

## Bufalin Suppresses Migration and Invasion of Hepatocellular Carcinoma Cells Elicited by Poly (I:C) Therapy

Yinglu Feng<sup>a,b</sup>, Yongan Chen<sup>a</sup>, Yongbin Meng<sup>a</sup>, Qingxin Cao<sup>a</sup>, Qun Liu<sup>a</sup>, Changquan Ling<sup>a</sup>, and Chen Wang<sup>a</sup>

<sup>a</sup>Department of Traditional Chinese Medicine, Changhai Hospital, Second Military Medical University, Shanghai, China; <sup>b</sup>Department of Traditional Chinese Medicine, 401 Hospital of the Chinese People's Liberation Army, Qingdao, Shandong, China.

### ABSTRACT

The Toll-like receptor 3 (TLR3) agonists as polyriboinosinic–polyribocytidylic acid (poly (I:C)) have been implicated as potential immunotherapy adjuvant for cancer whereas the exact roles of TLR3 agonists in hepatocellular carcinoma (HCC) treatment have not been clearly evaluated. In consistent with previous reports, we found that poly (I:C) triggering of TLR3 inhibited cell proliferation and induced apoptosis in HCC cells. However, poly (I:C), when used at lower concentration that cannot remarkably inhibit proliferation and induce apoptosis in HCC cells, enhanced the migration and invasion in vitro and the metastasis in vivo. More importantly, we found that bufalin, a prominent component of toad venom, could suppress poly (I:C)-inspired migration, invasion and metastasis of HCC cells despite that bufalin could not potentiate poly (I:C)-induced inhibition of proliferation and induction of apoptosis. In MHCC97 H cells, bufalin impaired poly (I:C)-induced activation of Tank-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3) pathway and NF- $\kappa$ B pathway. Inhibitor for TBK1 but not NF- $\kappa$ B suppressed poly (I:C)-inspired migration and invasion, which was further supported by using TBK1 deficient (*Tbk1*<sup>-/-</sup>) cells. In another model using poly (I:C) transfection, bufalin could also suppress the migration and invasion of HCC cells, which was not observed in *Tbk1*<sup>-/-</sup> MHCC97 H cells. Our data suggest that bufalin can suppress the metastasis of HCC cells in poly (I:C) therapy by impairing TBK1 activation, indicating that bufalin may be used in combination with poly (I:C) therapy in HCC treatment for the sake of reversing poly (I:C)-triggered metastasis of HCC cells.

### ARTICLE HISTORY

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### KEYWORDS

Bufalin; HCC; metastasis; poly (I:C); TLR3; TBK1

### Introduction


Hepatocellular carcinoma (HCC) is the sixth most common malignancy worldwide and the second leading cause of cancer-related deaths in Asia, particularly in China.<sup>1</sup> Surgical resection and liver transplantation are regarded as the firstline treatment of HCC. However, due to the low incidence of early diagnosis and the lacking of donor organs, the long-term prognosis of patients with HCC remains unsatisfactory because of tumor recurrence and a limited response to chemotherapy and radiotherapy.<sup>2</sup> Up to date, the only systemic therapy for HCC is the targeted therapy using multi-kinase inhibitor sorafenib, which is hindered by drug resistance among patients.<sup>2</sup> More importantly, recurrence and metastasis have been the major obstacles to improve survival for HCC patients.<sup>2</sup> Therefore, new therapies for HCC are in urgent need, and it may be expected that therapies dealing with HCC metastasis may help to improve the treatment status of HCC.

Toll-like receptors (TLRs) are typical pattern recognition receptors (PRRs) usually expressed by immune cells and initially implicated in innate immunity against pathogen-associated molecular patterns, and play critical roles in eliminating pathogens and causing inflammatory response as well.<sup>3</sup> In

recent years it has been recognized that TLRs play roles in cancer.<sup>4</sup> Both pro-tumor and anti-tumor effects have been reported for TLR activation in cancer.<sup>5-33</sup> However, activation of TLRs by agonists, such as polyriboinosinic–polyribocytidylic acid (poly (I:C)) for TLR3, small molecules specific for TLR7 or TLR8, and unmethylated oligodeoxynucleotide containing CpG motif (CpG-ODN) for TLR9, has been implicated in modulating tumor microenvironment by activating dendritic cells (DC), increasing Th1 cytokines, inhibiting regulatory T cells, or enhancing tumor infiltration of CD8<sup>+</sup> T cells and NK cells.<sup>4,24</sup> Based on the immunomodulatory and anti-tumor roles of TLR agonists, natural and synthetic TLR ligands have been used clinically or undergoing clinical trials.<sup>4</sup> However, up to date outcomes of clinical trials using TLR agonists have been unsatisfactory either due to limited efficacy or systemic toxicity.<sup>4</sup> Therefore, more investigations of TLR agonists as adjuvant immunotherapy are required to improve the efficacy and decrease the side effects of TLR-based therapies.

Among the TLRs, TLR3 has attracted our attention. TLR3 is cell surface and endosomal TLR receptors for double-stranded RNA from viruses, endogenous dsRNA released from dying

**CONTACT** Changquan Ling, M.D., Ph.D.  [changquanling@smmu.edu.cn](mailto:changquanling@smmu.edu.cn); Chen Wang, M.D., Ph.D.  [wangchenchina@126.com](mailto:wangchenchina@126.com) 

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cells, or synthetic dsRNA such as poly (I:C).<sup>3,4</sup> TLR3 signaling depends on the adapter protein TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF), leading to activation of the NF- $\kappa$ B and the Tank-binding kinase (TBK1)-phosphorylated interferon-regulatory factor 3 (IRF3) pathways, and finally production of inflammatory cytokines and type I interferons.<sup>3,4</sup> The role of TLR3 in cancer is still unresolved. Since TRIF itself exhibits proapoptotic activity,<sup>3,14</sup> it has been extensively demonstrated that TLR3 signaling can trigger apoptotic and necrotic cell death pathway in certain cancer cells.<sup>6,8,10-12,14-16,22,28,30</sup> TLR3 expression has been detected in human HCC samples and in HCC cell lines.<sup>11,17,22,24,25,28,31,32</sup> It has been demonstrated that TLR3 triggering can induce apoptosis, inhibit angiogenesis, retard tumor growth and promote infiltration of T cells and NK cells.<sup>11,17,22,24,25,28,32</sup> However, the exact effects of TLR3 triggering on migration and invasion of HCC have not been clearly evaluated to draw concordant conclusions.

In ancient China, the skin of toad has been used in the prescription for treatment of cancer including HCC.<sup>34-37</sup> Bufalin has been recognized as a prominent digoxin-like component of the Chinese medicine Chansu (venom of toad skin).<sup>38</sup> Previous studies have demonstrated that bufalin exerts antitumor activities in various cancer cells by inhibiting proliferation, inducing apoptosis and cell cycle arrest, reversing drug resistance, modulating immune response and inhibiting invasion and metastasis of HCC.<sup>34,39-44</sup> When investigating the effects of TLR3 triggering on HCC metastasis, we unexpectedly found that poly (I:C) could promote the migration and invasion of HCC cells. So we further explored whether bufalin could reverse the poly (I:C)-evoked metastasis of HCC while synergize with poly (I:C) in inducing apoptosis and inhibiting cell proliferation. We found that bufalin could inhibit poly (I:C)-inspired migration and invasion of HCC cells in vitro and the metastasis of HCC xenografts in vivo although synergistic effects of bufalin and poly (I:C) were not observed regarding proliferation and apoptosis of HCC. Our study suggests that bufalin could be used in combination with TLR3 agonists for the treatment of HCC for the sake of inhibiting metastasis of HCC.

## Materials and methods

### Mice, cells, antibodies and reagents

Wild type Balb/c nu/nu mice (6-8 weeks old) were purchased from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). All the animal experiments were approved by the Medical Ethics Committee of the Second Military Medical University and conducted according to the Declaration of Helsinki Principles. The HepG2 and human embryonic kidney 293 (HEK293) cells were obtained from ATCC (Manassas, VA). The metastatic HCC cell line MHCC97 H was from the Liver Cancer Institute, Zhongshan Hospital (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; heat-inactivated; Gibco of Thermofisher, Waltham, MA). Abs specific for IRF3 (#4302), Myc-tag, p65 (L8F6, #6956), phospho-IkBa (Ser32/Ser36) (5A5, #9246), TBK1 (#3013), phospho-IRF3 (Ser396) (4D4G, #4947), and phospho-TBK1 (Ser172) (#5483) were from Cell Signaling Technology (Beverly, MA).  $\beta$ -actin

antibody, poly (I:C), bufalin and other non-specified reagents were purchased from Sigma (St. Louis, MO). The TBK1 inhibitor BX-795 and the NF- $\kappa$ B inhibitor TPCA-1 were obtained from Selleck Chemicals (Houston, TX).

### Plasmid and transfection

Myc-tagged vector for human TLR3 was from Origene (Beijing, China) and subcloned into pcDNA3.1 vector as described.<sup>45</sup> For the transfection of expression vectors in HCC cells, the jet-PEI reagents were used (Polyplus-transfection Company, Illkirch, France). HepG2 cells transfected with TLR3 vector were selected under 500 ng/ml neomycin for 3 weeks as described previously.<sup>45</sup>

### mRNA quantification

Quantitative real-time RT-PCR (q-PCR) analysis was performed by LightCycler (Roche, Basel, Schweiz) and SYBR RT-PCR kit (Takara, Dalian, China) as described.<sup>45,46</sup> Data were normalized by the level of *Gapdh*. The primers used for *Tlr3* and *Gapdh* were as described previously.<sup>45,46</sup>

### CRISPR-Cas9-mediated depletion of TBK1

For the depletion of *Tbk1*, pc3-U6-guide RNA-CMV-RED (encoding guiding RNA and red fluorescent protein) and Cas9-IRES-EGFP (encoding Cas9 and green fluorescent protein) plasmids (kind gifts from Shanghai Biomodel Organism Science & Technology Development Co., Shanghai, China) were cotransfected into MHCC97 H cells as described.<sup>46</sup> Five target sequences for guiding RNA synthesis were tested, including 5' GCTACTGCAAATGTCTTTCG 3', 5' GAAGAA CCTTCTAATGCCTA 3', 5' TCTAATGCCTATGGACTACC 3', 5' GTGCACCGTGATATCAAGCC 3' and 5' CATGCGTG TTATAGGGGAAGA 3'. The target sequence (5' TCTAATGC CTATGGACTACC 3') was mostly efficient in TBK1 depletion. Cells with both red and green fluorescence were then sorted by using Gallios<sup>TM</sup> Flow Cytometer (Beckman Coulter, Brea, CA). Clones propagated from single cell were picked out. The depletion of TBK1 was confirmed by both Western blot and DNA sequencing.

### Cell proliferation, apoptosis, migration and invasion assays

Proliferation of the cells was evaluated by using the BrdU Cell Proliferation ELISA Kit (ab126556, Abcam Inc., Cambridge, MA) according to the manufacturer's instruction. Briefly,  $1 \times 10^4$  HCC cells (in 100  $\mu$ l complete DMEM) were plated in 96-well plate in triplicates, and cultured for 6 hours to allow adhesion. Then the cells were serum-starved in medium containing 0.5% FBS for 12 hours. After treatments with poly (I:C) and/or bufalin for 42 H, BrdU was added and cultured for 6 H. Incorporation of BrdU was finally determined by measuring optical density at 450 nm ( $OD_{450nm}$ ).

For apoptosis assays, cells were labeled with annexin V and propidium iodide (PI) provided by Molecular Probes (Eugene, OR). Samples were examined by fluorescence-activated cell sorter

(FACS) analysis, and the results were analyzed using CellQuest software (Becton Dickinson, San Jose, CA) as described.<sup>47</sup>

For the cell migration assay, the cells were assessed using the transwell assay (Boyden Chambers, Corning, and Cambridge, MA, USA). Cells ( $5 \times 10^4$ ) were seeded in a serum-free medium in the upper chamber and allowed to migrate towards the lower chamber that contained 10% FBS. After 48 hours, a cotton swab was used to get rid of the cells on the upper surface of the membrane. The cells were fixed using 4% paraformaldehyde and stained with 0.1% crystal violet. The migrated cells were counted in five selected fields under a microscope at a  $200 \times$  magnification. The cell invasion assay was performed similarly, except that 50  $\mu$ l of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was added to each upper well overnight before the cells ( $1 \times 10^5$ ) were seeded onto the membrane.

### Luciferase assays

The *Ifnb* and NF- $\kappa$ B luciferase reporter plasmids were as described.<sup>46</sup> Luciferase activities were measured with Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The determination of reporter activation was performed as described previously.<sup>46</sup>

### Western blotting

Total cell lysates were prepared by using cell lysis buffer (Cell Signaling Technology) containing phosphatase inhibitor cocktail (Sigma) as described<sup>45-47</sup> and protein concentration determined by the BCA protein assay (Pierce, Rockford, IL). Cell extracts containing equal amounts of proteins were subjected to SDS-PAGE, transferred onto nitrocellulose membrane and blotted per the standard protocol as described. The bands were revealed using Supersignal West Femto Maximum Sensitivity substrate (Pierce), and were imaged and analyzed by using SynGene Bio Imaging Systems (Frederick, MD).

### Quantification of signaling mediators by ELISA

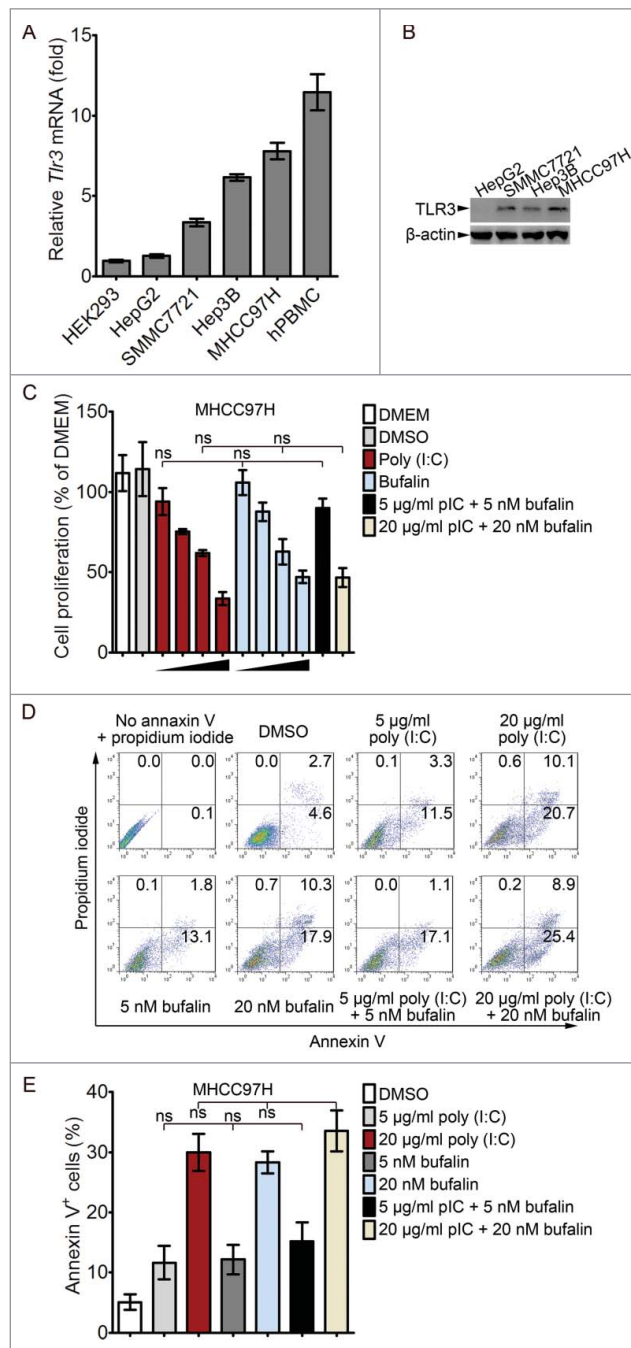
To analyze the active amounts of phospho-ERK1/2 (Thr202/Tyr204), phospho-JNK1/2 (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), and total *I $\kappa$ B $\alpha$*  in cell lysates, we used colorimetric ELISA kits (Upstate of Millipore, Billerica, MA) and performed the assays as instructed by the manufacturer.

### Animal models for metastases and treatments

The HCC cells ( $5 \times 10^6$ ) were subcutaneously inoculated into the right flanks of Balb/c nu/nu mice. After four weeks, the non-necrotic tumor tissue was cut into 1-mm<sup>3</sup> pieces and orthotopically implanted into the liver. The treatment was initiated one week later. The mice received intraperitoneal injections of 0.5, 1 or 2 mg/kg poly (I:C) (once a week) with or without 0.5, 1 or 2 mg/kg bufalin (5 days/week), whereas the control mice were injected with the vehicle alone (DMSO). The treatment was continued for six weeks. The mice were sacrificed, and the livers or lungs were also excised from each mouse for further analysis. For evaluation of metastases, the lungs were examined by H&E staining.

### Immunohistochemistry and TUNEL staining

Formaldehyde-fixed, paraffin-embedded sections of xenograft tumors were subjected to H&E staining and immunohistochemistry by following routine protocols. Immunostained sections were scored as previously described.<sup>45</sup> The degree of staining was



**Figure 1.** Effects of poly (I:C) and bufalin on cell proliferation and apoptosis of HCC cells. (A and B) Determination of TLR3 amounts in HCC cells by q-PCR (A) and Western blotting (B). HEK293 cells and human peripheral mononuclear cells (hPBMC) were used as negative and positive control, respectively. (C to E) MHCC97 H cells were treated with increasing doses of poly (I:C) (5, 10, 20 or 50  $\mu$ g/ml), bufalin (5, 10, 20 or 50 nM) or vehicles as indicated for 48 h. Cell proliferation was determined by BrdU incorporation as evaluated using OD<sub>450 nm</sub> (C), and apoptosis was determined by annexin V/PI staining as evaluated by FACS (D and E). In (A), (C) and (E), data are means  $\pm$  SD of triplicates. In (D), the proportion (%) numbers were shown. Data are representative of three independent experiments. ns, not significant (one-way ANOVA followed by Bonferroni multiple comparisons).



interpreted semiquantitatively by assessing the intensity and extent of staining for each field (400 x) on the entire section. The percent area of positively staining tumor cells was multiplied by their degree of staining (none [0], weakly [1], moderate [2], strong [3] staining cells). A staining score was then calculated (out of a maximum of 300). The repeatability and reproducibility of the staining profile were assessed by three pathologists and three positions were assessed for each section.

Apoptotic cells in xenograft tumors from nude mice were detected in situ by TUNEL method by using a TdT-FragEL DNA Fragmentation Detection Kit (Oncogene, Boston, MA).

### Statistical analyses

All the experiments were independently repeated at least three times. Results are given as mean  $\pm$  SE or mean  $\pm$  SD. Multiple comparisons were done with one-way ANOVA followed by Bonferroni multiple comparisons. Statistical significance was determined as  $P < 0.05$ .

## Results

### Both bufalin and poly (I:C) inhibit proliferation and induce apoptosis of HCC cells

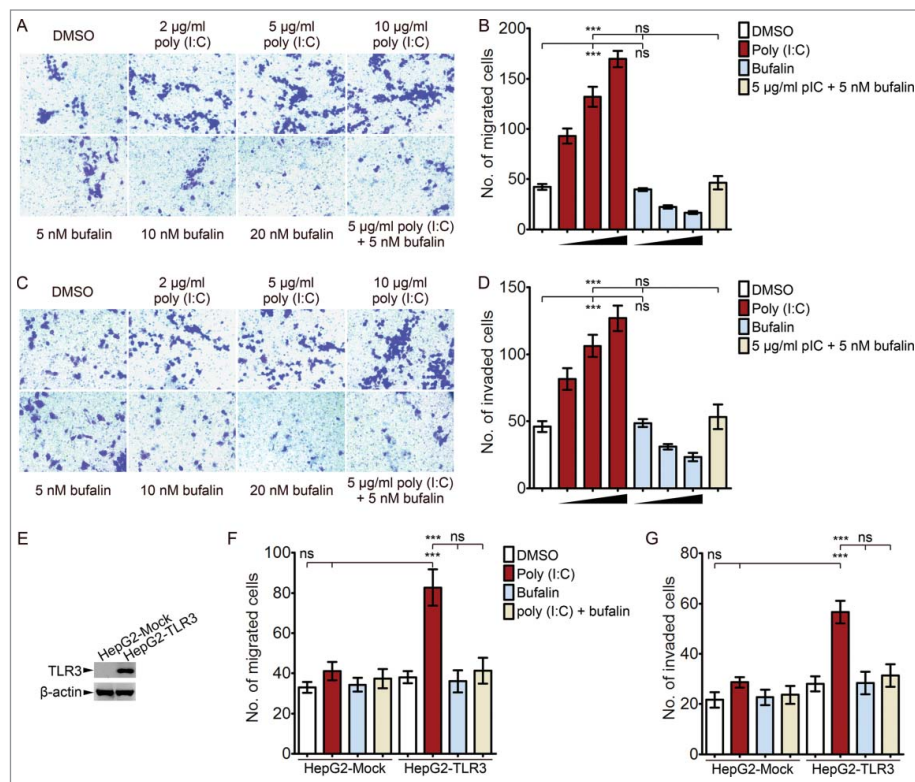
We analyzed the expression of TLR3 in HepG2, SMMC7721, Hep3B and MHCC97 H cells by q-PCR and Western blotting

assays. We found that TLR3 is highly expressed by MHCC97 H cells while HepG2 cells were faintly positive for TLR3 (Fig. 1A and 1B). So we selected MHCC97 H cells and HepG2 cells as the models to evaluate the effects of poly (I:C) on cell proliferation and apoptosis of HCC cells.

We found that poly (I:C) (higher than 10  $\mu\text{g/ml}$ ) was capable of inhibiting proliferation and inducing apoptosis of MHCC97 H cells (Fig. 1C-1E) and to a much lesser extent of HepG2 cells (Supplementary Fig. S1). Bufalin alone (higher than 10 nM) could also inhibit cell proliferation and induce apoptosis of HCC cells to different extents (Fig. 1C-1E, and Supplementary Fig. S1). However, we found that bufalin could not synergistically potentiate the effects of poly (I:C) on HCC cells. Notably, at lower concentrations of bufalin (5 nM) or poly (I:C) (5  $\mu\text{g/ml}$ ) that could not effectively elicit anti-tumor activity alone, the combination of both drugs could not remarkably inhibit growth or induce apoptosis of HCC cells in vitro (Fig. 1C-1E, and Supplementary Fig. S1). These data indicate that poly (I:C) and bufalin may act independently regarding cell proliferation and apoptosis of HCC cells.

### Bufalin suppresses Poly (I:C)-inspired migration and invasion of HCC cells

When examining the effects of poly (I:C) on migration and invasion of HCC cells, we found that poly (I:C) (as low as 2  $\mu\text{g/ml}$ ) could significantly enhanced the migration and



**Figure 2.** Effects of poly (I:C) and bufalin on migration and invasion of HCC cells. (A to D) MHCC97 H cells were treated with increasing doses of poly (I:C) (2, 5 or 10  $\mu\text{g/ml}$ ), bufalin (5, 10 or 20 nM) or vehicles as indicated for 48 h. Migrated cells (A and B) and invaded cells (C and D) were either photographed (A and C, 200 x magnification) or counted (cells of one field out of 5 fields of membrane; B and D, 200 x magnification). (E to G) HepG2 cells stably transfected with TLR3-Myc vector (HepG2-TLR3) or empty vector (HepG2-Mock) were treated with poly (I:C) (5  $\mu\text{g/ml}$ ), bufalin (5 nM) or vehicles as indicated for 48 h, and were examined for migration and invasion as in (A to D). Expression of TLR3 was examined by Western blotting using anti-Myc antibody (E). In (B), (D), (F) and (G), data are means  $\pm$  SD of triplicates. Data are representative of three independent experiments. ns, not significant; \*\*\*,  $P < 0.001$  (one-way ANOVA followed by Bonferroni multiple comparisons).

invasion of MHCC97 H cells but not HepG2 cells (Fig. 2A-2D, and Supplementary Fig. S2). More importantly, bufalin (higher than 10 nM) could reverse the effects of poly (I:C) on migration and invasion of HCC cells, even at lower concentration (as low as 5 nM; Fig. 2A-2D). To determine whether poly (I:C) elicited the effects through TLR3 triggering, we examined the effects of poly (I:C) on HepG2 cells stably overexpressing TLR3 (HepG2-TLR3; Fig. 2E). We found that the migration and invasion of HepG2-TLR3 cells were significantly increased while bufalin could reverse the effects (Fig. 2F and 2G). These data indicate that poly (I:C) triggering of TLR3 promotes migration and invasion of HCC cells while bufalin may be involved in the negative regulation of TLR3 signaling.

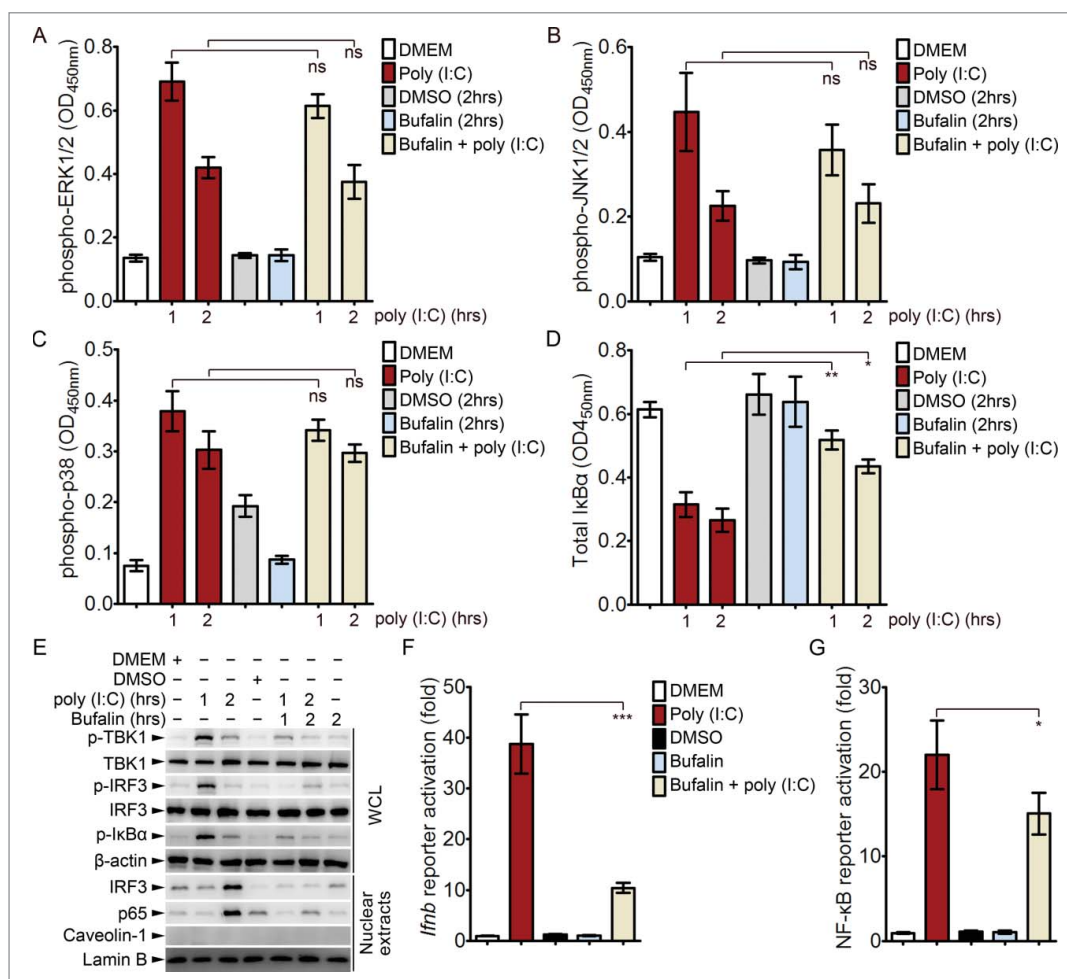
### Bufalin inhibits poly (I:C)-triggered activation of TBK1-IRF3 pathway in HCC cells

The above data of migration and invasion may suggest that bufalin negatively regulates TLR3 signaling, so we examined the activation of mitogen-activated protein kinases (phosphorylated

ERK1/2, JNK1/2 and p38 as indicators) and NF- $\kappa$ B (total I $\kappa$ B $\alpha$  as indicator) pathways by ELISA assays. We found that bufalin could not significantly affect the MAPK pathway activated by poly (I:C) but inhibited the degradation of I $\kappa$ B $\alpha$  (Fig. 3A-3D). More importantly, we found that bufalin significantly inhibited the phosphorylation of TBK1 and IRF3 triggered by poly (I:C) (Fig. 3E). As further evidence, we found that the nuclear levels of p65 and IRF3 were decreased by bufalin (Fig. 3E). In reporter assays, we found that bufalin significantly inhibited the activation of *Irfb* reporters and to a lesser extent NF- $\kappa$ B reporters by poly (I:C) (Fig. 3F and 3G). These data convincingly demonstrate that bufalin inhibits the activation of NF- $\kappa$ B and TBK1-IRF3 by TLR3 engagement.

### TBK1 is required for Poly (I:C)-inspired migration and invasion of HCC cells

To test the possibility that bufalin may inhibit migration and invasion of HCC cells via TBK1, we first examined the effects of NF- $\kappa$ B (TPCA-1) and TBK1 (BX-795) inhibitors on migration and invasion of HCC cells after TLR3 triggering. We found that



**Figure 3.** Bufalin inhibits poly (I:C)-triggered activation of TBK1-IRF3 pathway in HCC cells. (A to E) MHCC97 H cells were treated with poly (I:C) (5  $\mu$ g/ml), bufalin (5 nM) or vehicles (DMSO, 2hrs) as indicated. The amounts of indicated proteins contained in 100  $\mu$ g whole cell extracts (WCL) were determined by ELISA assays. In (E), equal amounts of WCL or nuclear extracts were examined for indicated proteins by using Western blotting. (F and G) MHCC97 H cells were transiently transfected with *Irfb* (F) or NF- $\kappa$ B (G) reporters for 24 H, and treated with poly (I:C) (5  $\mu$ g/ml), bufalin (5 nM) or vehicles for 6 H. Reporter activation was measured by dual-luciferase activity assays. In (A to D), (F) and (G), data are means  $\pm$  SD of triplicates. Data are representative of three independent experiments. ns, not significant; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (one-way ANOVA followed by Bonferroni multiple comparisons).

TBK1 inhibitor, but not NF- $\kappa$ B inhibitor, could block poly (I:C)-inspired migration and invasion of MHCC97 H cells (Fig. 4A and 4B). In HepG2-TLR3 cells in which poly (I:C) could promote the migration and invasion (Fig. 2F and 2G), TBK1 inhibitor could also impair the migration and invasion after poly (I:C) treatments (Fig. 4C and 4D). In *Tbk1*<sup>-/-</sup> MHCC97 H cells (Fig. 4E), poly (I:C)-inspired migration and invasion were impaired, and bufalin failed to further inhibit the migration and invasion after poly (I:C) treatments (Fig. 4F and 4G). In *Tbk1*<sup>-/-</sup> MHCC97 H cells rescued by TBK1 overexpression (Fig. 4H), bufalin could recover the ability to inhibit migration and invasion inspired by poly (I:C) (Fig. 4I and 4J), indicating that TBK1 signaling pathway may be the target for bufalin-elicited inhibition of migration and invasion. These data suggest that bufalin may inhibit migration and invasion of HCC cells by impairing the activation of TBK1 by TLR3 signaling.

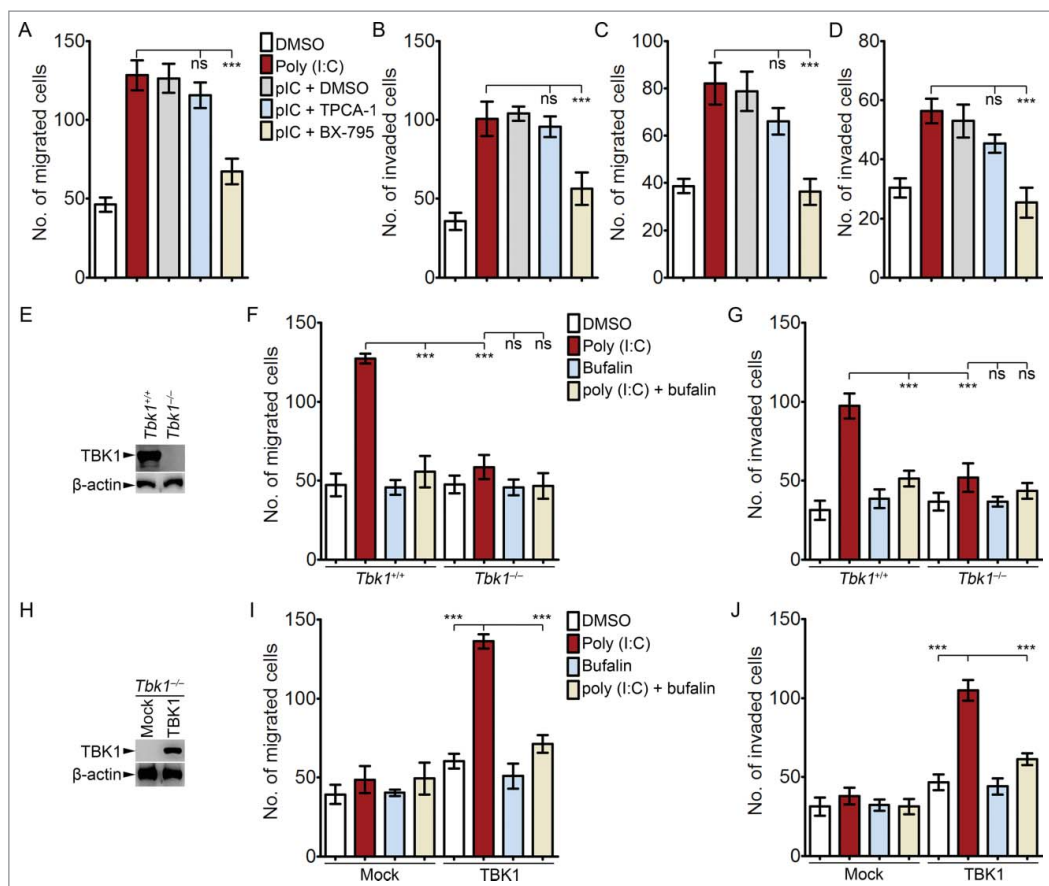
#### Bufalin inhibits the activation of TBK1 and suppresses migration and invasion of HCC cells triggered by poly (I:C) transfection

We used another model, in which poly (I:C) may trigger the activation of TBK1 pathway via cytosolic dsRNA sensors in

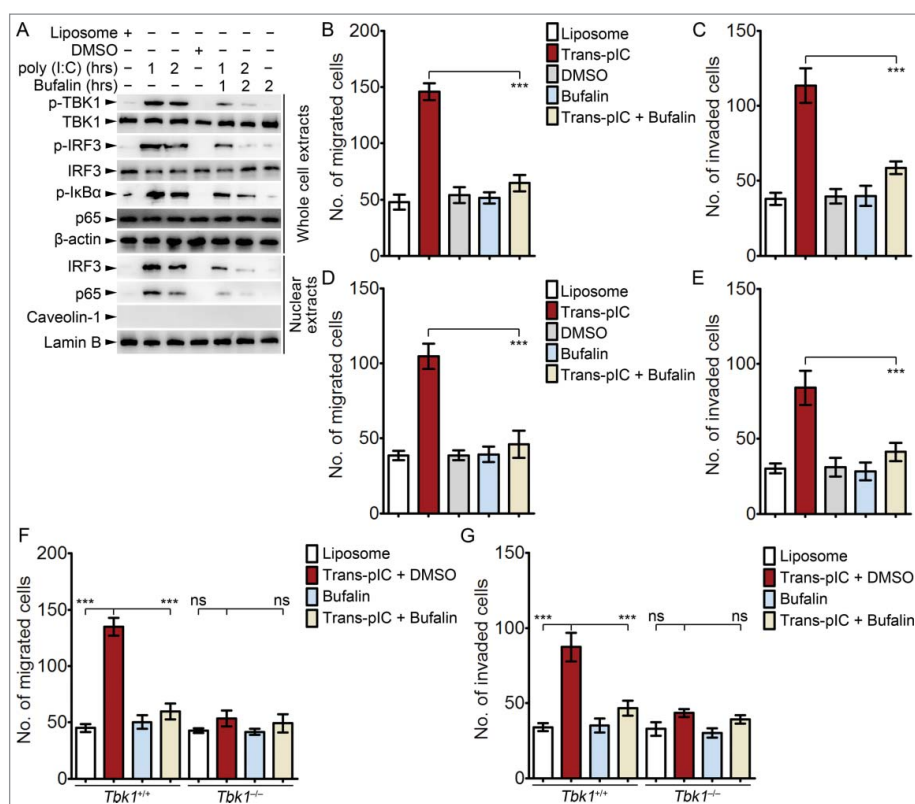
addition to TLR3<sup>3,48</sup>, to further test this hypothesis. As expected, we found that bufalin could still inhibit TBK1-IRF3 activation elicited by poly (I:C) transfection (Fig. 5A). Meanwhile, bufalin inhibited the migration and invasion of poly (I:C)-transfected MHCC97 H cells (Fig. 5B and 5C). Similar effects were observed in HepG2 cells for poly (I:C) transfection-inspired migration and invasion (Fig. 5D and 5E). Transfection of poly (I:C) in TBK1 deficient MHCC97 H cells could not significantly affect the migration and invasion, and bufalin failed to elicit inhibitory effects on migration and invasion (Fig. 5F and 5G). Therefore TBK1 activation may be the step by which bufalin inhibits migration and invasion of HCC cells triggered not only by TLR3 but also by cytosolic dsRNA sensors.

#### Bufalin inhibits poly (I:C)-inspired metastasis of HCC cells in vivo

To further examined the roles of poly (I:C) and bufalin in metastasis of HCC cells, we established orthotopical xenografts models in liver using MHCC97 H cells and HepG2-TLR3 cells. Since side effects, such as arthralgia, fever, erythema and sometimes endotoxin-like shock, have been



**Figure 4.** Roles of TBK1 in poly (I:C)- and bufalin-regulated migration and invasion of HCC cells. (A to D) MHCC97 H (A and B) or HepG2-TLR3 (C and D) cells were pre-treated with or without TPCA-1 (100 nM) or BX-795 (100 nM) for 30 min, and then treated with poly (I:C) (5  $\mu$ g/ml), bufalin (5 nM) or vehicles as indicated for 48 H. Migrated cells (A and C) and invaded cells (B and D) were counted (cells of one field out of 5 fields of membrane under 200 x magnification). (E to J) *Tbk1*<sup>+/+</sup> or *Tbk1*<sup>-/-</sup> MHCC97 H cells (E to G) or *Tbk1*<sup>-/-</sup> MHCC97 H cells rescued by transfection with empty (Mock) or TBK1 expression vectors for 24 H (H to J) were treated with poly (I:C) (5  $\mu$ g/ml), bufalin (5 nM) or vehicles as indicated for 48 H, and were examined for migration and invasion as in (A to D). Expression of TBK1 was examined by Western blotting using anti-TBK1 antibody (E and H). In (A to D), (F), (G), (I) and (J), data are means  $\pm$  SD of triplicates. Data are representative of three independent experiments. ns, not significant; \*\*\*,  $P < 0.001$  (one-way ANOVA followed by Bonferroni multiple comparisons).



**Figure 5.** Bufalin inhibits the activation of TBK1 and suppresses migration and invasion of HCC cells triggered by poly (I:C) transfection. (A to E) MHCC97 H (A to C) or HepG2 (D and E) cells were transfected with poly (I:C) (5  $\mu$ g/ml), or treated with bufalin (5 nM) or vehicles as indicated for 1–2 H (A) or 48 H (B to E). The amounts of indicated proteins in equal amounts of whole cell extracts or nuclear extracts were examined by Western blotting (A). Migrated cells (B and D) and invaded cells (C and E) were counted (cells of one field out of 5 fields of membrane under 200  $\times$  magnification). (F and G) *Tbk1*<sup>+/+</sup> or *Tbk1*<sup>-/-</sup> MHCC97 H cells were transfected with poly (I:C) (5  $\mu$ g/ml), or treated with bufalin (5 nM) or vehicles as indicated for 48 H. Migration and invasion were determined as in (B to E). In (B to G), data are means  $\pm$  SD of triplicates. Data are representative of three independent experiments. ns, not significant; \*\*\*,  $P < 0.001$  (one-way ANOVA followed by Bonferroni multiple comparisons).

reported for poly (I:C) therapy, we tested the efficiency of lower doses of poly (I:C) (0.5 mg/kg, half of usual doses) in combination with bufalin (0.5 mg/kg, half of usual doses) on apoptosis. We found that intraperitoneal administration of poly (I:C) at 0.5 mg/kg could not significantly induce the appearance of apoptotic cells in the liver orthotopical xenografts; and bufalin alone (0.5 mg/kg) or in combination with poly (I:C) was also ineffective in inducing apoptosis (Fig. 6A and 6B). So we used 0.5 mg/kg poly (I:C) or 0.5 mg/kg bufalin as the non-apoptotic dose to examine the effects of poly (I:C) and/or bufalin on lung metastasis of liver orthotopical xenografts.

After the complete of treatments (six weeks), we examined lung metastases by H&E staining. We found that poly (I:C) at 0.5 mg/kg could enhance the metastases of MHCC97 H cells in the lung while bufalin inhibited the appearance of metastases (Fig. 6C and 6D). In HepG2-TLR3 model, bufalin could also inhibit poly (I:C)-enhanced metastasis (Fig. 6E). In MHCC97 H model deficient in TBK1, poly (I:C) failed to induce metastasis formation, and bufalin failed to elicit the inhibitory effects on metastasis (Fig. 6F), indicating that TBK1 plays pivotal roles in promoting HCC metastases enhanced by poly (I:C) treatments. These data convincingly demonstrated that bufalin suppresses the metastasis of HCC cells by poly (I:C) in vivo.

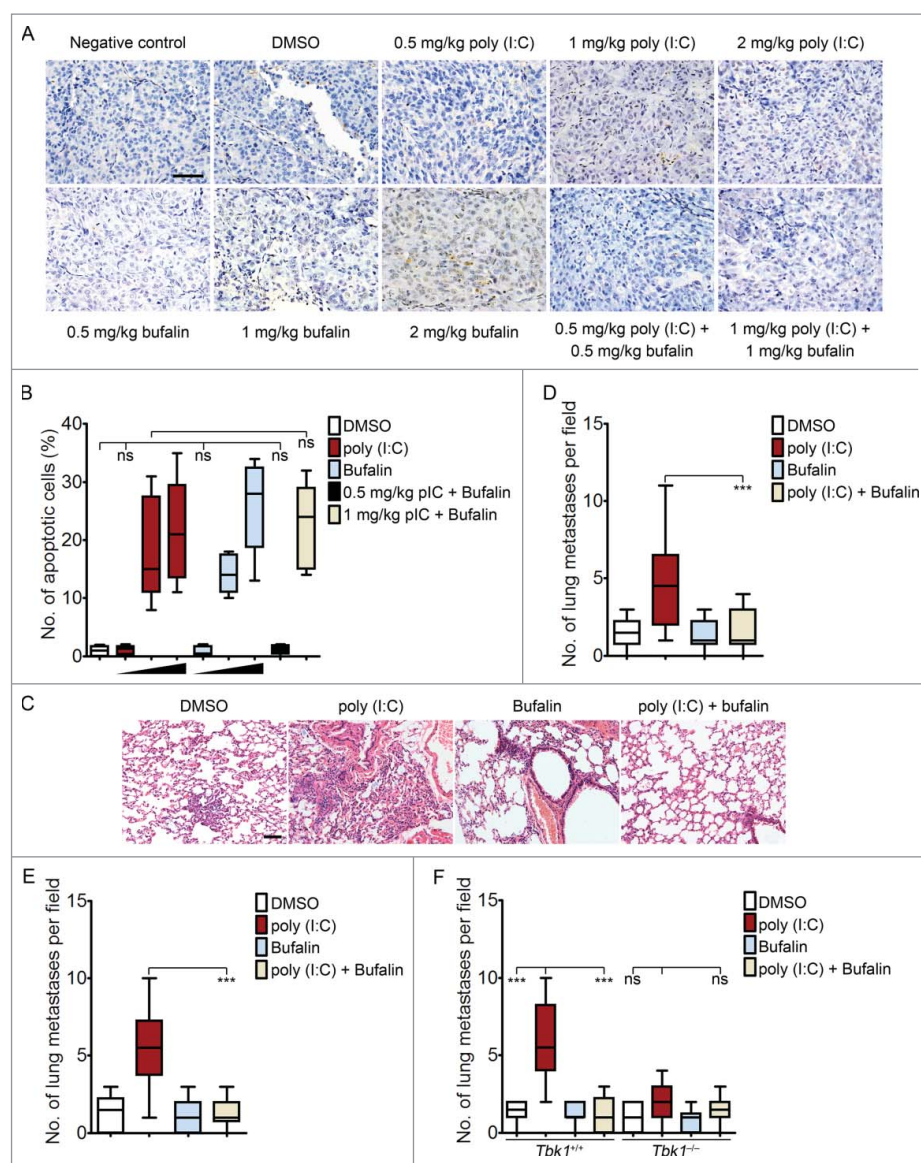
To test the possibility that TBK1 may also be affected by poly (I:C) plus bufalin treatments in vivo, we examined the phosphorylation of TBK1 in liver orthotopical xenografts by

immunohistochemistry. We found that in vivo administration of poly (I:C) could promote the phosphorylation of TBK1 while bufalin inhibits the phosphorylation of TBK1 induced by poly (I:C) both in MHCC97 H model (Fig. 7A and 7B) and in HepG2-TLR3 model (Fig. 7C and 7D). Therefore, it may be inferred that bufalin could inhibit the migration and invasion of HCC cells in vitro and the metastases of HCC in vivo by impairing poly (I:C)-triggered TBK1 phosphorylation.

## Discussion

TLRs play important roles in both innate and adaptive immunity.<sup>3</sup> TLR agonists have been recognized as potent immunostimulatory reagents by triggering signaling pathways in cells positive for TLRs, particularly immune cells.<sup>4</sup> Based on this immunomodulatory property of TLR agonists, they have been regarded as adjuvants for immunotherapy and been tested in clinical trials.<sup>4</sup> However, application of TLR agonists has encountered limitations in human settings, principally due to intolerable side-effects.<sup>4</sup> At present, the strategy to control the side-effects may rely on reduced doses, development of new TLR agonists, local administration, or management of the side-effects. In our study, we have unexpectedly found that low concentration of TLR3 agonist poly (I:C) enhanced the metastatic capacity of TLR3<sup>+</sup> HCC while bufalin can reverse this phenomenon, indicating that the risk of metastasis exists for poly (I:C)





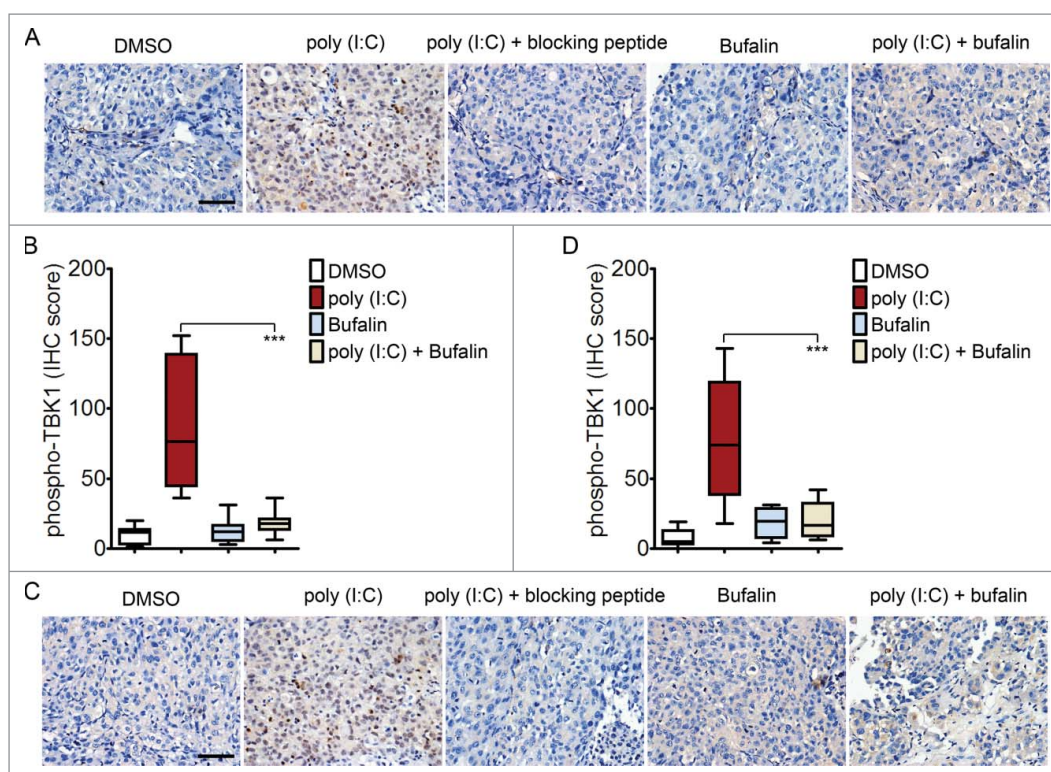
**Figure 6.** Bufalin inhibits poly (I:C)-inspired metastasis of HCC cells in vivo. (A and B) Balb/c nu/nu mice with MHCC97 H orthotopic xenografts were treated by intraperitoneal injection of indicated amounts of poly (I:C) (0.5, 1 or 2 mg/kg), bufalin (0.5, 1 or 2 mg/kg) or vehicles as indicated. For the combination treatments, bufalin was administrated at 0.5 mg/kg. On the third day after the second poly (I:C) treatment, liver were examined for apoptosis by TUNEL assays. The section stained with only the HRP-conjugated secondary antibody was used as negative control. The sections were photographed under 400 x magnification, and TUNEL positive cells under each field of 5 fields were counted. (C to F) After six weeks of treatment (0.5 mg/kg poly (I:C) or 0.5 mg/kg bufalin), the lungs were excised and examined for metastases by H&E staining. Typical metastases were shown in (C; 200 x magnification), and the metastases under each field of 5 fields were counted. The orthotopic xenografts examined were: MHCC97 H (C and D), HepG2-TLR3 (E) and *Tbk1<sup>+/+</sup>* or *Tbk1<sup>-/-</sup>* MHCC97 H (F). In (B), (D), (E) and (F), data are means  $\pm$  SD (n = 10 per group). In (A) and (C), bars indicate for 50  $\mu$ m. Data are representative of three independent experiments. ns, not significant; \*\*\*,  $P < 0.001$  (one-way ANOVA followed by Bonferroni multiple comparisons).

treatment of cancer even after reduction of poly (I:C) doses while bufalin may antagonize this unfavorable risk of poly (I:C). Therefore bufalin can be used in combination with TLR3 agonist as immunotherapy of cancer for the sake of decreased risk of metastasis but unaffected anti-tumor activity elicited by both bufalin and poly (I:C).

Expression of TLR3 has been detected in various cancer types.<sup>5-33</sup> Engagement of TLR3 usually elicits anti-proliferative and pro-apoptotic effects on cancer cells.<sup>6,8,10-12,14-16,22,28,30</sup> However, TLR3 has also been implicated in promotion of cancer survival and progression.<sup>9,18,19,21,27,29</sup> In clinical samples, it has also been reported that high TLR3 expression is a prediction marker for biochemical recurrence in prostate cancer, higher probability of metastasis in breast cancer patients, and

poorer overall survival and worse prognosis in patients with gastric cancer.<sup>19,27,29</sup> For human HCC, poly (I:C), either extracellularly or after transfection, has demonstrated anti-proliferative and pro-apoptotic activities both in vitro and in vivo.<sup>11,17,22,24,25,28,32</sup> TLR3 expression in HCC has been positively correlated with a longer survival for HCC patients, and the activation of TLR3 by poly (I:C) can cause apoptosis of the TLR3-positive HCC cell lines, promote NK cell proliferation and enhance NK cell antitumor activity.<sup>24,27,31</sup> However, TLR3 expression has also been correlated to poor survival in HCC patients.<sup>18,19,21,27,29</sup> Therefore TLR3 agonist may also act as double-edged sword in HCC therapy. In consistent with previous reports, we also found that poly (I:C) could inhibit proliferation and induce apoptosis of HCC cells. However, on the





**Figure 7.** Bufalin inhibits poly (I:C)-induced phosphorylation of TBK1 in vivo. Balb/c nu/nu mice with MHCC97 H (A and B) or HepG2-TLR3 (C and D) orthotopic xenografts were treated by intraperitoneal injection of poly (I:C) (0.5 mg/kg), bufalin (0.5 mg/kg) or vehicles as indicated. On the third day after the second poly (I:C) treatment, liver were examined for phospho-TBK1 expression by immunohistochemistry (IHC) assays. The sections were photographed under 400 x magnification (A and C), and the staining was semiquantitatively scored. The section derived from poly (I:C) group and stained in the presence of blocking peptide was used as negative control. In (B) and (D), data are means  $\pm$  SD ( $n = 10$  per group). In (A) and (C), bars indicate for 50  $\mu$ m. Data are representative of three independent experiments. \*\*\*,  $P < 0.001$  (one-way ANOVA followed by Bonferroni multiple comparisons).

other hand, it should be avoided that poly (I:C) may promote migration and invasion of HCC cells in vitro and enhance metastasis of HCC in vivo. In our study, we found that bufalin could suppress poly (I:C)-inspired metastatic behavior of TLR3-positive HCC cells even when poly (I:C) is used at usual dose or at higher dose that can elicit anti-proliferative and proapoptotic effects. Therefore, poly (I:C) may be cautiously used in the treatment of TLR3-positive HCC patients if bufalin is used in combination to retain the anti-tumor activity of poly (I:C) and avoid the pro-metastatic effects of poly (I:C).

TLR3 signals via TRIF-dependent pathway, leading to the activation of MAPK, NF- $\kappa$ B and especially TBK1-mediated IRF3 activation.<sup>3</sup> TRIF has been demonstrated to inspire a proapoptotic effects, possibly via receptor-interacting protein 1 (RIP1)-associated cell death pathway.<sup>3,14</sup> Activation of TLR3 has been implicated in the control of immune cell migration whereas the effects of TLR3 activation in cancer cell migration have not been clearly understood.<sup>3</sup> Our study indicates that TLR3 activation can also promote migration and invasion of TLR3-positive cells, evidenced by the data showing that poly (I:C) didn't enhance the migration of TLR3<sup>low/-</sup> HepG2 cells while potentiate the migratory capacity of HepG2 with TLR3 overexpression. The mechanisms by which TLR3 engagement enhances cell migration are not clear. In our study, we found that TBK1 pathway may be the key machinery to mediate cell migration. Although both MAPK and NF- $\kappa$ B pathways are activated by poly (I:C) in HCC cells, NF- $\kappa$ B inhibitor didn't affect migratory capacity of HCC cells, and MAPK activation

was not affected by bufalin, which may indicate that TBK1 pathway may be the key to control HCC cell migration at least in the context of bufalin-mediated suppression. More convincing evidence come from the experiments using TBK1 inhibitor and TBK1-deficient MHCC97 H cells. Our study using TBK1-deficient MHCC97 H cells after poly (I:C) transfection further implies that TBK1 plays a pivotal role in control of HCC cell migration, although we cannot clearly distinguish whether poly (I:C) transfection activates TLR3 pathway or cytosolic dsRNA sensor pathway at present.<sup>3,48</sup> Previous studies have indicated that TBK1 can promote the migration of melanoma cells and the migration of T cells.<sup>49,50</sup> Therefore, targeting TBK1 may eliminate the pro-metastatic effects of TLR3 agonists in cancer immunotherapy, and bufalin (or specifically other TBK1 inhibitors) may be used in combination with TLR3 agonists.

Bufalin, as a major component of the Chinese medicine Chansu, has long been clinically used in the treatment of HCC.<sup>34-37</sup> Previous studies have shown that bufalin can induce cell cycle arrest and apoptosis in many cancer types and inhibit angiogenesis.<sup>34,39-44</sup> Most recently, bufalin has been shown to inhibit the migration of HCC cells.<sup>42</sup> However, the molecular mechanisms for the anti-tumor activity of bufalin have not been clearly elucidated. Bufalin can induce apoptosis via Fas-, mitochondria- or autophagy-dependent pathway; and bufalin has been demonstrated to suppress the expression of cell cycle-regulating molecules, the activation of c-Myc and NF- $\kappa$ B, the activation of PI3 K-Akt pathway, and the secretion of VEGF.<sup>34,42,51,52</sup> All of these effects may contribute to the anti-tumor activity of

bufalin. What are the exact targets of bufalin in cancer cells has not come to a decision. A popular point is that bufalin is an inhibitor of sodium-potassium adenosine triphosphatases (ATPase), which may be due to its structure concordance to digoxin.<sup>34,38</sup> One of studies shows that bufalin can inhibit the ATPase activity of the dsRNA sensor RIG-I and thus decrease the virus-induced production of IFN- $\beta$  and expression of IFN-stimulated genes.<sup>53</sup> It may be possible that bufalin inhibits the ATPase activity of RIG-I and RIG-I-like MDA-5, thus leading to impaired TBK1 activation and decreased migration. But it is still not clear how bufalin impairs TLR3-triggered TBK1 activation and poly (I:C)-inspired migration in HCC cells. Unknown ATPase activity-possessing molecules that can regulate TLR3-triggered TBK1 activation may exist. Otherwise, bufalin may directly bind to TBK1 and inhibit TBK1 kinase activity, which may need further biochemical investigations.

In sum, our study has provided evidence for the risk of TLR3 agonists in HCC immunotherapy in that TLR3 activation may enhance the metastatic capacity of HCC cells. More importantly, our study indicates that bufalin may be a potential choice to avoid this side-effect of poly (I:C) treatments in HCC, given that bufalin does not affect the anti-proliferative and pro-apoptotic effects of poly (I:C).

## Authors' contributions

**Conception and design:** Chen Wang and Changquan Ling

**Provision of study materials:** Yinglu Feng, Qingxin Cao and Qun Liu

**Collection and assembly of data:** Yinglu Feng, Yongan Chen and Yongbin Meng

**Data analysis and interpretation:** Chen Wang and Changquan Ling

**Manuscript writing:** Chen Wang and Yinglu Feng

**Final approval of manuscript:** All authors.

## Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest.

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