

Letter to the Editor

Mechanisms of human eosinophil migration induced by the combination of IL-5 and the endocannabinoid 2-arachidonoyl-glycerol

To the Editor:

How eosinophils migrate to the tissues in eosinophilic diseases is not completely defined but is recognized to involve chemokines and/or bioactive lipids, noteworthy prostaglandin D₂ and 5-oxo-eicosatetraenoate (5-KETE). In mice, eosinophils might play an important role in adipose tissue maintenance.¹ The chemoattractants recruiting eosinophils to the latter are unknown, but IL-5 might be involved.^{1,2} The endocannabinoid 2-arachidonoyl-glycerol (2-AG), found in the adipose tissue, activates eosinophils³; is recognized to activate the cannabinoid receptors CB₁ and CB₂; and can regulate leukocytes' functions through its metabolites.⁴ Herein, we investigated the mechanisms of the 2-AG-induced migration of human eosinophils. **Methods** are provided in this article's Online Repository at www.jacionline.org.

IL-3, IL-5, and GM-CSF but not IL-13 or IFN- γ robustly increased the 2-AG-induced eosinophil migration from ~3% to ~40% (Fig 1, A). IL-3, IL-5, and GM-CSF receptors share a common $\beta\gamma$ subunit that activates within seconds the Src-like lyn kinase.⁵ Consistent with this, the effect of IL-5 was dependent on concentration, immediate, and blocked by the Src-like inhibitor PP2 but not its negative control PP3 (see Fig E1, A-D, in this article's Online Repository at www.jacionline.org).

Importantly, IL-5 potentiated the effect of 2-AG but not those of 5-KETE and prostaglandin D₂ (Fig 1, B). All the following migration experiments were performed in the presence of 10 ng/mL IL-5 because 2-AG alone did not induce significant eosinophil chemotaxis. 2-AG induced a migration rate comparable to that induced by CCL11 (eotaxin-1), higher than that induced by arachidonic acid (AA), but lower than that induced by 5-KETE in IL-5-treated eosinophils (Fig 1, C). In contrast, the CB₁/CB₂ receptor agonist CP 55,940 and the selective CB₂ receptor agonist L-759,633 induced minimal eosinophil migrations; AA mimicked 2-AG only at 30 μ M (Fig 1, D). When tested at a similar concentration, the 2-AG position isomer 1-AG was as potent as 2-AG whereas the effects of the endocannabinoid (and CB₂ agonist) arachidonyl-ethanolamide (AEA) and of 2-AG ether (nonhydrolysable 2-AG) were minimal (Fig 1, E). Human eosinophils express the CB₂ receptor,^{3,4} and both CB₂ receptor antagonists tested in this study inhibited the effects of 2-AG but not those of 5-KETE (Fig 1, F). The contrasting effects of CB₂ agonists and antagonists suggested that additional mechanisms, apart from CB₂ receptor activation, were involved in the effects of 2-AG. Human eosinophils express the monoacyl-glycerol (MAG) lipase, which hydrolyzes 2-AG into AA (see Fig E2, A, in this article's Online Repository at www.jacionline.org). Consistent with this, 2-AG and AA, alone or in combination with platelet-activating factor (PAF), induced similar cysteinyl-leukotrienes and eoxin C₄ (EXC₄) biosynthesis (Fig 2, A and B). Concentration-response

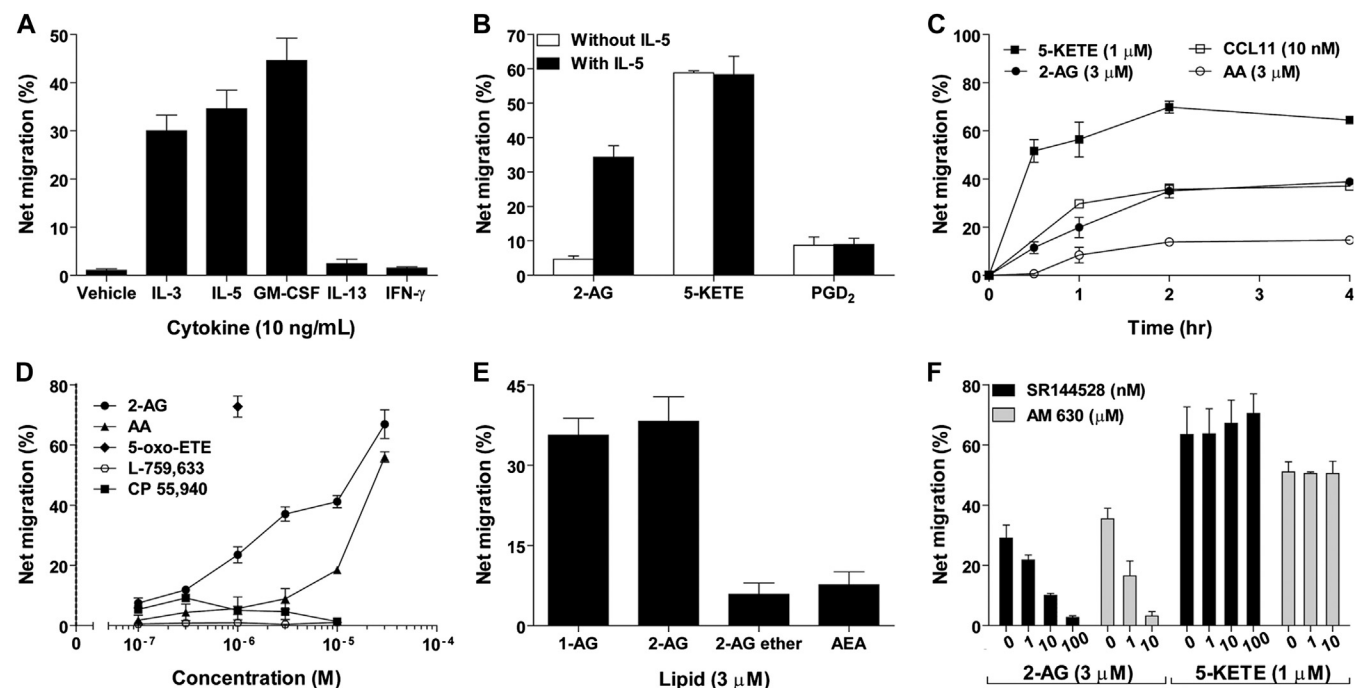


FIG 1. Impact of IL-5 and the CB₂ receptor on the 2-AG-induced eosinophil migration. **A-F**, IL-5 (or cytokine)-treated eosinophils migrated for 2 hours unless stated otherwise. Eosinophils were treated or not with IL-5 and then lipid-induced migration was performed (Fig 1, B). Migration of IL-5-treated eosinophils was performed during the indicated times (Fig 1, C). Migration of IL-5-treated eosinophils in response to different agonists (Fig 1, D and E). IL-5-treated eosinophils were incubated for 10 minutes with SR144528 or AM630 and then lipid-induced migration was performed (Fig 1, F).

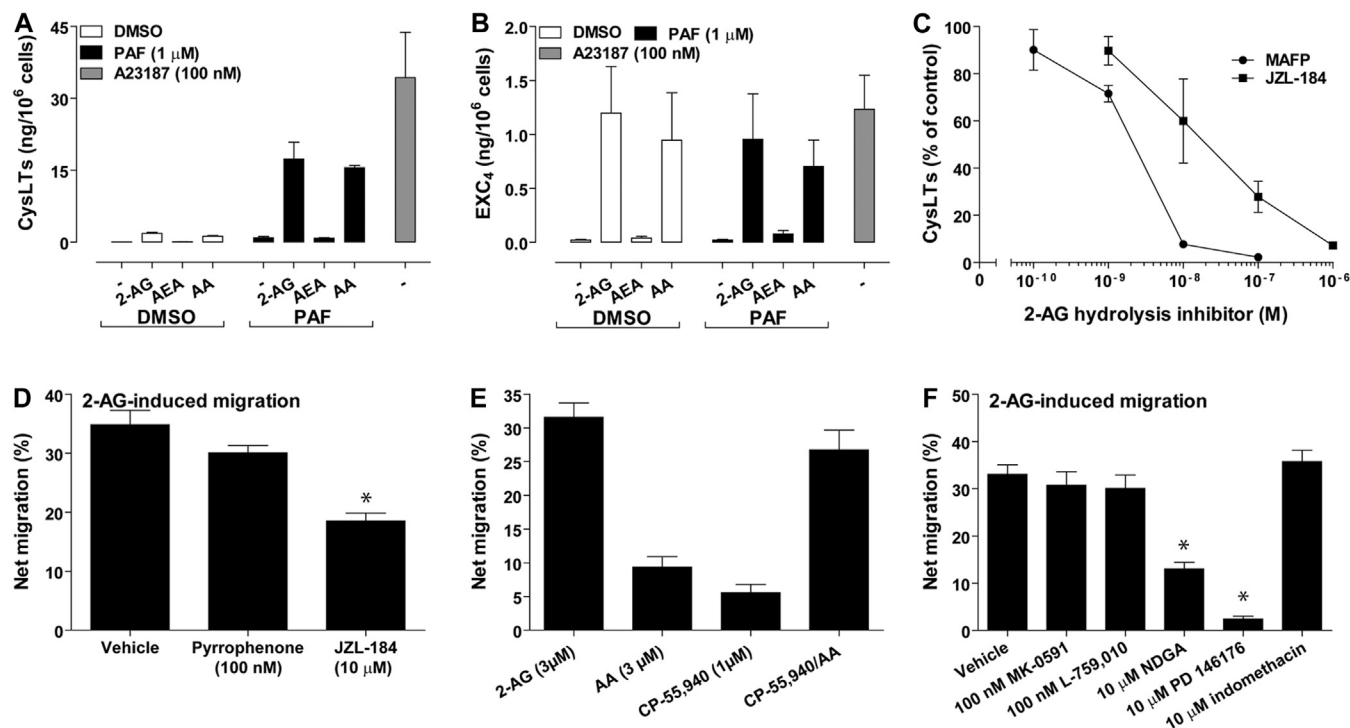


FIG 2. Metabolism of 2-AG by eosinophils and its role during migration. **A** and **B**, Eosinophils were stimulated with 1 μ M of 2-AG, AA, or AEA, alone or in combination with PAF. **C**, Eosinophils were stimulated for 15 minutes with 1 μ M of PAF and 2-AG in the presence or absence of the indicated inhibitors. **D** and **F**, Migration of IL-5-treated eosinophils in response to 3 μ M 2-AG in the presence of different inhibitors. **E**, Migration of IL-5-treated eosinophils in response to the indicated agonists. * $P < .001$. DMSO, Dimethyl sulfoxide.

experiments in PAF-stimulated eosinophils unraveled an optimal 2-AG concentration of 1 μ M (Fig E2, B). Noteworthy, the PAF-/2-AG-induced eicosanoid biosynthesis was blocked by the MAG lipase inhibitors methyl-arachidonoyl-fluorophosphonate and JZL-184 with IC₅₀ values of ~ 3 and ~ 30 nM, respectively (Fig 2, C). Consequently, we assessed whether 2-AG hydrolysis inhibition affected eosinophil migration. MAG lipase inhibition partially blocked ($\sim 40\%$) the 2-AG-induced eosinophil migration (Fig 2, D) while having no effect on the 5-KETE-induced migration (Fig E2, C). The inability of other CB₂ agonists to mimic 2-AG (Fig 1, D), and the inhibitory effect of CB₂ receptor antagonists (Fig 1, F) and JZL-184 (Fig 2, D), suggested that both CB₂ receptor activation and AA were involved in the regulation of eosinophil migration by 2-AG. In this respect, the combination of the CB₁/CB₂ receptor agonist CP 55,940 and AA led to migration rates similar to those of 2-AG (Fig 2, E). We next assessed whether eicosanoids biosynthetic enzymes were involved in the effects of 2-AG. While 5-lipoxygenase or cyclooxygenase inhibitors did not affect eosinophil migration, the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) and the 15-lipoxygenase inhibitor PD146176 significantly decreased the 2-AG- but not the 5-KETE-induced eosinophil migration (Fig 2, F, and Fig E2, D). Altogether these results indicate that the 15-lipoxygenase pathway is very likely involved in the regulation of eosinophil migration induced by 2-AG. However, we could not recapitulate the effect of 2-AG with 15(S)-hydroxyl-eicosatetraenoate, lipoxin A₄, EXC₄, or 15(S)-hydroxyl-eicosatetraenoate-*sn*1-glycerol, alone or combined with CP 55,940 (not shown).

We provide herein evidence that (1) 2-AG alone induces minimal eosinophil chemotaxis; (2) the 2-AG-induced migration is blocked by CB₂ receptor antagonists; (3) the 2-AG-induced eosinophil migration is enhanced by IL-5 in a Lyn-dependent manner; (4) 2-AG, but not AEA, is metabolized into eicosanoids by eosinophils; (5) the migration of eosinophils induced by 2-AG is sensitive to the MAG lipase inhibitor JZL-184; and (6) 15-lipoxygenase inhibitors block the effect of 2-AG on eosinophil migration. Noteworthy, the 2-AG-induced migration of eosinophils occurred at 2-AG concentrations that can be found *in vivo*.⁴ 2-AG was shown to induce a modest ($\sim 10\%$) and CB₂-dependent eosinophil migration in the presence of NDGA and in the absence of cytokine.³ Our data demonstrate that 2-AG alone induces minimal chemotaxis ($\sim 3\%$; Fig 1, A) but underscore that the 2-AG-induced eosinophil migration is complex and involves many mechanisms, notably cytokines, 2-AG hydrolysis, and 15-lipoxygenase metabolites. The inefficacy of natural (AEA) and synthetic CB₂ receptor agonists to mimic the effect of 2-AG indicates that the CB₂ receptor is required but not sufficient for the 2-AG-induced migration of eosinophils. Given the involvement of MAG lipase and 15-lipoxygenase in the 2-AG-induced migration, it is possible that CB₂ engagement on eosinophils activates these enzymes by yet to be documented mechanisms. IL-3, IL-5, and GM-CSF profoundly enhanced the 2-AG-induced eosinophil migration, and this was unique among the tested lipids (Fig 1, A and B). The Lyn inhibitor PP2 inhibited the effect of IL-5, which is in line with its rapid phosphorylation/activation in IL-5-treated eosinophils.⁶ Feltenmark et al⁷ showed

that IL-5 was the best stimulus for EXC₄ biosynthesis during short incubation periods, indicating that IL-5 quickly regulates 15-lipoxygenase. Given that the 15-lipoxygenase inhibitors NDGA and PD146176 decreased the 2-AG-induced migration, it is possible that Lyn directly/indirectly regulates 15-lipoxygenase activity by phosphorylation. Forsell et al⁸ and Brunnstrom et al⁹ recently documented the production of novel 2-AG-derived 15-lipoxygenase metabolites. Thus, the 15-lipoxygenase-derived lipidome of human eosinophils is likely more complex than initially thought. The use of a mixture of 2-AG- and AA-derived 15-lipoxygenase metabolites from eosinophils might have been a better strategy to recapitulate the effect of 2-AG on the migration of eosinophils than testing individual 15-lipoxygenase metabolites. In this regard, we are planning to analyze the 2-AG- and AA-derived lipidomes of eosinophils linked to 15-lipoxygenase and perform migration assays using this lipidome. Finally, we cannot exclude that the inhibitors we used in this study might inhibit off targets. In conclusion, this study confirms that the endocannabinoid 2-AG, in combination with IL-5, has the ability to activate and modulate eosinophil functional responses and, consequently, to play a significant role in the regulation of inflammatory processes such as asthma and possibly obesity.

*Marie-Chantal Larose, BSc**

*Caroline Turcotte, BSc**

François Chouinard, PhD

Claudine Ferland, BSc

Cyril Martin, MSc

Véronique Provost, PhD

*Michel Laviolette, MD**

*Nicolas Flamand, PhD**

From the Faculté de médecine, Département de médecine, Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec, Université Laval, Québec City, Québec, Canada G1V 4G5. E-mail: Nicolas.Flamand@criucpq.ulaval.ca.

*These authors contributed equally to this work.

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METHODS

Materials

15(S)-hydroxyl-eicosatetraenoate (HETE), 1-AG, 2-AG, 2-AG ether, 5-KETE, AA, AEA, CP 55,940, cysteinyl-leukotrienes enzyme immunoassay kit, EXC₄, EXC₄ EIA kit, His-tagged human MAG lipase, JZL-184, L-759,633, lipoxin A₄, methyl-arachidonyl-fluorophosphonate, NDGA, PAF, PD 146176, PD 98059, and pyrrophenone were purchased from Cayman Chemical (Ann Arbor, Mich). AM-630, SR144528, indomethacin, PP2, and PP3 were purchased from Tocris Bioscience (Ellisville, Mo). A23187 and dimethyl sulfoxide were obtained from Sigma-Aldrich (St Louis, Mo). The magnetic bead-conjugated anti-CD16 mAbs for the magnetic cell sorting were purchased from Miltenyi Biotec (Auburn, Calif). HBSS, FBS, and RPMI 1640 were obtained from Wisent Laboratories (St-Bruno, Quebec, Canada). The ECL detection kit was purchased from GE Healthcare (Mississauga, Ontario, Canada). The 5-LO inhibitor L 739,010 and the 5-LO-activating protein antagonist MK-0591 were kindly provided by Dr Denis Riendeau from Merck Frosst (Kirkland, Quebec, Canada). All cytokines and chemokines were purchased from Peprotech (Dollard des Ormeaux, Quebec, Canada). 15(S)-HETE-glycerol (G) was synthesized by Cayman Chemical and represents a mixture of 15(S)-HETE-*sn*1-G (81.6%) and 15(S)-HETE-*sn*2-G (14.6%).

Volunteers and isolation of eosinophils

The study was approved by the Ethic Committee of the Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec. All volunteers signed an informed consent form for the study. Human eosinophils were purified from the venous blood of rhinitic volunteers as described earlier.^{E1} The purity of the resulting eosinophil suspensions was always 98.5% or more and the viability was 98% or more, as assessed by Diff Quik staining and trypan blue exclusion, respectively.

Migration assay

Migration assays were performed with 3 μ m pore inserts (BD Bioscience, Mississauga, Ontario, Canada) as recommended by the manufacturer. In brief, 700 μ L of prewarmed (37°C) RPMI containing 10% FBS, the agonist of interest, was put in the lower chambers. Then, 200 μ L of prewarmed eosinophil suspensions (37°C, 2.5×10^6 cells/mL) in RPMI containing

10% FBS was added in the upper chamber of the migration apparatus. Eosinophils were allowed to migrate for 2 hours at 37°C unless stated otherwise (see Figures). The upper chambers were then removed, and migrated cells in the lower chambers were enumerated by using the Scepter 2 automated cell counter (EMD Millipore, Billerica, Mass). In experiments in which enzyme inhibitors or receptor antagonists were used, compounds were added to both the eosinophil suspensions and the lower chamber 5 minutes before the migration assay. Experiments were performed in the presence of 10 ng/mL of IL-5, which was added to eosinophils 10 minutes before the beginning of the migration assays (unless stated otherwise). Net migrations were calculated as follows: (stimulus-induced migrated cells – unstimulated cells)/total cells used in the assay. When a cytokine was present, net migration was calculated as follows: (cytokine-treated-stimulus-induced migrated cells) – (cytokine-treated cells). The eosinophil migration observed in the presence of 10 ng/mL of IL-5 alone typically represented approximately 10% of all cells.

Cell stimulations and analyses of cysteinyl-leukotrienes and EXC₄ biosynthesis

Prewarmed (37°C), freshly isolated human eosinophil suspensions (10^6 cells/mL) in HBSS containing 1 mM of CaCl₂ were stimulated with vehicle (dimethyl sulfoxide) 1-AG, 2-AG, AEA, AA, A23187 alone or in combination with 1 μ M of PAF for 15 minutes. Incubations were stopped by the addition of 1 volume of cold (4°C) incubation buffer, then rapidly centrifuged (4°C; 700g). Supernatants were harvested for the analysis of lipid mediator biosynthesis by ELISA according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were done using mixed-effects ANOVA, followed by the Tukey a posteriori test and were considered significant when *P* values were <.05.

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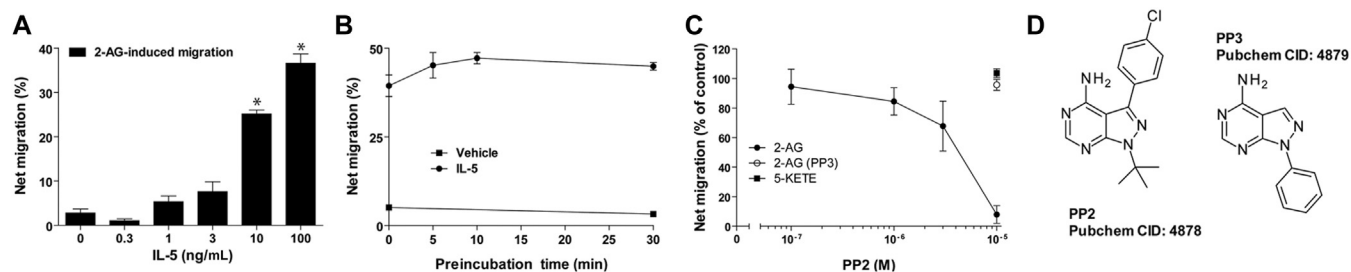


FIG E1. Impact of IL-5 and its signaling on the 2-AG-induced eosinophil migration. **A**, Eosinophils were treated for 10 minutes with IL-5 and then migration was performed for 2 hours. **B**, Eosinophils were treated with IL-5 for the indicated times and then migration was induced by 2-AG for 2 hours. **C**, Eosinophils were treated for 10 minutes with IL-5 (10 ng/mL) and then migration was induced with 2-AG or 5-KETE for 2 hours. PP2 or PP3 was added 10 minutes before the migration. **D**, Chemical structures of PP2 and PP3. * $P < .0001$.

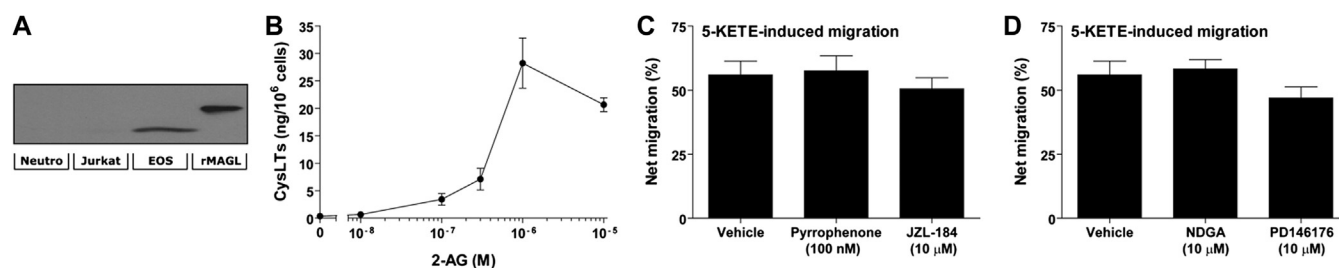


FIG E2. Involvement of metabolism for the 2-AG-mediated eosinophil migration. **A**, Immunoblot of MAG lipase with cell lysates from neutrophils, Jurkat T cells, eosinophils, or human recombinant His-tagged MAG lipase. **B**, PAF-stimulated eosinophils were incubated in the presence of the indicated concentration of 2-AG for 15 minutes. Cysteinyl-leukotriene biosynthesis was then assessed as described in Methods. **C**, Migration of IL-5-treated eosinophils in response to 1 μ M of 5-KETE in the presence of the group IVA phospholipase A_2 pyrrophenone or the MAG lipase inhibitor JZL-184. **D**, Migration of IL-5-treated eosinophils in response to 1 μ M of 5-KETE in the presence of compounds that inhibit the 15-lipoxygenase.