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## TNF- $\alpha$ promotes cell survival through stimulation of K<sup>+</sup> channel and NF $\kappa$ B activity in corneal epithelial cells

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### Abstract

Tumor necrosis factor (TNF- $\alpha$ ) in various cell types induces either cell death or mitogenesis through different signaling pathways. In the present study, we determined in human corneal epithelial cells how TNF- $\alpha$  also promotes cell survival. Human corneal epithelial (HCE) cells were cultured in DMEM/F-12 medium containing 10% FBS. TNF- $\alpha$  stimulation induced activation of a voltage-gated K<sup>+</sup> channel detected by measuring single channel activity using patch clamp techniques. The effect of TNF- $\alpha$  on downstream events included NF $\kappa$ B nuclear translocation and increases in DNA binding activities, but did not elicit ERK, JNK, or p38 limb signaling activation. TNF- $\alpha$  induced increases in p21 expression resulting in partial cell cycle attenuation in the G<sub>1</sub> phase. Cell cycle progression was also mapped by flow cytometer analysis. Blockade of TNF- $\alpha$ -induced K<sup>+</sup> channel activity effectively prevented NF $\kappa$ B nuclear translocation and binding to DNA, diminishing the cell-survival protective effect of TNF- $\alpha$ . In conclusion, TNF- $\alpha$  promotes survival of HCE cells through sequential stimulation of K<sup>+</sup> channel and NF $\kappa$ B activities. This response to TNF- $\alpha$  is dependent on stimulating K<sup>+</sup> channel activity because following suppression of K<sup>+</sup> channel activity TNF- $\alpha$  failed to activate NF $\kappa$ B nuclear translocation and binding to nuclear DNA.

### Keywords

Patch clamp; K<sup>+</sup> channel blockers; Cell death; Cell cycle mapping

### Introduction

TNF- $\alpha$  has pleiotropic effects in many cell types that include activation of apoptosis, inflammation, and immune responses. These responses underlie the pathogenesis of various diseases, including diabetes and cancer. Conversely, TNF- $\alpha$  can also induce through different signaling pathway module proliferation and differentiation [1]. TNF- $\alpha$  is one of a number of cytokines found in tears and its expression is elevated in allergic conjunctivitis [2]. TNF- $\alpha$  is also secreted by corneal epithelial cells in response to inflammatory stimulation caused by bacterial and viral infections [3–6]. However, a cell survival-promoting role for TNF- $\alpha$  has not been described in this tissue.

The TNF- $\alpha$ -linked cell signaling pathways that elicit apoptosis have been extensively characterized. Upon binding of TNF- $\alpha$  to Tumor Necrosis Factor Receptor 1 (TNF-R1), a type I membrane protein in the TNFR superfamily in mammalian cells, TNF-R1 monomers cluster resulting in death signal initiation [7–10]. There is a common structure among these receptors known as the “death domain” (DD) on the cytoplasmic side of the membrane [10]. Activation

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of a DD-containing receptor by TNF- $\alpha$  can lead to recruitment of intracellular DD-containing adaptors such as Fas associated death domain (FADD) and TNFR associated death domain (TRADD). TNF- $\alpha$  activates TNF-R1 coupled receptor DDs and they recruit several signaling molecules (adaptor proteins) to the activated receptor including: FADD, TRAFs, and receptor-interaction protein (RIP) leading to activation of caspase 8, NF $\kappa$ B, and c-Jun N-terminal kinase (JNK) signaling pathways [11,12]. NF $\kappa$ B protein is a member of the cytoplasmic heterodimeric transcription factor family associated with cell survival [13,14]. Upon phosphorylation by IKK, NF $\kappa$ B dissociates from I $\kappa$ B and I $\kappa$ B is then degraded by proteasome complexes [15]. Unbound NF $\kappa$ Bs dimerize and become active transcription factors. They then translocate into the nucleus to initiate gene transcription in response to TNF- $\alpha$ .

Even though TNF- $\alpha$  induces proliferation in some cell types, a cell signaling role for TNF- $\alpha$ -induced K<sup>+</sup> channel activation in eliciting this response has not been described. Their involvement is tenable because K<sup>+</sup> channels are a component of signaling pathways that mediate mitogenic responses to serum-containing growth factors in many cell types including corneal epithelial cells [16–19]. K<sup>+</sup> channel activity belonging to the Kv3.4 family in these cells is essential for EGF-induced proliferation [16,20]. Inhibition of K<sup>+</sup> channel activity with specific channel blockers results in attenuation of the cell cycle in the G<sub>1</sub> phase [16–19]. On the other hand, UV irradiation also induces robust increases in channel activity in these cells, which in turn triggers apoptosis through activation of JNK signal cascades [20–22]. TNF- $\alpha$ -stimulated increases in K<sup>+</sup> channel activities are important for TNF- $\alpha$ -induced cellular effects in cortical neurons, kidney epithelial, and liver cells [23–25]. TNF- $\alpha$  also induces mRNA expression of various K<sup>+</sup> channel types during a systemic inflammatory response as well as tumor cell proliferation in brain and cancer cells [26–28]. However, TNF- $\alpha$  suppresses particular types of K<sup>+</sup> channel activity in other cell types, indicating that the effect of TNF- $\alpha$  on K<sup>+</sup> channel activity is dependent on the channel type and cell origins [26,29]. In the present study, we investigated the role of TNF- $\alpha$ -induced changes in K<sup>+</sup> channel activity in determining human corneal epithelial cell fate. Our results reveal that such changes are requisite for TNF- $\alpha$ -induced NF $\kappa$ B nuclear translocation and activation, which in turn improves corneal epithelial cell survival. On the other hand, TNF- $\alpha$ -induced cellular responses in these cells do not involve activation of any of the MAP kinase limb pathways. It is currently unclear why these MAP kinase pathways are not involved in mediating improved cell survival in response to TNF- $\alpha$  exposure.

## Methods

### Cell culture of corneal epithelial cells

SV40-immortalized human corneal epithelial (HCE) cells were grown in DMEM/F-12 culture medium containing 10% fetal bovine serum, 5  $\mu$ g/ml insulin, and 10,000 units/ml penicillin and 10,000  $\mu$ g/ml streptomycin, and maintained in an incubator supplied with 95% air and 5% CO<sub>2</sub> at 37°C. Before experimentation, cells were synchronized in the G<sub>1</sub> phase of the cell cycle by serum deprivation for at least 24 h. The medium was replaced every 2 days and the cells were passed by treatment with 0.05% trypsin-EDTA. For apoptosis induction, cells were placed 6 cm away from a safe light containing UV-C in a tissue culture hood and irradiated with 40  $\mu$ J/cm<sup>2</sup>. After irradiation, they were collected and rinsed with phosphate-buffered saline (PBS). To suppress K<sup>+</sup> channel activity, the cells were exposed to a K<sup>+</sup> channel blocker, 4-aminopyridine (4-AP) and preincubated for 20 min prior to exposure to TNF- $\alpha$  dependent on the time requirement of different experiments. All chemicals unless otherwise specified were obtained from Sigma Chemicals.

### Nuclear staining with ethidium bromide and acridine orange

Cell viability and apoptosis were determined by nuclear staining to detect nuclear DNA condensation. A dye mixture containing ethidium bromide and acridine orange, each of which was present at 100 µg/ml (EB/AO), was added to each cell culture dish at a final density of  $3 \times 10^5$ /ml. Cell populations were scored according to their staining color and intensity using a UV-fluorescence microscope (Nikon). Nuclei staining green have not lost membrane integrity. In contrast, corneal epithelial cells in which the nuclei stained orange have lost membrane integrity. Apoptotic cells can be distinguished from viable cells on the basis of the absence or presence of nuclear condensation/fragmentation.

### Immunoblot analysis

The cells ( $2 \times 10^5$ ) were rinsed twice with ice-cold PBS and solubilized in sodium dodecyl sulfate polyacrylamide (SDS) sample buffer containing 62.5 mM Tris-HCl pH 6.8, 2% W/V SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue, or phenol red. The resulting suspensions were denatured by boiling for 5 min. After fractionation of cell lysates with 12% polyacrylamide gel (PAGE), proteins were electrotransferred to PVDF membranes. Membranes were exposed to blocking buffer containing 5% nonfat milk in TBS-0.1% Tween 20 (TBS-T) for 1 h at room temperature (RT, 21–23°C), and then incubated overnight or for 1 h with antibodies of interest at 4°C. All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). After three washes with TBS-T buffer, membranes were incubated with alkaline phosphatase (AP)-linked secondary antibody for 1 h at RT. The proteins were detected with a Phototope-Star Western Blot Detection kit (Cell Signaling Technology, Beverly, MA). In addition, caspase 3 activity was detected to determine cell apoptosis by using an antibody against cleaved caspase 3. This antibody recognizes endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175 (Cell Signaling Technology, Beverly, MA).

### Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted by centrifugation at 25 K rpm for 20 min. The consensus oligonucleotide for NFκB transcription factor (5'-AGTTGAGGGGACTTTCCAGGC-3') was 5'-end-labeled with  $\gamma$ - $^{32}$ P by using T4 poly-nucleotide kinase. After labeling with  $^{32}$ P, DNA probes were used to hybridize with extracted nuclear proteins. Competitive reactions were conducted by adding 1 pM nonlabeled DNA probe into each sample. DNA-protein complexes were displayed by electrophoresis on a 6% nondenaturing polyacrylamide gel. For the supershift assay, DNA-protein complexes were further incubated with a specific antibody against NFκB-p65. Specific competition experiments were performed by using unlabeled oligonucleotide to compete with the labeled oligonucleotide for NFκB. Nonspecific competition experiments were performed by using unlabeled Sp1 oligonucleotides to interact with the labeled NFκB oligonucleotides.

### Cell-attached and whole-cell patch clamps

Glass pipettes with a resistance of 3–4 MΩ were manufactured with a two-stage puller (PP-83, Narishige). For the cell-attached single channel patch clamp, electrode pipettes and bath chamber solutions contained (mM): 140 KCl, 5 NaCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 1 EGTA, and 10 HEPES (pH 7.4). Single channel currents were recorded with an Axonpatch 200A amplifier (Axon Instruments, Foster City, CA) and filtered with a 4-pole low-pass filter at 1 kHz and digitized at 22 kHz with a pulse-code modulator (A.R. Vetter, Rebersburg, PA). The nystatin-perforated patch clamp technique was used for whole cell K<sup>+</sup> current recording. Pipettes with a resistance of 3–4 MΩ when filled with 150 mM KCl solution were manufactured with a two-stage puller (PP-83, Narishige) and fire-polished before use. The pipette tip was filled with a solution containing (in mM) 140 KCl, 2 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 2 ATP, 0.05 GTP, 1 EGTA, and

10 HEPES (titrated with KOH to pH 7.2). The remainder of the pipette was back-filled with the same pipette solution supplemented with 200  $\mu\text{g/ml}$  nystatin. Cell membrane potential was held at  $-60$  mV and cells were stimulated by a voltage pulse applied from  $-80$  mV to  $+60$  mV at 20 mV increments. The duration of each pulse was 1000 ms. The bath solution was composed of (in mM) 140 NaCl, 2 KCl, 1  $\text{CaCl}_2$ , 10 HEPES (pH 7.4). A pCLAMP program (Axon Instruments, Foster City, CA) was used to analyze single and whole-cell patch clamp channel data. Channel activity was determined as  $N\text{Po}$ , where  $N$  represents the number of channels in the patch and  $\text{Po}$  represents the open channel probability. Patched cells were held for 2 to 3 min after the seal is stabilized before adding TNF- $\alpha$ . All experiments were performed at room temperature ( $21$ – $23^\circ\text{C}$ ). Channel blockers, including 4-AP and blood-derived substance-I (BDS-I), were applied into the patch pipettes to inhibit channel activity.

### Cell cycle and statistical analysis

Cell cycle analysis was performed using flow cytometry. After cells were trypsinized and fixed with 70% ethanol and 50 mM glycine, they were resuspended in PBS containing RNase A (100  $\mu\text{g/ml}$ ) and propidium iodide (PI, 25  $\mu\text{g/ml}$ ). Cell cycle phases were mapped with a FACScan and analyzed with Cell Quest software (Becton Dickinson, Mountain View, CA). For statistical analysis of Western blots, autoradiographic films were scanned and relative densities of each signal band were analyzed using an Image program. The data are represented as mean  $\pm$  SE and statistical significance was determined with the paired Student's  $t$  test at  $P < 0.05$ .

## Results

### TNF- $\alpha$ -induced attenuation of cell cycle progression

In corneal epithelial cells, serum-containing factors transiently stimulate ERK and p38 resulting in stimulation of proliferation and migration [30,31]. On the other hand, exposure to UV-C induces JNK and p38 activation resulting in apoptosis [21]. Time- and concentration-dependent effects of TNF- $\alpha$  on MAP kinase signaling pathways were studied because it has been shown that TNF- $\alpha$  induces JNK signaling pathway in many cell types. Exposure to TNF- $\alpha$  stimulation at different concentrations up to 50 ng/ml had no effect on either JNK, Erk, or p38 MAP kinase activities, respectively. Similarly, none of these pathways were activated following exposure to TNF- $\alpha$  for up to 60 min (Figs. 1A and B). Even after 16 h of exposure to TNF- $\alpha$ , TNF- $\alpha$  stimulation failed to phosphorylate JNK (Fig. 1C). On the other hand as described, exposure to UV-C irradiation caused rapid activation of both JNK isoforms after 10 min (Fig. 1A), which was sustained for a subsequent 16 h (Fig. 1C). These results were highly reproducible from 3–4 independent experiments. To assess whether TNF- $\alpha$  could affect another parameter associated with cell fate, we next determined if it alters an aspect of cell cycle progression induced by exposure to FBS. This was done by measuring with Western analysis the effects of TNF- $\alpha$  on the level of protein expression of a cell cycle inhibitory protein, p21. Fig. 2A compares the time course of p21 expression in TNF- $\alpha$ -induced cells with their untreated counterpart. The data are shown graphically in Fig. 2B, indicating that TNF- $\alpha$  increased p21 expression up to 16 h. Progression of the cell cycle in response to TNF- $\alpha$  stimulation was detected by cell cycle mapping using a flow cytometer (Fig. 2C). The cells were synchronized by serum-depletion for at least 24 h and then stimulated with 10% FBS in the absence and presence of TNF- $\alpha$ . In TNF- $\alpha$ - and FBS-stimulated cells, the cell population of serum-starved cells in the  $G_1$  phase decreased from 56% to 50% whereas in those only exposed to FBS, the decline reached 44% (Fig. 2D). On the other hand, the S phase population following FBS starvation contained 23% of the total whereas FBS exposure caused the number to rise from 23% to 34%. With those exposed to FBS and TNF- $\alpha$ , the S phase population rose to only 29%. In addition, sub- $G_0$  stage of the cell cycle was analyzed by comparing the differences among starved, FBS-stimulated, and TNF- $\alpha$ -induced cells (Fig. 2E). These changes suggest that TNF- $\alpha$  attenuates cell cycle progression. This interpretation is consistent with the decline in

population of TNF- $\alpha$  treated cells in the S phase as well as the rise in p21 protein expression induced by this cytokine.

### TNF- $\alpha$ -induced K<sup>+</sup> channel activity in HCE cells

In corneal epithelial cells, exposure to either mitogens or UV light induces increases in K<sup>+</sup> channel activity followed by increases in proliferation or apoptosis [16,20]. Such activation is requisite for these responses to occur because they are attenuated if K<sup>+</sup> channel activity is suppressed by 4-AP or other K<sup>+</sup> channel blockers. As TNF- $\alpha$  also affects cell fate, we determined whether changes in K<sup>+</sup> channel activity are a component of a signaling pathway mediating such a response. Accordingly, the nystatin-perforated whole-cell patch clamp was used to study the effect of TNF- $\alpha$  on K<sup>+</sup> channel activity by recording the whole-cell current resulted from depolarization of the membrane potential from a holding potential of -80 to +60 mV in 20 mV increments. Upon exposure of RCE cells to 40 ng/ml TNF- $\alpha$  following a time course up to 25 min, the amplitude of the K<sup>+</sup> current was markedly increased within 10 min and then gradually decreased (Figs. 3A and B). The long-term effect of TNF- $\alpha$  on K<sup>+</sup> channel activity was determined by patching these cells 18 h after exposure of cells to 20 ng/ml TNF- $\alpha$ . TNF- $\alpha$ -induced increases in K<sup>+</sup> current amplitude appeared after 18 h (Fig. 3C). K<sup>+</sup> currents in response to 20 ng/ml TNF- $\alpha$  stimulation were plotted as a function of membrane potentials (*I-V* curve) with/without TNF- $\alpha$  stimulation (Fig. 3D). To further confirm the effect of TNF- $\alpha$  on K<sup>+</sup> channel activity, K<sup>+</sup> channel activity was then investigated by using the cell-attached patch clamp. Serum-starved HCE cells were used in control experiments to record baseline K<sup>+</sup> channel activity. Application of 20 ng/ml of TNF- $\alpha$  onto the patched cells induced robust K<sup>+</sup> channel activity at a membrane potential of -60 mV (Fig. 3E). TNF- $\alpha$ -induced K<sup>+</sup> channel activities were recorded up to 30 min and there was no running down in channel activity. The channel opening properties were analyzed as long as the recording period. TNF- $\alpha$ -induced K<sup>+</sup> channel activity significantly increased to  $30.2 \pm 3.1\%$  ( $n = 8$ ), compared to K<sup>+</sup> channel activity in control cells ( $9.1 \pm 1.6\%$ ,  $n = 14$ ). However, TNF- $\alpha$ -induced K<sup>+</sup> channel activities were effectively suppressed by 4-AP (1 mM) and BDS-I (400 nM) in the patch pipette. Application of 4-AP and BDS-I markedly suppressed TNF- $\alpha$ -induced K<sup>+</sup> channel activity resulting in the channel opening to decline to  $8.4 \pm 0.8\%$  and  $7.6 \pm 0.9\%$ , respectively (Fig. 3F). Such inhibition is consistent with our previous study that showed that there is 4-AP and BDS-I sensitive K<sup>+</sup> channel activity in these cells in response to mitogen and stress stimulations.

### Dependence of TNF- $\alpha$ -enhanced cell survival on K<sup>+</sup> channel activity

As TNF- $\alpha$  induced attenuation of cell cycle progression by resulting a rise in the cell population in the G<sub>1</sub> phase, we determined if this effect is associated with an increase in cell viability. Accordingly, the dependence was evaluated by TNF- $\alpha$ -induced cell cycle attenuation on K<sup>+</sup> channel activity. This was done by evaluating viability of cells that were stimulated with 20 ng/ml TNF- $\alpha$  in the absence and presence of various dosages of 4-AP (Fig. 4). Cell death was determined 36 h after TNF- $\alpha$  induction by detection of chromatin condensation using nuclear staining with ethidium bromide (EB) and acridine orange (AO). Treatment of cells with just TNF- $\alpha$  (20 ng/ml) or 4-AP (1 mM) did not affect HCE cell viability. However, suppression of K<sup>+</sup> channel activity with 4-AP in TNF- $\alpha$ -induced cells triggered 4-AP dose-dependent programmed cell death. In addition, cell apoptotic response was also measured by detection of caspase 3 activity by detection of cleaved caspase 3 Tsp175 fragment (Fig. 4B). Caspase 3 activity was increased in TNF- $\alpha$ -induced and K<sup>+</sup> channel-inhibited cells. These data suggest that TNF- $\alpha$  promotion of cell survival is dependent on its ability to induce increases in K<sup>+</sup> channel activity.

### TNF- $\alpha$ -induced NF $\kappa$ B nuclear translocation and activation

To determine signaling downstream events linked to TNF- $\alpha$ -induced K<sup>+</sup> channel activation events in HCE cells, we first probed for NF $\kappa$ B nuclear translocation and activation. This was done by separating nuclear proteins from their cytosolic counterparts and by evaluating with Western analysis NF $\kappa$ B density in these two fractions. Nuclear translocation of NF $\kappa$ B was markedly increased in a dose-dependent manner from 20 to 100 ng/ml of TNF- $\alpha$  concentration (Fig. 5A). With 20 ng/ml of TNF- $\alpha$ , NF $\kappa$ B nuclear translocation became evident during the first 15 min of a 60 min measuring period (Fig. 5B). These results indicate a variation of TNF-R1-linked signaling pathway because TNF- $\alpha$  stimulation induces nuclear translocation of NF $\kappa$ B without activating JNK cascades.

### Effect of altered K<sup>+</sup> channel activity on TNF- $\alpha$ -induced NF $\kappa$ B activation

Activation of NF $\kappa$ B in TNF- $\alpha$ -induced cells was further assessed by measuring its capability to undergo nuclear translocation and selectively bind to DNA fragments. Time-dependent activation of NF $\kappa$ B in response to TNF- $\alpha$  induction was determined using electrophoresis gel shifting assay (EMSA). Nuclear proteins were extracted from HCE cells before and after TNF- $\alpha$  stimulation following a time course. NF $\kappa$ B-specific and <sup>32</sup>P-labeled DNA probes were hybridized with gel-displayed nuclear NF $\kappa$ B proteins (Fig. 6A). TNF- $\alpha$  induced two strong DNA binding bands from 15 to 60 min and their formation was competitively blocked by unlabeled NF $\kappa$ B-specific probes, but not with unrelated DNAs. A supershift band was observed in the lane where the sample was preincubated with anti-NF $\kappa$ B antibody, suggesting that these bands are indeed NF $\kappa$ B-specific bands. The dependence of TNF- $\alpha$ -induced NF $\kappa$ B nuclear translocation on K<sup>+</sup> channel activity was determined by treating HCE cells with various dosages of 4-AP. TNF- $\alpha$ -induced DNA binding activity of NF $\kappa$ B was markedly inhibited by suppression of K<sup>+</sup> channel activity with 4-AP in a dose-dependent manner (Fig. 6B). Suppression of K<sup>+</sup> channel activity with 4-AP also inhibited TNF- $\alpha$ -induced NF $\kappa$ B nuclear translocation analyzed by Western blot in nuclear and cytosolic separated proteins (Fig. 6C). These results provide evidence that TNF- $\alpha$ -induced NF $\kappa$ B nuclear translocation is dependent on upstream increases in K<sup>+</sup> channel activity.

## Discussion

The present study explores mechanisms mediating TNF- $\alpha$  control of corneal epithelial cell survival and apoptosis. It has important physiological relevance to corneal epithelial wound healing because TNF- $\alpha$  is increased in tears during infections and inflammations of the cornea [3,4,32]. Interestingly, TNF- $\alpha$  may have a dual effect of inducing cell death, but its predominant effect is to affect an increase in cell viability through K<sup>+</sup> channel activation [6]. Recent studies show that TNF- $\alpha$  is able to activate NF $\kappa$ B in many different cell types and this response is important to determine cell fate. The ability of NF $\kappa$ B to suppress apoptosis can be overridden by protein synthesis inhibitors such as cycloheximide resulting in sensitizing the cells to TNF- $\alpha$ -induced apoptosis [33–35]. We found in corneal epithelial cells that TNF- $\alpha$ -induced NF $\kappa$ B activation is ligand dose-dependent and time-dependent (Figs. 5 and 6). NF $\kappa$ B activation by TNF- $\alpha$  was identified based on NF $\kappa$ B nuclear translocation and increases in the ability of NF $\kappa$ B to bind to its target DNA. Some members of the TNF superfamily induce apoptosis, whereas others act as survival factors or induce cell proliferation. In corneal epithelial cells, TNF- $\alpha$  did not induce apoptosis, but instead increases in expression of p21 and cell cycle attenuation in the G<sub>1</sub> phase (Fig. 2).

TNF-stimulation activates MAP kinase cascades in some cell types, especially, the JNK signaling pathway [36–38]. There are crosstalks between JNK and NF $\kappa$ B in the TNF- $\alpha$ -induced signaling network. TNF- $\alpha$ -induced JNK activation may not be involved in induction of apoptosis when NF $\kappa$ B protects against apoptosis [39]. In the absence of NF $\kappa$ B activity, TNF-

$\alpha$ -induced apoptosis increased, whereas direct activation of NF $\kappa$ B protects cell against apoptosis. Similarly, in different cell types, there is an inverse relationship between TNF- $\alpha$ -induced activation of the JNK module and NF $\kappa$ B. In cases where there is JNK module activation, NF $\kappa$ B stimulation is suppressed whereas in other cases increased NF $\kappa$ B stimulation is associated with less JNK activation [40–43]. In the present study, we found that Erk, JNK, and p38 MAP kinase pathways were not activated by application of TNF- $\alpha$ . Lack of TNF- $\alpha$ -induced activation of JNK in HCE cells may enhance the antiapoptotic effect of NF $\kappa$ B. This notion is supported by recent studies in hepatocytes showing that the JNK inhibitor SP600125 minimizes TNF- $\alpha$ -induced apoptosis through suppression of caspase 3 activation and DNA laddering [43].

K<sup>+</sup> channels are universally present in the cell membrane and important in maintaining growth factor-stimulated cell growth and UV irradiation-induced apoptosis. K<sup>+</sup> channel activity has been found to be a key determinant for cell progression through the G<sub>1</sub> checkpoint of the cell cycle [16,18,44,45]. In K<sup>+</sup> channel activity-suppressed cells, retinoblastoma protein (pRB) is dephosphorylated and effectively inhibits the cell from progressing through the G<sub>1</sub>/S transition [18]. These results suggest that changes in K<sup>+</sup> channel activity can modulate the magnitude of a mitogenic response to a growth factor or cytokine. On the other hand, increases in K<sup>+</sup> channel activity are likely involved in mediating programmed cell death (apoptosis). In corneal epithelial cells, UV irradiation can induce hyper-activation of K<sup>+</sup> channels with a magnitude of 2-fold greater than that of mitogen-induced channel activity [20]. The hyper-activation of K<sup>+</sup> channels at this magnitude may cause an excessive loss of intracellular K<sup>+</sup> ions, resulting in a rapid cell shrinkage, activation of JNK cascades, and other catastrophic cellular effects [20,22]. Their diverse control suggests that there exists a crosstalk at various levels between K<sup>+</sup> channel activity in the cell membrane and growth factor receptor-linked signaling cascades or stress-induced signaling pathways [16,17,19,22,46]. In earlier studies, our lab and others have shown that the K<sup>+</sup> channel activity is the major conductance in corneal epithelial cells. In the present study, we found in HCE cells that TNF- $\alpha$  induces increases in activity of a 4-AP sensitive K<sup>+</sup> channel (Fig. 3). Such stimulation elicits activation through possibly a myriad of changes that include initially volume shrinkage leading to changes in cytoskeletal configuration and scaffolding protein conformation. In addition, TNF- $\alpha$  may also activate other pathways besides K<sup>+</sup> channel pathways. However, the exact details cannot be dealt with here because it is a topic for future investigation.

In previous studies, we have identified subtypes of K<sup>+</sup> channel in corneal epithelial cells using a specific panel of K<sup>+</sup> channel antibodies. The TNF- $\alpha$ -induced K<sup>+</sup> channel is similar to those K<sup>+</sup> channels in the Kv3.4 family because it is sensitive to 4-AP and BDS-I and has a similarity in the single channel conductance [47]. The K<sup>+</sup> channel subtype Kv3.4 is highly expressed in whole cell lysates and in cell membrane preparations of these cells. TNF- $\alpha$ -induced increases in K<sup>+</sup> channel activity are upstream from activation and nuclear translocation of NF $\kappa$ B because exposure to 4-AP prevented TNF- $\alpha$ -induced NF $\kappa$ B binding to DNA and its nuclear translocation (Fig. 6). In our previous studies, 4-AP inhibited K<sup>+</sup> channel at  $\mu$ M levels (IC<sub>50</sub> = 106  $\mu$ M), indicating that there is a good agreement between the inhibitory effects of 4-AP on K<sup>+</sup> channel activity and those on NF $\kappa$ B activation [20,47]. Furthermore, such suppression obviated the protective effect of TNF- $\alpha$  on cell death. Overriding this protective effect results in an increase in apoptosis of these cells, while application of TNF- $\alpha$  or 4-AP alone to cells does not induce apoptosis (Fig. 4). Our results further indicate that this cell membrane channel activity is responsible for initiation of TNF- $\alpha$ -induced activation of NF $\kappa$ B signaling that is essential for cell survival in response to cytokine and stress stimulations. In conclusion, our results reveal for the first time that TNF- $\alpha$ -induced stimulation of a 4-AP-sensitive K<sup>+</sup> channel is required for NF $\kappa$ B nuclear translocation and DNA binding activity, which in turn promotes cell survival.

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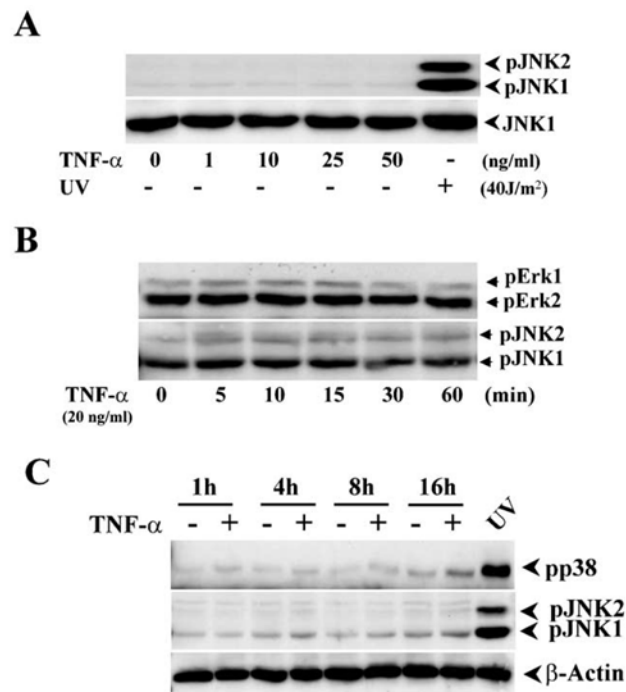
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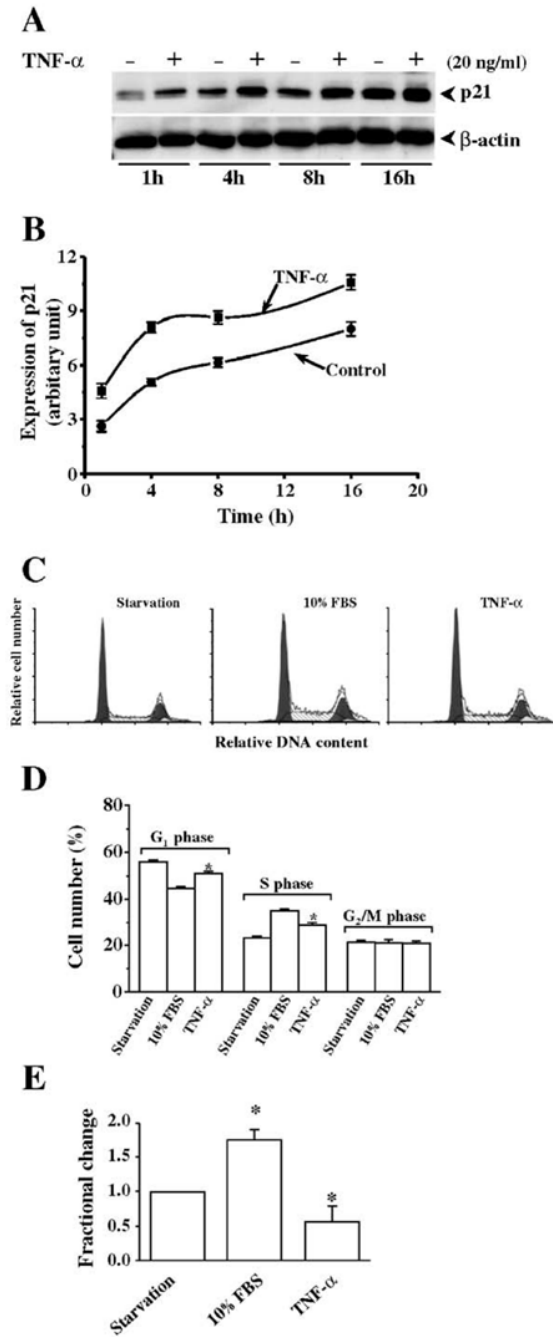


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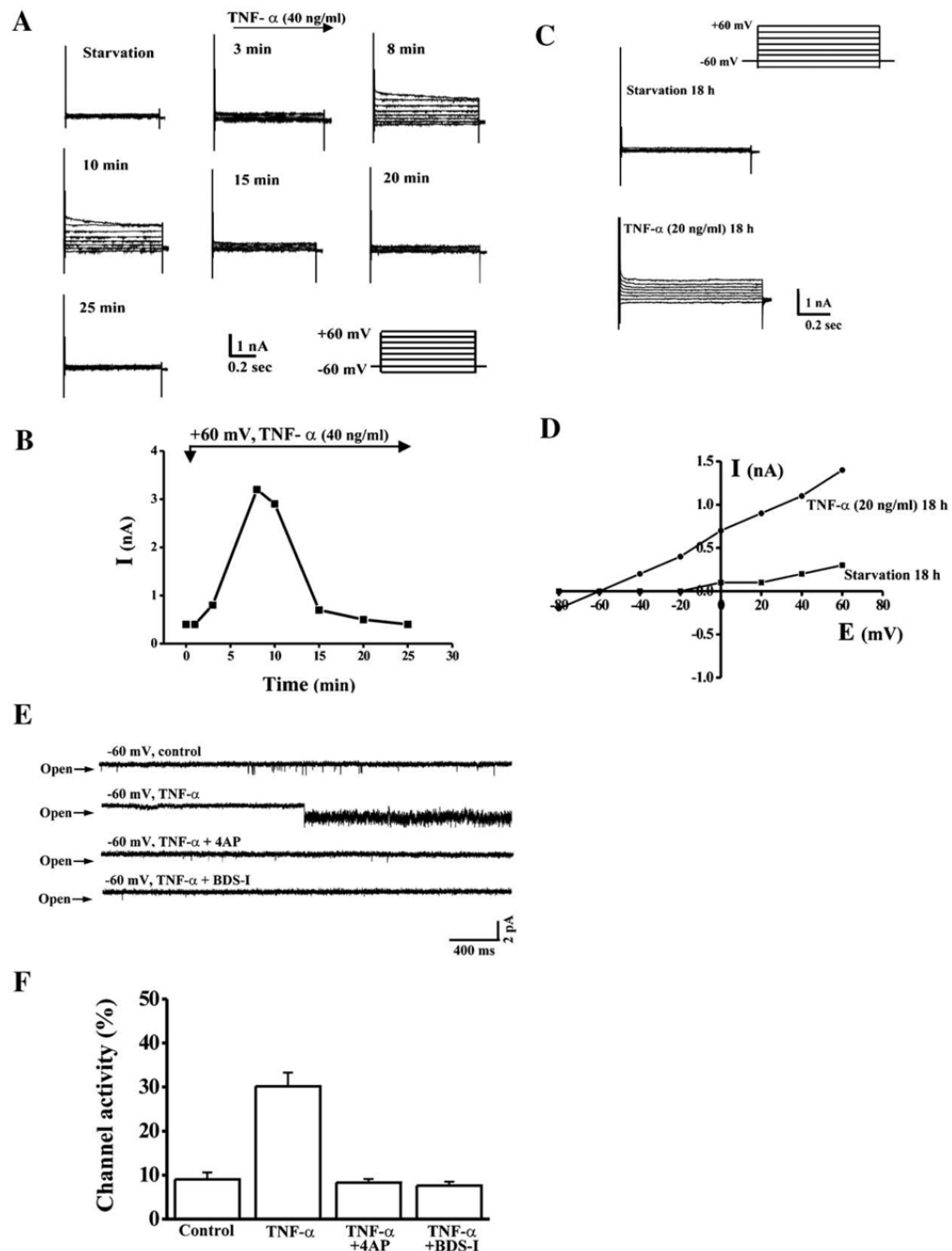


**Fig 1.** Effect of TNF- $\alpha$  on MAK kinase activation in HCE cells. (A) Dose-dependent effects of TNF- $\alpha$  stimulation on JNK activity. TNF- $\alpha$  was added into HCE cells at different concentrations. UV irradiation was used to activate JNK as a positive control. JNK activation was analyzed by Western blots using Western analysis with antibodies against phospho-JNK 30 min after the stimulation. (B) Effect of TNF- $\alpha$  stimulation on Erk activity. Effects of TNF- $\alpha$  stimulation on Erk and JNK activation were measured at indicated time points using Western analysis with antibodies against phospho-JNK and phospho-Erk. (C) Effect of TNF- $\alpha$  stimulation on p38 activity. Effects of TNF- $\alpha$  stimulation on p38 and JNK activation with/without UV irradiation were detected following a time course. HCE cells were incubated with 20 mg/ml TNF- $\alpha$  for different incubation periods. Whole cell lysates from UV exposed HCE cells were used as a positive control of phospho-JNK and phospho-p38.



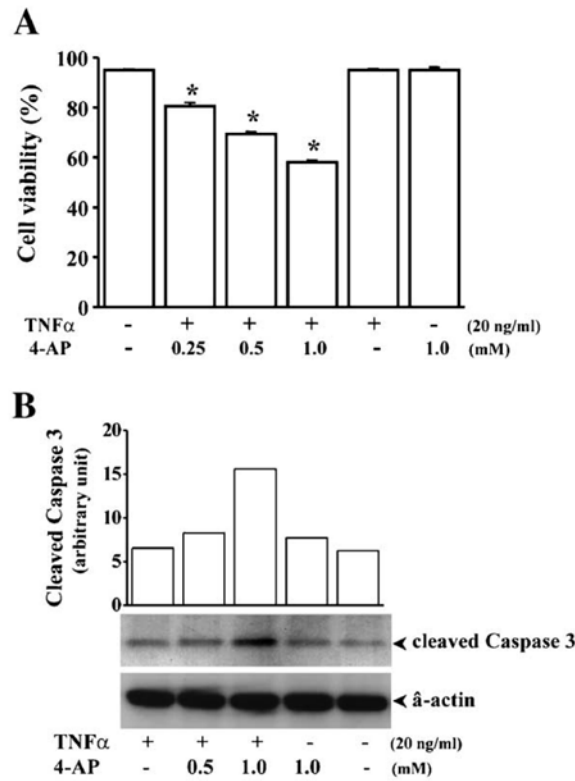
**Fig 2.** Effects of TNF- $\alpha$  stimulation on p21 expression and cell cycle progression. (A) Effect of TNF- $\alpha$  stimulation on p21 expression in HCE cells. TNF- $\alpha$  (20 ng/ml) was applied to activate p21 expression detected by Western blots following a time course up to 16 h. Expression of  $\beta$ -actin was detected as a loading control. (B) TNF- $\alpha$  stimulation-induced p21 expression plotted as a function of time. Statistical analysis revealed significant increases in p21 expression in response to TNF- $\alpha$  stimulation ( $n = 3$ ,  $P < 0.05$ ). (C) Effect of TNF- $\alpha$  stimulation on cell cycle progression. HCE cells were synchronize in G<sub>1</sub> phase by serum-starvation for at least 24 h and then grown in normal DMEM/F12 medium containing 10% FBS in the absence or presence of 20 ng/ml TNF- $\alpha$ . HCE cells were harvested 18 h later for cell cycle mapping experiment

using a flow cytometer. (D) Statistical analysis of cell cycle progression. (E) Statistical analysis of change in sub-G<sub>0</sub> stage of the cell cycle. Symbol “\*” indicates the significant difference between TNF- $\alpha$ -stimulated and unstimulated cells ( $n = 4$ ,  $P < 0.05$ ).



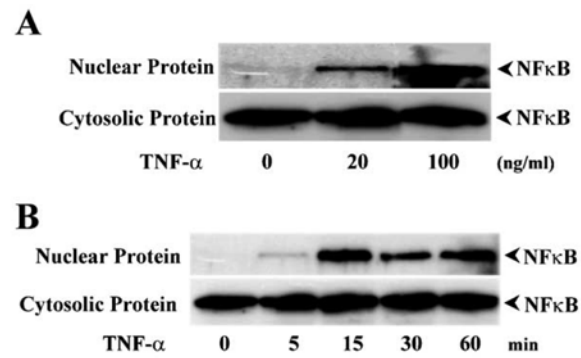
**Fig 3.** Effect of TNF- $\alpha$  on increases in K<sup>+</sup> channel activity. (A) Whole-cell current recorded from TNF- $\alpha$ -stimulated cells. Nystatin-perforated whole-cell patch clamp was performed to record K<sup>+</sup> currents in response to TNF- $\alpha$  stimulation. (B) Time course recorded from a TNF- $\alpha$ -stimulated cell. Amplitudes of K<sup>+</sup> current were recorded after 40 ng/ml TNF- $\alpha$  was applied in the patch chamber. (C) Long-term effect of TNF- $\alpha$  on K<sup>+</sup> currents. Whole-cell currents were recorded 18 h after TNF- $\alpha$  stimulation. (D) Current-voltage (*I*-*V*) relationship of TNF- $\alpha$ -stimulated K<sup>+</sup> currents. *I*-*V* curves of K<sup>+</sup> currents were obtained in cells 18 h after serum starvation and TNF- $\alpha$  stimulation. (E) Single channel activity recorded in TNF- $\alpha$ -stimulated cells. Single channel activity was recorded by the cell-attached patch clamping in HCE cells

in the absence and presence of 20 ng/ml TNF- $\alpha$ . The membrane potential was held at  $-60$  mV and 4-AP (1 mM) or BDS-I (400 nM) was used to suppress K<sup>+</sup> channel activity. (F) Comparison of single channel activity in the absence and presence of TNF- $\alpha$ . Single channel open probability was calculated using a pCLAMP program. Symbol “\*” indicates the significant difference between TNF- $\alpha$ -stimulated and unstimulated cells ( $n = 4$ ,  $P < 0.05$ ).

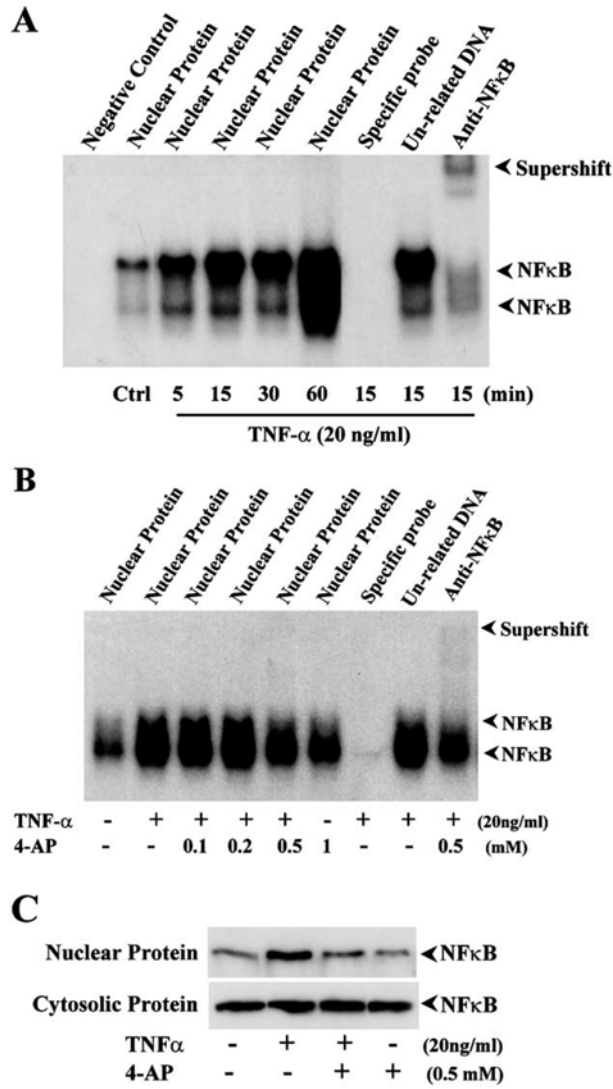


**Fig 4.** Effect of suppressing  $K^+$  channel activity on  $TNF-\alpha$  induced alterations of cell viability. HCE cells were treated with different dosages of 4-AP 30 min prior to  $TNF-\alpha$  stimulation (20 ng/ml). (A) HCE cell apoptosis detected by nuclear staining with ethidium bromide and acridine orange (EB/AO). HCE cell apoptosis was determined 36 h after  $TNF-\alpha$  stimulation by EB/AO to detect chromatin condensation/fragmentation. Cell population was scored according to the color using a UV-fluorescence microscope (Nikon). Symbol “\*” indicates the significant difference between cells with and without  $TNF-\alpha$  stimulation and 4-AP treatment ( $P < 0.05$ ). (B) HCE cell apoptosis detected by measuring caspase 3 activity. HCE cell apoptosis was determined by Western blot analysis of extracts from  $TNF-\alpha$ -stimulated and  $K^+$  channel-inhibited HCE cells using Cleaved Caspase-3 (Asp175) (5A1) Rabbit mAb.





**Fig 5.** Effect of TNF- $\alpha$  on NF $\kappa$ B translocation in HCE cells. (A) Dose-dependent response of TNF- $\alpha$ -induced NF $\kappa$ B nuclear translocation. Nuclear and cytosolic proteins were isolated from TNF- $\alpha$ -stimulated cells and expressions of NF $\kappa$ B were detected by Western blots. (B) Time course of TNF- $\alpha$  induced NF $\kappa$ B translocation in the nuclei. HCE cells were stimulated with 20 ng/ml TNF- $\alpha$  and collected for Western analysis at indicated time points.



**Fig 6.** Effects of TNF- $\alpha$  and suppression of K<sup>+</sup> channel activity on NF $\kappa$ B activation. (A) Time-dependent activation of NF $\kappa$ B in response to TNF- $\alpha$  stimulation. HCE cells were simulated with 20 ng/ml TNF- $\alpha$  and then lysed at indicated time points. (B) Dose-dependent effect of suppressing K<sup>+</sup> channel activity on TNF- $\alpha$ -induced NF $\kappa$ B activation. Nuclear extracts were displayed in EMSA as described in method. Unlabeled NF $\kappa$ B consensus oligonucleotides (specific competitor), unlabeled SP1 oligonucleotides (nonspecific competitor), anti-NF $\kappa$ B (p65) antibody (for supershift lane), and various concentrations of 4-AP were added to reaction, respectively. (C) Inhibition of TNF- $\alpha$  induced NF $\kappa$ B transnuclear activity by suppressing K<sup>+</sup> channel activity. HCE cells were treated with 1 mM 4-AP for 30 min prior to the stimulation of 20 ng/ml TNF- $\alpha$  for 15 min. The cell nuclear and cytosolic extracts were displayed in PAGE.