Human leukocytes differentially express endocannabinoid-glycerol lipases and hydrolyze 2-arachidonoyl-glycerol and its metabolites from the 15-lipoxygenase and cyclooxygenase pathways

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Accepted for publication in *J Leukoc Biol*, September 2019

Keywords: endocannabinoid, 15-lipoxygenase, 2-arachidonoyl-glycerol, prostaglandin

Abbreviations: 15-HETE, 15-hydroxy-eicosatetraenate; 15-HETE-G, 15-hydroxy-eicosatetraenoyl-glycerol; 2-AG, 2-arachidonoyl-glycerol; AA, arachidonic acid; ABHD, *α*/*β*-hydrolase domain; AMs, alveolarmacrophages; CES, carboxylesterase; G, glycerol; LYPLA, lysophospholipase; MAFP,methylarachidonoyl-fluorophosphonate; MAG, monoacylglycerol; PGE_2, prostaglandin; PGE_2-G, prostaglandin E_2-Glycerol; PPT, palmitoyl-protein thioesterase.

Abstract

2-Arachidonoyl-glycerol (2-AG) is an endocannabinoid with anti-inflammatory properties. Blocking 2-AG hydrolysis to enhance CB_2 signaling has proven effective in mouse models of inflammation. However, the expression of 2-AG lipases has never been thoroughly investigated in human leukocytes. Herein, we investigated the expression of seven 2-AG hydrolases by human blood leukocytes and alveolar macrophages (AMs) and found the following protein expression pattern: monoacylglycerol (MAG lipase; eosinophils, AMs, monocytes), carboxylesterase (CES1; monocytes, AMs), palmitoyl-protein thioesterase (PPT1; AMs), *α*/*β*-hydrolase domain (ABHD6; mainly AMs), ABHD12 (all), ABHD16A (all), and LYPLA2 (lysophospholipase 2; monocytes, lymphocytes, AMs).We next found that all leukocytes could hydrolyze 2-AG and its metabolites derived from cyclooxygenase-2 (prostaglandin E_2-glycerol [PGE_2-G]) and the 15-lipoxygenase (15-hydroxy-eicosatetraenoyl-glycerol [15-HETE-G]). Neutrophils and eosinophils were consistently better at hydrolyzing 2-AG and its metabolites than monocytes and lymphocytes. Moreover, the efficacy of leukocytes to hydrolyze 2-AG and its metabolites was 2-AG ≥ 15-HETE-G >> PGE_2-G for each leukocyte. Using the inhibitors methylarachidonoyl-fluorophosphonate (MAFP), 4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate (JZL184), Palmostatin B, 4′-carbamoylbiphenyl-4-yl methyl(3-(pyridin-4-yl)benzyl)carbamate, Nmethyl-N-[[3-(4-pyridinyl)phenyl]methyl]-4′-(aminocarbonyl) [1,1′-biphenyl]-4-yl ester carbamic acid (WWL70), 4′-[[[methyl[[3-(4-pyridinyl)phenyl]methyl]amino]carbonyl]oxy]-[1,1′-biphenyl]-4-carboxylic acid, ethyl ester (WWL113), tetrahydrolipstatin, and ML349, we could not pinpoint a specific hydrolase responsible for the hydrolysis of 2-AG, PGE_2-G, and 15-HETE-G by these leukocytes. Furthermore, JZL184, a selective MAG lipase inhibitor, blocked the hydrolysis of 2-AG, PGE_2-G, and 15-HETE-G by neutrophils and the hydrolysis of PGE_2-G and 15-HETE-G by lymphocytes, two cell types with limited/no MAG lipase. Using an activity-based protein profiling (ABPP) probe to label hydrolases in leukocytes, we found that they expressmanyMAFP-sensitive hydrolases and an unknown JZL184-sensitive hydrolase of ~52 kDa. Altogether, our results indicate that human leukocytes are experts at hydrolyzing 2-AG and its metabolites via multiple lipases and probably via a yet-to-be characterized 52 kDa hydrolase. Blocking 2-AG hydrolysis in humans will likely abrogate the ability of human leukocytes to degrade 2-AG and its metabolites and increase their anti-inflammatory effects in vivo.

1. INTRODUCTION

2-Arachidonoyl-glycerol (2-AG) is an endogenous endocannabinoid activating the cannabinoid receptors CB_1 and CB_2.1 Its involvement in physiologic processes such as appetite, adipogenesis, and nociception is well established.[2–4] Furthermore, the CB_2 receptor is widely expressed by leukocytes,[1,5] implying that 2-AG is also an immunomodulator. As such, numerous studies in CB_2 receptor-deficient mice have reported an enhanced inflammatory phenotype,1,5 supporting the concept that the CB_2 receptor is mainly anti-inflammatory. However, there is a paucity of evidence supporting the anti-inflammatory potential of the cannabinoid/endocannabinoid system in humans.[1,5] Thus, there is an urgent need to decipher the molecular and physiologic roles of the cannabinoid system in humans and to confirm whether the data obtained in experimental models

are translatable to humans. Two main strategies to increase CB_2 receptor signaling during the inflammatory process could be utilized: the use of selective CB_2 receptor agonists or the use of endocannabinoid hydrolysis inhibitors in order to increase 2-AG levels.

The inhibition of 2-AG hydrolysis is of great interest because it would not only enhance CB_2 receptor signaling, but it would also possibly decrease the production of other proinflammatory effectors such as leukotrienes (LT) and prostaglandins (PG). Indeed, 2-AG is very labile and rapidly hydrolyzed into arachidonic acid (AA) by numerous serine hydrolases. This is notably the case in human neutrophils and eosinophils in which the resulting AA is metabolized, in part, by eicosanoid biosynthetic enzymes to form LTB_4 and LTC_4, respectively.[6–8] Although monoacylglycerol (MAG) lipase is the most recognized lipase involved in 2-AG hydrolysis in the brain of mice,[9] other lipases with distinct sensitivities to pharmacologic inhibitors can also hydrolyze this endocannabinoid into AA.[10–16] The current knowledge about 2-AG lipases is summarized in Table 1.

Aside from its hydrolysis into AA, 2-AG can be metabolized by eicosanoid biosynthetic enzymes from the cyclooxygenase (COX)-2 and 15-lipoxygenase (15-LO) pathways, leading to eicosanoidglycerols (G), such as PG-Gs or 15-hydroxy-eicosatetraenoyl-glycerol (15-HETE-G).[17–19] In this regard, 15-HETE-G, PGD_2-G, and PGE_2-G activate cell surface or nuclear receptors.[18,20–24] Noteworthy, some of these metabolites exert anti-inflammatory effects,[1,25] although additional investigations are required to completely understand their biologic effects in health and disease. PG-Gs are hydrolyzed into PGs by human neutrophils[26] and in several murine tissues including the lungs.[6–8,27] In this regard, some 2-AG hydrolases can hydrolyze PG-Gs to some extent in cellulo or in vivo.[14,15,28,29] In addition, lysophospholipase 2 (LYPLA2) can hydrolyze PGE_2-G into PGE_2 although showing no hydrolytic activity toward 2-AG,[30] indicating that the complete set of enzymes involved in the hydrolysis of 2-AG and its metabolites from the COX-2 and 15-LO pathway is not totally defined yet.

The importance of each lipase in regulating the levels of 2-AGand its metabolites derived from the COX-2 and 15-LOpathways likely varies among tissues and cell types, due to their different proteomes. Herein, we defined the expression of 2-AG-related lipases in human leukocytes. Using a pharmacologic approach, we investigated their possible involvement in the hydrolysis of 2-AG, 15-HETE-G, and PGE2-G. We also used an activity-based protein profiling (ABPP) probe to visualize additional targets for some of the inhibitors we used.

2. MATERIAL AND METHODS

2.1 Materials

HBSS, RPMI, 1640 Leibovitz's L-15 medium, trypsin, and FBS were obtained from Wisent Laboratories (St-Bruno, Quebec City, Canada). Ficoll was obtained from Corning (Tewksbury, MA, USA). Dextran was purchased from Fisher Scientific (Ottawa, ON, Canada). The CD14 and CD16 magnetic bead-coupled antibody and the MACS magnetic separation system were purchased from Miltenyi Biotec (Auburn, CA, USA). THP-1 and MDA-MB-231 cells were obtained from ATCC (Manassas, VA, USA). Human hypothalamus samples were provided by the Douglas-Bell Canada Brain Bank (Montréal, Québec, Canada). Protease inhibitor cocktail tablets were purchased from Roche (Laval, QC, Canada). Aprotinin, leupeptin, and 4′-[[[methyl[[3-(4-pyridinyl)phenyl]methyl]amino]carbonyl]oxy]-[1,1′-biphenyl]-4-carboxylic acid, ethyl ester (WWL113) were purchased from Sigma-Aldrich (St-Louis, MO, USA). Diisopropyl fluorophosphate (DFP) was purchased from BioShop Canada (Burlington, ON, Canada). The HRP-linked anti-mouse IgG and anti-rabbit IgG secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies for *α/β*-hydrolase domain (ABHD) ABHD4, ABHD6, ABHD12, and palmitoyl-protein thioesterase (PPT1) were purchased from Abcam (Toronto, ON, Canada). 1-AG-D_5, PGE_2 -D_4, 15-HETE-D_8, PGE_2-G, 15-HETE-G, 2-AG, methylarachidonoyl-fluorophosphonate (MAFP), 4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate (JZL), tetrahydrolipstatin (THL), 4′-carbamoylbiphenyl-4-yl methyl(3-(pyridin-4-yl)benzyl)carbamate, N-methyl-N-[[3-(4-pyridinyl)phenyl]methyl]-4′-(aminocarbonyl) [1,1′-biphenyl]-4-yl ester carbamic acid (WWL70), as well as the primary antibodies for MAG lipase, were purchased from Cayman Chemical (Ann Arbor, MI, USA). The LYPLA2 primary antibody, the ABHD16A primary antibody, dextran and the fluorophosphonate (FP)-TAMRAprobewere from Thermo Fisher Scientific (Waltham, MA, USA) and the primary antibody for carboxylesterase (CES)1 was from R&D Systems (Minneapolis, MN, USA). PMSF, Palmostatin B, and the ECL detection kit were from EMD Millipore (Billerica, MA, USA). ML349 was a generous gift from Dr. Lawrence J. Marnett (Vanderbilt University, Nashville, TN, USA).

2.2 Isolation of human leukocytes

Human eosinophils, neutrophils, lymphocytes, and monocytes were isolated from the peripheral blood of healthy volunteers. A sample of 160 ml of blood was collected in tubes containing K_3-EDTA as an anticoagulant. Samples were centrifuged at 200 × g for 15 min and the supernatant consisting of platelet-rich plasma was discarded. Red blood cells were sedimented using HBSS containing 3% dextran. Leukocytes were centrifuged at 350 × g for 17 min on a discontinuous Ficoll-Paque gradient to separate granulocytes from PBMCs. Monocytes and lymphocytes were separated using a CD14 negative selection kit according to the manufacturer's instructions. Neutrophils and eosinophils were separated using aCD16positive selection kit, according to the manufacturer's instructions. The purities of the resulting cell suspensions were typically >98% for neutrophils and eosinophils, and >95% for lymphocytes and monocytes. We only utilized cell suspensions in which viability was greater than 99%, as determined by trypan blue exclusion. Cells were either used immediately for 2-AG metabolism assays, or lysed and kept frozen until western blotting experiments were conducted.

Human alveolar macrophages (AMs) were obtained by bronchoalveolar lavage of healthy volunteers as documented before.[31] In brief, volunteers underwent local anesthesia before a total of 300 ml of saline (5 syringes of 60 ml each) was injected in a segmental bronchi of the right middle lobe. The lavages were centrifuged (4°C, 350 × *g*, 10 min) and cells were washed twice with HBSS. Viability and purity were always greater

than 95%, as assessed by trypan blue exclusion and Diff Quick staining, respectively. AMs were either used immediately for 2-AG metabolism assays, or lysed and kept frozen until immunoblotting experiments were conducted.

2.3 Ethics approval

All volunteers gave informed consent and protocols were approved by the *Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec* Ethics Committee.

2.4 2-AG, PGE_2-G, and 15-HETE-G hydrolysis assays

Isolated leukocytes were suspended at a concentration of 2.5×10^6 cells/ml in HBSS containing 1.6 mM $CaCl_2$, and preheated at 37°C for 10 min.DMSOor serine hydrolase inhibitors (3 μM) were added to cell suspensions 5 min prior to the addition of 2-AG, PGE_2-G, or 15-HETE-G (1 μM) for 5min. Incubations were stopped by adding one volume of ice-cold MeOH containing 2 ng of 1-AG-D_5, PGE_2-D_4, and 15-HETE-D_8 as internal standards. For the control condition used to determine the initial amount of 2-AG in cell suspensions ($T = 0$), 2-AG, PGE_2-G, or 15-HETE-G were added to a cell suspension already containing MeOH. Samples were kept at −30◦C° until further processing.

2.5 Measurement of endocannabinoids and their metabolites by liquid chromatography-tandemmass spectrometry

Samples were thawed and centrifuged to eliminate cellular debris, then supernatants were diluted with water to obtain a final MeOH concentration of 10%. The pH of samples was adjusted at 3 by the addition of acetic acid. Samples were loaded on solid phase extraction cartridges (Strata-X Polymeric Reversed Phase, 60 mg/1 ml, Phenomenex, Torrance, CA, USA), washed with acidified water, and lipids were eluted with 1 mlMeOH. The eluates were dried under a nitrogen stream, and then reconstituted in 25 μl of HPLC solvent A (H2O optima LC/MS + 1 mM ammonium acetate + 0.05% acetic acid) and 25 μl of solvent B (MeCN/$H_2$0, 95/5, v/v containing 1 mM ammonium acetate + 0.05% acetic acid). A 25 μl aliquot was injected onto an RP-HPLC column (Kinetex C8, 150 × 2.1 mm, 2.6 μm, Phenomenex). Samples were eluted at a flow rate of 400 μl/min with a linear gradient of 10% solvent B that increased to 35% in 2 min, up to 75% in 10 min, from 75% to 95% in 0, 1 min, and held at 98% for 5 min before reequilibration to 10% solvent B for 2 min. The HPLCsystemwas directly interfaced into the electrospray source of a triple quadrupole mass spectrometer (Shimadzu 8050, Japan) and mass spectrometric analyses were performed in the positive (+) or the negative (−) ion mode using multiple reaction monitoring for the specific mass transitions of each lipid (2-AG (+), 379.30→287.25; 1-AG-D_5 (+) 384.50→287.20, 15-HETE (−) 319.40 → 301.20; 15-HETE-D_8 (−) 327.20 → 226.30; PGE2 (−) 351.20→271.15; PGE_2-D_4 (−) 355.20→275.35).

Each compound was diluted to prepare a calibration curve and was spiked with 2 ng of the appropriate deuterated standards. The samples were extracted as described above, and analyzed on the LCMS/MS system three times. The slope was then calculated using the ratio between the peak areas of the compound and its corresponding deuterated standard. Given that the fatty acid chain of 2-AG rapidly migrates from the sn-2 position to sn-1 position in aqueous solutions,[32] 2-AG levels represent the sum of 1-AG and 2-AG.

2.6 Analysis of proteins by immunoblot

Cells were lysed by sonication at 4°C in sucrose buffer containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 3 mM DFP, and 1 tablet protease inhibitor cocktail (for 10 ml of buffer). Laemmli sample buffer (5×; 62.5mMTRIS-HCl [pH 6.8], 2% SDS, 10% G, 0.01% bromophenol blue) was added to sonicated cell lysates and samples were boiled for 10 min. Buffer volumes were adjusted to obtain a final concentration of 2×10^6 cells/50 μl of lysate for all cell types with the exception of AMs, which were adjusted to 5×10^5 cells/50 μl. Proteins were separated by SDS-PAGE on 12% polyacrylamide gels and transferred onto PolyVinyliDene Fluoride (PVDF) membranes. Transfer efficiency and equal protein loading were verified by Ponceau Red staining. Membranes were placed in TBS-Tween buffer (25 mM Tris-HCl [pH 7.6], 0.2MNaCl, 0.15% Tween 20) containing 5% nonfat dried milk (w/v) for 30 min at room temperature, then probed with the primary antibody (4°C, overnight). The membranes were revealed by chemiluminescence using an HRP-coupled secondary antibody and the ECL detection kit.

2.7 FP-TAMRA labelling of serine hydrolases

Prewarmed leukocyte suspensions (37°C, 2×10^7 cells/ml) in HBSS containing 1.6mM $CaCl_2$ were incubated with DMSO, MAFP (10 μM), or JZL184 (10 μM) for 10 min. FP-TAMRA (3 μM) was then added for 30 min, and incubations were stopped using two volumes of ice-cold HBSS before transferring the samples in an ice-water bath. Samples were centrifuged (4°C, 350 × g, 5 min) and cell pellets were lysed and denatured using the same procedure as for immunoblots. Samples were loaded on a polyacrylamide gel, migrated and FP-TAMRAstained proteins were visualized using a Typhoon fluorescence imager (GE Healthcare, Toronto, ON, Canada). Enzyme sensitivity for the inhibitors was assessed by comparing the TAMRA fluorescence intensity with that of a labeled control without inhibitors.

2.8 Statistical analyses

For Figures 1 to 3, data were transformed as percentage of positive control. This was done using the following formula: 100 (experimental condition – baseline)/(positive control – baseline). Statistical analyses (1-way ANOVA with Dunnett's multiple comparisons test) were performed on the transformed data using the GraphPad Prism 7 software. *P* values <0.05 were considered significant.

3 RESULTS

3.1 Expression of 2-AG hydrolases in human leukocytes

In the first series of experiments, we assessed the expression of the 2-AG and PGE_2-G hydrolases documented so far (Table 1) in human neutrophils, eosinophils, monocytes, AMs, and lymphocytes. Lipase expression was assessed by immunoblot using lysates of freshly isolated leukocytes. Although we present the immunoblots from three different donors for each leukocyte population, they were not necessarily obtained from the same individuals.We also included a positive control for each target.Human hypothalamus lysateswere utilized for ABHD6 and ABHD12, lysates of the monocytic cell line THP-1 were utilized for ABHD16A, CES1 and PPT1, and lysates from the breast cancer cell line MDA-231 were utilized for LYPLA2. For MAG lipase, we noticed that protein integrity was dramatically affected by freezing (data not shown). For this reason, we used a histidine-tagged recombinant MAG lipase from Cayman Chemicals as a positive control and only probed freshly denatured leukocyte preparations.

Two of the enzymes, ABHD12 and ABHD16A, were found in all cell types tested (Fig. 4).MAGlipasewas not ubiquitous among leukocytes: neutrophils and lymphocytes showed very weak expression for this lipase in some donors, and no staining at all in other donors. ABHD6 was also not abundantly expressed in leukocytes, and its expression profile had important variations among donors. AMs are the cell type that express this enzyme most consistently, with two positive samples out of the three samples analyzed. As for LYPLA2, it was detected in all our monocyte, AMs and lymphocyte samples. As for eosinophils and neutrophils, they displayed a weak LYPLA2 signal in some samples and none in others, again underscoring that lipase expression profiles can vary between individuals.

Some of the enzymes displayed more distinct expression pattern. A good example of this is CES1, an enzyme initially described as a 2-AG hydrolase in THP-1 monocytes,[14] and that we only detected in human monocytes and AMs. Similarly, PPT1, which was characterized as an endocannabinoid hydrolase in THP-1 cells as well,15 could not be detected in human primary leukocytes with the exception of a weak signal in two out of three AM samples. Granulocytes and lymphocytes were clearly negative for these two enzymes. Overall, each cell type was found to express at least two of the documented hydrolases, suggesting that the hydrolysis of 2-AGby leukocytesmight involve more than one lipase and might not be entirely blocked by selective inhibitors.

3.2 Pharmacologic blockade of 2-AG hydrolysis in human leukocytes

Knowing that each leukocyte type expresses at least two 2-AG hydrolases, we next performed a series of experiments to confirm that all leukocytes could hydrolyze 2-AG into AA, and to pinpoint which hydrolase(s) is (are) most likely involved in this process. We thus treated leukocyte preparations with the various 2-AG hydrolysis inhibitors (or vehicle) for 5 min, added 2-AG (or vehicle) for an additional 5 min, and then stopped the reaction to quantitate 2-AG levels by liquid chromatography-tandem mass spectrometry (LC-MS/MS). In absence of inhibitors, all leukocyte preparations hydrolyzed 2-AG during the 5 min incubation time (Fig. 1A). Neutrophils were the most efficient at hydrolyzing 2-AG after 5 min (~90%). The hydrolysis of 2-AG was comparable between eosinophils, monocytes and AMs with 25% 2-AG remaining after 5 min. Lymphocytes were the least efficient at hydrolyzing 2-AG, with 50% of 2-AG still being detected after 5 min.

THL, WWL70, WWL113, and ML349, at 3 μM, did not prevent the hydrolysis of 2-AG in any cell type, indicating that their targets (ABHD6, CES1, and LYPLA2) were likely not involved in the 2-AG hydrolysis we observed. MAFP, the least selective compound,was consistently effective with an increase in 2-AG levels over baseline that was statistically significant in all cell types. JZL184 and Palmostatin B also blocked the hydrolysis of 2-AG, at least partially, in all cell types except for lymphocytes. MAFP was the most effective in neutrophils, eosinophils, and lymphocytes whereas Palmostatin B was more effective in monocytes and AMs. As for JZL184, it was developed as a selective MAG lipase inhibitor[33] and has few documented off target activities at the concentration used in these experiments, although it was reported to potently inhibit CES1.[14,34] The inability of JZL184 to prevent 2-AG hydrolysis in lymphocytes is thus not surprising given that they do not express MAG lipase nor CES1 (Fig. 4). However, the significant inhibitory effect JZL184 has on human neutrophils was puzzling, given the weak, almost absent, MAG lipase signal we obtained by immunoblot in these cells.

3.3 Impact of serine hydrolase inhibitors on PGE_2-G and 15-HETE-G hydrolysis by human leukocytes

We previously documented that both PGE_2-G and PGD_2-G are hydrolyzed into PGE_2 and PGD_2 by neutrophils, whereas others showed that JZL184 increased PGE_2-G levels in the brain of mice.[29] We thus sought to determine if and by which means PGE_2-G was also hydrolyzed into PGE_2 by leukocytes. PGE_2 levels were minimal in all our leukocyte preparations and increased from ~10-200 nM after treating the cells with 1 μM PGE_2-G during 5 min (Fig. 2A). This represents a 1–20% hydrolysis within 5 min. Figure 2B-E shows the effect of the inhibitors on the hydrolysis of PGE_2-G in each cell type. In neutrophils, eosinophils, and monocytes, MAFP, JZL184, and Palmostatin B almost completely inhibited PGE_2 formation and thus PGE_2-G hydrolysis. This was also the case in lymphocytes, although the effect of JZL184 in these cells was somewhat less prominent. Unsurprisingly, THL, which also lacks selectivity, significantly reduced PGE_2-G hydrolysis in most leukocytes. WWL113 had a modest

but statistically significant effect in neutrophils and monocytes, indicating that CES1, which was only found in monocytes and AMs, may not be its only target in human leukocytes. As for the LYPLA2 inhibitor ML349, it blocked ~35% of PGE_2 production in lymphocytes and also had a partial effect in neutrophils, although this did not reach statistical significance.

The hydrolytic pathway(s) of the PPARα agonist 15-HETE-G is (are) almost unknown. To our knowledge, the only study showing a possible effect of MAFP on 15-HETE-G hydrolysis inhibition was published recently by our group when we showed that 2-AG-treated neutrophils and eosinophils synthesized 15-HETE-G from exogenous 2-AG only when MAFP was present.[31] However, that study did not explore whether endocannabinoid hydrolysis inhibition with MAFP prevented 2-AG hydrolysis, 15-HETE-G hydrolysis, or both.We investigated that issue in the different leukocyte populations by assessing the impact of each serine hydrolase inhibitor on 15-HETE-G integrity. The hydrolysis of 15-HETE-G by each leukocyte was done by measuring the amounts of 15-HETE-G (1 μM) remaining after a 5 min incubation with the cells. Although it followed the same trend among leukocytes than for PGE_2-G(eosinophils >monocytes > neutrophils ≈ lymphocytes), 15-HETE-G hydrolysis into 15-HETE was more important than that of PGE_2-G in all leukocyte preparations (Fig. 3A). We next performed similar experiments to evaluate whether the different inhibitors impacted 15-HETE-G hydrolysis as we did for PGE_2-G, that is, by assessing 15-HETE levels. Once again, MAFP and Palmostatin B almost completely blocked 15-HETE-G hydrolysis in every cell type. JZL184, however, was less effective but had a significant effect in inhibiting the hydrolysis of 15-HETE-G. In all cases, the least selective compounds (MAFP and Palmostatin B) had a greater effect, almost completely blocking the hydrolysis of 15-HETE-G and the subsequent accumulation of 15-HETE. This underscores that 15-HETE-G hydrolysis occurs via numerous enzymes, although the contribution of each lipase appears to vary among cell types. As for THL, WWL70, WWL113, and ML349, they did not have a significant effect on 15-HETE-G hydrolysis in any cell type, in contrast to the hydrolysis of PGE-G (Fig. 2). Of note, PGE_2-G and 15-HETE-G hydrolyses were assessed in parallel, on the same leukocyte preparations, with the same inhibitors solutions.

3.4 Visualization of inhibitor targets in leukocytes by ABPP

Pharmacologic inhibitors are key tools to study metabolic pathways in human cells, but they often hit off-targets, especially when used at higher concentrations. Although the relevant documented secondary targets of each inhibitor are documented in Table 1, JZL184 inhibited the hydrolysis of 2-AG metabolites despite the lack of the MAG lipase protein in neutrophils and lymphocytes (Figs. 1–3). Thus, we could not exclude the possibility that additional enzymes were targeted by the inhibitors utilized in this study. In this respect, we used the ABPP method to visualize the targets of MAFP and JZL184 in human leukocytes. The rationale of this choice of inhibitors was to compare the targets of a nonselective inhibitor (MAFP) with those of a MAG lipaseselective inhibitor (JZL184), and to confirm the possibility that JZL184 has at least one additional target in human leukocytes, especially those that do not abundantly express MAG lipase such as neutrophils and lymphocytes. Figure 5A shows the principle of the FP-TAMRA labeling method. FP-TAMRA is, like MAFP, a fluorophosphonate. It covalently binds to serines in the active site of serine hydrolases. When a serine hydrolase inhibitor is added before FP-TAMRA, the latter will no longer label the enzyme, resulting in a decreased FP-TAMRA labeling (and fluorescence). The assay is very sensitive as 2 pg of FP-TAMRA MAG lipase could be detected (data not shown).

To confirm that FP-TAMRA was cell permeable, a series of experiment was undertaken in which we assessed its impact on the 2-AG-and the AA-induced LTB4 in neutrophils, as we reported previously with MAFP and JZL184.[6] In these experiments, FP-TAMRA inhibited the 2-AG-induced LTB4 biosynthesis of neutrophils in a concentration-dependent manner, with a complete inhibition at 100 nM. Furthermore, it did not affect the AA-induced LTB4 biosynthesis (data not shown). Next, serine hydrolases labeling with FP-TAMRA was done in human neutrophils, eosinophils, monocytes, lymphocytes, and AMs. Figure 5B shows a typical result of a FP-TAMRA labeling in human eosinophils and Figure 5C recapitulate the target we found in other leukocytes. MAFP prevented the labeling, by FP-TAMRA, of multiple targets (highlighted in blue). JZL184 also inhibited the labeling of some proteins (highlighted in purple). Of note, none of the labeled lipases was a target of JZL184 without being a target ofMAFP. In eosinophils, we only visualized two targets of JZL184. The first band migrated approximately around 30 kDa and was consistent with the height of the band that we usually obtain for MAG lipase (predicted molecular weight of 34 kDa) in immunoblot experiments. Moreover, this band was only present in eosinophils, monocytes, and AMs, but not in neutrophils and lymphocytes, which is consistent with the MAG lipase expression profile we established in Figure 4. The second JZL184 target, which weighs approximately 52 kDa, was observed in all cell types. This does not match the molecular weight of any of the enzymes we studied in this manuscript, strongly pointing to a JZL184-sensitive protein that has yet to be characterized.

4. DISCUSSION

Over the last decades, many proteins and enzymes have been suggested as promising anti-inflammatory targets. Among them is the MAG lipase which, as a main documented function, degrades the potentially anti-inflammatory 2-AG.[1,5] As such, inhibitors targeting this enzyme were developed and tested in animals for their efficacy at down-regulating inflammatory processes and for their safety. One of the most notorious compounds fulfilling these requirements is JZL184. It effectively inhibits MAG lipase and also diminishes the synthesis of eicosanoids such as PG and LT both in human cells and in vivo models involving mice, when 2-AG is the source of AA.[6–8,27,35,36] Of note, JZL184 does not induce the side effects that nonsteroidal anti-inflammatory drugs display on gastrointestinal integrity in mice.[37]

It has become clear, over the last decade, that human leukocytes can hydrolyze 2-AG and its metabolites from the COX-2 pathway. Indeed, we and others reported that leukocytes, notably neutrophils and eosinophils, readily and rapidly hydrolyze 2-AG and/or its metabolites.[6–8,26] One way to maximize the effects of 2-AG toward CB receptors is to increase its half-life. This concept has therapeutic potential in animal models of inflammation, as using MAG lipase deficient animals or JZL184 results in decreased inflammation.[1] Only one MAG lipase inhibitor, ABX-1431, is currently being investigated in humans.[38] Thus, translating this concept to humans requires a clear understanding of the 2-AG metabolic pathways involved in this species. In this study, we addressed the lack of knowledge regarding these pathways by characterizing the hydrolysis of 2-AG and its oxidation metabolites in human leukocytes.

We show that (i) leukocytes express at least two 2-AG hydrolases; (ii) leukocytes hydrolyze 2-AG, PGE_2-G, and 15-HETE-G; (iii) serine hydrolase inhibitors impair the hydrolysis of 2-AG, PGE_2-G, and 15-HETE-G; (iv) JZL184 inhibits the hydrolysis of 2-AG and its metabolites; (v) JZL184 displays inhibitory effects in leukocytes that do not express the MAG lipase; and (vi) ABPP profiling indicates that a ~52 kDa JZL184-sensitive protein is expressed in all leukocyte types.

Given that MAG lipase is the best known 2-AG hydrolase and that it is a frequently used genetic or pharmacologic target (using JZL184) to increase 2-AG levels in animal models, it was crucial to assess its expression and involvement in human leukocytes. Our data show that neutrophils and lymphocytes do not have detectable amounts of the MAG lipase protein, in contrast to eosinophils, monocytes, and AMs. By contrast, other lipases were detected in all leukocyte types (Fig. 4). In this regard, ABHD12 and ABHD16A were detected in all our leukocyte preparations, whereas CES1 was restricted to monocytes and AMs although its role seems limited, in agreement with Szafran and colleagues.[35] Interestingly, LYPLA2 was also found in monocytes, AMs, and in lymphocytes. Finally, PPT1was undetectable in leukocytes, with the exception of AMs.

This expression profile is consistent with our observation that all leukocyte population hydrolyzed 2-AG, PGE_2-G, and 15-HETE-G. In these experiments, we used the same amount of substrates (1 μM) and the same cellular concentration (2.5 million cells/ml) in the various leukocyte preparations. 2-AG hydrolysis was important in all cell types, with 50–90% 2-AG being hydrolyzed after 5 min. 15-HETE-G hydrolysis was also important with 50–100% hydrolysis after a 5 min treatment, eosinophils being the most efficient. In contrast, PGE_2-G hydrolytic activity in the same time frame was lower, reaching ~20% in human eosinophils.

Interestingly, the various lipase inhibitors used in this study did not impact the hydrolysis of 2-AG, 15-HETE-G, and PGE_2-G in the same manner. For instance, the only compound that maximally and consistently prevented the hydrolysis of 2-AG, 15-HETE-G, and PGE_2-G was MAFP, Palmostatin B being second. Both are unselective inhibitors. This supports the notion that 2-AG hydrolysis in leukocytes relies on several hydrolases in each cell types.We also cannot exclude that some enzymes are differentially involved in the hydrolysis of 2-AG, 15-HETE-G, and PGE_2-G. Indeed, whereas THL, WWL70, and WWL113 had a limited, yet significant impact on PGE_2-G hydrolysis in some leukocytes, they did not display significant inhibitory activities for the hydrolysis of 2-AG and 15-HETE-G. Of note, we assessed the hydrolysis of PGE_2-G and 15-HETE-G using the same leukocyte preparation, supporting the concept that the differential inhibitory effects we observed were not the consequence of individual variability or inhibitor solutions. It could reflect the involvement of different lipases, a substrate preference by the hydrolases, or even an inhibitor selectivity toward a given substrate, as previously documented with R-flubiprofen, which selectively inhibits the COX-2-mediated synthesis of PGE_2-G while not affecting that of PGE_2.[39] Finally, the inhibitory effects of THL, WWL70, WWL113 on PGE_2-G were limited compared to MAFP, Palmostatin B, and JZL184 and might also be the consequence of the limited PGE_2-G hydrolysis (vs. 2-AG and 15-HETE-G hydrolysis) wewere confronted to.We previously showed that PGE_2-G inhibited human neutrophils functions via its hydrolysis into PGE_2, an effect that was prevented by MAFP, palmostatin B, JZL184, and WWL113, but not WWL70, THL, or ML349.[26] The data presented herein support our initial findings and raise the possibility that WWL113 targets an additional lipase involved in the hydrolysis of PGE2-G in neutrophils.

Over the last century, research allowed us to appreciate that the selectivity of an inhibitor inversely correlates with the amount of time elapsed since its initial characterization. As such, the inhibitory effects of JZL184 on the hydrolysis of 2-AG, PGE_2-G, and 15-HETE-G were, at times, unexpected. For instance, neutrophils and lymphocytes barely or do not express MAG lipase. As a consequence, JZL184 and Palmostatin B did not significantly prevent 2-AG hydrolysis in lymphocytes. In contrast, JZL184 and Palmostatin B did inhibit 2-AG hydrolysis in neutrophils, suggesting that other enzyme(s) or the trace amounts of MAG lipase we found in neutrophils were probably inhibited by JZL184 and Palmostatin B. Even more intriguing is the fact that JZL184 significantly inhibited the hydrolysis of both PGE_2-G and 15-HETE-G hydrolysis by human lymphocytes (Figs. 2 and 3), even though MAG lipase was not found in these cells, and where accordingly the inhibitors did not significantly prevent, although diminished the hydrolysis of 2-AG. This indicated to us that JZL184 had other enzymatic targets in leukocytes, notably in neutrophils and lymphocytes and that the inhibitory effect of JZL184 on 2-AG, PGE_2-G, and 15-HETE-G reflects its action on multiple targets.

In an attempt to solve that issue, ABPP was performed with MAFP and JZL184 on human leukocytes and JZL184 indeed prevented the labeling of a ~30 kDa protein, very likely the MAG lipase, in the same leukocytes that were positive for MAG lipase expression by immunoblot (Figs. 4 and 5), but also prevented the labelling of a ~52 kDa protein by FP-TAMRA in all cell types tested (Fig. 5). Although successful at labeling our leukocytes with FP-TAMRA and preventing that labelling with MAFP and JZL194, we were unsuccessful at performing immunoprecipitation (IP). experiments with an anti-TAMRA on those labelled cells to identify the higher molecular weight, JZL184-sensitive protein that we consistently found in our leukocyte preparations.

The inefficacy of WWL113 to inhibit the hydrolysis of 2-AG in monocytes andAMswas surprising becauseWWL113is an inhibitor of CES1,which is expressed by these two cell types. This likely reflects the limited role that CES1 plays in the hydrolysis of 2-AG in these cells, at least in our model. However, its significant impact on PGE_2-G hydrolysis in neutrophils and monocytes raise the possibility that another lipase, somewhat sensitive to WWL113, is expressed by these two cell types.

The data provided in this paper were obtained with freshly isolated primary leukocytes. Working with freshly isolated primary leukocytes remains a challenge with some limitations, notably the use of chemical inhibitors vs. transfection/silencing. Herein, we utilized a pharmacologic approach because (i) in clinics, enzymes are mostly targeted by chemical compounds; (ii) human primary leukocytes are rarely transfectable and, when they are, this requires an overnight treatment; (iii) we cannot guarantee that incubating human primary leukocytes will not alter the expression of the several lipases we have been investigating nor their post-translational modifications, which could affect their activity; and (iv)we cannot exclude that co-factors or steric hindrance competitors are present in different amount during long incubation period, which could even further complicate and or mislead our conclusion.

In conclusion, we herein demonstrate that human leukocytes express at least two documented endocannabinoid hydrolases, are experts at hydrolyzing 2-AG and 15-HETE-G, and have a limited, yet significant impact on PGE_2-G hydrolysis (see also Turcotte et al.[26]). Our data emphasizes the lack of selectivity of the inhibitors that are currently available, and unravel a yet-to-be identified target of JZL184 that appears to be involved in 2-AG hydrolysis by leukocytes. Given that these lipases participate in determining the fate of G-containing endocannabinoids in an inflammatory setting, and that these metabolic pathways regulate the levels of several pro- and anti-inflammatory eicosanoids[6,26,27,29] our

findings will help to develop potent inhibitors that will increase the beneficial effects of endocannabinoids-G and their metabolites in inflammatory diseases.

ACKNOWLEDGMENTS

We thank Johane Lepage, Joanne Milot, and Hélène Villeneuve for providing the blood samples. We also thank Dr. Lawrence J. Marnett for providing the LYPLA2 inhibitor ML349. C.T., ÉD, A-S.A., C.M., V.DM., and N.F. are members of the Chaire d'excellence en recherche du Canada sur l'axe microbiome-endocannabinoïdome dans la santé métabolique.

This work was supported by grants to N.F. from the Natural Sciences and Engineering Research Council of Canada, the Quebec RespiratoryHealthNetwork, and the Chaire d'excellence en recherche du Canada sur l'axe microbiome-endocannabinoïdome dans la santé métabolique held by V.DM. C.T. and A-S.A. are the recipients of doctoral awards from the Canadian Institutes of Health Research and C.T. also received support from the Canadian Consortium for the Investigation of Cannabinoids.

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TABLE 1 Mains enzymes involved in the hydrolysis of 2-AG, PGE2-G, and 15-HETE-G

Enzyme	Documented to hydrolyze	Documented inhibitors (IC_{50})	Reference
ABHD6	2-AG	MAFP (16.9 nM)	40
	PGE_2-G	JZL184 (>10 μM)	33
		Palm B (52.5 nM)	16
		THL (48 nM)	40
		WWL70 (70 nM)	41
		WWL113 (ND)	42
ABHD12	2-AG	MAFP (87 nM)	40
	PGE_2-G	Palm B (1.8 μM)	16
		THL (193 nM)	40
ABHD16A	2-AG	Palm B (100 nM)	16
	PGE_2-G	THL (170 nM)	16
CES1	2-AG	MAFP (ND)	14,15
	PGE_2-G	JZL184 (ND)	14
		WWL113 (46 nM)	42
LYPLA2	PGE_2-G	JZL184 (29 μM)	40
		Palm B (37.7 nM)	43
		ML349 (904 nM)	40,44
MAG lipase	2-AG	MAFP (ND)	28
	PGE_2-G	JZL184 (8 nM)	33
		Palm B (93.3 nM)	16
PPT1	2-AG	MAFP (ND)	15
	PGE_2-G	Palm B (ND)	43,45

JZL184, 4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl) piperidine-1-carboxylate; Palm B, Palmostatin B; WWL70, 4′-carbamoyl biphenyl-4-yl methyl(3-(pyridin-4-yl)benzyl)carbamate, N-methyl-N-[[3-(4-pyridinyl)phenyl]methyl]-4′-(aminocarbonyl)[1,1′-biphenyl]-4-yl ester carbamic acid; WWL113, 4′-[[[methyl[[3-(4-pyridinyl)phenyl]methyl] amino]carbonyl]oxy]-[1,1′-biphenyl]-4-carboxylic acid, ethyl ester; ND, not determined.

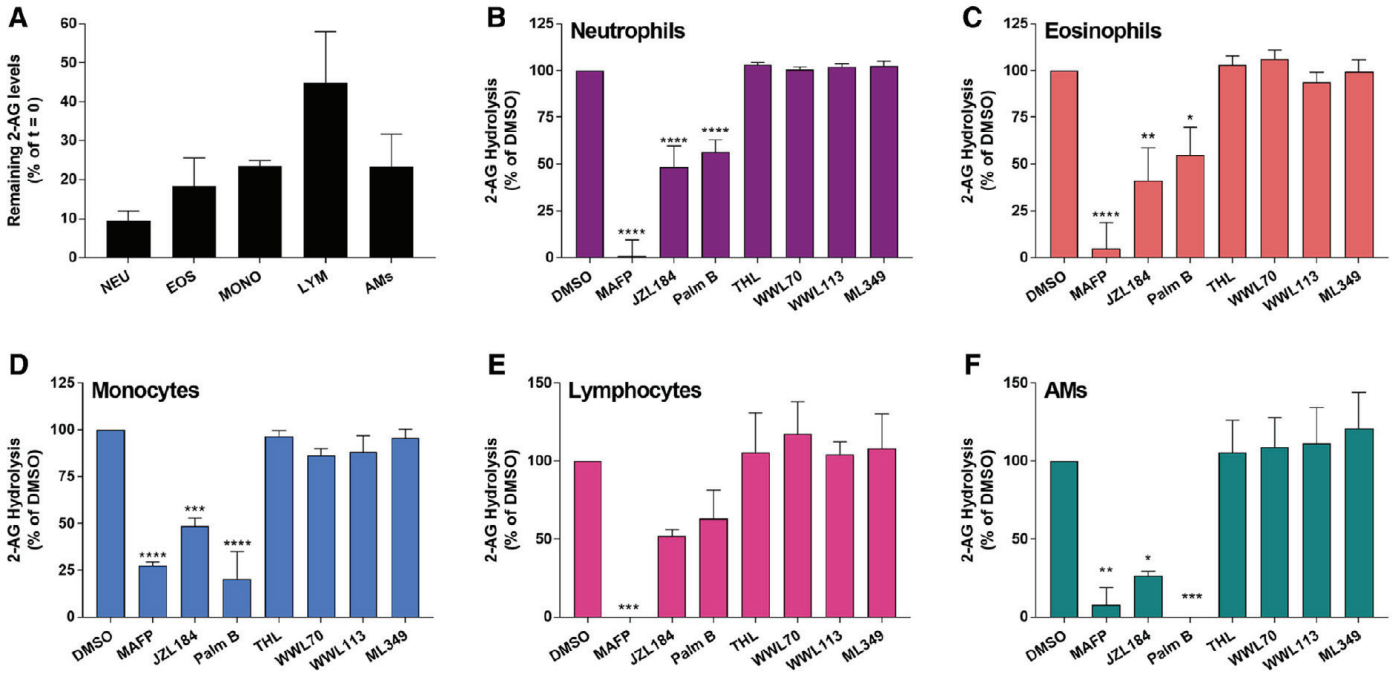


FIGURE 1 **Impact of various serine hydrolase inhibitors on 2-AG hydrolysis in human leukocytes. (A)** Pre-warmed human leukocyte suspensions (37°C, 2.5 × 10^6 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were treated with 1 μM 2-AG for 5 min. **(B)-(F)** Pre-warmed human leukocyte suspensions (37°C, 2.5 × 10^6 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were treated with DMSO or 3 μM inhibitors for 5 min then incubated with 1 μM 2-AG for another 5 min. **(A)-(F)** Incubations were stopped by the addition of one volume of cold (−20°C) MeOH containing 2 ng of 1-AG-D_5 as an internal standard. Samples were processed for the analysis of 2-AG by LC-MS/MS as described in Section 2. Results are the mean (±SD) of 3–5 independent experiments. Statistical analyses (1-way ANOVA with Dunnett's multiple comparisons test) were performed using the GraphPad Prism 7 software. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$ (vs. DMSO, $T = 5$ min)

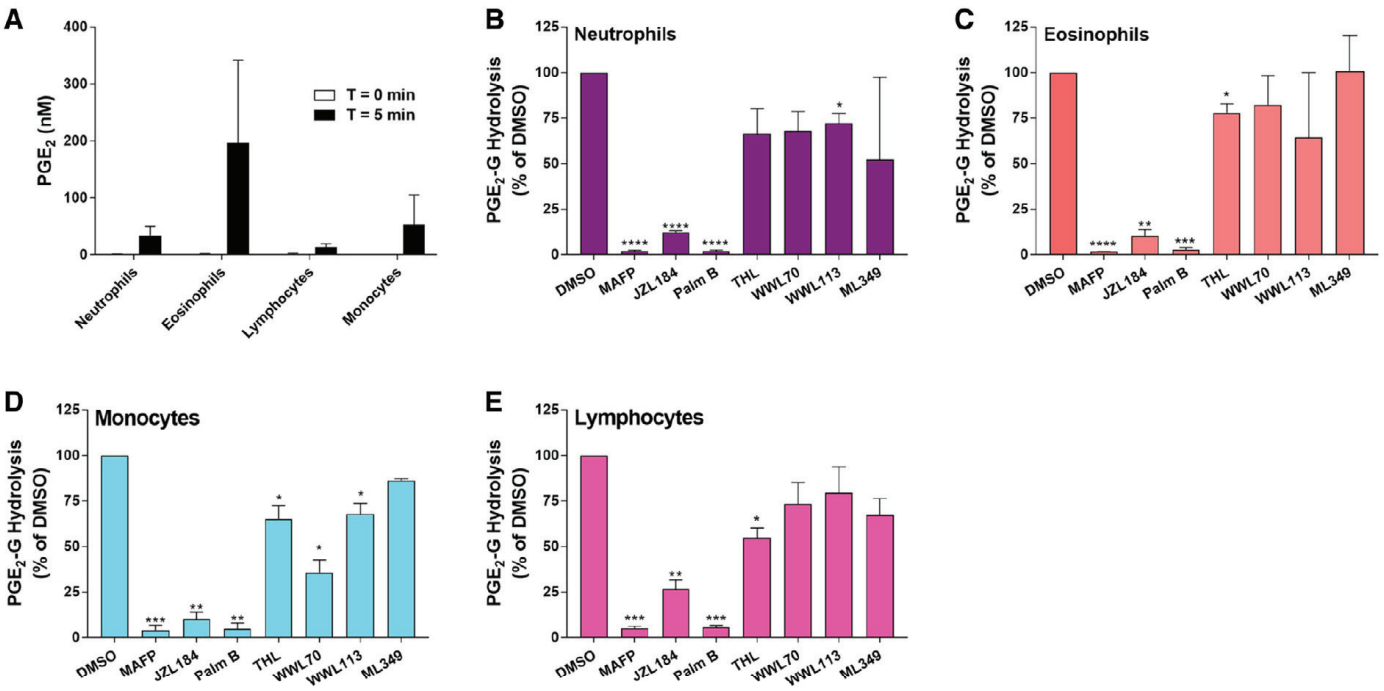


FIGURE 2 **Impact of various serine hydrolase inhibitors on PGE_2-G hydrolysis in human leukocytes.** **(A)** Pre-warmed leukocyte suspensions (37°C, 2.5×10^6 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were treated with DMSO or 1 μM PGE_2-G during 5 min. **(B)-(E)** Pre-warmed leukocyte suspensions (37°C, 2.5×10^6 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were treated with DMSO or 3 μM inhibitors for 5 min then incubated with 1 μM PGE_2-G for another 5 min. **(A)-(E)** Incubations were stopped by the addition of one volume of cold (−20°C) MeOH containing 2 ng of PGE_2-D_4 as an internal standard. Samples were processed and analyzed for PGE_2 levels by LC-MS/MS as described in Section 2. Results are the mean (±SD) of 3–5 independent experiments. Statistical analyses (1-way ANOVA with Dunnett's multiple comparisons test) were performed using the GraphPad Prism 7 software. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$ (vs. DMSO)

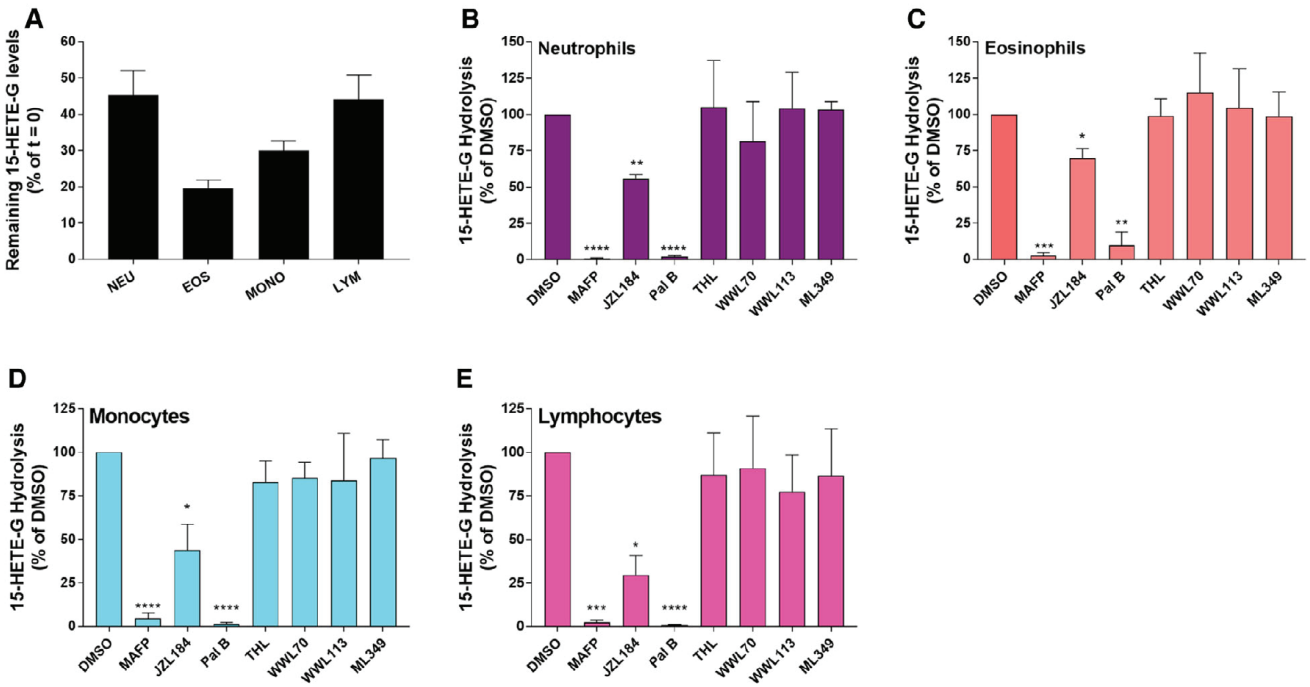


FIGURE 3 **Impact of various serine hydrolase inhibitors on 15-HETE-G hydrolysis in human leukocytes. (A)** Pre-warmed leukocyte suspensions (37°C, 2.5×10^6 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were treated with DMSO or 1 µM 15-HETE-G during 5 min. **(B)-(E)** Pre-warmed leukocyte suspensions (37°C, 2.5×10^6 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were treated with DMSO or 3 µM inhibitors for 5 min then incubated with 1 µM 15-HETE-G for another 5 min. **(A)-(E)** Incubations were stopped by the addition of one volume of cold (−20°C) MeOH containing 2 ng 15-HETE-D_8 as an internal standard. Samples were processed for the analysis of 15-HETE-G (**A**) and 15-HETE (**B)-(E**) by LC-MS/MS as described in Section 2. Results are the mean (±SEM) of 3 independent experiments. Statistical analyses (1-way ANOVA with Dunnett's multiple comparisons test) were performed using the GraphPad Prism 7 software. $^*P < 0.05$ and $^{****}P < 0.0001$ (vs. DMSO, $T = 5$ min)

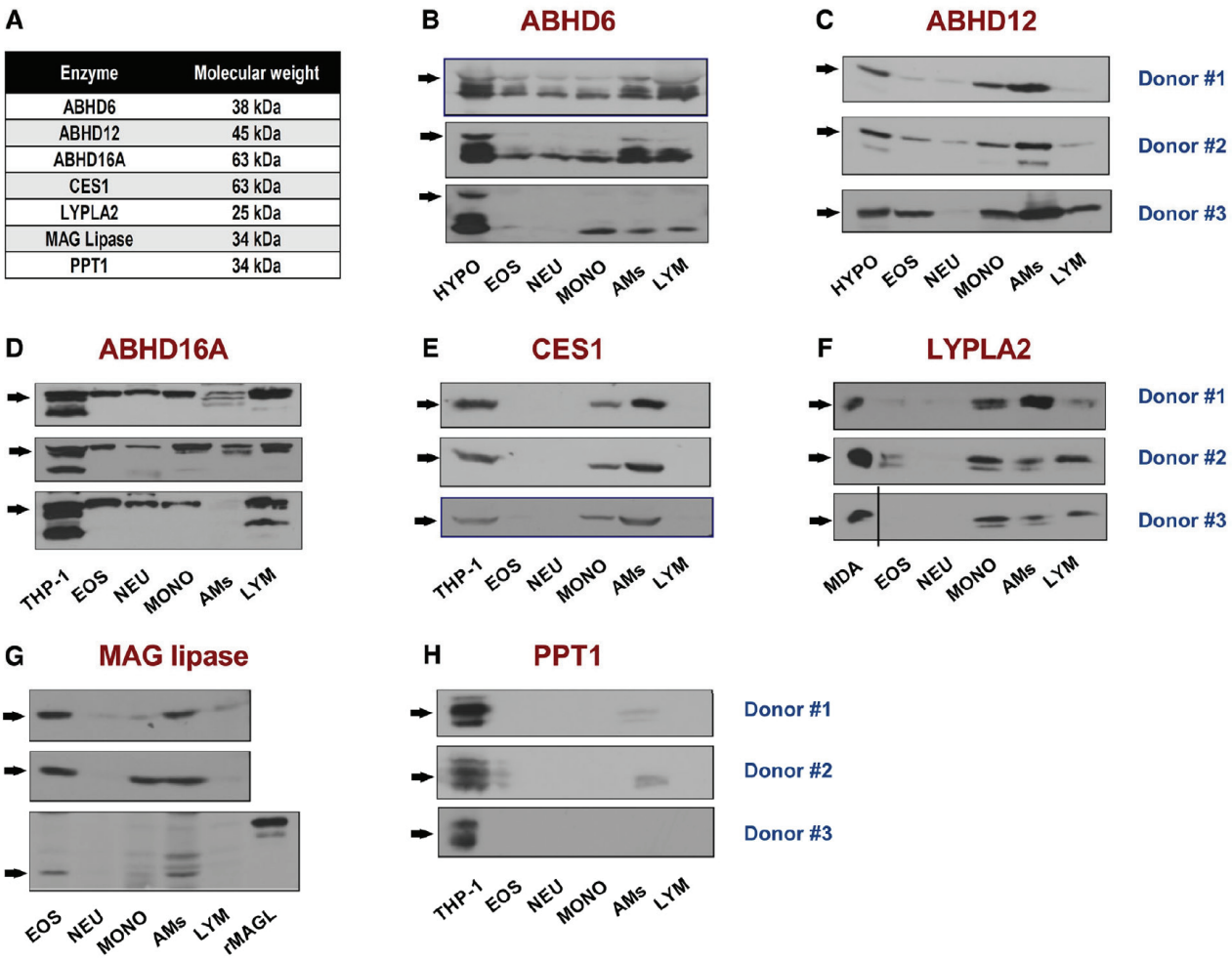
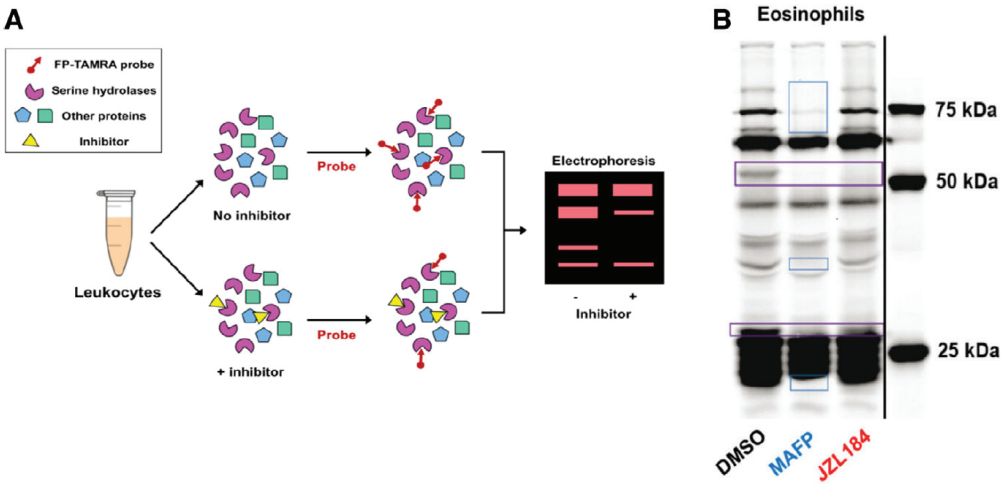


FIGURE 4 **Expression of 2-AG hydrolases in human leukocytes. (A)-(G)** Protein samples were prepared from freshly isolated human leukocyte preparations, as described in Section 2. Immunoblot experiments were conducted as described in Section 2 by loading the denatured proteins of 2 million cells per well. Each image represent a single experiment and donors are different in the three images. HYPO = hypothalamus; EOS = eosinophils; NEU = neutrophils; MONO = monocytes; LYM = lymphocytes; MDA = MDA-231; and rMAGL = His-tagged recombinant MAG lipase



C

Cell type	MAFP-sensitive proteins (kDa)	JZL184-sensitive proteins (kDa)
Neutrophils	>50, 60, 65, 85	>50
Eosinophils	20, 30, 40, >50, 60, 75, 85	30, >50
Monocytes	20, 30, 45, >50, 60, 65, 75, 85	30, >50
AMs	20, 25, 30, 40, 45, >50, 60, 75, 85	30, >50
Lymphocytes	20, 45, >50, 60, 65, 75, 90	>50

FIGURE 5 **Activity-based protein profiling** (ABPP) **reveals numerous inhibitor targets in human leukocytes. (A)** Leukocytes were incubated with 10 μM MAFP or JZL184 for 10 min. FP-TAMRA was then added to leukocytes for 30 min. Incubations were stopped by the addition of one volume of cold incubation buffer. Samples then were processed and analyzed for FP-TAMRA-labeled proteins as described in Section 2. (**B**) Example of the ABPP of human eosinophils, using DMSO, MAFP, or JZL184. Data are from one experiment that is representative of three. (**C**) Summary of the MAFP- and/or JZL184-sensitive proteins visualized by ABPP in each leukocyte (representative of 3 independent experiments)