약학석사학위논문

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15-Ketoprostaglandin E_2 에 의한 대식세포의 efferocytic 활성 증강

2018년 8월

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지도교수 서 영 준

이 논문을 약학석사학위논문으로 제출함

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Ishrat Aklima Muna의 약학석사 학위논문을 인준함

2018년 8월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

Abstract

Potentiation of Efferocytic Activity of Macrophages by 15-Ketoprostaglandin E_2

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College of Pharmacy

Graduate School

Seoul National University

Resolution of inflammation is an essential component of orchestrated host defense responses that counterbalances proinflammatory insults to restore homeostasis. One of the key features of resolution is the phagocytic clearance of superfluous cells which is termed efferocytosis. Over the years, researchers have found various endogenous lipid mediators with potential proresolving and anti-inflammatory properties. An example is 15-ketoprostaglandin E_2 (15-ketoPGE_2) which has recently been shown to modulate activity of some key transcription factors involved in pro- or anti-inflammatory processes. In the present study, I investigated the

proresolving activity of 15-ketoPGE$_2$ in a zymosan A-induced mouse peritonitis model. I found that 15-ketoPGE$_2$ treatment enhanced the efferocytic activity of macrophages for the removal of apoptotic neutrophils. 15-KetoPGE$_2$ stimulated expression of T-cell immunoglobulin- and mucin-domain-containing molecule 4 (TIM4), a phosphatidylserine receptor which is known to be involved in pathogen recognition by macrophages. 15-KetoPGE$_2$ also upregulated expression of nuclear factor-erythroid-2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) in peritoneal macrophages of zymosan A-treated mice. In another experiment, 15-ketoPGE$_2$ treatment potentiated efferocytic activity of bone marrow-derived macrophages (BMDM) and RAW 264.7 cells co-incubated with apoptotic thymocytes. Notably, 15-ketoPGE$_2$ treatment facilitated degradation of kelch-like ECH-associated protein 1 (Keap1) which accounts for activation of Nrf2 and subsequent upregulation of HO-1 expression in BMDM. 15-KetoPGE$_2$-induced expression of HO-1 in BMDM appears to contribute to upregulation of LC3-II protein. In summary, 15-KetoPGE$_2$ potentiates efferocytic activity of macrophages via the Nrf2/HO-1 axis. The induction of HO-1 upregulates TIM4 receptor and LC3-II protein which facilitates the efferocytosis mediated by macrophages.

Keyword: 15-Ketoprostaglandin E_2, Efferocytosis, Nuclear factor-erythroid-2-related factor 2, Heme oxygenase-1, T-cell immunoglobulin- and mucin-domain-containing molecule, LC3-associated phagocytosis.

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Introduction

Initial responses to infection or injury are characterized by the early signals of inflammatory events including release of proinflammatory cytokines, and migration/recruitment of leukocytes[1,2]. These events are counterbalanced in a timely manner by several proresolving mechanisms that involve release of anti-inflammatory cytokines and proresolving lipid mediators, which facilitate the phagocytic removal of apoptotic neutrophils, leading to complete resolution with restoration of host homeostasis. An imbalance between these two active biological processes can precipitate to deleterious consequences for the host such as atherosclerosis, autoimmune disorders, asthma, psoriasis, etc[3].

One of the key features of resolution is the promotion of inflammatory cell egress and clearance of apoptotic cells by phagocytes, a process termed as efferocytosis[4]. In this process, professional phagocytes such as macrophages are briskly recruited to the inflamed site where they engulf and digest the apoptotic corpse and other cellular debris[5,6]. Effective migration of macrophages is governed by 'find me' signals and uptake of the superfluous cells is facilitated by an array of receptors recruited by the macrophages that specifically recognize "eat me" signals transmitted by the apoptotic cells[5]. Among several characterized 'eat me' signals, phosphatidylserine (PtdSr) remains mostly elucidated[7]. A distinct set of

recently described PtdSr receptors such as TIM4 represents a family of small transmembrane proteins that bind to PtdSr through their Igv domain and plays a pivotal role in efferocytosis[8,9]. Once internalized, autophagic machinery is used to conjugate lipids to LC3 bound to phagosomes, a process known as LC3-associated phagocytosis (LAP).

Prostaglandin E_2 (PGE_2), a prototypic proinflammatory lipid mediator, has been reported to have context dependent proresolving effects[10]. The amount of biologically active PGE_2 is regulated not only by cyclooxygenase responsible for its synthesis from arachidonic acid, but by 15-hydroxyprostaglandin dehydrogenase (15–PGDH) that catalyzes oxidation of the 15(S)-hydroxyl group of PGE_2 to produce 15-ketoPGE_2[11,12]. 15-KetoPGE_2 has long been known as an inactive metabolite of PGE_2, but recent studies have uncovered that it acts as an endogenous ligand of anti-inflammatory transcription factor, peroxisome proliferator receptor-γ and inhibits cancer cell growth[13]. It has also been reported to induce apoptosis of pancreatic adenocarcinoma through production of reactive oxygen species (ROS)[14]. Moreover, 15-ketoPGE_2 attenuates necro-inflammatory responses in the liver of 15-PGDH transgenic mice[15]. Owing to its reactive α,β-unsaturated carbonyl group, 15-ketoPGE_2 has the potential to modify a nucleophilic thiol group of cysteine residue(s) in several proteins[16–18]. 15-KetoPGE_2 is further processed by prostaglandin reductase 2 (PTGR2). PTGR2 catalyzes the

NADPH-dependent reduction of 15-ketoPGE_2 to 13,14-dihydro-15-ketoPGE_2. The PTGR2-15-ketoPGE_2 axis has been reported to mitigate inflammatory responses in experimental sepsis model[19].

Nrf2, a stress-responsive transcription factor, is known to play vital roles against inflammatory insults. For activation, Nrf2 needs to be released from its cytosolic repressor Keap1[20]. Nrf2 is translocated to the nucleus after oxidation or covalent modification of distinct Keap1 cysteine residues and enhanced degradation of Keap1. Multiple lines of evidence suggest that deficiency in Nrf2 activation augments pro-inflammatory tissue damage, leading to inflammatory associated disorders[21]. Induction of heme oxygenase-1 (HO-1), a major target protein of Nrf2, has been implicated as a protective mechanism against inflammatory damage and oxidative stress[22]. Several endogenous prostaglandins and metabolites of neutrophils have been reported to modulate phagocytic activity of macrophages through activation of Nrf2 and upregulation of HO-1 [23–25]. Altogether this prompted me to investigate proresolving effects of 15-ketoPGE_2 in a zymosan A-induced murine peritonitis model and to elucidate underlying molecular mechanisms with particular focus on the Nrf2/HO-1 axis.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) medium, penicillin, streptomycin and fetal bovine serum were obtained from Gibco-BRL (Grand Island, NY, USA).15-KetoPGE$_2$ was supplied from Cayman Chemical (Cat. No.14720). Zymosan A, dexamethasone and antibodies against actin and acetylated tubulin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Recombinant mouse M-CSF were purchased from Biolegend Inc. (San Diego, CA, USA). pHrodo red was provided by Thermo Fisher Scientific Inc. (Waltham, MA, USA). Red blood cell lysis buffer was a product of iNtRON biotechnology. Zinc protporphyrin IX (ZnPP) was purchased from Enzo Life Sciences, Inc. (USA). Antibodies against LC3B and HO-1 were supplied by Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against Nrf2 and Keap1 were purchased from Abcam (ab-137550) and Santa Cruz Biotechnology (SC-365636), respectively. The anti-rabbit and anti-mouse horseradish peroxidase conjugated secondary antibodies were obtained from Zymed Laboratories (San Francisco, CA, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from Gelman Laboratory (Ann, Arbor, MI, USA). The enhanced Chemiluminescent (ECL) detection kit was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Cell culture

RAW 264.7 cells were cultured in DMEM which was supplemented with 10% v/v FBS and 100 units/ml penicillin and 100 μg/ml streptomycin at 37^{o}C in an incubator with humidified atmosphere of 95% O2/5% CO2.

Zymosan A-induced peritonitis

C57BL/6 mice (6-8 weeks of age) were purchased from Orient Bio, Inc. (Seoul, South Korea). All mice were maintained according to the Institutional Animal Care Guidelines. Animal experimental procedures were approved by the Institutional Animal Care and Use Committee at Seoul National University. Zymosan A (30mg/kg) was administered intraperitoneally at 12 h before giving 15-ketoPGE$_2$ [20 mg/kg, suspended in 10% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS)] or vehicle, and mice were sacrificed 12 h later. Peritoneal leukocytes were collected by washing with 3 ml of PBS containing 3M methylenediaminetetraacetic acid (EDTA).

Preparation of bone marrow-derived macrophages (BMDM)

Bone marrow-derived macrophages (BMDM) were generated from bone marrow progenitors obtained from both male and female littermates (8–12 weeks of age). In brief, the femur and tibia were isolated from C57BL/6

mice. Bone marrow was flushed out by cold PBS containing 2% heat-inactivated fetal bovine serum (FBS) using a 3 ml syringe. Cells were dissociated to be single by passing through 23G syringe needle for 4-6 times. The cells from bone marrow were then filtered through 70 μm FALCON nylon cell strainer (Thermo Fisher Scientific Inc.) to remove bone, hair, cell clumps, and other cells/tissues, and then centrifuged at 500x *g* for 5 min at 4^{o}C. Cell suspension were incubated with RBC-lysis buffer for 1 min on ice to remove unwanted RBCs. After removing lysis buffer, the remaining cells were re-suspended in DMEM containing 10% heat-inactivated FBS and 20 ng/ml M-CSF, seeded in petri dishes at a density of around 2x10^{6} cells/plate and incubated at 37^{o}C for 4 days in a humidified incubator containing 5% CO_2. Media containing M-CSF (10 ng/ml) were changed once after 3 days of incubation. BMDMs were detached from petri dishes and seeded again into new culture plates for additional experiment.

Isolation of mouse thymocytes and generation of apoptotic cells

The thymus was collected from C57BL/6 mouse and ground between the frosted ends of two microscope glass slides until completely homogenized without destroying cells. Collected cells were filtered with 40 μm FALCON nylon cell strainer (Thermo Fisher Scientific Inc.) to remove remaining tissue and clumped cells. Cells were washed twice with PBS, followed by resuspension in DMEM medium at 107 cells/ml, and then cultured at 37°C

in a humidified incubator containing 5% CO_2 (Miksa et al., 2009). Apoptosis of cells was triggered by treatment of 0.1 μM dexamethasone (DEX) to thymocytes for 16 h.

Efferocytosis assay

The apoptotic cells were collected and stained with 1 μl of 1 mg/ml pHrodo-SE in 50 ml cell suspension (final concentration 20 ng/ml and 106 cells) for 30 min at room temperature. The labeled cells were collected by centrifugation and washed twice with PBS. For 1×10^6 BMDMs on a 60 mm plate, 3×10^6 pHrodo-SE-labelled apoptotic thymocytes were added in 4 ml medium and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 1 h. Efferocytosis was verified under the light microscope prior to labeling and detaching cells. After incubation, the BMDMs were washed two times with PBS to remove free apoptotic cells, treated with 5mM EDTA, fixed with 2% formaldehyde and analyzed by flow cytometry assay or confocal microscopy. To quench fluorescence from non-phagocytized pHrodo-SE-labelled apoptotic cells, cells were washed and re-suspended in basic buffer (pH 8.8) before the flow cytometry assay[26].

Flow cytometry assay

The samples were prepared for flow cytometry assay as described earlier[27]. Briefly, cells ready to analyse were detached from plates and prepared by spinning down 5 min at 300 *g*, 4°C. For the assessment of intracellular proteins, cells were fixed with 2% formaldehyde in PBS for 30 min at 4°C. A permeabilization of cells was performed by 0.2% Tween-20 in PBS for 15 min at 37°C. For staining cell surface proteins, it is optional to fix cells but necessary to avoid permeabilization. To block nonspecific antibody binding, the cells were incubated with an unlabelled isotype control antibody in staining buffer containing 1% bovine serum albumin (BSA; fraction V) and 0.1% sodium azide (NaN3) in PBS for 15 min. Following complete removal of unwanted tissues and debris free isotype control antibody, an additional incubation with CD16/32 antibody was performed to block non-specific binding to Fc receptors. Specific antibodies without fluorophore conjugation were added to samples in the presence of CD16/32 antibody and incubated for 30 min. For use of non-conjugated primary antibodies, the fluorophore-conjugated secondary antibodies were used to stain for another 30 min. Samples were analyzed by a FACSCalibur™ flow cytometer (BD, Franklin Lakes, NJ, USA) and Flowjo software.

Western blotting

Cells were washed in PBS and collected, and lysed in ice-cold lysis buffer (2% Triton X-100, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM NaN_3, and 2 mM EDTA containing protease inhibitors (Roche Life Science) overnight at 4°C. Lysates were harvested and centrifuged at 12,000 *g* to eliminate nuclei. The protein concentration was determined using the BCA protein assay reagents (Thermo Fisher Scientific, Pierce, USA), and 20 μg of protein was electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to PVDF membranes. The membranes were then blocked with 5% w/v nonfat dry milk in PBST (PBS containing 0.1% Tween-20) buffer for 1 h at room temperature. Afterward, the blots were incubated with the primary antibody diluted in 3% non-fat milk PBST buffer overnight at 4°C. After washing to remove primary antibodies, the blots were incubated with proper horseradish peroxidase-conjugated secondary antibody. The immunoblot with protein-antibody complexes was detected by using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). When necessary, membranes were stripped with Restore PLUS Western Blot Stripping Buffer (Life Technologies), re-blocked in 1X PBST with 5% w/v non-fat dry milk, and probed with fresh antibodies. Images were captured with an Amersham Imager 600 and saved as tiff files. Representative images from reproducible, independent experiments are shown.

Statistical analysis

All data are analyzed by means ± SE and are based on experiments performed at least in triplicate. Statistical significance was calculated with the Student's *t*-test and Sigmaplot software.

Results

15-KetoPGE$_2$ potentiates efferocytic activity of macrophages

Among several features of resolution that can be modulated by 15-ketoPGE$_2$, I focused on the process of efferocytosis which was assessed using a self-resolving murine peritonitis model. Peritonitis was induced in mice by an intraperitoneal (*i.p.*) injection of zymosan A (30 mg/kg). In zymosan A-treated mice, total leukocyte infiltration in peritoneal fluid reaches the maximum at 12 h[28]. When the number of total leukocytes was maximal, 15-ketoPGE$_2$ (20mg/kg) was administered into the peritoneum of mice. Twelve hours later, peritoneal exudates were collected. Peritoneal cells with positive staining for both F4/80 (macrophage marker) and Gr-1 (neutrophil marker) were selectively identified by flow cytometry. The mice treated with zymosan A and 15-ketoPGE$_2$ showed a significantly higher proportion of peritoneal macrophages engulfing apoptotic PMNs (F4/80$^+$Gr-1$^+$) than those challenged with zymosan A alone (**Fig.1A**). For an *in vitro* efferocytosis assay, BMDM and RAW 264.7 cells were incubated with 40 μM of 15-ketoPGE$_2$. After 24 h incubation, they were treated with pHrhodo-stained apoptotic thymocytes at a 1:3 ratio. After 1 h treatment, cells were collected and analyzed using flow cytometry. Macrophages with positive staining of pHrhodo and F4/80 were selectively identified. Both the cell types showed an increased

efferocytic activity compared to the non-treated ones (**Fig.1 B, C**). Taken together, these results demonstrated that 15-ketoPGE$_2$ treatment potentiated efferocytic activity of macrophages.

15-KetoPGE$_2$ upregulates TIM4 expression in macrophages

Macrophages express several different types of PtdSr receptors involved in the removal of waste materials to avoid unnecessary tissue damage. TIM4 is one such PtdSr receptor responsible for recognizing apoptotic cells by macrophages[29]. Mice treated with zymosan A and 15-ketoPGE$_2$ showed a higher proportion of peritoneal macrophages with elevated TIM4 expression than those challenged with zymosan A alone (**Fig.2 A**). BMDM and RAW 264.7 cells were incubated with 40 μM of 15-ketoPGE$_2$. After 24 h incubation, flow cytometry analysis was conducted to detect (F4/80$^+$tim4$^+$) macrophages. (**Fig. 2 B, C**). Both types of macrophages showed an increased TIM4 expression compared to the non-treated ones. These results indicate that 15-ketoPGE$_2$ enhanced TIM4 expression which facilitates the recognition of apoptotic cells.

15-KetoPGE$_2$ activates Nrf2/HO-1 signalling in macrophages

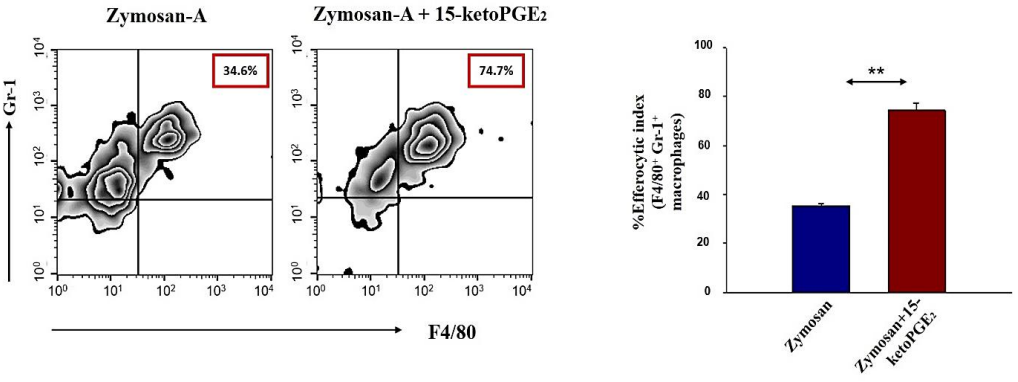
Nrf2 induces the expression of several cytoprotective genes in response to oxidative stress and noxious stimuli. Nrf2 deficiency is reported to delay resolution in several

inflammatory models[30]. Compared to mice challenged with zymosan A alone, those treated with zymosan A plus 15-ketoPGE$_2$ exhibited an increase in the proportion of macrophages expressing Nrf2 (**Fig. 3A**). In another *in vitro* study, 15-ketoPGE$_2$ treatment upregulated the Nrf2 protein expression in BMDM and RAW 264.7 cells. (**Fig. 3C**). Keap1 is a repressor protein that binds to Nrf2 and promotes its degradation by ubiquitin proteasome degradation pathways[31]. In this study, we found that 15-ketoPGE$_2$ decreased the Keap1 protein level in BMDMs. (Fig. 3C). HO-1 is one of the major target proteins of Nrf2. The Nrf2/HO-1 axis is largely known to combat oxidative stress and inflammation. Several studies have demonstrated significant anti-inflammatory effects of HO-1 and its by-products [32-34]. In a zymosan A-induced peritonitis model, 15-KetoPGE$_2$ (20 mg/kg) treatment resulted in an increased level of HO-1 (**Fig 3 B**). An *in vitro* study with BMDM and RAW 264.7 cells revealed that 15-ketoPGE$_2$ treatment elevated the HO-1 protein level in both cell types (**Fig. 3C and D**). These data suggest that 15-ketoPGE$_2$ activates the Nrf2/HO-1 axis, and this might contribute to the enhanced dead cell clearance by macrophages through efferoytosis.

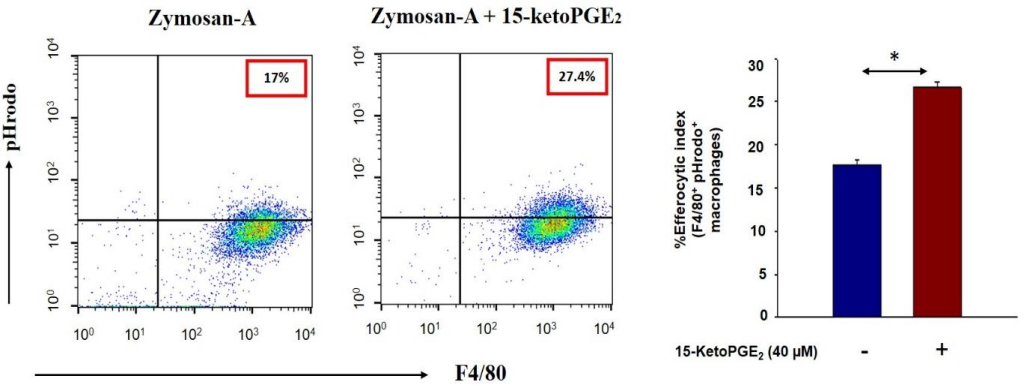
15-KetoPGE$_2$ upregulates LC3-II and TIM4 in a HO-1 dependent manner

Despite its relevance to the resolution of inflammation, there is very little information on whether lipid mediators contribute to the degradation of apoptotic cells upon engulfment. LAP is a recently described process which mediates the degradation of engulfed pathogens and apoptotic cells[35]. Molecular characterization of LAP revealed critical roles for the PtdSr receptor TIM4 and LC3-II[36]. In this study, we found that 15-ketoPGE$_2$ upregulates LC3-II protein level expression (**Fig.4 A**), which is the lipidated form of LC3 and indispensable for autophagosome closure and membrane fusion during LAP. Although studies depicting While there is paucity of data demonstrating the relation between HO-1 and LAP, is not yet done to that extent, in this study I found that upon pre-treatment with an HO-1 inhibitor (ZnPP), 15-ketoPGE$_2$ repressed the expression of TIM4 and LC3-II (**Fig 4 B** and **C**), indicating a possible relation between involvement of HO-1 induction and in LAP. These results show that 15-ketoPGE$_2$ enhances expression of TIM4 and LC3-II in a HO-1- dependent manner.

A)


Zymosan-A
Zymosan-A + 15-ketoPGE2
34.6%
74.7%
Gr-1
F4/80
%Efferocytic index (F4/80+ Gr-1+ macrophages)
**
Zymosan
Zymosan+15-ketoPGE2

B)


Zymosan-A
Zymosan-A + 15-ketoPGE2
17%
27.4%
pHrodo
F4/80
%Efferocytic index (F4/80+ pHrodo+ macrophages)
*
15-KetoPGE2 (40 µM)
-
+

C)

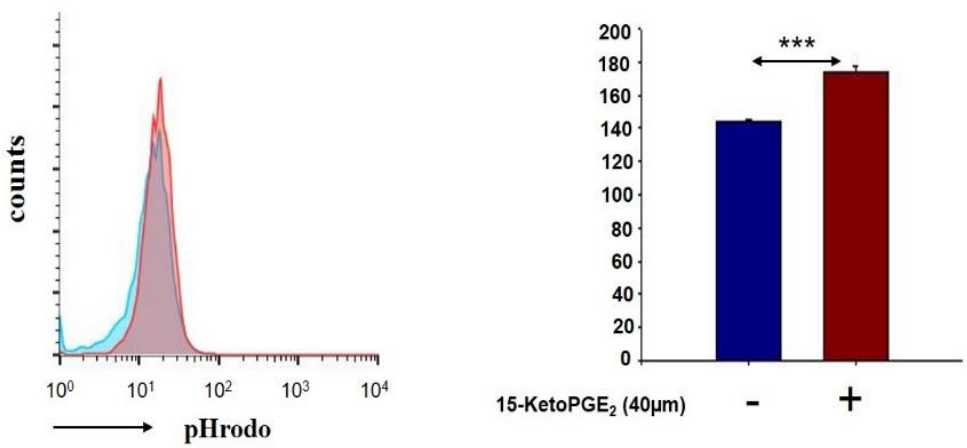
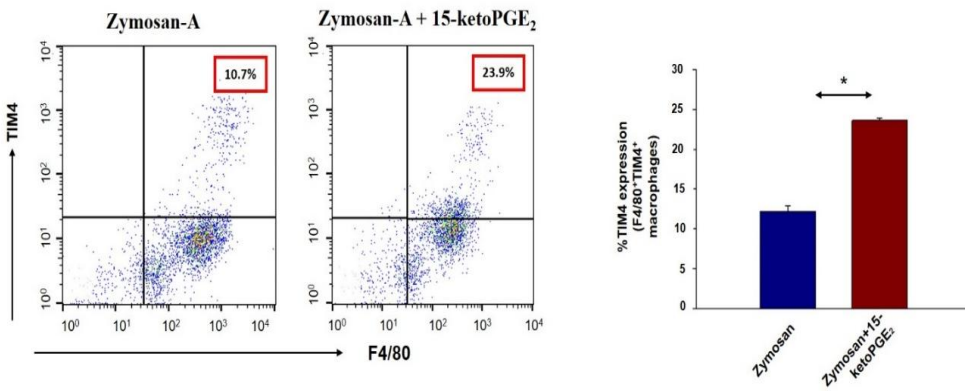


Figure 1. 15-KetoPGE$_2$ potentiates efferocytic activity of macrophages.

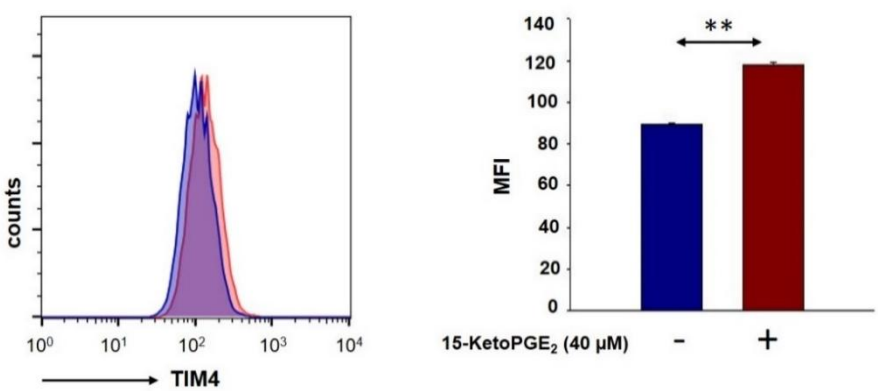
Mice were treated with an intraperitoneal (i.p.) dose (30 mg/kg) of zymosan A for 12 h, followed by i.p. injection of vehicle or 15-ketoPGE$_2$ (20 mg/kg). Twelve hours later, peritoneal exudates were collected. (A) In a spontaneous resolving zymosan A-treated peritonitis model, the proportion

of macrophages engulfing apoptotic neutrophils ($F4/80^{+}Gr\text{-}1^{+}$) was determined by flow cytometry. (B and C) In another *in vitro* study, efferocytic activity of macrophages induced by 15-ketoPGE$_2$ was assessed. (B) BMDMs and (C) RAW264.7 cells were treated with 15-ketoPGE$_2$ for 24 h and co-incubated with pHrhodo-stained apoptotic thymocytes for additional 1 h. The proportion of macrophages engulfing apoptotic thymocytes was determined by flow cytometry. All data represent mean ± SD (n = 3); *p <0.05, **p <0.01, and ***p < 0.001. Macrophages engulfing apoptotic neutrophils are shown inside the dotted square. SD; standard deviation, MFI; mean fluorescence intensity.

A)


Zymosan-A
Zymosan-A + 15-ketoPGE2
10.7%
23.9%
TIM4
F4/80
% TIM4 expression (F4/80+TIM4+ macrophages)
*
Zymosan
Zymosan+15-ketoPGE2

B)


counts
TIM4
MFI
**
15-KetoPGE2 (40 µM)
-
+

C)

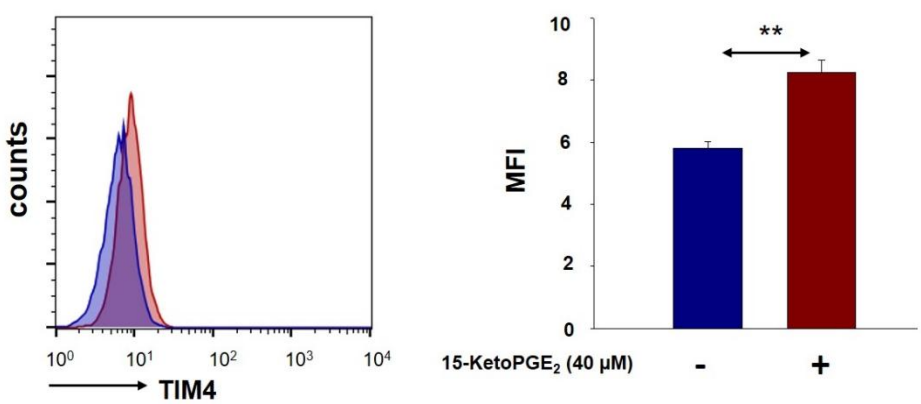
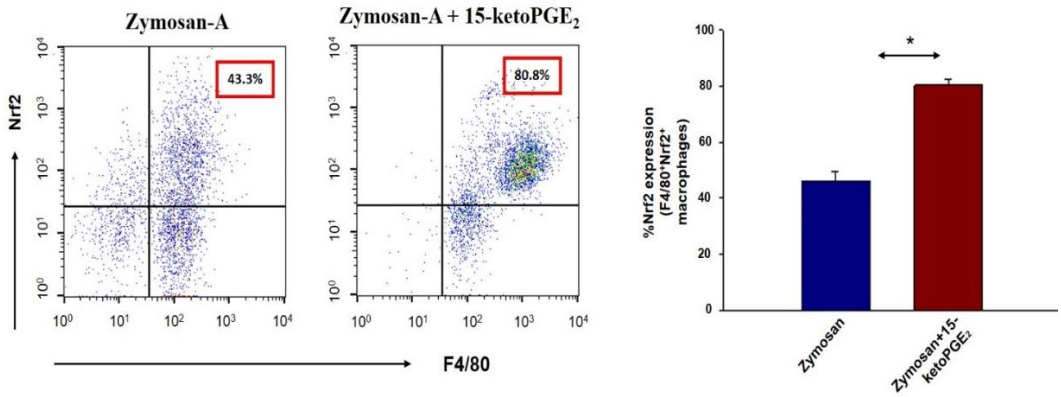


Figure 2. 15-KetoPGE$_2$ upregulates TIM4 expression in macrophages.

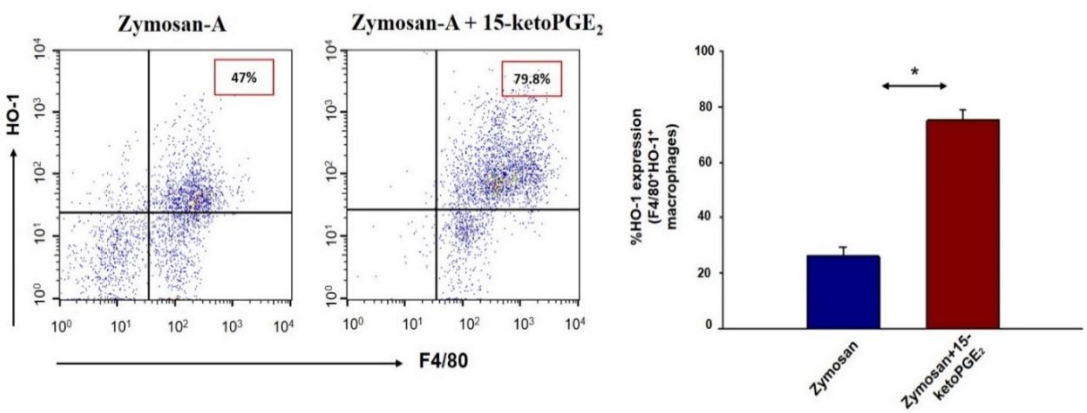
Mice were administered with an intraperitoneal dose (30 mg/kg) of zymosan A for 12 h, followed by i.p. injection of vehicle or 15-ketoPGE$_2$ (20 mg/kg,). Twelve hours later, peritoneal exudates were collected. (A) The proportion of peritoneal macrophages expressing TIM4 receptor (TIM4$^+$F4/80$^+$) was determined by flow cytometry. For an *in vitro* study, (B) BMDM and (C)

RAW264.7 cells were treated with 40 µM of 15-ketoPGE$_2$. After 24 h treatment, cells were collected and macrophages expressing TIM4 receptor were determined by flow cytometry. All data represent mean ± SD (n = 3); *p <0.05, **p <0.01, and ***p< p < 0.001 0.001

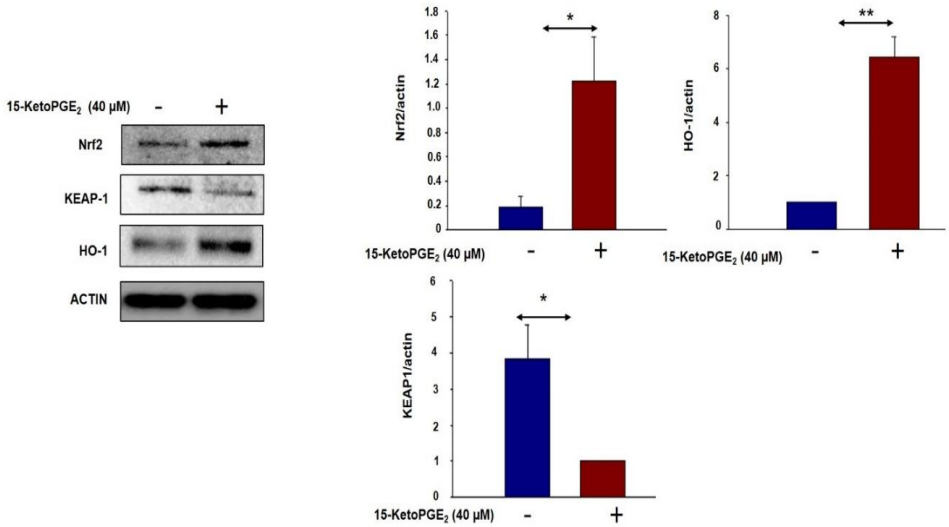
A)



B)



C)


15-KetoPGE2 (40 μM) - +
Nrf2
KEAP-1
HO-1
ACTIN
Nrf2/actin
HO-1/actin
KEAP1/actin
*
**
15-KetoPGE2 (40 μM) - +

D)

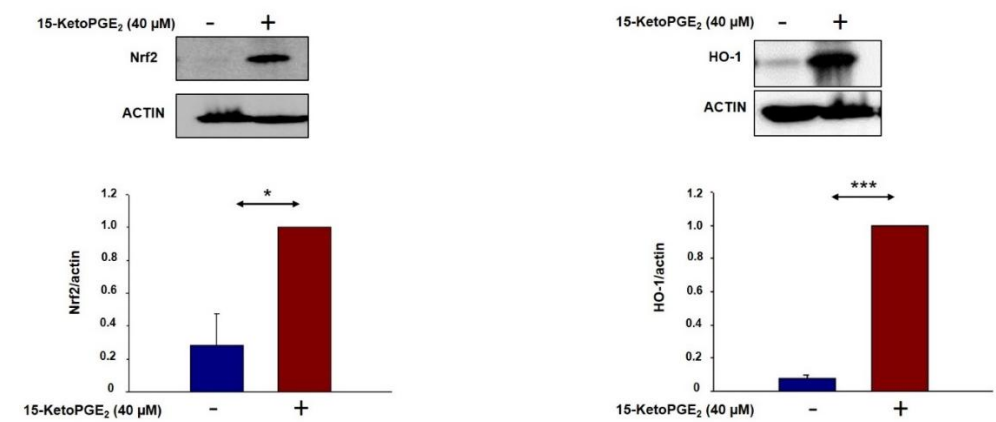
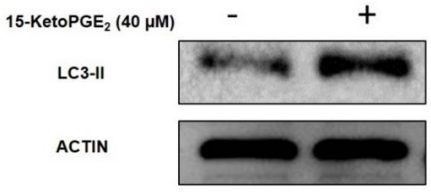
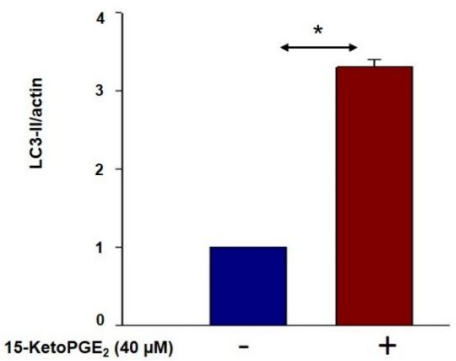

15-KetoPGE2 (40 μM) - +
Nrf2
ACTIN
HO-1
Nrf2/actin
HO-1/actin
*
***
15-KetoPGE2 (40 μM) - +

Figure 3. 15-KetoPGE$_2$ activates Nrf2/HO-1 signalling in macrophages.

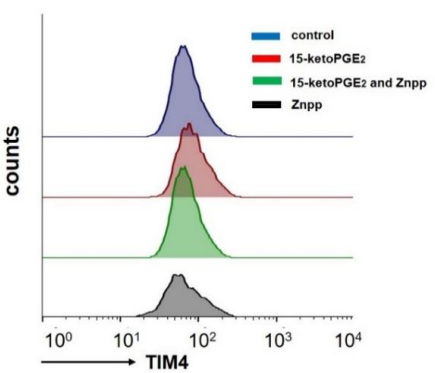
Mice were injected with an intaperitoneal dose (30 mg/kg) of zymosan A for 12 h, followed by i.p. injection of vehicle or 15-ketoPGE$_2$ (20 mg/kg). Twelve hours later, peritoneal exudates were collected. (A) Nrf2 and (B) HO-1 expression of peritoneal macrophages (Nrf2$^+$F4/80$^+$, HO-1$^+$F4/80$^+$) were determined by flow cytometry. For an *in vitro* study, Nrf2 and HO-1 protein levels of (C) BMDM and (D) RAW 264.7 cells were analyzed. Both cell types were treated with 40 μM of 15-ketoPGE$_2$ for 24 h. After 24 h treatment, cells were collected and lysed. Nrf2, HO-1 and Keap1 protein levels were determined using Western blot analysis. All data represent mean ± SD (n = 3); *p <0.05, **p <0.01, and ***p < 0.001.

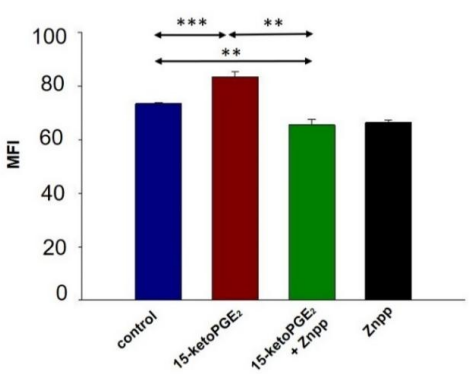
A)


15-KetoPGE2 (40 μM)
-
+
LC3-II
ACTIN


LC3-II/actin
4
3
2
1
0
*
15-KetoPGE2 (40 μM)
-
+

B)


control
15-ketoPGE2
15-ketoPGE2 and Znpp
Znpp
counts
10^0
10^1
10^2
10^3
10^4
TIM4


MFI
100
80
60
40
20
0
***
**
**
control
15-ketoPGE2
15-ketoPGE2 + Znpp
Znpp

C)

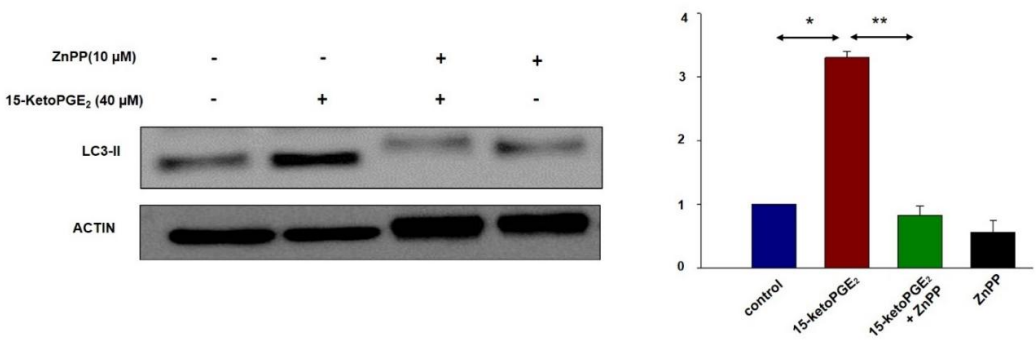


Figure 4. 15-KetoPGE$_2$ enhances the LC3-II and TIM4 expression of macrophages in a HO-1-dependent manner

BMDMs were pre-incubated with 10 μM of ZnPP, followed by incubation with 40 μM conc. of 15-ketoPGE$_2$. After 24 h incubation, cells were collected. (A and C) Western blot analysis was done for the measurement of the LC3-II protein level. (B) TIM4 expression of macrophages was determined by flow cytometry. TIM4 expression is presented as mean fluorescence intensity. All data represent mean ± SD (n = 3); *p <0.05, **p

<0.01, and *** p <0.001.

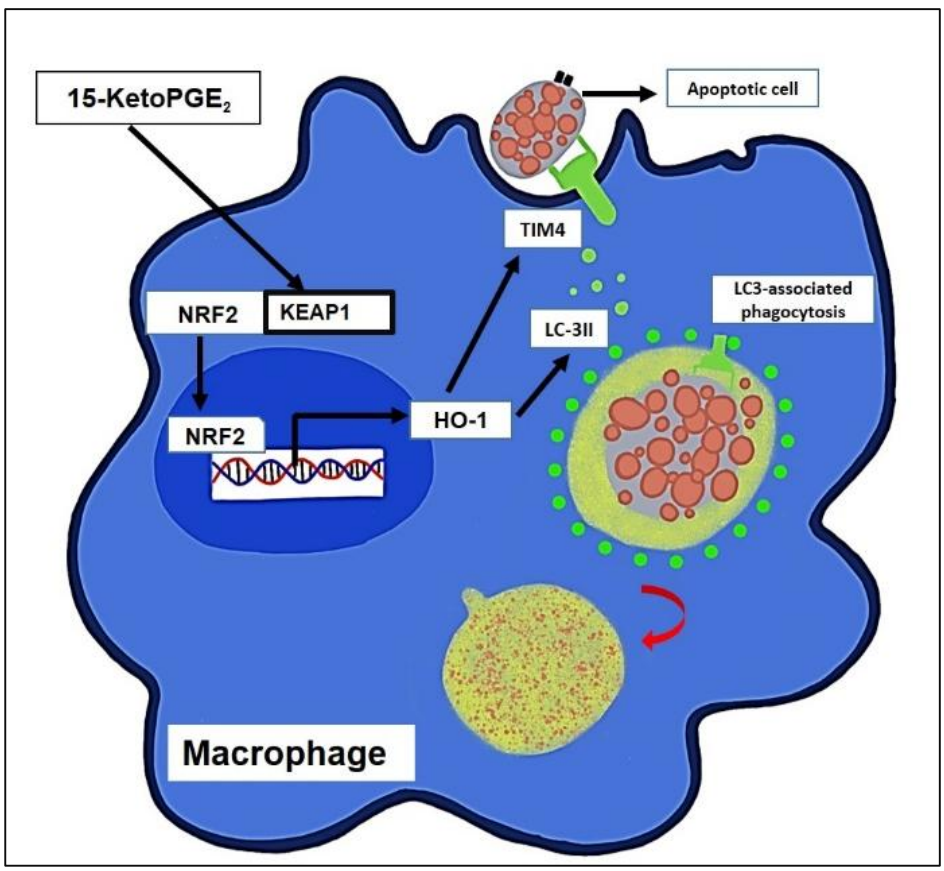


Figure.5. A proposed scheme illustrating the potentiation of efferocytic activity of macrophages by 15-ketoPGE$_2$

15-KetoPGE$_2$ treatment enhances efferocytic activity of macrophages. It activates the Nrf2/HO-1 axis. This, in turn, upregulates TIM4 receptor and LC3-II protein expression of macrophages, which potentiates the efferocytic activity of macrophages and contributes to the distinct non-canonical autophagy termed LC3-associated phagocytosis (LAP).

Discussion

Several proresolving lipid mediators are known to stimulate resolution of inflammation by expediting removal of effete cell debris by macrophages[18,37–39]. The demotion of efferocytic activity can cause apoptotic cells to be necrotic cells. These necrotic cells upon engulfment by macrophages can further trigger the proinflammatory signals which leads to chronic inflammation[40,41]. Following efferocytosis, macrophages produce high levels of IL-10, TGF-β, and other factors to facilitate tissue repair[42]. Therefore, efferocytosis is viewed as being central to the resolution of inflammation[43]. Understanding the molecular mechanisms of efferocytosis modulation by lipid mediators hence provides a novel therapeutic strategy for inflammation-associated disorders.

15-PGDH catalyzes conversion of PGE$_2$ to 15-ketoPGE$_2$ and is known to be essential for eliminating the biological activity of PGE$_2$[44]. As a downstream metabolite of PGE$_2$[45], 15-ketoPGE$_2$ was given relatively low

attention compared to other prostanoids. Recently its role in anti-inflammatory responses and cancer cell survival become unraveled. It is known to be an endogenous ligand of PPARγ, one of the major transcription factors known to promote phagocytosis and resolution of inflammation[46,47]. It has been reported that 15-ketoPGE$_2$ protects against liver injury through activation of PPARγ[15]. 15-KetoPGE$_2$ shares structural and functional similarity with another extensively studied PPARγ ligand, 15-deoxy-$\Delta^{12,14}$-Prostaglandin J$_2$ (15d-PGJ$_2$), a lipid mediator which has anti-inflammatory and proresolving activities. The structure of 15d-PGJ$_2$ is characterized by an electrophilic carbon present in the side chain with an additional α,β-unsaturated carbonyl moiety within the cyclopentenone ring[48,49]. Both prostaglandins have been shown to attenuate production of proinflammatory cytokines such as IL-6 and TNFα,[50,51] thereby inhibiting inflammation and protecting against experimentally induced sepsis[51,52]. 15d-PGJ$_2$ potentiates efferocytic activity of macrophages by stimulating the degradation of Keap1 with concomitant activation of the Nrf2/HO-1 axis in murine peritonitis model[18]. As 15-ketoPGE$_2$ is an electrophilic prostaglandin like 15d-PGJ$_2$, it is speculated that 15-ketoPGE$_2$ can facilitate resolution of inflammation by accelerating efferocytic activity through activation of Nrf2. In this study, exogenous treatment of 15-ketoPGE$_2$ activated the Nrf2/HO-1 axis and enhanced the efferocytic activity of macrophages.

A plethora of promising data from preclinical studies support the role of Nrf2 in the resolution of inflammation. It can induce transcription of anti-

inflammatory genes while repressing transcription of several proinflammatory genes[53]. Recent research has revealed that activation of Nrf2 through degradation of its cytosolic suppressor Keap1 by natural compounds[54] leads to attainment of the anti-inflammatory phenotype of macrophages (also called M2 type of macrophage)[55,56] and $CD8^{+}$T cells[57]. Among the target enzymes of Nrf2, HO-1 has been considered to be the most critical modulator of acute inflammation. HO-1 and its by-product carbon monoxide (CO) have been reported to accelerate resolution of inflammation by promoting apoptotic neutrophil clearance[24,58]. HO-1 induction is considered to ameliorate experimentally induced inflammation such as the endotoxic septic shock[59], vascular endothelium[60], neuro-inflammation[61], and skin inflammation[62,63]. The Nrf2/HO-1 axis upregulates various scavenger receptors such as CD36[24] and dectin-1[23]. In this study I found that activation of Nrf2/HO-1 signalling by 15-ketoPGE_2 enhanced expression of TIM4, a potent PtdSr receptor, thereby potentiating the efferocytic activity of macrophages.

The aftermath of efferocytosis includes degradation of engulfed debris by LAP, a novel non-canonical autophagy pathway which utilizes components of the canonical autophagy machinery to conjugate LC3 to phagosome membranes. This drives phagolysosomal fusion and subsequent apoptotic cell degradation[64–66], resulting in an 'immunologically silent' clearance of cell debris[67,68]. Deficiency of the LAP pathway is reported to develop autoimmune disorders such as systemic lupus erythematosus and

atherosclerosis[69,70]. Molecular characterization of LAP has revealed distinct roles of TIM4, LC3-II, and NADPH oxidase 2 (NOX2) in resolution of inflammation[71,36]. In this study, I found that 15-ketoPGE$_2$ upregulates TIM4 and LC3-II in BMDMs. TIM4 has previously been reported to inhibit nitric oxide. During phagosome formation, the function of NOX2 in regulating oxidative stress is suppressed by nitric oxide[72,73]. Therefore, upregulation of TIM4 by 15-ketoPGE$_2$ might, in turn, lead to the enhancement of NOX2 activation. Further detailed studies will be necessary to clarify the involvement of 15-ketoPGE$_2$ in the regulation of LAP.

In summary, 15-ketoPGE$_2$ potentiates the efferocytic activity of macrophages by activating the Nrf2/HO-1 axis and upregulating TIM4 expression which contributes to enhanced dead cell clearance. Precise clearance of dead cells by macrophages is critical for the resolution of inflammation, and its failure can cause inflammation-associated disorders[74]. As exogenous treatment of 15-ketoPGE$_2$ potentiated the efferocytic activity of macrophages and modulated expression of an anti-inflammatory transcription factor, Nrf2 along with its major target protein HO-1, this lipid mediator might have therapeutic potential in the management of inflammatory ailments.

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국문초록

염증의 해소는 염증 반응을 상쇄하여 염증 부위에서 항상성과 숙주 방어를 유지하는 과정이다. 우리의 면역 체계는 다양한 형태로 병원균을 인식, 제거 및 기억할 수있는 능력을 가지고 있으며 손상되고 감염된 부위의 기능을 복원할 수 있다. 사멸된 세포에 대한 식세포 작용은 염증 해소의 주요 특징 중 하나로 efferocytosis라고 불린다. Efferocytosis 는 적절한 염증 해소를 위한 항 염증성을 향상시키기 위해 대식세포 분극을 유도한다. 이 과정은 이러한 위험 분자의 제거만으로 멈추지 않고 세포 파편을 분해한다. 수년에 걸쳐 연구자들은 염증 해소의 메커니즘에 기여하는 다양한 내인성 및 외인성 분자를 발견했다. 그중 몇몇 지질 매개체들이 우리의 면역 체계의 분해 작용을 조절하는데 유망하다는 것이 증명되었다. 15-KetoprostaglandinE_2 (15-KetoPGE_2)는 아라키돈 산으로부터 유도된 지질 매개체로, 항염증 과정과 관련된 중요한 전사 인자의 작용을 촉진하는 것로 밝혀지고 있다. 본 연구에서는 Zymosan A 를 주입하여 유도한

마우스 복막염 모델에서 15-ketoPGE$_2$의 염증해소 활성을 조사하였다. Zymosan A 유도 마우스 복막염 모델에서 15-ketoPGE$_2$ 처리가 세포 사멸성 호중구 제거를 위한 대식세포의 efferocytic activity를 증가시켰다. 15-ketoPGE$_2$는 대식세포에 의한 사멸세포 인식과 관련된 것으로 알려진 포스파티딜 세린 수용체인 TIM4 (T 세포 면역 글로불린 및 뮤신 도메인 함유 분자 4) 발현을 증가시켰다. 15-ketoPGE$_2$가 zymosan-A 유도 마우스 복막염 모델에서 Nrf2와 HO-1 발현을 증가 시켰다. 또한 골수 유래 대식 세포 (BMDM)에서 15-ketoPGE$_2$ 처리는 세포 사멸 흉선 세포와 함께 공동 배양시 efferocytic 활성을 강화시켰다. 골수 유래 대식 세포에서 15-ketoPGE$_2$ 처리는 Keap1의 분해 및 Nrf2 및 HO-1 발현의 후속적인 상향 조절을 촉진시키는 것으로 밝혀졌다. 15-ketoPGE$_2$는 골수 유래 대식세포에서 TIM4 발현을 증가 시켰는데, 이는 HO-1 활성에 의존적인 것으로 밝혀졌다. 또한 TIM4가 매개하는 대식세포의 강화된 efferocytic 활성은 LC3 관련 식균 작용으로 알려진 독특한 비표준 autophagy 경로를 유발했다. 이것은 LC3의 지질화된 형태 인 LC3-II 단백질의 발현을 증가 시켰으며, 이는 LC3 관련 식균 작용 동안 autophagosome 형성에 없어서는 안될 필수 요소이다. 결론적으로15-ketoPGE$_2$ 매개 Nrf2 / HO-1 의 유도가 HO-1 의존적 방식으로 TIM4 수용체를 증가시킴으로써 대식세포의 efferocytic 활성을 증가시키며 또한 LC3 관련 식균 작용의 구성 요소를 불러들여 phagosome 분해 과정에 기여하는 것을 확

인하였다. 그러므로, 15-ketoPGE$_2$에 의한 염증 해소의 조절은 염증성 질환에 대한 새로운 치료 접근법을 제시 할 수 있을것으로 사료된다.

주요어: 염증의 해소, 15-ketoprostaglandinE$_2$, Efferocytosis, Nuclear factor-erythroid-2-related factor 2 (Nrf2), Heme Oxygenase-1 (HO-1), T-cell immunoglobulin- and mucin-domain-containing molecule (TIM4), LC3-associated phagocytosis. T- 세포 면역 글로불린 및 점액 - 도메인 - 포함 분자 (TIM4), LC3- 관련 식균 작용.