Biosynthesis of prostaglandin 15dPGJ2-glutathione and -cysteine conjugates in macrophages and mast cells via MGST3

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Abbreviations:

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>mPGES-1</td>
<td>microsomal prostaglandin E synthase-1</td>
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<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>GSH</td>
<td>glutathione</td>
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<tr>
<td>MGST3</td>
<td>microsomal glutathione S-transferase 3</td>
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<tr>
<td>15dPGJ</td>
<td>15-deoxy-Δ^12,14^-prostaglandin J2</td>
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<tr>
<td>15dPGJ2-GS</td>
<td>15-deoxy-Δ^12,14^-prostaglandin J2-glutathione</td>
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<tr>
<td>15dPGJ2-Cys</td>
<td>15-deoxy-Δ^12,14^-prostaglandin J2-cysteine</td>
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<tr>
<td>NSAIDs</td>
<td>non-steroidal anti-inflammatory drugs</td>
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<tr>
<td>PGD2</td>
<td>prostaglandin D2</td>
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<tr>
<td>PGE2</td>
<td>prostaglandin E2</td>
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<tr>
<td>PGH2</td>
<td>prostaglandin H2</td>
</tr>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>Keap1</td>
<td>kelch-like ECH-associated protein 1</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor “kappa-light-chain-enhancer” of activated B-cells</td>
</tr>
<tr>
<td>HIF1α</td>
<td>hypoxia-inducible factor 1-alpha</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>BMDM</td>
<td>bone-marrow derived macrophages</td>
</tr>
<tr>
<td>CBMC</td>
<td>cord-blood derived mast cells</td>
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<tr>
<td>MQ water</td>
<td>MilliQ water</td>
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Abstract

Inhibition of microsomal prostaglandin E synthase-1 (mPGES-1) results in decreased production of pro-inflammatory PGE2 and can lead to shunting of PGH2 into the PGD2/15-deoxy-Δ^12,14^-prostaglandin J2 (15dPGJ2) pathway. 15dPGJ2 forms Michael adducts with thiol-containing biomolecules such as glutathione (GSH) or cysteine residues on target proteins and is thought to promote resolution of inflammation. We aimed to elucidate the biosynthesis and metabolism of 15dPGJ2 via conjugation with GSH, to form 15dPGJ2-GS and 15dPGJ2-cysteine (15dPGJ2-Cys) conjugates and to characterize the effects of mPGES-1 inhibition on the PGD2/15dPGJ2 pathway in mouse and human immune cells. Our results demonstrate the formation of PGD2, 15dPGJ2, 15dPGJ2-GS, and 15dPGJ2-Cys in RAW264.7 cells after lipopolysaccharide (LPS) stimulation. Moreover, 15dPGJ2-Cys was found in LPS-activated primary murine macrophages as well as in human mast
cells following stimulation of the IgE-receptor. Our results also suggest that the microsomal glutathione S-transferase 3 (MGST3) is essential for the formation of 15dPGJ₂ conjugates. In contrast to inhibition of cyclooxygenase (COX), which leads to blockage of the PGD₂/15dPGJ₂ pathway, we found that inhibition of mPGES-1 preserves PGD₂ and its metabolites. Collectively, this study highlights the formation of 15dPGJ₂-GS and 15dPGJ₂-Cys in mouse and human immune cells, the involvement of MGST3 in their biosynthesis, and their unchanged formation following inhibition of mPGES-1. The results encourage further research on their roles as bioactive lipid mediators.

Keywords: PGD₂/15-deoxy-Δ¹²,¹⁴-prostaglandin J₂, cyclopentenones, lipid metabolism, GSH, resolution, inflammation, mPGES-1 inhibition, microsomal glutathione S-transferase-3, Michael adducts, immune cells

Introduction

There is growing evidence that selective mPGES-1 inhibitors represent an alternative therapeutic strategy to inhibit pro-inflammatory PGE₂ production. Unlike non-steroidal anti-inflammatory drugs (NSAIDs), which target cyclooxygenases (COX) and suppress all downstream prostaglandins, selective mPGES-1 inhibition prevents the formation of induced PGE₂ biosynthesis but spares other prostaglandins and may also lead to increased biosynthesis of anti-inflammatory arachidonic acid metabolites. The resulting changes in prostaglandin profiles observed in various cell types including cancer cells, fibroblasts, macrophages and vessels, raise the possibility of cardiovascular protection and enhanced anti-inflammatory effects following mPGES-1 inhibition (1-7). Previously, we showed that the eicosanoid profile is altered in LPS treated peritoneal macrophages from mPGES-1 knockout (KO) mice compared with cells from wild-type (WT) mice. Specifically,
levels of the PGD$_2$ metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15dPGJ$_2$) were increased after deletion of mPGES-1 (8). PGD$_2$ and 15dPGJ$_2$ are both synthesized during the inflammatory response in the inflammatory exudates of mice with experimental peritonitis and contribute to the resolution of inflammation (9). In addition, 15dPGJ$_2$ has been suggested to play a role in polarizing macrophages toward the anti-inflammatory M2 phenotype (10) and controlling the resolution phase of inflammation by inducing apoptosis in activated macrophages (11). Moreover, mPGES-1 was shown to regulate macrophage polarization toward a pro-inflammatory phenotype, whereas deletion of mPGES-1 resulted in upregulation of anti-inflammatory gene expression in macrophages (12). Furthermore, deletion of mPGES-1 as well as treatment with 15dPGJ$_2$ protected against influenza A infection in rodents (13, 14). The extent to which inhibition of mPGES-1 might contribute to activation of the PGD$_2$/15dPGJ$_2$ pathway and macrophage polarization remains unclear.

15dPGJ$_2$ is a bioactive product of PGD$_2$ metabolism. PGD$_2$, produced by COX/hematopoietic PGD synthase during innate and adaptive immune responses can be further metabolized to 15dPGD$_2$ and PGJ$_2$ (9). The latter gives rise to two downstream cyclopentenone-PG metabolites of the J series, namely $\Delta^{12}$-PGJ$_2$ and 15dPGJ$_2$ (15). Among the PGD$_2$ metabolites, 15dPGJ$_2$ is the best studied and has gained increasing interest since its initial description in 1983 (16). 15dPGJ$_2$ has been shown to function as a ligand of PPAR$_\gamma$ (17, 18) and is linked to a variety of anti-inflammatory, anti-proliferative, cytoprotective and pro-resolving activities (19-21). However, its physiological role has been questioned because only very low levels of free 15dPGJ$_2$ have been detected in-vivo, which contrasts with its activation of PPAR$_\gamma$ in-vitro at micromolar concentrations (22). Cyclopentenone prostaglandins are reactive lipid electrophiles that contain functional groups that can bind rapidly to the cysteine-containing tripeptide glutathione (GSH) and thiol groups in proteins such as Keap1, NF-$\kappa$B or HIF1$\alpha$ via Michael addition (23, 24). The electrophilic center at C9 within the cyclopentenone ring of 15dPGJ$_2$ has been characterized as the primary site of Michael addition with GSH and proteins (25, 26). Glutathionylation of a variety of electrophiles has been described in-vitro and in-vivo where GSH conjugation can protect the cell from the accumulation of harmful electrophiles but also leads to the formation of bioactive metabolites such as the cysteinyl leukotrienes (LTC$_4$, LTD$_4$, LTE$_4$) (27). Similar to the biosynthesis of the four-series leukotrienes, 15dPGJ$_2$ has been shown to be metabolized in cell culture via the conjugation to GSH (25, 28).
treated HepG2 cells, 15dPGJ2-GS has been described to be further metabolized via reduction at the cyclopentenone ring, removal of glutamic acid and glycine into a cysteine-conjugate (15dPGJ2-Cys) within 24 h (28). However, descriptions of their biosynthetic formation in immune cells are lacking and the function of these metabolites remains unclear. The cyclopentenone reactivity might be a reasonable explanation for the difficulty in detecting and quantifying free 15dPGJ2 in biological samples which challenged its role as an endogenous mediator.

The pleiotropic mechanisms that follow the inhibition of mPGES-1 lead to a decrease in pro-inflammatory PGE2 and as discussed here, to an enhancement of the PGD2/15dPGJ2 pathway. Thus, inhibition of mPGES-1, which is associated with anti-inflammatory and pro-resolving effects, holds great therapeutic potential and requires a detailed understanding of these pathways. Therefore, in this study, we investigated the biosynthesis of PGD2 metabolites, namely 15dPGJ2, 15dPGJ2-GS and 15dPGJ2-Cys in mouse and human immune cells and examined the effects of mPGES-1 inhibition on the formation of these metabolites.

Material and Methods

Materials

Prostaglandins including 15-deoxy-\(\Delta^{12,14}\)-PGJ2 (CAY-18570-1) and 15-deoxy-\(\Delta^{12,14}\)-PGJ2 glutathione (CAY-18580-100) were purchased from Cayman Chemicals Co. (Ann Arbor MI). LPS from E. Coli (126M4087V), and reduced GSH were purchased from Sigma Aldrich (Germany). Recombinant murine Macrophage Colony-Stimulating Factor (M-CSF) (0914245) was purchased from Peprotech (Germany). 1 x RBC lysis buffer (4314838) obtained from eBioscience. The COX inhibitors NS-398 and Diclofenac were purchased from Merck KGaA, Darmstadt, Germany and Sigma Aldrich (Germany) respectively. Selective mPGES-1 inhibitors compound III and compound 118 were described previously in (3, 29) and were produced by Gesynta pharma AB, Stockholm, Sweden. Inhibitors were reconstituted in DMSO to be used in experiments, whereby DMSO concentration did not exceed 0.05% in cell culture.
**In-vitro** preparation of 15dPGJ$_2$-Cys conjugate with reduced carbonyl at position C11

15dPGJ$_2$-Cys was prepared in a 1 mL phosphate buffered saline solution (PBS) containing 0.5 mg/mL L-Cysteine (Sigma), 1 mg GSH transferase from equine liver (Sigma) and 100 µg 15dPGJ$_2$ (Cayman Chemical) at 37 °C on a thermoblock, shaking for 2 h. The addition of glutathione-S-transferase (GST) to the 15dPGJ$_2$-L-Cysteine mixture has been described previously (28) but was found to be expandable in order to achieve full conjugation. The reaction was stopped by addition of formic acid to a final concentration of 0.3 % and the sample was subsequently loaded on a polymeric reverse phase sorbent column (StrataX-C18, Phenomenex). After the sample was loaded, the cartridge was rinsed with 1 mL 0.1% formic acid/ 5% methanol (MeOH) and the sample was eluted with 0.1% formic acid in MeOH. Eluates were dried in a vacuum concentrator and the product obtained was dissolved in 0.5 mL MeOH containing 1 mg/mL CeCl$_3$·H$_2$O and an aqueous solution of NaBH$_4$ (12% w/w). The reduction reaction from a carbonyl to a hydroxyl at position C11, was carried out on ice for 1 h. Subsequently the sample was acidified and applied to a polymeric reverse phase sorbent column and cleaned as described above. The eluate was dried and stored at -20 °C until LC-MS/MS analysis. Conjugation quality and efficiency was assessed by LC-MS/MS analysis and full conjugation was assumed when no non-conjugated 15dPGJ$_2$ could be detected.

**Plasma stability and pharmacokinetic analysis of 15dPGJ$_2$-Cys**

Plasma from freshly drawn heparinized (25 IU/mL) blood of healthy volunteers (in accordance with the Declaration of Helsinki and approved by the Regional Ethics committee, Stockholm Dnr 02-196) was obtained by centrifugation at 3000 rpm for 10 min. PGD$_2$, 15d-PGJ$_2$, 15d-PGJ$_2$-GS and 15d-PGJ$_2$-Cys were prepared in DMSO and spiked into 100 µL plasma at a final concentration of 0.5 µM in a 96-well plate and incubated at 37 °C for 0, 1, 3, 24 and 48 h. The samples were collected after incubation and stored at -20 °C until preparation for LC-MS/MS analysis. Plasma samples were thawed on ice, spiked with 400 µL of 100% MeOH containing the deuterated internal standards 6-keto-PGF$_{1α}$-d4, PGF$_{2α}$-d4, PGE$_2$-d4, PGD$_2$-d4, TxB$_2$-d4, and 15-deoxy-$\Delta^{12,14}$-PGJ$_2$-d4 (Cayman Chemical), followed by vortexing and centrifugation at 3000 g for 10 min at 4 °C.
Supernatants were collected and evaporated until complete dryness. Samples were reconstituted in 0.05% formic acid and subjected to solid phase extraction as described below.

For the pharmacokinetic analysis of 15dPGJ₂-Cys, mice (n=3 per time-point) were injected subcutaneously with 200 µL in-house generated 15dPGJ₂-Cys (dissolved in 0.1% DMSO/ saline solution to reach a final concentration of 5µg/mouse). Blood (~200µL) was collected after 0.5, 1 and 2 h from the left ventricle using EDTA-collection tubes (BD Vacutainer®, 367841), while the mice were anesthetized by isoflurane (Sigma-Aldrich, Y0000858). Blood samples were stored at 4 °C until centrifugation at 13000 rpm at 4 °C, followed by addition of MeOH (600 µL). Protein precipitation and solid phase extraction were performed as described above and in the following. Animal experiments were approved by the regional Ethics Committee, Stockholm (Dnr. 7886-2018).

**Cloning, expression and purification of MAPEG proteins**

The various MAPEG protein genes were cloned into pPICZA yeast vector (Invitrogen) with six histidine-tags at N terminus as described earlier (30, 31). In brief, all plasmids were transformed into competent cells of the *Pichia pastoris* KM71H strain using *Pichia* Easy Comp Transformation kit (Invitrogen). The transformed yeast cells were cultivated in baffled flasks containing 2*2 L minimal yeast medium containing glycerol at 27 °C at 110 rpm shaking. The cells were harvested and again resuspended in 2 L of fresh minimal yeast medium supplemented with 0.6% (v/v) MeOH every 24 h. The pH of the yeast medium was monitored and adjusted to 6.5 using 8% (v/v) NH3. Cells were harvested after 48 h and resuspended in breaking buffer (50mM Tris-HCl pH 7.8, 100 mM KCl, 10% (v/v) glycerol). Cells were lysed by homogenization with glass beads in a Bead-Beater ((BioSpec Products, Inc.) 6 times with 5 min intervals at 4 °C. Then broken cells were filtered through a nylon net and centrifuged at 1500 g for 10 min to remove cell debris. The supernatant was solubilized in 1% (w/v) Triton X-100, 0.5% (w/v) sodium deoxycholate and 5 mM β-mercaptoethanol under constant stirring at 4 °C for 1 h and further centrifuged at 10,000 g for 15 min at 4 °C. The clear supernatant was passed through a Ni-Sepharose Fast Flow (GE Healthcare) column pre-equilibrated with 25 mM Tris/HCl pH 7.8,150 mM NaCl, 15mM imidazole pH 7.8. The column was then washed with 15 volumes (CV) of wash buffer (25 mM Tris/HCl...
pH 7.8, 300 mM NaCl, 5% (v/v) glycerol, 5 mM β-mercaptoethanol, 0.03% (w/v) n-dodecyl β-D-maltoside (DDM), and 50 mM imidazole, pH 7.8) and protein was eluted with the same buffer containing 400 mM imidazole, pH 7.8. Pooled fractions were passed twice through S-hexylglutathione agarose column (Abcam and GE Healthcare). After passing the supernatant, the column was washed with buffer containing 25 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% (v/v) glycerol, 5 mM β-mercaptoethanol, 0.03% DDM. Protein was eluted with and the same buffer containing 30 mM probenecid. The eluted protein was concentrated using amicon Ultra 50-kDa cutoff membrane (Millipore) and loaded on size exclusion chromatography (SEC) with Superdex 200, 16/600 (GE Healthcare) column equilibrated with 25 mM Tris/HCl pH 7.5, 150 mM NaCl, 5% (v/v) glycerol, 0.1 mM tris (2-carboxyethyl) phosphine (TCEP), 0.03% DDM. Fractions containing the respective protein were combined and used directly for kinetics measurements. In some cases, protein was concentrated up to 0.8 to 1.0 mg/ml with 100 kDa cut off membrane (Millipore). Proteins were aliquoted, flash frozen in liquid nitrogen and stored at −80 °C until further use.

**Enzyme activity measurements of MAPEG proteins**

Enzymatic formation of 15dPGJ₂-conjugates by microsomal glutathione S-transferase 1 (MGST1), microsomal glutathione S-transferase 2 (MGST2), microsomal glutathione S-transferase 3 (MGST3), leukotriene C4 synthase (LTC4S), microsomal prostaglandin E₂ synthase 1 (mPGES-1) and 5-lipoxygenase activating protein (FLAP) was tested. Briefly, 5µg of each protein was added to 95 µL reaction buffer (25 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.03% DDM), containing reduced GSH (0.06 mM). The reaction was started when 15dPGJ₂ (1 µg) was added and incubation continued for 5 min with gentle shaking. All reactions were performed in triplicates. After incubation for 5 min the reactions were stopped with 800 µL 0.5% formic acid and reaction tubes were immediately placed on ice until lipid extraction. Control reactions without supplementing enzyme or substrate (15dPGJ₂) were carried out in parallel. Assay conditions were optimized for incubation times (0.25 min – 60 min) and GSH concentrations (0.06 mM – 1 mM). Samples were then spiked with 50 µL deuterated internal standard containing 6-keto-PGF₁α-d₄, PGF₂α-d₄, PGE₂-d₄, PGD₂-d₄, TxB₂-d₄, and 15-deoxy-Δ¹²,¹⁴-PGJ₂-d₄ (Cayman Chemical) in 100% MeOH and loaded on a pre-activated and equilibrated Oasis-HLB 1cc 30mg cartridge (Waters), washed with 5% MeOH, containing 0.05% formic acid and subsequently lipids were
eluted in 100% MeOH. Samples were evaporated and stored at -20 °C until LC-MS/MS analysis of the formation of 15dPGJ₂-GS. Experiments with the soluble Mu class glutathione S-transferase (Human, GSTM4-780H from Creative Bio Mart) were carried out under similar conditions. Assay conditions were varied for incubation times (up to 60 min) and protein concentrations (up to 10 µg per reaction). The MGST3 protein was tested in parallel as reference.

**Km determination of MGST3 protein**

For determination of the Michaelis constant (\(K_m\)) of MGST3, 5 µg enzyme was added to 95 µL reaction buffer (25 mM Tris-HCL, pH 7.5, 100 mM NaCl, 0.03% DDM), containing reduced GSH (0.1 mM). The reaction was started when 15dPGJ₂ (1 µg – 90 µg) was added and incubation continued for 5 min with gentle shaking at room temperature. Control reactions without supplementing enzyme or substrate (15dPGJ₂) were carried out in parallel under similar conditions. All reactions were performed in triplicates. After incubation for 5 min at room temperature, the reactions were stopped with 200 µL 0.5% formic acid and reaction tubes were placed on ice immediately until analysis by high-performance liquid chromatography (HPLC). The enzymatic conjugation of 15dPGJ₂ with GSH was monitored with a UV detector at 306 nm with 1:1:0.003 (v/v) acetonitrile/MQ water/acetic acid as mobile phase. Area units for 15dPGJ₂-GS were collected, subtracted from background and quantified based on spiked prostaglandin B₂ (PGB₂, 560 pmol, monitored as internal standard) with a correction coefficient of 0.4 (extinction coefficient 15dPGJ₂ (12,000) / extinction coefficient of PGB₂ (30,000)). Data were expressed as pmol/µg/min and the \(K_m\) value was calculated using hyperbolic regression analysis.

**Macrophage cell line culture**

The murine macrophage cell line RAW264.7 (ATCC) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 1 mM sodium pyruvate and 2 mM L-glutamine at 37 °C in a humidified 5% CO₂ atmosphere. Passaging of cells was performed using PBS-EDTA (5 mM) solution and passages 5-10 were used for experiments. For stimulation, cells were plated in 24-well plates, 12-well plates, or 6-well plates at concentrations of 6-10 x 10⁴ cells/cm² if not indicated differently. Cells were allowed to adhere for 24 h, then medium was aspirated and replaced with fresh culture
medium containing 2 µg/mL LPS and respective treatments (NS-398, 0.1 µM; CIII, 10µM; 118, 1 µM) for various time points. For transcription and translation inhibition, RAW 264.7 cells were pre-treated for 4 h with 5 µg/mL actinomycin D (Cayman Chemical, 11421) or 100 µg/mL cycloheximide (Cayman chemical, 14126) prior addition of 2 µM 15dPGJ₂ for various time-points.

Primary macrophage cell culture

Wild type (WT) and mPGES-1 knock-out (KO) mice were on an inbred DBA/1lacJ genetic background and generated by breeding heterozygous littermates as described previously (32). All animal experiments were approved by the regional Ethics Committee, Stockholm (Dnr. 7886-2018). For the preparation of bone marrow derived macrophages (BMDM), mice were sacrificed by CO₂ inhalation and femoral and tibia bones from hind legs were dissected and cleaned from all remaining tissue. Bone ends were cut open and bone marrow was flushed out. The bone marrow suspension was filtered through a 70 µm cell strainer, red blood cells were lysed with 1 x RBC lysis buffer and bone marrow cells were subsequently reconstituted in DMEM cell culture medium supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 1 mM sodium pyruvate, 2 mM L-glutamine and 2 mM HEPES in the presence of 20 ng/mL MCSF. Cells were cultured in low attachment cell culture flasks at 37 °C in a humidified 5% CO₂ atmosphere for 6 days. After 3 days, half of the cell culture medium was exchanged with fresh culture medium supplemented with 40 ng/mL M-CSF and incubation was continued. After 6 days in culture, the medium was aspirated and cells were detached from the cell culture flasks using PBS-EDTA (5 mM), counted and plated at various densities for stimulation experiments.

For macrophage activation, cells were stimulated with 2 µg/mL LPS if not indicated otherwise. When cells were co-incubated with GSH, 0.5 mM GSH in PBS was supplemented to the culture medium. For kinetic experiments in Figure 2 and 4, BMDM were directly seeded to 24-well plates, differentiated for 6 days and then treated. In these experiments prostanoid levels were normalized to protein concentrations. Protein concentration determination of cell pellets after lysis (50 mM Tris-HCl, 150 mM NaCl, 1% SDS, pH 7.9) and sonication for 15 min was measured by the bicinchoninic acid (BCA, Thermo Fisher) assay according to manufacturer’s instructions.
Primary human mast cell culture

For the generation of cord-blood derived mast cells (CBMCs), CD34⁺-hematopoietic progenitors were isolated from cord-blood and cultured as previously described (33), (in accordance with the Declaration of Helsinki and approved by the regional Ethics Committee, Stockholm, Dnr. 2019-01729,). When the cells reached about 90% tryptase positivity they were activated. Prior anti-IgE-activation, the cells were treated with 10 ng/mL human recombinant IL-4 (PeproTech) for four days, and the day before activation 1 µg/mL human IgE (Calbiochem) was added. Cells were plated to 24-well plates at a density of 0.5 x 10⁶ cells/mL or 1 x 10⁶ cells/mL, and the cells were stimulated with 2 µg/mL anti-IgE (Sigma) with or without 118 (1 µM) or diclofenac (1 µM), or only culture medium for unstimulated controls and incubated for 24 h at 37 °C. Subsequently, cells and supernatants were transferred to reaction tubes, centrifuged at 300 g for 10 min and supernatants were collected and frozen at -20 °C until LC-MS/MS analysis of lipid mediators. Cells were collected and frozen at -80 °C until RNA extraction.

Generation of RAW264.7 cells lacking MGST3

The CRISP-Cas9 system was used to generate MGST3 knock-out cells. The sgRNA was designed using the Green Listed software (http://greenlisted.cmm.ki.se/) (34), and the Brie reference library (35) selecting the sgRNA with the highest on-target score. A 2'-O-methyl modified and phosphorothioate stabilized version of the sgRNA (Mgst3: CGACACTCACGTGTTCTGGT) was ordered from Sigma-Aldrich. For sequencing, forward primer: ACCAATGCCCTCGTTCACAT and reverse primer: CAGAAAACCAGGCGCTCAT were designed to generate a 500-700 bp amplicon with the sgRNA binding site in the middle of the amplicon.

RAW264.7 were cultured in DMEM supplemented with 10 % fetal bovine serum and Penicillin-Streptomycin. The Neon Electroporation system was used to deliver CRISPR components according to manufacturer suggestions (pulse voltage: 1680, pulse width: 20 ms, and pulse number: 1). CRISPR reaction with ~500 pmol sgRNA and 20 µM spCas9 (Sigma-Aldrich) were delivered into 5x10⁶ cells using the Neon 10 µL kit. Single
cell clones were generated and screened for complete knock out of the gene. Typically, cells were harvested for PCR amplicon generation and sequenced using Sanger sequencing and analysed by ICE (https://ice.synthego.com) for indels. Cells with >90% of indel were used for further experiments.

**Lipid extraction**

Supernatants were thawed on ice, acidified with 350 µL 0.5% formic acid and spiked with 50 µL deuterated internal standard containing 6-keto-PGF₁α-d₄, PGF₂α-d₄, PGE₂-d₄, PGD₂-d₄, TXB₂-d₄, and 15-deoxy-Δ¹²,¹⁴-PGJ₂-d₄ (Cayman Chemical) in 100% MeOH. Samples were loaded on a pre-activated and equilibrated Oasis-HLB 1cc 30mg cartridge (Waters), washed with 5 % MeOH, containing 0.05 % formic acid and subsequently lipids were eluted in 100 % MeOH. Samples were evaporated and stored at -20 ºC until LC-MS/MS analysis.

Cell pellets were thawed on ice and re-suspended in 400 µL ice cold 100 % MeOH spiked with 50 µL deuterated internal standard containing 6-keto-PGF₁α-d₄, PGF₂α-d₄, PGE₂-d₄, PGD₂-d₄, TXB₂-d₄, and 15-deoxy-Δ¹²,¹⁴-PGJ₂-d₄ (Cayman Chemical). Samples were mixed (10 x by pipetting) and vortexed prior to a 20 min incubation on ice. Thereafter, samples were centrifuged (9000 g, 10 min at 4 ºC) and supernatants were transferred to a fresh reaction tube. Remaining pellets were suspended with 100 µL ice cold 100 % MeOH, mixed, vortexed and subsequently centrifuged (9000 g, 10 min at 4 ºC). Supernatants were transferred to already collected supernatants. Samples were evaporated and reconstituted in 50 µL of 20 % acetonitrile/MQ water and incubated at 4 ºC for 20 min. Samples were centrifuged (13000 g, 10 min at 4 ºC) and supernatants were transferred to injection vials for LC-MS/MS analysis.

**LC-MS/MS analysis of prostanoids**

Samples were reconstituted in 50 µL of 20 % acetonitrile/MQ and separated on a 50 × 2.1 mm Acquity UPLC BEH C18, 1.7 µm column (Waters) in a 13 min linear gradient with 0.05 % formic acid/MQ water as mobile phase A and 0.05 % formic acid/acetonitrile as mobile phase B at a flow rate of 0.6 mL/min. Analytes were quantified by MRM in negative mode for all prostanoids as described previously (8), except of the 15dPGJ₂-conjugates which were primarily analyzed in positive mode (Supplementary Table S1), using a triple quadrupole
mass spectrometer (Acquity TQ detector, Waters). Data presented in Figure 2e and S2e were acquired on an Acquity Xevo TQ-XS UPLC/MS system (Waters) with analyte separation on a 50 × 2.1 mm Acquity UPLC BEH C18, 1.7 μm column (Waters) in a 17 min linear gradient with 0.05 % formic acid/MQ water as mobile phase A and 0.05% FA/10% isopropanol in acetonitrile as mobile phase B at a flow rate of 0.5 mL/min. MRM transitions applied were m/z 624.0 > 308.0 (ES+) for 15dPGJ2-GS and m/z 438.0>351.0 for 15dPGJ2-Cys (ES-). Raw data were processed and analyzed using MassLynx software (version 4.1 and 4.2) and quantified against external standard curve with internal standard calibration for 6-keto-PGF1α, PGF2α, PGE2, PGD2, TXB2, and 15-deoxy-Δ12,14-PGJ2. For the quantification of 15dPGJ2-GS and 15dPGJ2-Cys, standard calibration curves were generated with the commercial standard or in-house generated metabolite respectively at serial concentrations ranging from 0 to 48 pmol injected. Deuterated internal standard was not available for these conjugates. Peak areas were recorded and the concentration of the metabolites in unknown samples were determined using the obtained calibration curves for each metabolite respectively.

Quantitative RT-PCR for MGST3

Cells were cultured and treated as described earlier. After 24 h the cells were washed once with PBS and lysed (RLT lysis buffer, QUIAGEN) in the culture vessel according to manufacturer’s instructions. Cell culture plates were subsequently frozen at -80 °C until RNA extraction. RNA was isolated following manufacturer’s instructions using the RNeasy Plus Mini Kit (250), from QUIAGEN and mRNA concentrations were measured with a Nanodrop spectrophotometer (Thermo Fischer). Subsequently, 0.5-1 μg template RNA was reverse transcribed into cDNA using the SuperScript™ Vilo™ cDNA synthesis kit (Thermo Fisher). Prior to reverse transcription of the extracted RNA from human mast cells, heparinase treatment was performed. RNA (1 μg) was incubated for 2 h at 25 ºC in 5 mM Tris-HCl pH 7.5, 1 mM CaCl2 (0.1 % BSA) with 2.5 U heparinase (Sigma Aldrich) and 2 U RNase inhibitor (Ambion).

Quantitative real-time PCR was performed on ABI 7300 Real-Time PCR system using the TaqMan™ Gene Expression Master Mix (# 4369514, Thermo Fisher) and TaqMan™ Gene Expression assays for the target genes mouse MGST2 (Mm00723390_m1), mouse MGST3 (Mm00787806_s1) and mouse LTC4S
Biosynthesis of 15dPGJ₂ conjugates. The relative mRNA expression was quantified by the ∆∆Ct method comparing treated samples to unstimulated controls after normalizing with the endogenous control gene β-actin (Mm00607939_s1). For the target gene analysis in human mast cells, the TaqMan™ Gene Expression Assays for human MGST2 (Hs00992727_g1), human MGST3 (Hs01058946_m1), human LTC4S (Hs00168529_m1) and human β-actin (Hs99999903_m1) were used.

**Statistical analysis**

Results are expressed as mean ± standard deviation (SD) of n independent experiments or n number of animals or donors. Calculations and graphs were prepared using GraphPad Prism version 9.0 (GraphPad software Inc.). Comparisons between two groups were performed using unpaired two-tailed student’s t-test. Statistical significance level was indicated as * p < 0.05.

**Results**

**LC-MS/MS method for the detection of 15dPGJ₂-GS and 15dPGJ₂-Cys**

To study the metabolism of 15dPGJ₂ via the conjugation to GSH, we set-up a targeted LC-MS/MS method for the analysis of 15dPGJ₂-GS and 15dPGJ₂-Cys conjugates (Figure 1 and Supplementary information Table S1). Compounds were separated in a 10 min linear gradient with 0.05% formic acid/MQ water as mobile phase A and 0.05% formic acid/acetonitrile as mobile phase B. Collection of the full scan total ion chromatogram (m/z 200-650) for the commercial 15dPGJ₂-GS standard showed a major peak eluting at 4.2 min and m/z 624.4, corresponding to [M+H]⁺ of 15dPGJ₂-GS (calculated formula weight 623.3) (Figure 1b, 1c). The smaller peak at 7 min corresponds to unconjugated 15dPGJ₂. The major fragments in a fragment spectrum of m/z 624.4 were m/z 308 [M-15dPGJ₂+H]⁺ and m/z 317 [M+H-GSH]⁺ which represent GSH and 15dPGJ₂ respectively (Figure 1d, left panel). The m/z 179 fragment represents the cysteine-glycine residue of GSH (Figure 1d, left panel). Analysis for the precursor mass eluting at 4.0 min for 15dPGJ₂-Cys [M+H]⁺ revealed the precursor ion at m/z 440.4 (calculated formula weight 439.2) (Figure 1f, 1g). The fragmentation spectrum for m/z 440.4, represents
the reduced conjugate m/z 440.4 and m/z 422 [M+H-H2O]+ as well as the free reduced 15dPGJ2 with m/z 301 which probably corresponds to loss of water (Figure 1h, left panel). In a longer 13 min analytical gradient 15dPGJ2-GS eluted at 7.25 min (Figure 1d, right panel) and 15dPGJ2-Cys eluted at 7.14 min (Figure 1h, right panel). The obtained MRM transitions for 15dPGJ2-GS were m/z 624.4>308.3, m/z 624.4>317.4, m/z 624.4>179.2, for 15dPGJ2-Cys m/z 440.4>301.2, m/z 422.4>301.2, and for 15dPGJ2 m/z 315.1>271.1 was used. Extraction of prostaglandins including the 15dPGJ2-conjugates was performed by solid phase extraction. Losses due to matrix effect were 17 % for 15dPGJ2-GS and 26 % for 15dPGJ2-Cys compared to non-extracted standard. The recovery rate comparing internal standards spiked into cell culture medium matrix before and after solid phase extraction was about 100% for 15dPGJ2-GS and 86% for 15dPGJ2-Cys. The lower limit of quantification defined as signal to noise >10 was determined to 0.1 pmol in solution injected on column (Supplementary Figure S1). Analysis of synthetic standards for leukotriene (LT)-C4 and LTE4 with the same m/z as the 15dPGJ2-metabolites showed clear separation in retention times (Supplementary Figure S1g). Stability measurements in plasma demonstrated superior stability of the 15dPGJ2-Cys conjugate up to 48 h compared to PGD2 which was reduced to 1% of its initial concentration after 24 h as well as 15dPGJ2 and 15dPGJ2-GS which were significantly diminished already after 3 h (Supplementary Figure S2). However, in-vivo pharmacokinetics showed poor stability of the 15dPGJ2-Cys conjugate in mouse plasma within 1 h post injection (Supplementary Figure S2e).

Metabolism of 15dPGJ2 into 15dPGJ2-GS and 15dPGJ2-Cys in RAW264.7 cells and BMDM

To study the metabolism of exogenous 15dPGJ2 by mouse macrophages, RAW264.7 cells or bone marrow derived macrophages (BMDM) were incubated in the presence of 15dPGJ2 (2 µM) for 0, 0.5, 1, 3, 6, 12, 24, 32 and 48 h and the supernatants were analyzed for the formation of 15dPGJ2 metabolites. No exogenous GSH was added to the culture medium in these experiments. In RAW264.7 cells, we observed the formation of 15dPGJ2-GS, which reached a plateau 24 h after treatment. Production of 15dPGJ2-Cys increased after 12 h, and levels continued to increase over time (Figure 2a). Depletion of serum albumin did not reduce the formation of 15dPGJ2-conjugates, it rather accelerated the formation of 15dPGJ2-GS (data not shown). This could be because
serum albumin could act as a carrier for fatty acids in extracellular fluids, it might trap 15dPGJ₂ in an albumin-fatty acid complex or covalently bind 15dPGJ₂ via the free –SH group in cysteine 34 of albumin delaying the cellular uptake (36, 37). Moreover, inhibition of transcription and translation with actinomycin D and cycloheximide blocked the formation of 15dPGJ₂-GS (Figure 2e). In BMDM treated with 15dPGJ₂, the 15dPGJ₂-GS conjugate increased after 3 h and peaked at 12 h, whereas the 15dPGJ₂-Cys conjugate increased continuously after 6 h (Figure 2b). In cell-free cell culture medium aspirated from untreated RAW264.7 cells and centrifuged prior to incubation with 15dPGJ₂, we observed significantly reduced amounts of 15dPGJ₂-GS and 15dPGJ₂-Cys (Figure 2a, 2c and 2d). Co-incubation of 15dPGJ₂ with an excess of GSH in cell-free cell culture medium, resulted in the formation of 15dPGJ₂-GS to the same levels as in the presence of RAW264.7 cells, measured after 24 h. However, the addition of GSH did not result in the formation of equivalent levels of 15dPGJ₂-Cys, which, together with the observed inhibition of 15dPGJ₂-GS after transcription and translation inhibition suggests enzyme involvement in the metabolism of GSH-conjugates of 15dPGJ₂.

MGST3 is involved in 15dPGJ₂-conjugate formation

To study the possible involvement of glutathione-S-transferases (GSTs) in the metabolism of 15dPGJ₂ we screened the enzyme activity of MGST1, MGST2, MGST3, LTC4S, FLAP and mPGES-1, members of the MAPEG family towards conjugation of 15dPGJ₂ with GSH. MGST3 was found to significantly enhance the formation of 15dPGJ₂-GS after 5 min, compared with control reactions without enzyme. MGST1, MGST2 and mPGES-1 showed weak activity towards 15dPGJ₂-GS conjugate formation, without reaching significance. LTC4S and FLAP had no effect on 15dPGJ₂-GS formation (Figure 3a). The apparent Michaelis constant (Km) for MGST3 was determined to 9.2 µM (Figure 3b). Parallel measurements of 15dPGJ₂-Cys indicated no direct activity of the MAPEG proteins towards generation of 15dPGJ₂-Cys in the activity assay. In addition, a soluble glutathione S-transferase (GST) of the Mu class was tested for the formation of 15dPGJ₂-GS and 15dPGJ₂-Cys under similar conditions. No enzyme activity was detected regrading production of both conjugates.
Biosynthesis of 15dPGJ\textsubscript{2} metabolites

(Supplementary Figure S7, representative results for 15dPGJ\textsubscript{2}-GS), even after longer incubation times and higher protein amounts (data not shown).

To understand whether MGST3 might play a role in the metabolism of 15dPGJ\textsubscript{2} \textit{in-vitro}, we analyzed MGST3 mRNA levels in RAW264.7 cells and primary human mast cells (CBMCs). We also assessed whether inhibition of mPGES-1 or COX affects MGST3 mRNA expression. In RAW264.7 cells, MGST3 expression was relatively high compared with MGST2 and LTC4S regardless of the treatment (Figure 3c). Treatment with the mPGES-1 inhibitors CIII and 118 or the COX-2 inhibitor NS-398 had no effect on MGST3 expression compared with the LPS control. Decreased MGST2, MGST3 and LTC4 mRNA levels were observed upon LPS treatment compared with unstimulated control. In CBMCs MGST3 expression, similarly to MGST2 and LTC4S, was not affected by anti-IgE stimulation and diclofenac treatment (Figure 3d). Moreover, we found upregulation of MGST3 expression after treatment of RAW264.7 cells with 15dPGJ\textsubscript{2} for 12 or 24 h (Supplementary Figure S3) which may serve as an explanation for the delayed formation of 15dPGJ\textsubscript{2}-conjugates in the kinetic experiments in Figure 2 and the inhibition seen after actinomycin D and cycloheximide treatment.

We next tested the formation of 15dPGJ\textsubscript{2}-GS and 15dPGJ\textsubscript{2}-Cys in RAW264.7 cells lacking MGST3. RAW264.7 WT and MGST3 KO cells were treated with 15dPGJ\textsubscript{2} (2 µM) for various time points, and the supernatants were analyzed for the formation of 15dPGJ\textsubscript{2}-GS and 15dPGJ\textsubscript{2}-Cys. The formation of both conjugates was significantly reduced in cells lacking MGST3, indicating that MGST3 is an essential enzyme in this metabolic pathway (Figure 3e and f). The formation of 15dPGJ\textsubscript{2}-GS was significantly reduced in MGST3 KO cells compared with WT cells at 24 h. The formation of 15dPGJ\textsubscript{2}-Cys was significantly reduced in MGST3 KO cells compared with WT cells at 12 h, 24 h and 48 h.

\textbf{Endogenous formation of the 15dPGJ\textsubscript{2} metabolites in macrophages and mast cells}

To identify endogenous 15dPGJ\textsubscript{2}-metabolites we studied their production by the murine monocyte-macrophage cell line RAW264.7, primary murine macrophages, and primary human mast cells.
Analysis of cell supernatants and cells showed that 15dPGJ\(_2\)-GS and the 15dPGJ\(_2\)-Cys conjugates were endogenously produced by RAW264.7 cells upon LPS stimulation, whereby the supernatants showed higher levels of both conjugates than the cell pellets (Supplementary Figure S4).

To better understand the biosynthesis and metabolism of PGD\(_2\), we performed kinetic experiments in LPS-stimulated macrophages. LPS treatment of RAW264.7 cells revealed that PGD\(_2\) levels were highest after 12 h of incubation and declined thereafter (Figure 4b), whereas PGE\(_2\) levels increased steadily until the 24 h time point (Figure 4a). We found that PGD\(_2\) is the predominant prostaglandin produced by RAW264.7 cells with levels in the cell supernatants two-fold higher than those of PGE\(_2\). 15dPGJ\(_2\), 15dPGJ\(_2\)-GS, 15dPGJ\(_2\)-Cys, PGF\(_{2\alpha}\), and low levels of TXB\(_2\) were also detected (Figure 4 and Supplementary Figure S4). 15dPGJ\(_2\) and its GSH metabolites were formed and released at later time-points starting at 12 h after treatment, with significant formation of 15dPGJ\(_2\)-Cys after 32 h (Figure 4c, d, e).

We next assessed the prostaglandin profile in LPS-treated BMDM from wild type female and male mice. LPS-treated BMDM produced primarily PGE\(_2\) and PGD\(_2\) as well as low levels of PGF\(_{2\alpha}\) and TXB\(_2\) after 24 h (Figure 4f and Supplementary Figure S5a). Female mice produced higher levels of prostanoids and were therefore used for further analysis (Figure 4f and Supplementary Figure S5a). PGE\(_2\) production reached highest levels at 24 and 32 h, whereas PGD\(_2\) levels began to increase at 12 h and peaked at 32 h where we also identified 15dPGJ\(_2\)-Cys (Figure 4g and 4h). Levels of 15dPGJ\(_2\) and 15dPGJ\(_2\)-GS were below quantification limit.

To identify endogenously formed 15dPGJ\(_2\)-metabolites in human primary cells, we analyzed supernatants from human CBMCs. PGD\(_2\), 15dPGJ\(_2\), 15dPGJ\(_2\)-GS and 15dPGJ\(_2\)-Cys were identified in anti-IgE stimulated cells, with levels of 15dPGJ\(_2\) and 15dPGJ\(_2\)-GS below the limit of quantification. 15dPGJ\(_2\)-Cys was significantly increased in response to stimulation with anti-IgE for 24 h (Figure 4i, 4j and Supplementary Figure S6). No PGE\(_2\) was detected in the analyzed supernatants.

**Effects of mPGES-1 inhibition on the PGD\(_2\)/15dPGJ\(_2\) pathway**
We investigated whether treatment with mPGES-1 inhibitors affected the PGD<sub>2</sub> pathway. RAW264.7 cells were stimulated for 24 h with LPS in combination with the selective mPGES-1 inhibitors CIII and 118 or the COX-2 inhibitor NS-398. Treatment with mPGES-1 inhibitors resulted in a 40 - 50% reduction of PGE<sub>2</sub> levels, with compound 118 reaching similar efficacy at a 10-fold lower concentration than CIII, indicating greater efficacy of compound 118 compared to CIII. PGD<sub>2</sub> levels were not affected by inhibition of mPGES-1. The levels of 15dPGJ<sub>2</sub>, 15dPGJ<sub>2</sub>-GS and 15dPGJ<sub>2</sub>-Cys tended to increase upon treatment with both mPGES-1 inhibitors, although the difference was not statistically significant. In contrast, COX-2 inhibition abolished the production of PGE<sub>2</sub> and the analyzed PGD<sub>2</sub> metabolites (Figure 5a-c).

In addition, we probed the shunting to the PGD<sub>2</sub> pathway in primary macrophages derived from bone marrow, by comparing WT and mPGES-1 KO mice. Primary macrophages from mPGES-1 KO mice treated with LPS or LPS in combination with GSH showed significantly reduced PGE<sub>2</sub> levels (Figure 5d). This was accompanied by a significant increase in PGD<sub>2</sub> levels in mPGES-1 KO macrophages after treatment with LPS in combination with GSH (Figure 5e). No significant shunting to PGF<sub>2α</sub> or TXB<sub>2</sub> formation was observed in macrophages from mPGES-1 KO mice (Supplementary Figure S5b, c). The levels of 15dPGJ<sub>2</sub> and 15dPGJ<sub>2</sub>-conjugates were below quantification limit in these experiments.

Finally, we tested how COX inhibition affects the formation of PGD<sub>2</sub> and the 15dPGJ<sub>2</sub>-Cys conjugate in human mast cells. COX-1/COX-2 inhibition with diclofenac blocked the formation of both PGD<sub>2</sub> and 15dPGJ<sub>2</sub>-Cys (Figure 5f and 5g). Human mast cells did not produce PGE<sub>2</sub> and consequently the mPGES-1 inhibitor 118 did not affect PGD<sub>2</sub> and 15dPGJ<sub>2</sub>-Cys formation (data not shown).

**Discussion**
mPGES-1 has shown promise as an alternative target for anti-inflammatory treatment strategies with improved selectivity and safety compared to traditional NSAIDs. The protective effect of mPGES-1 inhibition is thought to be due to the sole reduction of induced PGE$_2$ and the associated upregulation of anti-inflammatory and cardio-protective prostanoids (38). In the present study, we demonstrate the biosynthesis of metabolites downstream of PGD$_2$ in a murine macrophage cell line and in primary murine and human immune cells. 15dPGJ$_2$ is an anti-inflammatory and pro-resolving lipid mediator with potent bioactivity, and our data contribute to a better understanding of its biosynthesis and metabolism under inflammatory conditions and upon inhibition of mPGES-1.

We observed that 15dPGJ$_2$ is conjugated to GSH and converted to a 15dPGJ$_2$-Cys conjugate by RAW264.7 cells and mouse primary macrophages. GSH is synthesized in the cytosol, and more than 98% of GSH is in the thiol-reduced form, which allows intracellular nucleophile-electrophile interaction (39). In order to be conjugated to GSH and further metabolized, 15dPGJ$_2$ must be exposed to cells, as demonstrated in our experiments by incubation of 15dPGJ$_2$ in cell-free culture medium, which did not result in equivalent formation of 15dPGJ$_2$-GS or 15dPGJ$_2$-Cys, the latter even when GSH was added. The conversion of PGD$_2$ to PGJ$_2$ and 15dPGJ$_2$ was shown to be independent of albumin (15). Similarly, we observed that the formation of 15dPGJ$_2$ conjugates in RAW264.7 cells occurred independently of albumin (data not shown). Furthermore, inhibition of cellular transcription and translation blocked the formation of 15dPGJ$_2$-GS in RAW264.7 cells. Taken together, these observations propose an enzyme-dependent conversion of 15dPGJ$_2$ to the 15dPGJ$_2$-GS and the 15dPGJ$_2$-Cys metabolites. However, it is not clear which steps of 15dPGJ$_2$ metabolism (GSH conjugation, reduction of the cyclopentenone ring or removal of glutamic acid and glycine) are enzymatically regulated and which enzymes are involved. In addition, we cannot exclude the possibility that 15dPGJ$_2$-Cys may be formed by direct reaction of 15dPGJ$_2$ with cysteine. Several GSTs were suggested to be responsible for the formation of cyclopentenone-GS conjugates and in analogy to the leukotriene pathway γ-glutamyl transpeptidases and dipeptidases might be catalyzing the generation of the 15dPGJ$_2$-Cys metabolite (26, 40, 41). We found significantly enhanced formation of 15dPGJ$_2$-GS catalyzed by the microsomal glutathione S transferase 3 (MGST3), an enzyme belonging to the membrane associated proteins involved in eicosanoid and glutathione metabolism (MAPEG).
family (42). MGST3 shows both glutathione transferase and glutathione peroxidase activities (43) and RAW264.7 cells that lacked MGST3 were used to investigate if MGST3 is involved in the conjugation of 15dPGJ$_2$ with GSH in intact cells. Knock out of MGST3 significantly reduced the formation of 15dPGJ$_2$-GS and 15dPGJ$_2$-Cys, demonstrating the catalytic activity of MGST3 in this pathway. We have focused here mainly on members of the MAPEG family, but there may be other GST, cytosolic and mitochondrial GST, involved in the metabolism of 15dPGJ$_2$ with GSH (44). MGST3 expression has been described in human and mouse macrophages (44, 45) and in various tissues (e.g. human heart, brain, liver, kidney, pancreas, thyroid, testis, and ovary) (43). Because detection of MGST3 protein by Western blot is difficult, we assessed mRNA levels of MGST3, LTC4S, and MGST2 in RAW264.7 and human mast cells. We found MGST3 mRNA expression in both RAW264.7 cells and human mast cells. Treatment with an mPGES-1 inhibitor or a COX-inhibitor did not affect the expression levels of MGST3, LTC4S and MGST2, in stimulated RAW264.7 cells, with an overall reduction observed upon LPS stimulation. LPS has previously been shown to reduce LTC4S and MGST3 mRNA (46, 47). Others also showed that MGST3 protein levels were not affected by Kdo$_2$-lipid A (KLA) treatment in RAW264.7 cells, tending to decline over time (45). In CBMCs, anti-IgE and treatment with diclofenac had no effect on the expression levels of MGST2 and MGST3, which may indicate baseline expression of the enzymes as previously described (48). Even though mRNA expression of MGST3 decreased with LPS treatment, these result together with our observation that 15dPGJ$_2$ can induce MGST3 mRNA and that the formation of the 15dPGJ$_2$-conjugates requires de-novo protein biosynthesis suggest the involvement of MGST3 in the metabolism of 15dPGJ$_2$ in immune cells.

Consistent with previous studies, we detected PGD$_2$ as the predominant endogenous prostaglandin produced by RAW264.7 cells, with levels at 24 h twice that of PGE$_2$. At the earlier time points (6-12 h) we found a comparably lower PGD$_2$/PGE$_2$ ratio, which may be explained by different stimuli and stimulus concentrations (49). We found the formation of 15dPGJ$_2$, 15dPGJ$_2$-GS, and 15dPGJ$_2$-Cys in LPS-stimulated RAW264.7 cells following a kinetic profile consistent with the conversion of PGD$_2$ to 15dPGJ$_2$ and further to the GS-metabolites. However, we found lower levels of both metabolites in extracted cell pellets than in the supernatants. This could be explained by the rapid secretion of prostaglandins by RAW264.7 cells (50).
To further investigate the PGD₂/15dPGJ₂ pathway and identify 15dPGJ₂ metabolites we studied their production in primary mouse and human immune cells. In primary mouse macrophages, we found that PGD₂ levels were higher than PGE₂ levels 12 h after LPS stimulation, which converted to higher PGE₂ levels after 24 h, similar to what was previously reported (49). We identified endogenous 15dPGJ₂-Cys in primary murine macrophages upon LPS stimulation after 32 h. In addition, we found endogenously produced PGD₂ and 15dPGJ₂-Cys in human anti-IgE-stimulated mast cells. The 15dPGJ₂-Cys conjugate was detected after 24 h of mast cell stimulation, likely due to superior stability or delayed formation of this metabolite as we did only detect traces of 15dPGJ₂ and the 15dPGJ₂-GS conjugate at this time point.

The formation of GSH conjugates with cyclopentenone prostaglandins and metabolites (i.e., PGA₂, 9-deoxy-PGD₂, PGJ₂ and 15dPGJ₂) have been described previously in various cells (26, 51-53). HepG2 cells have been shown to produce the 15dPGJ₂-Cys as final metabolite when incubated with exogenous 15dPGJ₂ (28). However, with the exception of two studies in which the 15dPGJ₂-GS was detected in vehicle treated MCF7 cells and 15dPGJ₂-like metabolites were detected in rat liver, respectively, there are no reports on the identification of endogenously formed 15dPGJ₂-GS and 15dPGJ₂-Cys metabolites (25, 53). Here we described endogenous 15dPGJ₂-GS and 15dPGJ₂-Cys metabolites in murine macrophages and human mast cells and characterized the \textit{in-vitro} kinetics of 15dPGJ₂ metabolism under inflammatory conditions. Although GSH is the most abundant thiol in the cell, we cannot exclude retro-Michael addition reactions as well as conjugation of 15dPGJ₂ to other thiol-containing proteins. Given that no internal standard normalization was applied and quantification of the 15dPGJ₂-Cys conjugate was based on an in-house prepared standard, the levels we report should be considered as semiquantitative. Thus, in line with results by others (9, 52, 54), our data support the endogenous formation of 15dPGJ₂ and GSH-conjugation under inflammatory conditions. Whether the metabolism of 15dPGJ₂ into the 15dPGJ₂-GS and 15dPGJ₂-Cys conjugate is mainly a detoxification process or results in bioactive compounds that potentially activate nuclear receptors such as PPARγ and RXR and how this metabolism might be controlled and related to pathogenesis remains to be further elucidated.

Depending on the inflammatory milieu, PGD₂ and its metabolites are considered as pro-resolving lipids (55-57), and suppression of these lipids by COX inhibitors might interfere with successful resolution, contributing to
chronic inflammation. Recent studies highlight a possible impact of mPGES-1 inhibition and PGD\(_2\) metabolites on the polarization of macrophages toward an anti-inflammatory phenotype (9, 10, 12). Here we show that, in contrast to COX-2 inhibition, inhibition of mPGES-1 did not reduce PGD\(_2\) metabolite formation. Instead, after treatment with mPGES-1 inhibitors, we observed a tendency for increased production of these lipids in RAW264.7 cells and significant shunting to PGD\(_2\) in primary macrophages from mPGES-1 KO mice stimulated with LPS and GSH. Further, in human mast cells, we found COX-derived PGD\(_2\) and the metabolite 15dPGJ\(_2\)-Cys, while no PGE\(_2\) was detected. These results are consistent with previous publications showing that expression of mPGES-1 in human mast cells is not induced by cytokines or IgE stimulation (58, 59).

**Conclusion**

In conclusion, our data show the formation of 15dPGJ\(_2\)-conjugates in activated mouse macrophages and human mast cells, and we identify MGST3 as essential for 15dPGJ\(_2\)-conjugates formation. Moreover, inhibition of mPGES-1 maintained PGD\(_2\) metabolites, in contrast to inhibition of COX, which abolished their formation. Overall, the results presented provide new insights into the biosynthesis of lipid metabolites derived from the PGD\(_2\)/15dPGJ\(_2\) pathway and motivate further research on their role in inflammation.

**Data availability statement**

The data supporting the findings of this study are contained within the manuscript and the supplementary information file.

**Conflict of Interest**

P-J.J. is engaged in Gesynta Pharma AB, a company that develops mPGES-1 inhibitors.

**Author Contributions**
P-J.J., M.K., J.S.-S., D.S., and K.L. contributed to study conception and design. J.S.-S., F.B., and H.I. set up the analytical method for the cyclopentenone metabolites. J.S.-S. and J.L. carried out the cell culture experiments with RAW264.7 cell line and primary macrophages. S.B. generated MGST3 knock-out cells. J.L. performed cell culture experiments with MGST3 knock-out cells. M.E. and E.R carried out the mast cell experiment. J.S.-S. and J.L. performed the mass-spectrometry analysis and associated data analysis. R.S. purified MAPEG proteins. J.S-S. and R.S. performed the enzyme assays. J.S.-S. carried out the mRNA extraction and X.T. the qPCR experiments. J.S.-S., M.K. and P-J.J., drafted the manuscript. The manuscript was critically revised through the contribution of J.S.-S., M.K., P-J.J., K.L., J.L., H.I., R.S., X.T., F.B., M.E., E.R., J.Z.H., G.N., P.H., D.M., S.B., F.W., D.S. All authors have given approval of the final version of the manuscript.

References


25. Journal Pre-proof


Biosynthesis of 15dPGJ₂ metabolites


Figure legends
Figure 1: Analysis of 15dPGJ$_2$ conjugates by LC-MS/MS. Structures for 15dPGJ$_2$-GS (a) and 15dPGJ$_2$-Cys (e). Full scan total ion chromatogram ($m/z$ 200-650) for the commercial 15dPGJ$_2$-GS standard (b) eluting at 4.2 min and for 15dPGJ$_2$-Cys (f) eluting at 4.0 min. Mass spectra ($m/z$ 200-650) for 15dPGJ$_2$-GS (c) and for 15dPGJ$_2$-Cys (g). (d) Fragmentation spectra ($m/z$ 100-630) of precursor ion $m/z$ 624 (left panel) and representative multiple reaction monitoring (MRM) chromatograms (right panel) for 15dPGJ$_2$-GS with the fragments $m/z$ 308, $m/z$ 317 and $m/z$ 179. (h) Fragmentation spectra ($m/z$ 100-445) of precursor ion $m/z$ 440 (left panel) and representative multiple reaction monitoring (MRM) chromatograms (right panel) for 15dPGJ$_2$-Cys with the precursor mass $m/z$ 440 and $m/z$ 422 and the fragment $m/z$ 301. The intensity corresponds to counts per second (cps) measured.

Figure 2: Analysis of 15dPGJ$_2$ metabolism in macrophages treated with 15dPGJ$_2$. (a) Time dependent formation of prostaglandin (PG)-metabolites in RAW264.7 cells or cell-free culture media. The data show 3-6 independent experiments. (b) Time dependent formation of 15dPGJ$_2$-metabolites in BMDM from WT mice (n=3). (c, d) Cell dependent formation of 15dPGJ$_2$-GS and 15dPGJ$_2$-Cys in the presence of RAW264.7 cells or cell-free culture media in the presence or absence of GSH after 24 h. The data show 5-6 independent experiments. *p<0.05 indicates significant difference compared to cells incubated with 15dPGJ$_2$. (e) Time dependent formation of 15dPGJ$_2$-GS in RAW264.7 cells incubated with 15dPGJ$_2$ (2µM) and actinomycin D or cycloheximide. The data show three independent experiments. *p<0.05 indicates significant difference compared to cells incubated with the DMSO control.

Figure 3: Conversion of 15dPGJ$_2$ to 15dPGJ$_2$-GS by enzymes of the MAPEG family. (a) Enzymatic formation of 15dPGJ$_2$-GS in the presence of MGST1, MGST2, MGST3, LTC4S, mPGES-1 and FLAP. Measurements of 15dPGJ$_2$-GS after incubation of enzymes with the substrate 15dPGJ$_2$ in the presence of excess GSH for 5min by LC-MS/MS. The data show three independent experiments. Analytes were quantified using the MRM transition $m/z$ 624.4>317.4. *p<0.05 indicates significant difference to the control (CTRL, without MGST3 enzyme). Reactions with MGST3 but without substrate supplementation (15dPGJ$_2$) were carried out in parallel and referred to as negative control (NEG). (b) 15dPGJ$_2$ (0.75 to 90 µM) was incubated in the presence of MGST3 (5 µg) and GSH (0.1 mM) for 5min. Control reactions (CTRL) without MGST3 enzyme were carried
out in parallel. Vmax and apparent $K_m$ were calculated using hyperbolic regression analysis after subtraction of background (CTRL incubations) and quantification to reference compound PGB$_2$. The data show three independent experiments. Relative expression of MGST2, MGST3 and LTC4S were measured by qRT-PCR in (c) RAW264.7 cells and (d) CBMCs (cord-blood derived mast cells) after different treatments. Relative mRNA expression to LTC4 in control cells is presented. RAW264.7 cells were treated with LPS for 24 h in the presence or absence of the COX-2 inhibitor NS-398 (0.1 µM), and the mPGES-1 inhibitors CIII (10 µM) or 118 (1 µM). CBMCs were stimulated with anti-IgE for 24 h in combination with a COX-1/COX-2 inhibitor diclofenac (1 µM). In one experiment mRNA levels in the control samples were below detection limit and therefore removed. β-actin was used as an internal control to normalize target gene expression. The data show three independent experiments. Formation of 15dPGJ$_2$-GS (e) and 15dPGJ$_2$-Cys (f) in supernatants of RAW264.7 cells lacking MGST3. RAW264.7 WT cells or RAW264.7 MGST3 KO cells (2 different clones were analyzed, referred to as MGST3 KO1 and MGST3 KO2) were incubated with 15dPGJ$_2$ (2µM) for 12-48 h. Data are expressed as percentage of levels measured at the 24 h time point and presented as triplicates. Significant difference of KO cells compared to WT cells is indicated (*p<0.05 for MGST3 KO1, *#p<0.05 for MGST3 KO2).

**Figure 4:** Endogenous production of PGD$_2$ metabolites in RAW264.7 cells, BMDM and mast cells. (a-e) RAW264.7 cells were treated with LPS (●) for 4, 6, 12, 24 or 32 h and PG levels in supernatants were compared to respective time controls (Control represents untreated cells, ○). Data show four independent experiments. *p<0.05 indicates significant difference to the control. (f) Prostaglandins were analyzed in supernatants of LPS treated BMDM from wild type mice after 24 h (n=3). (g) PGE$_2$ and PGD$_2$ formation over time in BMDM from wild type mice (n=3), CTRL represents untreated cells. (h) Quantification of 15dPGJ$_2$-Cys (422.4>301.2) after 32 h, n=3. (i, j) PGD$_2$ (n=5) and 15dPGJ$_2$-Cys (422.4>301.2) (n=5) were quantified in supernatants from CBMCs stimulated with anti-IgE for 24 h. In three donors the PGD$_2$ levels were below quantification limit (data points set to 0). *p<0.05 indicates significant difference to the control (CTRL).

**Figure 5:** Effect of mPGES-1 inhibition on the formation of PGE$_2$, PGD$_2$ and PGD$_2$ metabolites in RAW264.7 cells, BMDM and mast cells. (a-c) RAW264.7 cells were treated with LPS for 24 h in the presence or absence of mPGES-1 inhibitors CIII (10 µM) or 118 (1 µM). For comparison the COX-2 inhibitor NS-398
(0.1 µM) was used. Prostaglandin (PG) levels are expressed as relative amounts to LPS stimulation in the absence of inhibitors. CTRL represents unstimulated cells. Data show four independent experiments. Comparison of PGE\(_2\) (d) and PGD\(_2\) (e) formation in BMDM from wild-type (WT, n=3) and mPGES-1 knock-out (KO, n=5) mice treated with LPS or LPS and GSH for 24 h. CTRL represents unstimulated cells. *p<0.05 indicates significant difference to WT. (f, g) CBMCs were stimulated with anti-IgE for 24 h in combination with the COX-1/COX-2 inhibitor diclofenac (1 µM). CTRL represents unstimulated cells. Supernatants were extracted, and PGD\(_2\) and 15dPGJ\(_2\)-Cys (422.4>301.2) were quantified (n=3). *p<0.05 indicates significant difference to CTRL, #p<0.05 indicates significant difference to anti-IgE.

**Figure 6:** Schematic overview of prostaglandin biosynthesis and changes in metabolism upon pharmacological inhibition of COX-1/COX-2 and mPGES-1. Arachidonic acid, released from membrane phospholipids by PLA\(_2\), is converted by cyclooxygenases (COX-1, COX-2) to PGH\(_2\). PGH\(_2\) serves as precursor for downstream synthases generating PGE\(_2\) (cytosolic PGE synthase, microsomal PGE synthase 1, microsomal PGE synthase 2), PGD\(_2\) (lipocalin PGD synthase, hematopoietic PGD synthase), PGF\(_{2\alpha}\) (PGF synthase), PGI\(_2\) (PGI synthase) and TXB\(_2\) (TXA synthase). Inhibition of cyclooxygenases by NSAIDs targeting COX-1 and/or COX-2 blocks the formation of all downstream prostaglandins. Inhibition of mPGES-1, the inducible PGE\(_2\) synthase, blocks solely the formation of pro-inflammatory PGE\(_2\) while a redirection of PGH\(_2\) into PGD\(_2\), PGF\(_{2\alpha}\), PGI\(_2\) or TXB\(_2\) may occur in a cell type and tissue dependent manner. In macrophages inhibition of mPGES-1 reinforces the PGD\(_2\) pathway including its downstream metabolite 15dPGJ\(_2\), which can be conjugated to GSH by MGST3 (microsomal glutathione S-transferase 3) generating 15dPGJ\(_2\)-GS and 15dPGJ\(_2\)-Cys.
Arachidonic acid

- COX-1
- COX-2

mPGES-1 inhibitor

PGH₂

- L-PGDS
- L-PGDS
- PGIS
- TXAS

PGD₂

PGF₂α

- PGFS
- mPGES-1
- mPGES-2
- PGF₁α

PGJ₂

PGE₂

- 6-keto PGF₁α
- TXA₂

15dPGJ₂

MGST3

15dPGJ₂-GS

15dPGJ₂-Cys

TRAIDs
**Author Contributions**

P-J.J., M.K., J.S.-S., D.S., and K.L. contributed to study conception and design. J.S.-S., F.B., and H.I. set up the analytical method for the cyclopentenone metabolites. J.S.-S. and J.L. carried out the cell culture experiments with RAW264.7 cell line and primary macrophages. S.B. generated MGST3 knock-out cells. J.L. performed cell culture experiments with MGST3 knock-out cells. M.E. and E.R carried out the mast cell experiment. J.S.-S. and J.L. performed the mass-spectrometry analysis and associated data analysis. R.S. purified MAPEG proteins. J.S.-S. and R.S. performed the enzyme assays. J.S.-S. carried out the mRNA extraction and X.T. the qPCR experiments. J.S.-S., M.K. and P-J.J., drafted the manuscript. The manuscript was critically revised through the contribution of J.S.-S., M.K., P-J.J, K.L., J.L., H.I., R.S., X.T., F.B., M.E., E.R., J.Z.H., G.N., P.H., D.M., S.B., F.W., D.S. All authors have given approval of the final version of the manuscript.