobtained withanOlympus IX71 microscope. Red and

green fluorescence intensities inthesame visual areawereanalyzed with ImageJ software,andthemean fluorescence intensity (MFI) was calculated from 4 areas chosen randomly in each slide.Tofurtherinvestigatethe proportion of cells with high△ψm, cells in various groups were collected by trypsinization and then transferred to 5-mL polystyrene tubes for flow cytometry. After being washed with fluorescence-activated cell sorting (FACS) buffer (10% fetal calf serum, 0.5 M EDTA in 0.1 M PBS) once, the cells were incubated with 0.2 μM JC-1 in FACS buffer for 30 minutes at 37 °C inadark box. The cells were analyzed by flow cytometry after being washedtwicewith warm FACS buffer. Fluorescence was measured in the FL-1 (green fluorescence) and FL-2 (red fluorescence) channels after gating on live cells. The mean intensities of red fluorescence (PE) and green fluorescence (FITC) were analyzed using FlowJo 7.6.1 software,and the ratio of red/green fluorescence intensity was analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA). The cells withahigh red/green ratio were gated relative totheNC group.

5. Flow cytometric analysis of ROS generation

The changesinROS production in naïve control and treated cellswere assessedusing flow cytometry after 2’-7’-dichlorodihydrofluoresceindiacetate (DCF, Invitrogen, Paisley, UK) staining. In brief, astrocytes were harvested by trypsinization and then incubated in 2μM DCF diluted in FACS buffer for 30 minutes at37 °C. The fluorescence intensity was assessed by flow cytometry and analyzed with FlowJo 7.6.1 software. Each assay included at least 10,000 gated events.

Allexperiments wererepeated at least 4 times independently.

Statistical Analysis

All numerical data are presented as the mean ± SD or box-whisker plots. Comparisons between the treatment groups were analyzed by one-way analysis of variance followed by Tukey’s multiple group comparisons (equal variances) or the Kruskal–Wallis (nonparametric) test followed by Dunn multiple group comparisons (unequal variances or scoring) (GraphPad Prism 5, San Diego, CA). A p value of 0.05 was considered statistically significant.

Results

1. Dex attenuated TNFα-induced cell cycle arrest.

Using double-labeled immunochemistry,theadenosine α2 receptor was shownto be coexpressedwith GFAPin1321N1 cells (Fig.1 A). Afterchallenge with 20 nMTNFα for 24 hours, detached or broken cells were found intheculture medium underaninverted microscope (Fig.1 B). TNFα induced asignificantincreaseintheproportionof G0-G1 phase cells (44.15±2.96%)comparedwith that inthenaïve control group (NC, 31.77±2.44%, p<0.001). The increaseinG0-G1 phase cells induced by TNFα was inhibited bypretreatmentwith1 nMDex(22.12±3.24% intheDT group, p<0.001) and was reversed by10 nMAti (α2 receptor competitive antagonist) (32.95±2.36% intheADT group, p<0.001) (Fig. 1 C and D).

2. Dex reversed the TNFα-induced downregulation of various cell cycle-related regulatory proteins.

Cell cycle-related regulatoryproteins,including Ki67 and cyclins (A, B, D and E),arebelievedto playcrucial roles in variousphasesofthecell cycle. By using immunochemistry, we foundthatthe ratio of Ki67-, cyclin A-, and cyclin B-positive cells but not cyclin D- or E-positive cells intheTNFα group was significantly lower than that intheNC group and ADT group ($p<0.01$). In the absence of Ati, Dex reversed the decreaseinthe ratios of Ki67-, cyclin A- and cyclin B-positive cells. Notably, Dex upregulated the fluorescenceintensitiesof Ki67-, cyclin A-, B-, D-, and E-positive cells ($p<0.05$) (Figs. 2, 3 and 4).

3. Dex prevented the TNFα-induced decline in △ψm.

A normal△ψmprovidesthe necessary mitochondrial conditions to produce adenosine triphosphate (ATP) and provide energyforcell survival. Fluorescence microscopy showed that, following JC-1 staining, there was adecrease inred fluorescence intensityand an increase ingreen fluorescence intensityin theTNFα group. The ratio of red/green fluorescence intheTNFα group was significantly lower than that intheNC group (46.57±5.27 compared to 158.24±8.81, $p<0.001$). The ratio of red/green fluorescence intheDT group was 198.62±10.31, which was significantly higher than that intheTNFα and Dexgroups(155.99±9.80) ($p<0.001$, Fig. 5 A and B). Moreover, flow cytometry showed that therelativelyhigher ratio of red/green fluorescence intensity after JC-1 staining indicated cells with high△ψm. Consistent with the microscopy, theproportionofcells with ahigh△ψmin theDT group was 52.08±2.23%, which was significantly higher than that intheTNFα group(12.90±1.12%), NC group(34.53±3.80%) and ADT group (27.78±3.74%) ($p<0.001$) (Fig. 5 C and D).

4. Dex inhibited TNFα-induced ROS generation

Highconcentrationsof ROS in cellsarebelievedtocontribute to TNFα-induced cell cycle arrest, apoptosis and necrosis. Flow cytometryindicated thatthe ratio of DCF-positive cells intheTNFα group was significantly higher than that intheNC group (57.15±3.62% compared to 43.82±1.34%, $p<0.001$). Furthermore,pretreatmentwith Dex inhibited theincrease in theratio of DCF-positive cells from 57.15±3.62% (TNFα group) to 48.73±3.50% (DT group) ($p<0.001$). Ati reversed theinhibitoryeffect of Dex ontheTNFα-induced ratio of DCF-positive cells from 48.73±3.50% (DT group) to 55.68±3.20% (ADT group) ($p<0.001$) (Fig. 6 A and B).

5. Dex inhibited the TNFα-induced upregulation of cleaved caspase 9 but not cleaved caspase 8.

Cleaved caspase 8 and 9 are the proteolytic cleavages products of procaspase 8 and 9, respectively, and immunochemistry was used to investigate these factors[12]. The immunofluorescence intensity of cleaved caspase 9 in the TNFα group was 23.91±1.95, which was significantly higher than that in the NC group (5.14±0.99, $p<0.01$). Pretreatment with Dex reduced the immunofluorescence intensity from 23.91±1.95 (TNFα group) to 9.38±1.45 (DT group) ($p<0.001$). The effect of Dex was attenuated by Ati (4.68±0.42%) ($p<0.001$). The immunofluorescence intensity of cleaved caspase 8 in the TNFα group was 12.00±1.44, which was significantly higher than that in the NC group (8.06±1.33, $p<0.01$). There was no significant difference in the immunofluorescence intensity among the TNFα group, DT group (10.96±1.32) and ADT group (9.90±0.98) ($p>0.05$) (Fig. 7 A and C).

Discussion

As a potent agonist oftheadenosine α2 receptor, Dex has been shownto haveprotective effects against injury in certain organs and tissues[13]. In this study, the effects of Dex on TNFα-induced cell injurywereexplored. Our resultsfirstdemonstrated that (1)Dex has potent protective effects against TNFα-induced mitochondrial dysfunction and cell cycle arrest in astrocytes. (2)The protective effect of Dex against TNFα-induced cell injuryispartly dependent on the activation of adenosine α2 receptors. (3)The effect of Dex onastrocytes mayinvolve improvements in mitochondrial function and the inhibition ofthemitochondrial stress-induced apoptosis pathway.

TNFαplaysa pivotal role inthemaintenance and homeostasis of the immune system, inflammation and host defense[14]. Activation of TNFα receptors has dual effects on mammalian cell proliferation and survival, which are dependent on the activation of the nuclear factor κB (NF-κB)signalingpathway. NF-κBactivationinducesnegative regulators of apoptosis,such as cellular FLICE-like inhibitory protein(c-FLIP), B-cell lymphoma-2 (Bcl-2) and superoxide dismutase[15]. Otherwise, apoptosis is mediated by caspase 8throughtheaccumulation of intracellular reactive oxygenspecies(ROS), sustained Jun N-terminal kinase activation and mitochondrial pathways[16,17]. In our study,adecreaseinmitochondrial function (△ψm decline) andanincreaseinROS generationwereobserved following TNFαchallenge, whichmaycontribute to the upregulation of cleaved caspase 8 and cleaved caspase 9 andinducethe arrest ofthecellcyclein astrocytes.

Cell cycle regulation is tightly linked to the control of cell death. Cell cycle-related regulatoryproteinsplay important roles in thisprocess. Cyclins,although originally characterized as cyclin-dependent kinase (CDK) partners, have CDK-independent roles that include regulating DNA damage repair and controlling cell death, differentiation, the immune response and metabolism[18]. Cyclin A is particularly activated by two different CDKs (CDK1 and CDK2) and functions in both S phase and mitosis (M phase).The complex of cyclin A and CDK2 controls DNA replication through the phosphorylation of a set of chromatin factors, which critically influences the S phase transition[19]. Cyclin B produces an almost identical activated conformation in CDK1 as that produced by cyclin A andcontrols theM phase ofthecell cycle[20]. Cyclin D, includingtheD1, D2 and D3subtypes, activates CDK4 and CDK6 and controls the length oftheG1 phase

ofthecell cycle[21]. Cyclin E accumulates during the G1/S transition, where it promotes S phase entry and progression by binding to and activating CDK2[22].Unlikecyclins, Ki67ispresent during all active phases ofthecell cycle (G1, S, G2 and M phase) but not during the resting G0 phase[23]. It was notable that Dex promoted the ratio of positive cells and the intensity of cyclin A, B and Ki67 expression in the TNFα-induced cells but not normal cells, which was partly dependent on the activation of α2 adenosine receptors. Thesefindingsmay be helpful in determiningthe underlying mechanism by which Dex protects injured cells.

The mechanism of the protective effects of Dex against cell injury is still not clear. Based on pilot studies, Dex and α2 receptors have potent protective effects on mitochondria in injured cells but not normal cells, restoring the△ψm, improvingATP production and inhibitingnoxious ROS generation[24]. In this study, Dexreversed the TNFα-inducedupregulation ofcleaved caspase 9 but not cleaved caspase 8. In the two best-described apoptotic pathways in mammals, caspase 9 functions as the initiator caspase intheintrinsic or mitochondrial stress-induced apoptosis pathway, while, caspase 8uniquelyacts as an environmental sensor and functions in the extrinsic or receptor-induced pathway in late apoptosis[25]. The effects of Dex on caspase 8 and 9 indicated that the protective effects of Dex against cell apoptosismay occurthroughtheintrinsic or mitochondrial pathway duringtheearly stage of injury.

Dexisnormally used as an analgesicandsedative agent intheoperating room and intensive care unit. An increasing number ofstudies haveindicatedthatDexisbeneficial in reducing the consumption ofopioidsand local anesthetics inpatientsusing patient-controlled spinal or intravenous analgesia[26,27]. In pilot studies,we foundthatDex has protective effects on lung and kidney cells induced by ischemia and reperfusion injury, oxidative stress and systematic inflammation[28-30].Mostadenosine α2 receptorsthat bindto Dexareexpressed in central nervous system cells, including neurons, astrocytes and microglia[31,32]. The results of our study indicated that Dex exerted a protective effect on injured astrocytes by improving the function of mitochondria and inhibiting noxious ROS generation, which allowed mitochondria to provide more energy for survival. Although our study only focused on the effect of Dex on astrocytes in vitro and there are some differences between cell lines and primary cultured cells, the results still give us a strong indication that Dex may be preferentially chosen as a sedative and analgesic for those individuals with or who are likely to suffer iatrogenic nervous cell injury. Otherwise, further studies of Dex need to be done on other nervous system cells, animal models and clinical trials to explore its profound effects and underlying mechanism.

Conclusion

Dex exerted a potent protective effect against cell injury induced by TNFα, possibly by improving the function of mitochondria and inhibiting the intrinsic caspase pathway during the early stage of apoptosis.

Declarations

Acknowledgments

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Declaration of interest statement

The authors declare that they have no competing interests.

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Figures

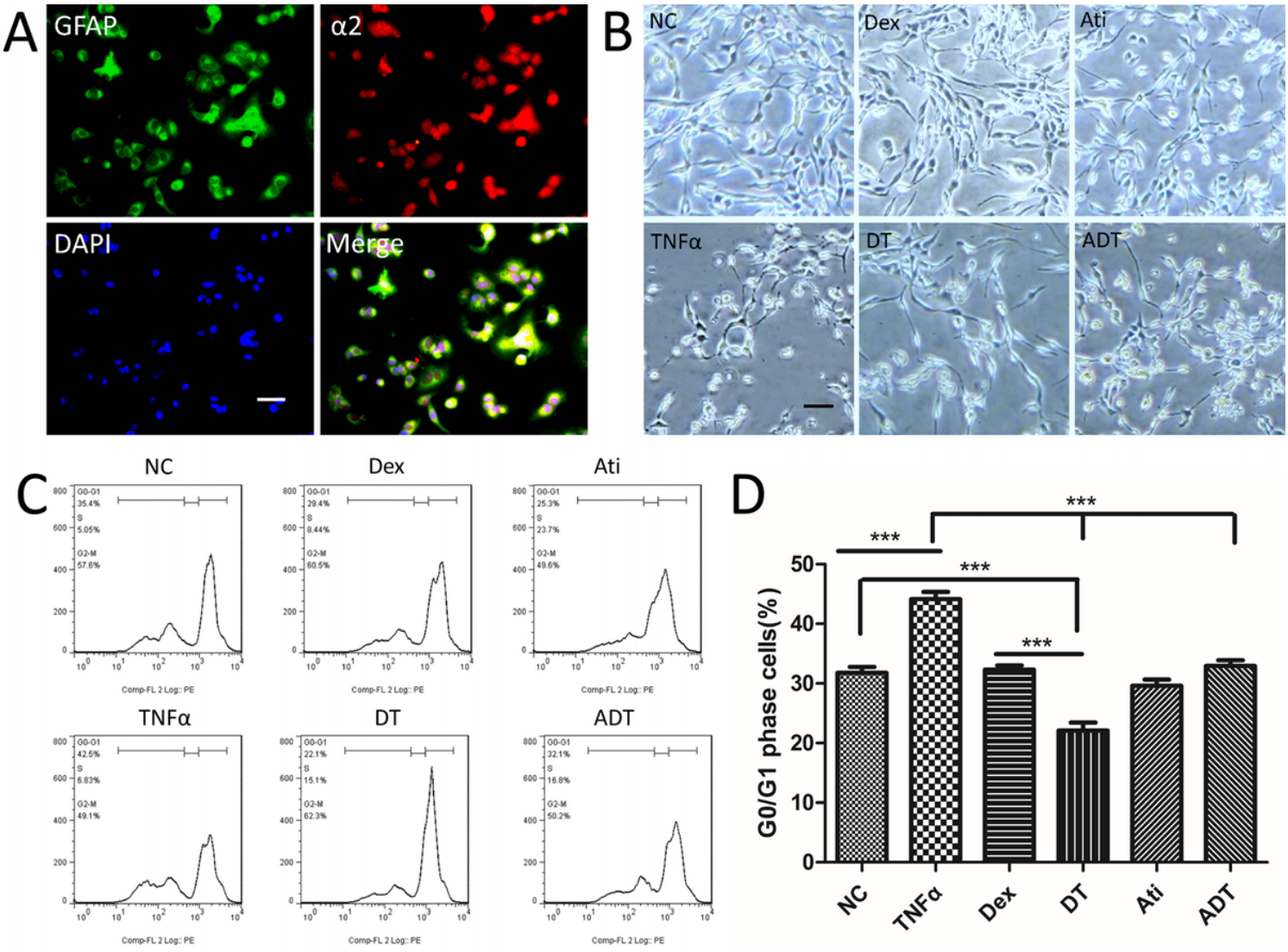

Figure 1

Dex attenuated cell cycle arrest induced by TNFα. A: Coexpression of the α2 receptor and GFAP on 1321N1 cells. B: Inverted microscopy showed that the number of broken cells in the medium in the TNFα group was increased compared to that in the NC, Dex, Ati and DT groups but not the ADT group. C: The ratio of G0-G1 phase cells was investigated by flow cytometry. D. G0-G1 phase cells increased following TNFα challenge for 24 hours, and this effect was inhibited by pretreatment with Dex in the absence of Ati. Scale bar = 50 μm. *** P<0.001.

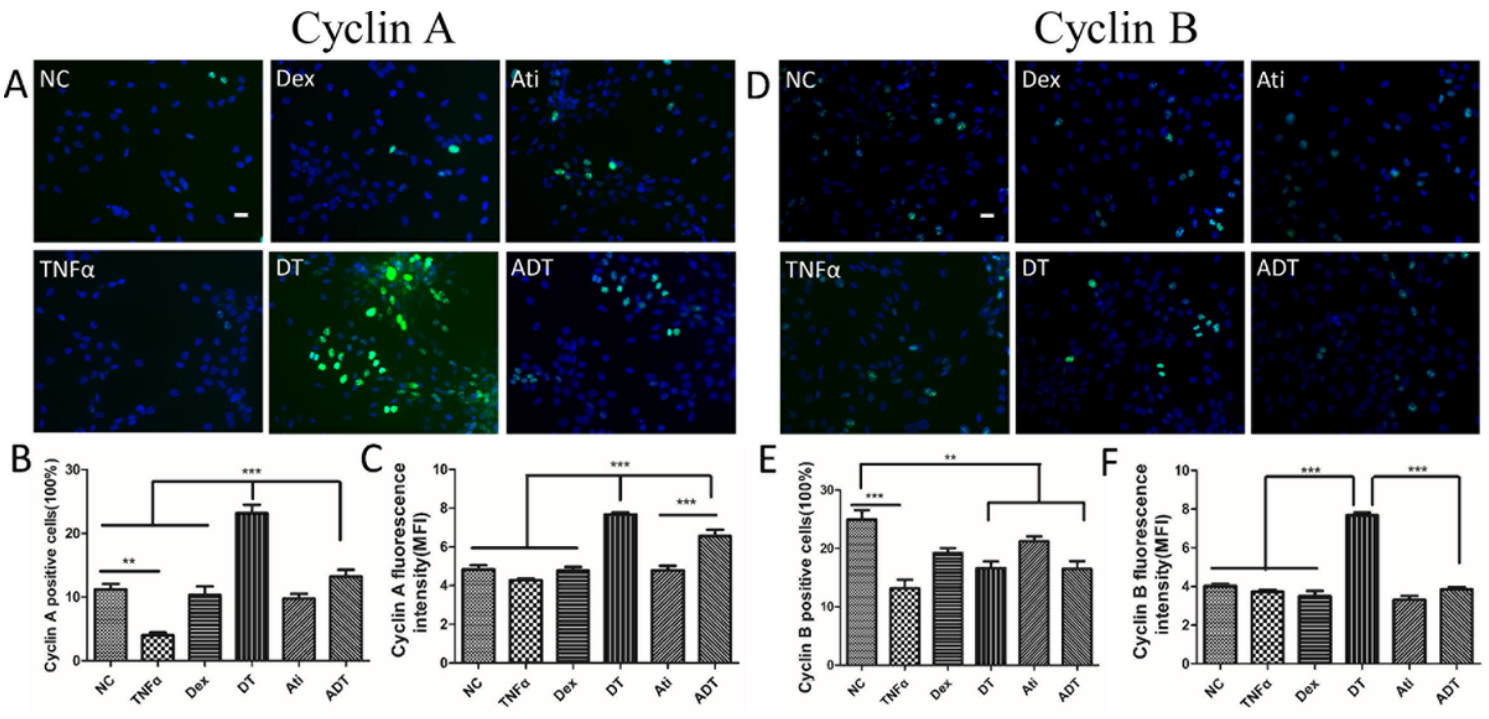

Figure 2

Dex reversed the downregulation of cyclin A and cyclin B induced by TNFα. A: The expression of cyclin A in astrocytes. B: Pretreatment with Dex upregulated the ratio of cyclin A-positive cells. C: Pretreatment with Dex increased the mean fluorescence intensity (MFI) of cyclin A expression in astrocytes. D: The expression of cyclin B in astrocytes. E: Pretreatment with Dex upregulated the ratio of cyclin B-positive cells. F: Dex pretreatment increased the MFI of cyclin B expression in astrocytes. Scale bar = 50 μm. **P<0.01, ***P<0.001.

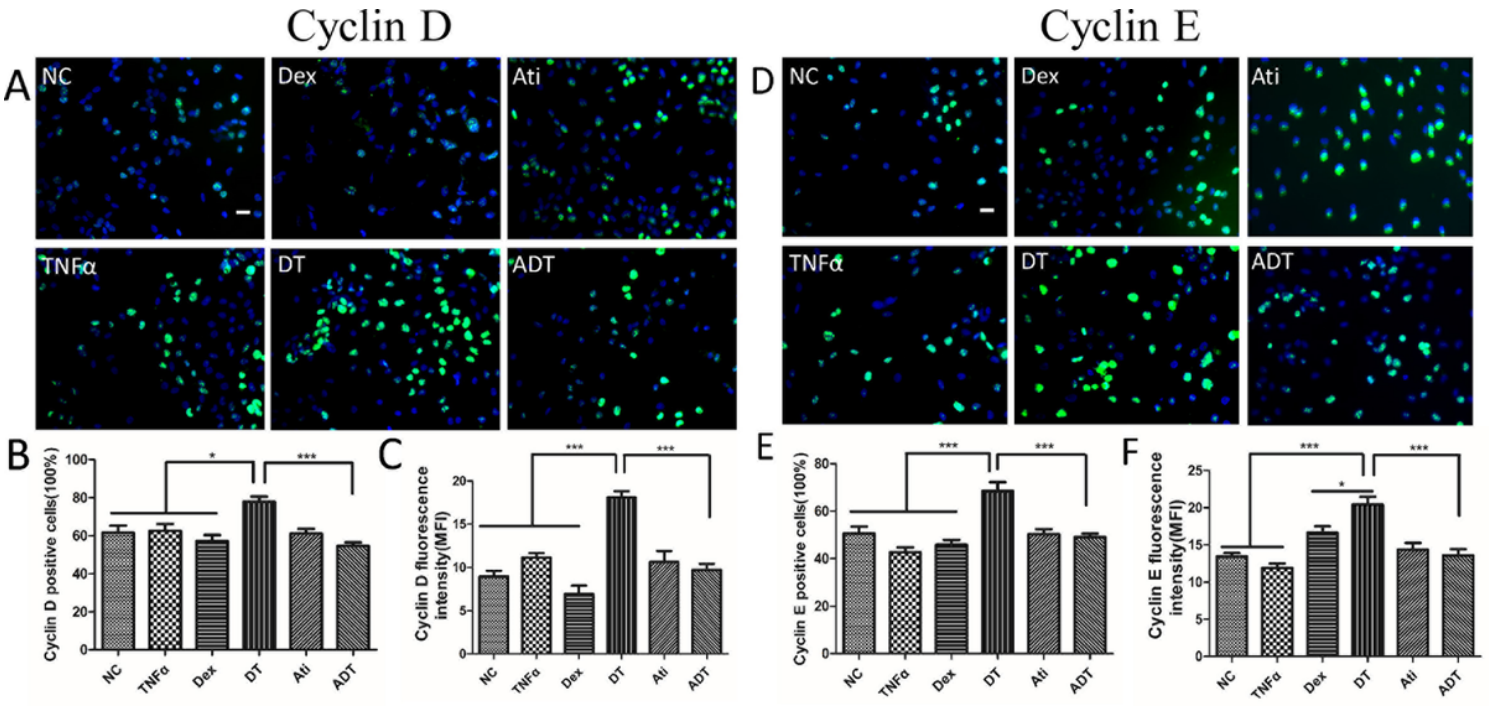

Figure 3

Dex upregulated the expression of cyclin C and cyclin D following TNFα challenge. A: The expression of cyclin C in astrocytes. B: TNFα had no effect on the expression of cyclin C, but pretreatment with Dex upregulated the ratio of cyclin C-positive cells. C: Dex pretreatment before TNFα challenge increased the mean fluorescence intensity (MFI) of cyclin C expression in astrocytes. D: The expression of cyclin D in astrocytes. E: TNFα had no effect on the expression of cyclin D, but pretreatment with Dex upregulated the ratio of cyclin D-positive cells. F: Pretreatment with Dex before TNFα challenge increased the MFI of cyclin D expression in astrocytes. Scale bar = 50 μm. *P<0.05, ***P<0.001.

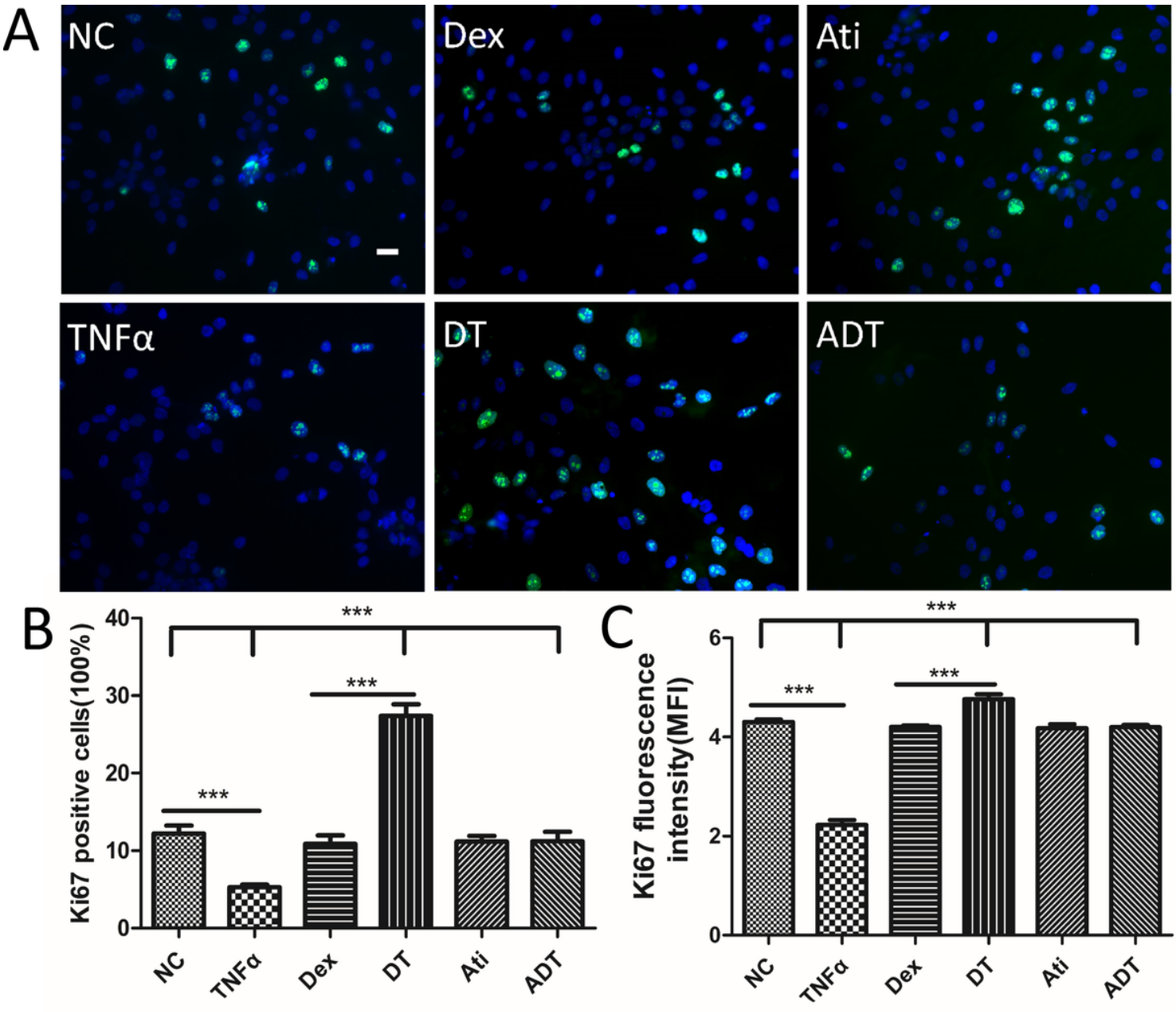

Figure 4

Dex upregulated the expression of Ki67 in TNFα-challenged astrocytes. A: The expression of Ki67 in astrocytes. B. The ratio of Ki67-positive cells was significantly decreased in the TNFα group and significantly increased in the DT group compared with the NC, Dex, Ati and ADT groups. C. Pretreatment with Dex reversed the Ki67 MFI to the same level as that in the NC, Dex, Ati and ADT groups. Scale bar = 50 μm. *** P<0.001.

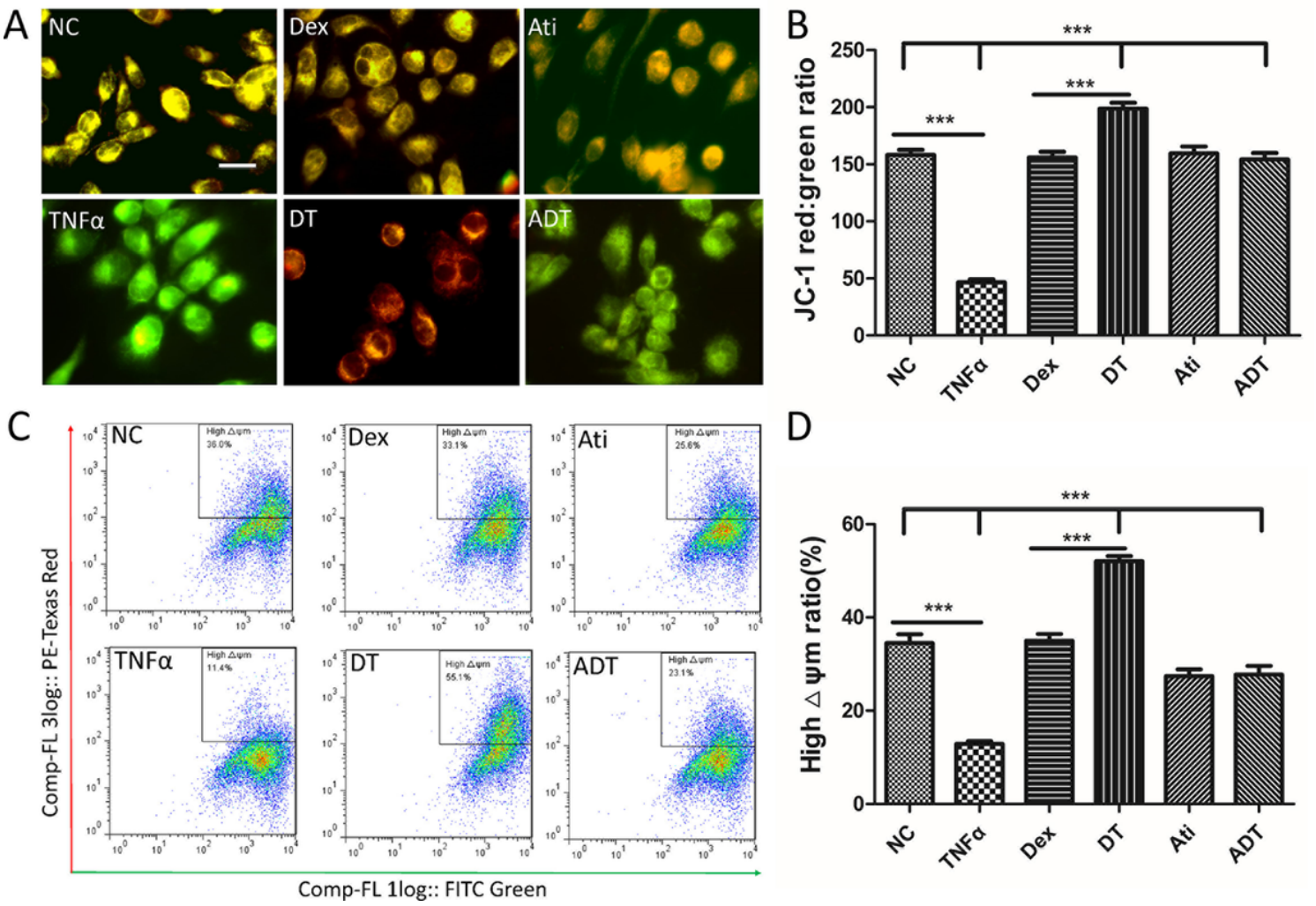

Figure 5

Dex restored the △ψm of astrocytes induced by TNFα in a manner dependent on the α2 receptor. A: Merged fluorescence microscopy images showing JC-1 staining. Red represents cells with a high △ψm, and green represents cells with a low △ψm. B: Ratio of red/green fluorescence MFI. B. The △ψm was measured by flow cytometry in the FL1 (FITC) and FL2 (PE) channels after staining with JC-1. The cells in the gating areas with a high ratio of red/green fluorescence intensity had relatively high △ψm, D: The ratio of the fluorescence intensity of JC-1 (red/green), was assessed by flow cytometry. Scale bar = 50 μm. ***P<0.001.

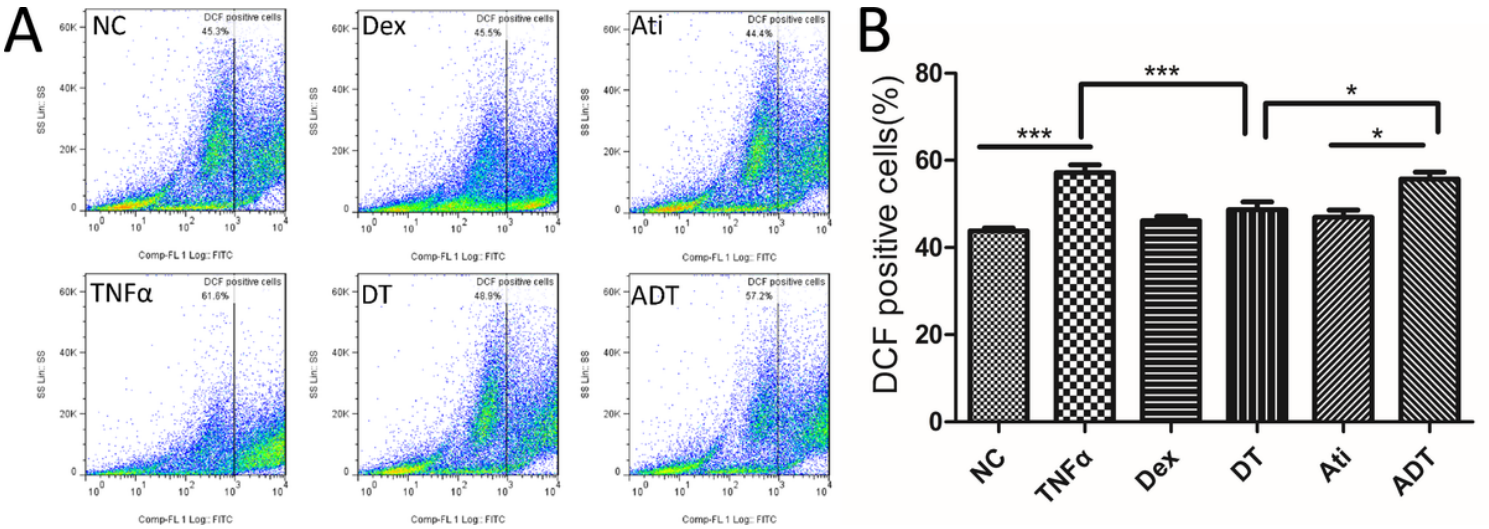

Figure 6

Effects of Dex on TNFα-induced ROS generation. A. DCF-positive cells were examined by flow cytometry. DCF-positive cells represent an increase in the production of ROS. B. The proportion of DCF-positive cells was assessed by flow cytometry. *P<0.05, ***P<0.001.

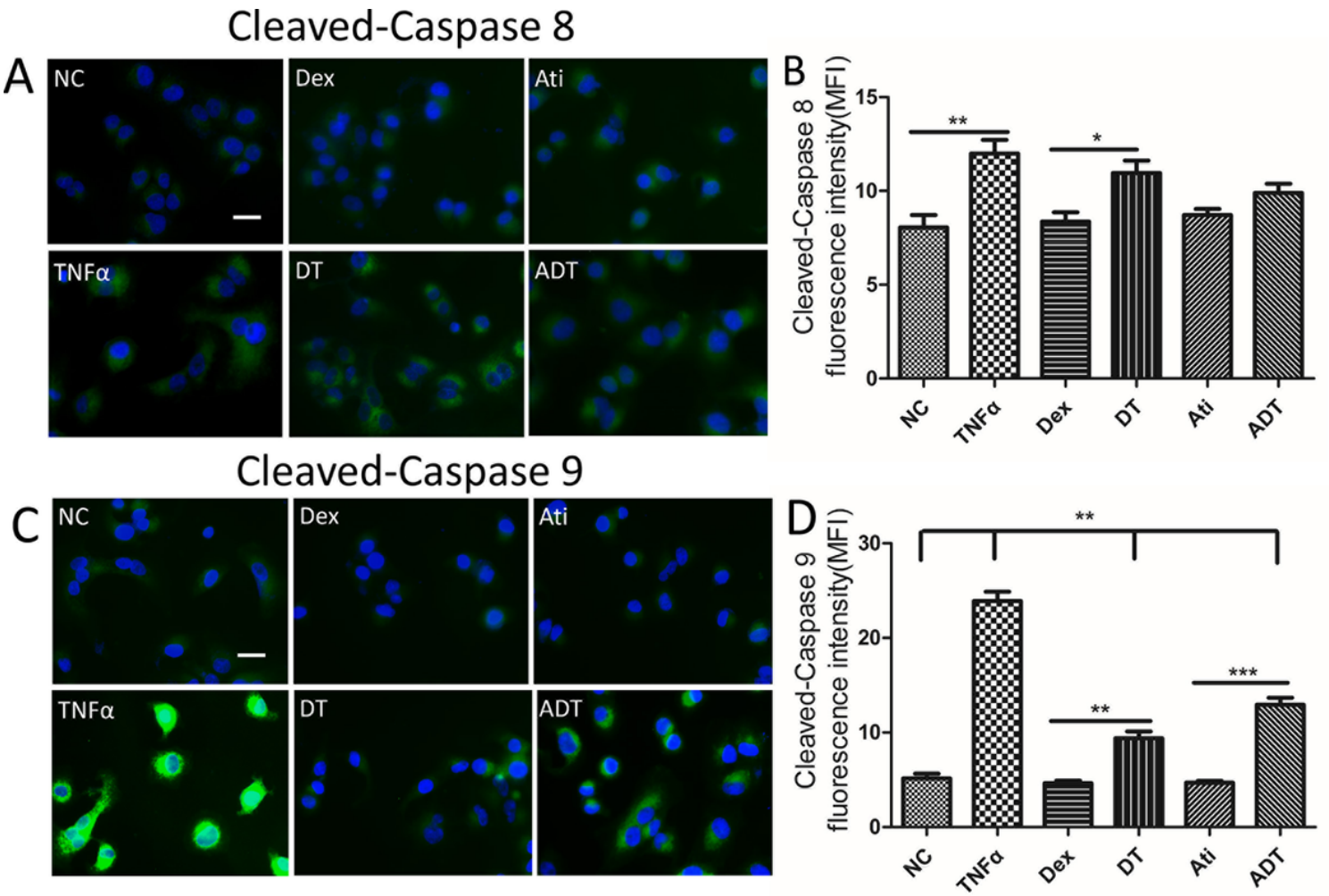

Figure 7

Effects of Dex on the TNFα-induced upregulation of cleaved caspase 8 and cleaved caspase 9. A: The expression of cleaved caspase 8 (green) was assessed by immunofluorescence staining. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). B: The MFI of cleaved caspase 8 was assessed by immunofluorescence staining, which indicated that Dex had no significant effect on the overexpression of cleaved caspase 8 induced by TNFα. C: The expression of cleaved caspase 9 (green) was assessed by immunofluorescence staining. D: The MFI of cleaved caspase 9 was assessed by immunofluorescence staining and indicated that Dex significantly inhibited the overexpression of cleaved caspase 9 induced by TNFα.