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Collection
15-Deoxy-Δ^{12,14}-prostaglandin J\(_2\) and taurine chloramine generated during the inflammatory response promote resolution of inflammation

염증반응시에서 생성되는 15-deoxy-Δ\(^{12,14}\)-prostaglandin J\(_2\) 와 taurine chloramine의 염증해소 효과

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藥学科 醫藥生命科學專攻
金 原 基
15-Deoxy-\(\Delta^{12,14}\)-prostaglandin J\(_2\) and taurine chloramine generated during the inflammatory response promote resolution of inflammation

by

Wonki Kim

A thesis submitted in partial fulfillment of the requirements for the degree of

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at the
College of Pharmacy,
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ABSTRACT

15-Deoxy-\(\Delta^{12,14}\)-prostaglandin J\(_2\) and taurine chloramine generated during the inflammatory response promote resolution of inflammation

Wonki Kim

Under the supervision of Professor Young-Joon Surh
at the College of Pharmacy, Seoul National University

Acute inflammation, a cellular immune response, is essential process to protect cells against invading pathogens and other noxious stimuli. It should be appropriately completed through effective resolution and tissue repair by phagocytes such as neutrophils, dendritic cell and macrophages. Resolution of inflammation is the removal of dead neutrophils, pathogens and other debris from inflamed sites, returning to homeostasis. Although the resolution of inflammation was previously considered to be a passive process, recent studies have demonstrated that resolution of inflammation is rather actively regulated by endogenous mediators which possess anti-inflammatory and pro-resolving effects. Theses endogenous mediators counteract excessive inflammatory responses and stimulate or potentiate anti-inflammatory mechanisms. The disruption of timely resolution can lead to chronic inflammation which implicated in the pathogenesis
of many prevalent human diseases, such as inflammatory bowel diseases, rheumatoid arthritis, diabetes and cancer. In this regard, identification of novel proresolving molecules and signaling pathways they regulate will provide opportunities to prevent the development of chronic inflammatory disorders.

Inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn’s disease, are representative chronic inflammatory disorders. Excessive and persistent inflammation are considered to increase the risk of IBD, and inappropriate macrophage activation has been implicated as one of major reasons for failure to resolve intestinal inflammation. However, the precise pathologic mechanism underlying inflammatory macrophage activation in intestinal inflammation has not been well investigated. Using a dextran sodium sulfate (DSS)-induced murine colitis model that mimics human IBD, I revealed that 15-deoxy-\(\Delta^{12,14}\)-prostaglandin J\(_2\) (15d-PGJ\(_2\)), one of endogenous lipid mediators of the cyclooxygenase-2 -mediated arachidonic acid cascade, was produced during resolution of inflammation, and then promoted resolution of intestinal inflammation. The pro-resolving effects of 15d-PGJ\(_2\) on intestinal inflammation were involved in not only inhibition of neutrophils infiltration and inflammatory macrophages (M1), but also an increase in alternative macrophage (M2) polarization. Moreover, 15d-PGJ\(_2\) suppressed DSS-induced overproduction of pro-inflammatory cytokines, especially interleukin-6 (IL-6), which is considered to play a key role in chronic inflammation and cancer. Inhibition of IL-6 expression induced by 15d-PGJ\(_2\) treatment, in turn, blocked STAT3 activation in CCD 841 CoN colon epithelial cells, facilitating the resolution of intestinal inflammation.
Engulfment of apoptotic neutrophils by macrophages, which is called efferocytosis, is essential for the resolution of inflammation. Through effective efferocytosis, macrophages prevent the release of cytotoxic waste from dying neutrophils to the inflammatory microenvironment, thereby preventing additional pro-inflammatory disruption of other cells. Although 15d-PGJ₂ is known to act as a local autacoid which stimulates the anti-inflammatory action of macrophages, the molecular mechanism underlying 15d-PGJ₂-mediated enhancement of efferocytosis remains largely unresolved. In the present study, 15d-PGJ₂ injected into the peritoneum of mice facilitated the resolution of zymosan A-induced peritonitis. 15d-PGJ₂ administration reduced the number of total leukocytes, and attenuated polymorphonuclear leukocytes infiltration. Furthermore, 15d-PGJ₂ increased the proportion of macrophages engulfing apoptotic neutrophils. In another experiment, RAW264.7 murine macrophages treated with 15d-PGJ₂ exhibited markedly increased phagocytic clearance of apoptotic cells. Under these conditions, expression of CD36 and heme oxygenase-1 (HO-1) was enhanced along with increased accumulation of the nuclear factor E2-related factor 2 (Nrf2) in the nucleus. Knockdown of Nrf2 abolished 15d-PGJ₂-induced expression of CD36 and HO-1, and silencing of CD36 and HO-1 attenuated 15d-PGJ₂-induced efferocytosis. Moreover, peritoneal macrophages isolated from Nrf2-null mice failed to upregulate 15d-PGJ₂-induced expression of CD36 and HO-1, and to mediate efferocytosis. These findings indicate 15d-PGJ₂ stimulates the resolution inflammation by potentiating the efferocytic activity of macrophages through Nrf2-induced expression of CD36 and HO-1.

Phagocytosis of pathogens by macrophages is also crucial for the successful
resolution of inflammation induced by microbial infection. Taurine chloramine (TauCl), an endogenous anti-inflammatory mediator, is produced by reaction between taurine and hypochlorous acid by myeloperoxidase activity in the cytosol of neutrophils under inflammatory conditions. Although TauCl released from activated neutrophils has been reported to act as anti-inflammatory mediator in inflammatory microenvironment, the molecular mechanism underlying the pro-resolving effects of TauCl remains overlooked.

In this study, I investigated the effects of TauCl on resolution of zymosan A-induced peritonitis. TauCl administration reduced the number of the total peritoneal leukocytes, while increased the number of peritoneal monocytes. Furthermore, TauCl promoted clearance of pathogens remaining in the inflammatory environment by macrophages.

When the macrophages isolated from thioglycollate-treated mice were treated with TauCl, their phagocytic activity was enhanced. In the murine macrophage-like RAW264.7 cells treated with TauCl, the proportion of macrophages clearing zymosan A particles was also increased. TauCl administration resulted in elevated expression of HO-1 in the peritoneal macrophages. Pharmacologic inhibition of HO-1 activity or knockdown of HO-1 in the murine macrophage RAW264.7 cells abolished the TauCl-induced phagocytosis, whereas the overexpression of HO-1 augmented the phagocytic ability of macrophages. Furthermore, peritoneal macrophages isolated from HO-1 null mice failed to mediate TauCl-induced phagocytosis. Our results suggest that TauCl-induced HO-1 expression is required for enhancement of phagocytic activity, thereby promoting resolution of inflammation.

Taken together, these observations suggest that 15d-PGJ₂ and TauCl generated
during inflammatory response promote resolution of inflammation by regulating macrophage polarization and promoting phagocytosis of macrophages, thereby preventing chronic inflammatory diseases.

**Keywords**
15-Deoxy-\(\Delta^{12,14}\)-prostaglandin, Inflammatory bowel diseases, Resolution of inflammation, Macrophages, Efferocytosis, Nrf2, Heme oxygenase-1, Taurine, Taurine chloramine

**Student Number: 2010-21670**
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<th>Description</th>
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<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>ARE</td>
<td>antioxidant response element</td>
</tr>
<tr>
<td>BAI-1</td>
<td>brain-specific angiogenesis inhibitor 1</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CD36</td>
<td>cluster of differentiation 36</td>
</tr>
<tr>
<td>CD</td>
<td>crohn’s disease</td>
</tr>
<tr>
<td>CO</td>
<td>carbon monoxide</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic pulmonary obstructive disease</td>
</tr>
<tr>
<td>CORM-2</td>
<td>CO-releasing molecule</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-responsive element-binding protein</td>
</tr>
<tr>
<td>DAI</td>
<td>disease activity index</td>
</tr>
<tr>
<td>DCF-DA</td>
<td>dichlorofluorescein diascetate</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sulphate sodium</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>Hb</td>
<td>hemoglobin</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>HIFs</td>
<td>hypoxia-inducible factors</td>
</tr>
<tr>
<td>HO-1</td>
<td>heme oxygenase-1</td>
</tr>
<tr>
<td>HOCl</td>
<td>hypochlorous acid</td>
</tr>
<tr>
<td>H-PGDS</td>
<td>hematopoietic-PGDS</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>ICAM 1</td>
<td>intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<td>IBD</td>
<td>Inflammatory bowel diseases</td>
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<tr>
<td>IRFs</td>
<td>interferon-regulatory factor</td>
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<tr>
<td>Keap1</td>
<td>Kelch-like ECH association protein 1</td>
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<td>KCl</td>
<td>potassium chloride</td>
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<td>L-PGDS</td>
<td>lipocalin-PGDS</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LXR</td>
<td>liver X receptor</td>
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<tr>
<td>MEF</td>
<td>mouse embryonic fibroblasts</td>
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<tr>
<td>MerTK</td>
<td>c-mer proto-oncogene tyrosine kinase</td>
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</table>
MgCl₂  magnesium chloride
MMPs  matrix metalloproteases
MPO  myeloperoxidase
NAC  N-acetyl-L-cysteine
NF-κB  Nuclear factor-kappa B
NO  nitric oxide
Nrf2  nuclear factor E2-related factor 2
pcDNA  plasmid construct DNA
PBS  heme oxygenase-1
PE  phycoerythrin
PGE₂  prostaglandin E₂
PGI₂  prostaglandin I₂
PI  propidium iodide
PMA  phorbol 12-myristate 13-acetate
PMNs  polymorphonucleocytes
PMSF  phenylmethylsulfonyl fluoride
PPARs  peroxisome proliferator-activated receptors
PPAR-γ  peroxisome proliferator-activated receptor-gamma
RT-PCR  reverse transcription-polymerase chain reaction
RXR  retinoid X receptor
PVDF  polyvinylidene difluoride
SD  standard deviation
SDS  sodium dodecyl sulfate
STATs  signal transducers and activators of transcriptions
siRNA  small interfering RNA
TauCl  taurine chloramine
Tim4  T-cell immunoglobulin and mucin-domain-containing molecule
TNF-α  tumor necrosis factor alpha
VCAM 1  vascular cell adhesion molecule 1
ZnPP IX  zinc protoporphyrin IX
15d-PGJ₂  15-Deoxy-Δ₁₂,₁₄-prostaglandin J₂
Chapter I

Novel endogenous pro-resolving mediators: a new therapeutic potential in the management of chronic inflammatory disorders
1. Introduction

Acute inflammation is a physiologically essential response to protect the host against microbial infection and tissue injury (Shinohara and Serhan 2016). Despite its critical function in host protection, acute inflammation should be properly resolved to avoid chronic and systemic inflammation responsible for the pathogenesis of many prevalent diseases such as cancer, obesity and inflammatory bowel diseases (IBD) (Fig. 1-1) (Serhan et al., 2007). It is characterized by activation of inflammatory cells, alterations in vascular permeability and production of pro-inflammatory mediators including cytokines, chemokines, lipid mediators, steroids and growth factors (Serhan et al. 2008). The harmful stimuli, including microbial peptide and lipid mediators, act as chemoattractants to recruit polymorphonuclear cells (PMNs) to the inflamed site, where they phagocytose invading microbes or cellular debris. Recruited PMNs release the toxic contents of their granules, which contain degradative enzymes, reactive oxygen species (ROS) and reactive nitrogen species (RNS), to kill trapped microbes or degrade cellular debris (Nathan 2006). However, PMNs not only eliminate invading harmful stimuli, but can also damage host tissues and amplify acute inflammatory signals if persistently released (Bian, Guo et al. 2012). Once activated PMNs undergo apoptosis, macrophages accumulate in the inflamed tissue. They release factors that prevent further trafficking of PMNs, and then engulf apoptotic neutrophils or cellular debris. The process of clearing apoptotic cells is also an important step in preventing tissue necrosis and chronic inflammation and is active process controlled by the endogenous mediators during inflammation (Fadok, Bratton et al. 1998). Upon termination of phagocytosis,
macrophages exit the inflamed site by lymphatic drainage, completing resolution of inflammation (Serhan et al., 2007). Thus, the successful completion of inflammatory response in the early stage leads to the return to non-inflammatory status, the process known as resolution of inflammation (Serhan, 2005; Spite, 2010).

The cellular and molecular events involved in the acute inflammation have been unraveled, but much less is known about the mechanisms underlying resolution of inflammation. Resolution of inflammation had been considered as a passive process, which accompanies a decrease in pro-inflammatory mediators such as prostaglandins (PGs) and leukotrienes (LTs) (Serhan et al., 2002). However, in recent years, new lipid mediators have been suggested as active modulators of resolution of inflammation. These include lipoxins, resolvins, marexins, and protectins, resolution of inflammation is accelerated (Serhan, et al. 2000).

The typical therapeutic treatment of chronic inflammatory disease and inflammation-associated cancer primarily have aimed to inhibit the production of proinflammatory chemical mediators, such as TNF-α, but in many cases such approaches are not effective (Lawrence et al., 2002). The proresolving lipid mediators not only dampen inflammation but also promote to restore tissue homeostasis after acute inflammation. Thus, understanding the role of endogenous anti-inflammatory and pro-resolving lipid mediators for resolving inflammation is now recognized as a promising therapeutic strategy for preventing and treating inflammatory disorders.
2. Resolution of inflammation

2.1 The process of inflammation

Acute inflammation is a protective reaction against infection or tissue injury. Upon encountering inflammatory insults, neutrophils recruited to the inflamed site are activated and undergo oxidative burst, a critical event in the host defense. This leads to overproduction of reactive oxygen species (ROS) with which the injurious agent is eliminated by the neutrophils. Subsequently, the activated neutrophils undergo apoptosis, and then are removed by the macrophages which are also recruited at sites of inflammation immediately following the neutrophil infiltration. Furthermore, macrophages target uncleared inflammatory pathogens remaining in the inflammatory environment. Upon phagocytosis, macrophages are stimulated to generate high levels of anti-inflammatory and pro-resolving mediators, which eventually promote resolution of
inflammation by activating macrophages to leave the inflamed site via the nearest lymph nodes (Serhan, 2008; Medeiros, 2009; Michlewska, 2009).

The process of resolution is actively controlled by a number of endogenous anti-inflammatory and pro-resolving mediators. A physiologic process termed ‘lipid mediator class switching’ in eicosanoid production, from LTs and PGs to lipoxins is required for transition from inflammation to resolution (Levy, 2001; Schwab, 2006; Medzhitov, 2008). Novel endogenous pro-resolving lipid mediators have been identified in resolving exudates by liquid chromatography with tandem mass spectrometry (Arita et al., 2005). These signaling molecules have important roles in orchestrating the resolution of acute inflammation.

2.2 Endogenous proresolving mediators from omega-3 polyunsaturated fatty acids; resolvins, protectins, and maresins

Endogenous pro-resolving mediators biosynthesized from the omega-3 polyunsaturated fatty acids (PUFAs) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) during self-limited inflammatory responses exert diverse biological effects (Levy et al., 2001).

Resolvins

Resolvins derived from EPA or DHA are endogenously produced during the resolution phase of inflammation. EPA is converted to the E-series resolvins (RvE) by acetylated COX-2 and 5-LOX during inflammation when endothelial cells interact with
leukocytes. DHA is converted to the D-series resolvins (RvD), which is catalyzed by either acetylated COX-2 or 15-LOX and sequentially by 5-LOX. (Serhan et al., 2011). Resolvins possess potent multiple protective effects against inflammation. These endogenous mediators inhibit not only neutrophil recruitment and proinflammatory gene expression but also promote clearing apoptotic cells by macrophages and lymphatic removal of phagocytes from the inflamed site, thereby facilitating the resolution of colitis, ischemia-reperfusion-induced kidney damage and peritonitis. (Hong, 2003; Arita, 2005; Duffield, 2006; Schwab, 2007; Sun, 2007; Isobe, 2013).

**Protectins**

Protectins (NPD1/PD1) derived from DHA are biosynthesized by enzymatic epoxidation and hydrolysis. protectin D1, a representative member of protectins, proceeds via 15-LOX-catalyzed epoxide intermediates. The protectins have important roles in anti-inflammatory and protective actions. Like resolvins, the protectins also regulate chemokine/cytokine production and promote lymphatic removal of phagocytes. Protectin D1 promotes T-cell apoptosis, reduces retinal and corneal injury and stroke damage, and improves corneal wound healing (Ariel, 2005; Marcheselli, 2010; Kenchegowda, 2013; Belayev, 2017).

**Maresins**

Maresins (macrophage mediators in resolving inflammation) are formed from DHA during the resolution phase by macrophages. Maresin 1 (MaR1) is produced via
oxidation of endogenous DHA by 12-lipoxygenase. MaR1 was also obtained from synthetic 7,14-dihydroxy-docosa-4Z,8Z,10,12,16Z,19Z-hexaenoic acid prepared by total organic synthesis (Buckley et al., 2014). This new molecule acts directly on macrophages. MaR1 regulates neutrophil infiltration as well as enhancement of clearing apoptotic neutrophils by human macrophage, thereby stimulating tissue regeneration and subsequently promoting resolution of inflammation (Serhan, 2012; Buckley, 2014; Gong, 2015; Dalli, 2016).

### 2.3 Novel endogenous mediator from eicosanoids

Endogenous mediators derived from omega-3 polyunsaturated fatty acids (PUFAs) including resolvins, protectins, and maresins promote resolution of inflammation, reduction of pain, clearance of microbes, and promotion of tissue generation (Buckley, et al., 2014). Recently, attention has been focused on eicosanoids biosynthesis in inflammation, which are key factors, and their function in the physiological regulation of acute inflammation. Eicosanoids are formed as a consequence of the enzymatic oxygenation of arachidonic acid and related polyunsaturated fatty acids (PUFAs) by cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) enzymes, or via non-enzymatic free radical mechanisms (Dennis et al., 2015).

**Lipoxins**

Lipoxins serve as ‘stop signals’ for infiltration of leukocytes, such as PMNs and eicosanoids and also promote recruitment of macrophages into inflamed sties (Serhan
Lipoxins are LOX-derived eicosanoids, derived enzymatically from arachidonic acid. During the initiation of inflammation, arachidonate-derived eicosanoids should switch from prostaglandins and LTs to lipoxins, which activate infiltration of mononuclear cells without stimulating activation of proinflammatory pathways and inhibit further neutrophil infiltration into an inflamed area (Levy, et al., 2001). Within the inflammatory stimuli, the activated neutrophils undergo apoptosis. Lipoxins signal macrophages to enhance their uptake of apoptotic neutrophils, contributing to resolution of inflammation. Lipoxins are also potent anti-inflammatory mediators which act as local autacoids to stimulate resolution of inflammation within human tissues and in animal disease models (Norling et al., 2010). These molecules have the specific proresolving actions by limiting PMN recruitment, chemotaxis and adhesion to the site of inflammation (Morris et al., 2009).

**Electrophilic cyclopentenone PGs**

Cyclooxygenase (COX) has two isoforms referred to as COX-1 and COX-2 and mainly catalyzes the synthesis of PGs. COX-1, is constitutively expressed in most tissue, where it maintains physiological processes (Allison et al., 1992), whereas COX-2 induced by inflammatory stimuli, hormones and growth factors is considered to have pro-inflammatory properties (Herschman et al., 1996). However, some studies have questioned the pro-inflammatory role of COX-2. The administration of the selective COX-2 inhibitor exacerbated gastrointestinal inflammation induced by 2,4,6-Trinitrobenzenesulfonic acid (TNBS) in rats as well as delayed the resolution of gastric
ulcers induced by acetic acid in mice (Reuter, 1996; Shigeta, 1998). Likewise, inhibition of COX-2 significantly exacerbated inflammation in a murine peritonitis (Gilroy DW et al., 1999). COX-2-derived cyclopentanone PGs are thought to enhance resolution of inflammation. Some cyclopentanone PGs reversed the increases in inflammatory cell counts induced by a COX-2 inhibitor (Gilroy, 1999; Kim, 2017). Thus, COX-2 is considered to have a dual role in the inflammatory process, initially stimulating onset of inflammation and later contributing to resolution of inflammation (Fig. 1-2).

**Fig. 1-2. COX-2 has a dual role in the inflammatory process**

15-Deoxy-Δ12,14-PGJ2 (15d-PGJ2), a representative J-series cyclopentenone PG, is generated as a consequence of a series of dehydration of PGD2. 15d-PGJ2 is characterized by the presence of a reactive α, β-unsaturated carbonyl group present in cyclopentenone ring. Through Michael addition reaction, 15d-PGJ2 forms a covalent adduct with a cysteine thiol group of intracellular regulatory proteins, such as nuclear factor-kappa B (NF-κB), signal transducer and activator of transcription 3 (STAT3), hypoxia-inducible factors (HIFs) and the activator protein 1 (AP-1), enabling them to inhibit their pro-inflammatory effects (Fig. 1-3) (Kim and Surh 2006).
15d-PGJ$_2$ has been shown to modulate conserved cysteine residues of NF-κB as well as elements of the upstream kinase complex such as IκB kinase, thereby inhibiting pro-inflammatory and anti-apoptotic gene expression. 15d-PGJ$_2$ controls the balance of cytokines and chemokines that regulate not only leukocyte trafficking during acute inflammation but also the efflux of macrophages to draining lymphatics. 15d-PGJ$_2$ regulates pro-inflammatory gene expression through macrophage activation and also induces leukocyte apoptosis through a caspase-dependent mechanism. 15d-PGJ$_2$ inhibits NF-κB-mediated expression of vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 in endothelium and the inducible enzymes inducible nitric oxide synthase and COX-2 and NF-κB-induced apoptosis of CD4$^+$ T cells (Khoshnan, 2000; Lawrence, 2002; Migita 2005).

**Taurine chloramine (TauCl)**

Taurine is one of the most abundant free amino acids in the cytosol of neutrophils. It plays important roles in several essential biological processes, such as osmoregulation,
membrane stabilization, calcium mobilization and immunity (Huxtable 1992). Taurine stored in neutrophils is chlorinated to form TauCl. It reacts stoichiometrically with hypochlorous acid (HOCl), a strong antibacterial oxidant produced from hydrogen peroxide (H₂O₂) by the myeloperoxidase activity of the activated neutrophils in the presence of chloride ion. This results in production of TauCl which is released to the surrounding inflammatory tissue when apoptotic neutrophil undergoes apoptosis. TauCl acts as a local autacoid in the inflamed tissues. TauCl has anti-inflammatory and pro-resolving properties. TauCl inhibits the activation of NF-κB in macrophages, and then reduces the production of pro-inflammatory mediators, such as nitric oxide, tumor necrosis factor, interleukin (IL)-6 and IL-8, thereby exerting the anti-inflammatory effect (Kim and Kim 2005). Furthermore, TauCl has been reported to have microbicidal activity due to its antioxidant and anti-inflammatory properties (Nagl, Hess, 2000; Nagl, 2001; Gruber, 2017). TauCl has also been shown to activate nuclear factor E2-related factor 2 (Nrf2) and thereby stimulating resolution of inflammation by regulating the transcriptional induction of antioxidant enzyme or several scavenger receptors responsible for efferocytosis (Kim et al., 2015).

3. A new therapeutic strategies in the management of chronic inflammatory disorders

Current therapeutic and preventive strategies for the prevention and treatment of the chronic inflammatory disorders have focused on ‘block or inhibit’ accumulation of particular mediators involved in exacerbated inflammation such pro-inflammatory PGs
and cytokines. However, such approaches may provoke some adverse effects (Baillie and Digard 2013). Chronic blockages of COX also lead to some undesirable side effects in gastrointestinal inflammation (Allison et al., 1992). Therefore, a new therapeutic strategy in the management of inflammation is to balance the inflammatory response, and focuses on activating or intensifying cellular processes such as prostaglandin synthesis switching, macrophage polarization, induction of neutrophil apoptosis, and efferocytosis.

3.1 A switch in prostaglandin synthesis

Lipid class switching in eicosanoid production from pro-inflammatory PGs and LTs to lipoxin and specialized proresolving mediators (SPMs) including resolvins, protectins and maresins has been considered as one of essential processes for resolution to be initiated (Levy, 2001; Bagga, 2003; Serhan, 2003). Furthermore, the anti-inflammatory cyclopentenone PGs are generated to resolve inflammation. They limit not only the extent of inflammation, but also increase tissue remodeling and repair to return to normal physiological state; in other worlds, a switch in PG synthesis from PGE\textsubscript{2} to cyclopentenone PGs such as 15d-PGJ\textsubscript{2} and its precursor PGD\textsubscript{2} is also actively regulated during resolution of inflammation (Rajakariar, 2006; Trivedi, 2006; Rajakariar, 2007). Gilroy et al. demonstrated a shift from PGE\textsubscript{2} to PGD\textsubscript{2} and 15d-PGJ\textsubscript{2} in in resolving exudates, which stimulated resolution of inflammation by their immunomodulatory and anti-inflammatory effects to switch off inflammatory response. Thus, the development of therapeutic strategies based on facilitating PG switching into PGD\textsubscript{2} and 15d-PGJ\textsubscript{2} is
a new area of pharmacology (Fig. 1-4).

![Diagram: Prostaglandin switching](image)

**Fig. 1-4. Prostaglandin switching is required for resolution of inflammation**

### 3.2 Macrophage phenotype switching; M1 to M2

Macrophages are key regulators of inflammatory response (Wynn and Vannella 2016). Macrophages have been classified into two main types; either classically activated (M1) macrophages or alternatively activated (M2) macrophages (Serhan and Savill 2005). The M1 macrophages are characterized by the production of pro-inflammatory cytokines and ROS or RNS, which have a strong microbicidal and tumoricidal activity. In contrast, the M2 macrophages possess anti-inflammatory and pro-resolving properties, which are thought to be involved in enhancement of phagocytic activity, tissue remodeling and tumor progression (Gordon and Martinez 2010). A diverse of transcription factors including STATs, interferon-regulatory factor, NF-κB,
AP-1, proliferator-activated receptor-gamma and cAMP-responsive element-binding protein is involved in M1 or M2 macrophages polarization, which can be converted into each other depending on their specific microenvironment (Liu et al., 2014). Notably, a switching from M1 to M2 macrophages is required for resolution of inflammation (Fig. 1-5). M2 macrophages have a key role in resolution of inflammation in diabetes, atherosclerosis and infection by various pathogens; inhibition of inflammation, clearance of tissue debris and apoptotic cells and stimulation of angiogenesis (Mallat, 2001; Kosteli, 2010; Hoeve, 2012). Functional M1 to M2 switching might be one of therapeutic strategies for the management of inflammatory diseases (Nofer, 2007; Odegaard, 2007; Martinez, 2009; Tabas, 2010; Lopez-Castejon 2011).

Fig. 1-5. M2 polarization is required for resolution of inflammation

3.3 Induction of neutrophil apoptosis and efferocytosis

Resolution of inflammation is an active process that requires inhibition of further leukocyte recruitment and elimination of leukocytes from inflamed sites. Extravasation and emigration of neutrophils are required for host defense against invading pathogens. Following clearance of pathogens, infiltrated neutrophils undergo apoptosis, which is removed by macrophages through the process of efferocytosis (Savill et al., 2002) (Fig. 1-6). Recently, timely induction of neutrophil apoptosis has been considered as a critical
process in resolving inflammation. Accelerated neutrophil apoptosis has significant pathological consequences such as infection and autoimmune diseases (Courtney, 1999; Allen, 2005; Elbim, 2009). On the other hand, delayed neutrophil apoptosis causes inflammatory diseases such as chronic pulmonary obstructive disease (COPD), sepsis and rheumatoid arthritis (Ertel, 1998; Wong, 2009; Brown, 2012). Indeed, anti-inflammatory and pro-resolving lipid mediators such as RvD1 and lipoxin A4 promote the resolution of inflammation through induction of neutrophil apoptosis (El Kebir, 2008; El Kebir, 2012). Subsequently, apoptotic neutrophils are eliminated by macrophages. Macrophages recognize apoptotic cells which have phosphatidylserine exposed on their surface for efferocytosis. Phosphatidylserine serves as the ‘eat-me’ signal upon apoptosis and is recognized by several scavenger receptors on the surface of macrophages, which facilitates the ingestion of apoptotic cells. After efferocytosis, macrophages produce anti-inflammatory mediators such as TGF-β and IL-10 that help terminate inflammation (Fadok et al., 1998). Impaired efferocytosis causes accumulation of dead cells, provoking pathological consequences including diabetes, arthritis atherosclerosis and COPD (O'Brien, 2002; Nathan, 2010; Hamon, 2014).
4. Conclusion and future directions

Acute inflammation is a protective mechanism, but excessive uncontrolled inflammatory responses cause chronic inflammation, resulting in chronic inflammatory disorders and inflammation-induced cancer. It is clear that inflammatory response contains key checkpoints that regulate its onset and resolution. Resolution of inflammation is an actively orchestrated by specific pro-resolving mediators involved in diverse cellular responses; apoptosis of PMNs, macrophage polarization, efferocytosis and exfiltration of inflammatory cells, all of which are regulated by a concomitant increase of pro-resolving mediators. Biosynthesis of pro-resolving mediators is a key event to drive the resolution of inflammation. Defects in endogenous pro-resolving pathways will undoubtedly predispose to the development of chronic inflammation. Therefore, the identification of novel pro-resolving mediators and understanding mechanisms underlying their pro-resolving effects are required.

Classical drugs for chronic inflammatory diseases mostly aim to block or inhibit particular pro-inflammatory mediators involved in tissue damage. They have been shown to have therapeutics effects, while they have undesirable side effect referred to as ‘resolution-toxic’ in that complete inhibition of inflammatory response is equally detrimental for resolution of inflammation. Considering pro-resolving mediators balance the inflammatory response and boost endogenous pro-resolving pathways, resolution-
based therapeutics are expected as good alternatives for side effects caused by the conventional anti-inflammatory drugs. Therefore, to more successfully develop therapeutic potential in the management of chronic inflammatory disorders, it would be better to identify novel endogenous pro-resolving mediators which can be exploited for drug discovery.

5. References


"Circulating mediators in serum of injured patients with septic complications inhibit neutrophil apoptosis through up-regulation of protein-tyrosine phosphorylation." J Trauma 44(5): 767-775; discussion 775-766.


PURPOSE OF THE STUDY

Nonresolving inflammation progress from acute to chronic inflammation, contributing to the development of chronic inflammatory disorder including obesity, IBD and cancer. Therefore, timely resolution of inflammation is important for preventing chronic inflammatory diseases. Classical drugs for chronic inflammatory diseases mostly aim to block or inhibit particular pro-inflammatory mediators involved in tissue damage. They have been shown to have therapeutics effects, while provoking undesirable side effect. Specific lipid mediators such as resolvin series, maresin, lipoxin, which are generated endogenously during resolution of inflammation, have therapeutic potential in management of chronic inflammatory. 15d-PGJ$_2$ and TauCl are also produced upon inflammatory insults. In the present study, the pro-resolving effect of these mediators and the underlying molecular mechanisms were investigated.
Chapter II

15-Deoxy-\(\triangle^{12,14}\)-prostaglandin J\(_2\) promotes resolution of intestinal inflammation
1. Abstract

Uncontrolled macrophage activation has been implicated in pathogenesis of inflammatory bowel diseases (IBD) as it can cause failure to resolve gut inflammation. 15-Deoxy-\(\Delta^{12,14}\)-prostaglandin J\(_2\) (15d-PGJ\(_2\)), one of endogenous lipid mediators formed from arachidonic acid during inflammatory process, has been reported to terminate inflammation due to its anti-inflammatory and pro-resolving properties. However, molecular mechanisms underlying the pro-resolving effects of 15d-PGJ\(_2\) on intestinal inflammation had not been investigated. In the present study, 15d-PGJ\(_2\) was found to be generated during resolution phase of intestinal inflammation and to participate in the resolution of inflammation. When biosynthesis of 15d-PGJ\(_2\) was inhibited by a hematopoietic prostaglandin D synthase (HPGDS) activity inhibitor, intestinal inflammation was barely resolved. 15d-PGJ\(_2\) injected into the peritoneum of mice exerted the resolution of dextran sulfate sodium (DSS)-induced colitis. 15d-PGJ\(_2\) treatment reduced the number of neutrophils and M1 macrophages, while increased the proportion of M2 macrophages. In particular, 15d-PGJ\(_2\) treatment significantly decreased the proportion of macrophages expressing IL-6. In another experiment, the conditioned media (CM) from human THP-1-differentiated macrophages exposed to bacterial lipopolysaccharide (LPS) with or without 15d-PGJ\(_2\) are used. When CM of from LPS-treated macrophages plus 15d-PGJ\(_2\) were co-incubated with normal colon epithelial cells, phosphorylation of STAT3 was significantly inhibited, compared with normal colon epithelia cells with CM from macrophages stimulated with LPS alone. Taken together, these findings clearly indicate that 15d-PGJ\(_2\), endogenously generated
from arachidonic acid during inflammatory process, promotes resolution of intestinal inflammation by regulating macrophage polarization.

**Keywords**
15-Deoxy-Δ^{12,14}-prostaglandin J₂, M1 macrophage, M2 macrophages, IL-6, STAT3, Resolution of intestinal inflammation

2. Introduction
Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn’s disease (CD), are chronic inflammatory disorders of the intestine characterized by inflammation of the gastrointestinal tract typically with a relapsing and remitting clinical course. (Kaser, 2010; Cader, 2013). In IBD, the immune response is initiated by interaction among the components of the innate immune system, including macrophages and dendritic cells (Saleh and Trinchieri 2011). The relapsing inflammatory disorder is associated with sustained overproduction of pro-inflammatory cytokines in the intestinal lamina propria (MacDonald et al., 2011). In particular, mucosal macrophages represent the largest population of mononuclear phagocytes in the intestine and play an important role in the mucosal immune system (Lee et al., 1985). Inappropriate macrophage activation has been failure to resolve acute inflammation in the gut (Neurath 2014). Intriguingly, in the inflamed gut of patients with IBD, activated macrophages produce significantly more pro-inflammatory cytokines such as TNF-α, IL-23 and IL-6 (Sanchez-Munoz et al., 2008).
The transcription factor STAT3 is known to play a key role in inflammation, and is activated by IL-6 family cytokines (Pickert et al., 2009). Prolonged or excessive inflammatory response by STAT3 activation contributes to chronic inflammation, resulting in inflammatory disorders including intestinal cancer. Phosphorylation of STAT3 facilitates its dimerization, and the dimerized STAT3 then translocates into nucleus, where it regulates transcription of genes involved in inflammation (Levy and Darnell 2002). Elevated STAT3 phosphorylation is found in the murine dextran sulphate sodium (DSS)-induced colitis as well as in inflamed colon tissue from IBD patients (Aggarwal, 2009; Bromberg, 2009). The genome-wide association studies of CD have revealed that STAT3 gene is one of the susceptibility loci in IBD (Barrett et al., 2008). Moreover, persistent activation of STAT3 results in IBD and colorectal cancer (Corvinus, 2005; Li, 2012). However, the mechanism underling STAT3 activation in the context of pathogenesis of IBD is still not clear.

The process of resolution is actively controlled by endogenous anti-inflammatory and pro-resolving mediators (Serhan and Levy 2003). Prostaglandins (PGs) are also key modulators of inflammation. Their production changes during the different stages of inflammation. In the initiation of inflammation, prostaglandin E2 (PGE2) and prostaglandin I2 (PGI2) are generated to initiate inflammation, while cyclopentenone PGs are produced to terminate inflammation in the late phase of inflammation. Gilroy et al. demonstrated a shift from PGE2 to cyclopentenone PGs, which promoted resolution of inflammation (Gilroy et al., 1999).

15-Deoxy-Δ^{12,14}-prostaglandin J2 (15d-PGJ2), a representative J-series
cyclopentenone PGs, plays important roles in regulating inflammation through inhibition of pro-inflammatory mediators. 15d-PGJ2 inhibits nuclear factor-kappa B (NF-κB)-mediated expression of vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 in endothelium (Khoshnan, 2000; Lawrence, 2002; Migita, 2005). 15d-PGJ2 regulates pro-inflammatory gene expression through macrophage activation and also induces leukocyte apoptosis through a caspase-dependent mechanism (Hortelano, 2000; Rossi, 2000). Although 15d-PGJ2 has been reported to protect against acute inflammatory tissue injury (Mochizuki et al., 2005), detailed molecular mechanisms underlying the pro-resolving effect of 15d-PGJ2 in intestinal inflammation remained unexplored. Here, I report that 15d-PGJ2 stimulates resolution of DSS-induced colitis through regulating macrophage polarization.

3. Materials and Methods

Animals
Male C57/BL6 mice (5 weeks of age) were purchased from Central Lab. Animal, Inc. (Seoul, South Korea). They were acclimated for 7 days with tap water and a pelleted basal diet before the start of the experiments. The animals were housed in plastic cages under controlled conditions of temperature, humidity, and light.

DSS-induced colitis
To induced colitis, mice were given drinking water containing 2.5% DSS (MW 36,000-
50,000; MP Biomedicals, USA) for 7 days. For evaluation of colitis resolution, mice were given normal drinking water for additional days. 15d-PGJ2 (2 mg/kg/day) suspended in 10% DMSO in phosphate-buffered saline (PBS) or vehicle was administered intraperitoneally. Mice were killed by cervical dislocation and their colorectal parts were taken out, cut longitudinally, and washed with PBS. For histopathological examination, the distal section of colon tissues was fixed in 10% buffered formalin, whereas another portion was flash-frozen in lipid nitrogen and kept at -70°C for Western blot analysis.

**Tissue lysis and protein extraction**

Colon tissues were homogenized in an ice-cold lysis buffer [150 mM NaCl, 0.5% Triton-X 100, 50 mM Tris–HCl (pH 7.4), 20 mM ethylene glycol tetra-acetic acid (EGTA), 1 mM dithiothreitol (DTT), 1 mM and protease inhibitors, 1 mM phenylmethylsulfonylfluoride (PMSF), and ethylenediaminetetraacetic acid (EDTA) -free cocktail tablet], followed by a periodical vortex for 30 min at 0°C. The lysates were centrifuged at 14,000 rpm for 15 min at 4°C. The supernatants were collected and stored at -70°C until use.

**Western blot analysis**

For Western blot analysis, the total protein concentration was quantified by using the bichinconinic acid (BCA) protein assay kit (Pierce). Cell lysates (20-50 μg protein) were mixed and boiled in a sodium dodecyl sulfate (SDS) sample buffer for 5 min before 8%–
17% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). They were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Gelman Laboratory). The blots were blocked in 5% fat-free dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature. The membranes were incubated for 12–24 h at 4°C with dilutions of primary antibodies for HPGDS, LPGDS, COX-2 (Cayman Chemical), p-STAT3, STAT3, (Cell Signaling Technology), and actin (Sigma Aldrich). The membranes were washed, followed by incubation with 1:3000 dilution of respective HRP-conjugated secondary antibodies (rabbit or mouse) (Zymed Laboratories) for 1 h, and again washed with TBST. Protein expressed was visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) and LAS-4000 image reader (Fugi film) according to the manufacturer’s instructions.

Immunohistochemical analysis

The dissected colon tissues were prepared for immunohistochemical (IHC) analysis of the expression patterns of p-STAT3. Four-μm sections of 10% Formalin-fixed paraffin-embedded tissues were cut on silanized glass slides and deparaffinized three times with xylene and rehydrated through graded alcohol bath. The deparaffinized sections were heated by using microwave and boiled twice for 6 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. To diminish nonspecific staining, each section was treated with 3% hydrogen peroxide and 4% peptone casein blocking solution for 15 min. For the detection of respective protein expression, slides were incubated with p-STAT3 antibodies at room temperature for 40 min in Tris-buffered saline containing 0.05%
Tween 20, and then developed using respective horseradish peroxidase (HRP)-
conjugated secondary antibodies (rabbit) EnVision™ System (DAKO EnVision™+
System, ). The peroxidase binding sites were detected by staining with 3,3-
diaminobenzidine tetrahydrochloride (DAKO EnVision™+ System, ). Finally,
counterstaining was performed using Mayer’s hematoxylin.

**Isolation of murine lamina propria immune cells from colonic tissues**

Isolation of colonic lamina propria cells was performed according to the protocol
(Weigmann, Tubbe et al. 2007). Entire colons from each group were longitudinally cut
and washed to remove feces. They were then cut into 1 cm pieces, followed by
incubation with predigestion solution containing 5 mM EDTA (Sigma-Aldrich, St Louis,
MO, USA), 0.145 mg/mL DTT (Sigma-Aldrich) for 20 min at 37°C on a shaking
platform. After removal of EDTA by three washes in PBS and passing through a cell
strainer (100 µm), the suspension of epithelial, subepithelial and villus cells was
removed. The remaining colon pieces including lamina propria cells and muscle layer
cut by using scissors, and then incubated in digestion media containing of collagenase D
(Roche), of DNase I (Sigma) and of dispase II (Roche) for 25 min at 37°C on a shaking
platform. After digestion, the lamina propria cells were enriched using Percoll density
gradient centrifugation. The resulting cells were then used for flow cytometry analysis.

**Flow cytometry**

Lamina propria cells were stained with a cocktail of antibodies to various markers
including CD45, F4/80, CD11B, CD86, CD206, and GR-1 for 30 mins at 4 °C. For intracellular staining, cells were stained with cell surface markers, and incubated Fixation/Permeabilization working solution to each sample for fixation and permeabilization according to the manufacturer’s instructions. The cells were stained with antibody specific to IL-6 for 1h at 4°C. All of those antibodies were purchased from Biolegend (San Diego, CA, USA) or eBioscience (San Diego, CA, USA). Cells were analyzed using FACS Aria III Flow Cytometer (BD, Franklin Lakes, NJ, USA), and the results were analyzed using FlowJo software.

**Preparation of bone marrow derived macrophages (BMDMs)**

Bone marrow (BM) cells were isolated from femurs and tibias of C57/BL6 mice. BMDMs are differentiate from BM cells in RPMI1640 medium supplemented with 10% FBS and 20 ng/ml M-CSF for 7 days. On day 7, after removing non-adherent cells, BMDMs were detached from the plate using Accutase (Innovative Cell Technologies, San Diego, CA, USA) and used for the further experiments. To induces M1 macrophages, BMDMs were induced by LPS (100 ng/ml) for 4 hours.

**Real-time RT-PCR**

Total RNA was isolated from RAW264.7 cells using TRizol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. RNA was then used to synthesize complementary DNA (cDNA) and further analyzed by using RealHelix qPCR kit.
(Nanohelix) with Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems) following the protocol described elsewhere.

4. Results

15d-PGJ\(_2\) is produced during resolution of intestinal inflammation

To investigate whether 15d-PGJ\(_2\) is endogenously produced during resolution of intestinal inflammation, the DSS-induced murine colitis model was used. Mice were given drinking water containing 2.5\% DSS for 7 days, which causes severe damage to colonic epithelium triggering intestinal inflammation (Park et al., 2015). This was followed by exposure to normal drinking water for an additional 15 days to allow DSS-induced intestinal inflammation to subside. Mice began to lose body weight after 5 days of DSS treatment, and the body weight loss continued 3 days after termination of DSS administration. Mice began to regain body weight from day 11. The weight of the mice in intestinal inflammation was completely restored after 15 days of drinking normal water (Fig. 2-1A). DSS-treated mice suffered from injury and acute colitis with massive colon ulceration, crypt damage, and severe inflammation. Based on the severity of stool consistency and rectal bleeding, DSS-induced pathogenic conditions were scored from 0 to 3. The sum was given into a form of the disease activity index (DAI). Mice treated with DSS exhibited serious symptoms with liquid stool and large amount of rectal bleeding. DAI score reached a maximal value on day 10, and decreased during resolution phase (Fig. 2-1B). In addition, mice exposure to DSS displayed shortening of colon length, but it was recovered after 15 days (Fig. 2-1C). I also performed histological
evaluation of dysplasia in the colonic crypts using hematoxylin and eosin (H&E) staining. While exposure to DSS completely disrupted the architecture of colonic mucosa, DSS-induced severe disruption of the colonic architecture was recovered 15 days later (Fig. 2-1D).

15d-PGJ₂ is normally derived from arachidonic acid during inflammatory response (Ricciotti and FitzGerald 2011). Two key enzymes involved in 15d-PGJ₂ biosynthesis are COX-2 and PGDS. There are two distinct isoforms of PGDS, hematopoietic-PGDS (H-PGDS) and lipocalin-PGDS (L-PGDS). The expression of COX-2, H-PGDS and L-PGDS was measured during onset and resolution of inflammation. COX-2 expression was gradually increased at the beginning of inflammation, and decreased thereafter during resolution of inflammation (Fig. 2-1E). Interestingly, H-PGDS expression was significantly upregulated, while L-PGDS expression was not affected in the resolution phase of inflammation (Fig. 2-1E). To determine whether 15d-PGJ₂ is produced during the resolution of inflammation or not, the level of 15d-PGJ₂ was measured and quantified by LC/MS/MS analysis. As illustrated in (Fig. 2-1F), 15d-PGJ₂ was detected in resolution phase of intestinal inflammation, compared with control group.

**Inhibition of H-PGDS exacerbates DSS-induced colitis.**

H-PGDS metabolizes COX-derived PGH₂ to PGD₂ and 15d-PGJ₂. The increased expression of H-PGDS responsible for production of 15d-PGJ₂ plays an essential role in regulating cellular immune response (Rajakariar, Hilliard et al. 2007, Gandhi, Kaushal et al. 2011). In order to determine the pro-resolving effects of 15d-PGJ₂, I used HQL-79,
a commonly used H-PGDS inhibitor (Aritake, Kado et al. 2006, Virtue, Masoodi et al. 2015). The mice were treated with 2.5% DSS in drinking water *ad libitum* for 10 days. HQL-79 (30 mg/kg) was given via gavage during the resolution phase (Fig. 2-2A). Under these conditions, HQL-treated mice exhibited a significant decrease in survival (Fig. 2-2B). In addition, inhibition of HPGDS activity severely compromised resolution of intestinal inflammation. HQL-79 administration hampered the recovery of body weight (Fig. 2-2C) and augmented the severity of diarrhea and rectal bleeding (Fig. 2-2D). Likewise, DSS-induced shortening of the colorectal length was barely recovered, compared with mice without HQL-79 treatment (Fig. 2-2E). These data demonstrate that 15d-PGJ$_2$ plays an important role in resolution of intestinal inflammation.

**15d-PGJ$_2$ attenuates DSS-induced colitis in mice**

To assess the effect of 15d-PGJ$_2$ on the resolution of DSS-induced colitis, the mice were treated with 2.5% DSS in drinking water *ad libitum* for 7 days. Mice were divided into two groups. One group of mice was allowed to resolve intestinal inflammation by providing drinking water for additional 4 days after termination of DSS administration. Mice in the other group were given intraperitoneally 15d-PGJ$_2$ (2 mg/kg) during the 4-day recovery daily (Fig. 2-3A). 15d-PGJ$_2$ treatment significantly improved body weight recovery, compared with mice provided water alone (Fig. 2-3B). In addition, 15d-PGJ$_2$ administration ameliorated the severity of diarrhea and rectal bleeding (Fig. 2-3C) and shortening of the colorectal length (Fig. 2-3D) caused by DSS.
15d-PGJ\textsubscript{2} regulates macrophage polarization during resolution of intestinal inflammation

Macrophages are key regulators controlling the onset and the resolution of acute inflammation (Wynn and Vannella 2016). Macrophages have been classified into two types; classically activated (M1) macrophages and alternatively activated (M2) macrophages. The M1 macrophages produce pro-inflammatory cytokines and reactive nitrogen and oxygen intermediates. In contrast, the M2 macrophages have anti-inflammatory and pro-resolving properties, which are involved in enhancement of phagocytic activity and tissue remodeling (Gordon and Martinez 2010). To assess the capability of 15d-PGJ\textsubscript{2} to resolve the intestinal inflammation, lamina propria cells (CD45\textsuperscript{+}) were isolated from colon tissue as illustrated in Fig. 2-4A. The proportion of infiltrated leukocytes (CD45\textsuperscript{+}) was significantly increased during the inflammation phase, but decreased during the resolution phase (Fig. 2-4B). Next, I determined the proportion of neutrophils by flow cytometry. The number of neutrophils (CD45\textsuperscript{+} CD11b\textsuperscript{+} Gr-1\textsuperscript{-} F4/80\textsuperscript{-}) in lamina propria of 15d-PGJ\textsubscript{2}-treated mice was decreased significantly, compared with that from mice given normal water alone. However, while the proportion of macrophages (CD45\textsuperscript{+} CD11b\textsuperscript{+} Gr-1\textsuperscript{-} F4/80\textsuperscript{+}) was increased during inflammatory phase, that of colonic macrophages was reduced during the resolution phase regardless of 15d-PGJ\textsubscript{2} administration (Fig. 2-5A). To assess the effects of 15d-PGJ\textsubscript{2} on macrophage polarization, I measured the number of M1 and M2 macrophages by flow cytometry. Interestingly, 15d-PGJ\textsubscript{2} administration reduced the proportion of M1 macrophages (CD45\textsuperscript{+} CD11b\textsuperscript{+} Gr-1\textsuperscript{-} F4/80\textsuperscript{-} CD86\textsuperscript{-}) (Fig. 2-5B), while significantly increased the
number of M2 macrophages (CD45⁺ CD11b⁺ Gr-1⁻ F4/80⁺ CD206⁺) during resolution of intestinal inflammation (Fig. 2-5C). To further examine the effect of 15d-PGJ₂ on M1 polarization, 15d-PGJ₂-treated bone marrow derived macrophages (BMDMs) were stimulated with LPS, which has been known to induce the expression of M1 macrophage markers. LPS upregulated the mRNA expression of M1 markers including iNOS, IL-1β and IL-12. 15d-PGJ₂ (10 μM) significantly inhibited the expression of M1 markers in LPS-stimulated macrophages (Fig. 2-5D). In addition, I investigated whether 15d-PGJ₂ could affect M2 macrophage polarization. 15d-PGJ₂ alone could not significantly induce expression of M2 macrophage markers, but synergistically increased M2 macrophages polarization when macrophages were co-treated with IL-4, a well-known inducer of M2 makers (Fig. 2-5E). These findings suggest that 15d-PGJ₂ regulates leukocyte trafficking and renders macrophages polarized into the M2 type while it suppresses manifestation of M1 macrophages.

15d-PGJ₂ inhibits IL-6-induced STAT3 activation in resolution of intestinal inflammation

Increased proinflammatory cytokine production is a hallmark of intestinal inflammation (Garside, 1999; Egger, 2000). In particular, Overproduction of IL-6 has been reported to trigger chronic intestinal inflammation and subsequently colon cancer. In order to determine whether the pro-resolving effects of 15d-PGJ₂ is responsible for inhibition of IL-6 in macrophages, mice were given 2.5% DSS in drinking water for 7 days, followed by normal water given for another 6 days to allow spontaneous recovery
from acute intestinal inflammation. DSS-induced intestinal inflammation was significantly ameliorated in 15d-PGJ2-treated mice, as indicated by the improvement of body weight loss, DAI score and shortening of colon length compared with mice given water alone (Fig. 2-6). Next, macrophages expressing IL-6 expression are selectively identified by flow cytometry. 15d-PGJ2 lowered the proportion of macrophages expressing IL-6 (CD45+ CD11b+ Gr-1+ F4/80+ IL-6+) during resolution of intestinal inflammation (Fig. 2-7A). To further determine whether 15d-PGJ2 could reduce the proportion of macrophages expressing IL-6, macrophages were first stimulated with LPS, which has been known to induce IL-6 expression. 15d-PGJ2 treated macrophages exhibited a decrease in IL-6 mRNA (Fig. 2-7B) and protein levels (Fig. 2-7C). STAT3 is one of representative transcription factors that plays a key role in intestinal inflammation and is mainly activated by IL-6. Next, I determined whether the inhibition of IL-6 expression in macrophage by 15d-PGJ2 could block STAT3 activation in CCD 841 CoN colon epithelial cells, THP-1 human leukemic monocytes were differentiated into macrophages by treatment with 10 nM of phorbol 12-myristate 13-acetate PMA. THP-1-differentiated macrophages were then stimulated with LPS for 6 hours in the absence or presence of 15d-PGJ2, and conditioned media (CM) were collected. When normal colon epithelial cells were incubated with CM from LPS-stimulated macrophages, there was a robust induction of STAT3 phosphorylation at 1 hour (Fig. 2-7D). However, when CM from LPS-stimulated macrophages in the presence of 15d-PGJ2 were co-incubated with normal colon epithelial cells, phosphorylation of STAT3 was significantly inhibited (Fig. 2-7E).
15d-PGJ2 induces resolution of intestinal inflammation through inhibition of DSS-induced STAT3 activation and COX-2 expression in mouse colon

I also examined the effects of 15d-PGJ2 treatment on STAT3 activation. 15d-PGJ2 treatment exerted a significant inhibitory effect on DSS-induced phosphorylation of STAT3. It also abolished the DSS-induced expression of COX-2 in the mouse colon, one of major proinflammatory enzymes in the inflamed colonic mucosa of patients with IBD (Kolios, Valatas et al. 2004, Wang and Dubois 2010) (Fig. 2-8A). Furthermore, immunohistochemical analysis revealed the inhibition of DSS-induced the phosphorylation of STAT3 (Fig. 2-8B) and DSS-induced the expression of COX-2 by 15d-PGJ2 (Fig. 2-8C). Moreover, expression of pro-inflammatory cytokines such as IL-6, IL-1β, INF-γ, IL-23, IL-12 and IL-17 was suppressed by 15d-PGJ2 treatment in the resolution phase of inflammation (Fig. 2-8D). Taken together, these results suggest that 15d-PGJ2 exerts resolution of intestinal inflammation through inhibition of IL-6-induced STAT3 activation in a DSS-induced colitis model.
Fig. 2-1. 15d-PGJ$_2$ is produced during resolution phase of DSS-induced murine colitis

Mice were administrated with drinking water containing 2.5% DSS for 7 days, followed by normal water for another 15 days. “No DSS” indicates mice without DSS administration. (A) Gradual change of body weight (B) Disease activity index (DAI) based on the severity of stool consistency and rectal bleeding was scored daily during the experiment period. (C) The comparison of the colon length in the inflammation phase and resolution phase of intestinal inflammation. (D) Representative distal colon sections stained with hematoxylin and eosin (H&E) (E) COX-2, H-PGDS and L-PGDS levels in colon tissue of the inflammation phase and resolution phase of inflammation were determined by immunocytochemical analysis. (F) The induction of 15d-PGJ$_2$ in colon tissue was measured by LC/MS/MS. All data represent mean ± S.D. (n=3), *p<0.05, **p<0.01, and ***p<0.001
Fig. 2-2. Inhibition of 15d-PGJ2 biosynthesis provokes the failure in resolution of intestinal inflammation

(A) Mice were given with drinking water containing 2.5% DSS for 7 days, followed by normal water for another 10 days. HQL-79 (30 mg/kg), a H-PGD inhibitor, was administrated orally for 10 days every other day. (B) The survival rate, (C) the change in the body weight and (D) DAI score were monitored every day during the entire experiment period. The survival rate of the mice was expressed by using Kaplan–Meier plot. (D) The colon length was measured when mice were sacrificed. All data represent mean ± S.D. (n=3), *p<0.05, **p<0.01, and ***p<0.001
A

Group 1
No DSS
0 0
H2O 2.5% DSS
11 (Days) 7 (Days)

Inflammation
Resolution

Group 2
0 0
2.5% DSS 2.5% DSS
7 7
H2O 2.5% DSS
11 (Days) 11 (Days)

Inflammation
Resolution

Group 3
0 0
2.5% DSS 2.5% DSS
7 7
H2O 2.5% DSS
11 (Days) 11 (Days)

Inflammation
Resolution

Group 4
0 0
2.5% DSS 2.5% DSS
7 7
15d-PGJ2 (2 mg/kg) daily i.p injection
11 (Days) 11 (Days)

Inflammation
Resolution

B

Percent of weight (%)

0 2.5% DSS 7 H2O 11 (Days)

Inflammation
Resolution

C

DMS score

0 2.5% DSS 7 H2O 11 (Days)

Inflammation
Resolution

D

No DSS
DSS
-15d-PGJ2 + 15d-PGJ2
Inflammation phase
Resolution phase

No DSS
DSS
-15d-PGJ2 + 15d-PGJ2
Inflammation phase
Resolution phase

Cafeteria length (cm)

0 2.5% DSS 7 H2O 11 (Days)

Inflammation
Resolution

** Seoul National University **
Fig. 2-3. 15d-PGJ₂ ameliorates pathological symptoms by stimulating resolution of intestinal inflammation

(A) Mice were administrated with drinking water containing 2.5% DSS for 7 days, followed by normal drinking water for another 4 days. 15d-PGJ₂ (2 mg/kg) or vehicle was intraperitoneally injected to mice every day during resolution phase of inflammation. (B) The change of body weight was monitored and (C) DAI was scored. (D) On day 7 (inflammation phase) and day 11 (resolution phase), the colon length was measured. All data represent mean ± S.D. (n=3), *p<0.05, **p<0.01, and ***p<0.001
Fig. 2-4. Lamina propria immune cells from the colon of mice are collected

Lamina propria immune cells were isolated from the colon of mice in each group. (A) Lamina propria immune cells were stained with CD45, CD11b, Gr-1, F4/80, CD206, and CD86 antibodies and then the cells of interest were gated according to the manufacturer’s protocol. The number and the percentage of total immune cells (CD45+) were determined by flow cytometry. (B) Lamina propria immune cells were isolated from the colon of DSS-treated mice, and the number and the percentage of total immune cells (CD45+) was determined by flow cytometry. All data represent mean ± S.D. (n=3), *p<0.05 and ***p<0.001
Fig. 2-5. 15d-PGJ₂ regulates leukocyte infiltration and macrophage polarization

Lamina propria immune cells were isolated from the colons of DSS-treated mice, collected on day 7 and day 11 as described in Materials and Methods. (A-C) The percentage of neutrophils (CD45⁺ CD11b⁺ Gr-1⁺ F4/80⁻), macrophages (CD45⁺ CD11b⁺ Gr-1⁻ F4/80⁻), M1 macrophages (CD45⁺ CD11b⁺ Gr-1⁻ F4/80⁺ CD86⁺), and M2 macrophages (CD45⁺ CD11b⁺ Gr-1⁻ F4/80⁺ CD206⁺) was determined by flow cytometry. The graphs show the percentage of each cell population. (C) BMDMs were treated with LPS (100 ng/ml) for 4 h in the absence or presence of 15d-PGJ₂ (10 μM). The mRNA levels of M1 markers including inos, IL-1β and IL-12 were measured by real-time PCR. (D) BMDMs were co-treated with IL-4 and 15d-PGJ₂ for 4 h. The mRNA levels of M2 markers including, Arg-1, CD36, TGF-β, and YM-1 were determined by real-time PCR. All data represent mean ± S.D. (n=3), *p<0.05, **p<0.01, and ***p<0.001
Fig. 2-6. 15d-PGJ2 stimulates resolution of DSS-induced colitis

(A) Mice were given with drinking water containing 2.5% DSS for 7 days, followed by normal drinking water for another 6 days with or without 15d-PGJ2 treatment every day during the resolution phase of inflammation. (B) The change of body weight was monitored and (C) DAI were scored. The colon length was measured on day 7 (inflammation phase) and day 13 (resolution phase). (D) Representative distal colon sections stained with hematoxylin and eosin (H&E). Magnifications, ×200. All data represent mean ± S.D. (n=3), **p<0.01 and ***p<0.001
Fig. 2-7. Inhibition of IL-6 expression in macrophages by 15d-PGJ\(_2\) treatment abrogates STAT3 activation

Intestinal macrophages were collected on day 7 and day 13 as described in Fig. 2-6. The percentage of macrophages expressing IL-6 (CD45\(^+\) CD11b\(^+\) Gr-1\(^+\) F4/80\(^+\) IL-6\(^+\)) was measured by flow cytometry. (A) BMDMs were treated with LPS (100 ng/ml) in the absence or presence of 15d-PGJ\(_2\) (10 \(\mu\)M). The mRNA and protein levels of IL-6 were determined by real-time PCR (A) and flow cytometry, respectively (B). THP-differentiated macrophages were stimulated by LPS for 6 hours, and then conditioned media (CM) from LPS-treated macrophages were collected. The CM were incubated with CCD 841 CoN colon epithelial cells for indicated time periods (C) and for 1 h (D). p-STAT3 (Tyr705) levels were determined by immunoblot analysis. Actin was used as an equal loading control for normalization. All data represent mean ± S.D. (n=3), **\(p<0.01\) and ***\(p<0.001\)
A

<table>
<thead>
<tr>
<th></th>
<th>Inflammation phase</th>
<th>Resolution phase</th>
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<tbody>
<tr>
<td>No DSS</td>
<td>pStat3(V705)</td>
<td>- 15d-POJ5</td>
</tr>
<tr>
<td></td>
<td>STAT3</td>
<td>+ 15d-POJ5</td>
</tr>
<tr>
<td></td>
<td>COX-2</td>
<td></td>
</tr>
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<td></td>
<td>Actin</td>
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B

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<tr>
<th></th>
<th>Inflammation phase</th>
<th>Resolution phase</th>
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<tbody>
<tr>
<td>No DSS</td>
<td></td>
<td>- 15d-POJ5</td>
</tr>
<tr>
<td>DSS</td>
<td></td>
<td>+ 15d-POJ5</td>
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C

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<th>Inflammation phase</th>
<th>Resolution phase</th>
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<tbody>
<tr>
<td>No DSS</td>
<td></td>
<td>- 15d-POJ5</td>
</tr>
<tr>
<td>DSS</td>
<td></td>
<td>+ 15d-POJ5</td>
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D

- No DSS
- DSS + H2O with intraperitoneally 16d-POJ5 injection
- DSS + H2O

COX-2: Green
DAPL: Blue
Fig. 2-8. 15d-PGJ₂ inhibits DSS-induced expression of STAT3 and COX-2 in mouse colon

Colon tissue were collected on day 7 and day 13 as described in Fig. 2.6 (A) The levels of p-STAT3 (Tyr705) and COX-2 were determined by immunoblot analysis. Actin was used as an equal loading control for normalization. (B) Immunohistochemical detection of pSTAT3 (brown spots) in colon tissue. To verify the expression of COX-2 in colon tissue, (C) immunocytochemical analysis was conducted using anti-COX-2 antibody. (D) mRNA expression of pro-inflammatory cytokines including IL-6, IL-1β, INF-γ, IL-23, IL-12 and IL-17 was determined in colon tissue by real time-PCR.
5. Discussion

Acute inflammation is a protective reaction against microbial infection and tissue injury (Shinohara and Serhan 2016). Despite its critical function in host defense, acute inflammation should be properly resolved to avoid chronic inflammation (Serhan et al., 2007). In this study, I investigated the effects of 15-dPGJ2 on intestinal inflammation by using a murine DSS-induced colitis model which mimics human IBD (Kaser, 2010; Perse, 2012). The process of onset and resolution of intestinal inflammation is regulated by endogenous checkpoints to avoid progression to chronic inflammation, indicative of the existence of contrasting endogenous processes that is actively orchestrated by endogenous pro-inflammatory and pro-resolving mediators (Serhan, 2007; Fiocchi 2008). PGs are key lipid mediators/modulators derived from arachidonic acid that have diverse functions in resolution of inflammation. It has been reported that some of pro-inflammatory PGs released in inflamed tissue amplify acute inflammation, whereas anti-inflammatory PGs, including cyclopentenone PGs, are generated to promote resolution of inflammation (Rajakariar, 2006; Trivedi, 2006; Rajakariar, 2007; Scher, 2009). Although 15d-PGJ2, a representative J-series cyclopentenone PG, was detected in self-resolving exudates, the molecular mechanism underlying pro-resolving effects of 15d-PGJ2 on intestinal inflammation has not been investigated. In the present study, I identified increased production of 15d-PGJ2 in colonic tissue during the resolution phase of DSS-induced colitis. Notably, the inhibition of 15d-PGJ2 impaired resolution of intestinal inflammation. From these findings, it is speculated that 15d-PGJ2 promotes resolution of intestinal inflammation by switching off inflammatory response.
Resolution of inflammation is an active process that requires inhibition of further leukocyte recruitment and elimination of leukocytes from inflamed sites. As part of the gut inflammatory response, neutrophils recruited to the inflamed site are activated and undergo oxidative burst, a critical event in the host defense. This leads to overproduction of reactive oxygen species (ROS) with which the neutrophils kill and eliminate the infectious pathogens (Serhan, 2008; Medeiros. 2009; Michlewsksa, 2009). Based on their function, infiltrated neutrophils are essential for host defense against invading pathogens, but excessive neutrophil infiltration is limited, and neutrophils appropriately undergo apoptosis. Accelerated neutrophil apoptosis have severe pathological consequences such as infection and autoimmune diseases (Courtney, 1999; Allen, 2005; Elbim, 2009). On the other hand, delayed neutrophil apoptosis causes chronic inflammatory disorders, such as sepsis, chronic pulmonary obstructive disease (COPD), and rheumatoid arthritis (Ertel, 1998; Wong, 2009; Brown 2012). It has been reported that 15d-PGJ$_2$ promotes endothelia cell apoptosis as well as granulocyte apoptosis (Bishop-Bailey, 1999; Ward, 2002). Furthermore 15d-PGJ$_2$ induces synoviocyte apoptosis and suppresses arthritis in rats (Ward et al., 2002). Our present results indicate that 15d-PGJ$_2$ inhibits neutrophil infiltration during resolution phase of inflammation. I speculate that 15d-PGJ$_2$ might regulate not only excessive neutrophils infiltration into inflamed area, but also neutrophils apoptosis, thereby promoting the resolution of intestinal inflammation.

IBD is characterized by persistent infiltration of inflammatory immune cells such as neutrophils and macrophages within the gut and in the circulation (Hanauer 2006). In particular, intestinal macrophages contribute to the gut homeostasis by balancing pro-
inflammatory and anti-inflammatory cytokines (Kuhl et al., 2015). The M1 macrophages produce pro-inflammatory cytokines and oxidative stress, resulting in initiation and progression of inflammation, while the M2 macrophages have anti-inflammatory and pro-resolving properties, promoting resolution of inflammation (Gordon and Martinez 2010). It has been reported that phenotypic shift from M1 macrophages to the M2 macrophage plays a critical role in the resolution of inflammation and tissue repair (Tabas, 2010; Shalhoub, 2011). The number of M1 macrophages which release pro-inflammatory cytokines is increased in the colonic tissue of IBD patients (Ferretti, 1994; Berndt 2007; Tabas, 2010). Our present study demonstrates that 15d-PGJ2 counteracts excessive inflammatory responses and promotes resolution of inflammation through regulating macrophage polarization.

A diverse transcription factors including STATs, interferon-regulatory factor, NF-κB, activator protein 1 (AP-1), proliferator-activated receptor-gamma (PPAR-γ) and cAMP-responsive element-binding protein are involved in M1 or M2 macrophages polarization (Liu et al., 2014). As reported by other investigators, PPAR-γ plays a key role in inflammation by regulating both M1 and M2 polarization. PPAR-γ negatively regulated NF-κB and AP-1 signaling, resulting in inhibition of M1 macrophage polarization. In contrast, PPAR-γ activation is also involved in M2 polarization by induction of arg-1 and CD36 expression (Ricote, 1998; Liu, 2014). In our previous study, 15d-PGJ2 was found to stimulate efferocytosis of macrophages through induction of CD36 expression, thereby resolving zymosan A-induced peritonitis. 15d-PGJ2 is a natural ligand that activates PPAR-γ and has been reported to inhibit pro-inflammatory signaling as well as
exert anti-inflammatory activity (Jiang, 1998; Gilroy, 1999). Based on these findings, I speculate that regulation of macrophage polarization by 15d-PGJ2 is thought to mediate PPAR-γ activation. Thus, it would be worthwhile to investigate whether PPAR-γ can regulate intestinal inflammation.

Overproduction of IL-6 has been considered to occur as a result of failure in resolution of intestinal inflammation. It is clearly defined that failure of intestinal resolution leads to IBD (Abraham, 2009; Neurath, 2014). IL-6 production by lamina propria macrophages and CD4+T cells was increased in experimental colitis and in patients with IBD (Atreya, 2000; Kai, 2005). Blockade of IL-6 signaling with monoclonal antibodies was effective in suppressing chronic intestinal inflammation in mouse models (Atreya, 2000; Yamamoto, 2000). However, detailed molecular mechanisms responsible for resolution of intestinal inflammation induced by IL-6 remain poorly understood.

In the present study, I notably found that intraperitoneal injection of exogenous 15d-PGJ2 expedited the resolution of DSS-induced colitis by lowering the proportion of macrophages expressing IL-6. I also found that IL-6 released from macrophages increased STAT3 activation, and this effect was significantly inhibited by 15d-PGJ2 treatment. 15d-PGJ2 contains a highly reactive α, β-unsaturated carbonyl present in the cyclopentenone ring and hence can form covalent adduct with cysteine thiol groups of diverse intracellular regulatory proteins, such as hypoxia-inducible factors, NF-κB, STAT3, and AP-1, thereby inhibiting their pro-inflammatory effects (Straus, 2001; Kim, 2006). Due to its characteristic cyclopentenone moiety, it is likely that 15d-PGJ2 can also directly inhibit STAT3 activation through Michael addition reaction, thus altering the
pro-inflammatory function of this transcription factor.

In conclusion, 15d-PGJ₂, generated during inflammatory process, promotes resolution of intestinal inflammation. It is evident that macrophage polarization is one of the important mechanisms responsible for resolution of intestinal inflammation by 15d-PGJ₂. 15d-PGJ₂ decreased the proportion of M1 macrophages and macrophages expressing IL-6, which accounts for its inhibition of STAT3 activation. In addition, 15d-PGJ₂-induced M2 macrophages polarization is speculated to contribute to the resolution of intestinal inflammation (Fig. 4-9). Unresolved inflammation caused by inappropriate macrophage polarization can result in inflammation-associated disorders like inflammatory bowel disease, diabetes, and arthritis (Korns, 2011; Liu, 2014). Regulating macrophages polarization is a crucial process in resolving inflammation, preventing the development of chronic inflammatory disorders. In this context, 15d-PGJ₂, generated during resolution of intestinal inflammation, is considered one of the novel endogenous proresolving molecules, and this lipid mediator might have a therapeutic potential in the management of IBD associated with macrophage dysfunction.
Fig. 2-9. A proposed mechanism underlying the pro-resolving effects of 15d-PGJ₂ on intestinal inflammation

15d-PGJ₂, is endogenously generated from arachidonic acid by COX-2 and subsequently HPGDS during resolution of intestinal inflammation. 15d-PGJ₂ promotes intestinal inflammation by regulating macrophage polarization.
6. References


Fiocchi, C. 2008. 'What is "physiological" intestinal inflammation and how does it differ from "pathological" inflammation?', *Inflamm Bowel Dis*, 14 Suppl 2: S77-8.

hematopoietic prostaglandin D2 synthase in macrophages is mediated through the activation of peroxisome proliferator-activated receptor (PPAR) gamma', *J Biol Chem*, 286: 27471-82.


Scher, J. U., and M. H. Pillinger. 2009. 'The anti-inflammatory effects of prostaglandins',


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that is mediated via inhibition of I kappa B alpha degradation using a peroxisome proliferator-activated receptor-gamma-independent mechanism', *J Immunol*, 168: 6232-43.


Chapter III

15-Deoxy-\(\triangle^{12,14}\)-prostaglandin J\(_2\) exerts pro-resolving effects through Nrf2-induced expression of CD36 and heme oxygenase-1
1. Abstract

The macrophage-mediated phagocytic removal of apoptotic neutrophils, termed efferocytosis, is required for the resolution of inflammation to prevent chronic inflammation. 15-Deoxy-\(\Delta^{12,14}\)-prostaglandin \(J_2\) (15d-PGJ\(_2\)), one of terminal products of the COX-2-mediated arachidonic acid cascade, has been reported to stimulate the anti-inflammatory action of macrophages. However, the pro-resolving effects of 15d-PGJ\(_2\) remained largely unexplored. In the present study, 15d-PGJ\(_2\) has been shown to participate in the resolution of acute inflammation. 15d-PGJ\(_2\) injected into the peritoneum of mice facilitated the resolution of zymosan A-induced peritonitis. 15d-PGJ\(_2\) administration reduced the number of total leukocytes, and attenuated polymorphonuclear leukocytes infiltration. Furthermore, 15d-PGJ\(_2\) increased the proportion of macrophages engulfing apoptotic neutrophils, a process called efferocytosis. In addition, when the thioglycollate-elicited mouse peritoneal macrophages were stimulated with 15d-PGJ\(_2\), their efferocytic activity was amplified. In another experiment, RAW264.7 murine macrophages exposed to 15d-PGJ\(_2\) conducted phagocytic clearance of apoptotic cells to a greater extent than the control cells. Under these conditions, expression of CD36 and heme oxygenase-1 (HO-1) was enhanced along with increased accumulation of the nuclear factor E2-related factor 2 (Nrf2) in the nucleus. Knockdown of Nrf2 abolished 15d-PGJ\(_2\)-induced expression of CD36 and HO-1, and silencing of CD36 and HO-1 attenuated 15d-PGJ\(_2\)-induced efferocytosis. Moreover, peritoneal macrophages isolated from Nrf2-null mice failed to upregulate 15d-PGJ\(_2\)-induced expression of CD36 and HO-1, and to mediate efferocytosis. Unlike
15d-PGJ₂, its non-electrophilic analogue 9,10-dihydro-15d-PGJ₂ lacking the α,β-unsaturated carbonyl group could not induce CD36 expression and efferocytosis. Taken together, these findings clearly indicate that 15d-PGJ₂ facilitates resolution of inflammation by inducing Nrf2-induced expression of CD36 and HO-1 in macrophages.

**Keywords**

15-Deoxy-Δ12,14-prostaglandin J₂, Efferocytosis, Nuclear factor E2-related factor, Heme oxygenase-1, Resolution of inflammation

**2. Introduction**

Acute inflammation is the cellular immune response essential for effective host defense against invading pathogens and other insults (Haworth and Buckley 2007). However, failure in resolving acute inflammation in a timely manner can result in various disorders, such as arthritis, inflammatory bowel disease and cancer. Prostaglandins (PGs) are key mediators/modulators of inflammation, and the profile of their production changes during the different stages of inflammation. In the initiation of acute inflammation, prostaglandin E₂ (PGE₂) and prostaglandin I₂ (PGI₂) are rapidly produced from membrane-derived arachidonic acid by cyclooxygenase-2 (COX-2) activity of leukocytes (Serhan 2014). These PGs regulate blood flow required for stimulating the migration of polymorphonuclear neutrophils (PMNs) to the site of injury, comprising the first line of defense against infection. Upon encountering inflammatory insults, PMNs undergo oxidative burst and apoptosis. The macrophage-mediated phagocytic removal
of apoptotic neutrophils, a process called ‘efferocytosis’, is required for resolution of inflammation to prevent tissue necrosis and chronic inflammation (Serhan, 2008; Buckley, 2014).

Recently, attention has been focused on a physiologic process termed ‘lipid mediator class switching’ in the resolution phase of inflammation, which promotes production of anti-inflammatory and pro-resolving eicosanoids (Levy et al., 2001).

15-Deoxy-\(\Delta^{12,14}\)-prostaglandin \(J_2\) (15d-PGJ\(_2\)), a representative J-series cyclopentenone PG, is one of the terminal products of the COX-2-mediated arachidonic acid cascade (Straus and Glass 2001). 15d-PGJ\(_2\) controls the balance of cytokines and chemokines that regulate leukocyte trafficking during acute inflammation as well as the efflux of macrophage to draining lymphatics, facilitating the resolution of inflammation (Rajakariar et al., 2007). It has been demonstrated that 15d-PGJ\(_2\) inhibits nuclear factor-kappa B (NF-\(\kappa\)B)-mediated expression of vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 in endothelium (Migita and Morser 2005). Although 15d-PGJ\(_2\) has been reported to protect against acute inflammatory tissue injury (Mochizuki et al., 2005), detailed molecular mechanisms underlying the pro-resolving effect of this cyclopentenone PG remain largely unresolved.

Nuclear factor E2-related factor 2 (Nrf2) is the transcription factor regulating the expression of cytoprotective proteins involved in the cellular defense against oxidative stress and inflammatory damage (Saw et al., 2014). Activation of Nrf2 triggers anti-inflammatory response, while disruption of Nrf2 signaling exacerbates inflammation. Among the proteins regulated by Nrf2, heme oxygenase-1 (HO-1) has an essential
function in physiological stress response to infection and inflammatory insults (Alcaraz, et al., 2003). Thus, HO-1 plays a fundamental role in the anti-inflammatory response in atherosclerosis (Orozco et al., 2007), cardiac ischemia-reperfusion injury (Hinkel et al., 2015) and arterial hypertension (Wenzel et al., 2015).

The macrophages play important roles in the clearance of apoptotic neutrophils for complete resolution of inflammation. This is achieved by recognition of phosphatidylserine (PS) present in the outer leaflet of apoptotic cells. Blocking PS suppresses the engulfing capacity of macrophages (Asano, 2004; Kim, 2015), whereas overexpression of PS receptor or bridge molecules enhances phagocytosis (Liu, 2013; Tian, 2014). Scavenger receptors expressed in macrophages participate in the PS recognition and subsequent engulfment of apoptotic cells (Fadok et al., 2000). An example is CD36 that is a membrane glycoprotein present in several types of immune cells, including monocytes or macrophages (Park, et al., 2014). Here, I report that 15d-PGJ2 potentiates the efferocytic capability of macrophages through Nrf2-mediated upregulation of CD36 and HO-1.

3. Materials and Methods

Materials

15d-PGJ2, 9,10-dihydro-15d-PGJ2 and NS-398 were purchased from Cayman Chemical CO. (Ann Arbor, MI, USA). Bacterial lipopolysaccharide (LPS; Escherichia coli O111:B4) was obtained from Sigma-Aldrich (St Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM) medium, fetal bovine serum and antibiotics were purchased
from Gibco-BRL (Grand Island, NY, USA). Zymosan A, NAC and an antibody against actin were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Antibodies against Nrf2 and CD36 and ZnPP were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). YM-1 and Relm-α antibodies were supplied by Abcam (Cambridge, UK). HO-1 antibody was obtained from Stressgen Biotechnologies Co. (Victoria, BC, Canada). FITC-conjugated CD36 antibody was a product of eBioscience (San Diego, CA, USA). The anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies and laminB1 antibody were purchased from Zymed Laboratories (San Francisco, CA, USA). Polyvinylidene difluoride (PVDF) membranes were supplied from Gelman Laboratory (Ann Arbor, MI, USA). The enhanced Chemiluminescent (ECL) detection kit was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK).

**Zymosan-A-induced peritonitis**

C57BL/6 mice (8 weeks of age) were purchased from Central Lab Animal Inc. (Seoul, South Korea). Nrf2 knockout mice (C57BL6/129SV) were kindly supplied by Dr. Jeffrey Johnson of the University of Wisconsin-Madision. HO-1 knockout mice (BALB/c) were provided by Dr. Perrela MA (Harvard Medical Center). 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 (30 mg/kg) was administered intraperitoneally at 12 h before giving 15d-PGJ2 (2 mg/kg, suspended in 10% DMSO in PBS) or vehicle and mice were
sacrificed 6 h later. Peritoneal leukocytes were collected by washing with 3 ml of PBS containing 3 mM EDTA.

**Total and differential leukocyte counts**

Cells from peritoneal exudates were incubated for 1 h by Turk’s solution (0.01% crystal violet in 3% acetic acid) to eliminate red blood cells, and then the number of the total leukocytes was counted by the hematocytometer. For the differential leukocyte counts, a cytospin centrifuge was used to concentrate cells from peritoneal exudates onto microscope slides in a circle with 6 mm diameter. The cells were then subjected to Wright-Giemsa staining.

**Cell culture**

Murine macrophage RAW264.7 cells and human lymphoblastic Jurkat T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). RAW264.7 cells were cultured in DMEM and peritoneal macrophages, and Jurkat T cells were maintained in RPMI 1640 with 10% FBS, 100 mg/ml streptomycin and 100 U/ml penicillin in humidified 5% CO\textsubscript{2} at 37°C.

**Efferocytosis assay**

To assess the percentage of macrophages engulfing apoptotic neutrophils *in vivo*, peritoneal exudate cells from mice were labeled with allophycocyanin (APC)-conjugated F4/80-antibody (eBioscience, San Diego, CA, USA), permeabilized with 0.1%
Triton X-100, and then labeled with the fluorescein isothiocyanate (FITC)-conjugated Gr-1 antibody (eBioscience, San Diego, CA, USA). The proportion of macrophages engulfing apoptotic neutrophils (F4/80+/Gr-1+) was determined by flow cytometry or immunocytochemistry. A sterile irritant, thioglycollate has been used to enhance the yield of peritoneal macrophages. For measuring efferocytosis ex vivo, mice were administered intraperitoneally injection of thioglycollate medium (3%). After three days, peritoneal macrophages were collected from peritoneal exudates of thioglycollate-treated mice and incubated in six-well flat-bottomed microtiter plates for 24 h. Non-adherent cells were collected and incubated for additional 24 h to induce apoptosis. Adherent monolayer cells were co-incubated for 1 h with apoptotic non-adherent cells, which were mostly composed of neutrophils. Apoptotic neutrophils engulfed by macrophages was visualized under a Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan).

The annexin V-FITC kit uses annexin V conjugated with fluorescein isothiocyanate (FITC) to label phosphatidylserine sites on the membrane surface. To determine the efferocytic activity of macrophages in vitro, apoptotic Jurkat T cells were labelled with FITC-annexin-V. Apoptosis of Jurkat T cells was induced by serum withdrawal and UVB (180 mJ/cm²) irradiation, followed by incubation for 8 h at 37°C in an atmosphere of 5% CO₂. FITC-labelled Jurkat T cells were then co-incubated with RAW264.7 cells, and the proportion of RAW264.7 cells containing apoptotic Jurkat T cells (FITC-positive cells) was assessed by flow cytometry.

**Flow cytometry**
Peritoneal macrophages and RAW264.7 cells were stained with the FITC-conjugated – CD36 antibody for 20 min. After washing with PBS, Cells were analyzed using FACSCalibur™ Flow Cytometer (BD, Franklin Lakes, NJ, USA).

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was isolated from RAW264.7 cells using TRizol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Total RNA (1 µg) was reverse-transcribed with murine leukemia virus reverse. PCR for Nrf2, HO-1, CD36 and for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was carried out with each primer (forward and reverse, respectively): Nrf2, 5’-CCTCTGTCACCAAGCTCAAGG-3’ and 5’-TTCTGGGCGGCGACTTTATT-3’; HO-1, 5’-GTCTATGCCCCACTCTACTT-3’ and 5’-TGGAAACGATATCAAAGTG-3’; CD36, 5’-ATAATGGCAGACGACGC and 5’-CTGGATTCTGGAGCGTGAT-3’; GAPDH, 5’-TGTGAACGGATTTGGCCGTA-3’ and 5’-GGTCTCGCTCCTGGAAAGTG-3’. PCR products were resolved by 2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

**Immunocytochemistry analysis of Nrf2 and CD36**

RAW264.7 macrophages were placed in an eight-chamber plate and cultured in medium. The cells were incubated in the absence or presence of 15d-PGJ2 and then fixed with 4% buffered formalin solution (20 min). The cells were then washed in PBS (twice for 5 min each), permeabilized with 0.2% Triton X-100 (5 min), washed in PBS (twice for 5 min each), and stained with ethidium bromide.
each), and blocked with 10% BSA in TBST (30 min). Polyclonal rabbit anti-Nrf2 or anti–CD36 antibodies, diluted 1:100 in 10% BSA in TBST, was applied overnight at 4°C. This was followed by washing cells in PBS (twice for 5 min each) and then incubation for 1 h at room temperature with FITC-conjugated anti-rabbit IgG secondary antibody diluted at 1:1000 in 1% BSA–TBST. Propodium iodide (PI) was used as a nuclear marker. After washing (twice for 5 min each), cells were treated with PI. Nrf2 and CD36 were visualized under a microscope.

**Preparation of nuclear proteins and Western blot analysis**

Cytosolic extracts were obtained by suspending the cells in hypotonic buffer A [10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES; pH 7.9), 10 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethanesulfonyl fluoride (PMSF)] and Nondiet P-40 solution (0.1%). To obtain the nuclear fraction, the cell pellets were suspended in hypertonic buffer C [50 mM HEPES (pH 7.9), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% glycerol]. Whole cell extracts were prepared according to the manufacturer’s instructions by using the cell lysis buffer (10 x Cell Signaling Technology #9803). The protein concentration of the supernatant was measured by using the BCA reagents (Pierce, Rockford, IL, USA). Protein (30 µg) was separated by running through 7–10% SDS–PAGE gel and transferred to the PVDF membrane (0.22 mm thickness; Gelman Laboratory, Ann Arbor, MI, USA). To block the non-specific binding of proteins with primary antibodies, the blots were blocked with 5% nonfat dry
milk/TBST buffer (TBS containing 0.1% Tween-20) for 1 h at room temperature. The membranes were then incubated with the primary antibody suspended in 3% non-fat milk TBST buffer overnight at 4°C. The blots were rinsed three times with TBST buffer for 10 min each and incubation using appropriate secondary antibody coupled to horseradish peroxidase. Proteins tagged with specific primary antibodies were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

**Chromatin immunoprecipitation (ChIP) assay**

RAW264.7 cells were incubated with 15d-PGJ2 (5 μM) for 6 h. After harvesting the cells, the ChIP assay was conducted with normal rabbit IgG or anti-Nrf2 antibody. The following PCR primers were used: CD36 1A-ARE1 TGCTGAGTAAGACCATGGCA/TGA TCAGACATAGTTTCCCT, CD36 1A-ARE2 GTTTGGGACCACTTGCTTGA/GAGTAGGTCAGTTCCCTGGG

**Measurement of the intracellular ROS accumulation**

To measure the ROS production in macrophages in vivo, peritoneal exudate cells collected were labeled with APC-conjugated F4/80-antibody (eBioscience, San Diego, CA, USA) for flow cytometry or anti-mouse F4/80 antibody (Santa Cruz, CA, USA) for immunocytochemical analysis and exposed to 10 μM dichlorofluorescein diacetate (DCF-DA; Molecular Probes, Invitrogen, USA) for 30-min incubation. To assess the in vitro intracellular ROS accumulation, RAW264.7 cells were incubated with DMSO,
9,10-dihydro-PGJ$_2$ or 15d-PGJ$_2$ for 1 h, and washed with PBS, followed by exposure DCF-DA (10 µM). After 30-min incubation at 37°C, ROS accumulation was examined under microscope.

**Statistical analysis**

All data were expressed as means ± SD of at least three independent experiments, and statistical analysis for single comparison was performed using a One-way ANOVA. The criterion for statistical significance was *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

4. Results

15d-PGJ$_2$ potentiates the efferocytic activity of macrophages by modulating leukocyte trafficking in a murine peritonitis model

To investigate the cellular events related to resolution of inflammation in vivo, 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 normally reaches the maximum at approximately 12 h, and gradually decreases thereafter (Bannenberg, et al., 2005). When the number of total leukocytes was maximal, 15d-PGJ$_2$ (2 mg/kg) was administrated into peritoneum of mice. Six hours later, peritoneal leukocytes were collected. The total leukocyte counts in the peritoneal exudates of zymosan A plus 15d-PGJ$_2$-treated mice were decreased significantly, compared with those from mice given zymosan A alone (Fig. 3-1A). To determine the proportion of PMNs/neutrophils and monocytes in the peritoneal exudates, differential cell counting was conducted. While
the proportion of PMNs was increased, that of peritoneal monocytes was reduced in the mice challenged with zymosan A (Fig. 3-1B). 15d-PGJ2 administration attenuated zymosan A-induced increases in PMN counts, and restored the proportion of monocytes. All together, these findings indicate that systemic administration of 15d-PGJ2 facilitates resolution of zymosan A-induced inflammation in the peritoneum of mice.

In the next experiment, efferocytic activity of peritoneal macrophages was determined *in vivo*. Peritoneal cells with positive staining of both F4/80 (macrophage marker) and Gr-1 (neutrophil marker) were selectively identified by flow cytometry. The mice treated with zymosan A plus 15d-PGJ2 showed a significantly higher proportion of peritoneal macrophages engulfing apoptotic PMNs (F4/80^+^Gr-1^+^) than those challenged with zymosan A alone (Fig. 3-1C). I then performed *ex vivo* experiments to measure efferocytic activity of macrophages in the absence and present of 15d-PGJ2. Primary macrophages isolated from the murine peritoneal cavity are commonly used to produce pro-inflammatory cytokines and test anti-inflammatory agents (Turchyn et al., 2007). However, the yield of these peritoneal macrophages are relatively low. The recovery of macrophages from the peritoneal cavity can be improved by injecting mice with sterile Brewer thioglycollate broth although such elicitation results in a mild inflammatory response and can alter physiological properties of cells collected (Turchyn, 2007; Ray, 2010).

When the thioglycollated-elicited macrophages were treated with 15d-PGJ2 for 12 h, the ability of macrophages to take up apoptotic neutrophils was increased (Fig. 3-1D). 15d-PGJ2-induced efferocytic activity of macrophages was also assessed in in vitro
experiments. Annexin V conjugated with fluorescein isothiocyanate (FITC) was used to label PS sites on the membrane surface of apoptotic cells. RAW264.7 cells were co-incubated with FITC-annexin V-labelled apoptotic Jurkat T cells for 1 h. Representative bar graphs reflect fold changes in the proportion of macrophages engulfing FITC-annexin V stained-apoptotic cells. In 15d-PGJ2-treated RAW264.7 macrophages, their engulfment of apoptotic cells was potentiated (Fig. 3-1E). These findings suggest that 15d-PGJ2 regulates leukocyte trafficking and increases efferocytic activity of macrophages.

15d-PGJ2 is an important lipid mediator generated by COX-2 activity during the resolution of zymosan A-induced murine peritonitis

COX-2 is known to regulate PGs synthesis. In order to elucidate the role of COX-2 in experimentally induced peritonitis, NS-398 (10 mg/kg) which is a specific inhibitor of COX-2 was injected into peritoneum of mice 1 h prior to zymosan A administration. The total leukocyte counts in the peritoneal exudates obtained from zymosan A plus NS-398-treated mice were elevated, compared with those from mice given zymosan A alone. However, administration of 15d-PGJ2 reduced the total number of leukocytes in the peritoneal exudates (Fig. 3-2A). Likewise, administration of NS-398 to mice further increased the number of the neutrophils in the peritoneal exudates from zymosan A-treated mice, and this effect was attenuated by 15d-PGJ2 administration (Fig. 3-2B). In contrast, the NS-398 treatment lowered the proportion of macrophages, which was restored by 15d-PGJ2 (Fig. 3-2C).
15d-PGJ$_2$ upregulates CD36 expression in macrophages

Macrophages are essential for the resolution of acute inflammation. Macrophages release anti-inflammatory cytokines, some of which are involved in the resolution of inflammation. 15d-PGJ$_2$ treatment to RAW264.7 murine macrophage cells resulted in upregulated expression of anti-inflammatory cytokines, such as transforming growth factor-beta (TNF-α) and interleukin (IL)-10 (Fig. 3-3A). The 15d-PGJ$_2$-induced expression of anti-inflammatory cytokine gene expression was verified in human U937 promonocytic cells subjected to differentiation by phorbol 12-myristate 13-acetate (PMA) (Fig. 3-3B).

Besides producing anti-inflammatory and pro-resolving cytokines, macrophages express several different types of scavenger receptors involved in the removal of waste materials to avoid unnecessary tissue damage. CD36 is one such scavenger receptor responsible for recognizing apoptotic cells by macrophages (Greenberg, 2006; Ferracini, 2013). Mice treated with zymosan A plus 15d-PGJ$_2$ showed a higher proportion of peritoneal macrophages with elevated CD36 expression than those challenged with zymosan A alone (Fig. 3-4A). I also demonstrated that 15d-PGJ$_2$-treated macrophages exhibited a transient increase in CD36 mRNA transcript in time- (Fig. 3-4B), and concentration-dependent (Fig. 3-4C) manners. In addition, 15d-PGJ$_2$ treatment enhanced the CD36 protein level in thioglycollate-elicited peritoneal macrophages and RAW264.7 cells as measured by flow cytometry (Fig. 3-4D). Immunocytochemical analysis also revealed that there was increased accumulation of CD36 in RAW264.7 cells
treated with 15d-PGJ$_2$ (Fig. 3-4E). To examine whether 15d-PGJ$_2$-induced CD36 expression is essential for enhancement of efferocytosis, I utilized small interfering RNA (siRNA) against CD36. Silencing of CD36 abolished the capability of 15d-PGJ$_2$ to induce the efferocytic activity of macrophages (Fig. 3-4F).

15d-PGJ$_2$-induced CD36 expression is mediated through Nrf2 activation

The transcription factor Nrf2 has been shown to regulate the expression of CD36 (Ishii, 2004; Maruyama, 2008). Compared with animals challenged with zymosan A alone, mice treated with zymosan A plus 15d-PGJ$_2$ exhibited a further increase in the proportion of macrophages expressing Nrf2 expression (Fig. 3-5A). Moreover, the 15d-PGJ$_2$-treated RAW264.7 cells exhibited an elevated accumulation of Nrf2 protein in the nucleus as measured by immunoblot (Fig. 3-5B) and immunocytochemical (Fig. 3-5C) analyses. When the transcriptional expression of Nrf2 was silenced in RAW264.7 cells by transfecting them with siRNA, 15d-PGJ$_2$ was unable to induce CD36 expression at both transcriptional (Fig. 3-5D) and translational (Fig. 3-5E) levels. Likewise, the thioglycollate-elicited peritoneal macrophages of Nrf2 knockout mice were not responsive to 15d-PGJ$_2$ in terms of expressing CD36 mRNA (Fig. 3-5F) and protein (Fig. 3-5G). To ensure that Nrf2 could mediate 15d-PGJ$_2$-induced CD36 expression, I conducted a chromatin immunoprecipitation (ChIP) assay. As illustrated in Fig. 3-5H, 15d-PGJ$_2$ enhanced the binding of Nrf2 to the antioxidant response element (ARE) consensus sequence of the CD36 promoter region.
**Nrf2-induced upregulation of CD36 and HO-1 expression is essential for pro-resolving effects of 15d-PGJ2**

I attempted to verify the role of Nrf2 in 15d-PGJ2-induced efferocytosis by use of Nrf2 knockout mice. 15d-PGJ2 administration increased the proportion of monocytes, with concomitant reduction in the proportion of PMNs in the peritoneal exudates during resolution of zymosan A-induced peritonitis, but these alterations were dampened in Nrf2 knockout mice (Fig. 3-6A). In the next experiment, the thioglycollate-elicited peritoneal macrophages isolated from wild-type and Nrf2 knockout mice were treated with 15d-PGJ2 for 12 h. As shown in Fig. 3-6B, the peritoneal macrophages from Nrf2-deficient mice exhibited reduced efferocytic activity compared to those from wild-type mice upon 15d-PGJ2 treatment. Likewise, the siRNA knockdown of Nrf2 gene expression abrogated 15d-PGJ2-induced efferocytic activity of RAW264.7 macrophage cells (Fig. 3-6C). In contrast, overexpression of Nrf2 further enhanced the efferocytosis (Fig. 3-6D). Taken together, these results suggest that Nrf2 is crucial in 15d-PGJ2-induced resolution of inflammation.

HO-1, one of the major target proteins of Nrf2, has potent anti-inflammatory effects in macrophages (Lee, 2002; Alcaraz, 2003), and drives the phenotypic shift from M1 to M2 macrophages, facilitating resolution of inflammation (Huang, 2014; Naito, 2014; Tu, 2014). I found that 15d-PGJ2 increased HO-1 expression in RAW264.7 cells (Fig. 3-7A). The 15d-PGJ2-induced HO-1 expression was abrogated in Nrf2 silenced RAW264.7 cells (Fig. 3-7B) and thioglycollate-elicited peritoneal macrophages from Nrf2 knockout mice (Fig. 3-7C). In addition, 15d-PGJ2 induced not only HO-1 expression but also the
upregulation of some M2 macrophage markers at both transcriptional (Fig. 3-8A) and translational (Fig. 3-8B) levels. 15d-PGJ$_2$ treatment to human monocyte-derived macrophages increased the proportion of cells expressing CD163, a monocyte/macrophage specific marker, which is also used to identify M2 macrophages (Fig. 3-8C). 15d-PGJ$_2$-induced M2 marker expression was inhibited by zinc protoporphyrin IX (ZnPP), a commonly used HO-1 inhibitor (Fig. 3-8D, E) (Bonkovsky et al., 1990). These results suggest that M2 macrophage polarization induced by 15d-PGJ$_2$ may contribute to its pro-resolving as well as anti-inflammatory effects.

To further ensure that the HO-1 expression upregulated by 15d-PGJ$_2$ is responsible for the increased efferocytic activity of macrophages in zymosan A-induced mouse peritonitis model, 15d-PGJ$_2$ was intraperitoneally administered in wild-type and HO-1 knockout mice. 15d-PGJ$_2$ treatment increased the number of monocytes, while it reduced the proportion of PMNs in the peritoneal exudates during resolution of zymosan A-induced peritonitis in wild-type mice. Theses alterations induced by 15d-PGJ$_2$ were less prominent in HO-1 knockout mice (Fig. 3-9A). The proportion of macrophages engulfing apoptotic neutrophils characterized by positive staining of both F4/80 and Gr-1 was then measured by flow cytometry. Notably, the efferocytic activity triggered by zymosan A with or without 15d-PGJ$_2$ was relatively low in the macrophages of HO-1 knockout mice (Fig. 3-9B).

In an ex vivo experiment, the thioglycollate-elicited peritoneal macrophages isolated from HO-1-deficient mice exhibited much weaker efferocytic activity than those from wild-type mice when treated with 15d-PGJ$_2$ (Fig. 3-9C). Knockdown of HO-1 by
interfering RNA abolished the 15d-PGJ$_2$-induced efferocytosis in RAW264.7 cells (Fig. 3-9D). When the transcriptional expression of HO-1 was silenced in RAW264.7 cells by transfecting them with siRNA, 15d-PGJ$_2$ failed to induce CD36 expression (Fig. 2-9E). In contrast, CD36 mRNA expression was upregulated in HO-1 overexpressing RAW264.7 cells (Fig. 3-9F). Consistent with this finding, the proportion of macrophages expressing CD36 was significantly elevated by HO-1 overexpression (Fig. 3-9G). In addition, overexpression of HO-1 further enhanced the efferocytic activity of macrophages (Fig. 3-9H).

*The $\alpha,\beta$-unsaturated carbonyl moiety of 15d-PGJ$_2$ is essential for its pro-resolving activity*

The cyclopentenone moiety of 15d-PGJ$_2$ has been considered to be critical for its physiologic effects. In agreement with this notion, 9,10-dihydro-PGJ$_2$ that lacks the $\alpha,\beta$-unsaturated carbonyl moiety in the cyclopentenone ring (Fig. 3-10A) failed to induce translocation of Nrf2 to the nucleus (Fig. 3-10B). In addition, CD36 expression was not evident in RAW264.7 cells treated with 9,10-dihydro-PGJ$_2$, compared to 15d-PGJ$_2$-treated cells (Fig. 3-10C, D). Likewise, efferocytic activity of RAW264.7 macrophages was enhanced by 15d-PGJ$_2$, but not by 9,10-dihydro-PGJ$_2$ (Fig. 3-10E).

Although maintaining a high level of reactive oxygen species (ROS) produced in macrophages is detrimental (Dey et al., 2014), ROS is responsible for regulating phagocytic functions of macrophages (Lo et al., 2013). 15d-PGJ$_2$ was reported to generate ROS (Song, 2011; Wang, 2011). I postulated that 15d-PGJ$_2$-derived ROS
accumulation may contribute to an increased efferocytic activity in macrophages. Mice treated with zymosan A plus 15d-PGJ$_2$ showed significantly higher accumulation of intracellular ROS in peritoneal macrophages, compared with those challenged with zymosan A alone (Fig. 3-11A, B). I found that 15d-PGJ$_2$ provoked ROS generation in RAW264.7 cells, but its non-electrophilic analogue 9,10-dihydro-PGJ$_2$ did not (Fig. 3-11C). As shown in Fig. 3-11D, the ROS scavenger, N-acetyl-L-cysteine (NAC) blocked nuclear translocation of Nrf2 induced by 15d-PGJ$_2$. In addition, 15d-PGJ$_2$-induced CD36 expression (Fig. 3-11E, F) and efferocytic ability of macrophages (Fig. 3-11G) were suppressed by NAC treatment. These results clearly indicate that the electrophilic carbon 9 located in the cyclopentenone ring is crucial in 15d-PGJ$_2$-induced potentiation of efferocytosis through Nrf2-induced ROS production and CD36 expression. As NAC also acts as a thiol reducing agent as well as an antioxidant, involvement of an ROS-independent mechanism, especially covalent modification of Keap1 cysteine thiol, in 15d-PGJ$_2$-induced Nrf2 activation and efferocytosis should not be excluded.
Fig. 3-1. 15d-PGJ$_2$ modulates leukocyte trafficking and increases efferocytic activity of macrophages in a peritonitis model

Mice were administered with an intraperitoneal dose (30 mg/kg) of zymosan A for 12 h, followed by intraperitoneal injection of vehicle or 15d-PGJ$_2$ (2 mg/kg, i.p.). Six hours later, peritoneal exudates were collected. (A) The number of total leukocytes in peritoneal exudates was counted. (B) The proportions of PMNs and mononuclear cells in collected peritoneal exudates were measured by differential cell counting. (C) In a spontaneous resolving zymosan A-initiated peritonitis model, the proportions of macrophages engulfing apoptotic neutrophils (F4/80$^+$Gr-1$^+$) were determined by flow cytometry as described in Materials and Methods. (D) To evaluate efferocytic activity of macrophages ex vivo, mice were administered intraperitoneally with thioglycollate medium (3%). The thioglycollate-elicited peritoneal macrophages were treated with 15d-PGJ$_2$ (5 µM) for 12 h, and then co-incubated with apoptotic peritoneal neutrophils for 1 h. The efferocytosis was detected by immunostaining using anti-F4/80 (green; macrophage marker) and anti-GR-1 (red; neutrophil marker) antibodies. A representative fluorescence micrograph shows macrophages (green) engulfing apoptotic neutrophils (red). (E) To confirm efferocytic ability of macrophages induced by 15d-PGJ$_2$ in vitro, RAW264.7 cells treated with 15d-PGJ$_2$ were co-incubated with FITC-annexin V-labelled apoptotic Jurkat T cells for 1 h. The proportion of macrophages engulfing apoptotic Jurkat T cells 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.
Fig. 3-2. 15d-PGJ₂ is an important lipid mediator generated by COX-2 activity during the resolution of zymosan A-induced murine peritonitis

Mice were administered intraperitoneally with NS-398 (10 mg/kg) 1 h before i.p. injection of zymosan A (30 mg/kg). Twelve-h later, mice were given, vehicle or 15d-PGJ₂ (2 mg/kg). After 6 h, peritoneal exudates were collected. (A) The number of total leukocytes was counted. (B, C) The proportions of neutrophils and macrophages were determined by flow cytometry using FITC-conjugated anti-Gr-1 or APC-conjugated anti-F4/80. All data represent mean ± SD (n=3); *p < 0.05, **p < 0.01, and ***p < 0.001.
Fig. 3-3. 15d-PGJ$_2$ induces the expression of anti-inflammatory cytokines in macrophages

(A) RAW264.7 murine macrophage cells were treated with 15d-PGJ$_2$ (5 µM) for indicated time periods. The mRNA levels of $Nrf2$, $TGF$-$\beta$, and $IL$-$10$ were determined by RT-PCR. (B) To determine whether 15d-PGJ$_2$ could increase expression of anti-inflammatory cytokines in human macrophages, U937 human promonocytic cells were stimulated with 100 nM of PMA for 3 days to differentiate into macrophages, followed by treatment with 15d-PGJ$_2$ (10 µM) for additional 24 h.
Fig. 3-4. 15d-PGJ2 augments efferocytosis through upregulation of CD36 expression in peritoneal macrophages of zymosan A-treated mice and RAW264.7 cells

(A) The proportion of peritoneal macrophages expressing CD36 was determined by flow cytometry as above. (B, C) RAW264.7 cells were treated with 15d-PGJ2 (5 µM) for indicated time periods (B) and with different concentrations of 15d-PGJ2 for 9 h (C). The mRNA level of CD36 was determined by RT-PCR. GAPDH was used as an internal control to ensure equal amounts of cDNA loaded. (D) Thioglycollate-elicited peritoneal macrophages or RAW264.7 cells were treated with 15d-PGJ2 (5 µM) for 12 h, and the proportion of cells expressing CD36 was measured by flow cytometry using a FITC-conjugated antibody. To further verify the expression of CD36 in macrophages, immunocytochemical analysis was conducted using anti-CD36 antibody. (E) RAW264.7 cells were stained for CD36 or PI as described in Materials and Methods. (F) RAW264.7 cells were transfected with scrambled or CD36 siRNA, and then incubated with or without 15d-PGJ2 (5 µM) for 12 h, followed by co-incubation with apoptotic Jurkat T cells labelled with FITC-annexin V for another 1 h. The assay for efferocytosis was conducted as described in Materials and Methods. All data represent mean ± SD (n=3); *p < 0.05 and **p < 0.01.
(F) Nrf2 WT Nrf2 KO
15d-PGJ2 - + - +
Nrf2
CD36
GAPDH

(G) Nrf2 WT Nrf2 WT
- 15d-PGJ2 + 15d-PGJ2
Nrf2 KO
CD36
- 15d-PGJ2 + 15d-PGJ2
Nrf2 KO

(H) CD36 promoter exon1A1
1A-ARE1 1A-ARE2
1A-ARE1 1A-ARE2

Input Nrf2 IgG
15d-PGJ2 - + - + - +
1A-ARE1
1A-ARE2

*** ***
Fig. 3-5. 15d-PGJ₂-mediated Nrf2 activation upregulates CD36 expression

(A) The proportion of peritoneal macrophages expressing Nrf2 in zymosan A-treated mice was determined by flow cytometry as described in Materials and Methods. (B) RAW264.7 cells were incubated with 15d-PGJ₂ (5 μM) for indicated time periods, and then nuclear extracts were prepared. Nuclear translocation of Nrf2 was measured by immunoblot analysis. Lamin B was used as a specific nuclear protein marker. (C) To verify nuclear translocation of Nrf2, immunocytochemical analysis was conducted using an antibody against Nrf2 after the treatment of RAW264.7 cells with 5 μM of 15d-PGJ₂ for 3 h. Cells were stained with PI as a nuclear marker. (D, E) RAW264.7 cells were transfected with scrambled or Nrf2 siRNA, and then treated with 15d-PGJ₂ for 9 h or 12 h, respectively to measure the mRNA (D) and protein (E) levels of CD36 by RT PCR and flow cytometry. (F, G) To determine whether 15d-PGJ₂-induced Nrf2 activation is required for CD36 expression, the thioglycollate-elicited peritoneal macrophages from wild-type and Nrf2 knockout mice were treated with 15d-PGJ₂ for 9 h. (H) To verify that Nrf2 binds to ARE consensus sequence in the CD36 promoter, the ChIP assay was conducted after the treatment of RAW264.7 cells with 15d-PGJ₂ for 9 h. All data represent means ± SD (n=3); **p < 0.01 and ***p<0.001.
Fig. 3-6. Nrf2 is the key transcriptional factor required for 15d-PGJ₂-mediated resolution of inflammation

Wild-type and Nrf2 knockout mice were administered intraperitoneally with zymosan A (30 mg/kg) for 12 h and subsequently with vehicle or 15d-PGJ₂ (2 mg/kg). Six hours later, peritoneal exudates were collected. (A) The proportions of PMNs and mononuclear cells in collected peritoneal exudates were determined by differential cell counting. (B) For measuring efferocytosis *ex vivo*, thioglycollate-elicited peritoneal macrophages from wild-type and Nrf2 knockout mice were collected and co-incubated with the apoptotic neutrophils separated from the same peritoneal exudates as described in Materials and Methods. A representative fluorescence micrograph shows macrophages (green) and engulfed apoptotic neutrophils (red). Macrophages engulfing apoptotic neutrophils are shown inside the dotted square. (C) RAW264.7 cells were transfected with scrambled or Nrf2 siRNA. (D) RAW264.7 cells were transfected with AAV-Nrf2 vector for 24 h. The efferocytic activity of macrophages was measured by flow cytometry as described previously. All data represent means ± SD (n=3); **p<0.01 and ***p<0.001.
Fig. 3-7. 15d-PGJ$_2$-induced expression of HO-1 is regulated through Nrf2 activation in macrophages

(A) RAW264.7 cells were treated with 15d-PGJ$_2$ (5 µM) for indicated time periods. (B) To determine whether 15d-PGJ$_2$-induced Nrf2 activation regulates HO-1 expression, RAW264.7 cells were transfected with scrambled or Nrf2 siRNA, and then incubated with or without of 15d-PGJ$_2$ (5 µM) for 6 h. (C) Peritoneal macrophages obtained from wild-type and Nrf2 knockout mice were treated with 15d-PGJ$_2$ for 6 h. The mRNA levels of HO-1 and/or Nrf2 (A-C) were measured by RT PCR.
RAW264.7 murine peritoneal macrophages

U937 human monocyte-derived macrophages

RAW264.7 murine peritoneal macrophages
Fig. 3-8. 15d-PGJ₂ enhances alternative macrophage polarization

(A) RAW264.7 cells were treated with 15d-PGJ₂ (5 µM) for indicated time periods. The mRNA levels of HO-1 and M2 marker genes (Arg-1 and Relm-α) were determined by RT-PCR. (B) The protein levels of HO-1 and M2 marker proteins (Arg-1 and Relm-α) were measured by Western blot analysis. Macrophages differentiated from U937 human promonocytic cells as described in the Supplementary Fig. S1B were treated with 15d-PGJ₂ (10 µM) for 24 h. (C) The proportion of CD163 expressing cells was measured by flow cytometry. The data represent mean ± SD (n=3); ***p<0.001. RAW264.7 cells were treated with ZnPP (10 µM) for 1 h before incubation with 15d-PGJ₂ (5 µM) for additional 6 h (D) and 12 h (E). The mRNA and protein levels of HO-1 and M2 marker genes were determined by RT-PCR and Western blot analysis, respectively.
Fig. 3-9. HO-1 plays a role in 15d-PGJ2-induced efferocytosis

The peritoneal exudates from wild-type and HO-1 knockout mice were harvested as described in the legend to Fig 1. (A) The proportions of PMNs and mononuclear cells in collected peritoneal exudates were measured by differential cell counting. (B) The proportion of macrophages engulfing PMNs (F4/80^+Gr-1^+) was determined by flow cytometry. (C) For determining efferocytosis ex vivo, the engulfment of apoptotic neutrophils by thioglycollate-elicited peritoneal macrophages was assessed by immunocytochemistry using anti-F4/80 and anti-Gr-1 antibodies. Macrophages (green) engulfing apoptotic neutrophils (red) are shown inside the dotted square. (D) RAW264.7 cells were transfected with scrambled or HO-1 siRNA, followed by treatment with 15d-PGJ2 (5 μM) for 12 h, and then efferocytic activity of macrophages was measured by flow cytometry. (E) RAW264.7 cells were transfected with scrambled or HO-1 siRNA, followed by treatment with 15d-PGJ2. The mRNA level of CD36 was determined by RT PCR. (G, H) RAW264.7 cells were transfected with pcDNA-mock or pcDNA-HO-1, and mRNA and protein levels of CD36 were determined by RT-PCR (F) and flow cytometry, respectively (G). (H) RAW264.7 cells were transfected with pcDNA-mock or pcDNA-HO-1 for 24 h, and their efferocytic activity was measured as described in Materials and Methods. Data represent mean ± SD (n=3); *p<0.05, **p<0.01 and ***p<0.001.
Fig. 3-10. The α,β-unstaturated carbonyl moiety is essential for pro-resolving effects of 15d-PGJ₂

(A) 15d-PGJ₂ possesses the α,β-unsaturated carbonyl moiety in the cyclopentenone ring structure, whereas 9,10-dihydro-PGJ₂ lacks this electrophilic moiety. (B, C, D) RAW264.7 cells were treated with 15d-PGJ₂ (5 μM) or 9,10-dihydro-15d-PGJ₂ (5 μM), and nuclear translocation of Nrf2 was measured at 3 h (B), and CD36 mRNA and protein levels at 9h or 12h, respectively (C, D). (E) RAW264.7 cells were treated with 15d-PGJ₂ or 9,10-dihydro-15d-PGJ₂ (5 μM each) for 12 h, and then efferocytic activity was measured as described previously. All data represent mean ± SD (n=3); *p<0.05, **p<0.01 and ***p<0.001.
Fig. 3-11. ROS is involved in 15d-PGJ₂-induced potentiation of efferocytic activity of macrophages

(A and B) To measure the ROS accumulation of macrophages in vivo, Mice were administered intraperitoneally with zymosan A (30 mg/kg) for 12 h and subsequently with vehicle or 15d-PGJ₂ (2 mg/kg). One hour later, peritoneal exudates were collected. Intracellular ROS in peritoneal macrophages with positive staining of both F4/80 (macrophage marker) and DCF-DA (indicator of ROS) was measured by flow cytometry (A) and immunocytological analysis (B). (C) To determine the in vitro intracellular ROS accumulation, RAW264.7 cells were exposed to 5 µM each of 15d-PGJ₂ or 9,10-dihydro-15d-PGJ₂ for 1 h, and then the intracellular ROS level was measured by DCF-DA staining. DCF-DA-stained cells were counted under a microscope with the aid of Image-Pro Plus Software. (D) RAW264.7 cells were pre-incubated for 1 h with NAC (10 mM) followed by treatment with 15d-PGJ₂ (5 µM) for additional 3 h. Nuclear accumulation of Nrf2 was determined by western blot analysis. (E, F, G) Cells were exposed to 15d-PGJ₂ in the absence or presence of NAC (10 mM), and mRNA (E), and protein levels (F) of CD36 and efferocytic activity of macrophages (G) were measured as described previously. All data represent mean ± SD (n=3); *p<0.05, **p<0.01 and ***p<0.001.
5. Discussion

Resolution is an active process orchestrated by a distinct set of endogenous anti-inflammatory and pro-resolving lipid mediators (Serhan 2007). It has been reported that PGs play an important role in the cellular inflammatory response (Ricciotti and FitzGerald 2011). 15d-PGJ$_2$ is produced from arachidonic acid in the late phase of acute inflammation. 15d-PGJ$_2$ is endogenously produced at sufficient levels to drive the resolution of zymosan A-induced peritonitis (Rajakariar et al., 2007) and also during the resolution phase of inflammation in carrageenan-induced pleurisy (Itoh et al., 2004). In the latter study, NS-398 administration caused persistence of neutrophil infiltration and also a delay in macrophage recruitment following carrageenan treatment in mice. These effects are attributable, at least in part, to attenuation of COX-2-mediated 15d-PGJ$_2$ production (Itoh et al., 2004). In addition, 15d-PGJ$_2$ has been known to inhibit monocyte trafficking and to regulate macrophage activation (Gilroy et al., 2004). 15d-PGJ$_2$ elicits potent immunomodulatory effects through peroxisome proliferator-activated receptor γ (PPAR)-γ-mediated macrophage activation (Jiang, 1998; Ricote, 1998; Majai, 2007). 15d-PGJ$_2$ also suppresses activation of pro-inflammatory transcription factors, such as NF-κB and STAT3 (Kim and Surh 2006). Although the role of 15d-PGJ$_2$ in the resolution of inflammation has been investigated, the molecular events associated with efferocytic activity of macrophages stimulated by this cyclopentenone PG are not clearly defined.

The resolution phase of inflammation limits excessive neutrophil infiltration into the inflamed site, while the macrophage-mediated engulfment of apoptotic neutrophils is facilitated by efferocytosis (Headland and Norling 2015). Failure in clearing apoptotic
cells leads to secondary necrosis, followed by additional disruption of other cells. Upon acute inflammatory response, pro-inflammatory cytokines and chemokines are produced, and these molecules counteract efferocytic ability of macrophages (Aronoff et al., 2004). However, at the late phase of inflammation, the production of pro-resolving lipid mediators facilitates elimination of apoptotic neutrophils. Our current study demonstrates that 15d-PGJ₂ administration inhibits neutrophil recruitment and promotes monocytes/macrophages infiltration in inflamed peritoneum of zymosan A-treated mice. In addition, intraperitoneal injection of exogenous 15d-PGJ₂ expedited the resolution of zymosan A-induced peritonitis by promoting macrophage-mediated efferocytosis. It has been reported that delivery of exogenously produced apoptotic cells protects against lethal septic shock (Ren et al., 2008). We attempted to assess the anti-inflammatory effects of 15d-PGJ₂ on bacterial lipopolysaccharide (LPS)-induced sepsis, another acute inflammation model. However, LPS treatment was quite toxic, resulting in dramatic body weight loss in 3 days after the treatment, and almost all the mice died. In this model, 15d-PGJ₂ was not protective against both body weight loss and mortality. Intriguingly, low levels of the circulating gram-negative bacterial endotoxin LPS is speculated to provoke a non-resolving low-grade inflammation (Glaros et al., 2013). These findings suggest that protection of zymosan A–induced murine peritonitis by 15d-PGJ₂ is a specific effect mediated through efferocytosis, whereas 15d-PGJ₂ is not protective against LPS-induced endotoxemia that may skew host immune environment toward a mild non-resolving pro-inflammatory state (Fig. 3-12).

To carry out the efferocytosis, macrophages which have an ability to engulf apoptotic
cells need to stimulate the expression of surface scavenger receptors. These receptors recognize PS exposed at the outer leaflet of the plasma membrane of apoptotic cells, and initiate the process of efferocytosis (Ravichandran 2010). One such scavenger receptor involved in efferocytosis is CD36. CD36 was characterized as an adhesion receptor for thrombospondin-1 (Asch et al., 1987) and platelet-collagen (Tandon et al., 1989). CD36 cooperating with thrombospondin-1 recognizes apoptotic cells for phagocytosis (Savill et al., 1992). CD36 is also involved in uptake of oxidatively modified low density lipoprotein, and regulates the macrophage-mediated inflammatory response (Silverstein and Febbraio 2009). Macrophages from CD36 knockout mice have decreased phagocytic activity, compared with those from wild-type mice (McGilvray et al., 2000). The recognition of apoptotic neutrophils and their engulfment by macrophages then trigger secretion of anti-inflammatory cytokines, such as TGF-β and IL-10, that inhibit the production of pro-inflammatory mediators by macrophages. We found that 15d-PGJ₂ triggered expression of these anti-inflammatory cytokines as well as CD36 in macrophages. By controlling the balance between pro-inflammatory and anti-inflammatory cytokines, 15d-PGJ₂ is likely to facilitate resolving inflammation.

Nrf2 is a key redox sensitive transcription factor which plays a central role in cellular protection against inflammatory insult as well as oxidative stress. In addition, Nrf2 modulates inflammatory-immune response (Cho, 2009; Harvey, 2011; Yageta, 2011). Nrf2 inhibits the acute inflammation by regulating the recruitment of inflammatory cells and the expression of anti-inflammatory and anti-oxidant genes (Itoh et al., 2004). Our present study demonstrates that Nrf2 activation in macrophages is essential for the pro-
resolving effects of 15d-PGJ2. Nrf2 is an important regulator of CD36 expression in murine macrophages (Ishii et al., 2004). In the present study, 15d-PGJ2 induced CD36 expression through Nrf2 activation in both peritoneal macrophages and cultured RAW264.7 cells, which accounts for its enhancement of efferocytosis. Although the promoter region of CD36 gene harbours the Nrf2 binding site, it is intriguing to note that its expression is also upregulated by HO-1, another Nrf2 target protein.

Nrf2 activation is negatively regulated by Keap1, which is a substrate adaptor for the Cullin-3 (Cul3)-dependent E3 ubiquitin ligase machinery (Kobayashi et al., 2004). Keap1 is a thiol-rich protein, and human Keap1 contains a total of 27 and mouse Keap1 has 25 cysteine residues (Kansanen et al., 2013). Some electrophiles or ROS can structurally alter some of reactive cysteines present in Keap1 that serve as redox sensors, thereby attenuating the ability of Keap1 to sequester Nrf2 in the cytoplasm. 15d-PGJ2 has an electrophilic \( \alpha,\beta \)-unsaturated carbonyl moiety and hence can act as a Michael acceptor capable of covalently modifying the Keap1 cysteine thiol(s). 15d-PGJ2 can also generate ROS which subsequently oxidize cysteine residue(s) of Keap1, releasing Nrf2 for nuclear translocation. Consistent with this notion, 15d-PGJ2-induced accumulation of Nrf2 protein, expression of its target gene CD36, and the efferocytic activity of macrophages were abolished by pretreatment with the antioxidant NAC. Moreover, we also found that the generation of ROS by 15d-PGJ2 was increased in zymosan A-induced peritoneal macrophages. The molecular mechanism responsible for 15d-PGJ2-generated ROS formation remains to be defined, but one plausible mechanism may involve Rac1/NADPH oxidase (Hong et al., 2008). Alternatively, ROS can be produced through
conjugation of 15d-PGJ$_2$ with the cellular reduced glutathione (GSH). A previous study from this laboratory has revealed that 15d-PGJ$_2$ binds and subsequently depletes GSH (Song et al., 2011). Such effects were not achievable with the non-electrophilic analogue 9,10-dihydro-PGJ$_2$. Notably, Zhang et al. have reported that ROS production is necessary for the M2 macrophages polarization (Zhang et al., 2013). Knockdown of Rac1, which is one of the critical ROS-producing components, efficiently blocked M2 macrophage differentiation (Zhang et al., 2013). 15d-PGJ$_2$ treatment resulted in transient upregulation of some M2 macrophage markers, Arg-1 and Relm-$\alpha$ in RAW264.7 murine macrophages and enhanced the proportion of CD163 expressing human monocyte-derived macrophages.

One of the key target proteins of Nrf2 is HO-1. HO-1 is generally expressed at very low levels in most tissues under normal physiologic conditions, but it is highly inducible in response to various inflammatory stimuli to protect cells and tissues from injury. For instance, HO-1 knockout mice are extremely prone to oxidative stress and severe inflammatory condition (Poss et al., 1997). Thus, HO-1-deficient mice are more susceptible than wild-type mice to LPS-induced endotoxemia and markedly vulnerable to experimentally induced autoimmune encephalomyelitis (Chora et al., 2007). HO-1 deficiency provokes a cascade of inflammatory reactions, and vascular endothelial damage may rapidly occur with various complications. In contrast, upregulation of HO-1 significantly inhibits skin allergy (Listopad, 2007; Kirino, 2008). Although HO-1 induction in macrophage by 15d-PGJ$_2$ has anti-inflammatory effects (Gong et al., 2002), the mechanism underlying pro-resolving effects exerted by this cyclopentenone
prostaglandin through upregulation of HO-1 expression remained overlooked.

The cytoprotective action of HO-1 is thought to be mediated by CO which is one of the by-products of heme degradation (Wang et al., 2009). Under stressful conditions, CO generated as a consequence of induction of HO-1 expression/activity enhances the host defense response to inflammatory insults (Chung et al., 2008) as well as exerts antioxidant effects (Brouard et al., 2000). Based on these findings, I speculate that CO may play a crucial role in mediating 15d-PGJ2-induced efferocytosis and resolution of inflammation. Thus, it would be worthwhile to examine whether HO-1-derived CO can stabilize or upregulate expression of CD36 and other scavenger receptors in macrophages.

In conclusion, 15d-PGJ2 generated during the inflammatory process from arachidonic acid by COX-2 activity, stimulates efferocytosis. It is evident that the activation of Nrf2 by 15d-PGJ2 is one of the important mechanisms responsible for its potentiation of engulfment of apoptotic cells by macrophages. Nrf2 directly upregulates expression of both HO-1 and CD36 by binding to the ARE region present in their promotor region, which accounts for efferocytosis facilitated by 15d-PGJ2-stimulated macrophages (Fig. 3-13). Precise control of efferocytosis by macrophages is critical for the resolution of inflammation, and its failure can cause inflammation-associated disorders (Korns, et al., 2011). Therefore, 15d-PGJ2, generated during resolution of inflammation, is one of the key molecules involved in cellular protection against inflammatory damage. As intraperitoneal injection of exogenous 15d-PGJ2 potentiated the efferocytic activity of macrophages, this lipid mediator might have a therapeutic potential in the management
of chronic inflammatory disorders associated with impaired efferocytosis.
Fig. 3-12. 15d-PGJ₂ failed to protect against LPS-induced sepsis

Mice were intraperitoneally injected with 15d-PGJ₂ (2 mg/kg) at 2 h, 24 h and 48 h after LPS (15 mg/kg, i.p.) administration. The change in the body weight (A) and the survival rate (B) were monitored up to 72 h. The survival rate of the mice was expressed by using Kaplan–Meier plot.
**Fig. 3-13. A proposed mechanism underlying 15d-PGJ$_2$-induced efferocytosis**

15d-PGJ$_2$ is endogenously produced under inflammatory conditions which can increase efferocytic activity of macrophages through Nrf2-induced the expression of CD36 and HO-1.
6. References


Gahlot, S., et al. (2017). "Heme Oxygenase-1 Deficiency Diminishes Methicillin-


Kim, J. W. and C. Kim (2005). "Inhibition of LPS-induced NO production by taurine
chloramine in macrophages is mediated through Ras-ERK-NF-kappa B." Biochem Pharmacol 70(9): 1352-1360.


Marcinkiewicz, J., et al. (2009). "Taurine haloamines and heme oxygenase-1 cooperate


macrophages contributes to LPS-induced IL-10 release." Immunobiology 215(9-10): 788-795.


Turchyn, L. R., et al. (2007). "Phenotypic and functional analysis of murine resident and


Chapter IV

Role of Heme Oxygenase-1 in Potentiation of Phagocytic Activity of Macrophages by Taurine Chloramine: Implications for the Resolution of Zymosan A-induced Murine Peritonitis
1. Abstract

Phagocytosis of pathogens by macrophages is crucial for the successful resolution of inflammation induced by microbial infection. Taurine chloramine (TauCl), an endogenous anti-inflammatory and antioxidative substance, is produced by reaction between taurine and hypochlorous acid by myeloperoxidase activity in neutrophils under inflammatory conditions. In the present study, we investigated the effect of TauCl on resolution of acute inflammation caused by fungal infection using a zymosan A-induced murine peritonitis model. TauCl administration reduced the number of the total peritoneal leukocytes, while it increased the number of peritoneal monocytes. Furthermore, TauCl promoted clearance of pathogens remaining in the inflammatory environment by macrophages. When the macrophages isolated from thioglycollate-treated mice were treated with TauCl, their phagocytic capability was enhanced. In the murine macrophage-like RAW264.7 cells treated with TauCl, the proportion of macrophages clearing the zymosan A particles was also increased. TauCl administration resulted in elevated expression of heme oxygenase-1 (HO-1) in the peritoneal macrophages. Pharmacologic inhibition of HO-1 activity or knockdown of HO-1 in the murine macrophage RAW264.7 cells abolished the TauCl induced phagocytosis, whereas the overexpression of HO-1 augmented the phagocytic ability of macrophages. Moreover, peritoneal macrophages isolated from HO-1 null mice failed to mediate TauCl-induced phagocytosis. Our results suggest that TauCl potentiates phagocytic activity of macrophages through upregulation of HO-1 expression.
Keywords: Heme oxygenase-1, Peritonitis, Resolution of inflammation, Taurine, Taurine chloramine, Zymosan A

2. Introduction

Chronic inflammation can occur when an intracellular microbial infection is not properly resolved. If microbial pathogens or their toxic components are not adequately eliminated, the infectious process result in considerable damage to the host and cause infectious disease such as respiratory allergy, skin diseases and inflammatory bowel diseases (Romani, 2004; Soehnlein, 2010). Phagocytes, such as neutrophils, monocytes and macrophages, play pivotal roles in the effective elimination of pathogens, thereby maintaining the tissue homeostasis. Upon encountering inflammatory pathogens, polymorphonuclear neutrophils (PMNs) recruited to the inflamed area undergo oxidative burst, comprising the first line of host defense against pathogen infection. This leads to overproduction of reactive oxygen species with which the neutrophils kill and eliminate pathogens. The activated neutrophils acquire significantly enhanced ability to phagocytose pathogens (Michlewska et al., 2009). Subsequently, macrophages, which are derived from monocytes circulating in the blood, are recruited, and then target uncleared inflammatory pathogens remaining in the inflammatory environment (Diamond, 1993; Cheng, 2012). The phagocytic removal of the pathogens by macrophages, a process called ‘phagocytosis’, is essential for resolution of inflammation caused by microbial infections.

In addition to their functions in the removal of uncleared pathogens by phagocytosis,
macrophages release pro-inflammatory cytokines, and this alarms cellular defense mechanisms of the host to fight the microbial infection. Moreover, they also produce anti-inflammatory substances, some of which are involved in the resolution of inflammation (Sasada and Johnston 1980). Heme oxygenase -1 (HO-1) is one of the major anti-inflammatory enzymes that plays a critical role in defending the body against microbial infection (Poss and Tonegawa 1997). Heme oxygenase -1 (HO-1) is one of the major anti-inflammatory enzymes that plays a critical role in defending the body against microbial infection (Chung et al., 2008) and tuberculosis (Sinnis and Ernst 2008). Although it has been reported that HO-1 has anti-inflammatory effects against invading pathogens, there is less certainty about the pro-resolving effects of HO-1 expressed in macrophages during microbial infection.

Taurine, a decarboxylation product of cysteine, is one of the most abundant free amino acids in inflammatory cells including neutrophils and plays important roles in several essential biological processes, such as osmoregulation, membrane stabilization, calcium mobilization and immunity (Huxtable, 1992). The stored taurine reacts stoichiometrically with hypochlorous acid (HOCl), a strong antibacterial oxidant produced from hydrogen peroxide (H2O2) by the myeloperoxidase activity of the activated neutrophils in the presence of chloride ion. This results in the generation of taurine chloramine (TauCl), which is then released from apoptotic neutrophils to the surrounding inflammatory tissue. In particular, TauCl has been reported to have microbicidal activity due to its antioxidant and anti-inflammatory properties (Nagl, 2000; Nagl, 2001; Gruber, 2017). However, molecular mechanisms underlying phagocytosis
of pathogens by TauCl have yet to be established. Here, we report that TauCl upregulates HO-1 expression in macrophages and thereby facilitates the engulfment of a fungal component to allow the resolution of inflammation.

3. Materials and methods

Animals
C57BL/6 mice (8 weeks of age) were purchased from Central Lab Animal Inc. (Seoul, South Korea). HO-1 knockout mice (BALB/c) were provided by Dr. M.A. Perrela (Harvard Medical Center). All mice were maintained according to the Institutional Animal Care Guidelines. Animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University.

Reagents and kits
Taurine was purchased from Sigma Aldrich (St Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), and fetal bovine serum (FBS) were obtained from GIBCO RBL (Grand Island, NY, USA). Zymosan A-FITC or PE particles were purchased from invitrogen (Carlsbad, CA, USA). Small interfering RNAs (siRNAs) against HO-1, and ZnPP were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody against actin was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-HO-1 was the product of Stressgen (Ann, Arbor, MI, USA), and anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were provided by Zymed Laboratories Inc. (San Francisco, CA, USA). Polyvinylidene difluoride (PVDF) membranes were supplied from Gelman Laboratory (Ann, Arbor, MI, USA). The Enhanced Chemiluminescent (ECL) detection kit was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Synthesis of N-chlorotaurine sodium
N-chlorotaurine sodium salt was synthesized by the reaction of taurine with an ethanolic
solution of chloramine-T as a source of electrophilic chlorine and sodium salt. To a well stirred suspension of finely powdered taurine (5 g) in absolute ethanol (100 ml) was added chloramine-T (12 g). The reaction mixture was stirred for about 5 h at ambient temperature. The resulting sodium salt of \( N \)-chlorotaurine was filtered off and washed with 40 ml of ethanol. Its purity and identity were verified by UV and IR spectral data and further evidence has been gained by \( ^{13} \text{C} \) NMR spectroscopy and mass spectrometry.

**Zymosan-A-FITC-induced peritonitis**

Zymosan A-FITC (30 mg/kg) was administered intraperitoneally at 12 h before giving TauCl (20 mg/kg, suspended in autoclaved water) or vehicle, and mice were sacrificed 6 h later. Peritoneal leukocytes were collected by washing with 3 ml of PBS containing 3 mM EDTA.

**Total and differential leukocyte counts**

Cells from peritoneal exudates were incubated for 1 h by Turk’s solution (0.01% crystal violet in 3% acetic acid) to eliminate red blood cells, and then the number of the total leukocytes was counted by the hematocytometer. For the differential leukocyte counts, a cytospin centrifuge was used to concentrate cells from peritoneal exudates onto microscope slides in a circle with 6 mm diameter. The cells were then subjected to Wright-Giemsa staining.

**Phagocytosis assay**

To assess the percentage of phagocytosis of macrophages \textit{in vivo}, peritoneal exudate cells from mice were labeled with allophycocyanin (APC)-conjugated F4/80-antibody (eBioscience, San Diego, CA, USA), permeabilized with 0.1% Triton X-100. The proportion of macrophages engulfing zymosan A-FITC particles (F4/80+/zymosan A-FITC\(^+\)) was determined by flow cytometry or immunocytochemistry. A sterile irritant, thioglycollate has been used to enhance the yield of peritoneal macrophages. For measuring efferocytosis \textit{ex vivo}, mice were administered intraperitoneally injection of
thioglycollate medium (3%). After three days, peritoneal macrophages isolated from thioglycollate-treated mice, and then incubated in six-well flat-bottomed microtiter plates for 24 h. Adherent monolayer cells were co-incubated for 1 h with zymosan A-FITC or zymosan A-PE particles. Zymosan A-FITC or PE particles engulfed by macrophages were visualized under a Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan). To determine the phagocytic activity of macrophages in vitro. FITC or PE-labelled zymosan A were then co-incubated with RAW264.7 cells, and the proportion of RAW264.7 cells containing zymosan A particles (FITC or PE-positive cells) was assessed by FACSCalibur™ Flow Cytometer (BD, Franklin Lakes, NJ, USA).

Flow cytometry
Cells were fixed with 10% neutral-buffered formation solution for 30 min at room temperature, permeabilized with 0.2% Triton X-100 for 5 min, and blocked with 2% BSA in PBS for 30 min. Anti-HO-1 antibody, diluted 1:100 in 2% BSA in PBS, was applied overnight at 4°C. After washing with PBS, cells were incubated with PE-conjugated anti-rabbit IgG secondary antibody diluted at 1:1000 for 1 h. Cells were analyzed using FACSCalibur™ Flow Cytometer (BD, Franklin Lakes, NJ, USA).

Immunocytochemical analysis of HO-1
Peritoneal exudates were spun in a cytocentrifuge onto a slide. The cells were then subjected to immunocytochemical analysis of HO-1. After fixation with 10% neutral-buffered formalin solution for 30 min at room temperature, cells were permeabilized with 0.2% Triton X-100, incubated with blocking agents [0.1% Tween-20 in PBS containing 5% bovine serum albumin], washed with PBS, and then incubated with a diluted (1:100) HO-1 antibody overnight at 4°C. After washing with PBS, cells were incubated with a diluted (1:1000) PE-goat anti-rabbit IgG secondary antibody for 1 h and with 4′,6-diamidino-2-Phenylindole (DAPI) for 5 min, and examined under a confocal microscope (Nikon, Tokyo, Japan).
Cell culture
The murine macrophage-like cell line (RAW264.7) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Primary macrophages were obtained from thioglycollate-elicited mice. RAW264.7 cells and primary peritoneal macrophages were cultured in DMEM with 10% FBS, streptomycin (100 μg/ml) and penicillin (100 U/ml). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

Transient transfection with HO-1 plasmid (pc-DNA-HO-1) or HO-1-small interfering RNA
RAW264.7 cells were transiently transfected as previously described (X. Zhang, et al., 2009) The RAW264. 7 cells were transfected with HO-1 plasmid (pc-DNA-HO-1) or HO-1 siRNA (Santa Cruz, CA, USA). For each electroporation, the concentration of 4 × 10⁶ cells in a 0.25-mL volume are added to the cuvette and then mixed with plasmid or siRNA. The mixture is allowed to stand at room temperature for 10 min. After electroporation cells, the cells are plated in plates and allowed to recover overnight. The media is removed and replenished with a fresh media (DMEM, 10 % FBS, 1 % AB) at which time the cells are ready for each experiment.

Western blot analysis
The whole cell extracts were prepared according to the manufacturer’s instructions by using the cell lysis buffer (Cell Signaling Technology #9803). The protein concentration of the supernatant was measured by using the BCA reagents (Pierce, Rockford, IL, USA). Protein (10 μg) was separated by running through 10% SDS–PAGE gel and transferred to the PVDF membrane (0.22 mm thickness; Gelman Laboratory, Ann Arbor, MI, USA). To block the non-specific binding of proteins with primary antibodies, the blots were blocked with 5% fat-free dry milk-TBST (Tris-buffered saline containing 0.1% Tween-20) buffer for 30 mins at room temperature. The membranes were then incubated with the primary antibody suspended in 3% non-fat milk TBST buffer for 4 h at room
temperature. The blots were washed three times with TBST buffer for 10 min each and incubation using appropriate secondary antibody coupled to horseradish peroxidase. Proteins tagged with specific primary antibodies were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Statistical analysis
All data were expressed as means ± SD of at least three independent experiments, and statistical analysis for single comparison was performed using the Student’s t test. The criterion for statistical significance was *p < 0.05, **p < 0.01, and ***p < 0.001.

4. Results

**TauCl regulates leukocyte trafficking against invading inflammatory pathogens in zymosan A-induced a murine peritonitis**

To investigate pro-resolving effects of TauCl on inflammation induced by microbial infection in vivo, a zymosan-induced murine peritonitis model was adopted. Acute peritoneal inflammation was induced in mice by an intraperitoneal injection of zymosan A, a particle derived from yeast cell wall, conjugated with fluorescein isothiocyanate (FITC) particles. In the peritoneum of zymosan A-injected mice, the total leukocyte count peaks at 12 h, which gradually decreases thereafter (Bannenberg et al., 2005). To determine the pro-resolving effect of TauCl, we used stable sodium salt form of TauCl prepared according to the method previously reported by Gottardi and Nagl (Gottardi, 2002; Nagl, 2010), and the purity was also confirmed by the UV spectrum and 13C-NMR spectrum (Fig. 4-1). When the number of total leukocytes was maximal, TauCl
(20 mg/kg) was administrated into the peritoneum of mice. Six hour later, peritoneal exudates were collected. The total leukocyte counts in the peritoneal exudates obtained from zymosan A plus TauCl-treated mice were markedly decreased, compared with those from mice given zymosan A alone (Fig. 4-2A). In the next experiment, the proportions of PMNs (neutrophils) and monocytes in the peritoneal exudates were determined by differential cell counting. While the number of peritoneal monocytes was decreased, the proportion of PMNs was increased in the mice challenged with zymosan A. TauCl administration partly restored the monocyte counts, and reduced the zymosan A-induced increases of PMN counts (Fig. 4-2B).

**TauCl facilitates engulfment of pathogens by macrophages in a murine peritonitis**

In our previous study, we have demonstrated that TauCl enhanced macrophages engulfing apoptotic neutrophils, in a process named efferocytosis, thereby facilitating resolution of inflammation (Martin et al., 2014). For the successful resolution of inflammation, macrophages mediate not only rapid engulfment and clearance of apoptotic cells including neutrophils, but also pathogens remaining in inflammatory environment by phagocytosis (Elliott et al., 2017). To assess the pro-resolving effect of TauCl on macrophages-mediated phagocytosis in vivo, the ability of peritoneal macrophages engulfing zymosan A particles was measured. Peritoneal macrophages were labeled with the allophycocyanin (APC)-conjugated F4/80 antibody, followed by permeabilization to identify macrophages taking up zymosan A particles. The phagocytic activity of macrophages (F4/80+zymosan A-FITC+) was selectively determined by flow
cytometry. Notably, the mice treated with zymosan A plus TauCl showed a significantly higher proportion of peritoneal macrophages engulfing zymosan A particles than those challenged with zymosan A alone (Fig. 4-3A). Moreover, the peritoneal macrophages engulfing zymosan A-FITC particles were stained with phycoerythrin (PE)-conjugated F4/80 antibody, and then subjected to immunocytochemical analysis. TauCl administration further enhanced the phagocytic activity of macrophages (Fig. 4-3B). Taurine, accumulated especially in neutrophils, protects host cells from inflammation associated with oxidative stress. In another experiment, RAW264.7 macrophages preincubated with either Taurine or TauCl (0.5 mM each) for 18 h were exposed to zymosan A particles for additional 1 h. TauCl enhanced the phagocytic activity of macrophages, but its parent compound Taurine was not effective (Fig. 4-4).

Besides evaluating phagocytic activity of macrophages in vivo, we performed an ex vivo experiment in which macrophages from murine peritoneum were treated with TauCl. Primary macrophages isolated from the murine peritoneal cavity are commonly used for biochemical and mechanistic studies (Turchyn et al., 2007). However, the yield of these peritoneal macrophages is relatively low. Thus, sterile Brewer thioglycollate broth is commonly injected into the peritoneal cavity increase the macrophage yield, although such elicitation results in a mild inflammatory response and may alter physiological properties of cells to be collected (Turchyn, 2007; Ray, 2010). When thioglycollated-elicited macrophages were treated with TauCl for 18 h, followed by co-incubation with zymosan A-particles for additional 1 h, the ability of macrophages to take up zymosan A particles was increased (Fig. 4-3C). These findings suggest that TauCl stimulates
engulfment of inflammatory pathogens by macrophages, facilitating resolution of inflammation.

**TauCl further enhances HO-1 expression during zymosan A-induced peritonitis**

HO-1 has potent cytoprotective and anti-inflammatory effects (Lee and Chau 2002, Alcaraz, Fernandez et al. 2003) and is involved in resolving inflammation through M2 macrophage polarization (Huang, 2014; Naito, 2014; Tu, 2014). Our previous study has demonstrated that TauCl-induced HO-1 expression is responsible for an increase of efferocytic activity of macrophages in vivo as well as in the murine macrophage-like RAW 264.7 cell line (Kim et al., 2015). Although HO-1 expression is significantly increased during microbial infection, the role of this anti-inflammatory enzyme in phagocytic activity of macrophages and underlying molecular mechanisms remain largely unresolved. To verify the role of HO-1 in pathogen-induced inflammation, HO-1 level expression in the peritoneal cells of zymosan A-treated mice was measured by immunocytochemical analysis. Moderate peritoneal expression of HO-1 was induced in mice treated with zymosan A alone as a host adaptive response, but TauCl treatment further enhanced the HO-1 protein level in peritoneal exudates (Fig. 4-5A). To assess HO-1 expression specifically in macrophages, peritoneal cells with positive staining of both F4/80 and PE-conjugated HO-1 antibody were selectively identified by flow cytometry. Again, mice treated with zymosan A plus TauCl showed a higher proportion of peritoneal macrophages expressing HO-1 than those challenged with zymosan A alone (Fig. 4-5B).
TauCl pretreatment induces HO-1 expression and potentiate phagocytic activity of macrophages

To determine the role of HO-1 induced by TauCl in phagocytic activity of macrophages, the mice was administrated with thioglycollate plus TauCl or thioglycollate alone. Three days later, the peritoneal exudates obtained from the mice were collected. Administration of TauCl significantly increased the proportion of macrophages expressing HO-1, compared with those from mice given thioglycollate alone as measured by flow cytometry (Fig. 4-6A). Immunocytochemical analysis also revealed that there was increased expression of HO-1 in peritoneal macrophages obtained from TauCl plus thioglycollated-treated mice (Fig. 4-6B). When peritoneal macrophages obtained from mice treated thioglycollate plus TauCl were co-incubated with zymosan A particles for additional 1 h, their phagocytic activity was enhanced, compared to those from mice treated with thioglycollate alone (Fig. 4-6C). In the next experiment, we pretreated the mice three times with an intraperitoneal dose (20 mg/kg) of TauCl before injecting FITC-labeled zymosan A into the mice peritoneum. Peritoneal exudates were collected 18 h later, and the proportion of macrophages engulfing zymosan A particles (F4/80+zymosan A-FITC+) was assessed by flow cytometry. The mice treated with TauCl showed a higher proportion of peritoneal macrophages carrying the engulfed zymosan A particles (Fig. 4-6D). Immunocytochemical analysis also revealed that macrophages from mice treated with TauCl showed higher phagocytic activity (Fig. 4-6E).
HO-1 expression is essential for pro-resolving effects of TauCl

In order to investigate whether the HO-1 expression upregulated by TauCl is essential for enhancement of phagocytosis, we utilized zinc protoporphyrin IX (ZnPP), a commonly used HO-1 inhibitor. RAW264.7 cells were incubated with ZnPP (10 μM) or vehicle for 1 h followed by treatment with TauCl (0.5 mM) for additional 18 h. RAW264.7 cells treated with TauCl were then co-incubated with zymosan A particles for another 1 h. Representative flow cytometric dot plots demonstrate changes in the percentage of macrophages engulfing zymosan A particles. Pharmacologic inhibition of HO-1 suppressed the capability of cultured macrophages to carry out phagocytosis upon TauCl treatment (Fig. 4-7A). Likewise, when the transcriptional expression of HO-1 was silenced in RAW264.7 cells by transfecting them with siRNA, TauCl failed to induce phagocytic activity of macrophages (Fig. 4-7B). In contrast, overexpression of HO-1 further enhanced the phagocytic activity of macrophages (Fig. 4-7C).

To further ensure that TauCl-induced HO-1 expression is responsible for the increased phagocytosis of macrophages, the thioglycollate-elicited macrophages isolated from wild-type (WT) or HO-1 knockout mice were treated with TauCl for 18 h, followed by co-incubation with zymosan A-particles for additional 1 h. The thioglycollate-elicited macrophages of HO-1 knockout mice were not responsive to TauCl-induced phagocytosis (Fig. 4-8A). Moreover, the peritoneal macrophages from HO-1-deficient mice exhibited much weaker phagocytic activity than those from WT mice upon TauCl treatment as measured by phase-contrast microscopy (Fig. 4-8B).
Likewise, immunochemical analysis revealed that the peritoneal macrophages from HO-1-knockout mice exhibited much lower phagocytic activity compared to WT mice when treated with TauCl (Fig. 4-8C). Taken together, these data clearly suggest that TauCl-induced HO-1 expression is crucial for enhancement of phagocytosis by peritoneal macrophages.
Fig. 4.1 Purity of N-clorotaurine was proved by spectral data

(A) NCT-Na displays one peak at 251.54 nm by UV spectrum. The UV spectrum was measured by a UV visible spectrometer Lambda 25 (Perkin-Elmer). (B) NCT-Na dissolved in D$_2$O shows two methylene singlets at 50.8 and 49.0 ppm by $^{13}$C-NMR spectrum. The NMR spectrum was recorded on a Varian Gemini-200 300MHz spectrometer.
Fig. 4-2. TauCl modulates leukocyte trafficking in zymosan A-induced murine peritonitis

Mice were administered with an intraperitoneal dose (30 mg/kg) of zymosan A for 12 h, followed by intraperitoneal injection of vehicle or TauCl (20 mg/kg). Six hours later, peritoneal exudates were collected. (A) The number of total leukocytes in peritoneal exudates was counted. (B) The proportions of PMNs and mononuclear cells in collected peritoneal exudates were determined by differential cell counting. All data represent mean ± SD (n=3); ***p<0.001.
Fig. 4-3. TauCl increases phagocytic activity of macrophages in a peritonitis model

(A) In a spontaneous resolving zymosan A-initiated peritonitis model, the proportions of macrophages engulfing zymosan A-FITC particles (F4/80<sup>+</sup>zymosan A-FITC<sup>+</sup>) were determined by flow cytometry as described in Materials and Methods. To further verify TauCl-induced phagocytosis, immunocytochemical analysis was conducted using anti-F4/80 antibody.  (B) Peritoneal macrophages taking up zymosan A-FITC particles were stained for F4/80 or DAPI as described in Materials and Methods. (C) To evaluate phagocytic activity of macrophages <i>ex vivo</i>, mice were administered intraperitoneally with thioglycollate medium (3%). The thioglycollate-elicited macrophages were treated with TauCl (0.5 M) for 18 h, and then co-incubated with zymosan A-FITC particles for 1 h. The macrophages engulfing zymosan A-FITC particles were detected by immunostaining using anti-F4/80 antibody. A representative fluorescence micrograph shows macrophages (red) engulfing zymosan A-FITC particles (green). The data represent mean ± SD (n=3); **<i>p</i><0.01. Macrophages engulfing zymosan A-FITC particles are shown inside the dotted square.
Fig. 4-4. Comparison of TauCl and its parent compound taurine for their capabilities to induce phagocytosis

(A) RAW264.7 cells treated with TauCl or taurine (0.5 mM each) were co-incubated with FITC-labelled zymosan A particles for 1 h, and their phagocytic activity was measured as described in Materials and Methods. Representative flow cytometric dot plots reflect fold changes in the proportion of macrophages engulfing FITC-labelled zymosan A particles. All data represent mean ± S.D. (n=3), *p<0.05 and **p<0.01.
Fig. 4-5. TauCl upregulates HO-1 expression in macrophages during the resolution of zymosan A-induced murine peritonitis

To verify the expression of HO-1 induced by TauCl in peritoneal leukocytes, immunocytochemical analysis was conducted using anti-HO-1 antibody in a zymosan A-induced peritonitis. (A) Peritoneal leukocytes were stained for HO-1 or DAPI as described in Materials and methods. (B) The proportion of peritoneal macrophages expressing HO-1 was determined by flow cytometry. The data represent mean ± SD (n=3); *p<0.05.
Fig. 4-6. TauCl-induced HO-1 expression is associated with phagocytosis of zymosan A by murine peritoneal macrophages.

Mice were injected intraperitoneally with thioglycollate alone or thioglycollate plus TauCl. Mice were euthanized on day 3 (A,B). The proportion of macrophages expressing HO-1 obtained from TauCl plus thioglycollated-treated mice was determined by flow cytometry and immunocytochemical analyses as above. (C) Macrophages from the peritoneal exudates were incubated with FITC-labeled zymosan A for 1 h, and then the phagocytic activity was determined by flow cytometry. Mice pre-administered with vehicle or TauCl thrice a week were treated with zymosan A-FITC (1 mg/mouse), and peritoneal exudates were collected 18 h later. The proportion of macrophages engulfing zymosan A–FITC (F4/80+zymosan A–FITC+) was determined by flow cytometry (D) and immunocytochemical analysis (E). A representative fluorescence micrograph shows macrophages (red) engulfing zymosan A-FITC (green). The data represent mean ± SD (n=3); **p<0.01.
Fig. 4-7. TauCl augments phagocytosis through upregulation of HO-1 expression in RAW264.7 cells

(A) RAW264.7 cells were treated with ZnPP (10 μM) for 1 h prior to incubation with TauCl (0.5 mM) for additional 18 h. RAW264.7 cells treated with TauCl were co-incubated with FITC-labeled zymosan A particles for 1 h. The proportion of macrophages engulfing zymosan A-FITC particles was determined by flow cytometry. (B) RAW264.7 cells were transfected with scrambled or HO-1 siRNA, followed by treatment with TauCl (0.5 mM) for 18 h, and then phagocytic activity of macrophages was measured by flow cytometry. (C) RAW264.7 cells were transfected with pcDNA-mock or pcDNA-HO-1 for 24 h, and their phagocytic activity was measured as described in Materials and Methods. All data represent mean ± SD (n=3); ***p<0.001.
Fig. 4-8. HO-1 is the key transcriptional factor required for TauCl-mediated resolution of inflammation

For measuring efferocytosis *ex vivo*, thioglycollate-elicited macrophages from wild-type (WT) or HO-1 knockout mice were collected and then co-incubated with the dye-labeled zymosan A particles separated from the same peritoneal exudates as described in Materials and Methods. (A) Representative flow cytometric dot plots demonstrate changes in the percentage of macrophages engulfing zymosan A-FITC particles was determined by flow cytometry. The phagocytic activity of macrophages was measured by phase-contrast microscopy (B) and immunochemical analysis (C). The data represent mean ± SD (n=3); **p<0.01 and ***p<0.001.

5. Discussion

TauCl is produced endogenously, and is then released by the activated neutrophils that infiltrate into the inflamed site. In our present study, peritoneal injection of exogenous TauCl potentiated the phagocytic activity of peritoneal macrophages engulfing zymosan A particles. This process accelerates resolution of the zymosan-A induced peritonitis. It has been reported that TauCl-induced HO-1 expression accounts for its cytoprotective effects and anti-inflammatory activities (Olszanecki, 2004; Sun Jang, 2009). TauCl-induced HO-1 expression in macrophages also plays a role in invading pathogens, through enhancement of phagocytosis.

Resolution is an active process governed by a distinct set of endogenous anti-inflammatory and pro-resolving mediators (Basil and Levy 2016). When inflammation
occurs, neutrophils infiltrated into infected tissues play important roles by phagocytosing invading microbes. As the activated neutrophils undergo apoptosis, TauCl, endogenously released into inflamed site, acts as a local autacoid which stimulates the anti-inflammatory action of macrophages (Barua, 2001; Kanayama, 2002; Kim, 2015). In addition to having anti-inflammatory activity, TauCl has a direct microbicidal effect by killing pathogens in inflammatory process (Nagl, 2000; Nagl, 2001; Gruber, 2017). It has been reported that TauCl does not induce apoptotic cell death in human neutrophils (Kim et al., 2015). Here, we note that TauCl has no toxicity to macrophages (data not shown), but stimulates engulfment of zymosan A particles.

Engulfment of pathogens is facilitated in microbial infections when pattern recognition receptors (PRR) expressed by most innate immune effector cells such as dendritic cells, neutrophils and macrophages recognize pathogen-associated molecular patterns (PAMPs) expressed by an invading microorganism (Mogensen, 2009; Thompson, 2011). Our data suggest that TauCl stimulates the engulfment of zymosan A particles containing PAMPs derived from yeast, which allows the resolution of inflammation caused by fungal infection. Previously, we found that TauCl potentiated the efferocytic activity of macrophages through induction of scavenger receptors, such as BAI-1, MerTK, and Tim4 (Kim et al., 2015). Dectin-1, a receptor for fungal β-1,3-glucans, is expressed predominantly on the surface of phagocytes, including macrophages and dendritic cells, and to mediate phagocytosis of particles, such as zymosan A, containing β-glucan (Brown and Gordon 2001). Our recent study has demonstrated that TauCl substantially increased the phagocytic efficiency of
macrophages through upregulation of dectin-1 (Kim et al., 2017). Moreover, mice treated with zymosan A plus TauCl showed a higher proportion of peritoneal macrophages with elevated dectin-1 expression than those challenged with zymosan A alone (data not shown).

HO-1 expression is rapidly increased after injury and increased its expression provokes cytoprotective and anti-inflammatory effects in acute inflammation (Oh, 2006; Marcinkiewicz, 2009). In particular, induction of HO-1 expression in macrophages is associated with their anti-inflammatory function in patients with type 2 diabetes, cardiovascular disease and intestinal disorders (Fantuzzi, 2008; Takagi, 2008). Recently, it has been also reported that HO-1 plays important roles in cellular protection against microbial infection. Overexpression HO-1 attenuated severe lung injury induced by microbial infection (Hashiba et al., 2001). In contrast, HO-1-deficient mice are susceptible to microbial infection (Silva-Gomes, 2013; Gahlot, 2017). Although HO-1 induction in macrophages by TauCl has anti-inflammatory effects (Olszanecki, 2004; Sun Jang, 2009), the mechanism underlying pro-resolving effects exerted by TauCl through upregulation of HO-1 expression remained overlooked in inflammation caused by microbial infections. Our present study demonstrates that TauCl-induced HO-1 expression in macrophages protects against zymosan A-induced inflammation by stimulating their engulfment of zymosan A particles.

The action of HO-1 is thought to be mediated by carbon monoxide (CO) which is one of the by-products of heme degradation (Wang et al., 2009). Under the inflammatory condition, CO generated as a consequence of induction of HO-1 expression enhances
the host defense response to microbial infections (Chung et al., 2008) as well as exerts antioxidant effects (Brouard et al., 2000). CO suppressed not only pro-inflammatory cytokines response, but also increased production of anti-inflammatory cytokines in macrophages challenged with bacterial lipopolysachharide (LPS) (Otterbein, 2000; Sarady, 2002) Moreover, inhaled CO gas has been shown to increase production of pro-resolving lipid mediators including RvD1, RvD2, RvE2, and MaR1 (Chiang et al., 2013). CO treatment also upregulates the expression of 15-lipoxygenase, which is a key enzyme in pro-resolving lipid mediator biosynthesis. These mediators stimulated by induction of the HO-1/CO system limited infiltration of PMNs and enhances phagocytic capability of macrophages. Based on these findings, we speculate that CO may play a crucial role in mediating TauCl-induced resolution of inflammation. TauCl may stimulate the production of novel anti-inflammatory and pro-resolving lipid mediators, which coordinately downregulate excessive PMN accumulation and stimulate clearance of both microbial pathogens and cellular debris, facilitating resolution of inflammation.

In summary, TauCl released into the inflammatory milieu from apoptotic neutrophils stimulates phagocytosis through upregulation of HO-1 expression. It is evident that the upregulated HO-1 accounts for TauCl-induced phagocytic activity of macrophages and resolution of inflammation (Fig. 4-9). Uncleared inflammatory pathogens remaining in the inflammatory environment can damage to tissue, resulting in chronic inflammation. Through effective phagocytosis, macrophages prevent the release of cytotoxic waste from pathogen to the inflammatory microenvironment, thereby preventing the development of chronic inflammatory diseases. Thus, TauCl might have a therapeutic
potential in the management of inflammatory disorders associated with impaired phagocytosis.

Fig. 4-9. A proposed mechanism underlying the pro-resolving effects of TauCl exerted by stimulating macrophages-mediated phagocytosis
6. References


Oxygenase-1 Deficiency Diminishes Methicillin-Resistant Staphylococcus aureus Clearance Due to Reduced TLR9 Expression in Pleural Mesothelial Cells', *PLoS One*, 12: e0169245.


CONCLUSION

Acute inflammation is a protective reaction against inflammatory stimuli or tissue injury, but excessive uncontrolled inflammatory responses lead to chronic inflammation, resulting in chronic inflammatory diseases as well as cancer. To prevent chronic inflammation, timely resolution of inflammatory response is important. The process of resolution is actively controlled by a number of endogenous anti-inflammatory and pro-resolving mediators. In the past, endogenous mediators derived from omgega-3 polyunsaturated fatty acids (PUFAs) such as resolvins, protectins, and maresins have been reported to promote resolution of inflammation. Recently, attention has been focused on Prostaglandins formed as a consequence of the enzymatic oxygenation of arachidonic acid and related polyunsaturated fatty acids (PUFAs) by cyclooxygenase (COX) as well as other enzymes during inflammatory response. Our present study shows 15-Deoxy-Δ^{12,14}-prostaglandin J₂, endogenously generated during inflammatory process, promotes resolution of intestinal inflammation. Since uncontrolled macrophages activation as well as sustained production of IL-6 is implicated in the pathogenesis of inflammatory bowel diseases, 15d-PGJ₂-mediated not only inhibition of M1 macrophages polarization and induction of M2 macrophage but also blockade of IL-6 overexpression should be essential for resolution of intestinal inflammation and prevention of chronic inflammatory diseases. Moreover, 15d-PGJ₂ shows the pro-resolving effects on macrophages-mediated efferocytosis. The results from our present study suggest a novel mechanism that the activation of Nrf2 by 15d-PGJ₂ is one of the
important mechanisms responsible for its potentiation of engulfment of apoptotic cells by macrophages which will have a therapeutic potential in the management of chronic inflammatory disorders. Not only 15d-PGJ$_2$ but also Taurine chloramine (TauCl) released into the inflammatory milieu from apoptotic neutrophils will be involved in resolution of inflammation. In the present study, TauCl, one of endogenous anti-inflammatory mediators, promotes resolution of inflammation through regulating leukocytes infiltration and clearance of pathogens remaining in the inflammatory environment by macrophages.

Collectively, these observations suggest that 15d-PGJ$_2$ and TauCl generated during inflammatory responses exert resolution of inflammation, thereby preventing chronic inflammatory diseases. 15d-PGJ$_2$ and TauCl might have a therapeutic potential in the management of chronic inflammatory disorders and is also anticipated as desirable therapeutics for the prevention of cancer.
국문초록

염증이란 생체 조직이 외부로부터 감염이나 자극을 받을 때 나타나는 생체 방어 작용으로써 우리 몸에 필수적인 생리적 작용이다. 이전까지만 해도 염증은 친 염증성 매개체가 분해되어 멈춘다고 알려져 있었지만, 최근에는 염증의 해소 과정에 이르는 일련의 과정 동안 다양한 항염증 효과를 가진 내인성 매개체들에 의해 조절되는 능동적 과정으로 인식되고 있다. 염증 해소가 완전히 이루어지지 않으면, 급성염증은 만성 염증으로 발전하여, 암뿐 아니라 인체 대부분의 다양한 염증 질환의 원인이 되기에, 항염증 후보 물질을 찾고 염증 해소 이해는 필수적이다.

과민성 대장 증후군은 지속적인 대장 염증에 의해 유발된 대표적인 대장 염증 질환 중 하나이며, 염증성 대식세포에 의해 유발된다고 알려져 있다. 그러나 자세한 분자적 기작은 연구가 미한 상태이다. 본 연구자는 대장 염증시에 생성되는 항염증 물질을 탐색하고, 이 물질의 염증 해소효과를 대식 세포에 중점을 두어 연구하였다. Dextran sulfate sodium (DSS) 유발 대장염 동물 모델을 사용하였으며, 염증 해소시기에 cyclooxygenase-2 (COX-2) 최종 산물인 15-Deoxy-Δ12, 14-prostaglandin J2 (15d-PGJ2)가 생성 됐을 확인하였다. 내인성으로 생성된 15d-PGJ2는 대장 염증 해소를 촉진시키고, 과민성 대장 증후군을 억제하는데 이바지할 것으로 예상한다. 이를 규명하
기 위해서 대장염이 유발된 마우스에 15d-PGJ2를 주입하여 그 효과를 살펴보았다. 그 결과 15d-PGJ2는 대장 염증을 유도하는 neutrophils과 염증 대식세포인 classical activated macrophages (M1)을 감소시켰을 뿐 아니라, 염증 해소에 중요한 alternative macrophages (M2)를 증가시켰다. 또한, 만성 염증 및 암화 과정의 원인 중 하나인 대식세포의 IL-6 과다 생성을 억제하며 그에 따른 STAT3의 활성을 감소시킴으로써 대장 염증 해소를 촉진하는 것으로 사료된다.

사멸된 백혈구를 제거하는 과정, efferocytosis는 염증 해소에서 필수적으로 일어나야 한다. 효과적인 efferocytosis를 통하여 대식세포는 사멸된 백혈구로부터 나오는 독성물질의 분비를 억제함으로써 다른 주변 세포의 피해를 저해한다. 생체내에서 생성되는 COX-2 최종 산물인 15d-PGJ2는 염증 반응을 억제하는 것에 보고되고 있지만, 대식세포에 영향을 주어 염증 해소에 관여하는지는 알려진 바가 없다. 본 연구자는 15d-PGJ2의 대식세포 efferocytosis 능력 촉진 여부를 중점적으로 연구하였다. 우선 zymosan A를 이용하여 마우스에 복막염을 유도한 후, 염증이 최대치에 도달한 순간 15d-PGJ2를 처리하여 15d-PGJ2의 염증 해소 효과를 살펴 보았다. 15d-PGJ2 처리한 그룹에서는 복강 내 유입된 염증세포의 수가 현저히 감소하였지만, 염증 해소에 있어 중요한 단핵구의 비율은 상대적으로 증가함을 보았다. 또한 15d-PGJ2에 의해 대식세포의 사멸된 백혈구를 제거하는 능력 (efferocytosis)이 향상함을 확인하였다. 이러한 15d-PGJ2의 염증 해소 효
과는 Nrf2의 활성화를 통하여 대식세포의 scavenger receptor의 발현과 HO-1을 증가시키고 efferocytosis를 촉진함으로써 나타났다. 이는 Nrf2와 HO-1 결핍 마우스에서는 15d-PGJ2에 의한 염증 해소 효과가 나타나지 않았다. 이를 통해서 생체 내에서 생성되는 15d-PGJ2는 대식세포의 efferocytosis 능력을 향상시켜 염증 해소를 촉진하는 것으로 사료된다.

병원균 감염에 의한 염증 조절 실패는 염증성 질환의 원인 중 하나이다. 병원균 또는 독성물질이 완전히 제거되지 않으면 친 염증성 매개체와 사이토카인의 분비가 지속되어 세포와 조직의 손상이 일어나며 이는 만성 염증을 유발한다. Taurine Chloramine (TauCl)은 염증시 호중구의 세포자살로부터 생성되는 대표적인 내인성 물질이다. 생체내에서 생성되는 TauCl은 염증환경에서 염증 반응을 억제하는 기로 보고되고 있지만, 염증 해소에 관여하는지 알려진 바가 없다. 본 연구자는 TauCl의 감염에 의한 염증에 대한 해소 작용을 연구하고자, 염증 유발물질인 zymosan A를 마우스 복강에 주입한 후 염증이 최대치에 도달한 시점에 TauCl을 복강 내로 주입하였다.

선행연구 결과들은 바탕으로 TauCl을 6시간 동안 처리한 후, 복강액을 추출하여 TauCl의 염증해소 촉진 여부를 확인하였다. TauCl을 처리한 그룹에서는 복강내 유입된 염증세포의 수가 현저히 감소하였지만, 염증 해소에 있어 중요한 단핵구의 비율은 상대적으로 증가함을 보였다. 또한 TauCl에 의해 대식세포의 병원균 포식작용 능력 (Phagocytosis)이 증가 됐음을 알 수 있었다. 이러한 TauCl의 염증해소 효과는 HO-1 발현을 증가시킴으로써 나타남을 알 수 있었다.
타났다. 복막염 모델에서 TauCl을 주입하였을 때에 염증 해소 단계에서 대식세포의 HO-1 발현이 증가하는 것을 확인하였으며, HO-1 결핍마우스에서는 TauCl에 의한 연증 해소 효과가 나타나지 않았다. 이를 통해서 생체내에서 형성되는 TauCl은 대식세포의 phagocytosis 능력을 증가시켜 염증해소를 촉진하는 것으로 사료된다.

본연구를 통해 제시된 15d-PGJ2와 TauCl 의한 염증 해소를 기반으로 15d-PGJ2와 TauCl는 다양한 염증성 질환 및 암 예방 치료제로 개발 가능할 것으로 사료된다.

주요어
15-데옥시 프로스타글란디J2 (15d-PGJ2), 만성염증 대장 증후군(IBD), 염증해소, 대식세포, 석세포작용, Nrf2, HO-1, TauCl.

학번: 2010-21670
BIOGRAPHICAL DATA

Wonki Kim, Ph.D. Candidate

Tumor microenvironment Global Core Research
College of Pharmacy, Seoul National University
599 Gwanak-ro, Gwanak-gu, Seoul 151-742, South Korea

Phone; +82-2-877-3730, Fax: +82-2-874-9775
E-mail: worldsadon@hanmail.net

Education

Mar 2010 – present: College of pharmacy, Seoul National University, Seoul, South Korea (Combined Master and Ph.D.)

Mar 2005 – Feb 2010: Department of Life science, Catholic University, Kyung gi, South Korea (B.S.)

Honor and Awards


Outstanding poster award: The 8th International Conference on Heme Oxygenases: BiolIron and Oxidative Stress (October, 2014, Australia)
Outstanding poster award: International Conference on Nutrition and Physical Activity (NAPA) in Aging, Obesity, and Cancer (Mar, 2012, Seoul, Korea)

**Full Papers in Peer-reviewed Journals**


Abstracts presented at the professional meetings-----------------------------

Oral presentation

Kim W and Surh YJ. 15-Deoxy-△12,14-prostaglandin J2 exerts pro-resolving effects through Nrf2-induced expression of CD36 and heme oxygenase-1 (The 10th Seoul-Kyoto-Osaka Joint Symposium on Pharmaceutical Sciences For Young Scientists, May, 2017, Japan)

Kim W and Surh YJ. 15-deoxy-delta 12 14-prostaglandin j2 exerts pro-resolving effects through Nrf2-induced expression of CD36 and heme oxygenase-1 (2016 Korean Society
of cancer prevention Osaek Symposium, July, 2016, South Korea)

Kim W and Surh YJ. 15-Deoxy-△12,14-prostaglandin J2 exerts pro-resolving effects through activation of Nrf2-mediated expression of CD36 and HO-1 in murine macrophages (Korean Society of Cancer Prevention, July, 2014, South Korea)

Poster presentation

Kim W and Surh YJ. 15-deoxy-△12,14-prostaglandin j2 exerts pro-resolving effects through Nrf2-induced expression of CD36 and heme oxygenase-1 (2017 International conference korean society for molecular and cellular biology, Sep, 2017)

Kim W and Surh YJ. 15-Deoxy-△12,14-prostaglandin J2 exerts pro-resolving effects through Nrf2-induced expression of CD36 and heme oxygenase-1 (APNNO 2016 Biennial conference Nutrigenomics and Nutrigenetics for Disease prevention and Optimal health, Dec, 2016)

Kim W and Surh YJ. Taurine chloramine exerts anti-inflammatory and proresolving effects through induction of heme oxygenase-1 expression (International conference on Hemeoxygenase 2016, Sep, 2016)

Kim W and Surh YJ. 15-Deoxy-△12,14-prostaglandin J2 exerts pro-resolving effects through activation of Nrf2-mediated expression of CD36 and HO-1 in murine macrophages (Korean Society of Cancer Prevention, July, 2016, South Korea)
Kim W and Surh YJ. Taurine Chloramine Stimulates Phagocytosis through Nrf2-mediated Heme Oxygenase-1 Expression; Possible Involvement Carbon Monoxide (The 7th International Congress of Asian Society of Toxicology, Jun, 2016)

Kim W and Surh YJ. Taurine chloramine exerts proresolving effects through Nrf2-mediated heme oxygenase-1 upregulation (The 20th International Taurine meeting, May, 2016)

Kim W and Surh YJ. 15-Deoxy-Δ12,14-prostaglandin J2 stimulates efferocytosis through Nrf2-mediated expression of CD36 and heme oxygenase-1 in murine macrophages (20th International Conference on Annual Meeting of Korean Society of Cancer Prevention, Dec, 2015)

Kim W and Surh YJ. Taurine Chloramine Stimulates Phagocytosis through Nrf2-mediated Heme Oxygenase-1 Expression: Possible Involvement of Carbon Monoxide (20th International Conference on Annual Meeting of Korean Society of Cancer Prevention, Dec, 2015)

Kim W and Surh YJ. 15-Deoxy-Δ12,14-prostaglandin J2 stimulates efferocytosis through Nrf2-mediated expression of CD36 and heme oxygenase-1 in murine
macrophages (The 31st Annual Meeting of KSOT/KEMS, Nov, 2015)

**Kim W** and Surh YJ. Taurine Chloramine Stimulates Phagocytosis through Nrf2-mediated Heme Oxygenase-1 Expression: Possible Involvement of Carbon Monoxide (The 31st Annual Meeting of KSOT/KEMS, Nov, 2015)

**Kim W** and Surh YJ. Taurine Chloramine Stimulates Phagocytosis through Nrf2-mediated Heme Oxygenase-1 Expression; Possible Involvement Carbon Monoxide (2015 SNU CRI cancer symposium, Apr, 2015)


**Kim W** and Surh YJ. Taurine Chloramine Stimulates Phagocytosis through Nrf2-mediated Heme Oxygenase-1 Expression; Possible Involvement of Carbon Monoxide (The 26th KSMCB Winter Conference 2015, Jan, 2015)

**Kim W** and Surh YJ. 15-Deoxy-Δ12,14-prostaglandin J2 stimulates efferocytosis through Nrf2-mediated expression of CD36 and heme oxygenase-1 in murine macrophages (The 8th International Conference on Heme Oxygenases: BioIron and Oxidative Stress, Oct, 2014)

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Kim W and Surh YJ. Taurine Chloramine Stimulates Phagocytosis through Nrf2-mediated Heme Oxygenase-1 Expression; Possible Involvement Carbon Monoxide (The 8th International Conference on Heme Oxygenases: BioIron and Oxidative Stress, Oct, 2014)

Kim W and Surh YJ. Taurine Chloramine Stimulates Phagocytosis through Nrf2-mediated Heme Oxygenase-1 Expression; Possible Involvement Carbon Monoxide (International Conference on the 19th Annual Meeting of Korean Society of Cancer Prevention, Dec, 2014)

Kim W and Surh YJ. 15-deoxy-delta12,14-prostaglandin J2 exerts pro-resolving effects through activation of Nrf2-mediated CD36 Expression in murine macrophages (The XII International congress of Toxicology, July, 2013)

Kim W and Surh YJ. 15-deoxy-delta12,14-prostaglandin J2 exerts pro-resolving effects through activation of Nrf2-mediated CD36 Expression in murine macrophages (2013 Shanghai Symposium on Polyunsaturated Fatty Acids and Metabolism, Jun, 2013)

Kim W and Surh YJ. 15-Deoxy-Δ12,14-prostaglandin J2 exerts pro-resolving effects through activation of Nrf2-mediated expression of CD36 and HO-1 in murine macrophages (The Environmental Response IV, Mar, 2013)
Kim W and Surh YJ. 15-deoxy-delta12,14-prostaglandin J2 exerts pro-resolving effects through activation of Nrf2-mediated CD36 expression in murine macrophages (한국프리라디칼학회 2012 년도 추계학술대회 및 제 4회 NCEED 국제 심포지엄, Nov, 2012)

Kim W and Surh YJ. 15-deoxy-delta12,14-prostaglandin J2 exerts pro-resolving effects through activation of Nrf2-mediated CD36 expression in murine macrophages (2012 Korean Society for Molecular and Cellular Biology, Oct, 2012)

Kim W and Surh YJ. Pro-resolving effects of 15-deoxy-delta12,14-prostaglandin J2 through activation of Nrf2-mediated CD36 expression in murine macrophages (3rd Nutrition and Physical Activity (NAPA) in Aging, Obesity, and Cancer, Mar, 2012)

Kim W and Surh YJ. Pro-resolving Effects of 15-deoxy-Δ12, 14-prostaglandin J2 through Nrf2-Mediated CD36 Expression in Murine Macrophages (PROCEEDINGS of the Fall International Convention of The Pharmaceutical Society of Korea, Nov, 2011)

Kim W and Surh YJ. Pro-resolving Effects of 15-deoxy-Δ12, 14-prostaglandin J2 through Nrf2-Mediated CD36 Expression in Murine Macrophages (102nd Annual Meeting (Innovation and Collaboration: The Path to Progress, Apr, 2011)