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The low molecular weight fraction of human serum albumin upregulates production of 15d-PGJ_2 in Peripheral Blood Mononuclear Cells

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ABSTRACT

Activation of the innate immune system involves a series of events designed to counteract the initial insult followed by the clearance of debris and promotion of healing. Aberrant regulation can lead to systemic inflammatory response syndrome, multiple organ failure, and chronic inflammation. A better understanding of the innate immune response may help manage complications while allowing for proper immune progression. In this study, the ability of several classes of anti-inflammatory drugs to affect LPS-induced cytokine and prostaglandin release from peripheral blood mononuclear cells (PBMC) was evaluated. PBMC were cultured in the presence of dexamethasone (DEX), ibuprofen (IBU), and the low molecular weight fraction of 5% albumin (LMWF5A) followed by stimulation with LPS. After 24 h, TNFα, PGE_2, and 15d-PGJ_2 release was determined by ELISA. Distinct immunomodulation patterns emerged following LPS stimulation of PBMC in the presence of said compounds. DEX, a steroid with strong immunosuppressive properties, reduced TNFα, PGE_2, and 15d-PGJ_2 release. IBU caused significant reduction in prostaglandin release while TNFα release was unchanged. An emerging biologic with known anti-inflammatory properties, LMWF5A, significantly reduced TNFα release while enhancing PGE_2 and 15d-PGJ_2 release. Incubating LMWF5A together with IBU negated this observed increased prostaglandin release without affecting the suppression of TNFα release. Additionally, LMWF5A caused an increase in COX-2 transcription and translation. LMWF5A exhibited a unique immune modulation pattern in PBMC, disparate from steroid or NSAID administration. This enhancement of prostaglandin release (specifically 15d-PGJ_2), in conjunction with a decrease in TNFα release, suggests a switch that favors resolution and decreased inflammation.

1. Introduction

The innate immune system is comprised of cellular machinery that provides immediate defense mechanisms to protect against infection. Cells of the innate immune system recognize and respond to various antigens without providing long-lasting immunity to the host. A major function of the innate immune system includes recruitment of immune cells to the site of infection via the production and secretion of various chemical factors, such as cytokines. Additionally, the innate immune system activates the complement cascade and leukocytes to promote identification of

Abbreviations: LPS, Lipopolysaccharide; PBMC, Peripheral blood mononuclear cells; DEX, Dexamethasone; IBU, Ibuprofen; LMWF5A, Low molecular weight fraction of 5% albumin; TNFα, Tumor necrosis alpha; PGE_2, Prostaglandin E2; 15d-PGJ_2, 15-Deoxy-Δ-12,14-prostaglandin J2; COX-2, Cyclooxygenase 2; mPGES1, Microsomal prostaglandin E synthase 1; mPGES2, Microsomal prostaglandin E synthase 2.

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bacteria and clearance of debris. Finally, the innate immune system is responsible for initiating the adaptive immune system, which is critical for immunological memory and amplification following future pathogenic insult. One of the initial responses of the innate immune system to infection is inflammation. Although inflammation causes pain, swelling, and fever to the host, it is important in halting the spread of infection and promotes healing followed by the clearance of pathogens. Properties of the ideal anti-inflammatory drug include regulation of inflammation without interfering with the recognition of foreign substances, clearance of cellular debris, and repair of damaged areas.

Experiments previously conducted in our laboratory demonstrated that the low molecular weight fraction (<5 kDa) of commercial human serum albumin (LMWF5A) possesses anti-inflammatory properties. Pro-inflammatory cytokine release from peripheral blood mononuclear cells (PBMC) and T-cell lines stimulated through the T-cell receptor complex was inhibited by the presence of the LMWF5A [1]. Additionally, LPS stimulation of PBMC from multiple donors caused a decrease in the pro-inflammatory mediator TNFα after LMWF5A administration [2]. The known components of LMWF5A include the cyclic compound derived from the N-terminus of human serum albumin (HSA), aspartate-alanine diketopiperazine (DA-DKP) [3]. Also, N-acetyl tryptophan (NAT) and sodium caprylate are added to commercial HSA as solution stabilizers. As a result, NAT degradation products have been identified in commercial HSA solutions [4]. Finally, various peptides derived from non-HSA sources have been characterized in commercial HSA solutions [5,6]. Therefore, it is conceivable that any combination of known and unknown small molecular weight components of commercial HSA contributes to the anti-inflammatory activity found in the LMWF5A. Since the presence of anti-inflammatory activity in the <5 kDa HSA fraction has been demonstrated *in vitro*, LMWF5A is currently under development as a therapeutic for osteoarthritis of the knee. In a recently completed clinical trial, it was found that a single intra-articular (IA) injection of LMWF5A resulted in a significant 42.3% reduction in pain that was observed at 4 weeks post-injection and persisted to the completion of the trial at week 20 versus saline controls [7].

In order to understand the anti-inflammatory mechanism of LMWF5A, comparison with known anti-inflammatory pharmaceuticals is warranted. Therefore, in this study, LMWF5A was compared to dexamethasone (DEX) and ibuprofen (IBU), two immunomodulators with diverse mechanisms of action. By comparing the patterns of cytokine (TNFα) and prostaglandin (PGE_2 and 15d-PGJ_2) release from LPS-stimulated PBMC pre-treated with said anti-inflammatory therapeutics, a potential mechanism of action of LMWF5A may be inferred from the data. Based on our findings, LMWF5A exhibited an unexpected immunomodulatory pattern unique from steroid or NSAID treatment.

2. Materials and methods

2.1. Materials

Cell culture reagents were purchased from Gibco, Life Technologies (Grand Island, NY). 5% human serum albumin (HSA) from Octapharma (Hoboken, NJ) was used for <5 kDa filtrate collection. All other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated.

2.2. Collection of LMWF5A

LMWF5A was isolated by Ampio Pharmaceuticals, Inc. (Englewood, CO, USA) using a tangential flow filtration (TFF) process with a 5 kDa MWCO PVDF filter membrane (Sartorius Stedim Biotech GmbH, Germany). In accordance with cGMP guidelines, the isolation process involved the removal of the >5 kDa component (primarily HSA) and the aseptic filling of sterile glass vials with 10 mL LMWF5A. Each vial was sealed with a rubber stopper and a proper metal closure. The vials were stored in the dark at ambient temperature.

2.3. Preparation of PBMC

Isolated human peripheral blood mononuclear cells (PBMC) from healthy donors were purchased from Astarte Biologics (Redmond, WA). PBMC stocks were thawed and then transferred drop wise to 9 mL of thaw medium (RPMI-1640 medium containing 10% human AB serum, 20 units/mL DNase, and 1% Pen/Strep) and centrifuged at 1,000 × g for 10 min.

2.4. TNFα and prostaglandin release assay

Working dilutions of test compounds were prepared in saline. 50 μL of each test compound (10 μM IBU, 1:2 LMWF5A, 0.1 μM DEX, 0.1 μM mifepristone, or 10 μM HQL 79; final concentrations) were added in quadruplicate to a 96 well U bottom tissue culture plate and incubated at 37 °C/5% CO2 for 1 h. An equal volume of PBMC (1×10^6 cells/mL) suspended in growth medium (RPMI + 20% fetal bovine serum, 2% Pen/strep, 1% L-glutamine, 1% sodium bicarbonate, 1% sodium pyruvate, 1% NEAA) was added to each well. After an hour incubation, 100 ng/mL LPS was added to the appropriate wells. After an overnight incubation, the plate was centrifuged at 1,000 × g for 10 min and the supernatants were collected. TNFα (Thermo Scientific, Rockford, IL) and prostaglandin (PGE_2 and 15d-PGJ_2; Abcam, Cambridge, MA) release were determined by ELISA. TNFα and PGE_2 assays were performed by diluting the supernatants 1:5 or 1:100 in PBS, respectively. 15d-PGJ_2 release was assayed using undiluted culture medium. To assure accurate quantification, separate standard curves were generated in culture medium containing saline or LMWF5A as described above and data analyzed accordingly. All other ELISA steps were performed as recommended by the manufacturer.

2.5. COX-2 western blot analysis

PBMC were cultured as described above then lysed at the indicated times in 50 μL lysis buffer (Qproteome Mammalian Protein kit; Qiagen) according to manufacturer's instructions. The lysates were centrifuged at 12,000 × g at 4 °C for 10 min to remove the cellular debris and prepared for western blot analysis by boiling in Bolt Reducing Buffer and Bolt LDS Sample Buffer (ThermoFisher Scientific, Waltham, MA). Proteins were separated by SDS-PAGE (8%) and subjected to western blot analysis using an anti-COX-2 rabbit monoclonal primary antibody (1:1,000, ab62331; Abcam, Cambridge, MA) and a goat anti-rabbit IgG secondary antibody (1:10,000, Cat# 7074P2, Cell Signaling, Danvers, MA). The membranes were normalized to α-tubulin by striping with Reblot Plus (Millipore, Billerica, MA) and reprobing with a horseradish peroxidase-conjugated α-tubulin antibody (1:5,000, DM1A, Cat# 12351S, Cell Signaling, Danvers, MA). Photography and densitometry were performed on a Carestream molecular imaging system with supplied software (Woodbridge, CT).

2.6. Quantitative reverse transcription PCR

Total RNA was isolated from PBMC cultures at the indicated times utilizing Qiagen (Valencia, CA) RNeasy plus columns. Complimentary DNA was then generated from 10 μl of the isolated RNA with Qiagen Omniscript kit reagents and quantitative PCR (qPCR)

performed on a Roche 480 lightcycler using SYBR green I master mix (Roche Diagnostics, Indianapolis, IN) with qPCR assay primers purchased from Qiagen following the manufacturer's protocol. Relative gene expression was calculated using ΔΔCt analysis versus saline controls normalized to housekeeping gene expression using an average of the ct calls for β-actin and GAPDH.

2.7. Data analysis

To calculate percent changes in cytokine and prostaglandin release, the following formula was employed: ((LPS with treatment - LPS only control)/LPS only control)*100. A paired Student t-test was applied to data sets (Microsoft Excel; Redmond, WA), with statistical significance accepted at $p \leq 0.05$.

3. Results and discussion

3.1. LMWF5A and dexamethasone reduce LPS-induced TNFα release from PBMC

In this study, both LMWF5A and dexamethasone (DEX) significantly reduced TNFα released by LPS-stimulated peripheral blood mononuclear cells (PBMC) from healthy donors. An average 51% decrease in TNFα release was observed in LPS-stimulated PBMC pre-treated with LMWF5A (see Fig. 1A). This result was similar to the amount of TNFα inhibition observed when LMWF5A was incubated with PBMC from 13 healthy donors [2]. Glucocorticoid treatment with DEX inhibited TNFα release by 71% at a concentration of 0.1 μM (see Fig. 1A). Interestingly, the glucocorticoid receptor antagonist, mifepristone (MIF), also reduced TNFα release by 20% (see Fig 1A). The inhibition of TNFα release by DEX after LPS stimulation is well documented. In murine macrophages, DEX was shown to inhibit LPS induction of Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) activity, thereby regulating TNFα translation [8]. Additionally, in human monocytes, DEX suppressed LPS-stimulated secretion of TNFα by decreasing the binding of c-Jun/ATF-2 and NF-κB complexes at the TNFα promoter [9]. It is possible that LMWF5A has a similar mechanism of action to DEX via c-Jun/ATF-2 since a component of LMWF5A was shown to decrease phosphorylated c-Jun/ATF-2 expression in T lymphocytes [1]. The non-steroidal anti-inflammatory drug (NSAID) ibuprofen (IBU) did not have a significant effect on TNFα release (see Fig. 1A). This was expected since the mechanism of action for IBU involves the inhibition of cyclooxygenases, which are not involved in TNFα production and release [10]. Interestingly, the combination of LMWF5A and IBU caused an inhibition of TNFα release similar to the LMWF5A only treatment (see Fig. 1A). This suggests that LMWF5A and IBU act through independent anti-inflammatory mechanisms.

3.2. LMWF5A potentiates while dexamethasone and ibuprofen reduce LPS-induced prostaglandin release

Differences in the anti-inflammatory effects of LMWF5A versus the other known drugs (DEX and IBU) emerge during the measurement of prostaglandin release from LPS-stimulated PBMC. Surprisingly, LMWF5A caused a 104% increase in PGE_2 release from LPS-stimulated PBMC (see Fig. 1B). DEX caused a significant 80% decrease in PGE_2 release from LPS-stimulated PBMC, while IBU caused a 75% decrease (see Fig. 1B). MIF treatment produced a minimal reduction in PGE_2 of 22% (see Fig. 1B). This was expected since DEX is known to indirectly inhibit phospholipase A_2 and cyclooxygenase activity, key components in the synthesis of PGE_2 [11]. As mentioned earlier, IBU inhibits PGE_2 production and release by inhibiting cyclooxygenase activity [10]. Historically, PGE_2 is generally regarded as a pro-inflammatory agent and is up-regulated in various diseases in which inflammation is prevalent. Recently, PGE_2 has been suggested to play a homeostatic role in the resolution of inflammation, thereby potentiating tissue repair [12]. The ability of LMWF5A to increase PGE_2 production and release is attenuated when IBU is added causing a decrease similar to the addition of IBU only (see Fig. 1B).

Similar to PGE_2 production and release, LMWF5A caused a greater than 2-fold increase in the release of 15d-PGJ_2 from LPS-stimulated PBMC (see Fig. 1C). Similarly, DEX and IBU caused a decrease in 15d-PGJ_2 release (see Fig. 1C). Again, IBU attenuated the increase in 15d-PGJ_2 release caused by LMWF5A (see Fig. 1C). This finding could be a positive distinction for LMWF5A versus glucocorticoids and NSAIDs since 15d-PGJ_2 is an important prostaglandin for the resolution of acute inflammation [13]. Of note, pre-treatment of LPS stimulated PBMC with 10 μM HQL 79, a specific inhibitor of hematopoietic PGD synthase (H-PGDS), reduced LMWF5A-induced 15d-PGJ_2 release by 37%. The concentration of HQL 79 used in our experimental model might be too low since some published methods use an HQL 79 final concentration as high as 300 μM to completely inhibit 15d-PGJ_2 synthesis and release [14].

3.3. LMWF5A alters LPS induced transcription in PBMC

To explore the potential effect of LMWF5A on transcription, RT-qPCR was performed on PBMC cultures following treatment and stimulation with LPS. Relative expression was calculated by ΔΔCt analysis versus saline treated controls with normalization to housekeeping genes (see Table 1). Fold changes greater than 2 fold from the controls were deemed biologically significant.

When COX-2 was examined, the enzyme that converts arachidonic acid to the parent prostanoid molecule, PGH_2, LMWF5A had a profound effect on message levels (see Table 1). One hour following stimulation, the presence of LMWF5A doubled the detectable amount of COX-2. A 10.4-fold increase for COX-2 was observed with LPS alone versus 19.4-fold for LPS with LMWF5A. COX-2 transcription is strongly induced 3 h following LPS stimulation with a 122.4-fold increase observed over the saline control. Even at this level of expression, the presence of LMWF5A increased transcription to 159.8-fold. Interestingly, cultures treated with LMWF5A alone exhibited almost a 4-fold increase in COX-2 transcription after 20 h in culture. This was observed when the transcription in LPS stimulated cells recovered to near background levels.

Evaluation of TNFα showed that relative expression increased 8.5-fold as compared to saline alone 1 h following exposure to LPS (see Table 1). Unexpectedly, LMWF5A treatment slightly enhanced LPS induced TNFα transcription to a relative expression of 12.2-fold. When examined 3 h post stimulation, however, a reduction in TNFα was observed in the presence of LMWF5A in the LPS stimulated cells. At this time point, expression increased 23-fold in cells treated with LPS alone compared to an 18.8-fold increase observed in the presence of LMWF5A. By 20 h, TNFα levels in LPS treated PBMC return to normal.

To explore the molecular mechanisms involved with the ability of LMWF5A to potentiate LPS induced prostaglandin release, primers for enzymes involved in PGE_2 and 15d-PGJ_2 biogenesis were also selected. mPGES1 and mPGES2, two isoforms of microsomal prostaglandin E synthase, were unaffected by LPS stimulation and/or LMWF5A treatment (see Table 1) even though threshold cycles indicate abundant transcript (data not shown). Hematopoietic PGD synthase (H-PGDS) was also evaluated, but mRNA levels were below the detection limit of this assay. This could explain the low 15d-PGJ_2 release observed and indicates that this

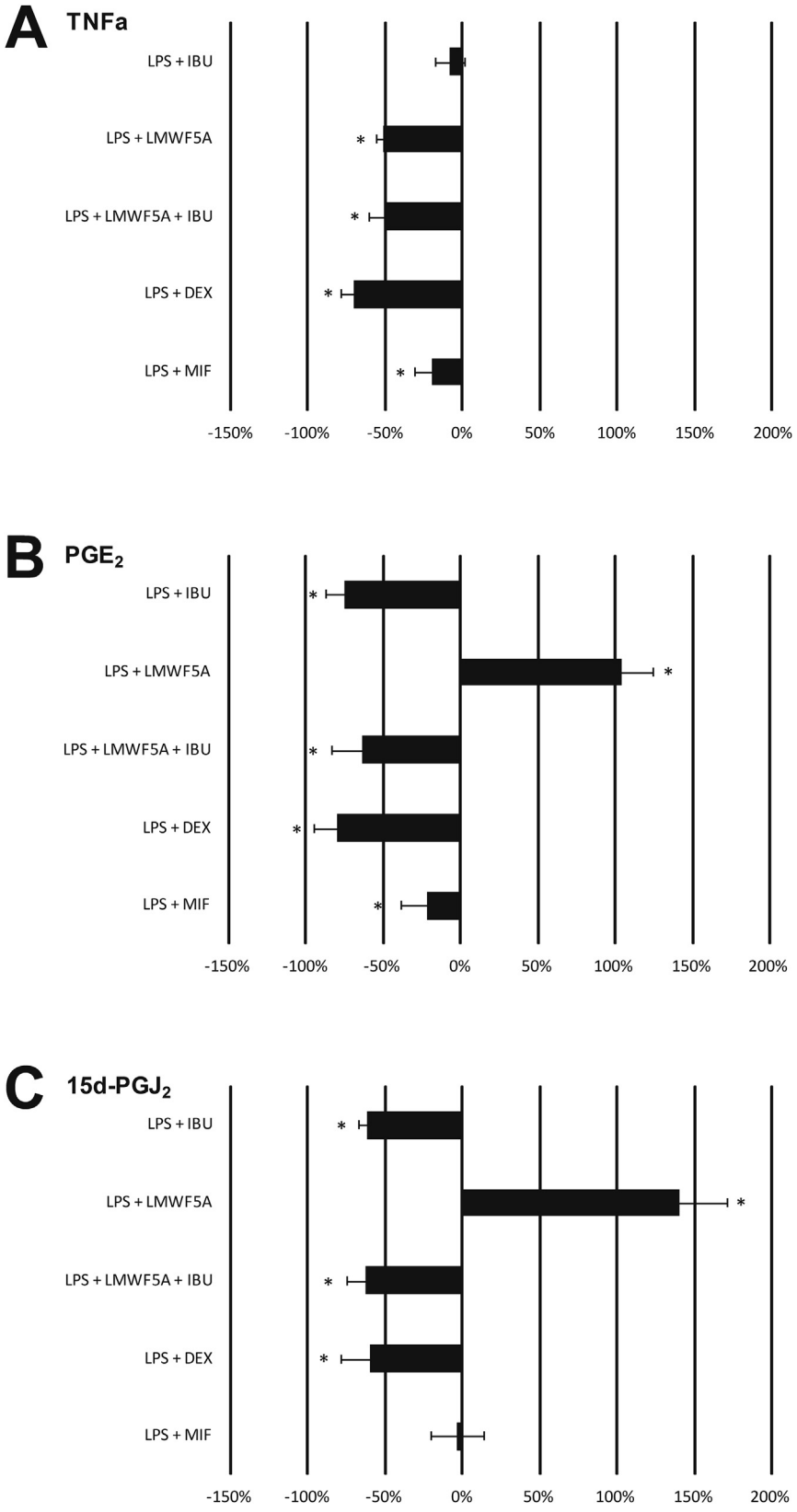

Fig. 1. TNFα release from LPS-stimulated PBMC pre-treated with various anti-inflammatory compounds (A). PGE_2 release from LPS-stimulated PBMC pre-treated with various anti-inflammatory compounds (B). 15d-PGJ_2 release from LPS-stimulated PBMC pre-treated with various anti-inflammatory compounds (C). PBMC were incubated with compounds for 1 h followed by overnight stimulation with 100 ng/mL LPS. TNFα, PGE_2, and 15d-PGJ_2 release were determined by ELISA and are presented as mean ± SD after repeating the experiment on 3 different occasions. * = $p \leq 0.05$ versus saline control.

Table 1
RT-qPCR results in PBMC cells stimulated with LPS and/or treated with LMWF5A for 30 min, 1 h, 3 h, and 20 h. Relative expression was calculated by ΔΔCt analysis versus saline treated controls normalized to β-actin and GAPDH. This experiment was repeated on 3 different occasions.

Primer target − cDNA sample	30 min	1 h	3 h	20 h
COX2- LMWF5A	1.7	1.4	1.5	3.6*
COX2- LPS	1.1	10.4*	122.4*	2.8
COX2- LPS + LMWF5A	1.4	19.4*	159.8*	1.8
TNFα- LMWF5A	1.4	1.1	0.7	1.7
TNFα- LPS	1.2	8.5*	23.0*	1.7
TNFα- LPS + LMWF5A	1.0	12.2*	18.8*	0.6
mPGES- LMWF5A	1.2	0.9	0.6	2.2
mPGES- LPS	1.3	1.8	0.6	1.5
mPGES- LPS + LMWF5A	1.2	2.4	0.9	0.9
mPGES2- LMWF5A	0.9	1.0	0.6	1.3
mPGES2- LPS	0.8	1.4	0.5	2.1
mPGES2- LPS + LMWF5A	0.7	1.4	0.4	1.2
CD49d- LMWF5A	1.2	0.7	0.6	0.6
CD49d- LPS	1.4	0.9	0.7	0.6
CD49d- LPS + LMWF5A	1.0	1.4	0.8	0.6

enzyme is only expressed by a rare subset of cells in the PBMC fraction. The transcription of CD49d, an integrin alpha subunit which mediates migration of T cells to extravascular spaces, was not affected by LPS and/or LMWF5A treatment (see Table 1).

3.4. LMWF5A induced changes in COX-2 protein levels in PBMC

To determine if the changes in COX-2 transcription observed following LPS stimulation of LMWF5A treated PBMC carry to protein translation, western blots were performed. COX-2 protein was undetectable in unstimulated as well as LPS stimulated PBMC cells 30 min or 1 h following exposure (data not shown). Trace amounts of COX-2 were observed 3 h after LPS stimulation with LMWF5A minimally increasing expression by 16% (see Fig. 3). Higher levels of COX-2 were observed in 20 h protein extracts, even though mRNA returned to background levels. Interestingly, saline treated controls also express detectable amounts of COX-2 at 20 h. When normalized to α-tubulin levels, COX-2 increased 5-fold following LPS stimulation as compared to saline controls. LMWF5A potentiated COX-2 protein translation by 76%. At 20 h, LMWF5A alone increased COX2 1.7-fold. This could be the result of LMWF5A working synergistically with autocrine or paracrine derived PBMC factors. While temporally different, these findings are consistent with our release and RT-qPCR data.

3.5. Importance of the LMWF5A cytokine/prostaglandin release pattern in PBMC

The cytokine/prostaglandin release patterns in LPS-stimulated PBMC for LMWF5A, DEX, and IBU are presented in Fig. 2. DEX causes an across-the-board decrease in TNFα and prostaglandin release. IBU only decreases prostaglandin release while having no effect on TNFα release. The addition of LMWF5A to the IBU-treated

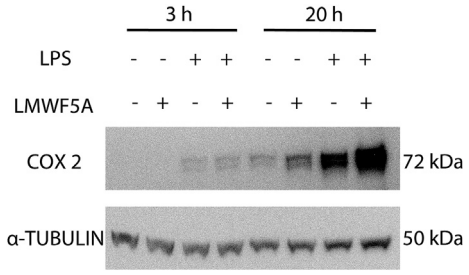

Fig. 3. Representative western blot of COX-2 protein translation in PBMC cells stimulated with LPS and/or treated with LMWF5A. Densitometry was performed on all detectable bands and normalized to α-tubulin. This experiment was repeated on 3 different occasions.

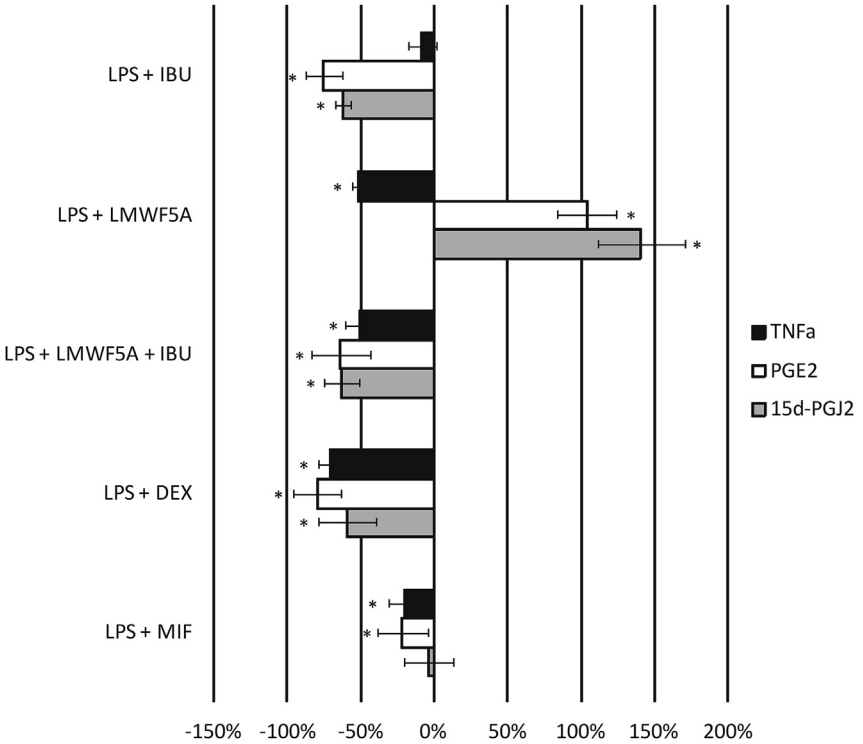

Fig. 2. Combined release data for TNFα, PGE_2, and 15d-PGJ_2. Data are presented as mean ± SD after repeating the experiment on 3 different occasions. * = $p < 0.05$ versus saline control.

cells results in a pattern similar to the DEX only treatment group. LMWF5A treatment causes a unique profile: decreased levels of the pro-inflammatory cytokine TNFα and increased prostaglandin release. Why is this release pattern, which is unique from the more commonly administered anti-inflammatory drugs, important to the novelty of LMWF5A as a potential anti-inflammatory and initiator of resolution?

LMWF5A is similar to DEX in that TNFα release is significantly inhibited after stimulation with LPS. TNFα promotes the inflammatory response and is elevated in various auto-immune and other inflammatory diseases. Therefore, the discovery of TNFα inhibitors is a high priority for pharmaceutical companies, and candidate compounds are currently undergoing clinical trials for the treatment of various pathological conditions, such as arthritis, inflammatory bowel disease, psoriasis, and asthma. However, severe side effects have been documented when using TNFα inhibitors. These include lymphomas, infections, congestive heart failure, demyelinating disease, a lupus-like syndrome, induction of autoantibodies, injection site reactions, and systemic side effects [15]. DEX treatment also has potential serious side effects that include allergic reactions, pancreatitits, heart problems, high blood pressure, and slow wound healing. Importantly, LMWF5A was used in a multicenter randomized, vehicle-controlled, double-blind study for the treatment of knee pain associated with osteoarthritis and demonstrated to have no adverse events after intra-articular injection [7].

This difference in adverse effects between LMWF5A and DEX might be attributed to the unique regulation of prostaglandin release in LPS-stimulated PBMC. TNFα exposure in brain endothelial cells caused an induction in cyclooxygenase-2 (COX-2) protein but not cyclooxygenase-1 (COX-1) [16] resulting in increased PGE_2 and other prostaglandin release concurrent with significant increases in permeability due to changes in cytoskeletal structure. Although LMWF5A causes an increase in COX-2 transcription and expression, resulting in an increase in PGE_2 and 15d-PGJ_2 release in PBMC, this does not translate into increased permeability and endothelial cell monolayer barrier dysfunction (submitted manuscript). Also, LMWF5A causes cytoskeleton changes via cortical F-actin rearrangement from a stress fiber morphology to a more diffuse, elongated phenotype in bone marrow-derived mesenchymal stem cells [17]. Therefore, the possible mechanism of action for LMWF5A seems to be related to inhibition in TNFα release combined with increased prostaglandin release. More importantly, increased 15d-PGJ_2 release may be critical since this prostaglandin is a potent anti-inflammatory agent [18].

In conclusion, LMWF5A demonstrates a unique cytokine/prostaglandin release pattern in PBMC distinct from two common anti-inflammatory agents, dexamethasone and ibuprofen. Further study is warranted to determine if LMWF5A causes a switch in prostaglandin release that favors anti-inflammation and pro-resolution as previously suggested [19].

Conflict of interest

The authors confirm that this article content has no conflicts of interest.

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Transparency document

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