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Apocynin prevents GM-CSF-induced-ERK1/2 activation and -neutrophil survival independently of its inhibitory effect on the phagocyte NADPH oxidase NOX2

Running title: Apocynin prevents GM-CSF-induced ERK1/2 activation

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ABSTRACT

Neutrophils are key cells in innate immunity and inflammation. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is known to enhance many neutrophil functions such as upregulation of reactive oxygen species (ROS) production, degranulation and cell survival via the activation of the ERK1/2 pathway. ERK1/2 activation pathway is redox sensitive and could be modulated by ROS. In order to investigate whether NADPH oxidase NOX2-derived ROS could contribute to GM-CSF-induced ERK1/2 phosphorylation, we tested the effect of two selective NOX2 inhibitors, diphenylene iodonium (DPI) and apocynin. Results showed that, while both DPI and apocynin strongly inhibited neutrophil ROS production, only apocynin, but not DPI, inhibited GM-CSF-induced ERK1/2 phosphorylation, suggesting that ROS are not involved in this process. Apocynin did not affect GM-CSF-induced p38MAPKinase phosphorylation, another redox sensitive kinase. Interestingly, apocynin inhibited GM-CSF-induced MEK1/2 and AKT phosphorylation without affecting fMLF-induced phosphorylation of these proteins. GM-CSF is known to inhibit neutrophils apoptosis and to promote cell survival via the AKT-ERK1/2 pathway. In this regard, we found that apocynin also inhibited GM-CSF-induced anti-apoptotic effect in neutrophils. These results suggest that NADPH oxidase NOX2-derived ROS are not involved in GM-CSF-induced ERK1/2 phosphorylation and that apocynin inhibits GM-CSF-induced ERK1/2 phosphorylation pathway independently of its inhibitory action on NADPH oxidase NOX2. Thus, apocynin can exert an anti-inflammatory effect not only by limiting neutrophil ROS production but also by decreasing neutrophil survival at inflammatory site.

Keywords: Apocynin; Neutrophils; NADPH oxidase NOX2; Reactive oxygen species; GM-CSF; ERK1/2

1. INTRODUCTION

Human polymorphonuclear neutrophils play an important role in host defence against microorganisms and in inflammation. During infection, neutrophils migrate from blood to the tissues, to recognize and engulf the pathogen. This mechanism called phagocytosis trigger the activation of several neutrophil functions, such as the release of proteases, bactericidal peptides and reactive oxygen species (ROS) via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, leading to the death and destruction of the microbe [1]. Although, neutrophils play a key role in host defence, excessive neutrophil activation is harmful to the adjacent tissues and is involved in inflammatory diseases, like rheumatoid arthritis, inflammatory bowel diseases and adult respiratory distress syndrome [2, 3]. The phagocytes NADPH oxidase NOX2 is composed of a heterodimer of two membrane proteins called cytochrome b_{558} (gp91phox, p22phox) and four cytosolic proteins, p47phox, p67phox, p40phox and the small G-proteins Rac1 (in monocytes) or Rac2 (in neutrophils) [4-6]. In resting cells, these NADPH oxidase NOX2 components are not assembled and are separated in the cytosol, granule and plasma membranes. Upon cell activation, p47phox, p67phox, p40phox, gp91phox, and p22phox are phosphorylated and the cytosolic components translocate to the membranes where they bind to p22phox and gp91phox to assemble the active NADPH oxidase NOX2 which then produces superoxide anions ($O_2^{\circ-}$) using cytosolic NADPH as the electron donor [4]. Superoxide anion ($O_2^{\circ-}$) is the precursor of other ROS, such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH°) and hypochlorous acid (HOCl) [4, 7].

Pro-inflammatory agents (cytokines and TLR agonists) such as granulocyte-macrophage colony-stimulating factor (GM-CSF), Tumor Necrosis Factor alpha (TNF α), lipopolysaccharide (LPS) and CL097- induce up-regulation or priming of neutrophil functions when they are stimulated by the bacterial peptide formyl-methionyl-leucylphenylalanine (fMLF) [6].

GM-CSF, is a 22 kDa cytokine produced by macrophages, T-cells, mast cells, endothelial cells and fibroblasts. It is involved in the growth, differentiation and maturation of myeloid precursor cells and enhances the function of mature neutrophils, eosinophils and monocytes [8-10]. GM-CSF binds to a specific receptor which is composed of two chains, a low-affinity alpha subunit (CD116) which is specific to GM-CSF, and a high-affinity beta chain (CD131) which is shared with IL-3 and IL-5 [9]. GM-CSF binding to its receptor induces activation of a number of signal transduction pathways including protein tyrosine kinases (PTK), PI3K and the MAPK family ERK1/2. GM-CSF binding to its receptor also induces and enhances the binding of the protein tyrosine kinase JAK2 and the src-tyrosine kinase Lyn to the intracellular domain of the receptor. These tyrosine kinases transduce the GM-CSF signal by phosphorylating other proteins such as STAT, PKB and PI3K. JAK2 is the only member of the JAK family to be activated by GM-CSF in neutrophils and it is involved, directly or indirectly, in the tyrosine phosphorylation of p85, the regulatory subunit of PI3K [8-10].

We have previously shown that GM-CSF induced priming of the NADPH oxidase NOX2 and excessive ROS production by human neutrophils via the activation of JAK2-ERK1/2-p47phox phosphorylation pathway [11, 12]. In this study we hypothesized that NADPH oxidase NOX2-derived ROS might contribute to GM-CSF-induced ERK1/2 phosphorylation thereby acting in a positive feedback loop to induce NADPH oxidase NOX2 hyper-activation. We thus tested the effect of two NADPH oxidase NOX2 inhibitors, diphenylene iodonium (DPI) and apocynin on GM-CSF-induced ERK1/2 phosphorylation. DPI is a flavoprotein

inhibitor which binds to gp91phox-FAD binding site and inhibits electron transfer from NADPH to heme and oxygen [13-15]. Apocynin (4-hydroxy-3-methoxyacetophenone) is a methoxy-substituted catechol isolated from *Picrorhiza kurroa*, a traditional medicinal plant found in the Himalayan mountains [16, 17]. Apocynin has been widely used as an inhibitor of the phagocyte NADPH oxidase NOX2 [17, 18] and is known to have anti-inflammatory activity in a variety of cell and animal models of inflammation [16]. In neutrophils, apocynin was found to be activated by H₂O₂ and myeloperoxidase (MPO) to form a symmetrical dimer, diapocynin, which oxidizes thiols in the NADPH oxidase NOX2 and thus inhibits p47phox translocation to the membranes and the assembly of this enzyme [17, 18].

In order to investigate the role of NADPH oxidase NOX2-derived ROS in GM-CSF-induced ERK1/2 phosphorylation and whether ROS could act via a positive feedback loop for NADPH oxidase NOX2 activation, we tested the effect of DPI and apocynin on ERK1/2 phosphorylation. Surprisingly, while apocynin and DPI both inhibited ROS production, only apocynin, but not DPI, inhibited GM-CSF-induced ERK1/2 phosphorylation. Furthermore, apocynin also inhibited GM-CSF-induced neutrophil survival which is known to be regulated by ERK1/2. Together these data suggest that the effect of apocynin on GM-CSF-induced ERK1/2 phosphorylation and cell survival is probably due to an effect unrelated to NADPH oxidase NOX2 inhibition.

2. MATERIALS AND METHODS

2.1. Reagents and antibodies

Apocynin (4-hydroxy-3-methoxyacetophenone), diphenyleneiodonium (DPI), 4-phorbol-12-myristate-13-acetate (PMA), formyl-methionyl-leucylphenylalanine (fMLF), Luminol (5-amine-2,3-dihydro-1,4-phtalazinedione), Hanks' balanced salt solution (HBSS), p-nitro blue tetrazolium (NBT) / 5-bromo-4-chloro-3-indolyl phosphate (BCIP) reagents, and all the others chemical product used were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Dextran T500 and Ficoll were purchased from GE Healthcare (Orsay, France). The reagents to make SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and Western blot were purchased from Bio-Rad (Marnes-la-Coquette, France). Human granulocyte/macrophage colony-stimulating factor (GM-CSF) was from PeproTech France (Neuilly-Sur-Seine, France). RPMI 1640 medium, fetal bovine serum (FBS), penicillin/streptomycin solution, trypan blue, turk blue were purchased from Invitrogen (LifeTechnologies, France). Antibodies against phospho-ERK1/2 and ERK1/2 were from R&D systems (Abingdon, UK). Antibodies against phospho-p38MAPK (T180/Y182), phospho-MEK1/2 (Ser221), MEK1/2, phospho-STAT5 (Y694), STAT5 (D206Y), phospho-AKT (Ser473) and AKT were from Cell Signaling Technology (Leiden, The Netherlands). Antibodies against p22phox, secondary HRP-conjugated goat anti rabbit or anti mouse and secondary alkaline phosphatase-conjugated goat anti rabbit or anti mouse antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany). Antibodies against p47phox were generated by our lab as previously described [19].

2.2. Ethics statement and human neutrophils isolation

All experiments were supported by the Inserm Institutional Review Board and ethics committee. The collection and analyses of data were performed anonymously. Neutrophils were isolated from venous fresh blood of healthy volunteers' donors, with their signed informed consent, by dextran sedimentation, to remove red blood cells, followed by differential centrifugation through a Ficoll-Hypaque density gradient to remove mononuclear cells, and hypotonic lysis to remove any remaining contaminating red blood cells [20, 21]. Neutrophils in the pellet were washed and resuspended in appropriate medium, such as HBSS. Cell counting was determined by Turks' blue staining and flow cytometry analysis (CD15, CD16 and CD33 markers) to evaluate the purity of neutrophils, and the viability by Trypan blue staining. Neutrophils were more than 97% pure and 99% viable.

2.3. Luminol-amplified chemiluminescence assay in activated neutrophils

Neutrophils (5×10^5) were suspended in HBSS (0.5 ml) and preincubated for 15 minutes at 37°C in the presence of luminol (10 μ M), with or without DPI (10 μ M) or apocynin (200 μ M). Cells were then treated with GM-CSF (25 ng/ml) for 20 min and stimulated by fMLF (10^{-7} M) or PMA (100 ng/ml). Luminol-amplified chemiluminescence was measured using a Berthold 953 apparatus for respectively 10 and 30 minutes at 37°C and light emission was expressed in counted photons per minute (c.p.m) [22].

2.4. Treatment of neutrophils and Western Blotting analysis

Neutrophils (10×10^6) resuspended in HBSS (400 μ l) were treated with two different NADPH oxidase NOX2 inhibitors, apocynin or DPI, for 15 minutes and at the indicated concentrations at 37°C with mild shaking. The stimulation was performed at 37°C with GM-CSF (25 ng/ml) for 20 minutes, fMLF (10^{-6} M) for 1 minute or PMA (100 ng/ml) for 8 minutes. The reaction was stopped by adding 5 \times concentrated Laemmli sample buffer containing 50 % glycerol, 12.5 % sodium dodecyl sulfate (SDS), 25 % beta-mercaptoethanol, 312.4 mM Tris-HCl pH 6.8, 12.5 mM EDTA, 12.5 mM EGTA, 0.75 mM bromophenol blue, protease and phosphatase inhibitors [23, 24]. Samples were then denatured for 3 minutes at 100°C and stored at -80°C until use. Neutrophils lysates were sonicated and subjected to 10% SDS-PAGE (equivalent of 8×10^5 cells/well). The separated proteins were transferred to nitrocellulose and blocked for 1 hr at room temperature in TBS-Tween (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk. After blocking, the membranes were incubated overnight at 4°C with the primary antibody at the following dilutions against: phosphoERK1/2 (T202/Y204) (1:1000), phospho38MAPK (T180/Y182) (1:1000), phospho-MEK1/2 (S221) (1:1000), phospho-AKT (S473) (1:1000), phosphoSTAT5 (Y694) (1:1000), ERK1/2 (1:1000), MEK1/2 (1:1000), AKT (1:1000), STAT5 (1:1000), p47phox (1:4000), p22phox (1:4000). After three washes with TBS-Tween of 5 minutes each, the membranes were incubated with horseradish peroxidase-conjugated goat anti rabbit or anti mouse antibodies for 1 hr (1:10000). After further washes, the protein bands were revealed by a chemiluminescence method with ECL western blotting reagents (GE Healthcare LifeSciences, Velizy, France) and then exposed to a camera Amersham Imager 600 (GE Healthcare LifeSciences, Velizy, France). Alternatively, the membranes were incubated with an alkaline phosphatase-conjugated goat anti mouse or goat anti rabbit and proteins were revealed with the NBT/BCIP reagents in the carbonate buffer (100 mM NaHCO₃, 2 mM

MgCl₂, pH 9.8). The intensity of bands was quantified by Image J program (Wayne Rasband, National Institute of Health, USA). Antibodies against total ERK1/2, MEK1/2, AKT, STAT5, p47phox and p22phox were used as control for protein loading and transfer.

2.5. Measurement of neutrophil apoptosis by flow cytometry

Freshly isolated neutrophils were resuspended in complete RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), and placed in 6-well tissue culture plates to a final concentration of 5×10^6 cells/ml. Neutrophils were incubated, for 48 hrs at 37°C in 5% CO₂ in presence or absence of 20 ng/ml human GM-CSF, and with or without 400 µM of apocynin [25]. The percentage of apoptotic neutrophils was quantified by PE annexin V/7-Amino-Actinomycin (7-AAD) binding kit (BD Biosciences, San Jose, CA), using a flow cytometer (FACS CANTO II, BD biosciences), before and after culture with or without addition of human GM-CSF and apocynin, following a protocol provided by the manufacturer. The following controls were used to set up compensation and quadrants: unstained cells, cells stained with PE Annexin V (no 7-AAD), cells stained with 7-AAD (no PE Annexin V). The untreated population was used to define the basal level of apoptotic and dead cells. 7-AAD was used as an indicator of viability and no distinction was made between early and late apoptosis. Cells that stained positively for PE-annexin-V were considered apoptotic. The combination of PE annexin V and 7-AAD differentiates between early apoptotic neutrophils (annexin V⁺, 7-AAD⁻) and late apoptotic neutrophils (annexin V⁺, 7-AAD⁺).

2.6. Statistical Analysis

All results are expressed as means \pm SEM. Data were analyzed with GraphPad Prism 7 software (GraphPad Software, San Diego, CA). Differences between groups were analyzed by One-way ANOVA test with Tukey's Multiple Comparison post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ values were considered as significant.

3. RESULTS

3.1. Effect of DPI and apocynin on GM-CSF-induced ERK1/2 phosphorylation and on ROS production in human neutrophils

To evaluate the role of NADPH oxidase NOX2-derived ROS on GM-CSF-induced ERK1/2 phosphorylation in human neutrophils, freshly isolated neutrophils were treated for 15 minutes with DPI or apocynin, two NADPH oxidase NOX2 inhibitors, prior to stimulating with GM-CSF. Results show that GM-CSF clearly induced ERK1/2 phosphorylation in human neutrophils (**Fig. 1A**). Interestingly, treatment of cells by DPI did not affect GM-CSF-induced ERK1/2 phosphorylation indicating that ROS are not involved, however surprisingly, treatment of cells by apocynin inhibited this process. We checked that both molecules were able to inhibit neutrophil ROS production induced by GM-CSF+fMLF and by PMA (**Fig. 1B-**

C and **1D-E**). Dose effect studies showed that apocynin inhibited GM-CSF-induced ERK1/2 phosphorylation between 100 and 400 μM (**Fig. 2A-B**), however DPI had no effect even at 20 μM (**Fig. 2C-D**). Interestingly, treatment of neutrophils first with DPI to completely inhibit NOX2, then the addition of apocynin also inhibited GM-CSF-induced ERK1/2 phosphorylation (data not shown). These results show that while both DPI and apocynin are able to inhibit neutrophil ROS production, only apocynin is able to inhibit GM-CSF-induced ERK1/2 phosphorylation, suggesting that apocynin inhibits this pathway independently of its inhibitory effect on NADPH oxidase.

3.2. Apocynin inhibits GM-CSF-induced ERK1/2 phosphorylation but does not affect GM-CSF-induced p38MAPK phosphorylation in human neutrophils

To check if apocynin action was specific for ERK1/2, we tested its effect on GM-CSF-induced p38 phosphorylation. Results showed that while apocynin was able to block GM-CSF-induced ERK1/2 phosphorylation, it had no effect on p38MAPK phosphorylation even at high concentrations (**Fig. 3**).

3.3. Effect of apocynin on ERK1/2 phosphorylation induced by different stimuli

As ERK1/2 was also activated by other agents like the bacterial peptide fMLF and PMA, we analysed the effect of apocynin on ERK1/2 phosphorylation induced by these agents. Results

showed that while apocynin was able to inhibit GM-CSF-induced ERK1/2 phosphorylation, it was without effect on fMLF-induced phosphorylation even at high concentrations (**Fig. 4A-B**). Surprisingly, apocynin inhibited PMA-induced ERK1/2 phosphorylation.

3.4. Effect of apocynin on upstream kinases involved in ERK1/2 phosphorylation induced by GM-CSF, fMLF and PMA

As ERK1/2 was phosphorylated by MEK1/2, our results suggested that apocynin could inhibit MEK1/2 or an upstream kinase. The kinase known to phosphorylate MEK1/2 is Raf. Therefore, in order to verify if apocynin could inhibit Raf activity, we examined its effect on MEK1/2 phosphorylation. Results showed that apocynin inhibited GM-CSF-induced MEK1/2 phosphorylation, while having no effect on fMLF-induced phosphorylation at 200 μ M of apocynin and a moderate effect at 400 μ M of apocynin (**Fig. 5A-B**). Apocynin also inhibited PMA-induced MEK1/2 phosphorylation. These results suggested that apocynin could inhibit Raf kinase or an upstream kinase. It is known that AKT is upstream Raf-MEK1/2 and ERK1/2 in human neutrophils [26-29]. We thus tested the effect of apocynin on AKT phosphorylation. Results showed that apocynin strongly inhibited GM-CSF-induced AKT-phosphorylation, without having any effect on fMLF-induced phosphorylation even at high concentrations (**Fig. 5C-D**). PMA did not induce AKT phosphorylation. Moreover, PKC activity was not inhibited by apocynin in vitro (data not shown). GM-CSF-receptor is associated with different protein tyrosine kinases, like lyn and JAK2 which control the activation of MAPKinases. To identify the target of apocynin in GM-CSF-stimulated neutrophils, we first determined the effect of apocynin on the phosphorylation of STAT5, the

endogenous substrate of JAK2. Results showed that apocynin did not affect GM-CSF-induced STAT5-phosphorylation (**Fig. 5E-F**). fMLF and PMA did not induce STAT5 phosphorylation. Lyn phosphorylation was not inhibited by apocynin (data not shown). Moreover, Phosphoinositide 3-kinase (PI3K) which is activated by tyrosine kinase was not inhibited by apocynin (data not shown). These results strongly suggest that apocynin inhibits AKT phosphorylation induced by GM-CSF but not the one induced by fMLF, suggesting the presence of two different kinases able to phosphorylate AKT, one of them being apocynin-sensitive.

3.5. The anti-apoptotic effect of GM-CSF is inhibited by apocynin

GM-CSF is a pro-inflammatory cytokine known to delay apoptosis of neutrophils. AKT and ERK 1/2 are known to control the anti-apoptotic effect of GM-CSF [27, 29]. We therefore analysed whether apocynin was able to prevent GM-CSF-induced delay of neutrophil apoptosis as a consequence of AKT- ERK1/2 pathway inhibition. Results showed that GM-CSF was able to inhibit apoptosis of neutrophils, confirming the anti-apoptotic effect of GM-CSF (**Fig. 6A-B**). Apocynin alone had no effect on apoptosis but it inhibited the anti-apoptotic effect of GM-CSF.

4. DISCUSSION

Human neutrophils play an important role in host defence and in inflammation. During infection, these cells allow the destruction of infectious agent by activation of the NADPH oxidase NOX2 and ROS production. However, excessive ROS production is involved in development of tissues injury and inflammatory diseases [3, 6]. Thus, the development of drugs targeting the NADPH oxidase system is an important anti-inflammatory strategy. Apocynin and DPI are among the most used NADPH oxidase inhibitors of the phagocyte NADPH oxidase NOX2 in vitro and in vivo [30]. Apocynin is believed to be a specific inhibitor for NADPH oxidase NOX2, among other NOXs, however off-target effects of this molecule are less known. In this study we showed that while apocynin and DPI both inhibited neutrophil ROS production, only apocynin was able to inhibit GM-CSF induced AKT-MEK1/2-ERK1/2 phosphorylation cascade and GM-CSF-induced anti-apoptotic effect. DPI inhibited neutrophil ROS production, but had no effect on GM-CSF induced ERK1/2 phosphorylation pathway. Thus apocynin inhibits GM-CSF-induced ERK1/2 pathway independently of its inhibitory action on NADPH oxidase NOX2.

In addition to ERK1/2 pathway, GM-CSF can also activate p38MAPKinase pathway in human neutrophils [31]. Interestingly, apocynin did not inhibit GM-CSF induced p38 phosphorylation suggesting that it specifically targeted the ERK1/2 pathway. We also wanted to check if apocynin inhibited the JNK phosphorylation pathway, however GM-CSF did not activate this latter pathway in neutrophils (data not shown). ERK 1/2 is also activated by other agonists such as the chemotactic peptide fMLF and the PKC activator PMA. Interestingly apocynin inhibited GM-CSF- and PMA-induced ERK1/2 phosphorylation but not fMLF-induced ERK1/2 phosphorylation. This indicates that apocynin inhibits a common enzyme

required for GM-CSF- and PMA-induced ERK1/2 phosphorylation. This apocynin target is not the tyrosine JAK2, because the phosphorylation of STAT5 was not affected by apocynin. On the basis of our observations, we suggest the presence of a kinase specific for GM-CSF pathway able to phosphorylate AKT and that is sensitive to apocynin (**Fig. 7**).

GM-CSF can regulate several neutrophil functions including priming of neutrophil, ROS production and inhibition of neutrophil apoptosis. Since apocynin inhibited AKT and ERK1/2, two kinases known to control the anti-apoptotic effect of GM-CSF [26-29], we choose to test its effect on GM-CSF-induced neutrophil apoptosis. We showed that GM-CSF inhibited neutrophil apoptosis and that apocynin prevented this effect. These results suggest that apocynin could be a promising molecule to inhibit GM-CSF- and other cytokines-induced survival at inflammatory sites and that it could exert anti-inflammatory effect by favoring the resolution of inflammation. In this context, several studies demonstrated that apocynin represented a therapeutic candidate for the treatment of inflammatory diseases such rheumatoid arthritis [32, 33], inflammatory bowel diseases [34, 35] and lung inflammation [36]. Apocynin had also beneficial effects in many other diseases, including cancer [37], cardiovascular diseases [38], diabetic nephropathy [39] and neurodegenerative diseases [40]. These effects could be related to its action on NADPH oxidase NOX2 and on ERK1/2 phosphorylation pathway as reported in this study.

In addition to the inhibitory effect of apocynin on p47phox translocation and NADPH oxidase NOX2 activation [17], little is known about its impact on other processes. It was suggested that apocynin is an antioxidant by reacting with hydrogen peroxide [41]. The same study by Heumuller S et al, showed that apocynin inhibited H₂O₂-induced AKT, p38 and ERK1/2 phosphorylation in rat smooth muscle cells and suggested that this effect is probably due to its antioxidant effect [41]. In this report, we showed that in contrast to apocynin, DPI, a powerful inhibitor of neutrophil ROS production, had no effect on GM-CSF-induced ERK1/2

phosphorylation, supporting the conclusion that the inhibitory action of apocynin on GM-CSF-induced ERK1/2 phosphorylation was neither related to its inhibitory effect on NADPH oxidase NOX2 nor on its possible antioxidant properties. Moreover, in the current study, we showed that apocynin did not have any effect on phosphorylation of p38 MAPK, STAT5, Lyn and PKC in human neutrophils stimulated by GM-CSF, suggesting that apocynin could inhibit a specific isoform of PI3K or an upstream protein kinase which phosphorylates AKT on Ser373. To check if apocynin has an effect on the phosphorylation of PI3K, we used an anti-Phospho-PI3Kinase-p85(Tyr458) antibody and an anti-p55(Tyr199) antibody. The results showed that apocynin did not inhibit GM-CSF-induced PI3K phosphorylation on these sites (data not shown). To ascertain that apocynin inhibits or not PI3K activity, enzymatic activity assay should be performed. Our results clearly show that apocynin inhibited GM-CSF-induced AKT phosphorylation on Ser473, thus apocynin inhibits the kinase which phosphorylates this site. AKT could be phosphorylated by auto-phosphorylation, mTORC and PDK1. PDK1 phosphorylates AKT on Thr308 and mTORC2 phosphorylates AKT on Ser373 [42]. Apocynin could inhibit mTORC2 or AKT rather than PDK1, more studies are required to check this possibility.

Jan Kucera et al, performed with mouse embryonic stem cells suggested that apocynin at much higher concentration could inhibit PI3K [43]. Other studies showed that the food additive divanillin, the homodimer of vanillin and apocynin decreased the metastatic potential of HepG2 liver cancer cells by inhibiting of FAK/PI3K/Akt signalling pathway [44], and apocynin can induce vasodilation of aortic rings by inhibiting Rho Kinase activity [45].

In conclusion our study shows that apocynin, a known NADPH oxidase inhibitor can have other effects unrelated to inhibition of NADPH oxidase NOX2 such as inhibition of GM-CSF-induced AKT-MEK1/2-ERK1/2 pathway and inhibited GM-CSF-pro-survival effect.

Apocynin can thus exert an anti-inflammatory effect by limiting not only neutrophil ROS production but also neutrophil survival at inflammatory site.

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Author contributions

CP designed and performed the experiments. MBK and DL performed the experiments. PMCD, MHN and JEB designed the experiments and analyzed the data. All authors contributed to writing the manuscript.

Conflicts of Interest

The authors declare no competing conflict of interest.

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FIGURE LEGENDS

Fig. 1: Effect of DPI and apocynin on GM-CSF-induced ERK1/2 phosphorylation and on ROS production in human neutrophils

Neutrophils (10×10^6) were treated with GM-CSF (25 ng/ml) for 20 minutes at 37°C in the absence or presence of DPI (10 μ M) or apocynin (200 μ M) and lysed in Laemmli sample buffer. Activation of ERK1/2 was analysed by SDS-PAGE and western blot using phospho-ERK1/2 antibody. Total p47phox antibody was used as control for protein loading and transfer (**A**). Neutrophils (5×10^5) were resuspended in HBSS (0.5 ml) and preincubated for 15 minutes at 37°C in the presence of luminol (10 μ M), with or without DPI (10 μ M) or apocynin (200 μ M). Cells were then stimulated with GM-CSF (25 ng/ml) + fMLF (10^{-7} M) for 10 minutes (**B and C**) or PMA (100 ng/ml) for 30 minutes (**D and E**). ROS production was measured by Luminol-amplified chemiluminescence for 10 or 30 minutes at 37°C and the light emission is expressed in counted photons per minute (c.p.m). (**B**) and (**D**) show result of 1 representative experiment. (**C**) and (**E**) show a quantification analysis of 3 independent experiments. Values are expressed as percentage of control (condition named “buffer”: neutrophils stimulated without inhibitor). Mean \pm SEM of 3 independent experiments. *****: $p < 0.0001$ as compared to the control (100% of ROS production corresponds to $4.73 \pm 0.5 \times 10^8$ cpm for GMCSF + fMLF stimulation, and $1.51 \pm 0.25 \times 10^9$ cpm for PMA stimulation).

Fig. 2: Dose effect of apocynin and DPI on GM-CSF-induced ERK1/2 phosphorylation in human neutrophils

Neutrophils (10×10^6) were treated with GM-CSF (25 ng/ml) for 20 minutes at 37°C in the absence or presence of apocynin at different concentrations (100 μ M, 200 μ M and 400 μ M) and lysed in Laemmli sample buffer. Activation of ERK1/2 was analysed by SDS-PAGE and western blot using phospho-ERK1/2 antibody. Total p22phox antibody was used as control for protein loading and transfer (A). Protein bands were quantified using Image J software and the ratio of phospho-ERK1/2 to the total amount of p22phox was calculated. Values are expressed as means \pm SEM of 3 independent experiments. ns (not significant): $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$ as compared to the control (neutrophils stimulated with GM-CSF without inhibitor) (B). Neutrophils (10×10^6) were treated with GM-CSF (25 ng/ml) for 20 minutes at 37°C in the absence or presence of DPI at different concentrations (5 μ M, 10 μ M and 20 μ M) and lysed in Laemmli sample buffer. Activation of ERK1/2 was analysed by SDS-PAGE and western blot using phospho-ERK1/2 antibody. Total p22phox antibody was used as control for protein loading and transfer (C). Protein bands were quantified using Image J software and the ratio of phospho-ERK1/2 to the total amount of p22phox was calculated. Values are expressed as means \pm SEM of 3 independent experiments. ns (not significant): $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$ as compared to the control (neutrophils stimulated with GM-CSF without inhibitor) (D).

Fig. 3: Apocynin inhibits GM-CSF-induced ERK1/2 phosphorylation but does not affect GM-CSF-induced p38MAPK phosphorylation in human neutrophils

Neutrophils (10×10^6) were treated with GM-CSF (25 ng/ml) for 20 minutes at 37°C in the absence or presence of apocynin at different concentrations (100 μ M, 200 μ M and 400 μ M) and lysed in Laemmli sample buffer. Activation of p38MAPK and ERK1/2 were analysed by western blot using phospho-p38MAPK (T180/Y182) and phospho-ERK1/2 antibodies. Total

p22phox antibody was used as control for protein loading and transfer. Experiment repeated three times.

Fig. 4: Effect of apocynin on ERK1/2 phosphorylation induced by different stimuli

Neutrophils (10×10^6) were stimulated with GM-CSF (25 ng/ml), fMLF (10^{-6} M) or PMA (100 ng/ml) for respectively 20 minutes, 1 minute or 8 minutes at 37°C in the absence or presence of apocynin at different concentrations (200 μ M and 400 μ M). Activation of ERK1/2 was analysed by western blot using phospho-ERK1/2 antibody. Total ERK1/2 and p22phox antibodies were used as control for protein loading and transfer (A). The ratio of phospho-ERK1/2 to the total amount of p22phox was quantified using Image J software. Values are expressed as means \pm SEM of 3 independent experiments. ns (not significant): $p > 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$ as compared to the control (neutrophils stimulated with GM-CSF, fMLF or PMA without inhibitor) (B).

Fig. 5: Effect of apocynin on upstream kinases involved in ERK1/2 phosphorylation induced by GM-CSF, fMLF and PMA

Neutrophils (10×10^6) were treated with GM-CSF (25 ng/ml), fMLF (10^{-6} M) or PMA (100 ng/ml) for respectively 20 minutes, 1 minute or 8 minutes at 37°C in the absence or presence of apocynin at different concentrations (200 μ M and 400 μ M). Activation of MEK1/2 was analysed by western blot using phospho-MEK1/2 (S221) antibody. Total MEK1/2 and p22phox antibodies were used as control for protein loading and transfer (A). The ratio of phospho-MEK1/2 to the total amount of p22phox was quantified using Image J software. Values are expressed as means \pm SEM of 3 independent experiments. ns (not significant):

$p > 0.05$, **: $p < 0.01$, ****: $p < 0.0001$ as compared to the control (neutrophils stimulated with GM-CSF, fMLF or PMA without inhibitor) **(B)**. Activation of AKT was analysed by western blot using phospho-AKT (S473) antibody. Total AKT and p22phox antibodies were used as control for protein loading and transfer **(C)**. The ratio of phospho-AKT to the total amount of p22phox was quantified using Image J software. Values are expressed as means \pm SEM of 3 independent experiments. ns (not significant): $p > 0.05$, *: $p < 0.05$, ****: $p < 0.0001$ as compared to the control (neutrophils stimulated with GM-CSF, fMLF or PMA without inhibitor) **(D)**. Activation of STAT5 was analysed by western blot using phospho-STAT5 (Y694) antibody. Total STAT5 (D206Y) and p22phox antibodies were used as control for protein loading and transfer **(E)**. The ratio of phospho-STAT5 to the total amount of p22phox was quantified using Image J software. Values are expressed as means \pm SEM of 3 independent experiments. ns (not significant): $p > 0.05$ as compared to the control (neutrophils stimulated with GM-CSF, fMLF or PMA without inhibitor) **(F)**.

Fig. 6: Effect of apocynin on the anti-apoptotic effect of GM-CSF

Neutrophils (10×10^6) were incubated, for 48 hrs at 37°C in 5% CO_2 in presence or absence of 20 ng/ml GM-CSF, and with or without $400 \mu\text{M}$ of apocynin. Neutrophils were stained with PE annexin V and 7-Amino-Actinomycin (7-AAD) and the fluorescence was measured by flow cytometry. **(A)** show result of 1 representative experiment. Values represent the percentage of late apoptotic neutrophils (PE annexin V and 7-AAD positive). Live neutrophils (L) are both PE annexin V and 7-AAD negative. Early apoptotic neutrophils (EA) bind PE annexin V but exclude 7-AAD. Late apoptotic neutrophils (LA) are both PE annexin V and 7-AAD positive. Necrotic neutrophils (N) stain with 7-AAD only. **(B)** show a quantification analysis of 3 independent experiments. Values show the percentage of late apoptotic

neutrophils (PE annexin V and 7-AAD positive) and are expressed as means \pm SEM of 3 independent experiments. Value of 100% corresponds to 1×10^5 neutrophils in late apoptotic phase. **: $p < 0.01$ and ***: $p < 0.001$

Fig. 7: Summary diagram of the effect of apocynin on GM-CSF-induced ERK1/2 activation pathway in human neutrophils

Activation of human neutrophils by GM-CSF, fMLF or PMA leads to the phosphorylation of several pathways. Binding of GM-CSF to its receptor induced the activation of JAK2 and Lyn tyrosine kinases which activate PI3Kinase-AKT-Raf1-MEK1/2 and ERK1/2. fMLF induced the activation of PI3Kinase-AKT-Raf1-MEK1/2 and ERK1/2. PMA induced the activation of PKC which phosphorylates raf1. Apocynin strongly inhibits GM-CSF-induced AKT-ERK1/2 activation, but doesn't have any effect on p38 MAPK nor on STAT5 and PKC. Apocynin target is localized in the PI3K-AKT module.

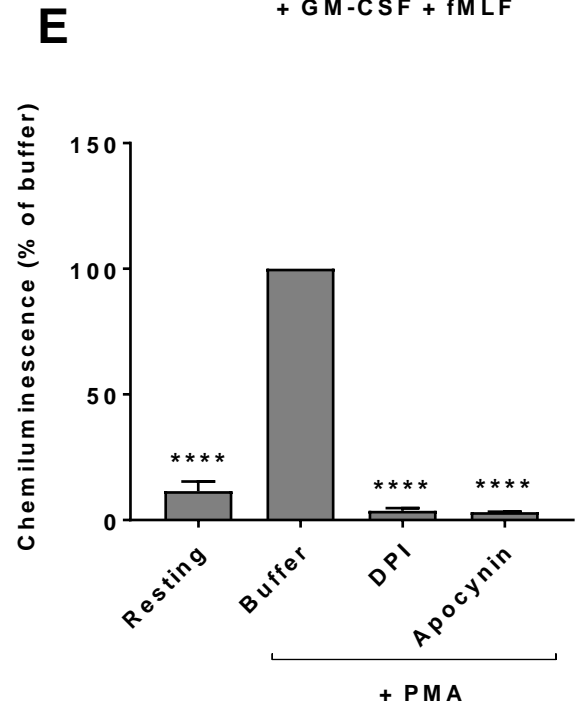
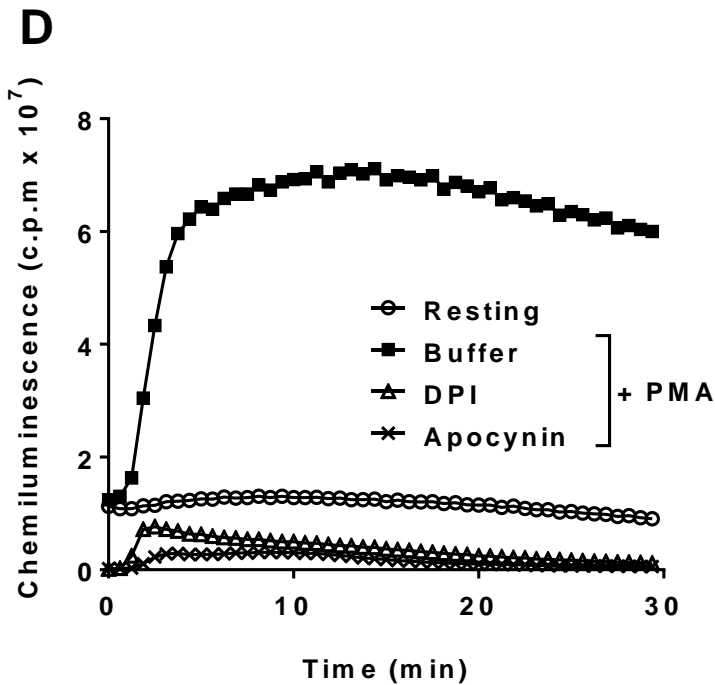
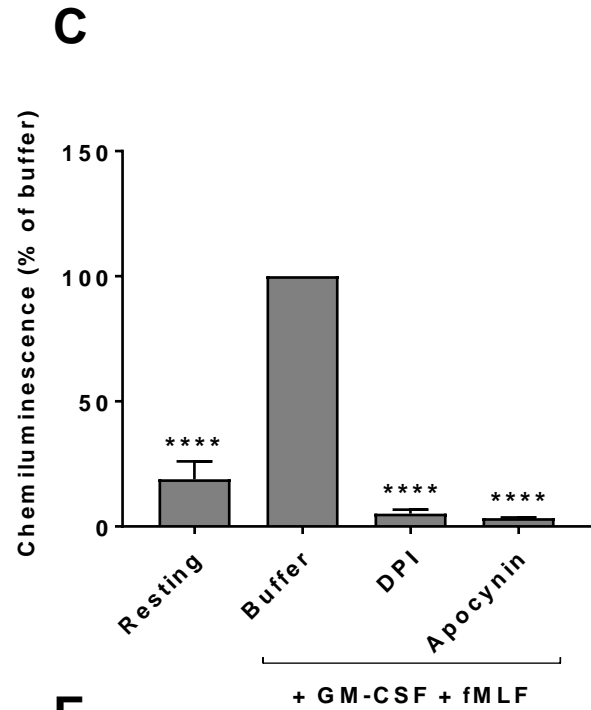
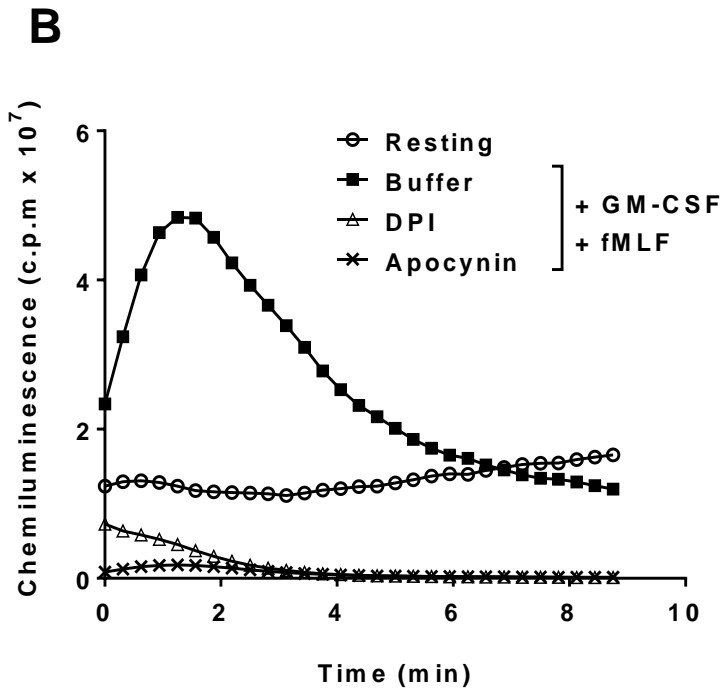
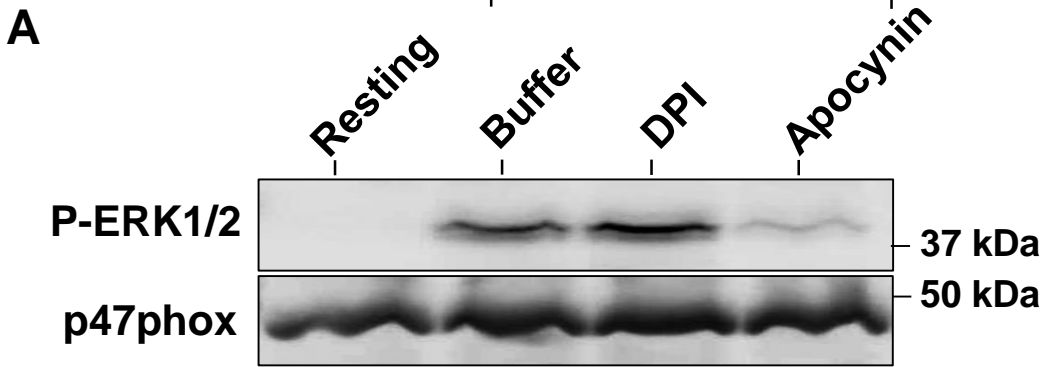
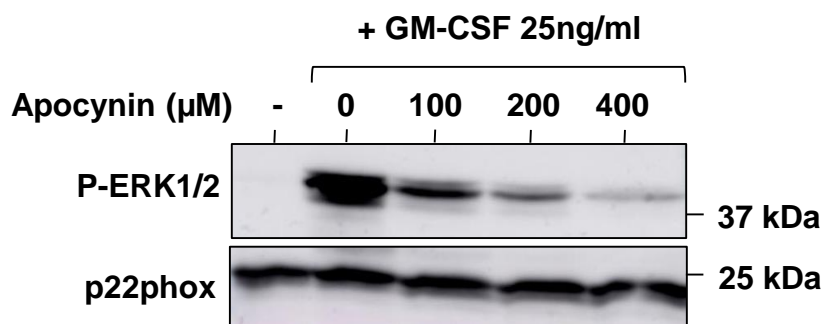
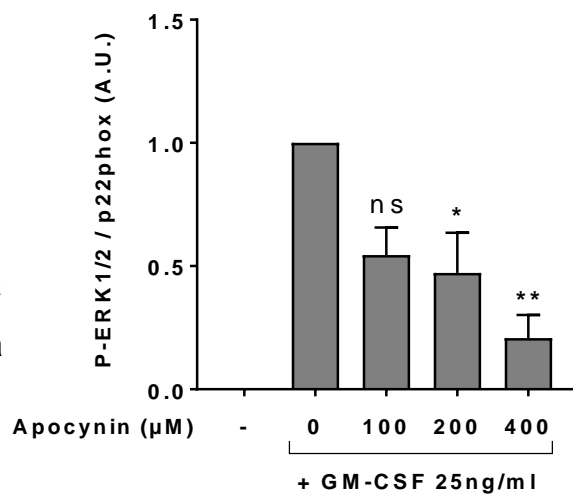
Fig. 1**GM-CSF**

Fig. 2

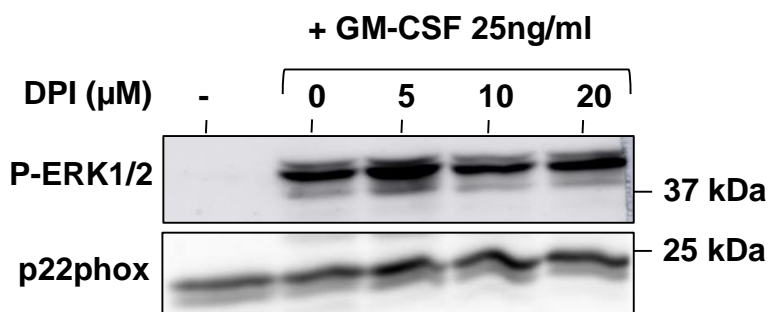
A



B



C



D

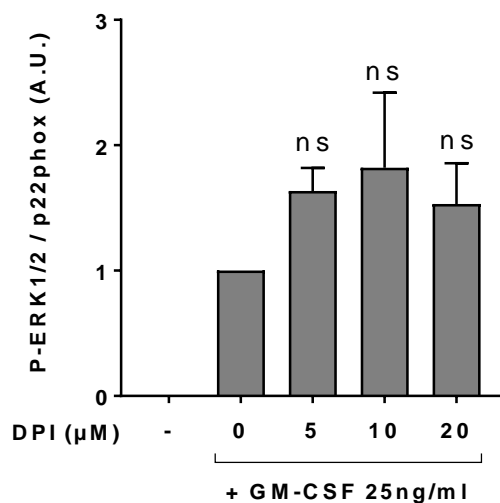


Fig. 3

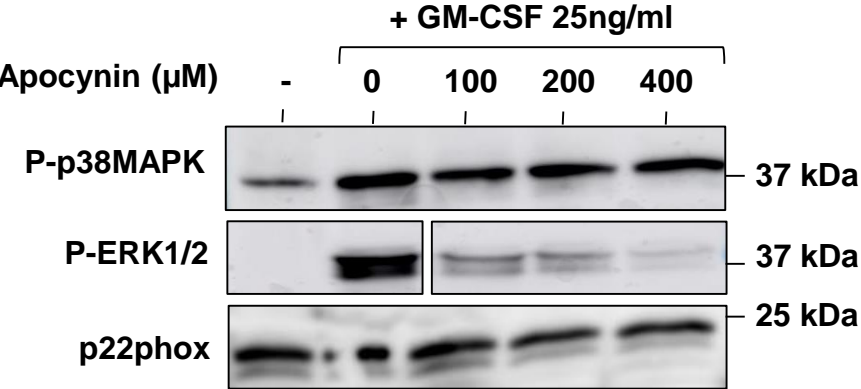
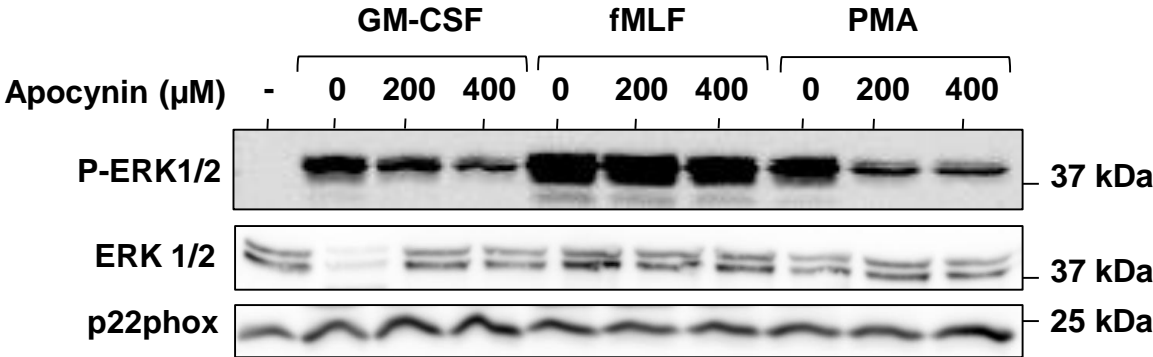


Fig. 4

A



B

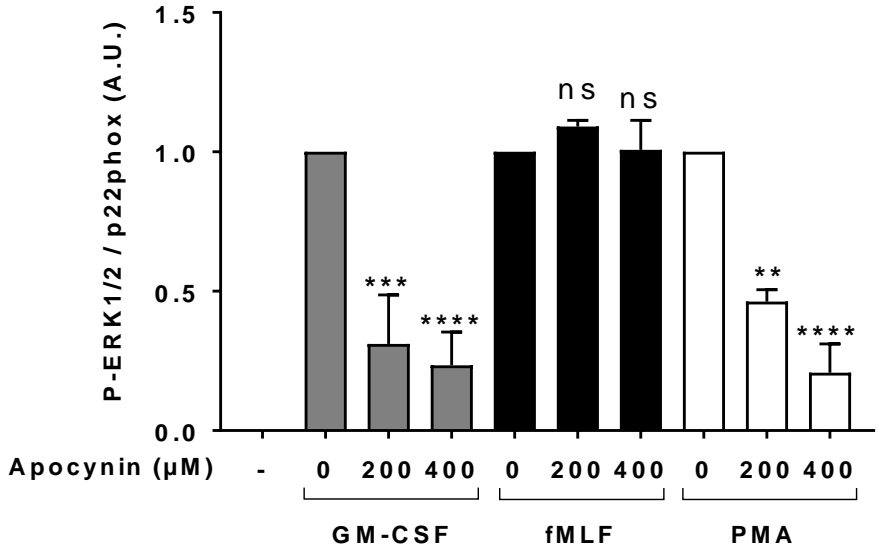
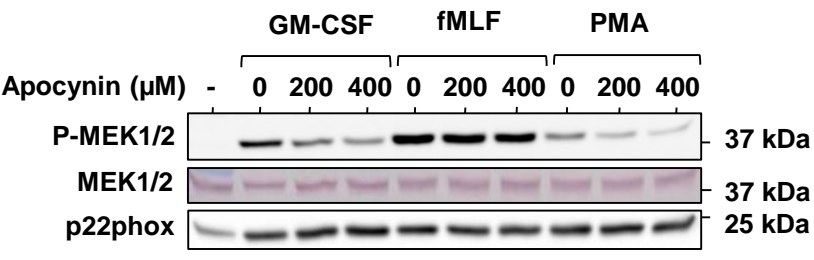
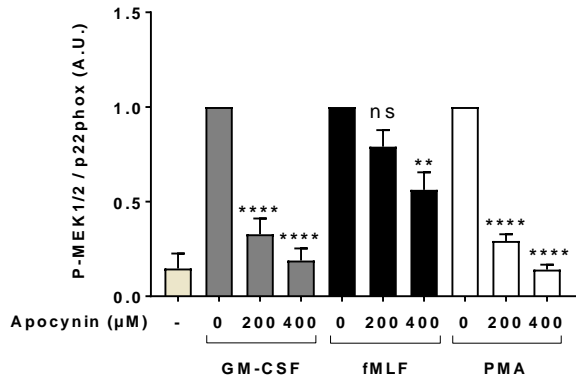


Fig. 5

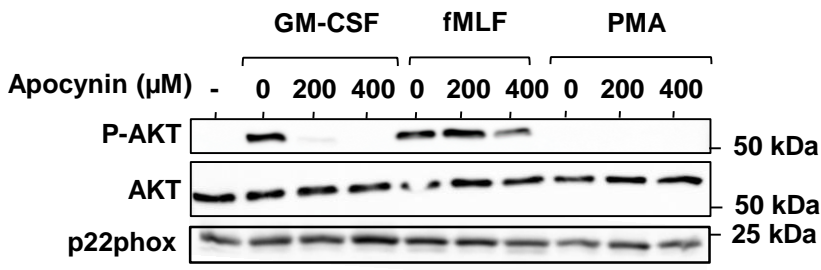
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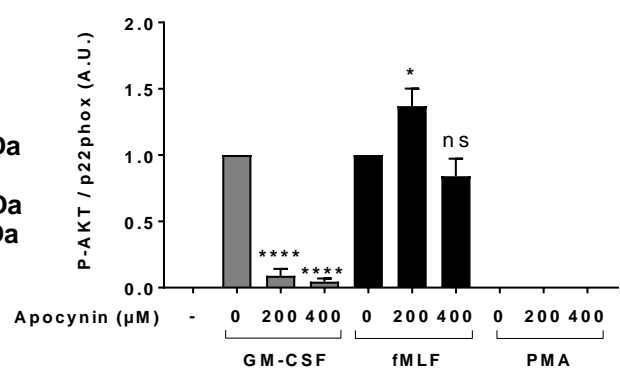
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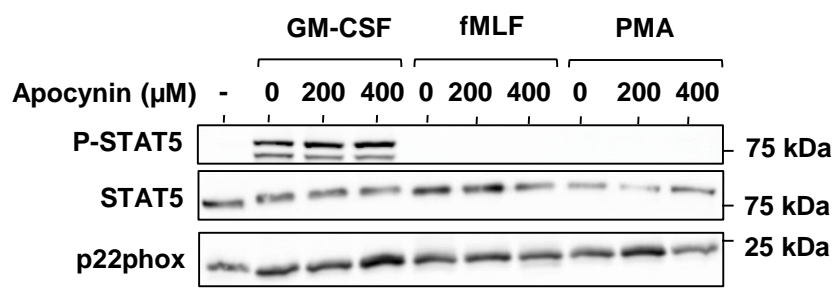
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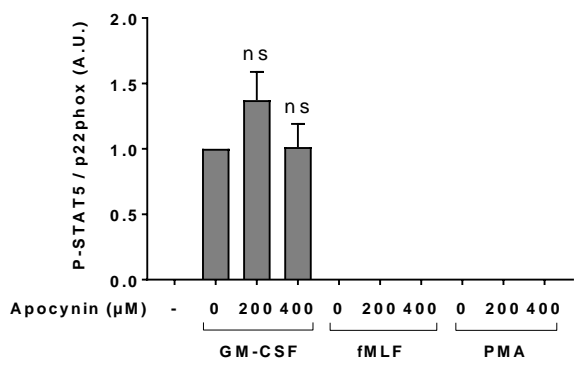
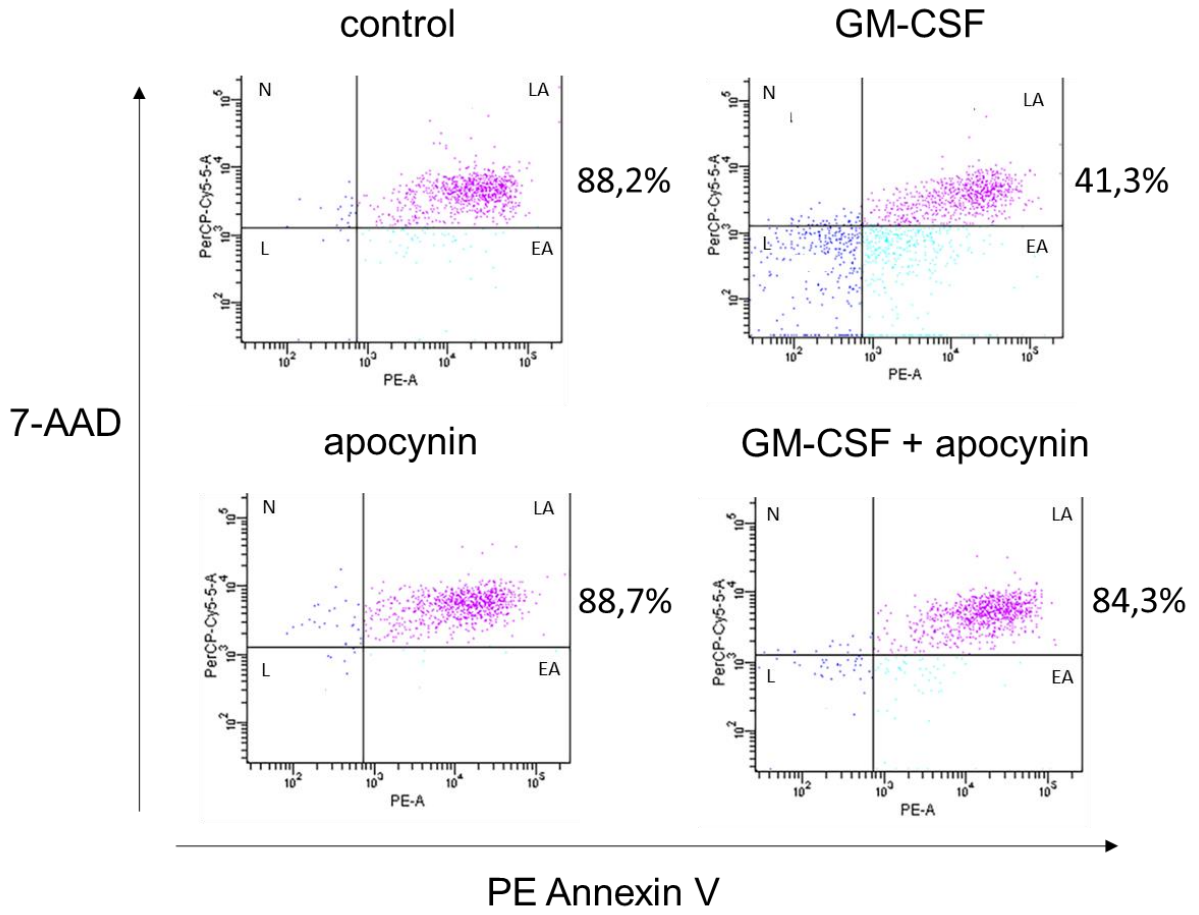


Fig. 6

A



B

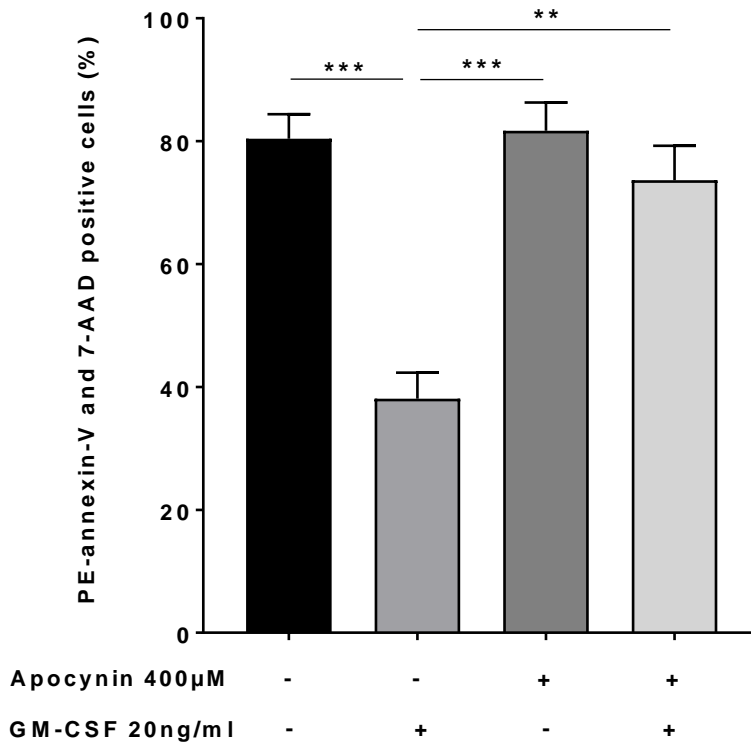
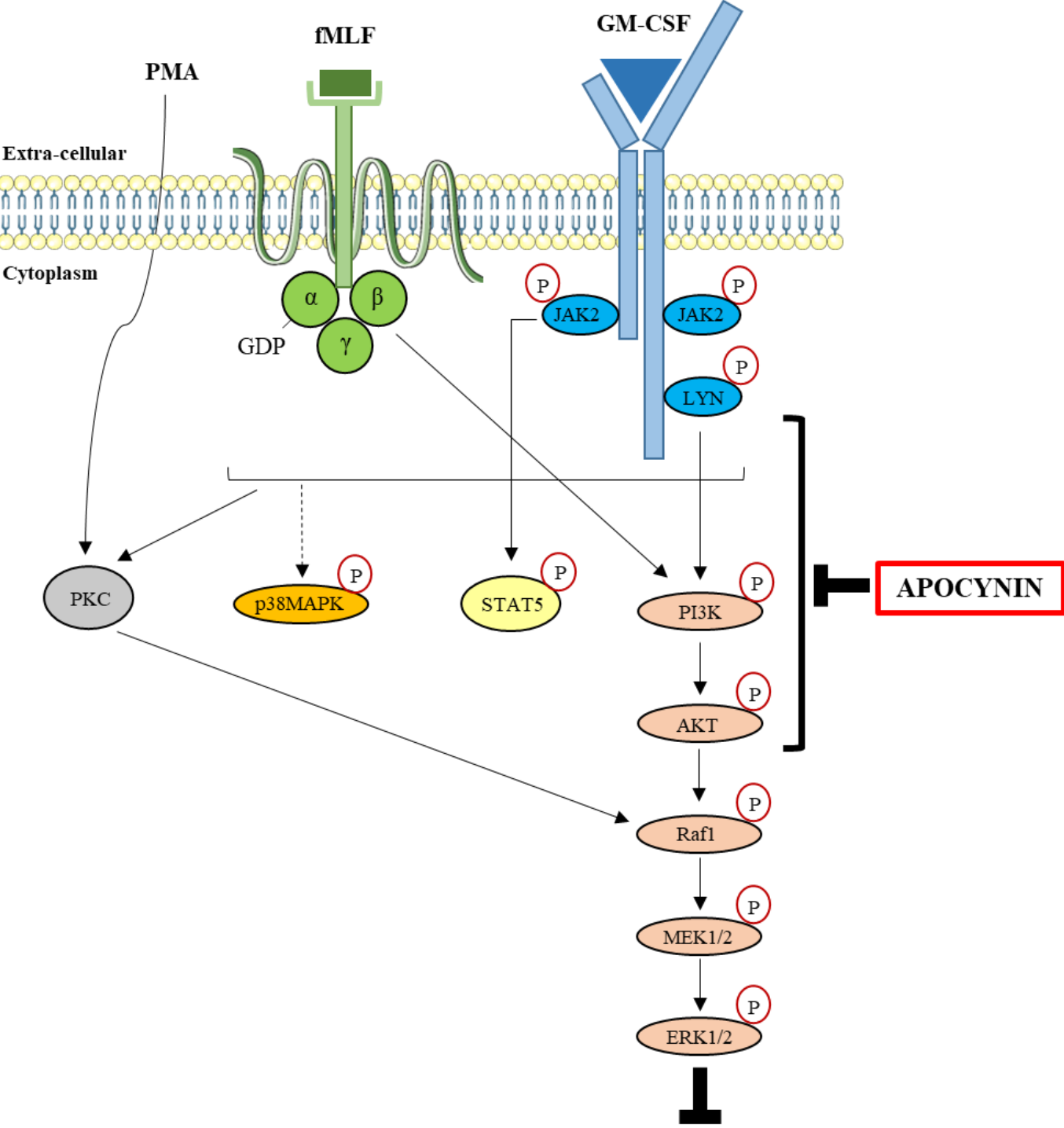


Fig. 7



anti-apoptotic effect of GM-CSF