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2-Arachidonoylglycerol enhances platelet formation from human megakaryoblasts

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Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, anandamide; APC, allophycocyanin; CB_1, type-1 cannabinoid receptor; CB_2, type-2 cannabinoid receptor; CD, cluster of differentiation; DAGL, diacylglycerol lipase; eCB, endocannabinoid; FAAH, fatty acid amide hydrolase; FITC, fluorescein isothiocyanate; HEL, human erythroleukemia; MAGL, monoacylglycerol lipase; PE, phycoerythrin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Platelets modulate vascular system integrity, and their loss is critical in haematological pathologies and after chemotherapy. Therefore, identification of molecules enhancing platelet production would be useful to counteract thrombocytopenia. We have previously shown that 2-arachidonoylglycerol (2-AG) acts as a true agonist of platelets, as well as it commits erythroid precursors toward the megakaryocytic lineage. Against this background, we sought to further interrogate the role of 2-AG in megakaryocyte/platelet physiology by investigating terminal differentiation, and subsequent thrombopoiesis. To this end, we used MEG-01 cells, a human megakaryoblastic cell line able to produce *in vitro* platelet-like particles.

2-AG increased the number of cells showing ruffled surface and enhanced surface expression of specific megakaryocyte/platelet surface antigens, typical hallmarks of terminal megakaryocytic differentiation and platelet production. Changes in cytoskeleton modeling also occurred in differentiated megakaryocytes and blebbing platelets. 2-AG acted by binding to CB_1 and CB_2 receptors, because specific antagonists reverted its effect. Platelets were split off from megakaryocytes and were functional: they contained the platelet-specific surface markers CD61 and CD49, whose levels increased following stimulation with a natural agonist like collagen. Given the importance of 2-AG for driving megakaryopoiesis and thrombopoiesis, not surprisingly we found that its hydrolytic enzymes were tightly controlled by classical inducers of megakaryocyte differentiation.

In conclusion 2-AG, by triggering megakaryocyte maturation and platelet release, may have clinical efficacy to counteract thrombocytopenia-related diseases.

Introduction

Platelets are cytoplasmic fragments released from megakaryocytes within bone marrow, which play a crucial role in haemostasis, by controlling thrombus formation and vascular tone, as well as by releasing soluble molecules needed for leukocyte-leukocyte and leukocyte-endothelium interactions.[1,2] Platelets are also receiving growing attention in senescence, as platelet-derived growth factor has been reported to induce both senescence and cellular transformation in human fibroblasts;[3] given that senescence plays a key role during oncogenesis and inflammation,[3,4] the role of these anucleated cells is starting to be further investigated. Loss of platelets, due either to decreased survival in the periphery or to reduced production, can occur in several diseases, including thrombocytopenic purpura, acute leukemia, aplastic anaemia, multiple myeloma, HELLP (hemolysis, elevated liver enzymes, low platelets) and Scott (a rare bleeding disorder) syndromes.[5-12] Thrombocytopenia is also a detrimental side effect of chemo- and radio-therapies, often resulting in bleeding episodes, chemotherapy dose reductions or re-scheduling.[13-15] Up to date, the most effective approaches for treating the life-threatening complications of thrombocytopenia are platelet transfusion and supplementation with cytokines or thrombopoietic agents.[16-19] Nonetheless, their use is limited by side effects, costs and availability of blood donors. Therefore, more efficacious treatments to raise platelet count are needed.

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Endocannabinoids (eCBs) are lipid mediators, which exert their biological action by binding to type-1 (CB_1) and type-2 (CB_2) cannabinoid receptors, both localized to different extents in the central nervous system and peripheral tissues.[20,21] To date, the most biologically active eCBs, *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), together with other plant-derived CBs, are receiving growing attention due to their ability to regulate biological events, including cell death and differentiation.[22-25]

To date, both AEA and 2-AG can be listed among regulators of megakaryocyte/platelet functions, although they are endowed with distinct biological activity. AEA is a co-agonist of classical aggregating agents,[26] and extends platelet survival by inhibiting pro-apoptotic cascades.[27] Conversely, 2-AG is a true platelet agonist,[28] and is an active megakaryopoietic agent.[29] We have previously demonstrated that 2-AG (but not AEA) drives human erythroleukemia (HEL) cells toward megakaryocytic differentiation: physiological concentrations of 2-AG up-modulate the expression of megakaryocyte/platelet surface antigens, while down-modulating the expression of markers of erythroid phenotype.[29] Although 2-AG has been proven to play a role in haematopoietic lineage determination, we could not investigate its role on terminal steps of the differentiation program, because HEL cells are not competent for platelet shedding.

Against this background, here we went further to interrogate the role of 2-AG on megakaryopoiesis and thrombopoiesis, exploiting human megakaryocytic MEG-01 cells that are able to produce platelet-like particles, structurally and functionally similar to freshly isolated human platelets, and commonly used to study megakaryocyte biology.[30,31]

Results

2-AG promotes terminal megakaryocytic differentiation

To evaluate the effect of eCBs on megakaryopoiesis, proliferating MEG-01 cells were grown for 24 hours in the presence of increasing concentrations (0.1–10 μM) of 2-AG or AEA, as well as of 10 nM TPA, a known inducer of megakaryocytic differentiation.[31] Much alike TPA, 2-AG induced morphological changes associated with terminal differentiation: in the presence of 2-AG, cells increased adherence and showed irregular surface membrane with enhanced beaded extensions (**Figs. 1A** and **B**). The effect was dose-dependent, being already evident at 0.1 μM, and reaching a peak at 1 μM 2-AG (**Fig. 1A**). Conversely, AEA did not exert any effect (**Fig. 1A**).

To check whether the pro-differentiating effect of 2-AG could be mediated by its hydrolysis product arachidonic acid, we pretreated cells with JZL184, a selective inhibitor of the 2-AG-degrading enzyme MAGL.[32] Inhibition of MAGL led to a significant enhancement of differentiation, confirming the role of intact 2-AG itself (**Fig. 1A**). Cells were also pre-incubated with AM281 or SR144528 (specific antagonists of CB_1 and CB_2 receptors, respectively).[33] Both compounds reduced the number of differentiated cells (**Fig. 1A**), with AM281 being more effective. Neither AM281 nor SR144528 affected cell morphology, when incubated alone (data not shown). Remarkably, the effects of both antagonists were not additive (**Fig. 1A**), speaking in favor of the main engagement of CB_1 receptor subtype in 2-AG-dependent megakaryopoiesis. Additional proofs were obtained through incubation with ACEA (a CB_1 agonist)[34] and JWH015 (a CB_2 agonist),[35] which both increased the number of differentiated cells (**Fig. 1A**). Again, the effect was more evident in the presence of the CB_1 agonist. Altogether, these data suggest that CB_1 receptor, and to a lesser extent CB_2 receptor, mediate 2-AG-dependent megakaryopoiesis.

Cell cycle was also analyzed. Although less potently than TPA, 2-AG significantly increased the portion of MEG-01 cells in the G0/G1 phase, while decreasing the percentage of cells in the S phase (**Fig. 1C**), thus suggesting that 2-AG lowered the proliferation rate and increased differentiation.

2-AG induces cytoskeleton remodelling in differentiating megakaryocytes

Microtubule and actin filaments play a crucial role in proplatelet elongation and branching, respectively.[36,37] This process depends on elaboration of a dense and highly organized array of cytoskeletal polymers, thus we investigated 2-AG-triggered morphological shape changes by confocal microscopy, after F-actin and tubulin staining. On the basis of membrane morphology, cells were categorized in 3 sub-populations, representing different stages of maturation (see also **Fig. 1B**):[37] cells with smooth edges (undifferentiated and immature megakaryocytes), cells with membrane blebbing (rough cells) and cells extending cytoplasmic processes (spinous cells). The majority of control cells (77±10 %) displayed an undifferentiated phenotype, with a low percentage of rough (14±2 %) and spinous (9±2 %) cells (**Fig. 2**). TPA induced MEG-01 cells to become flat with the appearance of polylobulated nuclei and bleb-like structures in the plasma membrane (59±8 %) (**Fig. 2**) and cells exhibiting the spinous-phenotype represented 7±2 % of total cells. By contrast, cells cultured with 2-AG mostly assumed the spinous phenotype (52±8 %), characterized by marked cytoskeletal reorganization with formation of a circumferential band of microtubules just below the plasma membrane, loss of the centrosome, and accumulation of F-actin bundles in spiky filopodia-like protrusions (**Fig. 2**). The frequency of 2-AG-treated cells displaying membrane blebbing was below 10±3 %. Pre-incubation with AM281 partially reversed the effects of 2-AG, while SR144528 had only a small, yet not significant, effect (**Fig. 2**), suggesting that 2-AG triggered cytoskeletal re-organization mainly occurred via CB_1 receptor.

2-AG enhances expression of platelet-related markers

Next, we evaluated the expression of specific megakaryocyte/platelet surface antigens. Like TPA, 2-AG slightly increased (1.4 fold) the percentage of cells positive for α (CD41) and β (CD61) chains of fibrinogen receptor,[38] and doubled surface expression of the very late antigen α 2 chain (CD49) of the collagen receptor (**Fig. 3**).[39] Interestingly, the major effect was seen with expression of P-selectin (CD62P), a membrane glycoprotein exposed on megakaryocytes and activated platelets,[40] that increased 3 fold over untreated cells (**Fig. 3**) and that we have already shown to be

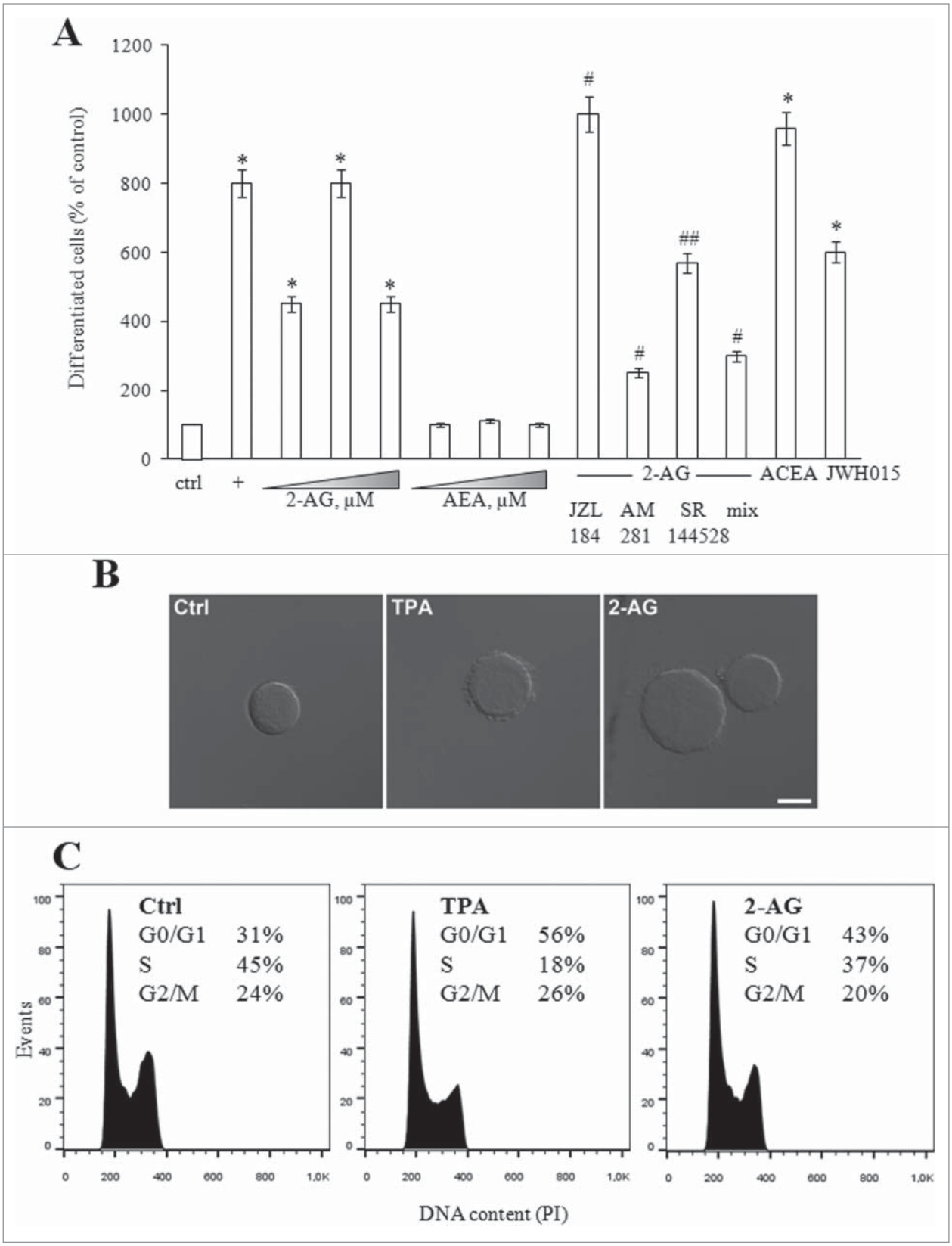

Figure 1. For figure legend, see page 3941.

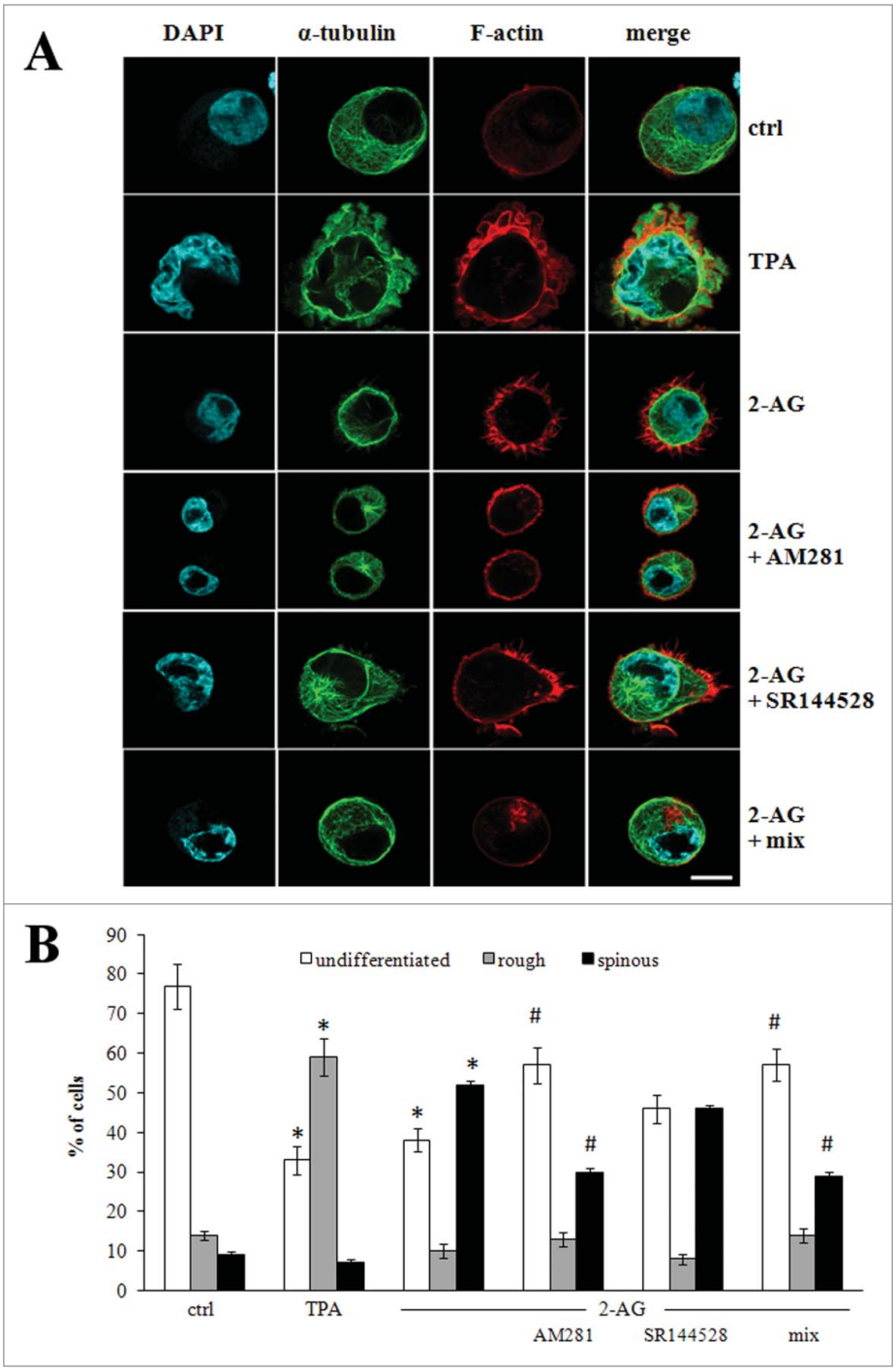

Figure 2. Cytoskeleton reorganization of MEG-01 cells. (**A**) Representative confocal microscopy images of cells left untreated (ctrl) or treated with 10 nM TPA or 1 μM 2-AG, the last being used alone or after pre-treatment with 0.1 μM AM281 or SR144528, incubated alone or in combination (mix). Cyan: nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI). Green: α-tubulin. Red: F-actin. The last panel on the right is merging of the 3 fluorescence signals. Scale bar, 10 μm. Note the smooth surface in undifferentiated cell, the ruffled surface (rough cell) in the presence of TPA, and the thin cytoplasmic projections (spinous cell) in the presence of 2-AG. (**B**) Percentage of undifferentiated (white bars), rough (gray bars) and spinous (black bars) cells visualized in MEG-01 cells treated as in (A). Data refer to n = 27 cells analyzed in 3 independent experiments. * $P < 0.001$ *vs* the corresponding bar in ctrl cells; # $P < 0.01$ *vs* the corresponding bar in 2-AG-treated cells.

sensitive to 2-AG.[41] Similarly to what we observed in endothelial cells,[41] the positive effect on P-selectin exposure depended on activation of both CB receptors, as shown by AM281 and SR144528 treatment (**Fig. 3**). Conversely, the 2 antagonists seemed to have no effect on 2-AG-triggered modulation of other surface markers (**Fig. 3**), suggesting a mechanism of action specific for each molecule.

2-AG enhances production of functional platelet-like particles

We went further insight by evaluating the effect of 2-AG on the early stages of thrombopoiesis. To this aim, we evaluated production and release of platelet-like particles from mature megakaryocytes incubated for 24 hours with 2-AG. MEG-01 cells stimulated either with TPA or 2-AG significantly increased (about 10 fold) the number of platelet-like particles split off from megakaryocytes into the culture medium (Table 1). The use of specific agonists (ACEA and JWH015) and antagonist (AM281 and SR144528) confirmed, once again, the involvement of both CB receptors (data not shown). Next, we investigated whether platelets released from MEG-01 cells upon 2-AG stimulus were active and responsive to classical activators.

Figure 1 (**See previous page**). Effect of eCBs on mekagaryocyte differentiation. (**A**) MEG-01 cells were left untreated (ctrl) or treated with increasing concentrations (0.1-10 μM) of 2-AG or AEA, or with 10 nM TPA (+), or with 0.1 μM ACEA (CB_1 agonist) or JWH015 (CB_2 agonist); cells were also incubated with 1 μM 2-AG, after pre-incubation with 0.1 μM JZL184, 0.1 μM AM281 or 0.1 μM SR144528 [the last 2 compounds used alone or in combination (mix)]. After 24 hours, the percentage of differentiated cells was counted by an inverted microscope. Values are reported as percentage of control, set to 100% (absolute value = 10.0 ± 0.1% of differentiated cells). * $P < 0.001$ *vs* ctrl; # $p < 0.01$ and ## $P < 0.05$ *vs* 2-AG-treated cells. (**B**) Differential interference contrast micrographs of cells left untreated (ctrl) or treated with 10 nM TPA or 1μM 2-AG for 24 hours. Scale bar, 10 μm. **(C)** Cell-cycle analysis performed on cells treated as in (B); percentages of cells in G0/G1, S and G2/M phases are given in each panel.

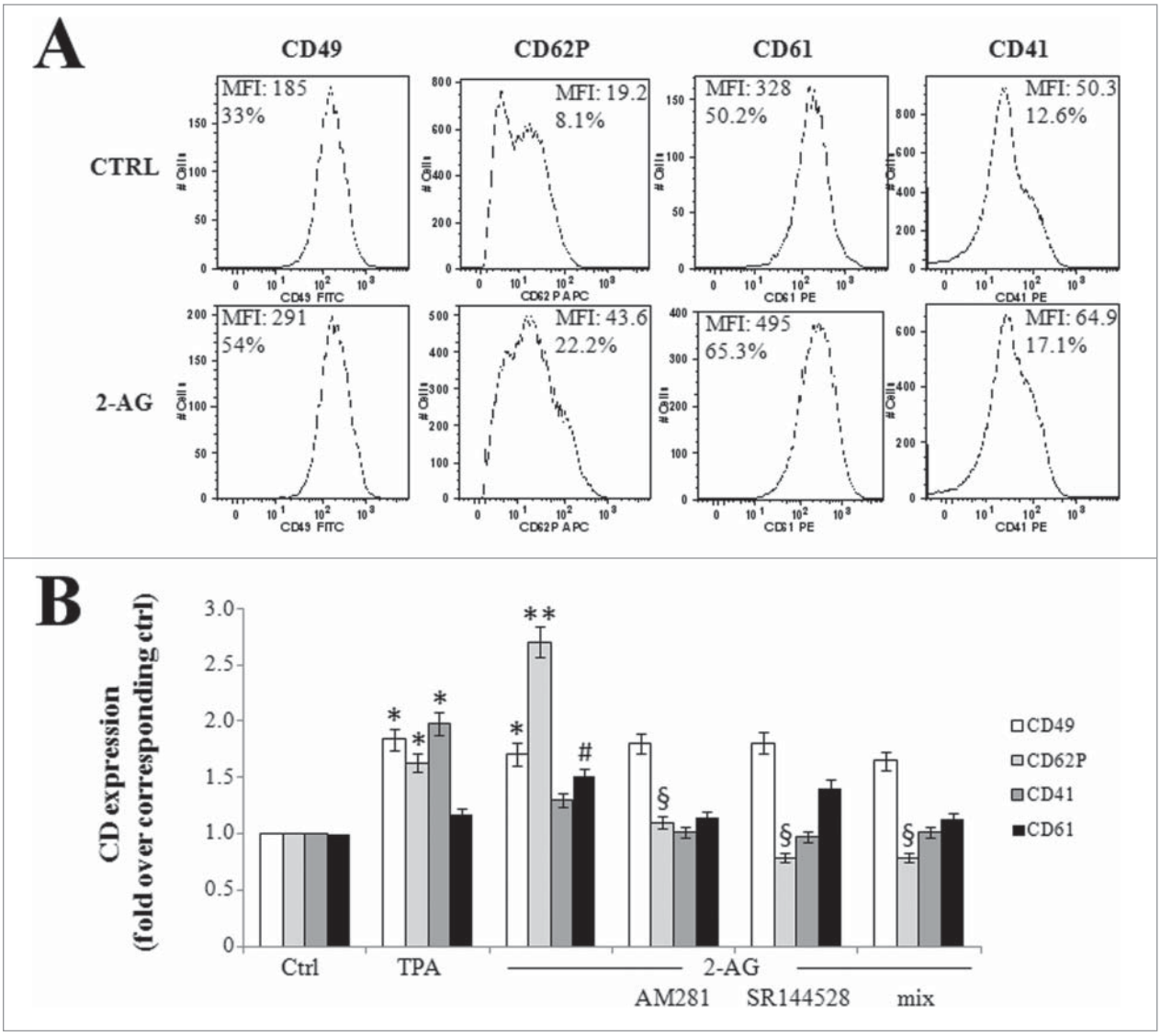

Figure 3. Expression of megakaryocyte/platelet surface antigens. (**A**) Cells were left untreated (ctrl) or treated with 1 μM 2-AG for 24 hours, before staining with specific CD antibodies. The antibodies used were: FITC-conjugated CD49, APC-conjugated CD62P, PE-conjugated CD61, and PE-conjugated CD41. Mean fluorescent intensity (MFI) and percentage of positive cells are given for each panel. (**B**) Cells were left untreated (ctrl) or treated with 10 nM TPA, or with 1 μM 2-AG, used alone or after pre-incubation with 0.1 μM AM281 or SR144528 [the last 2 drugs used alone or in combination (mix)]. After 24 hours, immunophenotyping was performed by FACS analysis. Values are reported as fold over control, set to 1. * $P < 0.01$, ** $P < 0.001$ and # $P < 0.001$ *vs* the corresponding bar in ctrl cells; § $P < 0.01$ *vs* the corresponding bar in 2-AG-treated cells.

Table 1. Immuno-phenotypic characteristics of MEG-01-derived platelets.

	Platelets from control cells	Platelets from TPA-treated cells	Platelets from 2-AG-treated cells	Platelets from TPA-treated cells plus collagen	Platelets from 2-AG-treated cells plus collagen
Parameter					
Number	1	10.0 ± 0.8*	9.2 ± 0.5*	-	-
Side scatter (SSC)[a]	1	1.8 ± 0.1**	1.8 ± 0.05**	$2.1 \pm 0.1^{\S}$	$2.2 \pm 0.1^{\S}$
Forward scatter (FSC)[a]	1	0.6 ± 0.05	0.8 ± 0.02	$0.7 \pm 0.05^{\S}$	$1.0 \pm 0.2^{\S}$
CD49[a]	1	$2.2 \pm 0.1^{\#}$	$3.3 \pm 0.1^{\#}$	$3.5 \pm 0.1^{\S}$	$4.0 \pm 0.1^{\S}$
CD61 [a]	1	$3.13 \pm 0.1^{\#}$	$2.1 \pm 0.1^{\#}$	$4.1 \pm 0.1^{\S}$	$2.7 \pm 0.1^{\S}$

Platelet activation was achieved by treatment with 10 μg/ml collagen and analyzed by FACS. Results are expressed as fold over untreated MEG-01-derived platelets, set to 1. [a] Mean fluorescent intensity. * $P < 0.001$ *vs* control; ** $P < 0.05$ *vs* control; # $P < 0.01$ *vs* control; § $P < 0.05$ *vs* the corresponding control in basal conditions.

To this end, isolated platelet-like particles were incubated with collagen, able to promote platelet activation and aggregation, and expression of specific surface markers was analyzed by FACS. Incidentally, platelets from both TPA- and 2-AG-treated MEG-01 cells seemed to be partially activated under basal conditions, maybe because of the prolonged exposure to the compounds. Nonetheless, collagen was able to increase granularity of platelet-like particles, as well as expression of fibrinogen and collagen receptors (**Table 1**), that were indicative of platelet activation because they play a key role during aggregation.[38-40]

Megakaryocytes self-regulate 2-AG metabolism during differentiation

Given the importance of 2-AG for driving megakaryopoiesis and thrombopoiesis, we investigated whether megakaryocyte maturation *per se* modulates 2-AG tone. To this aim, we analyzed the activity of the major enzymes involved in 2-AG metabolism. We found that, when compared to proliferating cells, TPA-differentiated cells showed a significantly increased activity of both MAGL (1.6 fold) and FAAH (2.8 fold), which are responsible for 2-AG hydrolysis; under the same experimental conditions, no changes in the activity of DAGL, the 2-AG-biosynthesizing enzyme, was observed (**Fig. 4A**). Differentiated megakaryocytes regulated 2-AG activity also by acting at the level of its receptors; indeed, reduced expression of both CB_1 and CB_2 receptors on plasma membrane occurred during differentiation (**Fig. 4B**), confirming the ability of megakaryocytes to self-regulate the endogenous tone of 2-AG. This finding suggested a specific regulation of CB receptors along differentiation, as immature megakaryoblasts showed the highest expression,[29] while platelets showed the lowest expression of these eCB-binding proteins.[42]

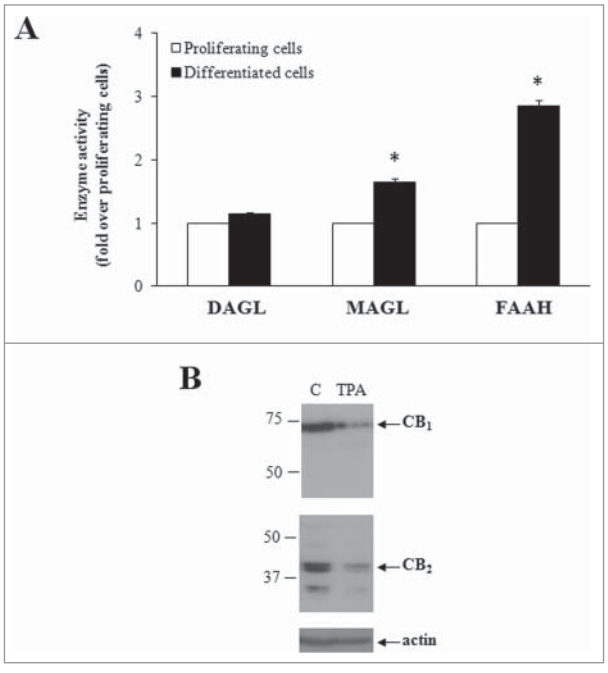

Figure 4. Modulation of the eCB system during differentiation. (**A**) Activity of 2-AG synthesising (DAGL) and degrading (MAGL, FAAH) enzymes, in proliferating (white bars) and TPA-differentiated (black bars) cells. Values are reported as fold over proliferating cells (absolute values: MAGL 69.1±9 .3; DAGL 8.1±0 .3; FAAH 28.5±1 .5 pmol/min/mg protein). * $P < 0.05$ *vs* proliferating cells. (**B**) Expression of CB receptors in proliferating (C) and TPA-differentiated cells by Western blotting. Molecular weights (kDa) are shown on the left-hand side. Gels are representative of 4 independent experiments.

Discussion

Megakaryocyte differentiation is composed of several consecutive stages, including formation of megakaryocytic progenitors, maturation of megakaryocytes, cell apoptosis, and production of platelets.[43-47] Cytological changes and modulation of surface receptor expression distinguish each step, making these features suitable markers for every stage of maturation.[48-50] In recent years, scientific progress has led to the production of functional platelets *in vitro*, in order to overcome problems related to platelet transfusion. However, large-scale production for clinical use is far from being achieved, because the number of platelets obtained *in vitro* is much lower than that *in vivo*, and manufactured platelets appear activated in the absence of agonists. Therefore, there is the need to define endogenously produced, regulatory factors essential for promoting platelet biogenesis (as well as to extend their lifespan), in order to develop novel strategies that generate functional platelets in adequate amounts.

By using an *in vitro* model, we provided evidence that 2-AG may be one of these modulators, as it is able to stimulate megakaryocyte maturation and enhance platelet production. The megakaryoblastic cell line MEG-01, established from the bone marrow of a patient with chronic myelogenous leukemia,[30,31] represents a useful model for the study of human megakaryopoiesis. MEG-01 cells treated with 2-AG underwent differentiation, characterized by loss of rounded morphology and gaining of a spinous phenotype with extended cytoplasmic protrusions, and by expression of surface markers specific for late stages of megakaryocyte maturation. These findings are in agreement with our previous data on the bi-potent erythroleukemia HEL cell line, showing that 2-AG was a specific signal for the megakaryocytic phenotype.[29]

2-AG not only stimulated MEG-01 maturation, but it also had a platelet production-enhancing effect. Indeed, platelet-like particles could be recovered from the culture medium of 2-AG-treated MEG-01 cells, with a yield that was 8–10 times higher than the spontaneous one. These particles expressed platelet-specific receptors on their plasma membrane, and appeared to be functional: in response to classical agonists, including collagen, they raised the surface levels of specific markers important for blood coagulation, changed shape and became activated.

The platelet-enhancing effect of 2-AG, together with the pro-survival effect of AEA on platelets[27] may have potential clinical significance in thrombocytopenias and myelosuppression triggered by radio- or chemo-therapy. The main advantage of 2-AG over classical thrombopoietic compounds resides in the fact that it is an endogenous lipid, whose tone can be finely-tuned by regulation of its synthesising and degrading enzymes. In this context,

it is noteworthy that eCBs can be counted among factors that govern haematopoietic stem cell biology. In bone marrow, CB receptors are highly expressed in haematopoietic stem cells,[51-53] and stromal cells release significant amounts of eCBs that modulate differentiation and migration, alone or in synergy with classical growth factors.[51,52] Therefore, it is tempting to speculate that pharmacological alteration of 2-AG tone in specialized niches of bone marrow would affect self-renewal and differentiation of haematopoietic cells, as well as lineage commitment.[54] In addition, owing to the specific effect of 2-AG on megakaryocyte precursors, it would be helpful to manage bone marrow failure and blood cell loss occurring in several pathological conditions. The flip side could be that 2-AG is a true platelet agonist, so its levels (as well as its acute *vs* chronic stimulation) should be kept tightly under control to avoid unwanted platelet activation, and to reduce some side-effects of 2-AG signaling at both central and peripheral levels. Indeed, activated platelets accumulate in the brain following injury and release factors that protect neurons from cell death, but the same anti-apoptotic cascade stimulated by activated platelets also provided chemo-resistance to several tumors.[55] Moreover, it has recently been reported that prostaglandin E2 glyceryl ester (a COX-2-derived 2-AG metabolite) exacerbated excitotoxic damage,[56] while epoxyeicosatrienoic acids (cytochrome P450 epoxygenase metabolites of arachidonic acid) protect from mitochondrial dysfunction and cell death.[57] The dual function of 2-AG should be taken into account, especially considering that platelet hyperactivation is related to aging, inflammation and cancer.[58,59] Age-related decline in cardiovascular function (and, therefore, susceptibility to thrombotic and inflammatory disorders) is often associated to increased levels of reactive oxygen species and oxidative stress;[60,61] since aged platelets display increased NADPH oxidase expression and hydrogen peroxide generation, platelet hyperactivity may contribute to this phenomenon.[62] Aging and activated platelets also modulate lineage-specific development, as well as the senescence program itself, thus promoting cell transformation and playing a role in the early progression to malignancy;[63-66] in particular, by shedding microRNA-containing microvesicles, activated platelets may have clinical relevance in promoting tumor growth and spread,[67] and potentially could be used as biomarkers, as circulating microRNA-containing microvesicles may differ according to cancer stage.[68] Noticeably, mature megakaryocytes drop down 2-AG levels in order to ensure a correct homeostasis of eCB effects.[29]

Although promising for the chance to broaden the field of investigation, the thrombopoietic activity of 2-AG will have to be assessed under authentic *in vivo* conditions, in order to better understand the molecular mechanisms underlying megakaryopoiesis and thrombopoiesis.

Materials and Methods

Reagents

Chemicals were of the purest analytical grade. AEA, 2-AG, AM281, 12-O-tetradecanoylphorbol-13-acetate (TPA) and collagen type I were from Sigma Chemical Co. ACEA and JWH015, SR144528 and JZL184 were from Alexis Corporation.

Cell cultures

Human megakaryoblastic MEG-01 cells (ATCC) were grown in DMEM:F12 (1:1) medium (Invitrogen) supplemented with 2 mM L-glutamine and 20% heat-inactivated foetal bovine serum, at 37°C in a humidified atmosphere of 5% CO_2. Differentiation was achieved by culturing cells in medium containing 10 nM TPA (positive control)[69] or increasing concentrations of the tested compounds, for the indicated periods of time.

Western blotting

Membrane fractions (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotted onto polyvinylidene difluoride membranes, incubated with primary antibodies and detected with chemiluminescence, as described.[27]

Enzymatic activities

Diacylglycerol lipase (DAGL) activity was evaluated following the release of [^{14}C]2-AG from [^{14}C]diacylglycerol, by thin layer chromatography and scintillation counting.[70] Fatty acid amide hydrolase (FAAH) activity was evaluated measuring the release of [^{14}C]ethanolamine from [^{14}C]AEA, by scintillation counting.[71] Monoacylglycerol lipase (MAGL) activity was assayed by measuring the release of [^{3}H]glycerol from [^{3}H]2-oleoyL-glycerol, by scintillation counting.[72]

Platelet isolation

Platelet-like particles were isolated from differentiated MEG-01 cells by sequential centrifugation, as reported.[30] Briefly, cells were pelletted at 150 x *g* for 10 min and the resulting supernatant was centrifuged again at 500 x *g* for 15 min to remove all contaminant cells. Platelets were further centrifuged at 1000 x *g* for 15 min and resuspended in Tyrode's buffer (100 mM Hepes, 1.3 M NaCl, 29 mM KCl, 120 mM $NaHCO_3$, pH 7.4), containing 1/10 (v/v) ACD (112 mM glucose, 130 mM citric acid, 152 mM sodium citrate) and 2 mM glucose and counted by an inverted microscope.

Flow cytometry

Immunophenotyping of cells was analyzed by flow cytometry in a FACSCanto instrument (Beckton Dickinson). Briefly, MEG-01 cells or platelet-like particles were fixed in 4% paraformaldehyde and stained with appropriate cluster of differentiation (CD) antibodies. The antibodies used were: phycoerythrin (PE)-conjugated CD61, fluorescein isothiocyanate (FITC)-conjugated CD49, PE-conjugated CD41 and allophycocyanin (APC)-conjugated CD62P (Beckton Dickinson). Platelets were also assessed for functionality, after treatment with 10 μg/mL collagen type I for 15 min at room temperature.

The cell cycle was evaluated by propidium iodide (50 μg/ml) staining, after prior incubation with 13 kunits/ml RNase A, as described.[73]

For each analysis, 10 thousand events were acquired and analyzed using the Flowjo software (TreeStar).

Confocal microscopy

MEG-01 cells, fixed on cover-glasses with 3% paraformaldehyde plus 4% sucrose for 20 min at room temperature, were blocked for 30 min with 5% bovine serum albumin in phosphate buffered saline, containing 0.1% saponin, before staining with primary anti-α-tubulin antibody (1:200; Sigma) for 1 hour. Then, cells were incubated with Alexa Fluor 488-conjugated secondary antibody (1:1000; Invitrogen) and Alexa Fluor 568-phalloidin (1:100; Invitrogen) for an additional 1 hour. Cellular localization of F-actin and α-tubulin was visualized by means of a Zeiss LSM400 confocal microscope, and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/). Differential interference contrast images were collected using a 40.0×1.25 objective.

Statistical analysis

All values are expressed as means ± SEM of at least 3 independent experiments, each performed in triplicate. The Student's unpaired *t* test or one-way ANOVA (followed by Bonferroni *post-hoc* analysis) were used to analyze experimental data by means of the InStat 3 program (GraphPAD). Significant differences were accepted at $p < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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