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Shock Waves Increase T-cell Proliferation or IL-2 Expression by Activating p38 MAP Kinase

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Abstract Shock waves were elicited by transient pressure disturbances, which could be used to treat musculoskeletal disorders. In present studies, we investigated whether the low-density shock waves (LDSWs), which are able to damage plasma membrane without impairing the vimentin or other organelles, might augment T-cell proliferation as well as IL-2 expression, and if mitogen activated protein kinase p38 (p38 MAPK) might be an underlying mechanism through which the LDSWs enhanced T-cell function. We found that the LDSWs increased activation of p38 MAPK in Jurkat T cells. The LDSWs alone didn't result in the T-cell proliferation and IL-2 expression. However, in combination with other stimuli, LDSWs could augment the T-cell proliferation and IL-2 expression. Inhibition of p38 MAPK using SB203580 reduced the stimulatory effects of the LDSWs, which indicated that the LDSWs enhanced IL-2 expression through a mechanism that involved p38 MAPK activation. We concluded that the p38 MAPK activation played a key role in the regulation of T cell function by the LDSWs.

Key words shock waves; p38 MAP kinase; IL-2; T-cell proliferation

As elicited by a transient pressure disturbance, shock waves are characterized by high positive pressure, a rise time lower than 10 ns and tensile wave [1,2]. The fast pressure transition of shock waves (high pressure, short rise time) causes very high tension on the exposed material surfaces so that the structure of the material cracks [1,2]. Because of this, extracorporeal-generated shock waves were introduced approximately 20 years ago to disintegrate kidney stones [3,4]. This therapy substantially changed the treatment of urolithiasis [3,4]. Urology, however, is not the only medical field for the potential use of shock waves to settle problems [3,4]. Shock waves subsequently have been used in orthopedics and traumatology to treat various insertional tendinopathies (enthesiopathies) and delayed unions and nonunions of fracture [3,4]. Thus, the subsequent researches on biologic mechanism of shock wave treatment on the musculoskeletal disorders were carried on all over the world [4]. Wang *et al.* [5] demonstrated that the low-density shock waves (LDSWs, an optimal dose of 500-impulse shock wave treatment at 0.16 mJ/mm^2) caused differentiation of bone marrow stromal cells (BMSCs) toward osteoprogenitor in 2 d, increase of bone alkaline phosphatase activation and collagen type I mRNA expression in 6 d, and osteocalcin mRNA expression in 12 d. Now that shock waves can influence the function of BMSCs, they may do the same to other cells such as lymphocytes. Haupt and Chvapil's studies showed that the upper dermis of partial-thickness wounds in piglets which received the shock wave treatment (10 shock waves at 14 kV) had increased the number of dilated microvessels and increased macrophages in the perivascular spaces histologically [6]. Therefore, we postulated that shock waves might enhance T-cell proliferation.

Because the shock-wave treatment cause high pressure on the exposed material surface in the short rise time [1.2], it can also be regarded as a kind of mechanical stimulation. The mechanical stimulation can activate multiple signaling enzymes, including mitogen activated protein kinase p38 (p38 MAPK) [7]. This kinase is structurally related to the yeast protein HOG-1 (the product of *Saccharomyces cerevisiae* osmosensing gene), which is part of the signaling system that allows yeast cells to regulate gene transcription in response to osmotic stress [8]. T cell p38

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MAPK signaling is involved in the activation of IL-2 gene expression in human [9]. Therefore, by analogy with mechanical stress, shock waves may enhance IL-2 expression by activating the p38 MAPK.

In present *in vitro* studies, we investigated if LDSWs might augment T-cell proliferation and IL-2 expression, if LDSWs enhanced IL-2 expressions through a mechanism that involved p38 MAPK activation.

Materials and Methods

Materials

Phytohemagglutinin (PHA), suramin, apyrase, and dimethylsulfoxide (DMSO) were from Sigma (St. Louis, MO). SB203580 was from Calbiochem (San Diego, CA). Anti-CD3 and anti-CD28 antibodies (clone CD28.2) were products of Santa Cruz Biotech Inc..

Cell lines

Jurkat T cells (clone E6-1) were obtained from Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences, and maintained in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 100 u/ml penicillin, 100 μg/ml streptomycin (University of California San Diego Core Facility, la Jolla, CA), and 10% heat-inactivated fetal calf serum (*V*/*V*, Gibco, Invitrogen Corporation, USA). Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized venous blood of healthy human volunteers and collected by Ficoll-Paque density gradient centrifugation (d=1.007 g/ml, Amersham Biosciences AB).

Unless indicated, cells were pretreated for 1 h at 37 °C with the p38 MAPK inhibitor SB203580 [10]. All materials and compounds used in these experiments were sterile and endotoxin-free.

Shockwave treatment

A KDE-2001 extracoporeal shock wave lithotripter (Beijing Zhongke Jian'an Meditechs Co., Beijing, China) was employed for studies. Shock waves were generated by underwater spark discharge from an electrode which was loaded in the first focus of a hemi-ellipsoid and the de-gassed water was jacketed with an UV-resistant coupling membrane. The propagation wave was focused to the second focus of the ellipsoid where the center of tube containing target cells was fixed as shown in Fig. 1. LDSWs were applied at a frequency of 50 Hz at 7 kV generator voltage with a 0.3 μF capacitance. Their positive pressure was (23.0 ± 1.4) MPa (n=3) as measured with PA membrane hydrophone (Precision Acoustics Ltd., UK) according to IEC 61846 (International Electrotechnical Commission 1998, Chicago, IL, USA), and their energy flux density (i.e., the derived pulse intensity integral at the focus) was (0.180 ± 0.009) mJ/mm^2 (n=3) as calculated from the waveform in the focus according to IEC 61846. Cells (1×10^6 cells/ml) were suspended in a 2-ml polystyrene round-bottom tube (Falcon, Becton-Dickson Co., NJ) containing 0.5 ml RPMI 1640 at pH 7.4 and exposed to LDSWs at 0.18 mJ/mm^2 for 0, 50, 100, 200, 250, 500 and 1000 impulses respectively. Duration of the LDSW treatment took 10–20 min depending on the dose applied. The ultrasound transmission gel (Pharmaceutical Innovations Inc., NJ) was used as contact medium between the apparatus and the target tube. The apparatus is covered with a UV-resistant membrane in order to avoid the UV light damage to the environment. After the LDSW treatment, cell number and viability were determined with a hemocytometer by a 0.4% trypan blue exclusion assay.

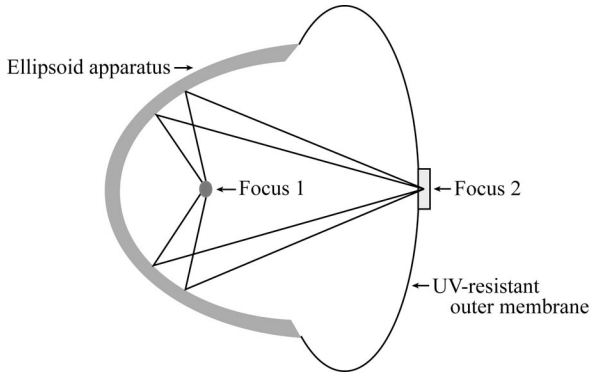

Fig. 1 Schematic diagram of the shock wave apparatus

Cell stimulation with PHA or anti-CD3 and anti-CD28 antibodies

Jurkat cells were stimulated by simultaneously activating the T cell receptor-CD3 complex and CD28 co-receptor with Dynabeads (Dynal Inc., Lake Success, NY). The beads (10^7), precoated with anti-mouse IgG, were coated with 5 μg anti-CD3 and anti-CD28 antibodies and incubated at room temperature for 1 h [11]. Then the beads were washed twice with RPMI containing 10% fetal calf serum and resuspended at 10^7 beads/ml, and 20 μl were added to 2×10^4 Jurkat cells (10 beads/cell). The cells were incubated in a final volume of 200 μl for 20 h at 37 °C under tissue culture conditions, and IL-2 in the superna-

tant was determined mentioned below.

PBMCs were suspended in the RPMI 1640 medium mentioned above (10^6 cells/ml) and stimulated under tissue culture conditions with a suboptimal dose of PHA (1 μg/ml) in a final volume of 200 μl. Dose-response curves (data not shown) indicated that the optimal dosages for inducing lymphoproliferation were 5 μg/ml for PHA. After 20 h culture, IL-2 concentrations in the supernatants were determined with the enzymelinked immunosorbent assay method illustrated below; or after 48 h culture, the T-cell proliferation was assessed as introduced below.

T-cell proliferation assay

PBMCs were exposed to the low-density shock waves at 0.18 mJ/mm^2 for 0, 100, 150, 200, 250, 300, 320, 360, and 400 impulses, then they were or were not stimulated with 1 μg/ml PHA. Cells were incubated at 37 °C in humidified air with 5% CO_2 for 48 h, pulsed with 1 μCi/well [methyl-^{3}H]thymidine ([^{3}H]TdR, Amersham-Life Science, Aylesbury, England), and then incubated for another 8 h. Subsequently, the cells were harvested onto glass fiber filter discs using a PHD cell harvester (Cambridge Technology, Inc., Watertown, Mass). The filter discs were transferred into scintillation vials, dried overnight, and covered with 4 ml/vial scintillation fluid (ScintiVerse BD. Fisher Scientific, Fair Lawn, NJ). Incorporated thymidine was counted with a liquid scintillation counter (Tri-Crab 2100TR, Packard Inc, USA). Proliferation was assessed by [methyl-^{3}H]thymidine uptake. Values were expressed as mean ± SD using triplicated determinations.

IL-2 expression

An enzyme-linked immunosorbent assay (ELISA) was performed as manufacturer's instructions to detect the released IL-2 in the supernatant with monoclonal mouse anti-human IL-2 as the primary antibody (clone 5355.111) and biotinylated goat anti-human IL-2 as the secondary antibody (both from R&D Systems Inc., Minneapolis, MN). The recombinant human IL-2 was as control (Genzyme Diagnostics, Cambridge, MA), and the horseradish peroxidase-conjugated streptavidin (Zymed Laboratories Inc., San Francisco, CA) as secondary antibody.

p38 MAPK expression and activation

The phosphorylation of p38 MAP kinase of Jurkat cells was measured with the PhosphoPlus® p38 MAP kinase antibody kit (Cell Signaling Technology, Inc., USA). Jurkat cells received physical shock wave treatment at 0.18 mJ/mm^2 for 0, 50, 100, 150, 200 or 250 impulses respectively, and the cells (1×10^6 cells/ml) were cultured in a 12-well plate of 1 ml RPMI 1640 medium containing 10% FBS for each well for 45 min. And then Jurkat cells (10^6 cells/sample) were placed on ice, centrifuged, resuspended in 100 μl of ice-cold SDS sample buffer containing 100 mM dithiothreitol, and lysed by boiling for 5 min. The cell lysates were separated on 8%–16% Tris-glycine polyacrylamide gradient gels (Novex, San Diego, CA). Lysed proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA), and these membranes were subjected to immunoblotting with phospho-specific antibodies that recognized the phosphorylated (on Thr^{180}/Tyr^{182}) form of p38 MAPK, or to immunoblotting with antibodies recognizing both active and inactive p38 MAPK. The activated p38 MAPK or the total p38 MAPK on the membranes was detected with X-ray film using LumiGLO® chemiluminescent reagent (Cell Signaling Technology, Inc., USA) according to the protocol provided by the manufacturer. Band intensities in the X-ray film were analyzed with commercially available UTHSCSA (The University of Texas Health Science Center in San Antonio) graphic software (Image Tool 3.00) and ratios between activated and total p38 MAPK were used to calculate p38 MAPK activation.

Statistical analyses

Unless otherwise indicated, data were presented as mean ± SD. Sets of data were analyzed by Student's *t*-test, and $P<0.05$ indicated significant difference.

Results

Optimal dose of LDSW treatment promoted activated-T-cell proliferation

We first investigated whether LDSW treatment could affect the viability of the human PBMCs or Jurkat cells. Cells exposed to different doses from 100 to 500 impulses of the 0.18 mJ/mm^2 LDSWs showed normal viability, whereas doses of more than 500 impulses significantly suppressed cell viability (Fig. 2). Then we tested if LDSW treatment exerts a co-stimulatory effect on T-cell proliferation. Human PBMCs were treated with 0.18 mJ/mm^2 LDSW impulses in presence or absence of PHA. Proliferation was significantly enhanced when the cells were stimulated with 0.18 mJ/mm^2 LDSWs ranging between 100 and 360 impulses in the presence of PHA (Fig. 3). The LDSWs of 320 impulses had the best promotion, whereas the treatments with more than 400 impulses exerted a suppressing effect (Fig. 3).

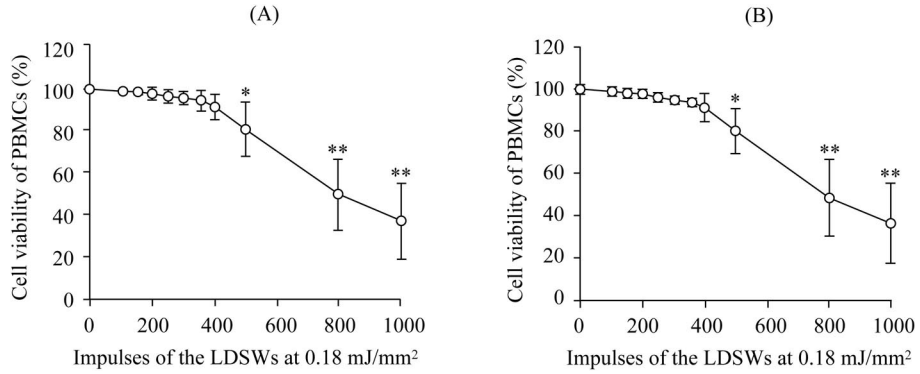

Fig. 2 Effects of LDSWs on cell viability

Physical shock wave treatment at 0.18 mJ/mm^2 for 100, 150, 200, 250, 300, 360, 400 or 500 impulses did not affect cell viability as determined by trypan blue exclusion (P>0.05), but doses higher than 500 impulses significantly decreased cell viability (*P<0.05, **P<0.01). There were not significant differences between PBMCs and Jurkat T-cell samples. Results were presented with mean ± SD calculated from six paired triplicate experiments.

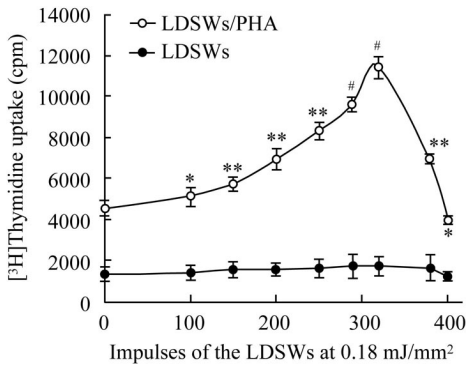

Fig. 3 Low-density shock waves enhance PHA-stimulated T-cell proliferation

Asterisks indicated statistically significant differences from control values of no shock-wave treatment in the presence or absence of PHA (*P<0.05, **P<0.01, $^{\#}P$<0.001).

Optimal dose of LDSW treatment enhanced IL-2 expression in T-cell

We hypothesized that co-stimulation with LDSWs might enhance T-cell proliferation by increasing IL-2 expression in T-cell. To test this hypothesis, PHA-activated human PBMCs or CD3/CD28-activated Jurkat cells were subjected to doses from 100 to 500 impulses of the 0.18 mJ/mm^2 LDSWs. 24 h after the treatment, IL-2 levels in the supernatants were determined by ELISA. It was found that the LDSW treatment with doses from 200 to 430 impulses enhanced PHA-activated IL-2 expression and that the LDSW treatment with doses from 150 to 300 impulses enhanced CD3/CD28-activated IL-2 expression (Fig. 4). The treatment with 320 impulses approximately trebled the PHA-activated IL-2 expression, and the one at 250

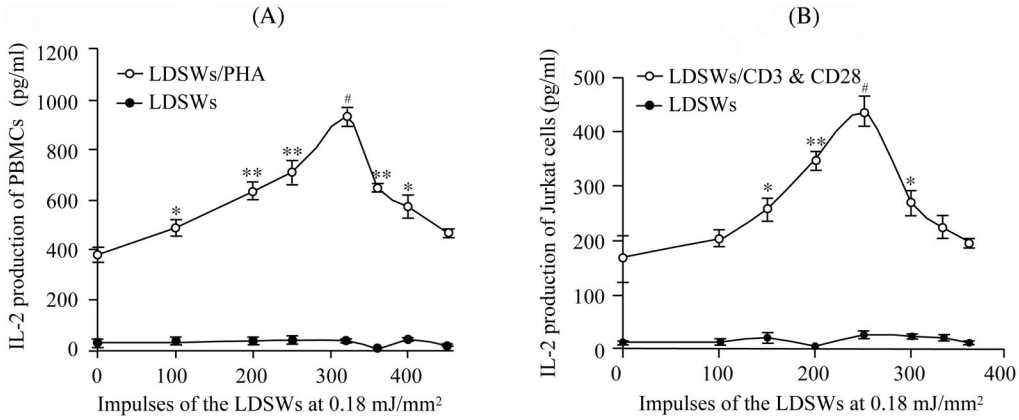

Fig. 4 LDSWs enhance stimulated-T-cell IL-2 expression

Asterisks indicated statistically significant differences from control values of no LDSW treatment (*P<0.05, **P<0.01, $^{\#}P$<0.001).

impulses nearly doubled the CD3/CD28-activated IL-2 expression. However, the treatments with more than 460 impulses suppressed the PHA-activated IL-2 expression, and the ones with more than 320 impulses prevented the CD3/CD28-activated IL-2 expression.

LDSWs activate p38 MAPK in T cells

The LDSW treatments with doses from 50 to 300 impulses activated p38 MAPK in Jurkat cells. The LDSW treatment with 250 impulses had the best promotion (Fig. 5).

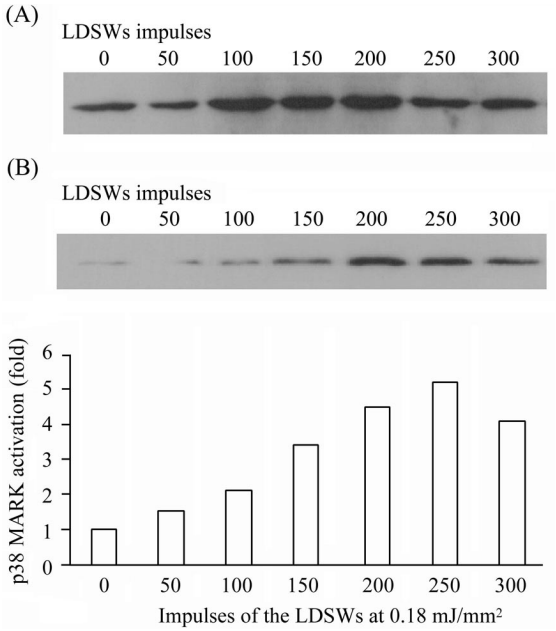

Fig. 5 LDSWs activate p38 MAPK in Jurkat cells

p38 MAPK activation was determined by immunoblotting with anti-p38 MAPK antibodies (A) or with anti-phospho-p38 MAPK antibodies recognizing inactive and active p38 MAPK activation (B). Band intensities were analyzed with UTHSCSA graphic software (Image Tool 3.00) and ratios between activated and total p38 MAPK were recorded. The data shown were representatives of 3 experiments with similar results.

p38 MAPK is an important element in the mechanism whereby LDSWs enhance activated-T-cell proliferation and IL-2 expression

Because p38 MAPK signaling targeted several nuclear factors that might be responsible for T cell gene regulation [9], we tested whether p38 MAPK contributed to the mechanisms through which LDSWs enhanced T-cell proliferation and IL-2 expression. We hence inhibited p38 MAPK with the inhibitor SB203580 and investigated if this would prevent the enhancing effect of LDSWs on PHA-stimulated T-cell proliferation and CD3/CD28-stimulated IL-2 expression. SB203580 completely abrogated the enhancement of T-cell proliferation and IL-2 expression by LDSWs (Fig. 6,7). These results implied that p38 MAPK played a major role in enhancing T-cell proliferation and IL-2 expression by the LDSWs.

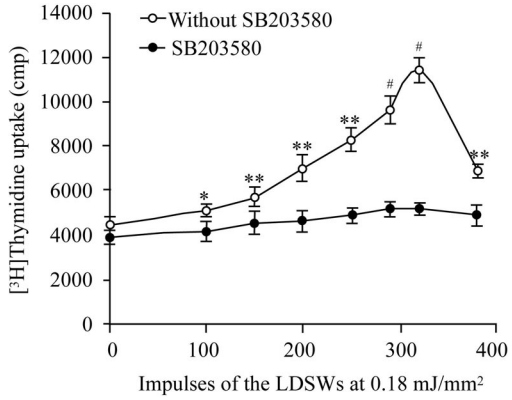

Fig. 6 SB203580 prevents the enhancing effect of LDSWs on PHA-stimulated-T-cell proliferation

T-cell proliferation was determined as indicated. Data were representatives of 3 different experiments and expressed as mean ± SD. There were significant differences between SB203580-treated and no SB203580-treated samples (P<0.001), which were compared with analysis of variance. Asterisks indicated statistically significant differences from control values (no shock-wave treatment) in the presence or absence of SB203580 (*P<0.05, **P<0.01, $^{\#}P$<0.001).

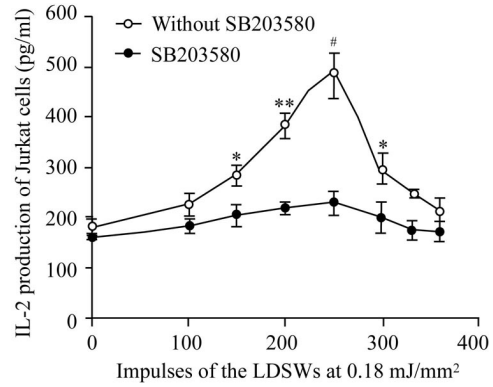

Fig. 7 SB203580 prevents the enhancing effect of LDSWs on CD3/CD28-stimulated IL-2 expression

T-cell IL-2 production was measured as indicated. Data were representative of 3 different experiments and expressed as mean ± SD. There were significant differences between SB203580-treated and no SB203580-treated samples (P<0.001), which were compared with analysis of variance. Asterisks indicated statistically significant differences from control values (no shock-wave treatment) in the presence or absence of SB203580 (*P<0.05, **P<0.01, $^{\#}P$<0.001).

Discussion

Shock waves are created by a high voltage spark discharge under water causing an explosive evaporation of water and producing high energy acoustic waves [1,2]. Shock wave treatment has been divergently applied for eukaryotic and prokaryotic biology systems. It is well known that shock waves provide a non-invasive biophysical strategy for breaking renal stones with minimal side effects [3,4]. Evidence also suggests that shock waves can potentially enhance gene transfer [12], suppress tumor growth [13], and promote the bactericidal effect of microorganisms [14]. Recently, it has been shown that shock wave treatment has a promising effect on the promotion of bone fracture healing and repair of tendonitis. The mechanism by which shock waves enhance fracture healing and repair of tendonitis remains to be confirmed. Researches showed that shock waves promoted bone marrow osteoprogenitor growth through transforming growth factor-β1 induction and superoxide induction [15] and that shock waves caused differentiation of bone marrow stromal cells toward osteoprogenitor [5,15].

We found that the LDSW treatment increased T-cell proliferation, as measured by thymidine uptake, when cells were stimulated with mitogens or other agents that ligate the T-cell receptor (Fig. 3). Enhanced T-cell proliferation seemed to be the result of increased IL-2 expression of T-cells [Fig. 4(A)]. Fig. 4(B) showed that the human Jurkat T-cell also responded to the LDSWs with increased IL-2 production. However, the optimal LDSW dose for increasing IL-2 production in Jurkat T cells or in PBMCs was different.

The LDSW exposure of T-cells without other stimuli did not increase thymidine uptake or IL-2 production. This finding indicated that the LDSWs served as a co-stimulatory factor, however, itself didn't trigger these T-cell responses. To gain insight in how LDSWs could co-stimulate T-cell functions, we investigated what effect the LDSWs might have on T-cell signaling. The human Jurkat T-cell line was used for these experiments to eliminate any possibility of interference by B-cell, monocyte, or any other cell types that might be present in human PBMC preparations.

p38 MAPK is activated by mechanical stimulation in a number of cell types [16,17]. Activated p38 MAPK in turn mediates cytokine production [10,18]. Matsuda *et al.* [9] show that p38 MAPK is activated by signals that lead to IL-2 production in T cells. The p38 signaling pathway in Jurkat T cells affects the recruitment of the p300 co-activator to the CD28 response element/activator protein 1 (CD28RE/AP1) and the combinatorial interaction of NF-κB and cAMP response element-binding protein (CREB) at the CD28RE/AP1 element coupled with the subsequent dynamic co-assembly and activation of p300 [19]. The IL-2 promoter has been shown to have multiple binding sites for NF-κB family members and members of the AP-1 and CREB family of transcription factors [20,21]. Thus, it is obvious that the p38 signaling pathway influences the IL-2 production in T cells. Therefore, we speculated that the LDSWs might co-stimulate IL-2 expression in T cells by activating MAPK p38. The present study demonstrated for the first time that the LDSW treatment greatly activated p38 MAPK in Jurkat T cells and inhibited p38 MAPK with SB203580 to prevent the enhancement of T cell proliferation or IL-2 expression by the LDSWs. In addition, significant activation of p38 MAPK was observed at the LDSW doses that increased IL-2 expression. This result was consistent with the idea that p38 MAPK signaling played a critical role in the T cell response to the LDSWs.

Phospho-p38 MAPK activates ATF-2, CHOP-1, MEF-2 and other transcription factors through phosphorylation [22–24]. Other immediate targets include MNK1, MSK2, Elk-1, MAPKAP-2, MAPKAP-K3, MSK1 and hnRNP [25, 26]. Of particular note for cancer therapy, p38 MAPK has been demonstrated to phosphorylate substrates directly, thus activating the key cell-cycle regulators p53 [27,28] and p73 [29]. Downstream activities attributed to these phosphorylation events include cell-cycle arrest, apoptosis, cytokine production, regulation of RNA splicing, and cell differentiation. These activities are frequently cell-type specific [30]. Thus, we speculate that the LDSW treatment may be useful for cancer therapeutics.

Steinbach *et al.* [31] exposed single-cell suspension of the prostate carcinoma cell line PCA to electromagnetically generated shock waves, and analyzed the intracellular damage of the individual cell components by laser scanning microscopy following specific fluorescence staining. They reported that defects on cell plasma membrane were induced by a shock wave with 0.12 mJ/mm^2 energy flux density, defects on vimentin by 0.21 mJ/mm^2, defects on mitochondria by 0.33 mJ/mm^2, and defects on nuclear membrane by 0.5 mJ/mm^2 [31]. According to that, the LDSWs at 0.18 mJ/mm^2, applied in our studies, can damage the plasma membrane of the cells without injuring the vimentin or other organelles.

Overall, our results suggested a regulatory pathway that the LDSWs, which were able to damage plasma membrane without impairing the vimentin or other organelles, increase the activitation of p38 MAPK. Finally, p38 MAPK

exerted a co-stimulatory signal that enhanced IL-2 expression of CD3/CD28-stimulated T cells, thereby enhancing T cell function.

So far, most of the researches about shock waves have targeted the effects of shock waves on musculoskeletal disorders. Our research suggested that the LDSWs at 0.18 mJ/mm^2 might be useful to modulate immune function. However, this possibility has not been tested clinically. Future clinical studies should examine the possible immunoregulatory role of the LDSWs at 0.18 mJ/mm^2 in tumor therapeutics. In addition, the dose and mode of administration of the LDSW treatment may have to be reconsidered to optimize its potential as an up-regulator of immune function, but also to investigate possible negative side effects of the LDSW treatment.

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