Endocannabinoid hydrolysis inhibition unmasks that unsaturated fatty acids induce a robust biosynthesis of 2-arachidonoyl-glycerol and its congeners in human myeloid leukocytes

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Abstract
The endocannabinoid (eCB) 2-arachidonoyl-glycerol (2-AG) modulates immune responses by activating cannabinoid receptors or through its multiple metabolites, notably eicosanoids. Thus, 2-AG hydrolysis inhibition might represent an interesting anti-inflammatory strategy that would simultaneously increase the levels of 2-AG and decrease those of eicosanoids. Accordingly, 2-AG hydrolysis inhibition increased 2-AG half-life in neutrophils. Under such setting, neutrophils, eosinophils, and monocytes synthesized large amounts of 2-AG and other monoacylglycerols (MAGs) in response to arachidonic acid (AA) and other unsaturated fatty acids (UFAs). Arachidonic acid and UFAs were ~1000-fold more potent than G protein-coupled receptor (GPCR) agonists. Triascin C and thimerosal, which, respectively, inhibit fatty acyl-CoA synthases and acyl-CoA transferases, prevented the UFA-induced MAG biosynthesis, implying glycerolipid remodeling. 2-AG and other MAG biosynthesis was preceded by that of the corresponding lysophosphatidic acid (LPA). However, we could not directly implicate LPA dephosphorylation in MAG biosynthesis. While...

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Abbreviations: 2-AG, 2-arachidonoyl-glycerol; 2-DHG, 2-docosahexaenoyl-glycerol; 2-DPG, 2-docosapentaenoyl-glycerol; 2-EPG, 2-eicosapentaenoyl-glycerol; 2-LG, 2-linoleoyl-glycerol; 2-OG, 2-oleoyl-glycerol; 2-PG, 2-palmitoyl-glycerol; AA, arachidonic acid; AEA, anandamide or N-arachidonoyl-ethanolamine; AMs, alveolar macrophages; BAL, bronchoalveolar lavage; DAG, diacylglycerol; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; eCB, endocannabinoid; EPA, eicosapentaenoic acid; FAAH, fatty acid amide hydrolase; GPCR, G protein-coupled receptor; LA, linoleic acid; LPA, lysophosphatidic acid; LT, leukotriene; MAFP, methylarachidonoyl-fluorophosphonate; MAG, monoacylglycerol; MBOAT, membrane bound O-acyl transferase; NAE, N-acyl-ethanolamine; OA, oleic acid; PA, palmitic acid; PAF, platelet activating factor; PL, phospholipase; UFA, unsaturated fatty acid.

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INTRODUCTION

2-Arachidonoyl-glycerol (2-AG) is a bioactive lipid and endocannabinoid (eCB) activating the cannabinoid receptors CB1 and CB2. As such, it modulates several physiological processes including appetite, pain, and adipogenesis.1-3 2-AG also modulates immune cell functions, usually leading to decreased inflammatory responses, at least in mice,4,5 very much likely by activating the CB2 receptor.6,7 Two main strategies are currently investigated to, respectively, mimic or promote the anti-inflammatory effects of 2-AG: the use of CB2 receptor agonists or the use of 2-AG hydrolysis inhibitors, which would enhance 2-AG half-life in vivo. While both strategies are attractive, using 2-AG hydrolysis inhibitors in a context of inflammation might have the additional benefit of decreasing eicosanoid levels because leukocytes efficiently hydrolyze 2-AG and its metabolites from the cyclooxygenase-2 and 15-lipoxygenase pathways.8-12

The synthesis of 2-AG by human leukocytes is not well documented. Furthermore, efforts to induce 2-AG biosynthesis in leukocytes led to 2-AG levels lower than those required to activate the CB2 receptor, for which 2-AG has a Ki of ~145 nM.13-16 The classic 2-AG biosynthetic pathway involves two enzymatic steps. First, a phospholipase C (PLC) will cleave a phosphatidylinositol-4,5-bisphosphate containing arachidonic acid (AA) in the sn-2 position into inositol-1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG). Next, the obtained DAG will be hydrolyzed by DAG lipase α or β into 2-AG.17-19 While the expression profile of DAG lipase α or β in leukocytes is ill defined, DAG lipase β blockade in murine peritoneal macrophages, led to a significant decrease in 2-AG.20 Two alternative routes for the biosynthesis of 2-AG have been documented. The first one likely involves phospholipase D (PLD), as it uses phosphatidic acid as a precursor for DAG synthesis, which is then converted into 2-AG by DAG lipases.21 The second one is the dephosphorylation of lysophosphatidic acid (LPA) into 2-AG.22 The importance of these alternative pathways in 2-AG biosynthesis in vivo remains to be elucidated in the periphery.

Herein, we assessed whether human leukocytes were a significant source of 2-AG as well as the underlying mechanism involved. We report that 2-AG hydrolysis inhibition strikingly prolongs 2-AG half-life, allowing to correctly assess the 2-AG biosynthetic capabilities of leukocytes. We also report that the classic 2-AG biosynthetic pathway (PLC/DAG lipase) does not lead to an important 2-AG biosynthesis in human leukocytes; that instead AA stimulates a robust biosynthesis of 2-AG; and that unsaturated fatty acids (UFAs) stimulate the synthesis of their monoacylglycerols (MAGs) and 2-AG congeners. The UFA-induced MAG biosynthetic pathway was found in neutrophils, eosinophils, and monocytes but not in alveolar macrophages (AMs), lymphocytes, platelets, or erythrocytes. This biosynthetic pathway is insensitive to DAG lipase inhibitors, sensitive to acyl-CoA synthase and transferase inhibitors, and is preceded by the biosynthesis of a corresponding LPA intermediate.

MATERIALS AND METHODS

2.1 Materials

Dextran and mass spectrometry-grade methanol and acetonitrile were purchased from Fisher Scientific. 1-AG-d₅, 2-AG, 2-AG-d₈, AA, AA-d₈, leukotriene (LT) LTB₄, Platelet-activating factor (PAF), fMet-Leu-Phe (fMLP), A23187, R848, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (n-3) (DPA), linoleic acid (LA), oleic acid (OA), palmitic acid (PA), 2-palmitoyl-glycerol (2-PG), 2-oleoyl-glycerol (2-OG), 2-linoleoyl-glycerol (2-LG), methylarachidonoyl-fluorophosphonate (MAFP), triascin C, and JZL184 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Lymphocyte separation medium was purchased from Corning (Corning, NY, USA). Palmostatin B was purchased from EMD Millipore (Billerica, MA, USA). Strata-X columns for solid phase lipid extraction were purchased from Phenomenex (Torrance, CA, USA). Dextran and mass spectrometry-grade methanol and acetonitrile were purchased from Fisher Scientific. 1-AG-d₅, 2-AG, 2-AG-d₈, AA, AA-d₈, leukotriene (LT) LTB₄, Platelet-activating factor (PAF), fMet-Leu-Phe (fMLP), A23187, R848, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (n-3) (DPA), linoleic acid (LA), oleic acid (OA), palmitic acid (PA), 2-palmitoyl-glycerol (2-PG), 2-oleoyl-glycerol (2-OG), 2-linoleoyl-glycerol (2-LG), methylarachidonoyl-fluorophosphonate (MAFP), triascin C, and JZL184 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Lymphocyte separation medium was purchased from Corning (Corning, NY, USA). Palmostatin B was purchased from EMD Millipore (Billerica, MA, USA). Strata-X columns for solid phase lipid extraction were purchased from Phenomenex (Torrance, CA, USA). The magnetic bead-conjugated anti-CD16 and anti-CD14 mAb and MACS columns were purchased from Miltenyi Biotec (Auburn, CA, USA). Dextran and mass spectrometry-grade methanol and acetonitrile were purchased from Fisher Scientific. 1-AG-d₅, 2-AG, 2-AG-d₈, AA, AA-d₈, leukotriene (LT) LTB₄, Platelet-activating factor (PAF), fMet-Leu-Phe (fMLP), A23187, R848, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (n-3) (DPA), linoleic acid (LA), oleic acid (OA), palmitic acid (PA), 2-palmitoyl-glycerol (2-PG), 2-oleoyl-glycerol (2-OG), 2-linoleoyl-glycerol (2-LG), methylarachidonoyl-fluorophosphonate (MAFP), triascin C, and JZL184 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Lymphocyte separation medium was purchased from Corning (Corning, NY, USA). Palmostatin B was purchased from EMD Millipore (Billerica, MA, USA). Strata-X columns for solid phase lipid extraction were purchased from Phenomenex (Torrance, CA, USA). The magnetic bead-conjugated anti-CD16 and anti-CD14 mAb and MACS columns were purchased from Miltenyi Biotec (Auburn, CA, USA). Adenosine deaminase (ADA) was purchased from Roche (Laval, QC, Canada). 1/2-Eicosapentaenoyl-glycerol (2-EPG), 1/2-docosapentaenoyl-glycerol (n-3) (2-DPG), and 1/2-docosahexaenoyl-glycerol (2-DHG) were either provided by Dr Samuel Fortin from SCF pharma or purchased from Nu-Chek...
2.2 Ethics committee approval

This work required the use of human cells from volunteers and was approved by our institutional ethics committee. All the experiments were conducted with the understanding and the signed consent of each participant.

2.3 Isolation of human leukocytes

For the isolation of neutrophils, eosinophils, lymphocytes, and monocytes, human venous blood was obtained from healthy or rhinitic volunteers and collected in tubes containing K$_3$EDTA as anticoagulant. Leukocytes were isolated as described previously with some modifications. In brief, the blood was centrifuged and the plasma was discarded. Erythrocytes were sedimented with 3% dextran, and granulocytes were separated from PBMCs using a discontinuous gradient. The PBMC layer was harvested and monocytes and lymphocytes were separated using a magnetic bead-conjugated anti-CD14, according to the manufacturer’s instructions. Residual erythrocytes were eliminated from the granulocyte pellet by hypotonic lysis with sterile water. Eosinophils were separated from neutrophils using anti-CD16-conjugated magnetic beads according to the manufacturer's instructions. The purity and viability of the resulting leukocyte suspensions were always ≥98%, as assessed by counting 500 cells by Diff Quik staining and trypan blue exclusion, respectively.

Human AMs were obtained by bronchoalveolar lavage (BAL) of healthy volunteers as previously described. The BAL fluid containing leukocytes was recovered and centrifuged (4°C, 350g, 10 minutes). Supernatants were discarded and cells were washed twice with cold HBSS then their viability was assessed by trypan blue exclusion. Viability and purity of AMs were always greater than 95%, as assessed by enumerating 500 cells with stained with trypan blue and Diff Quick staining, respectively.

2.4 Cell stimulations

Cells were suspended in HBSS containing 1.6 mM CaCl$_2$ and preheated at 37°C for 10 minutes. To better mimic their fate, adenosine deaminase (0.3 U/mL) was added 10 minutes before the addition of the stimuli in all experiments involving neutrophils. Inhibitors were added 5 minutes before the stimuli and/or the fatty acids, at the concentrations detailed in the figure legends. In experiments assessing their impact on eCB biosynthesis, PAF, fMLP, and LTB$_4$ were added to the cell suspensions simultaneously with the fatty acids. For the analysis of MAGs and N-acyl-ethanolamines (NAEs) by LC-MS/MS, incubations were stopped by the addition of one volume of cold (−20°C) MeOH containing 0.01% acetic acid and 2 ng of 1-AG-d$_5$ and as an internal standard. Samples were then kept at −20°C until further processing.

2.5 Analysis of DAG lipase expression by immunoblot

For the analysis of DAG lipase protein expression, cells were lysed with NP-40 in an hypotonic lysis 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 (5x; 62.5 mM TRIS-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.01% bromophenol blue) was added to cell lysates and samples were boiled for 10 minutes. Buffer volumes were adjusted to obtain a final concentration of 2 x 10$^6$ cells/50 µL of lysate for all cell types except for AMs, which were adjusted to 5 x 10$^5$ cells/50 µL. Proteins were separated by SDS-PAGE on 12% polyacrylamide gels and transferred onto PVDF membranes. Transfer efficiency and equal protein loading were confirmed by Ponceau Red staining. Membranes were placed in TBS-Tween buffer (25 mM Tris-HCl [pH 7.6], 0.2 M NaCl, 0.15% Tween 20) containing 5% nonfat dried milk (w/v) for 30 minutes at room temperature, then incubated with the primary antibody (4°C, overnight). The membranes were revealed by chemiluminescence using a HRP-coupled secondary antibody and an ECL detection kit (EMD Millipore; Billerica, MA, USA).

2.6 Analysis of MAGs, NAEs and LPA by liquid chromatography—Tandem mass spectrometry (LC-MS/MS)

For the analysis of MAGs and NAEs, the denatured samples containing the ISTD were thawed and centrifuged (1000g; 10 minutes) to remove cellular debris, then supernatants were diluted with water to a final MeOH concentration of 10%, and maintained at pH 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). Cartridges were washed with 2 mL of acidified water and lipids were eluted with 1 mL of MeOH. The eluates were evaporated to dryness under a stream of nitrogen. For the analysis of LPA, the denatured samples containing the ISTD were acidified with acetic acid (0.1 M final concentration). Lipids then
were extracted from the denatured samples by adding 1 mL of chloroform, vortexing for 1 minute, and centrifuging at 4000g for 5 minutes without brakes. This was repeated three times. The organic phases were collected, pooled, and evaporated to dryness under a stream of nitrogen. For the quantification of MAGs in human plasma, samples were extracted as documented before with slight modifications. 200 µL of plasma samples were mixed with 300 µL of TRIS (pH 7.4, 50 mM). Toluene (2 mL) containing the ISTD was then added to the samples, vortexed for 1 minute, centrifuged at 4000g for 5 minutes without brakes. Samples then were placed in an ethanol-dry-ice bath (−80°C) to freeze the aqueous phase (bottom). The organic phase (top) was then collected and evaporated to dryness under a stream of nitrogen. Samples were reconstituted in 25 µL of HPLC solvent A (H2O with 0.05% acetic acid and 1 mM NH4+) and 25 µL of solvent B (MeCN/H2O, 95/5, v/v, with 0.05% acetic acid and 1 mM NH4+). A 25 µL of aliquot was injected onto an RP-HPLC column (Kinetex C8, 150 x 2.1 mm, 2.6 µm, Phenomenex). Quantification was performed on a Shimadzu 8050 triple quadrupole mass spectrometer using the same LC program as described previously.

Quantification was achieved by generating calibration curves using pure standards and analyzed on the LC-MS/MS system three times. The slope was then calculated using the ratio between the peak areas of the compound and its standard (1-AG-d5 for MAGs, AEA-d4 for anandamide or N-arachidonoyl-ethanolamine (AEA) and C17:1-LPA for the various LPA species). The mass transition and retention times of each compound are provided in Table 1.

### Statistical analyses

Statistical analyses (one-way ANOVA with Dunnett’s multiple comparisons test) were done using the GraphPad Prism 7 software. *P* values < .05 were considered significant.

### Results

#### Expression of DAG lipase α and β

Previous attempts to stimulate 2-AG production by human immune cells were somewhat disappointing, leading to eCB levels below those required to activate the CB2 receptor. This raised the possibility that key biosynthetic enzymes, notably the DAG lipases, might be absent or poorly expressed. In the first series of experiments, the expression of DAG lipases was thus assessed by immunoblot in human leukocytes. A sharp difference between the expression patterns of DAG lipase α and β were found (Figure 1). DAG lipase α was detected in the hypothalamus samples, which were included as a positive control. In contrast, DAG lipase α was usually absent from our leukocyte preparations, with the exception of some eosinophil and AM samples in which we detected a weak signal. As for DAG lipase β, it was found in eosinophils, monocytes, and AMs while being almost absent in the hypothalamus samples, neutrophils, and lymphocytes. These data support the concept that the DAG lipase pathway might not be involved in the biosynthesis of 2-AG by all leukocyte types, particularly in neutrophils and lymphocytes.

#### 2-AG biosynthesis by leukocytes stimulated with PLC-activating agonists

Considering the obtained DAG lipases expression patterns (Figure 1), we postulated that human leukocytes, especially eosinophils, monocytes, and AMs, might be capable of generating 2-AG following PLC activation. Leukocytes hydrolyze 2-AG and its metabolites within seconds to minutes.

| Compound | ISTD | Q1 → Q3 | Retention time (minutes) |
|----------|------|---------|--------------------------|
| 1-PG     | 1-AG-d5 | 331.10 → 313.25 | 12.64 |
| 2-PG     | 1-AG-d5 | 331.10 → 313.25 | 12.44 |
| 2-OG     | 1-AG-d5 | 357.20 → 265.40 | 12.85 |
| 1-OG     | 1-AG-d5 | 357.20 → 265.40 | 12.65 |
| 1-LG     | 1-AG-d5 | 355.20 → 263.30 | 12.16 |
| 2-LG     | 1-AG-d5 | 355.20 → 263.30 | 11.98 |
| 1-AG     | 1-AG-d5 | 379.30 → 287.25 | 12.20 |
| 2-AG     | 1-AG-d5 | 379.30 → 287.25 | 12.07 |
| 2-AG-d4  | 1-AG-d5 | 387.30 → 82.10  | 12.01 |
| 1-EPG    | 1-AG-d5 | 377.10 → 285.25 | 11.19 |
| 2-EPG    | 1-AG-d5 | 377.10 → 285.25 | 11.06 |
| 1-DPG    | 1-AG-d5 | 405.20 → 313.30 | 12.52 |
| 2-DPG    | 1-AG-d5 | 405.20 → 313.30 | 12.37 |
| 1-DHG    | 1-AG-d5 | 403.20 → 311.20 | 12.04 |
| 2-DHG    | 1-AG-d5 | 403.20 → 311.20 | 11.94 |
| 1-AG-d5  | –    | 384.5 → 287.50 | 12.59 |
| AA       | AA-d5 | 303.20 → 259.30 | 13.22 |
| 1-AA-LPA | 17:1-LPA | 457.30 → 152.90 | 8.09 |
| 1-EPA-LPA | 17:1-LPA | 455.4 → 153.05 | 7.37 |
| 1-DHA-LPA | 17:1-LPA | 481.30 → 152.90 | 8.09 |
| 1-LA-LPA | 17:1-LPA | 433.3 → 153.15 | 8.04 |
| 1-OA-LPA | 17:1-LPA | 435.40 → 153.05 | 8.88 |
| 17:1-LPA | –    | 421.30 → 197.20 | 7.39 |
| LTB4     | LTB4-d4 | 335.30 → 195.25 | 7.47 |
| LTB5     | LTB5-d4 | 333.40 → 195.25 | 6.61 |
| LTB1-d4  | –    | 339.30 → 197.20 | 7.39 |
and the efficacy of 2-AG hydrolysis inhibitors at enhancing 2-AG half-life overtime has not been documented in human leukocytes. Thus, we first compared the effects of three 2-AG hydrolysis inhibitors with varying selectivity, namely MAFP, Palmostatin B, and JZL184, to determine which one would most effectively increase 2-AG half-life in neutrophil suspensions. We selected these three compounds because they consistently inhibited 2-AG hydrolysis in each leukocyte type compared to other compounds.12 In absence of inhibitor, ~90% of the added 2-AG in our neutrophil suspensions had disappeared after one minute (Figure 2A). JZL184, Palmostatin B, and MAFP all prolonged 2-AG half-life in neutrophils (Figure 2A). MAFP, at 1 µM, was the most efficient with ~75% of 2-AG remaining after 15 minutes. MAFP (100 nM) and Palmostatin B (10 µM) had comparable inhibitory effect on 2-AG hydrolysis with ~50% 2-AG remaining after 15 minutes. JZL184 (10 µM) was the least efficient with ~35% 2-AG remaining after 5 minutes and basically no 2-AG left at 10 minutes. Consequently, we selected MAFP to prevent 2-AG hydrolysis in our experimental model, in the hope of better characterizing the possible biosynthesis of 2-AG by human leukocytes. We next investigated whether leukocytes could generate 2-AG in response to two agonists previously documented to activate the PLC (and DAG lipase) pathway in leukocytes: the calcium ionophore A23187 or PAF.13,29 The stimulations were performed in the presence of MAFP, in order to document the impact of increasing 2-AG half-life and to correctly assess the biosynthetic capabilities of leukocytes. PAF did not significantly stimulate a robust biosynthesis of 2-AG in any cell type. A23187 stimulated the biosynthesis of 2-AG in monocytes and AMs, although the obtained levels were modest (Figure 2B-E). These data indicate that although A23187 has a stimulatory effect on 2-AG biosynthesis, human leukocytes do not generate large amounts of 2-AG via the classic biosynthetic pathway involving the sequential actions of PLC and DAG lipases.

3.3 | Arachidonic acid stimulates a robust biosynthesis of 2-AG in human neutrophils

We next treated human neutrophils with a larger panel of inflammatory effectors, notably AA (the product of 2-AG hydrolysis), TLR agonists and the recognized neutrophil activator fMLP. In absence of MAFP, human neutrophils did not synthesize detectable 2-AG levels (detection limit of 25 fmol) in response to fMLP, LPS, or the TLR 7/8 agonist R848 while they synthesized modest amounts of 2-AG in response to AA (Figure 3A). When these experiments were repeated in presence of MAFP, 2-AG was synthesized in very limited amounts upon stimulation with G protein-coupled receptor (GPCR) or TLR agonists. In contrast, MAFP-treated neutrophils synthesized large amounts of 2-AG in response to AA (Figure 3B). The effect of AA was concentration-dependent (Figure 3C).
FIGURE 2  Biosynthesis of 2-AG induced by PAF or A23187. A, Pre-warmed neutrophil suspensions (37°C, 5 x 10⁶ cells/mL) in HBSS containing 1.6 mM CaCl₂ were treated with DMSO, MAFP, Palmostatin B (Palm B), or JZL184 at the indicated concentration for 5 minutes then incubated with 1 µM 2-AG for up to 15 minutes. B-E, Pre-warmed leukocyte suspensions (37°C, 5 x 10⁶ cells/mL) in HBSS containing 1.6 mM CaCl₂ were treated with 1 µM MAFP for 5 minutes then treated with either DMSO, PAF (1 µM), or A23187 (100 nM) for 15 minutes. A-E, Incubations were stopped by adding 0.5 volume of cold (−20°C) MeOH containing 2 ng 1-AG-d₅ as internal standard. Samples were processed and analyzed for 2-AG levels as described in Materials and methods. Data are the mean (±SEM) of 3-4 independent experiments.

FIGURE 3  Impact of AA on the biosynthesis of 2-AG by human neutrophils. Pre-warmed human neutrophil suspensions (37°C, 5 x 10⁶ cells/mL) in HBSS containing 1.6 mM CaCl₂ were utilized for all experiments. Cells were incubated with (A) DMSO, (B-E) 1 µM MAFP or (F) JZL184, Palmostatin B or MAFP for 5 minutes before the addition of the stimuli. A,B, Cells were stimulated with fMLP (1 µM), R848 (10 µM), PAF (1 µM), A23187 (100 nM), or AA (10 µM) for 15 minutes. C, Cells were stimulated with 10 µM AA for the indicated times. D, Cells were stimulated with 10 µM of AA for 15 minutes. F, Cells were stimulated with 10 µM AA during 15 minutes. A-F, Incubations were stopped by the addition of 0.5 mL of cold (−20°C) MeOH containing 2 ng 1-AG-d₅ as internal standard. Samples then were processed and analyzed for 2-AG and other MAGs by LC-MS/MS as described in Material and methods. Data are the mean (±SEM) of three or four independent experiments.
and kinetic experiments unraveled that 2-AG biosynthesis/accumulation was maximal at 15 minutes, after AA had disappeared from the cell preparations (Figure 3D). AA selectively induced the biosynthesis of 2-AG, as no other MAG was detected (Figure 3E). We next performed an additional set of experiments in which we compared the efficacy of JZL184, Palmostatin B, and MAFP to unmask the AA-induced 2-AG biosynthesis. In these experiments, AA stimulated the biosynthesis of 2-AG in presence of the three inhibitors (Figure 3F). However, MAFP was the most efficient inhibitor at unmasking the AA-induced 2-AG synthesis. Importantly, these data perfectly fitted with the ability of the three inhibitors to prevent 2-AG hydrolysis in neutrophils (Figure 2A), further consolidating that MAFP was the inhibitor of choice to better assess the AA-induced biosynthesis of 2-AG by neutrophils. As for the eCB AEA, it was not detected in large amounts, from being below detection limit in untreated neutrophils to 0.28 ± 0.05 pmol/million cells in AA-stimulated neutrophils (n = 3), even though MAFP would have blocked its hydrolysis by inhibiting the fatty acid amide hydrolase (FAAH). This, combined with the results presented in Figure 4, prompted us to determine whether other fatty acids would stimulate the biosynthesis of their MAG counterparts. Human neutrophils were thus treated with various fatty acids for 15 minutes then processed for MAG quantification. Figure 5A shows that neutrophils metabolized UFAs into MAGs with varying efficacy, DHA being the most efficiently metabolized. Long chain fatty acids were better transformed than shorter chains fatty acids (C22>C20>C18). In addition, for a given fatty acid chain length, an increased in the number of double bonds led to a better efficacy (DHA vs DPA; EPA vs AA; LA vs OA). Of note, the 16-carbon saturated fatty acid palmitic acid did not induce the biosynthesis of palmitoyl-glycerol.

3.4 | Inhibition of the AA-induced 2-AG biosynthesis in human neutrophils by acylation inhibitors

Since 2-AG biosynthesis was maximal after most of the AA was cleared from the incubation media (Figure 3D), we postulated that AA was acylated into cellular membranes then released from the cell membranes to yield a MAG. To verify this, experiments were performed in which human neutrophils were treated with the fatty acyl-CoA synthase inhibitor triascin C or the acyl-CoA transferase inhibitor thimerosal. In agreement with the working hypothesis, triascin C and thimerosal both inhibited the AA-induced 2-AG biosynthesis by ~90% (Figure 4A). To further explore this recycling pathway, another set of experiments was done in which neutrophils were stimulated with AA-d₈ (Figure 4C) instead of AA (Figure 4B). AA-d₈ led to an almost inexistent biosynthesis of 2-AG, which was replaced by an equivalent biosynthesis of 2-AG-d₈ (Figure 4B,C). Altogether, these data indicate that in neutrophils, some of the exogenously-added AA is acylated into phospholipids and then further processed into 2-AG.

3.5 | Biosynthesis of MAGs by UFAs

While 2-AG is the MAG binding to the CB₂ receptor with the highest affinity, other MAGs can activate DMSO-differentiated HL60 cells (a cell model of neutrophil), which express the CB₂ receptor, with equal or better potency and efficacy than AEA. This, combined with the results presented in Figure 4, prompted us to determine whether other fatty acids would stimulate the biosynthesis of their MAG counterparts. Human neutrophils were thus treated with various fatty acids for 15 minutes then processed for MAG quantification. Figure 5A shows that neutrophils metabolized UFAs into MAGs with varying efficacy, DHA being the most efficiently metabolized. Long chain fatty acids were better transformed than shorter chains fatty acids (C22>C20>C18). In addition, for a given fatty acid chain length, an increased in the number of double bonds led to a better efficacy (DHA vs DPA; EPA vs AA; LA vs OA). Of note, the 16-carbon saturated fatty acid palmitic acid did not induce the biosynthesis of palmitoyl-glycerol.

**FIGURE 4** Impact of reacylation inhibitors and AA-d₈ on the AA-induced 2-AG biosynthesis by neutrophils. A-C, Pre-warmed human neutrophil suspensions (37°C, 5 × 10⁶ cells/mL) in HBSS containing 1.6 mM CaCl₂ were pre-incubated with 1 μM MAFP for 5 minutes then stimulated with 10 μM AA or AA-d₈ for 15 minutes. A, Triascin C or thimerosal were added 2 minutes before the addition of AA. A-C, Incubations were stopped by the addition of 0.5 mL of cold (−20°C) MeOH containing 2 ng 1-AG-d₅ as internal standard. Samples were processed and analyzed for 2-AG and 2-AG-d₈ levels as described in Material and methods. The data are the mean (±SEM) of at least three independent experiments.
Eosinophils and monocytes, but not AMs nor lymphocytes, also metabolize UFAs into MAGs

In the next series of experiments, the hypothesis that other leukocytes could also biosynthesize 2-AG and other MAGs in response to fatty acids was tested. UFAs also induced the biosynthesis of their MAG derivatives in human eosinophils and human monocytes (Figure 5B,C) but to a lesser extent than neutrophils. In contrast, human lymphocytes and human AMs only produced trace amounts of MAGs in response to UFAs (Figure 5D,E). Finally, AA, LA, or OA did not induce the synthesis of their respective MAGs by platelets or erythrocytes (data not shown). Again, palmitoyl-glycerol was not detected in any cell type treated with palmitic acid, suggesting that saturated fatty acids do not undergo this MAG biosynthetic pathway. Altogether, these data raise the possibility that the UFA-mediated synthesis of 2-AG and other MAGs is restricted to leukocytes from the myeloid lineage.

Possible mechanism of UFAs-induced biosynthesis of MAGs in leukocytes

The poor stimulatory effect of GPCR agonists such as PAF and fMLP on the biosynthesis of 2-AG by leukocytes, and the striking effect of triasacin C and thimerosal on the AA-induced 2-AG biosynthesis in neutrophils which we found here not to express DAG lipases compared to other leukocytes, suggested that AA stimulated 2-AG biosynthesis independently of the PLC/DAG lipase pathway (Figures 1-3). In further support to this hypothesis, the PLC inhibitor U73122 and the DAG lipase inhibitors KT109 and KT172, at 10 µM, did not inhibit the AA-induced 2-AG biosynthesis in neutrophils (data not shown), supporting the concept that AA induces the biosynthesis of 2-AG in a PLC/DAG lipase-independent manner and very likely via its remodeling into phospholipids.

Apart from the PLC/DAG lipase pathway, the biosynthesis of 2-AG (and possibly other MAGs) was documented to occur via other pathways: a PLD-mediated cleavage of phosphatidic leading to DAG, which is then converted into 2-AG by DAG lipases; or by the conversion of LPA into 2-AG by a phosphatase. Given the barely detectable expression of DAG lipases in neutrophils (Figure 1), the lack of effect of DAG lipase inhibitors, and the inability of fMLP to stimulate 2-AG biosynthesis in neutrophils (fMLP being the most recognized GPCR activating PLD in neutrophils), we concluded that the PLD/DAG lipase pathway was not involved in the AA-induced 2-AG biosynthesis. The next experiments were thus focused on testing whether AA stimulated 2-AG biosynthesis via a LPA intermediate. Human neutrophils were thus treated with AA for different times and the levels of AA-LPA were assessed. AA stimulated the biosynthesis of AA-LPA (Figure 6). While 2-AG levels are maximal at 15 minutes (Figure 3C), AA-LPA levels peaked at 5 minutes then declined overtime (Figure 6A). Other fatty acids also induced the biosynthesis of the corresponding LPA species in human neutrophils (Figure 6B). The biosyntheses of the different LPA species followed the same trend as their MAGs counterparts (Figure 5A), with longer fatty acid chains leading to more important
biosynthesis of the corresponding LPAs. Moreover, the treatment of neutrophils with AA and AA-d₈ led to the biosynthesis of AA-LPA and AA-d₈-LPA, respectively, indicating that the newly biosynthesized LPA originated from the added fatty acid (Figure 6C). Importantly, thimerosal and triascin C both inhibited the AA-, the EPA-, and the DHA-stimulated LPA biosyntheses to the same extent than their MAGs (data not shown). Finally, several phosphatase inhibitors were tested to evaluate whether they could inhibit the putative dephosphorylation of LPA into 2-AG. Sodium orthovanadate, AlF₃, propranolol, bromoenol lactone and XY-14, which all inhibit LPA dephosphorylation failed to prevent AA-induced 2-AG synthesis (data not shown).

3.8 | AA-induced 2-AG biosynthesis is decreased by pro-inflammatory effectors

As mentioned above, our attempts at stimulating the biosynthesis of 2-AG by agonists stimulating the PLC/DAG lipase pathway did not lead to a robust biosynthesis of 2-AG and other MAGs, as opposed to the UFA-stimulated 2-AG (and other MAGs) biosynthesis (Figures 3 and 4). We next examined whether these two pathways interacted with each other by treating human neutrophils with AA in combination with PAF, fMLP, or LTB₄. All three GPCR agonists significantly decreased the AA-induced 2-AG biosynthesis (Figure 7). PAF was the most potent with ~45% inhibition, followed by fMLP (~35%) and LTB₄ (~20%). Interestingly, PAF had a comparable effect on the EPA-stimulated biosynthesis of 2-EPG, while having little no effect on the OA-induced 2-OG biosynthesis (Figure 7B). PAF is a recognized neutrophil activator. As such, it induces an important mobilization of Ca²⁺ ions from internal stores. As a result, this activates enzymes capable of metabolizing fatty acids, notably the 5-lipoxygenase. We thus postulated that the decreased 2-AG biosynthesis in presence of PAF/AA (vs AA alone) was be the consequence of a greater metabolism of AA and EPA by the 5-lipoxygenase in neutrophils. In agreement with the latter, the combination of PAF with either AA or EPA led to a 6-fold increase in LTB₄ and a 26-fold increase in LTB₅ biosynthesis (Figure 7C), indicating that the inhibitory effect of PAF displays on the AA-induced 2-AG biosynthesis and the EPA-induced 2-EPG biosynthesis is likely the consequence of increased metabolism of exogenously-added AA and EPA into 5-lipoxygenase metabolites, thereby diminishing the amount of UFA being acylated and undergoing the MAG biosynthetic pathway we have unmasked. This also indicates that during the course of acute inflammation in which polyunsaturated fatty acids are present simultaneously with pro-inflammatory effectors such as PAF, MAG biosynthesis might be diminished by a pro-inflammatory entourage to the advantage of other pro-inflammatory effectors such as leukotrienes and prostaglandins.

3.9 | Levels of MAGs in human plasma

The levels of 2-AG in human plasma have been documented in humans, varying from 1 to 20 nM. However, the levels of the other MAGs documented herein have never been documented completely, although some studies reported the presence of 2-OG, 2-EPG, and 2-DHG. Herein, we quantitated the levels of MAGs derived from PA, OA, LA, AA, EPA, DPA, and DHA in the plasma from fasting healthy volunteers (five women, five men), matched for age (29 ± 12 and 30 ± 12, respectively; mean ± SD) and body mass index (21,89 ± 1,22 vs 22,43 ± 1,41; mean ± SD). The MAGs we investigated herein were all detected in the plasma (Figure 7D). 2-OG, 2-PG, and 2-LG were the most abundant. There
was no statistically significant difference between men and women for any of the MAGs we measured.

4 DISCUSSION

Endocannabinoids are natural anti-inflammatory mediators, at least in mice, and the pharmacological blockade of their degradation has great potential to treat inflammatory diseases, notably by preventing leukocytes of hydrolyzing them. However, whether human leukocytes are an important source of eCBs has been elusive. In this paper, we show that: (a) DAG lipase expression differs from a leukocyte subset to the other with DAG lipase β being more expressed than DAG lipase α, (b) leukocytes do not synthesize large amount of 2-AG via the PLC/DAG lipase pathway, (c) The non-selective 2-AG hydrolysis inhibitors MAFP and palmostatin B increase the half-life of 2-AG for a longer duration than JZL184 in neutrophils, (d) circulating myeloid leukocytes biosynthesized 2-AG and other MAGs in response to UFAs, (e) the UFA-mediated MAG synthesis is not inhibited by DAG lipase inhibitors but is inhibited by acyl-CoA synthase and an acyl-transferase inhibitors, (f) the UFA-mediated biosynthesis of MAGs is preceded by the buildup of their LPA congeners, (g) pro-inflammatory effectors activating PLC inhibit the AA-induced 2-AG biosynthesis, and (h) human plasma contains each of the investigated MAGs, some of which were never documented before.

The expression of the different DAG lipases by human leukocytes was ill defined. DAG lipase α was only found in trace amounts in leukocytes, notably eosinophils and AMs, in sharp contrast with human hypothalamus samples. This finding was expected, as DAG lipase α was described as the main 2-AG biosynthetic enzyme in mouse brain. As for DAG lipase β, it was found in larger levels in eosinophils, monocytes and AMs (Figure 1) vs low/undetectable levels in neutrophils and lymphocytes, supporting that these leukocytes do not synthesize 2-AG in a DAG lipase-dependent fashion, as underscored in Figure 2. The latter finding is in line with data showing the importance of this enzyme in 2-AG synthesis by mouse brain. As for DAG lipase β, it was found in larger levels in eosinophils, monocytes and AMs (Figure 1) vs low/undetectable levels in neutrophils and lymphocytes, supporting that these leukocytes do not synthesize 2-AG in a DAG lipase-dependent fashion, as underscored in Figure 2. The latter finding is in line with data showing the importance of this enzyme in 2-AG synthesis by mouse brain. As for DAG lipase β, it was found in larger levels in eosinophils, monocytes and AMs (Figure 1) vs low/undetectable levels in neutrophils and lymphocytes, supporting that these leukocytes do not synthesize 2-AG in a DAG lipase-dependent fashion, as underscored in Figure 2. The latter finding is in line with data showing the importance of this enzyme in 2-AG synthesis by mouse brain. As for DAG lipase β, it was found in larger levels in eosinophils, monocytes and AMs (Figure 1) vs low/undetectable levels in neutrophils and lymphocytes, supporting that these leukocytes do not synthesize 2-AG in a DAG lipase-dependent fashion, as underscored in Figure 2. The latter finding is in line with data showing the importance of this enzyme in 2-AG synthesis by mouse brain.

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we would have expected similar findings in eosinophils, which instead produced less than 0.1 pmol/10^6 cells in response to PLC activation (Figure 2C). Given that GPCR agonists inhibit 2-AG synthesis by neutrophils (Figure 7), GPCR activation was bound to fail to induce 2-AG (and other MAG) biosynthesis in leukocytes. We did not detect large amounts of DAG lipase β in neutrophils nor lymphocytes compared to eosinophils, supporting that these leukocytes do not synthesize 2-AG in a DAG lipase-dependent fashion, as underscored in Figure 6. Combined with the inhibitory effects of PAF, fMLP, and LTβ on the AA-induced 2-AG biosynthesis, we conclude that the DAG lipase pathway does not mediate a robust biosynthesis of 2-AG by human inflammatory cells.

Our data also underscore the importance of hydrolase inhibition when studying eCB biosynthesis in leukocytes. The data presented herein, combined with our previous work, show that human leukocytes are experts at hydrolyzing 2-AG.\(^8,9,12\) Indeed, in absence of hydrolase inhibitors, we did not observe detectable amounts of 2-AG in stimulated neutrophils with the exception of AA, which led to a minimal 2-AG biosynthesis (Figure 2). The use of MAFP unraveled that AA induces a robust biosynthesis of 2-AG by neutrophils. These findings might provide an explanation for the low amounts of 2-AG produced by human immune cells in previous studies, in which 2-AG hydrolysis was not prevented.\(^13,15,17\) However, we show that most leukocytes do not biosynthesize substantial levels of 2-AG in response to agonists recognized to activate the PLC pathway, either in the absence or in the presence of MAFP. One study showed that human lung macrophages stimulated with LPS synthesize 2-AG in the presence of a MAG lipase inhibitor but this increase of approximately 40% is modest compared to the impact MAFP had in our biosynthetic route involving UFAs.\(^14\)

Following the structure elucidation of prostaglandins and leukotrienes, AA has been typecast as a villain. The stimulatory effect it has on 2-AG biosynthesis by leukocytes might thus be surprising and counter-intuitive. However, the data provided herein indicate that the biosynthetic route involved in the AA-induced 2-AG biosynthesis is clearly different from the classical GPCR-PLC-PLA\(_2\) pathway involved in eicosanoid biosynthesis. Indeed, the use of the pharmacological inhibitors triascin C and thimerosal indicate that AA is transformed into AA-CoA, then incorporated either into a glycerol-moiety or into lysophospholipids, then further metabolized into 2-AG (Figure 4). This biosynthetic route, while incomplete, is also supported by the data obtained with AA-d\(_8\), the latter almost exclusively stimulating the synthesis of 2-AG-d\(_8\) (Figure 4C). Thimerosal is known to effectively block AA acylation in human neutrophils by inhibiting two acyltransferases, namely membrane bound O-acyl transferase 5 (MBOAT5) and MBOAT7.\(^33\) Given the sharp inhibitory effect of thimerosal on the AA-induced 2-AG biosynthesis, it is very likely that one, perhaps both of these enzymes are involved in this process.

The metabolism of AA into 2-AG does not involve the DAG lipase pathway, but might involve LPA as an intermediate (Figure 6). The dephosphorylation of AA-LPA to produce 2-AG was indeed described in rat brains,\(^22\) but its importance in 2-AG (and other MAG) biosynthesis in humans had not been investigated. In our experimental model, AA-LPA biosynthesis preceded that of 2-AG, with maximal levels being detected at 5 and 15 minutes, respectively (Figures 3 and 6). This suggests that a LPA buildup occurs first, which could be followed by its dephosphorylation into 2-AG overtime. However, we were unable to prevent the AA-induced 2-AG biosynthesis using a wide array of LPA phosphatase inhibitors. Furthermore, the addition of exogenous AA-LPA did not lead to the synthesis of 2-AG (data not shown). Altogether, the LPA data indicate the following possible outcomes: (a) the buildup of LPA preceding MAG biosynthesis is coincidental, (b) LPA dephosphorylation to MAG occurs via a phosphatase that is insensitive to the inhibitor used, and/or (c) LPA dephosphorylation occurs within the cells and exogenously-added LPA do not enter the cells and is not susceptible to dephosphorylation. Therefore, the involvement of LPA in this process requires further investigation. Moreover, the fact that the UFA-induced biosynthesis of MAGs and the corresponding LPA species occurs to a significantly higher extent when starting with polyunsaturated vs saturated fatty acids, that is, with fatty acids that are normally esterified to the 2-position of (lyso) phospholipids, clearly points to a biosynthetic mechanism that, via phospholipid remodeling, increases the levels of potential 2-acyl-(lyso)phospholipid precursors for MAGs.

The plasmatic levels of 2-AG we report here are in the low nM range, in line with previous studies.\(^27,44\) The MAGs from the ω-3 fatty acids EPA, DPA, and DHA were the least abundant among the compounds we detected. Diet is known to have a direct effect on the fatty acid content found in the circulation and therefore, it could influence the levels of MAGs as well.\(^47\) Given that we show here that circulating leukocytes isolated from the peripheral blood convert UFAs into MAGs, the dietary intake of fatty acids is probably a determinant of plasma eCB levels. Another possible determinant of 2-AG and other MAG levels in the blood might be their degradation by 2-AG hydrolases, which is also a reaction that occurs rapidly in the presence of leukocytes.\(^8,9,12\) However, we also show that the use of a lipase inhibitor (MAFP), drastically reduces this 2-AG (and other MAG) hydrolysis and allows for an impressive build-up in fatty acid-stimulated leukocytes. Therefore, the therapeutic use of hydrolase inhibitors would allow the eCB tone to be increased and for eCBs to have higher bioactive potential.

Altogether, our data show that human myeloid leukocytes biosynthesize significant amounts of 2-AG and other MAGs. This biosynthesis is preceded by the biosynthesis of a LPA intermediate, although the involvement of this intermediate in the biosynthesis of MAGs remains to be proven.
Our data also suggests that the development of MAG hydrolysis inhibitors might turn human myeloid leukocytes into anti-inflammatory effectors by increasing their ability to synthesize anti-inflammatory 2-AG (and other MAGs) and eventually activate the endocannabinoidome receptors, notably the anti-inflammatory CB2 receptor as well as other anti-inflammatory receptors for other MAGs.

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CONFLICT OF INTEREST
Authors declare they have no conflict of interest.

AUTHOR CONTRIBUTIONS
CT, ASA, ML, MRB, AV, VD, and NF designed research. CT, ASA, ÉD, ML, KY, TI, NO, LPB, and CM performed research. KY, NO, and TI contributed new reagents. CT, ASA, ÉD, and NF analyzed data. CT and NF wrote the original draft of the paper. All authors were involved in reviewing, editing, and revising the paper.

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