Punicalagin Suppresses LPS-induced Inflammatory Responses in Murine Macrophages via JAK/STAT Signaling Pathway and Zymosan-induced Mice Paw Edema

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ABSTRACT

Aim: To investigate the anti-inflammatory activity of punicalagin (PUN) on mouse macrophages and the underlying mechanisms. **Materials and Methods:** Mouse primary peritoneal macrophages (PMs) were treated with lipopolysaccharide (LPS; 10μg/ml) and/or PUN (50μM) for 4, 8 and 12 hr. The expression levels of inducible nitric oxide synthase (iNOS), interleukin (IL)-1β, IL-6, IL-12P40 and CCL-2 mRNAs were analyzed by qRT-PCR. Total iNOS, MAPK and JAK/STAT protein levels were measured by Western blotting and the secreted levels of IL-1β, IL-6, IL-12P40 and IL-1270 by ELISA. Acute paw edema was induced in mice with zymosan and the animals were treated with high (50 mg/kg), medium (25 mg/kg) and low (12.5 mg/kg) doses of PUN. The levels of IL-1β, IL-6, CCL-2 and other related inflammatory factors and chemokines in the mouse paw tissue homogenates were detected by ELISA. **Results:** Compared to the LPS-treated cells, PUN significantly decreased the levels of inflammatory cytokines and other factors ($P<0.05$). In addition, high dose of PUN down regulated p-ERK, p-JAK1, p-STAT1 and p-STAT3, without significantly affecting p-p38 and p-JNK. Finally, acute paw edema in the mouse model was alleviated within 12h of high-dose PUN administration, which also inhibited inflammatory cytokines and chemokines. **Conclusion:** PUN inhibits macrophage-mediated inflammation and the secretion of inflammatory cytokines/chemokines by targeting the ERK/MAPK and JAK/STAT signaling pathways.

Key words: Punicalagin, LPS-induced Inflammatory Responses, Macrophages, JAK/STAT, Paw edema.

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INTRODUCTION

Punicalagin (PUN) is the main active ingredient in pomegranate peel extract and has anti-inflammatory, antioxidant, anti-viral, anti-tumor, antibacterial and other pharmacological effects.[1-3] The mechanistic basis of its anti-inflammatory action is the inhibition of the NF-*κ*B signaling pathway via blocking phosphorylation of mitogen-activated protein kinase (MAPK), p38, c-Jun amino terminal kinase (JNK) and extracellular protein kinase (ERK).[4] Macrophages are the primary immune cells involved in triggering inflammation[5,6] and are regulated by multiple signaling pathways,[7,8] including the JAK/STAT signaling pathway that is activated in response to various external stimuli.[9,10] The inflammatory response is driven by several cytokines that are secreted from immune cells and act on the effect or cells through intracellular pathways, thereby affecting cellular activation, proliferation and survival. For instance, macrophages secrete various inflammatory factors such as IL-1β, IL-6, iNOS, IL-12P40, IL-12P70 and CCL-2

during acute inflammation.[11,12] The aim of our study was to assess the anti-inflammatory effects of PUN on lipopolysaccharide (LPS)-stimulated primary murine peritoneal macrophages (PMs) and in a zymosan-induced model of paw edema and acute inflammation.[13-15]

MATERIALS AND METHODS

Reagents

Punicalagin (>98 %) and LPS were purchased from Sigma (St.Louis, MO, USA). RPMI 1640 was obtained from GibcoBRL (Gaithersburg, MD, USA). ELISA kits for IL-1β, IL-6, CCL-2 and CCL-4 were purchased from R and D Systems (Minneapolis, MN, USA) and that for IL-12p40 and IL-12p70 from DaKeWe (ShenZhen, China). Antibodies targeting iNOS, p38, p-p38, ERK, p-ERK, JNK, p-JNK, JAK1, p-JAK1, STAT1, STAT3, SHP2, p-SHP and SOCS3 were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

Primary PMs were extracted from female C57BL/6 mice aged 8 weeks. The cells were cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) at 37°C in a humidified incubator under 5% CO_2. For LPS/PUN treatment, the PMs were seeded in 6-well plates at the density of 2×10^6 cells/well and treated with 10μg/ml LPS and/or 50μM PUN ($n = 3$ each) for 4, 8, 12 and 24 hr as appropriate.

Quantitative RT-PCR Analysis

Total RNA was extracted from the suitably treated cells, reverse transcribed and amplified using SYBR Premix (TaKaRa) on a BioRad CFX96 cycler. Primer sequences are Table 1.

Enzyme-Linked Immunosorbent Assay (ELISA)

Supernatants were collected from the suitably treated cells after 12h and 24h of culture and the levels of IL-1β, IL-6, IL-12p40, IL-12p70, CCL-2 and CCL-4 were measured using ELISA kits according to the manufacturer's instructions.

Western Blotting

The suitably treated PMs were washed twice with ice-cold PBS and lyzed in 200μl RIPA lysis buffer supplemented with protease inhibitors on ice for 10 min. The homogenates were gently scraped from the plate, transferred to 1.5 mL pre-cooled EP tubes and placed on ice for 15 min while overtaxing every 5 min. The cell extracts were centrifuged at 13,000×g at 4°C for 10 min and the supernatants were collected. Following quantification with the BCA kit, equal amounts of protein per sample were separated on 10% Tris-Glycine extended polyacrylamide gels. Densitometric analysis of the bands was performed using Image J.

Establishment of zymosan-induced mouse paw edema model

Acute inflammation was modeled *in vivo* by inducing edema in the left hind paws mice through a single injection of 50μl zymosan (1%). Four hours later, the animals were injected intraperitoneally with 25, 50 and 100 mg/kg of PUN or 5mM dexamethasone (positive control). Paw edema was monitored over a period of 24h and the animals were euthanized. The paw tissues were harvested and then stored at −80°C. IL-1b, IL-6, CCL-2 and CCL-4 content in the paw tissue homogenates were analyzed by ELISAkits.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7.0 software. Data was expressed as mean ± SEM from three experiments and compared using unpaired t-test. $P<0.05$ was considered statistically significant.

RESULTS

PUN inhibits the inflammatory response in LPS-stimulated macrophages

LPS challenge significantly increased the production of iNOS mRNA and protein in PMs compared to that in the untreated controls, which was inhibited by PUN in a time-dependent manner (Figure 1A, B). Consistent with this, LPS also markedly increased the mRNA levels of

Table 1: Primer sequences.		
Gene	**Reverse primer (5′ →→′)**	**Forward primer (5′ →→′)**
iNOS	GTGGACGGGTCGATGTCAC	GTTCTCAGCCCAACAATACAAGA
IL-1β	ATCTTTTGGGGTCCGTCAACT	GCAACTGTTCCTGAACTCAACT
IL-6	CTCATTTCCACGATTTCCCAG	ACCACGGCCTTCCCTACTTC
CCL-2	GCATTAGCTTCAGATTTACGGGT	TTAAAAACCTGGATCGGAACCAA
IL-12b	GACTGTAGTAGGTAGGGTGAGG	CATACCGTTGGGACAGACCG
β-actin	CTCTCAGCTGTGGTGGTGAA	AGCCATGTACGTAGCCATCC

several inflammatory cytokines. PUN intervention rapidly decreased the levels of CCL-2 and IL-12P40 (Figure 2C, D) and that of IL-1β and IL-6 after 8h and 12h (Figure 2A, B). Furthermore, LPS stimulation increased secretion of the above inflammatory cytokines was inhibited by PUN with the exception of IL-6 (Figure 3).

PUN inhibits ERK/MAPK and JAK/SAT pathways in LPS-stimulated PMs

To further elucidate the mechanism underlying the anti-inflammatory effect of PUN, we analyzed the levels of

ERK/MAPK pathway mediators in the LPS-stimulated cells. LPS induced phosphorylation of ERK, p38 and JNK in the murine PMs (Figure 4A): While PUN significantly inhibited p-ERK expression (Figure 4B), it had no effect on the total and phosphorylated JNK and p38 levels (Figure 4C, D). Since the JAK/STAT pathway is also involved in the process of inflammation, we analyzed the levels of JAK1, STAT1/3 and the negative regulatory
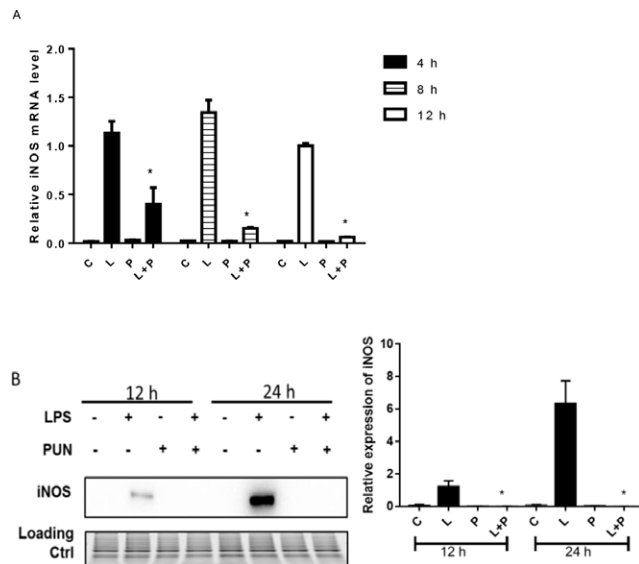

Figure 1: Effect of PUN on iNOS mRNA and protein in PMs. A. RT-PCR assay showing iNOS mRNA levels in PMs treated with 10µg/ml LPS and 50µM PUN for 4, 8 and 12h. **B.** Immunoblot and bar graph showing iNOS protein levels in the above treated cells. *p*<0.05 compared with the LPS-treated group.
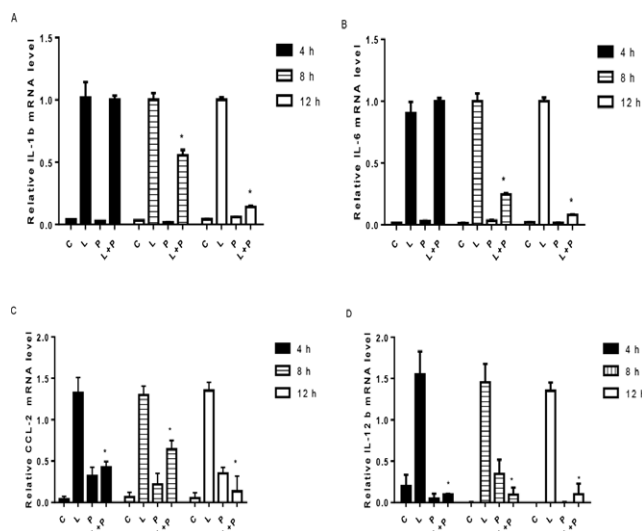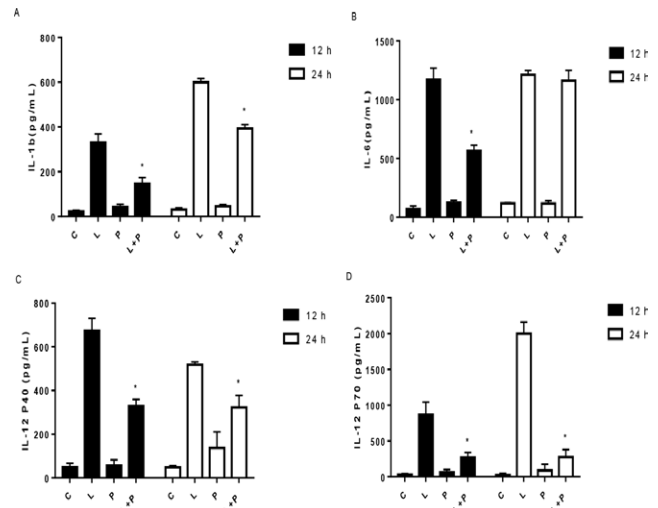
Figure 3: Effect of PUN on LPS-induced secretion of pro-inflammatory cytokines. IL-1β (A), IL-6 (B), IL-12P40 (C) and IL-12P70 (D) levels in the supernatants of PMs treated with 10µg/ml LPS and 50µM PUN for 12 and 24 h. *p*<0.05 compared with the LPS-treated group.

Figure 2: Effect of PUN on LPS-induced proinflammatory cytokine mRNAs. The levels of IL-1b (A), IL-6 (B). CCL-2 (C) and IL-12b(D) mRNAs in PMs treated with 10µg/ml LPS and 50µM PUN for 4, 8 and 12h. * *p*<0.05 compared with the LPS-treated group.
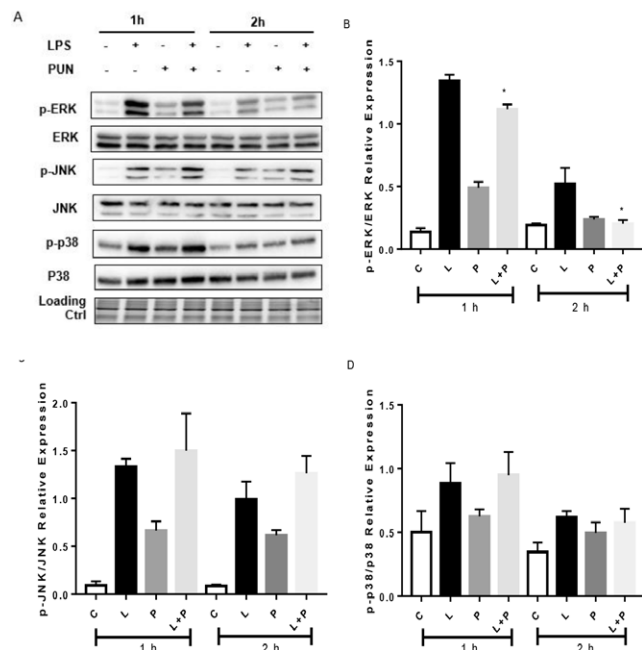
Figure 4: The effect of PUN on LPS-induced phosphorylation of MAPKs in PMs cells. Phosphorylation levels of p38, JNK and ERK in PMs treated with 10µg/ml LPS and 50µM PUN for the indicated time points. *p*<0.05 compared with the LPS-treated group.

proteins SOCS$_3$ and SHP. As shown in Figure 5A, LPS significantly increased JAK1, STAT1 and STAT3 phosphorylation, which was prevented by PUN treatment (Figure 5B-D). In addition, PUN slightly increased the levels of SOCS3 (Figure 5E) but had no effect on SHP2 (Figure 5E, F). Taken together, PUN inhibits the inflammatory response in PMs by blocking both the ERK/MAPK and JAK/STAT pathways.

Effect of PUN on Zymosan-induced mouse paw edema

The *in vivo* effects of PUN were assessed in a murine zymosan-induced paw edema model. An acute inflammatory reaction was triggered in the left hind paw within 1h of zymosan injection, characterized by redness and edema. As shown in Figure 6A, high-dose PUN alleviated swelling within 12h compared to that in the untreated model group, whereas the low and medium doses of PUN had minimal effect even after 24h. Consistent with this, the paw homogenates of the untreated model showed high levels of IL-6, CCL-2 and CCL-4 and all but IL-6 were reduced significantly after high-dose PUN intervention (Figure 6B-E). Thus, PUN can relieve symptoms of acute localized inflammation *in vivo*.

DISCUSSION

PUN accounts for about 10% of the tannin content of pomegranates[16] and has established anti-inflammatory effects. It inhibits the inhalable particulate matter (PM10)-induced adhesion of monocytes to endothelial cells *in vitro*. In addition, both pomegranate peel extract and PUN reduce the inflammatory response caused by particulate matter.[17] Studies[18,19] show that PUN also significantly mitigates LPS-induced neuroinflammation, oxidative stress and memory impairment by inhibiting nuclear factor-kappa (NFᴋ)-B.

Macrophages are the primary effectors of inflammation as well as tissue regeneration post bacterial infection through the production of pro- and anti-inflammatory cytokines respectively.[20] A previous study has shown the anti-inflammatory action of PUN on the RAW 264.7 macrophage cell line.[21] Consistent with this, we detected a significant decrease in the expression levels of iNOS, IL-1b, IL-6, IL-12p40, IL-12p70 and CCL-2, which are the hallmarks of activated macrophages,[22,23] in the LPS-stimulated primary PMs.

The MAPK pathway aggravates the inflammatory response by inducing production of effect or cytokines. MAPKs are serine/threonine protein kinases that are involved in
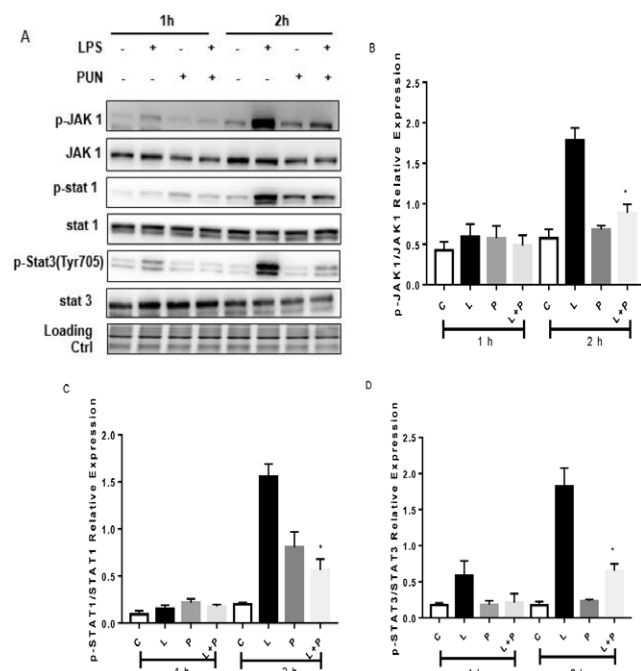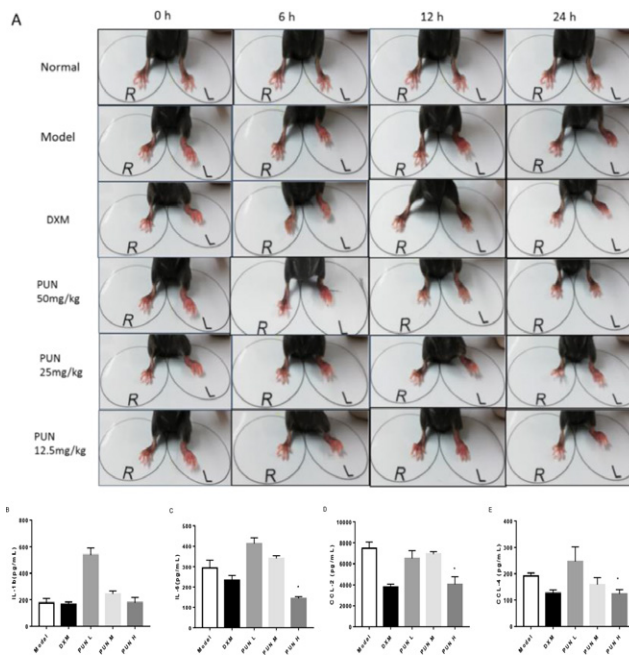
Figure 5: PUN inhibits LPS-induced activation of JAK/STAT signaling pathway in PMs Cells. Phosphorylation levels of JAK1, STAT1 and STAT3, as well as levels of SOCS$_3$ and SHP in PMs treated with 10μg/ml LPS and 50μM PUN for the indicated time points. *p<0.05 compared with the LPS-treated group.

Figure 6: Effects of PUN in a zymosan-induced paw edema model. Edema was induced in the left hind paw of mice by a single injection of 50μl Zymosan (1%), followed by administration of 12.5, 50 and 100 mg/kg PUN or 5mM dexamethasone (positive control) after 4h. A. Representative images showing paw edema in the indicated groups at different time points. B-E. IL-1b, IL-6, CCL-2 and CCL-4 levels in the paw tissue homogenates of different groups. *p<0.05 compared with the model group.

most cellular responses.[24,25] PUN inhibited phosphorylation of ERK in the LPS-challenged macrophages without affecting that of JNK and P38. The JAK/STAT pathway is frequently deregulated in various diseases and its constitutive activation is the basis of autoimmune diseases such as rheumatoid arthritis and ulcerative colitis.[26,27] Binding of LPS to its receptor activates the receptor-associated JAK., which then leads to phosphorylation and nuclear translocation of STAT. The latter binds to the promoter region of its target genes, including those encoding for pro-inflammatory mediators.[28-30] The JAK/STAT pathway is regulated by SOCS[31-33] that forms an inhibitory feedback loop by directly counteracting JAK and STAT activation. SHP2 is another inhibitor of this pathway that blocks the activity of JAKs. PUN not only inhibited JAK1, STAT1 and STAT3 phophorylation, but also slightly activated SOCS3 increasing its level.

The *in vivo* effect of PUN was finally assessed in a zymosan-induced model of acute inflammation. High dose PUN alleviated paw edema and also decreased the levels of pro-inflammatory cytokines in the paw homogenates. Taken together, PUN is a suitable candidate drug for inflammation-related diseases and future studies should focus on its clinical potential.

CONCLUSION

PUN inhibits secretion of pro-inflammatory cytokines from macrophages by suppressing the ERK/MAPK and JAK/STAT signaling pathways.

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CONFLICT OF INTEREST

We declare that all authors have read and agreed to the submitted version of the paper and all who have been acknowledged as contributors have agreed to their inclusion. The material of this manuscript is original and it has not been published and neither under consideration for publication elsewhere. If the manuscript is accepted, the paper will not be published elsewhere in the same form, in English or in any other language, without written consent of the copyright holder. In addition, all the authors have no conflicts of interest to disclose.

ABBREVIATIONS

LPS: lipopolysaccharide; **PUN:** Punicalagin; **PMs:** peritoneal macrophages; **MAPK:** mitogen-activated protein kinase; **JNK:** c-Jun amino terminal kinase; **ERK:** extracellular protein kinase.

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