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Inhibition of HSP90β by ganetespib blocks the microglial signalling of evoked pro-inflammatory responses to heat shock

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ABSTRACT

Although microglial reaction to heat shock is considered to be protective, heat shock is still a potential hazard caused by high temperatures. Recent studies indicate that the inhibition of the 90-kDa heat shock protein (HSP90) increasing the protective heat shock response and suppressing inflammatory signalling pathways in several diseases. Nevertheless, the effects of heat shock on microglial pro-inflammatory responses are not completely identical. Here, we aim to investigate the effect of the HSP90 inhibitor ganetespib on microglial pro-inflammatory responses following heat shock. HSP90 isoforms were determined by transfecting N9 microglial cells (N9 cells) with enzymatically prepared siRNA (esiRNAs). We found that heat shock significantly increased the secretion of tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6 and nitric oxide (NO), and the phosphorylation of extracellular signal–regulated kinase (ERK), Janus-activated kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκB-α) and p65 nuclear factor kappa-light-chain-enhancer of activated B cells (p65 NF-κB) in N9 cells. These increases, except for phospho-p65, were attenuated efficiently in a dose-dependent manner by ganetespib pretreatment. Furthermore, the suppression of heat shock–evoked cytokines and NO production, and the phosphorylation of ERK, JAK2 and STAT3 in cytosols and/or nuclei were also observed by administering esiRNA HSP90β, but not HSP90α, in heat shock–treated N9 cells. Taken together, our findings demonstrate that the HSP90 inhibitor ganetespib blocks pro-inflammatory responses in heat shock–treated N9 cells via a signalling mechanism involving HSP90β and STAT3.

1. Introduction

The trend of increasing global temperature brings environmental health hazards to humankind and other organisms (McMichael, 2003). The intensity, frequency and duration of extreme heat coincide with adverse effects on the central nervous system (CNS); cardiovascular, respiratory and endocrine systems and psychiatric disorders (Kirch et al., 2005). Considering that sudden elevated temperatures damage cellular functions and, on the contrary, cells induce a protective heat shock response (HSR) autonomously (Richter et al., 2010), increased attention is being paid to address the molecular basis of heat shock. Studies regarding effects of heat on the CNS have been greatly overlooked; however, the mechanisms behind heat injury are still unclear. Moreover, except for glial ability to respond to heat shock (Cervos-Navarro et al., 1998; Marcuccilli et al., 1996), very little information is available related to the relationship between the pro-inflammatory response and microglial activation after heat shock.

As the primary immune defenders in the CNS, macrophage-like microglia detect abnormal neuronal activity or tissue damage and maintain healthy functions in response to any deviation from physiological homeostasis (Hanisch and Kettenmann, 2007). Not surprisingly, microglial reactions are believed to be neuroprotective cells responsible for heat shock (Cervos-Navarro et al., 1998). However, uncontrolled and over-activated microglia are a prominent source of excess production of pro-inflammatory factors, triggering neurotoxicity by a possible autocrine loop (Block et al., 2007; Smith et al., 2012; Yang et al., 2010). By now, the signal transduction mechanisms involved in microglial activation and the neuroinflammatory factors released after heat shock are still largely unknown. Although mitogen-activated protein kinases (MAPKs), signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF–κB) pathways have crucial roles in the pathogenesis of several neuroinflammatory disorders (Jenkins, 2014; Kim et al., 2004), it is not known whether or how these pathways participate in microglial

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reaction to heat shock.

Ganetespib (formerly known as STA-9090) is a second-generation, resorcinol-containing, small-molecule inhibitor of heat shock protein 90 (HSP90) (Ying et al., 2012). Ganetespib has been proven highly effective against a diverse group of cancers through phase II–III clinical trials, and it also exerts anti-inflammatory properties currently (Lilja et al., 2015; Tukaj and Wegrzyn, 2016). Recent studies have provided increasing evidence to show that HSP90 inhibition attenuates the critical effector molecules of MAPKs, STAT3 and NF–κB signalling cascades in inflammatory diseases (Hosoi et al., 2016; Poulaki et al., 2007; Thangjam et al., 2016). Moreover, HSP90 has been the most widely tested target for neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Kakimura et al., 2002; Ou et al., 2014; Alam et al., 2017). It is reasonable to suggest that ganetespib treatment might exert anti-inflammatory activity against microglial inflammatory responses induced by heat shock. The present study demonstrated that HSP90 inhibitor ganetespib may dephosphorylate and inactivate extracellular signal–regulated kinase (ERK) and STAT3, leading to a depression in pro-inflammatory mediators following heat shock. Moreover, isoform HSP90β (also known as HSP90AA1) was found to play a key role in regulating the heat shock–induced activation of JAK2–STAT3 signalling. These results might provide critical information supporting the possible biological insights and therapeutic implications of HSP90 blockers as anti-inflammatory agents for heat injuries.

2. Materials and methods

2.1. Cell culture and treatments

The immortalized murine microglial cell line N9 was a gift from Dr. Yun Bai [Department of Genetics, Army Medical University (Third Military Medical University), China] and was cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μM 2-mercaptoethanol (Sigma-Aldrich, MO, USA). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Cells were passaged every 3 days with a 1:4 split ratio and used at passages 3–10. After a 24-h incubation, the cell culture medium was replaced with serum-free IMDM supplemented with or without a solvent control (dimethyl sulfoxide; Sigma-Aldrich), or a transient transfection control (DharmaFECT; GE Healthcare Dharmacon, CO, USA), ganetespib (25, 50, 100 and 200 nM; MedChemExpress, NJ, USA) or enzymatically prepared siRNA (esiRNA; Sigma-Aldrich) and incubated for 30 min or 12 h respectively. Subsequently, cells were immediately subjected to a 0.5-, 1-, 1.5-, 2- or 3-h process of heat shock in a pre-warmed incubator at 42 °C, followed by recovery at the normal growth temperature of 37 °C for the desired time, both in a humidified atmosphere of 5% CO_2.

2.2. Cell viability assay

The Cell Counting Kit-8 assay (CCK-8; Dojindo, Shanghai, China) was used to indirectly assess cell viability dependent of several proliferation-related elements, including dehydrogenase, NAD(H), NADP(H) and mitochondrial activity. Briefly, Cells were seeded at a density of 5×10^3 cells/well for 6 and 24 h in 96-well plates in the IMDM medium at 37 °C, which was followed by the cell treatments described earlier. At the end of the culture period, 10 μL of CCK-8 solution was added to each well in the culture plate. After a 2-h incubation at 37 °C, the absorbance at 450 nm was measured with a plate reader (Bio-tek Epoch, VT, USA).

2.3. ELISA and nitric oxide determination

After the designated treatment with ganetespib and/or heat shock, cell culture supernatants were collected for determining tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β and IL-6 using the enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, CA, USA) according to the manufacturer's protocol. The supernatant obtained was also used for measuring the level of nitric oxide (NO) production by indirectly determining NO metabolites (nitrates and nitrites) using the Griess reagent system (Promega, WI, USA) according to the manufacturer's protocol. The results of ELISA assay and NO determination were read using a plate reader (Bio-tek Epoch) at 450 nm and 540 nm respectively.

2.4. Flow cytometry

The expression of microglial marker CD11b (Kreutzberg, 1996) was measured to assess the activation state of microglial cells by the fluorescence-activated cell sorting (FACS) analysis. Briefly, cells were washed three times with flow buffer [phosphate-buffered saline (PBS) containing 0.1% (w/v) sodium azide and 1% (w/v) bovine serum albumin] and resuspended in 250 μL of ice-cold flow buffer. Cells were preincubated with goat serum [Zhongshan Goldenbridge Biotechnology (Zsbio), Beijing, China] for 20 min at 4 °C to block nonspecific binding to Fc receptors. Cells were then spun down at 5000 rpm, washed three times with flow buffer and incubated with rat anti-mouse antibody CD11b-Brilliant Violet 510™ (1:100; Biolegend, San Diego, CA, USA) or rat immunoglobulin G2b (IgG2b) isotype control (1:100; Biolegend) for 1 h at 4 °C in the dark. A quantitative analysis was performed using a flow cytometer (BD Accuri C6; BD Biosciences, CA, USA).

2.5. Quantitative real-time polymerase chain reaction

Cells were seeded in six-well plates. After treatment, the total RNA was isolated using TRIzol reagent (1 mL/well; Invitrogen, CA, USA). RNA was reverse-transcribed to cDNA using a PrimeScript RT reagent kit with a gDNA Eraser cDNA synthesis kit according to the manufacturer's protocol (Takara Biotechnology, Dalian, China). A quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using a CFX Connect Detection System (Bio-Rad, CA, USA) and a KAPA SYBR FAST qPCR Kit (Kapa Biosystems, MA, USA). The relative expression levels of mRNAs were normalized to an internal control hypoxanthine phosphoribosyltransferase using the $2^{-\Delta\Delta Ct}$ cycle threshold method (Schmittgen and Livak, 2008). Primers sequences are listed in Table 1.

2.6. Western blotting

Cells were washed with ice-cold PBS and scraped in the RIPA lysis buffer containing protease and phosphatase inhibitors (Roche, Penzberg, Germany). Nuclear and cytoplasmic fractionations were

Table 1
The sequences of RT-PCR primers.

Sequences		
HSP90α	Forward	TTGTGTCAAACCGACTGGTG
	Reverse	GCTGCCATGTAACCCATTGT
HSP90β	Forward	AAGATGACAGCGGCAAAGAC
	Reverse	CCATACTCCTCCTGCGTGAT
HSP70	Forward	GCGAGCTTTAGTGTGTCAAGG
	Reverse	CATGCCTACACCCAACTGTG
TNF-α	Forward	GACCCTCACACTCAGATCATCTTCT
	Reverse	CCTCCACTTGGTGGTTTGCT
IL-1β	Forward	TGGTGTGTGACGTTCCCATTA
	Reverse	CAGCACGAGGCTTTTTTGTTG
IL-6	Forward	ACAACCACGGCCTTCCCTACTT
	Reverse	CACGATTTCCCAGAGAACATGTG
iNOS	Forward	GGCAGCCTGTGAGACCTTTG
	Reverse	GCATTGGAAGTGAAGCGTTTC
HPRT	Forward	GTTAAGCAGTACAGCCCCAAA
	Reverse	AGGGCATATCCAACAACAAACTT

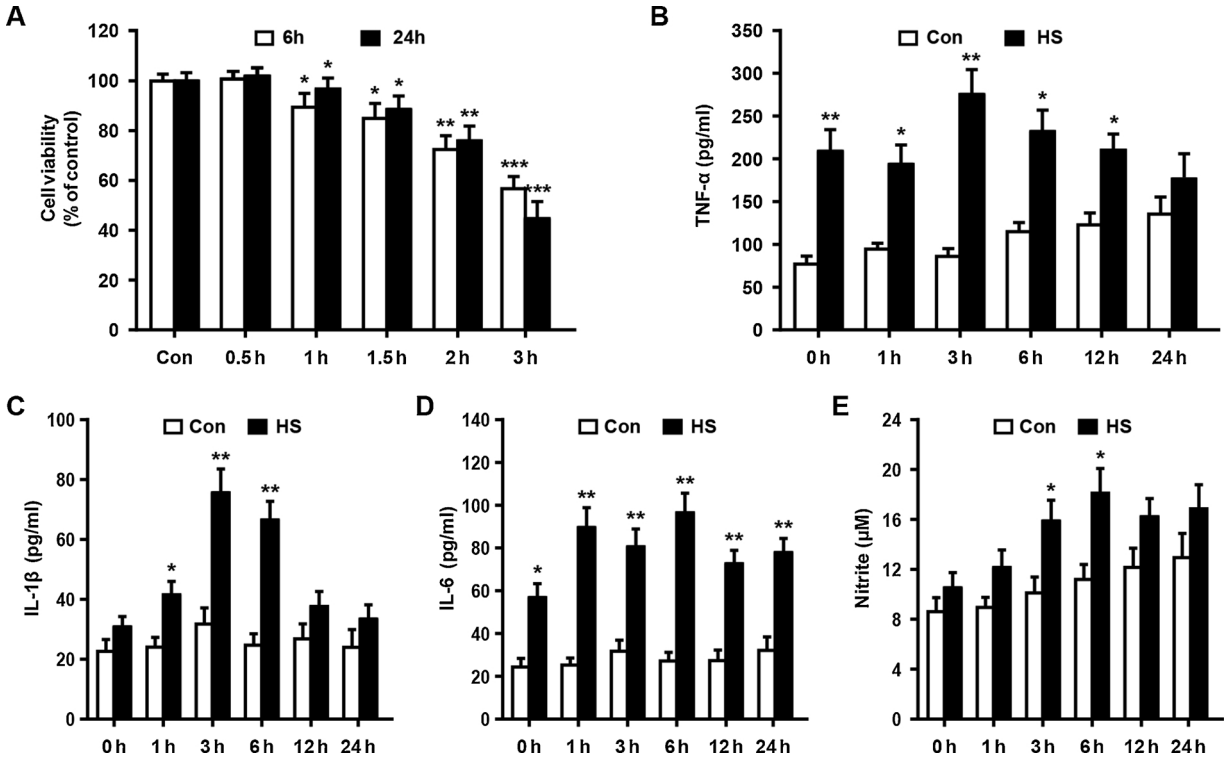

Fig. 1. Heat shock-induced pro-inflammatory responses in N9 cells. N9 cells were exposed to a 0.5-, 1-, 1.5-, 2-, or 3-h heat shock process at 42 °C, followed by the indicated recovery periods. (A) Heat shock-induced toxicity was determined using CCK-8 in the 6- and 24-h recovery period respectively in cultured N9 cells. An ELISA assay of TNF-α (B), IL-1β (C) and IL-6 (D) and Griess determination of nitrite (E) in the culture medium supernatants of N9 cells were determined after the cells were exposed to 42 °C heat shock or sham control for 1.5 h at the indicated recovery time points. Data are presented as means ± SEM of three independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's test. Statistical comparisons to the untreated control group are indicated by $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

performed with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Thermo Scientific, IL, USA). The protein concentration was determined by a bicinchoninic acid protein assay (Beyotime Biotech, Beijing, China). Whole-cell, cytoplasmic or nuclear extracts were separated by 10% TGX stain-free polyacrylamide gels (Bio-Rad) and transferred onto polyvinylidene difluoride membranes (Bio-Rad). After blocking with 5% nonfat skim milk, the membranes were immunoblotted with antibodies against HSP90α and HSP90β (Millipore, MA, USA), and with antibodies (Cell Signaling Technology, MA, USA) that recognize HSP70 and the phosphoprotein and total protein of p38 MAPK, p44/42 MAPK (ERK1/2), SAPK/Jun N-terminal Kinase (JNK), JAK2, STAT3, IκB-α and p65 nuclear factor (NF-κB). Subsequently, the blots were incubated with horseradish peroxidase –conjugated secondary antibodies (ZsBio, Beijing, China), protein bands were visualized by enhanced chemiluminescence reagent (Bio-Rad) and the signal was detected using a ChemiDoc MP gel imaging system (Bio-Rad).

2.7. siRNA transfection

Cells were seeded in six-well plates or 25 cm^2 T-flasks. Following 24-h incubation, cells were transfected with 20 nM esiRNAs (Sigma-Aldrich) oligonucleotide targeting HSP90α or HSP90β or a control esiRNA using DharmaFECT (GE Healthcare Dharmacon) in Opti-MEM (Invitrogen Life Technologies, CA, USA) following the manufacturer's protocol. At the end of the culture, the effects of esiRNA on mRNA and protein expression were analysed using qRT-PCR and Western blotting.

2.8. Immunofluorescence

Cells were grown on coverslips in 24-well plates. After being fixed and permeabilized, the cells were blocked with goat serum (ZsBio) for 20 min at room temperature and washed three times in PBS. Cells were incubated with rabbit anti-mouse HSP90β (Millipore) and mouse anti-mouse phospho–Tyr705–STAT3 antibody (Cell Signaling Technology) at 37 °C for 1 h to observe HSP90β expression and phospho–STAT3 nuclear translocation. After being washed, slides were incubated for 1 h at 37 °C with chicken anti-rabbit IgG (H + L) CF633 and donkey anti-mouse IgG (H + L) CF555 antibody (Sigma-Aldrich) in the dark. Slides were then washed and mounted with an aqueous-based anti-fade mounting medium. Images of stained cells were captured using an LSM 780 confocal laser-scanning microscope (Carl Zeiss GmbH, Jena, Germany). A semi-quantitative analysis was performed to analyze the fluorescence intensity using software ImageJ 1.50i. Pixel-by-pixel co-localization analysis was performed using an ImageJ plugin termed JACoP to determine the P with marker proteins.

2.9. Statistical analysis

A statistical analysis was performed using PASW Statistics 18.0 software (SPSS, Inc., Somers, NY, USA). Data were presented as the means ± standard error of the mean. Statistical differences between the groups were assessed by a one- or two-way analysis of variance followed by the Tukey's test. Statistical significance was established at $P < 0.05$, unless otherwise indicated.

3. Results

3.1. Pro-inflammatory responses are evoked in heat-shocked N9 cells

The cell viability of N9 microglial cell (N9) cells was assessed indirectly for the duration and recovery time of heat shock by a

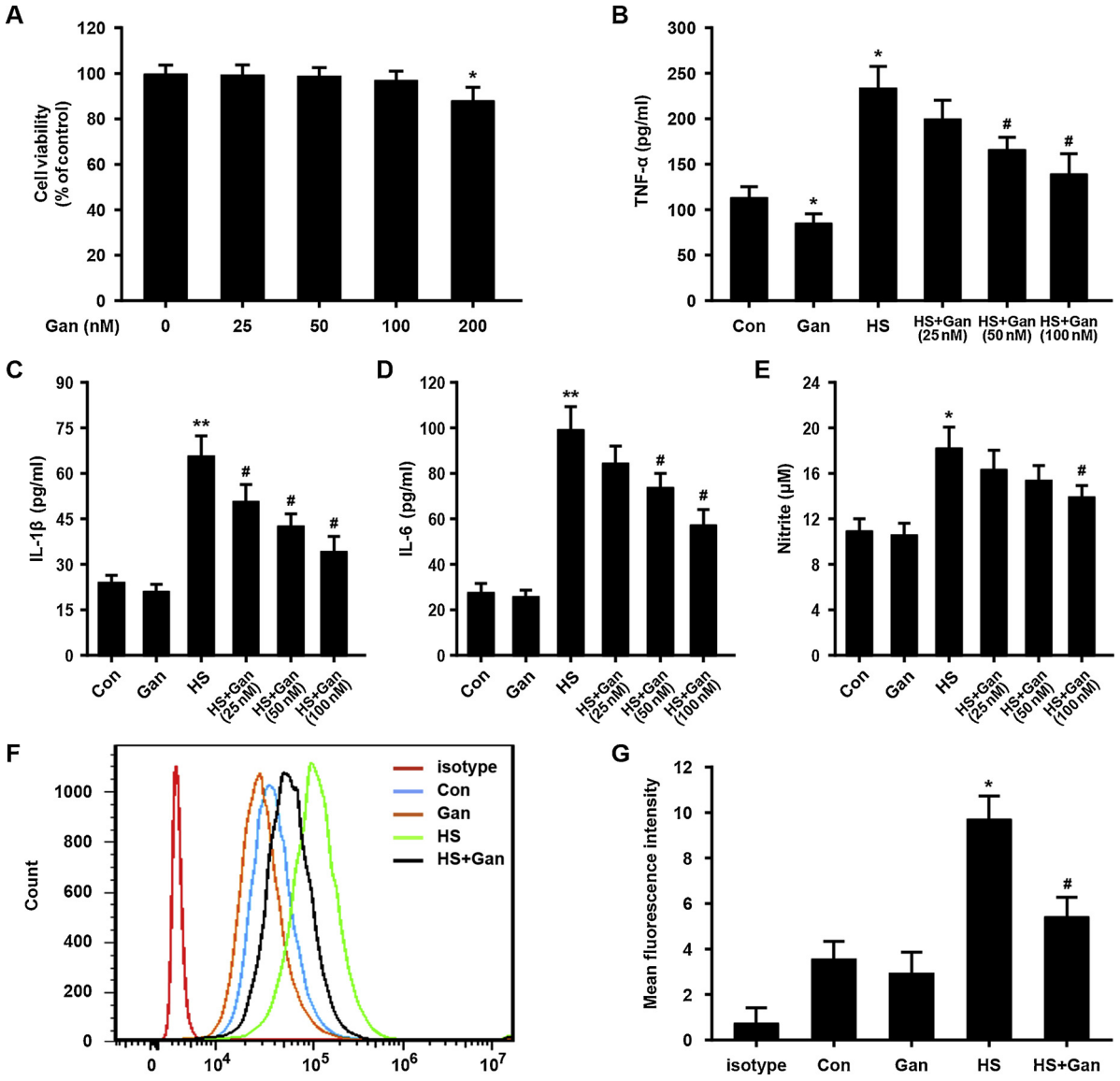

Fig. 2. Effects of ganetespib on heat shock-induced pro-inflammatory responses in N9 cells. N9 cells were pretreated with or without ganetespib and then subjected to heat shock or sham control. (A) Cell viability was measured using CCK-8 after 24-h treatment with ganetespib (0 (Control), 25, 50, 100, and 200 nM). An ELISA assay of TNF-α (B), IL-1β (C) and IL-6 (D) and Griess determination of nitrite (E) in the culture medium supernatants of N9 cells pretreated with or without ganetespib (25, 50, and 100 nM) 6 h after heat shock. (F) N9 cells were pretreated with or without 100 nM ganetespib. Representative flow cytometry histograms for CD11b expression 6 h after heat shock exposure. (G) Average values of the mean fluorescence intensity of CD11b. Data are presented as means ± SEM of three independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's test. Statistical comparisons to the untreated control group are indicated by $^{*}P < 0.05$; $^{**}P < 0.01$. Statistical comparisons to the heat shock group are indicated by $^{\#}P < 0.05$.

proliferation-based CCK-8 assay (Fig. 1A). Heat shock was shown to be cytotoxic to N9 cells when heat exposure was longer than 1 h at the recovery period after heat shock at 42 °C. Clearly, cytotoxicity was significantly intensified when the heating process lasted longer than 1.5-h heat exposure with a 6-h recovery time point, which was identified as a threshold condition representing the time of duration and recovery beyond which cytotoxicity significantly increases. Given the potential pro-inflammatory effects of the threshold duration time of heat shock on microglia, the levels of TNF-α, IL-1β, IL-6 and NO in the culture medium supernatants of N9 cells were measured at the indicated time points during recovery from heat shock (Fig. 1B–E). TNF-α and IL-6 were found to immediately increase, and IL-1β and NO significantly increased at 1 h and 3 h respectively after the heat shock (Fig. 1B–E). TNF-α and IL-1β levels peaked at 3 h and were even higher than the control at 6 h; IL-6 and NO levels peaked at 6 h (Fig. 1B–E). This study confirmed the threshold 6-h recovery time of heat shock with prominent pro-inflammatory activity. Consequently, these results indicate that heat shock may be the potential risk factor to induce aberrant microglial immunoregulation in these threshold experimental conditions.

3.2. Ganetespib suppresses the pro-inflammatory responses of heat-shocked N9 cells

Recent studies have demonstrated the protective effects of HSP90 inhibition in various inflammatory diseases and conditions (Lilja et al., 2015; Tukaj and Wegrzyn, 2016). Accordingly, the production of pro-inflammatory mediators from cellular supernatant sampling was determined to test whether the HSP90 inhibitor ganetespib prevents the pro-inflammatory responses evoked in heat shock–activated N9 cells. Preliminary tests were performed at different concentrations of ganetespib to establish the optimal dose in experimental conditions. As

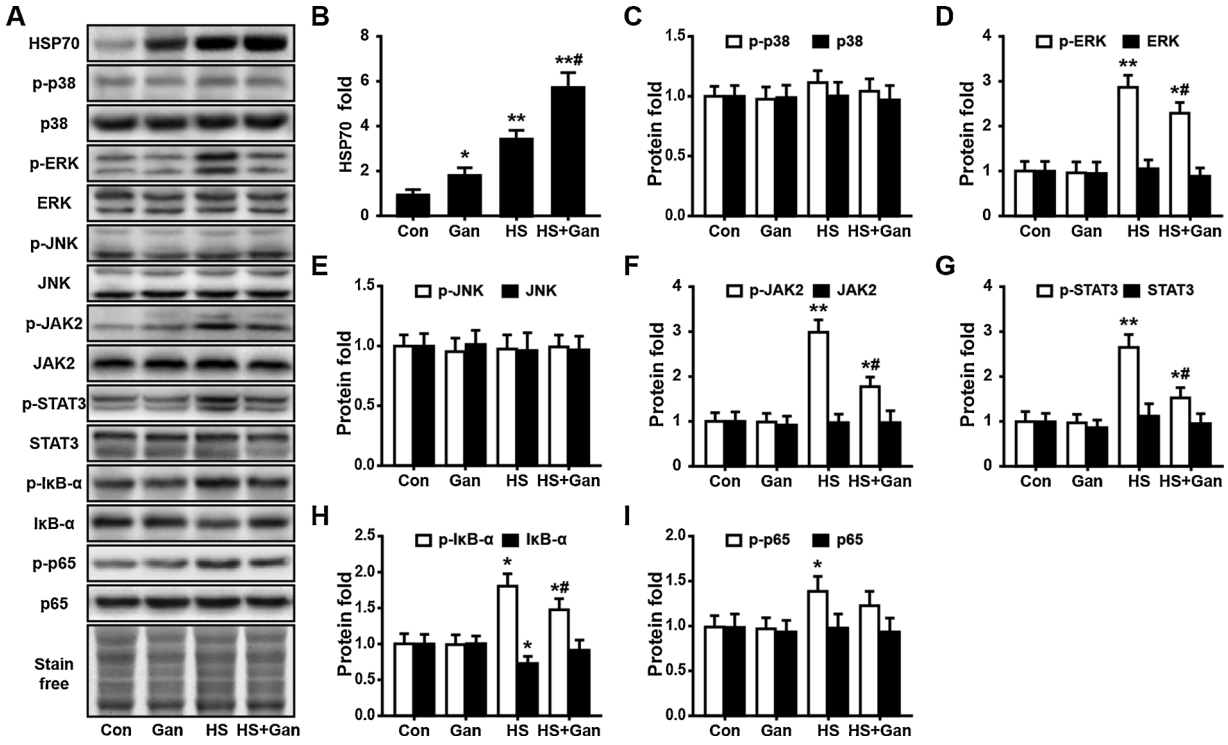

Fig. 3. Ganetespib regulates the heat shock-mediated signalling effectors in N9 cells. N9 cells were pretreated with or without ganetespib (100 nM) and then subjected to heat shock or sham control. (A) Levels of HSP70, and phosphorylation and total protein of p38, ERK, JNK, JAK2, STAT3, IκB-α and p65 in total cell lysates were analysed using Western blotting at 6-h recovery after heat shock. The densitometric analysis was performed for HSP70 (B), and phosphorylation and total protein of p38 (C), ERK (D), JNK (E), JAK2 (F), STAT3 (G), IκB-α (H) and p65 (I) respectively. Data are presented as means ± SEM of three independent experiments. Statistical significance was determined by two-way ANOVA followed by Tukey's test. Statistical comparisons to the untreated control group are indicated by $^{*}P < 0.05$; $^{**}P < 0.01$. Statistical comparisons to the heat shock group are indicated by $^{\#}P < 0.05$.

shown in Fig. 2A, no significant changes were observed in cell survival when N9 cells were exposed to ganetespib at concentrations lower than 200 nM for 24 h. Subsequently, the aforementioned threshold heat shock, 1.5-h duration with a 6-h recovery process, was employed to determine the anti-inflammatory effects of ganetespib. Indeed, ganetespib significantly suppressed the release of TNF-α, IL-1β, IL-6 and NO in a dose-dependent manner over a dose range of 25–100 nM 6 h after heat shock in N9 cells (Fig. 2B–E). Moreover, a FACS analysis revealed that heat shock–induced enhancement expression of CD11b was significantly attenuated by administering ganetespib (Fig. 2F–G). The findings of the present study suggest that the inhibition of microglial HSP90 might be capable of preventing the pro-inflammatory activation of microglia following heat shock treatment.

3.3. Signalling pathways involved in the inhibition of pro-inflammatory responses by ganetespib

A stain-free technology for the Western blotting analysis (Gurtler et al., 2013) was considered appropriate to avoid the probable alteration of internal control housekeeping proteins influenced by heat shock (de Gannes et al., 1998) and performed in the following experiments (Fig. 3A). Mechanisms for HSP90 inhibition could involve an altered abundance of HSP70, which was recognized as a surrogate marker for HSP90 inhibition (Powers and Workman, 2007). Western blots showed that ganetespib pretreatment dramatically augments the expression of HSP70 (Fig. 3A–B). Subsequently, the possible signalling elements involved in the anti-inflammatory activity of ganetespib in heat shock–-treated N9 cells were examined. Consistent with the inhibition of pro-inflammatory mediators by ganetespib, it was found that heat shock significantly increased the phosphorylation of ERK, JAK2, STAT3, IκB-α and p65 NF-κB (Fig. 3A, D, F–I). As expected, this increase was markedly reversed by ganetespib, except for phosphor-p65 NF-κB (Fig. 3A, D, F–I). In contrast, the phosphorylation and total protein of p38 and JNK remained at the basal level for each condition, including the ganetespib-treated group (Fig. 3A, C, E). Moreover, no significant change in total effector proteins, except for IκB-α, was observed in all the groups (Fig. 3A, C–I). These results suggest that the anti-inflammatory effects of ganetespib observed in heat shock–treated N9 cells might be mainly due to the reduced expression/phosphorylation of HSP90β, ERK, JAK2 and STAT3 with a minor role played by IκB-α and p65 NF-κB.

3.4. esiRNA HSP90β, not HSP90α, attenuates heat shock–induced pro-inflammatory responses

The ganetespib-related regulation of functional microglial activation may be largely attributed to HSP90 interference. Accordingly, the esiRNA approach was used to evaluate the effect of silencing HSP90α and HSP90β in heat shock–activated N9 cells. The knockdown of HSP90 by specific esiRNA was confirmed at 12 h post-transfection, and the effectiveness of HSP90α and HSP90β inhibition was confirmed by qPCR and Western blotting (Fig. 4A–B). HSP90α and HSP90β esiRNA correspondingly suppressed the increased expression of the two major isoforms of the HSP90 family in either sham- or heat shock–treated control cells (Fig. 4A–B). It was also found that cells treated only with HSP90α or HSP90β esiRNA alone exhibited no change in the noncorresponding HSP90 isoform (Fig. 4A–B). Interestingly, although both HSP90α and HSP90β esiRNA enhanced heat shock–induced HSP70 expression, HSP90β esiRNA seems to be more effective in the augmentation of HSP70 expression than that of HSP90α at mRNA and protein levels in N9 cells (Fig. 4A–B). Moreover, the mRNA levels of TNF-α, IL-1β, IL-6 and iNOS were significantly inhibited by HSP90β esiRNA in heat shock–treated N9 cells (Fig. 4C–F). In addition, ELISA and NO detection indicated that the secretion of TNF-α, IL-1β, IL-6 and NO in the cell

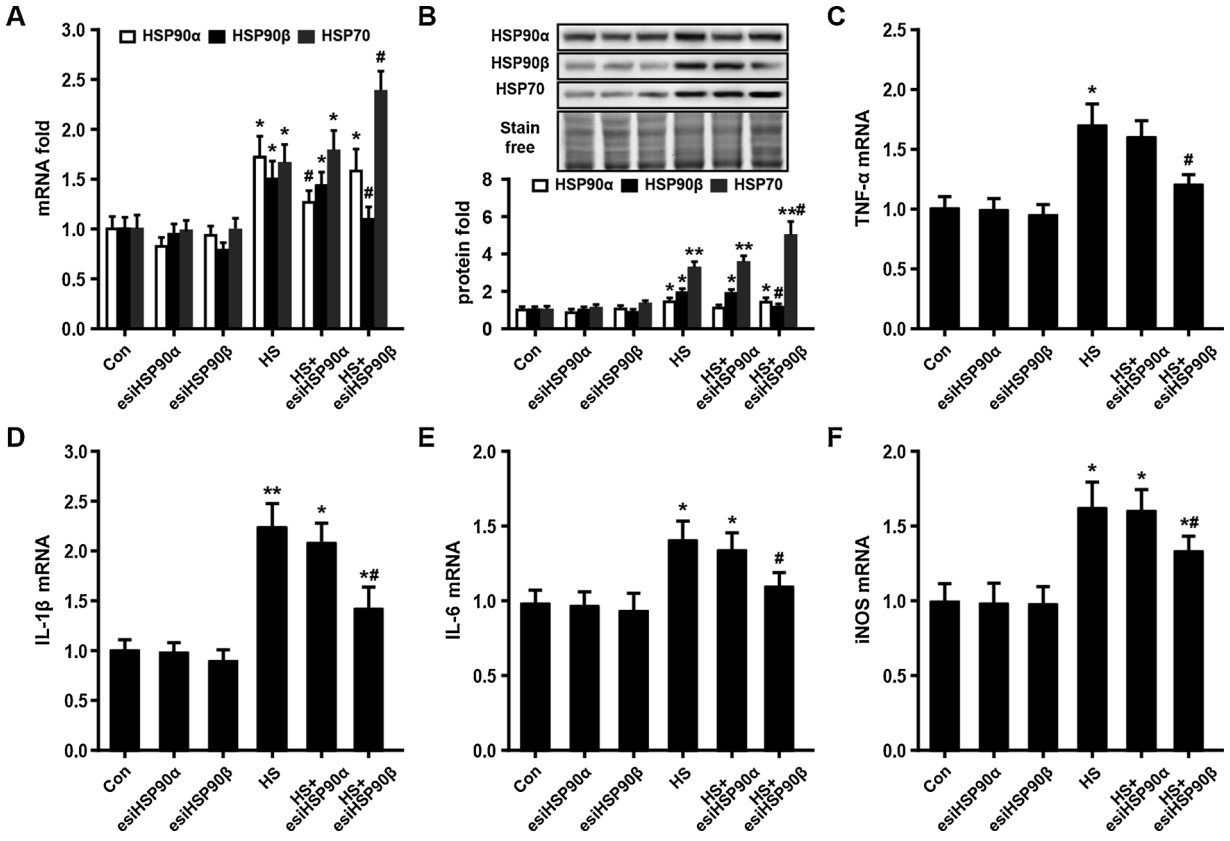

Fig. 4. Effects of HSP90α and HSP90β esiRNA on heat shock-induced transcription of pro-inflammatory mediators. N9 cells were transfected with or without HSP90α and HSP90β esiRNA and then subject to heat shock or sham control. RT-PCR (A) and Western blotting (B) quantification of HSP90α, HSP90β and HSP70 6 h after heat shock. Gene expression of TNF-α (C), IL-1β (D), IL-6 (E) and iNOS (F) was determined by qRT-PCR at 6-h recovery after heat shock. Data are presented as means ± SEM of three independent experiments. Statistical significance was determined by two-way ANOVA followed by Tukey's test. Statistical comparisons to the untreated control group are indicated by $^{*}P < 0.05$; $^{**}P < 0.01$. Statistical comparisons to the heat shock group are indicated by $^{\#}P < 0.05$.

culture medium supernatants were also significantly inhibited by HSP90β esiRNA (Fig. 5). Similarly, cell cultures treated with either HSP90α or HSP90β esiRNA alone showed no significant changes in the mRNA and protein levels of TNF-α, IL-1β, IL-6 and NO in the control groups (Fig. 4C–F, 5). All these results indicate that HSP90β, and not HSP90α, plays an important role in the pro-inflammatory responses of heat shock–treated N9 cells.

3.5. Inhibition of HSP90β blocks ERK- and STAT3- dependent pro-inflammatory responses

The aforementioned findings showed that the anti-inflammatory activity targeting HSP90 in heat shock–treated N9 cells might be largely dependent on the suppression of HSP90β, which also might be an anti-inflammatory mechanism of ganetespib. The indicated signalling effectors, ERK, JAK2 and STAT3, were determined using the HSP90α and HSP90β esiRNA approach to further reveal the preventive mechanism of ganetespib underlying the recovery from heat shock. esiRNA of HSP90β, not HSP90α, were found to attenuate the heat shock–evoked activation of ERK, JAK2 and STAT3 by suppressing their phosphorylation in the cytosol (Fig. 6A–D). The total amounts of ERK, JAK2 and STAT3 did not change for each condition. No alteration of these signalling elements in N9 cells was observed with pretreatment of either HSP90α or HSP90β esiRNA alone. The immunoblot analysis also revealed that the nucleus activation of STAT3 was abolished in heat shock–treated N9 cells with HSP90β esiRNA preconditioning (Fig. 6E–F). In addition, immunolocalization and confocal microscopy provided further evidence for HSP90β expression and STAT3 phosphorylation, showing a strong increase in fluorescence intensity and an expected correlation value of colocalization in heat shock–treated N9 cells (Fig. 7). In contrast, a low level of HSP90β and phosphor-STAT3 was observed in the sham-treated control group. Moreover, a similar inhibitory pattern of immunoreaction of HSP90β and phosphor-STAT3 was observed with the pretreatment of ganetespib and HSP90β esiRNA in the microscopy assay (Fig. 7). These results demonstrated that HSP90β inhibition by ganetespib and esiRNA might be responsible for the decrease of pro-inflammatory ERK and STAT3 pathways in N9 cells following heat shock.

4. Discussion

Cellular response to heat shock can be considered a protective mechanism which allows the restoration of damaged basic functions and cellular homeostasis. Probably microglial response to heat shock will not have similar fate in the CNS. Notably, as a harmful stimulus, severe heat shock attracted numerous studies before the inspiring application of clinical hyperthermia in the second half of the 20th century. Recent studies have also reported that following heat-shock treatment, microglia also show perturbations in phosphocreatine and ATP levels, membrane composition and mitochondrial distribution (de Gannes et al., 2000, 1998).Considering the dramatic shift in the pattern of protein synthesis during heat-shock recovery, the present study focused on inflammatory damage, studied the microglial pro-inflammatory response and anti-inflammatory properties of ganetespib, and paid special attention to the inhibition of HSP90 following heat shock. This study observed the immunoregulation of microglial pro-inflammatory responses by the HSP90 inhibitor ganetespib in heat shock–treated N9 cells. It found a significant inhibition of cytokine secretion by

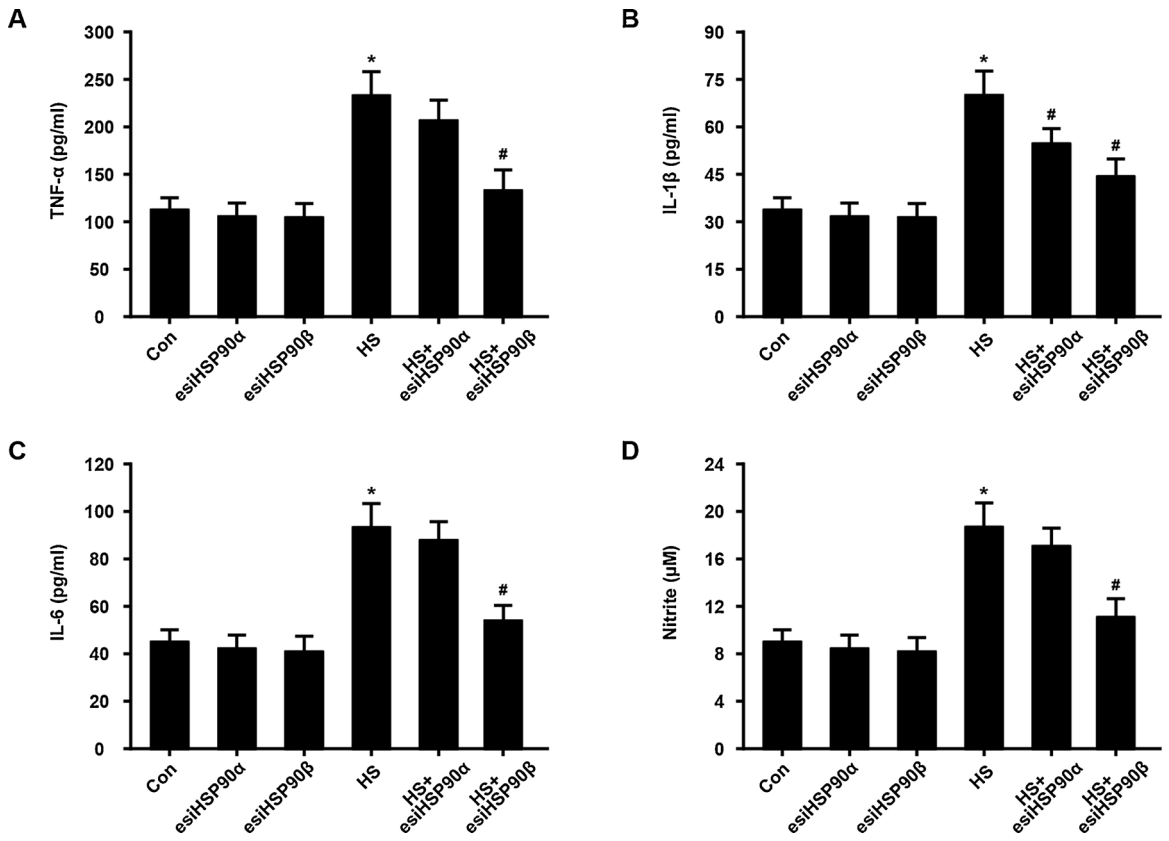

Fig. 5. Effects of HSP90α and HSP90β esiRNA on heat shock-induced pro-inflammatory secretion. N9 cells were transfected with or without HSP90α and HSP90β esiRNA and then subject to heat shock or sham control. The levels of TNF-α (A), IL-1β (B), IL-6 (C) and NO (D) production were detected by ELISA and Griess reaction at 6-h recovery after heat shock. Data are presented as means ± SEM of three independent experiments. Statistical significance was determined by two-way ANOVA followed by Tukey's test. Statistical comparisons to the untreated control group are indicated by $^{*}P < 0.05$. Statistical comparisons to the heat shock group are indicated by $^{\#}P < 0.05$.

ganetespib preconditioning. Importantly, ganetespib pretreatment significantly augmented the expression of HSP70, postulating the possible involvement of HSP90β in the inhibition of pro-inflammatory responses. Moreover, the HSP90β esiRNA experiment showed effective suppression of pro-inflammatory mediators and signalling effectors, ERK, JAK2 and STAT3, in heat shock–treated N9 cells. These results suggest that ganetespib reverses heat shock–induced pro-inflammatory responses in N9 cells via the immune-regulation of HSP90β, not HSP90α, linking to the phosphorylate activation of downstream pro-inflammatory signalling molecules.

It is well known that the rapid HSR of microglia increases HSC70 expression and HSP70 synthesis, accelerating their repair following short, sublethal heat shock (de Gannes et al., 1998; Foster and Brown, 1997; Voisin et al., 1996). HSR also prevents the initiation of inflammatory gene expression via the inhibition of NF-κB activation (Heneka et al., 2000). Interestingly, animal models and observations in humans have identified heat-induced inflammation and tissue injury (Chang et al., 2007; Maghsudlu and Farashahi Yazd, 2017), with less evidence of normal cells *in vitro* for the existence of inflammation directly induced by heat shock. Instead, it appears that temperature elevation and heat shock might affect a stimulus-dependent augmentation of cytokines and/or NO production through distinct mechanisms in inflammatory cells such as macrophages and microglia (Bernard et al., 1994; Goldring et al., 2000; Matsui et al., 2012). This apparent incongruity may be, in part, attributable to a decisive breakthrough defined by the report that demonstrates stress-induced genetic variations in *Drosophila* (Ritossa, 1962). Since then, numerous studies have focused on the enhancing inflammatory role of heat shock under stress conditions. Another interesting recent report has found that NO production increased in a time interval–dependent manner between heat shock and lipopolysaccharide (LPS) (Prestes-Carneiro et al., 2007). In this sense, the understanding of the biological effects of heat shock on pro-inflammatory responses in untreated normal cells attracts new interests to an old field. A recent study reported an upregulation of pro-inflammatory gene expressions when tenocytes were exposed to heat at 43 °C for 30 min (Maeda et al., 2017). The present study observed a significant increase in cytokine and NO production in N9 cells at 6-h recovery after a 1.5-h heat exposure at 42 °C. Taken together, these results suggest that as a physical environment factor, heat can exert both protective and destructive actions on brain cells, depending on the degree of the duration and intensity and the recovery of the heating (Li et al., 2009). This discrepancy might be due mainly to the balance of interactions between damaged molecules and stress proteins following different degrees, durations, intensities and recovery of heat shock, resulting in a similar, double-edged sword role in inflammation in the CNS (Gabai and Sherman, 1985).

Remarkably, the aforementioned balance of the initial effector molecules may affect the fate of cells exposed to heat shock. Except for the evoked cytokine and NO levels in the experimental conditions in the present study, a cytotoxic effect was also observed at a 1.5-h heat exposure with a 6-h recovery period. Given the rapid abundant induction and critical pro-inflammatory role of HSP90 in cancer and inflammatory diseases (Quintana and Cohen, 2005), pharmacologic agents targeting microglial HSP90 may be helpful for preventing the deleterious consequences of unregulated inflammatory responses following heat shock (Tukaj and Wegrzyn, 2016). Indeed, recent studies have shown that glial expression of HSP90 parallels the glial and inflammatory response previously described in a neuropathological condition induced by kainic acid (Jeon et al., 2004; Wang et al., 2005). The microglial expression of HSP90α and HSP90β was also found in the

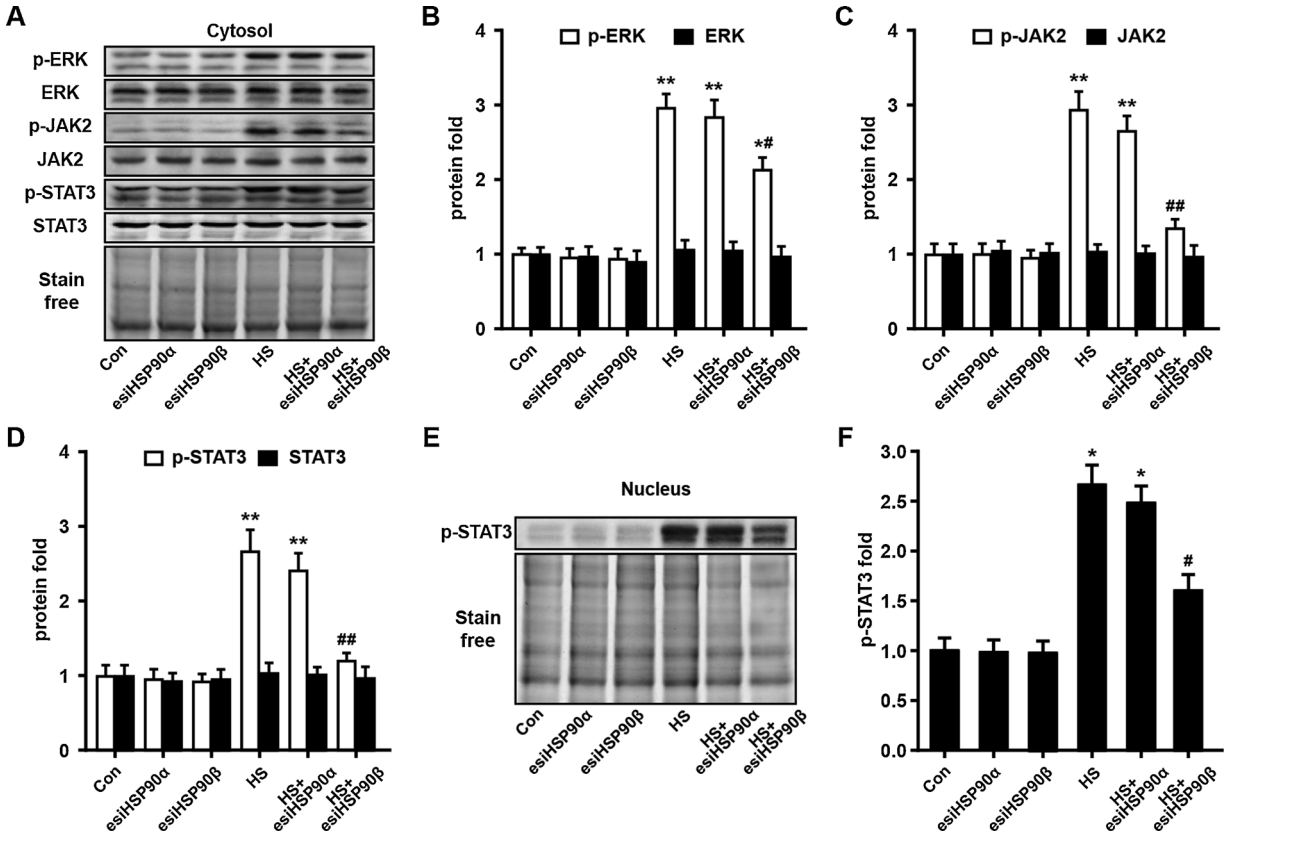

Fig. 6. Involvement of HSP90β in the regulation of the expression of ERK, JAK2 and STAT3 in heat shock-treated N9 cells. N9 cells were transfected with or without HSP90α and HSP90β esiRNA and then subject to heat shock or sham control. (A) Levels of phosphorylation and total protein of ERK, JAK2 and STAT3 in the cytosol were analysed using Western blotting at 6-h recovery after heat shock. The densitometric analysis was performed for ERK (B), JAK2 (C) and STAT3 (D). (E) The levels of STAT3 phosphorylation was measured by Western blotting in the nucleus 6 h after heat shock. (F) The densitometric analysis was performed for phospho-STAT3. Data are presented as means ± SEM of three independent experiments. Statistical significance was determined by two-way ANOVA followed by Tukey's test. Statistical comparisons to the untreated control group are indicated by $^{*}P < 0.05$; $^{**}P < 0.01$. Statistical comparisons to the heat shock group are indicated by $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

experimental conditions of the present study. Recently, Lilja et al. (2015) demonstrated that ganetespib, a novel non-geldanamycin HSP90 inhibitor, shows potent anti-inflammatory efficacy in LPS-induced lung inflammation. Apart from this, similar anti-inflammatory effects were found in the first-generation HSP90 inhibitor, geldanamycin (Bucci et al., 2000; Wax et al., 2003). Not surprisingly, in agreement with the earlier data, the present study found that the heat shock–induced secretion of pro-inflammatory cytokines and NO was efficiently suppressed by ganetespib preconditioning. In addition, the levels of the microglial activation marker CD11b were significantly attenuated by the addition of ganetespib to the culture medium. Therefore, understanding the molecular pathways of the anti-inflammatory activity of ganetespib in heat shock–activated microglia might open up new therapeutic options for preventing CNS injuries.

As an important molecular chaperone, HSP90 supports cellular activation and inflammatory responses by maintaining the stability and function of several key signalling molecules, such as p38, ERK, JNK, JAK2 and IκB-α kinases, and transcription factors such as STAT3 and NF-κB (Broemer et al., 2004; Kakimura et al., 2002; Schopf et al., 2017). The present study investigated the involvement of the aforementioned inflammatory pathways to reveal the precise HSP90 clients in the heat-shock process. Consistent with the suppression of cytokine and NO production, different inhibitory levels of the phosphorylation of ERK, JAK2, STAT3, IκB-α and p65 NF-κB and the transcription activity of STAT3 were observed by ganetespib pretreatment in heat shock–treated N9 cells. Although IκB-α was significantly attenuated by ganetespib, no significant suppression of phospho–p65 was reported, which is increased slightly following heat shock. This might be attributed to the linkage of the immunosuppressive action of HSP70 derived by HSP90 inhibition following heat shock and the subsequent blockade of transcription factor NF-κB activity (Feinstein et al., 1996; Heneka et al., 2000). Moreover, the inefficient inhibitory effect of HSP90 inhibition on NF-κB was also observed in another synthetic HSP90 inhibitor, EC144, which appears to not alter LPS-dependent activation of p50 NF-κB (Yun et al., 2011). It is notable that the results of this study also revealed the phosphorylation of ERK and its inhibitory regulation by ganetespib in heat shock–treated N9 cells. In agreement, previous studies showed that HSP90 inhibition effectively diminished the constitutive phosphorylation of ERK in different cell types (Hackl et al., 2010; Madrigal-Matute et al., 2012). Again, previous studies also showed that geldanamycin suppressed the phosphorylation of JAK2 and STAT3 (Hosoi et al., 2016; Sato et al., 2003). Taken together, although the beneficial effects of HSP90 inhibition can be observed under various experimental conditions, the underlying mechanism of ganetespib in microglia for the treatment of heat shock remains to be fully clarified.

HSP90 is known to exert a broader range of functional divergence mostly depending on two major cytoplasmic isoforms, HSP90α and HSP90β (Moore et al., 1989; Sreedhar et al., 2004). HSP90α is generally stress-inducible and may, consequently, be a more cytoprotective isoform of HSP90, while HSP90β has been reported to have a key role in cancer and trophoblast differentiation (Fujita et al., 2002; Sreedhar et al., 2004). This study found a microglial expression of HSP90α and HSP90β mRNA and protein following heat shock. A very strong correlation between HSP90β inhibition and pro-inflammatory reduction with the pretreatment of ganetespib following heat shock was also observed. As a result, HSP90β seems to be associated with the anti-

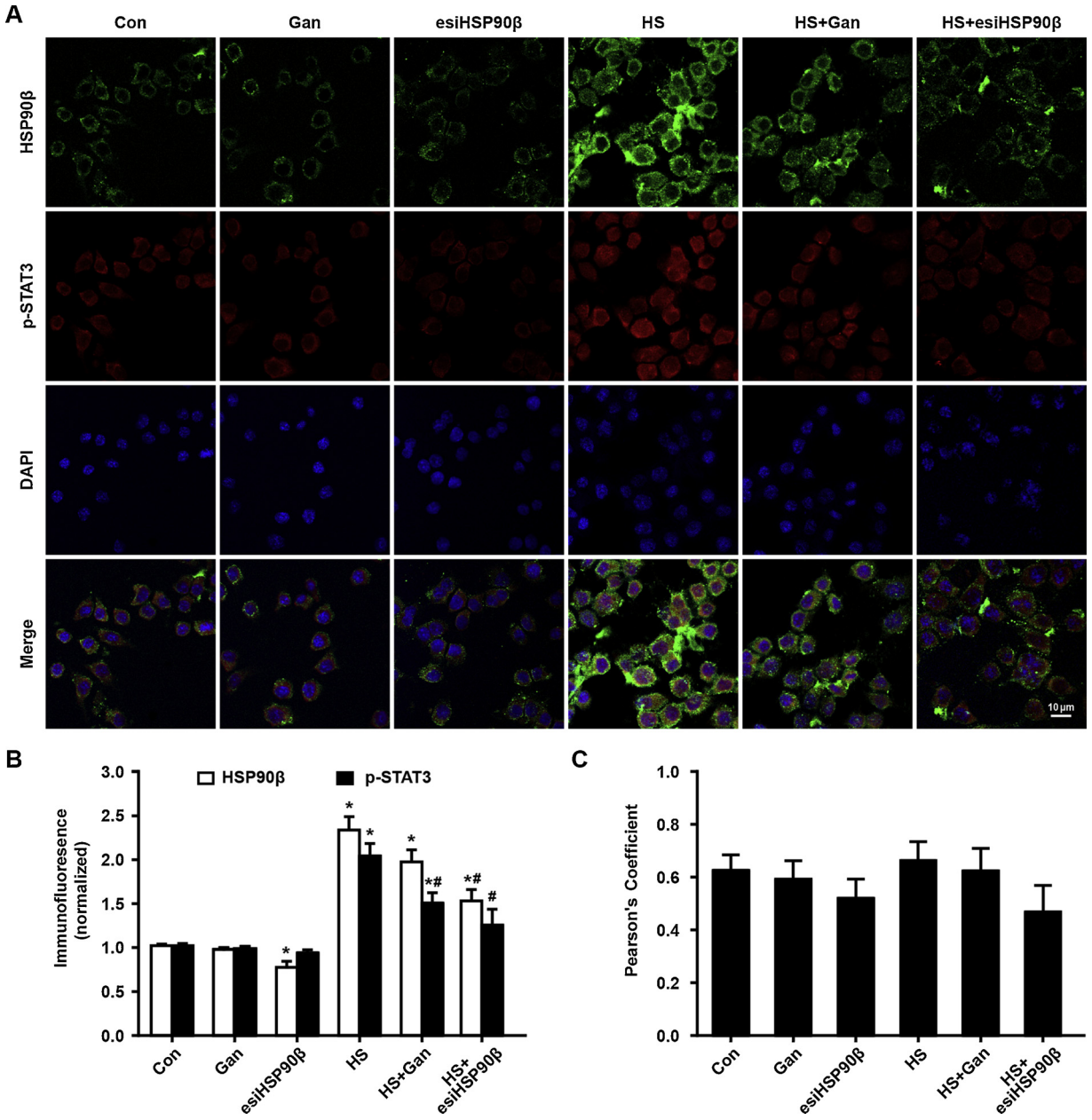

Fig. 7. Localization of HSP90β and phospho-STAT3 in heat shock-treated N9 cells with the addition of ganetespib and HSP90β esiRNA. N9 cells were pretreated with or without ganetespib (100 nM) and HSP90β esiRNA, and then subject to heat shock or sham control. (A) Confocal immunofluorescence microscopy was performed on cultures that were immunoreacted with antibodies against HSP90β and phospho-Tyr705-STAT3 at 6-h recovery after heat shock. (B) Bar graphs show semi-quantification of fluorescence intensity for HSP90β and phospho-STAT3 using ImageJ. (C) Pixel-by-pixel colocalization analysis yielded a Pearson's correlation coefficient of HSP90β with phospho-STAT3. Scale bar: 10 μm.

inflammatory effects of HSP90 inhibition underlying heat shock. Indeed, esiRNA of HSP90β, not HSP90α, significantly suppressed the mRNA and protein levels of TNF-α, IL-1β, IL-6 and NO in heat shock–treated N9 cells. In support of this, it is accepted that HSP90β bound each inhibitor with greater relative interaction strength than did HSP90α (Prince et al., 2015). Moreover, it has been reported that HSP90β upregulates the nuclear translocation of STAT3 in mouse embryonic stem cells (Setati et al., 2010), and HSP90 inhibitor 17-AAG treatment abolished the phosphorylation of STAT3 via at least the impaired chaperone activity of HSP90β (Okumura et al., 2011). Furthermore, the involvement of ERK, JAK2 and STAT3 phosphorylation in heat shock–treated N9 cells was verified in the esiRNA experiments in cytosol and/or nucleus. Confocal microscopy provided further evidence of the potent effect of ganetespib on STAT3-dependent inflammatory inhibition via HSP90β in N9 cells after heat shock.

In conclusion, the results of the present study strongly suggest that heat shock directly triggers an initial activation of microglia with obvious HSR and pro-inflammatory responses. The findings also indicate that ganetespib abolished microglial pro-inflammatory responses via the inhibition of HSP90β–STAT3 system. Regardless of the detailed mechanism, the data presented in this study may assist future studies that aim to determine the therapeutic potential of ganetespib.

Conflict of interest

The authors declare no conflict of interest.

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