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The resolution of acute inflammation induced by cyclic AMP is dependent on Annexin A1

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Running Title: *cAMP induces AnxA1-dependent inflammation resolution*

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Abstract

Annexin A1 (AnxA1) is a glucocorticoid-regulated protein known for its anti-inflammatory and proresolving effects. We have previously shown that cAMP enhancing compounds rolipram (ROL - a PDE4 inhibitor) and db-cAMP (cAMP mimetic) drive caspase-dependent resolution of neutrophilic inflammation. In this follow up study, we investigated whether AnxA1 could be involved in the proresolving properties of these compounds using a model of LPS-induced inflammation in BALB/c mice. The treatment with ROL or db-cAMP at the peak of inflammation shortened resolution intervals, improved resolution indices and increased AnxA1 expression. *In vitro* studies showed that ROL and db-cAMP induced AnxA1 expression and phosphorylation and this effect was prevented by PKA inhibitors, suggesting the involvement of PKA on ROL-induced AnxA1 expression. Akin to these *in vitro* findings, H89 prevented ROL and db-cAMP-induced resolution of inflammation, and it was associated with decreased levels of intact AnxA1. Moreover, two different strategies to block the AnxA1 pathway - by using BOC-1 a nonselective AnxA1 receptor antagonist or by using an anti-AnxA1 neutralizing antiserum - prevented ROL and db-cAMP-induced resolution and neutrophil apoptosis. Likewise, the ability of ROL or db-cAMP to induce neutrophil apoptosis was impaired in AnxA knockout mice. Finally, in *in vitro* settings ROL and db-cAMP overrode the survival-inducing effect of LPS in human neutrophils in an AnxA1dependent manner. Our results show that AnxA1 is at least one of the endogenous determinants mediating the proresolving properties of cAMP elevating agents and cAMP-mimetic drugs.

Annexin A1 (AnxA1, previously known as lipocortin-1) is a 37-kDa member of the

Annexin superfamily which is composed by proteins that bind to cellular membranes in a calcium-dependent manner. Originally described as an endogenous mediator of the anti-inflammatory effects of glucocorticoids, over the past 20 years AnxA1 has been shown to have a broad range of molecular and cellular actions, including modulation of leukocyte migration in acute and chronic inflammation, kinase activities in signal transduction, preservation of cytoskeleton and extracellular matrix integrity, tissue maintenance and apoptosis, cell growth and differentiation (1-5). AnxA1 is particularly abundant in cells of the myeloid lineage, including neutrophils, eosinophils, macrophages and mast cells (6). In resting cells, AnxA1 is by and large localized in the cytosol and, upon activation it can be secreted and then resynthesized. Once in the extracellular medium, this protein exerts autocrine, paracrine and juxtacrine effects which are mediated by the FPR2/ALX receptor (7-11).

AnxA1 exerts a variety of anti-inflammatory effects, including inhibition of leukocyte migration, direct inhibition of cytosolic phospholipase A2 (cPLA2), inhibition of COX-2 and iNOS expression and stimulation of IL-10 release (12-18). AnxA1 also possesses genuine proresolving properties by inducing neutrophil apoptosis (10,19,20) and increasing the clearance of apoptotic cells by efferocytosis (10,21-23). Both apoptosis and efferocytosis modulated by AnxA1 are crucial for resolution of inflammation (10,20).

In the context of the discovery of new mechanisms for known drugs, production and action of AnxA1 has been shown to be involved in the proresolution effects of histone deacetylase (HDAC) inhibitors (24), as well as in the anti-inflammatory effects of propofol (25) and cromoglycate-like compounds (6,26). We have previously shown that rolipram (ROL), a selective phosphodiesterase-4 (PDE4) inhibitor that increases intracellular levels of cyclic adenosine monophosphate (cAMP), induces resolution of neutrophilic inflammation and it was associated to increased accumulation of AnxA1 in inflammatory cells (20). However, the relevance and mechanisms underlying ROL-induced AnxA1 expression remain unknown.

In addition to the known anti-inflammatory properties of cAMP-elevating agents (27-29), emerging data support a role for cAMP in some steps of the resolution process (30-37). Indeed, cAMP elevation promoted by treatment with ROL or by cAMP mimetic drugs during established eosinophilic or neutrophilic inflammation induced resolution of inflammation via protein kinase A (PKA), the best known cAMP effector (3,32). Of note, modulation of cAMP may account for the proresolving abilities of melanocortins peptides (38) and lysophosphatidylserine (39). In this study, we investigated the ability of ROL and db-cAMP to modulate AnxA1 expression and wondered whether AnxA1 was involved in the proresolving ability of these compounds. Our results demonstrate that AnxA1 is induced by cAMP elevating agents and is indeed involved in the proresolving properties of these compounds, pointing them as potential therapeutic tools to control inflammatory diseases and induce resolution of inflammation.

Results

Rolipram and db-cAMP promote resolution of neutrophilic inflammation associated with increased AnxA1 expression. Initially, we evaluated whether ROL (PDE4 inhibitor) and db-cAMP (cAMP mimetic) would improve resolution indices during acute pleurisy. In this self-resolving model of inflammation the intrapleural injection of LPS induces a time-dependent influx of neutrophils into the pleural cavity of mice that is detectable at 2 h and reaches maximal at 8-24 h, decreasing thereafter with resolution occurring after 48hs, as previously reported (20,33,40,41). Therefore, we quantified the resolution interval (R_i) by defining profiles of acute inflammatory parameters (42,43). The treatment of mice with ROL or db-cAMP at the peak of LPS-inflammation significantly reduced the number of PMN recruited to the pleural cavity and shortened R_i in ~ 12 hours (Fig. 1A and 1B).

To investigate the potential relationship between cAMP and AnxA1, we carried out Western blot analysis in whole cell extracts recovered from the pleural cavity of mice treated 4 h after LPS challenge (when inflammatory cell influx was already established). Western blot was performed to quantify the overall AnxA1 content (ie. the sum of intracellularly localized or cell surface-bound). As seen in Fig 1C and 1D, treatment with ROL or db-cAMP decreased neutrophil numbers and increased levels of intact AnxA1 (Fig. 1E and 1F). AnxA1 was constitutively expressed on resident cells from the pleural cavity (PBS-injected). LPS injection

induced AnxA1 cleavage (as detected by presence of the 33 kDa breakdown product) and treatment with ROL or db-cAMP increased levels of intact AnxA1 (37 kDa form) and reduced AnxA1 cleavage in whole inflammatory extracts when compared to LPS.

Dexamethasone, used as an anti-inflammatory control drug, promoted resolution of neutrophilic inflammation and increased AnxA1 expression, as previously shown (20). Interesting, ROL was able to decrease neutrophil numbers and increase intact levels of AnxA1 as early as 1 h after treatment (Supplementary Fig. 1A and 1B).

The N-terminal region of AnxA1 is the major effector portion responsible for the anti-inflammatory action of the protein and its cleavage decreases the resolution-inducing effects of AnxA1 (10,44). Here, we observed that ROL partially decreased LPS-induced AnxA1 cleavage, as evidenced by the lower levels of the 33-kDa fragment when compared to LPS alone (Figure 1E). As AnxA1 can be cleaved by elastase *in vivo*, we evaluated whether inhibition of elastase activity by ROL could account for the observed effect. We found that ROL decreased LPS-induced elastase activity and expression in whole cell lysates recovered from pleural inflammatory cells (Supplementary Fig. 1C and 1D). The latter results suggest that ROL modulates AnxA1 levels at least by reducing its degradation.

Regulation of AnxA1 expression and phosphorylation by cAMP in macrophages. AnxA1 is present in many differentiated cell types (human and murine) but is particularly abundant in neutrophils, eosinophils, macrophages and mast cells (2,6). Protein expression can be modulated through several mechanisms such as the mobilization of intracellular pool of the protein to exportation and secretion, increased phosphorylation (45) or both. To explore the mechanisms underlying the effect of cAMP elevating agents on AnxA1 expression and localization, *in vitro* experiments using differentiated THP-1 cells, BMDMs and the murine macrophage cell line RAW264.7 were carried out. THP-1 was used in this work to evaluate the expression of AnxA1, since it has been shown to be a suitable cell line to study AnxA1 modulation (45,46). In these experimental settings, Dexa treatment induced dose-dependent induction of AnxA1 expression (data not show). As shown in the Fig 2, treatment of THP-1 cells with ROL increased AnxA1 levels in a concentration- (Fig 2A) and time- (Fig 2C) dependent fashion. This modulatory property was also observed in BMDM and RAW264.7 cells (Supplementary Fig 2A-C). For more quantitative data, similar experiments were performed to quantify AnxA1 message by qPCR. Significant increases in AnxA1 mRNA were observed in THP-1 cells treated with ROL, with optimal settings at 10 μM and 6 hours incubation (Fig 2B and 2D, respectively). Interesting, ROL also increased the level of phosphorylated AnxA1 in THP-1 cells (Fig 2E) and BMDMs (Supplementary Fig 2A). In line with the ability of ROL to inhibit degradation and increase intracellular levels of cAMP, there was strong phosphorylation of the CRE-binding protein (CREB), which followed the same kinetics of AnxA1 expression (Fig 2A and 2C). In accordance with the requirement of cAMP levels to induce AnxA1 expression, a cell permeable cAMP (db-cAMP) induced AnxA1 accumulation and phosphorylation (Fig 3A and 3B). Densitometry data for both, AnxA1 and P-AnxA1 were represented graphically (Fig 3C). We also measured the intracellular levels of cAMP in THP-1 cells after rolipram treatment (10μM) and we found an increase of 20% over basal levels, which return to the baseline 2h after. As expected for a cell permeable cAMP, db-cAMP greatly increased levels of intracellular cAMP 1h after cell treatment by 150% over basal, decreasing thereafter but still remain high until 2h (85% of increase over basal levels).

Moreover, Forskolin, a direct activator of adenylate cyclase, was also able to increase AnxA1 levels (Fig 3D). Noteworthy, physiological cAMP-elevating compounds such as PGE_2, norepinephrine and adenosine (36,47-51) were able to increase AnxA1 protein levels, as shown in the Supplementary Fig 3A-C. Likewise, monobutyryladenosine cAMP (6MB-cAMP), a membrane permeable analog of cAMP that activates protein kinase A and is resistant to degradation by phosphodiesterase, was able to increase AnxA1 accumulation as did db-cAMP (Supplementary Figure 3D). In contract, a naked cAMP or yet a cell-permeable cGMP analog (8-br-cGMP) did not increase AnxA1 accumulation, as analyzed by Western blot (See Supplementary Figure 3E).

Taken together, the data gathered so far suggested that ROL and other agents which elevate or mimic cAMP are acting in several

ways to regulate the dynamics of AnxA1 accumulation: they increase mRNA expression, protein accumulation and phosphorylation of AnxA1.

Rolipram and db-cAMP induce PKA-dependent AnxA1 expression. The promoter region of the AnxA1 gene contains one cAMP-responsive element (CRE) and this is functional since a CRE-binding protein (CREB) is required for either Dexa-induced or cAMP-induced AnxA1 synthesis (8,52). To investigate whether the observed effects of ROL occurred *via* PKA, the best-known cAMP downstream effector, THP-1 differentiated macrophages were treated with two PKA inhibitors, H89 (nonselective) or cAMPS-Rp (highly selective), 30 min before ROL or db-cAMP treatments. As shown in Supplementary Figure 4A-C, the blockade of PKA with H89 or cAMPS-Rp decreased AnxA1 mRNA and protein levels induced by both ROL and db-cAMP. Of note, the effect of PKA inhibitors on ROL-induced AnxA1 levels was also observed in RAW264.7 murine macrophages (Supplementary Fig 2C).

Since AnxA1 expression was associated to the proresolving role of cAMP (Fig 1) and the expression of AnxA1 *in vitro* was modulated *via* PKA (Supplementary Fig 2C and 4), we investigated if such pathway could also be engaged *in vivo*. In agreement with the *in vitro* findings, inhibition of PKA by H89 prevented ROL and db-cAMP-induced resolution of neutrophilic inflammation (Fig 4A and 4B) and this effect was associated with reduction of intact AnxA1 and increase of the cleaved form (Fig 4C and 4D).

A nonselective FPR antagonist prevents rolipram and db-cAMP-induced resolution of neutrophilic inflammation. FPR2/ALX, a G protein-coupled member of the formyl peptide receptor (FPR) family, conveys the biological functions of a variety of ligands, including the proresolving mediators AnxA1 and lipoxin A_4 (9). To investigate whether there was involvement of these receptors in our system, we used the non-selective antagonist BOC-1, which blocks also FPR1. Administration of BOC-1, before ROL or db-cAMP injection, prevented resolution of inflammation induced by these cAMP-elevating agents (Fig 5A and 5B) as seen by permanence of neutrophil and decreased apoptosis into the pleural cavity. Apoptosis was evaluated biochemically through Mcl-1 - the most important Bcl-2 family protein that governs neutrophil half-life (53,54) - (Fig 5C and 5D) and AnnexinV staining (Fig 5F) or by morphological criteria (Fig 5E). Mcl-1 is a key anti-apoptotic protein of the Bcl-2 family protein known to be modulated by ROL (33). Of note, treatment of mice with BOC-1 alone had no effect on neutrophil counts (data not show) and apoptosis (Fig 5E and 5F). Prevention of ROL-induced apoptosis by BOC-1 was associated with decreased levels of intact AnxA1 paralleled by increase of the cleaved form in cells from pleural exudates (Fig 5C and 5D).

Neutralization of endogenous AnxA1 prevents rolipram and db-cAMP-induced resolution of neutrophilic inflammation. Having established the effect of AnxA1 receptor blockade on ROL and db-cAMP-induced resolution (Fig 5), we evaluated the effects of an anti-AnxA1 neutralizing strategy by using a specific antiserum. The administration of the anti-AnxA1 antiserum prevented ROL-induced resolution (Fig 6A) and apoptosis, as assessed using either morphological criteria (Fig 6B and 6E) or biochemically by Mcl-1 (Fig 6C) and Annexin V staining (Fig 6D). Of note, treatment of mice with a goat nonimmune serum had no effect on the resolution of LPS-induced pleurisy (data not show) reinforcing previous report (20). AnxA1 neutralization was also able to prevent the effect of db-cAMP on neutrophil numbers (Fig 7A) and apoptosis (Fig 7B-E) similarly to the results obtained with ROL.

Furthermore, we carried out experiments using AnxA1 deficient mouse (24) and found results similar to those obtained by inhibition of AnxA1 actions with BOC-1 or AnxA1 neutralization. Indeed, the treatment with ROL or db-cAMP was able to induce neutrophil apoptosis in WT mice, and such an effect was impaired in AnxA KO mice (Fig 8). Therefore, we have shown by pharmacological and genetic strategies the importance of AnxA1 for the proresolving properties of ROL and db-cAMP.

Rolipram and db-cAMP override the survival-inducing effect of LPS in human neutrophils and such an effect is Anxa1 dependent. Neutrophil apoptosis is an integral modulatory mechanism that constrains inflammation and contributes to its successful resolution. The fate of neutrophils inside an inflammatory milieu - ie. whether they undergo apoptosis or remain viable - depends on the

balance of pro-survival stimuli such LPS, GM-CSF and oxygen availability, as well as the presence of pro-apoptotic stimuli including Fas ligand and TNF (55). Because ROL and db-cAMP induced neutrophil apoptosis in an inflammatory milieu *in vivo*, we investigated the ability of these cAMP elevating agents to counteract the prosurvival effects of LPS in vitro. As previously showed, LPS decreased the spontaneous apoptosis of cultured human neutrophils (41) and the treatment with ROL and db-cAMP prevented this effect, as evaluated by increased percentage of apoptotic neutrophils when comparing LPS-treated cells with LPS+ROL or LPS+db-cAMP (Fig 9A and 9G). There was no difference among the different doses used (Fig 9B and 9C). Sivelestat, a synthetic protease inhibitor, was used as a positive control for induction of neutrophil apoptosis (Fig 9A), as previously reported (41). In accordance with our *in vivo* data, the ability of ROL and db-cAMP to decrease the prosurvival effect of LPS was abolished by pretreatment with Anti-AnxA1 serum (Fig 9D and 9E) or by using WRW4, a selective FPR2 antagonist (Fig 9F). Therefore, our data show that cAMP elevating agents can effectively induce or accelerate a pro-apoptotic program in neutrophils leading to resolution of inflammation.

Discussion

Cyclic AMP is a fundamental second-messenger-molecule produced after adenylate cyclase activation in response to several stimuli, endowed with fundamental modulatory activities in cells involved in the inflammatory process, a property exerted primarily through PKA activity. Intracellular levels of cAMP result from a balance of modulatory pathways that involve elevation through agonist ligands (such as PGE2, adenosine and β-adrenergic drugs) and degradation by phosphodiesterases (PDEs) (27,28,56). There are different families of PDEs with various roles in different cells or tissues. The PDE4 isoenzyme family plays a particularly important role in the immune system and is the predominant PDE in inflammatory cells including mast cells, eosinophils, neutrophils, T cells and macrophages (56). Our group has previously shown that ROL and cAMP mimetics induce resolution of an established neutrophilic or eosinophilic inflammation (32,33) by inducing caspase-dependent apoptosis of polymorphonuclear cells.

In this follow up study, we reveal an important role for AnxA1 in the proresolving properties of ROL and db-cAMP, the cyclic AMP mimetic of choice. This conclusion is substantiated by the following major findings: (i) ROL and db-cAMP promoted resolution in a model of acute inflammation in mice challenged with LPS, and this process was associated with increased levels of intact AnxA1; (ii) ROL induced AnxA1 expression and phosphorylation in macrophages, an effect associated to CREB phosphorylation. Dibutyryl-cAMP, forskolin and physiological cAMP-elevating agents increased AnxA1 expression; (iii) The increase of AnxA1 induced by ROL was PKA-dependent in human (THP-1) and murine (RAW) macrophages; (iv) The effect of ROL and db-cAMP *in vivo* was via PKA as shown by using PKA inhibitors. The latter drugs not only prevented cAMP-induced resolution, but also prevented the increase in intact AnxA1 levels; (v) Two different pharmacological strategies were employed to inhibit the AnxA1 pathway, FPR antagonism and neutralizing AnxA1 antiserum: in both cases there was marked reduction of the resolution properties displayed by cAMP elevating agents. Importantly, in AnxA1 deficient mice, ROL or db-cAMP treatment could not induce neutrophil apoptosis. (vi) ROL and db-cAMP induced AnxA1-dependent apoptosis of human neutrophil in presence of prosurvival stimuli LPS. Therefore, our results show that the effects of ROL and db-cAMP on resolution of inflammation are at least in part due to modulation of AnxA1 expression, stabilization and mobilization to cell surface. These data identify AnxA1 as a proresolving molecule involved in pro-resolving actions of cAMP (Fig 10).

In addition to the role of AnxA1 in mediating anti-inflammatory properties of endogenous cortisol, AnxA1 is also an important mediator of anti-inflammatory and proresolving properties of pharmacological doses of glucocorticoids (20,57). During the initial steps of acute inflammation, AnxA1 limits the recruitment of leukocytes and the production of pro-inflammatory mediators (2). During the resolution phase, AnxA1 acts by promoting the apoptosis of neutrophils (10,20) and increasing their efferocytosis by macrophages (10,21). Recent studies indicate that modulation of AnxA1 disposition, levels and indeed

externalization in specific cell targets may represent a common mechanism evoked by anti-inflammatory agents, such as LXA_4 (58) and oestrogens (59). Interestingly, in this study we have found that physiological cAMP-elevating compounds such as adenosine increase AnxA1 protein levels. However, remain to be investigated whether AnxA1 account for the proresolving abilities of adenosine and which receptor is engaged to elicit this effect. Another group of drugs, HDAC inhibitors, endowed with multiple properties like the PDE4 inhibitor used here, also modulate AnxA1 expression and localization. Indeed, administration of HDAC inhibitors such as valproic acid and sodium butyrate at the peak of zymosan-induced peritonitis accelerated resolution in wild type mice, but much more modestly in AnxA1 null mice. These effects were consequence of the capacity of HDAC inhibitors to elevate AnxA1 levels, which then modulated leukocyte apoptosis and efferocytosis (24). During the resolving phase of LPS-inflammation, high levels of AnxA1 have been found in macrophages with resolutive phenotypes (40).

Phosphorylation and release of AnxA1 are central to the mechanism of action of the antiallergic cromoglycate-like drugs on mast cells and are essential for the inhibition of the release of histamine and PGD_2. The latter effects were abolished in the presence of neutralising anti-AnxA1 monoclonal antibody (6). In our studies, we demonstrated that ROL and db-cAMP promoted resolution of neutrophilic inflammation associated to high levels of intact AnxA1 and decreased levels of the cleaved form. Furthermore, ROL induced AnxA1 mRNA expression, accumulation and phosphorylation in macrophage lineages. More importantly, the blockage of the endogenous AnxA1 prevented the proresolving effects of these cAMP elevating agents and ROL and db-cAMP prevented the prosurvival effect of LPS on human neutrophils.

cAMP regulates apoptosis in several cell types, inhibiting or stimulating the process depending on the cell type and stage of differentiation (60). There are some *in vitro* studies using neutrophils that have shown that PDE4 inhibition or an increase of cAMP levels by other cAMP-increasing agents delay neutrophil apoptosis (61-64). In contrast with these *in vitro* studies, our group demonstrated that *in vivo* administration of ROL was clearly associated with resolution of neutrophilic inflammation by inducing caspase-3-dependent apoptosis (33). It is important to point out that our experimental settings were designed to investigate if these drugs could interfere with neutrophil accumulation (apoptosis+ efferocytosis/clearance) and not infiltration (migration), since we treated mice 4 h after LPS challenge, when inflammatory cell influx was already established. Indeed, we have previously shown that the neutrophil-active chemokines, such as CXCL1 and CXCL2 peak early in this model of inflammation (1-2h) and their levels are similar to basal at 4h after LPS injection (33). Also, injection of reparixin (an allosteric inhibitor of CXCR2) 4h-after LPS challenge failed to affect the accumulation of neutrophils, while in the same experiment, post-treatment with rolipram greatly decreased neutrophil accumulation in the cavity. Therefore, the effects of ROL and db-cAMP in our experimental settings are not in the migration process of the cells into the pleural cavity, since the cAMP elevating agents were given after the stimulus and after the peak of neutrophil-active chemokine production in the cavity (33).

Here, we went further and clearly showed that cAMP-elevating agents were able to induce AnxA1-dependent resolution of inflammation and increased neutrophil apoptosis associated with loss of Mcl-1. Importantly, the blockage of AnxA1 pathway prevented Mcl-1 loss and it was associated to neutrophil survival. The apoptotic effect of ROL and db-cAMP was also observed in cultured human neutrophils exposed to the prosurvival stimuli of LPS. The apparent contradictory actions of cAMP on isolated neutrophils of the previous studies (61-64) with our study can be explained because ROL and db-cAMP induce apoptosis in the presence of an inflammatory milieu, when these drugs were able to counteract the prosurvival stimuli such as LPS. These data presented here cluster with those generated with HDAC inhibitors and cromoglycate-like drugs to suggest that induction of AnxA1 may account for the anti-inflammatory and resolving mechanisms of a few known drugs. We propose that AnxA1 represents a central check-point mechanism regulating leukocyte survival and reactivity during on-going inflammatory reactions.

After cell activation, AnxA1 is externalized on the cell surface, the N-terminal region is exposed and interacts with its receptor named FPR2/ALX. Once in the extracellular

medium, AnxA1 can to be cleaved at the N-terminal region by proteases including NE and PR3, generating the 33-kDa isoform of poorly known properties. Intact AnxA1 (37 kDa) is the biological active form of the protein (13,44,65). Here, we showed that compounds which we have previously show to increase resolution of neutrophilic inflammation (33), are able to increase the levels of intact AnxA1 and prevent partially its degradation. A recent work of our group (66) showed that ROL could increase intact AnxA1 and prevent AnxA1 cleavage associated to ameliorated of inflammation of pneumococcal pneumonia. Interestingly, such an effect was more efficient when ROL was combined with the antibiotic ceftriaxone. Therefore, the effect of ROL on the cleaved levels of AnxA1 may be, at least in part, due to the decreased elastase activity and expression, and associated with decreased neutrophils number since elastase is an important protease present in neutrophils. In the inflammatory context, the decreased elastase levels may be important to resolution occurs. During the resolving phase of LPS-induced inflammation, in which was associated with decreased elastase expression and activity there were more intact AnxA1. Conversely, during the productive phase of inflammation there were high elastase expression/activity and higher proportion of AnxA1 cleavage. Indeed, inhibition of elastase by using synthetic (Sivelestat) or natural inhibitors (elafin or secretory leukocyte protease inhibitor - SLPI) was able to promote resolution of inflammation by protection of endogenous intact levels of AnxA1 and resulting neutrophil apoptosis (13,41,65). Furthermore, cleavage-resistant (CR) AnxA1 exhibited greater anti-inflammatory effect when compared with the parent protein in different animal models of inflammation (13,41,65).

The regulation of cAMP levels is a key feature to regulate a large number of events in the body (67). As a ubiquitous second messenger, cAMP regulates several processes in many cell types including cells from the immune system (30-32,34,35). Elevated cAMP levels were reported at the resolution point in a model of resolving peritonitis, and this was important to clear PMNs and regulate monocyte-derived macrophage functions (31). Sokolowska and colleagues (36) demonstrated that prostaglandin E_2, a potent lipid mediator involved in maintaining homeostasis, inhibits NLRP3 inflammasome activation through EPE4 receptor and an increase in intracellular cAMP in human macrophages. In teleost fish, prostaglandin E2 promoted M2 polarization macrophages via a cAMP/CREB signaling pathway (37). Bystrom and colleagues (30) examined whether macrophage phenotype was dictated by cAMP and whether this phenotype could be altered by changing intracellular levels of this potent intracellular second messenger. In M1 macrophages, TNFα production was attenuated by db-cAMP, whereas IL-10 production was increased, suggesting a reversion toward the anti-inflammatory or resolutive phenotype. Noteworthy, cAMP may function as an intermediate of the effects of other pro-resolving molecules, such as melanocortins peptides (38), lysophosphatidylserine (39) and resolving D1 (RvD1) (68,69). Recently it was reported the effect of cAMP on neutrophil extracellular trap (NET). Shishikura and colleagues have described the inhibitory action of PGE2 on PMA-induced NET formation *in vitro* through EP2 and EP4 Gαs-coupled receptors. Also, incubation with db-cAMP or inhibitors of PDE, also suppressed NET formation (70). Here, we described one more immunomodulatory function for cAMP, induction of AnxA1-dependent resolution of inflammation.

The biology of cAMP is mediated by downstream effector molecules and the most important one is protein kinase A (PKA): cAMP binds directly to PKA provoking a functional re-arrangement with enzymatic activity. PKA was shown to mediate, for example, the inhibition on macrophage inflammatory mediator generation induced by cAMP (71), and according to (32), the inhibition of PKA by H89 was able to limit the cAMP-mediated neutrophilic resolution. EPAC is another protein that, together with PKA, is the major binding partner of cAMP (72). In our model we investigated whether inhibition of PKA with H89 could inhibit the resolution induced by ROL and db-cAMP. In agreement with these previous studies, H89 reverted resolution induced by both ROL and db-cAMP *in vivo* and these events were followed by a decrease in the levels of intact AnxA1. Moreover, our *in vitro* experiments confirmed the non-redundant function of PKA since pre-treatment with H89 or a more selective cAMP-Rp thethylammonium, reduced cellular levels of AnxA1 below those measured in RAW264.7 or THP-1 cells treated with ROL alone. When this antagonist is used, the levels of AnxA1 are lower, what proves once again the importance of

cAMP to the action of ROL in this system. Altogether, these experiments led us to conclude that PKA is the major effector for cAMP in the processes evoked by ROL.

Obviously, by interfering with cAMP levels we may alter cGMP levels (because some PDEs hydrolyse both cAMP and cGMP, so PDEs that metabolize cGMP may be altered by intracellular cAMP levels) (73,74). In this sense, it will be import in the future to study the cross talk between cAMP and cGMP.

In conclusion, our study showed that cAMP elevating agents increase levels of AnxA1 and this is functionally involved in the pro-resolving abilities of cAMP. These results reinforce the hypothesis that AnxA1 acts at multiple regulatory levels to promote resolution of inflammation and may be a common mechanism that account for the pro-resolving actions of pro-resolving molecules. cAMP elevating drugs may represent a useful therapeutic strategy not only to block inflammatory processes (during onset of inflammation) but also, and equally important, to actively induce the mechanisms underlying the resolution of inflammation.

Experimental procedures

Animals. Male BALB/c mice (8-10 weeks) obtained from the Bioscience Unit of Instituto de Ciências Biológicas (Brazil) were housed under standard conditions of optimum light, temperature and humidity (12:12 h light–dark cycle, 22 ± 1°C, 50–60%) with food and water provided ad libitum. Annexin A1 knockout (BALB/c background) mice were generated as previously described (57) and bred at the Universidade Federal de Minas Gerais. All described procedures had prior approval from the Animal Ethics Committee of Universidade Federal de Minas Gerais (CEUA/UFMG, protocol number 15/2011).

Drugs, reagents and antibodies. Dibutyryl cAMP (db-cAMP), 6MB-cAMP, cAMP, Forskolin, Dexamethasone, Adenosine, (-)-norepinephrine, sodium butyrate, cGMP, Anti-β-actin (#A5316) and LPS (from Escherichia coli serotype O:111:B4) were from Sigma Aldrich (St. Louis, MO, USA). 8-Br-cGMP was from Calbiochem (EMD chemicals, San Diego, CA). Rolipram was purchased from Enzo Life Science (NY, USA). H89 dihydrochloride and cAMPS-Rp trethylammoniun salt were from Tocris (Ellisville, MO, USA). Prostaglandin E2 (PGE2) was from Cayman Chemical (Michigan, USA). Anti-AnxA1 antiserum was a king gift from Dr. Steve Poole (Biotherapeutics Group, National Institute for Biological Standards and Control, UK). Anti-AnxA1 (Sc-11387), anti-elastase (Sc-9521) or secondary anti-mouse (Sc-2005) peroxidase-conjugate antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti P-CREB (#9191) and secondary anti-rabbit peroxidase-conjugate antibody (#7074) were from Cell Signaling Technology (Danvers, MA, USA). We also used Anti-AnxA1 (#713400) from Invitrogen (Carlsbad, CA, USA). BOC-1 (N-t-Boc-Met-Leu-Phe) was from MP Biomedicals (California, USA). Polyclonal anti-Ser^{27}-AnxA1 antibody was generated as previously described (75).

LPS-induced pleurisy model and treatment with drugs. Mice received an intrapleural (i.pl.) injection of LPS (250 ng/cavity) or phosphate-buffered saline (PBS) as previously described (20,33). Four hours later, mice were treated with rolipram (6 mg/kg, i.p.), Dexa (2 mg/kg, i.p.) or with db-cAMP (4 mg/kg, i.pl.). These doses and route of administration were validated in our previous studies (20,32). AnxA1-knockout mice were also treated with these drug doses. To prevent the action of AnxA1, mice were treated with BOC-1, a nonselective AnxA1 receptor antagonist (5 mg/kg, i.p.) 30 min before the drugs or with anti-AnxA1 antiserum (0.1 mL hyperimmune serum diluted in 100 µL PBS/mice, i.p.) given 1 h before the challenge with LPS and again 1 h before ROL. In other cases, the PKA inhibitor H89 (4 mg/kg, i.pl.) was used. Compounds were diluted in DMSO or ethanol and further in PBS. db-cAMP was only diluted in PBS. Control mice received the respective vehicle only. Mice were euthanized by inhalation of CO_2. Cells in the pleural cavity were harvested by washing the cavity with 2 mL of PBS and total cell counts performed in a modified Neubauer chamber using Turk's stain. Differential cell counts were performed on cytocentrifuge preparations (Shandon Cytospin III) and the slides were stained with May-Grünwald-Giemsa using standard morphological criteria to identify cell types (20,33,57). Results are presented as the number of cells per cavity.

Calculation of resolution indices. We quantified the resolution indices as previously described (42,43). Murine pleural exudates were collected at 8, 24, 36 and 48 time points after LPS challenge. The number of PMN and mononuclear cells was determined by total and differential leukocyte counting. The resolution of acute inflammation was defined in quantitative terms by the following resolution indices: 1) magnitude (ψ_{max} and T_{max}), ψ_{max} (the maximal PMN numbers in the exudates), T_{max} (time point when PMN numbers reach maximum); 2) duration (T_{50}), T_{50} (time point when PMN numbers reduce to 50% of maximum) and 3) resolution interval R_i (the time period when 50% PMNs are lost from the pleural cavity, i.e., T_{50} -T_{max}).

Cell culture and in vitro assays. The human promonocytoid cell line THP-1 and murine macrophages RAW264.7 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). THP-1 cells were cultured in RPMI 1640 medium (Cultilab, São Paulo, Brazil) supplemented with 8% heat-inactivated fetal bovine serum (FBS) and antibiotics (Cultilab, São Paulo, Brazil) and RAW264.7 cells were cultured in DMEM (Cultilab, São Paulo, Brazil) in the same conditions. Cell cultures were maintained at 37˚C and 5% CO_2, and cell viability was determined using a trypan blue dye-exclusion assay. THP-1 cells were differentiated using 4α-phorbol 12-myristate 13-acetate (PMA - 20 ηg/mL, Sigma Aldrich, St. Louis, MO, USA) and deprived with FBS 1% for 24 h; subsequently cells were treated with drugs at different time intervals and concentrations as indicated in the specific figures. Dexamethasone (Dexa) was used as a positive control for AnxA1 induction.

cAMP measurements. cAMP levels in cellular extracts were measure using the cAMP Direct Immunoassay Kit, as described by the manufacturer (Cat#ab65355 Abcam, Cambridge, UK). Briefly, THP-1 cells were lysed completely with 0,1M HCl, following by centrifuging 10.000 RPM for 10 min. The supernatant was collected as the testing sample. To be ready for quantification, cAMP standards and samples were neutralized and acetylated using the neutralizing buffer and acetylating reagent supplied in the Kit, respectively. During the quantification, standard cAMP and testing samples were added to the Protein G coated 96-well plate. After blended with anti-cAMP antibody, the suspension was incubated for 1 h at room temperature with gentle agitation and for another hour with the adding of cAMP-HRP. Then the plate was washed for five times, following by incubation with HRP developer for 1 h. The reaction was stopped by 1M HCl and the absorbance was detected by a microtiter plate reader (Spectra Max 190, Molecular Devices) at 450 nm. The molar concentration of cAMP in cells was determined from standard curves generated using standard preparation. The cAMP levels were expressed by percentage above the control untreated cells.

Bone-marrow derived macrophages (BMDMs). Bone marrow cell suspensions were isolated by flushing femurs and tibias of 8-10 week BALB/c mice with complete DMEM (+10% FCS, + 1% Pen/Strep) and 20% L929 cell-conditioned medium (LCCM) as a source of macrophage colony-stimulating factor (M-CSF) (76). Aggregates were dislodged by gentle pipetting, and debris was removed by passaging the suspension through a cell strainer (BD Biosciences). Cells were seeded on 6-well plates and incubated at 37°C in a 5% CO_2 atmosphere. Five days after seeding, another 2 ml of DMEM containing 10% FBS and 20% LCCM was added. On the seventh day, cells were completely differentiated into macrophages. Cells were seeded on 24-well plates (5 x 10^5 cells/well) and later were pre incubated with rolipram (10 μM) for one hour and further stimulated with LPS (100 ng/mL) for 24 hours.

In vitro assay to evaluate neutrophil apoptosis. Neutrophils were isolated from human peripheral blood from healthy donors (Ethics Committee of the Universidade Federal de Minas Gerais, Brazil - Institutional Review Board Project number 0319.0.203.000-11) by using histopaque gradient (Histopaque 1119 and 1077 - from Sigma Chemicals, St. Louis, MO, USA) as described previously (41,77). Neutrophils (1 x 10^6 cells/well) were resuspended in RPMI 1640 medium, seeded in 96-well culture plates (BD Biosciences) and incubated at 37°C in a 5% CO_2 atmosphere. Cell viability was determined using a trypan blue dye-exclusion assay and the purity of

preparations was 95%. To evaluate the effect of ROL or db-cAMP on LPS-induced prosurvival/delayed apoptosis of neutrophils, isolated neutrophils were cultured in the presence of LPS (500 ng/mL) and 1 h after were treated with the drugs for further 5 h as indicated in the figures. In some experiments, neutrophils were pre-treated with an anti-AnxA1 antiserum (αAnxA1 - 100 μg/mL) or a selective antagonist of FPR2, WRW4 - 10 μM (number 344220; Calbiochem, San Diego, CA, USA) before addiction of LPS. Sivelestat - 100 μg/mL (number S7198; Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control for neutrophil apoptosis (41). Apoptosis was evaluated morphologically (as described above) and the experiments were performed in biological quadruplicates.

Assessment of leukocyte apoptosis. Apoptosis was assessed as previously reported (20,33). Briefly, cells (5 x 10^4) collected after LPS challenge or from in vitro experiments were cyto-centrifuged, fixed and stained with May-Grünwald-Giemsa and counted using oil immersion microscopy (x100 objective) to determine the proportion of cells with distinctive apoptotic morphology (cells with chromatin condensation, nuclear fragmentation and formation of apoptotic bodies out or inside macrophages). At least 500 cells were counted per slide and results are expressed as the mean ± SEM of percentage of cells with apoptotic morphology. Assessment of neutrophil apoptosis ($Ly6G^+/F4/80^-/AnxV^+/7AAD^-$) was also performed by flow cytometry using FITC-labeled annexin V and 7- aminoactinomycin D (BD Biosciences, San Jose, CA, USA) as previously reported (40,41). Antibodies used were F4/80 (PEcy7; eBioscience, San Diego, CA, USA) and Ly6G (V450; BD Bioscience, San Jose, CA, USA). Stained cells were acquired in BD FACSCanto II cell analyzer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Western blot analysis. Inflammatory cells harvested from the pleural cavity, THP-1, RAW264.7 or BMDMs were washed with PBS and whole cell extracts were prepared as described (32,78,79). The protein content of the lysate was determined by Bradford assay reagent (Bio-Rad, Hercules, CA, USA). Extracts (20 μg) were separated by electrophoresis on 10% SDS-PAGE and electrotransferred to nitrocellulose membranes, as described (78). Membranes were blocked overnight at 4°C with PBS containing 5% (w/v) nonfat dry milk and 0.1% Tween-20, washed three times with PBS containing 0.1% Tween-20 and then incubated with anti-AnxA1 (Santa Cruz, 1:1000 or Invitrogen, 1:3000), polyclonal anti-Ser^{27}-AnxA1 (1:1000), anti-P-CREB (1:1000), anti-Mcl-1 (1:1000), anti-elastase (1:1000) and anti-β-actin (1:5000) antibodies in PBS containing 5% (w/v) BSA and 0.1% Tween-20. After washing, membranes were incubated with appropriated horseradish peroxidase-conjugated secondary antibody (1:3000). Immunoreactive bands were visualized by using an ECL detection system, as described by the manufacturer (GE Healthcare, Piscataway, NJ). The values of AnxA1 or P-AnxA1 were quantified by using a densitometric analysis software (ImageJ, Image Processing and Analysis in Java; NIH, Bethesda, MD). Changes in protein levels were estimated by the control (untreated cells) and the results were expressed as fold increase of the arbitrary units of AnxA1 or P-AnxA1 normalized to the values of β-actin in the same sample.

RNA extraction and quantitative RT-PCR. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of RNA with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), following the manufacturer's recommended protocol. Synthesized cDNA was added to the relevant forward and reverse primer together with Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Real-time PCR was performed in duplicate, with 1 μL cDNA at a concentration of 100 ng, 0.5 mM primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, U.K.) using StepOne (Applied Biosystems, Foster City, CA, USA). The data were analyzed using StepOne Detection System software with a cycle threshold (Ct) in the linear range of amplification and then processed by the $2^{-\Delta\Delta Ct}$ method. Reactions were run in duplicates. Primers (IDT) used were as follows: human *AnxA1* (5'-ATCAGCGGTGAGCCCCTATC-3'/ 5'-TTCATCCAGGGGGCTTTCCTG-3') and human *Gapdh* (5'-AGAAGACTGTGGATGGCCCC-3'/ 5'-TGACCTTGCCCACAGCCTT-3'). A dissociation step was always included to confirm

the absence of unspecific products. Samples of all groups were run on one plate with two technical replicates. *Gapdh* was used as an endogenous control to normalize the variability in expression levels and results were expressed as fold increase.

Elastase activity assay. The elastase activity was measured in cell extracts prepared in the absence of proteases inhibitors by using an in-house procedure that relies on the use of MeO-Suc-AA-Pro-Val-pNA (M4765-Sigma Aldrich, St. Louis, MO, USA) as substrate. Cells obtained from pleural cavity of mice were lysed on appropriated buffer (200 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, pH 8.0). The lysate was centrifuged at 12.000 rpm in a microcentrifuge for 15 minutes at 4º C and supernatant (30 µL) was added to 20 µL of TBS (Tris-HCl, pH 8.0) and 50 µL of the substrate (1 mM) in a 96-well microplate. Following incubation for 2 h at 37º C, the absorbance of samples was analyzed in a spectrophotometer (Spectra Max 190, Molecular Devices) at 405 nm. A standard curve was performed with p-nitroanilide in accordance to the procedures supplied by the manufacturer (BioVision Inc., California, USA). The results are presented as elastase activity absorbance.

Statistical analysis. All results are presented as the mean ± SEM. Data were analyzed by One-way ANOVA, and differences between groups were assessed using the Student-Newman-Keuls post-test. A P value < 0.05 was considered significant. Calculations were performed using the Prism 5.0 software for Windows (GraphPad software, San Diego, CA).

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Conflicts of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions

LPS and MMT designed research, analyzed data and wrote the paper. KML and JPV performed the main experiments, analyzed data and helped to write the paper. RGA, BRCC, AAFC, KML, MAS and IG performed *in vitro* experiments. KML, TRC and FMS carried out PCR analyses. GLNL and LPT performed some *in vivo* experiments. VP provided expertise. ES provided the P-AnxA1 antibody and contributed to manuscript revision. MP provided guidance on experimental design and contributed to manuscript writing. All authors approved the final version of the manuscript.

References

1. Alessandri, A. L., Sousa, L. P., Lucas, C. D., Rossi, A. G., Pinho, V., and Teixeira, M. M. (2013) Resolution of inflammation: mechanisms and opportunity for drug development. *Pharmacology & therapeutics* **139**, 189-212
2. Perretti, M., and D'Acquisto, F. (2009) Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nat Rev Immunol* **9**, 62-70
3. Sousa, L. P., Alessandri, A. L., Pinho, V., and Teixeira, M. M. (2013) Pharmacological strategies to resolve acute inflammation. *Current opinion in pharmacology* **13**, 625-631
4. Gavins, F. N., and Hickey, M. J. (2012) Annexin A1 and the regulation of innate and adaptive immunity. *Frontiers in immunology* **3**, 354

5. Bizzarro, V., Belvedere, R., Dal Piaz, F., Parente, L., and Petrella, A. (2012) Annexin A1 induces skeletal muscle cell migration acting through formyl peptide receptors. *PLoS One* **7**, e48246
6. Yazid, S., Sinniah, A., Solito, E., Calder, V., and Flower, R. J. (2013) Anti-allergic cromones inhibit histamine and eicosanoid release from activated human and murine mast cells by releasing Annexin A1. *PloS one* **8**, e58963
7. Taylor, A. D., Philip, J. G., John, C. D., Cover, P. O., Morris, J. F., Flower, R. J., and Buckingham, J. C. (2000) Annexin 1 (lipocortin 1) mediates the glucocorticoid inhibition of cyclic adenosine 3',5'-monophosphate-stimulated prolactin secretion. *Endocrinology* **141**, 2209-2219
8. Castro-Caldas, M., Mendes, A. F., Duarte, C. B., and Lopes, M. C. (2003) Dexamethasone-induced and estradiol-induced CREB activation and annexin 1 expression in CCRF-CEM lymphoblastic cells: evidence for the involvement of cAMP and p38 MAPK. *Mediators of inflammation* **12**, 329-337
9. Bena, S., Brancaleone, V., Wang, J. M., Perretti, M., and Flower, R. J. (2012) Annexin A1 interaction with the FPR2/ALX receptor: identification of distinct domains and downstream associated signaling. *J Biol Chem* **287**, 24690-24697
10. Dalli, J., Consalvo, A. P., Ray, V., Di Filippo, C., D'Amico, M., Mehta, N., and Perretti, M. (2013) Proresolving and tissue-protective actions of annexin A1-based cleavage-resistant peptides are mediated by formyl peptide receptor 2/lipoxin A4 receptor. *J Immunol* **190**, 6478-6487
11. Cooray, S. N., Gobbetti, T., Montero-Melendez, T., McArthur, S., Thompson, D., Clark, A. J., Flower, R. J., and Perretti, M. (2013) Ligand-specific conformational change of the G-protein-coupled receptor ALX/FPR2 determines proresolving functional responses. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 18232-18237
12. Getting, S. J., Flower, R. J., and Perretti, M. (1997) Inhibition of neutrophil and monocyte recruitment by endogenous and exogenous lipocortin 1. *Br J Pharmacol* **120**, 1075-1082
13. Oliani, S. M., Paul-Clark, M. J., Christian, H. C., Flower, R. J., and Perretti, M. (2001) Neutrophil interaction with inflamed postcapillary venule endothelium alters annexin 1 expression. *The American journal of pathology* **158**, 603-615
14. Oh, J., Rhee, H. J., Kim, S., Kim, S. B., You, H., Kim, J. H., and Na, D. S. (2000) Annexin-I inhibits PMA-induced c-fos SRE activation by suppressing cytosolic phospholipase A2 signal. *FEBS Lett* **477**, 244-248
15. Wu, C. C., Croxtall, J. D., Perretti, M., Bryant, C. E., Thiemermann, C., Flower, R. J., and Vane, J. R. (1995) Lipocortin 1 mediates the inhibition by dexamethasone of the induction by endotoxin of nitric oxide synthase in the rat. *Proc Natl Acad Sci U S A* **92**, 3473-3477
16. Minghetti, L., Nicolini, A., Polazzi, E., Greco, A., Perretti, M., Parente, L., and Levi, G. (1999) Down-regulation of microglial cyclo-oxygenase-2 and inducible nitric oxide synthase expression by lipocortin 1. *Br J Pharmacol* **126**, 1307-1314
17. Ferlazzo, V., D'Agostino, P., Milano, S., Caruso, R., Feo, S., Cillari, E., and Parente, L. (2003) Anti-inflammatory effects of annexin-1: stimulation of IL-10 release and inhibition of nitric oxide synthesis. *Int Immunopharmacol* **3**, 1363-1369
18. Parente, L., and Solito, E. (2004) Annexin 1: more than an anti-phospholipase protein. *Inflammation research : official journal of the European Histamine Research Society ... [et al.]* **53**, 125-132
19. Solito, E., Kamal, A., Russo-Marie, F., Buckingham, J. C., Marullo, S., and Perretti, M. (2003) A novel calcium-dependent proapoptotic effect of annexin 1 on human neutrophils. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **17**, 1544-1546
20. Vago, J. P., Nogueira, C. R., Tavares, L. P., Soriani, F. M., Lopes, F., Russo, R. C., Pinho, V., Teixeira, M. M., and Sousa, L. P. (2012) Annexin A1 modulates natural and glucocorticoid-induced resolution of inflammation by enhancing neutrophil apoptosis. *Journal of leukocyte biology* **92**, 249-258

21. Maderna, P., Yona, S., Perretti, M., and Godson, C. (2005) Modulation of phagocytosis of apoptotic neutrophils by supernatant from dexamethasone-treated macrophages and annexin-derived peptide Ac(2-26). *J Immunol* **174**, 3727-3733
22. Scannell, M., Flanagan, M. B., deStefani, A., Wynne, K. J., Cagney, G., Godson, C., and Maderna, P. (2007) Annexin-1 and peptide derivatives are released by apoptotic cells and stimulate phagocytosis of apoptotic neutrophils by macrophages. *J Immunol* **178**, 4595-4605
23. Dalli, J., Jones, C. P., Cavalcanti, D. M., Farsky, S. H., Perretti, M., and Rankin, S. M. (2012) Annexin A1 regulates neutrophil clearance by macrophages in the mouse bone marrow. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **26**, 387-396
24. Montero-Melendez, T., Dalli, J., and Perretti, M. (2013) Gene expression signature-based approach identifies a pro-resolving mechanism of action for histone deacetylase inhibitors. *Cell death and differentiation* **20**, 567-575
25. Tang, J., Chen, X., Tu, W., Guo, Y., Zhao, Z., Xue, Q., Lin, C., Xiao, J., Sun, X., Tao, T., Gu, M., and Liu, Y. (2011) Propofol inhibits the activation of p38 through up-regulating the expression of annexin A1 to exert its anti-inflammation effect. *PLoS One* **6**, e27890
26. Yazid, S., Solito, E., Christian, H., McArthur, S., Goulding, N., and Flower, R. (2009) Cromoglycate drugs suppress eicosanoid generation in U937 cells by promoting the release of Anx-A1. *Biochem Pharmacol* **77**, 1814-1826
27. Teixeira, M. M., al-Rashed, S., Rossi, A. G., and Hellewell, P. G. (1997) Characterization of the prostanoid receptors mediating inhibition of PAF-induced aggregation of guinea-pig eosinophils. *British journal of pharmacology* **121**, 77-82
28. Soderling, S. H., and Beavo, J. A. (2000) Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. *Current opinion in cell biology* **12**, 174-179
29. Schudt, C., Hatzelmann, A., Beume, R., and Tenor, H. (2011) Phosphodiesterase inhibitors: history of pharmacology. *Handb Exp Pharmacol*, 1-46
30. Bystrom, J., Evans, I., Newson, J., Stables, M., Toor, I., van Rooijen, N., Crawford, M., Colville-Nash, P., Farrow, S., and Gilroy, D. W. (2008) Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by cAMP. *Blood* **112**, 4117-4127
31. Rajakariar, R., Newson, J., Jackson, E. K., Sawmynaden, P., Smith, A., Rahman, F., Yaqoob, M. M., and Gilroy, D. W. (2009) Nonresolving inflammation in gp91phox-/- mice, a model of human chronic granulomatous disease, has lower adenosine and cyclic adenosine 5'-monophosphate. *J Immunol* **182**, 3262-3269
32. Sousa, L. P., Carmo, A. F., Rezende, B. M., Lopes, F., Silva, D. M., Alessandri, A. L., Bonjardim, C. A., Rossi, A. G., Teixeira, M. M., and Pinho, V. (2009) Cyclic AMP enhances resolution of allergic pleurisy by promoting inflammatory cell apoptosis via inhibition of PI3K/Akt and NF-kappaB. *Biochemical pharmacology* **78**, 396-405
33. Sousa, L. P., Lopes, F., Silva, D. M., Tavares, L. P., Vieira, A. T., Rezende, B. M., Carmo, A. F., Russo, R. C., Garcia, C. C., Bonjardim, C. A., Alessandri, A. L., Rossi, A. G., Pinho, V., and Teixeira, M. M. (2010) PDE4 inhibition drives resolution of neutrophilic inflammation by inducing apoptosis in a PKA-PI3K/Akt-dependent and NF-kappaB-independent manner. *Journal of leukocyte biology* **87**, 895-904
34. Lee, H. N., and Surh, Y. J. (2013) Resolvin D1-mediated NOX2 inactivation rescues macrophages undertaking efferocytosis from oxidative stress-induced apoptosis. *Biochemical pharmacology* **86**, 759-769
35. Sheldon, K. E., Shandilya, H., Kepka-Lenhart, D., Poljakovic, M., Ghosh, A., and Morris, S. M., Jr. (2013) Shaping the murine macrophage phenotype: IL-4 and cyclic AMP synergistically activate the arginase I promoter. *J Immunol* **191**, 2290-2298
36. Sokolowska, M., Chen, L. Y., Liu, Y., Martinez-Anton, A., Qi, H. Y., Logun, C., Alsaaty, S., Park, Y. H., Kastner, D. L., Chae, J. J., and Shelhamer, J. H. (2015) Prostaglandin E2 Inhibits NLRP3 Inflammasome Activation through EP4 Receptor and Intracellular Cyclic AMP in Human Macrophages. *J Immunol* **194**, 5472-5487
37. Montero, J., Gomez-Abellan, V., Arizcun, M., Mulero, V., and Sepulcre, M. P. (2016) Prostaglandin E2 promotes M2 polarization of macrophages via a cAMP/CREB signaling pathway and deactivates granulocytes in teleost fish. *Fish & shellfish immunology*

38. Montero-Melendez, T., Patel, H. B., Seed, M., Nielsen, S., Jonassen, T. E., and Perretti, M. (2011) The melanocortin agonist AP214 exerts anti-inflammatory and proresolving properties. *The American journal of pathology* **179**, 259-269
39. Frasch, S. C., and Bratton, D. L. (2012) Emerging roles for lysophosphatidylserine in resolution of inflammation. *Progress in lipid research* **51**, 199-207
40. Vago, J. P., Tavares, L. P., Garcia, C. C., Lima, K. M., Perucci, L. O., Vieira, E. L., Nogueira, C. R., Soriani, F. M., Martins, J. O., Silva, P. M., Gomes, K. B., Pinho, V., Bruscoli, S., Riccardi, C., Beaulieu, E., Morand, E. F., Teixeira, M. M., and Sousa, L. P. (2015) The role and effects of glucocorticoid-induced leucine zipper in the context of inflammation resolution. *J Immunol* **194**, 4940-4950
41. Vago, J. P., Tavares, L. P., Sugimoto, M. A., Lima, G. L., Galvao, I., de Caux, T. R., Lima, K. M., Ribeiro, A. L., Carneiro, F. S., Nunes, F. F., Pinho, V., Perretti, M., Teixeira, M. M., and Sousa, L. P. (2016) Proresolving Actions of Synthetic and Natural Protease Inhibitors Are Mediated by Annexin A1. *J Immunol* **196**, 1922-1932
42. Bannenberg, G. L., Chiang, N., Ariel, A., Arita, M., Tjonahen, E., Gotlinger, K. H., Hong, S., and Serhan, C. N. (2005) Molecular circuits of resolution: formation and actions of resolvins and protectins. *J Immunol* **174**, 4345-4355
43. Chiang, N., Shinohara, M., Dalli, J., Mirakaj, V., Kibi, M., Choi, A. M., and Serhan, C. N. (2013) Inhaled carbon monoxide accelerates resolution of inflammation via unique proresolving mediator-heme oxygenase-1 circuits. *J Immunol* **190**, 6378-6388
44. Pederzoli-Ribeil, M., Maione, F., Cooper, D., Al-Kashi, A., Dalli, J., Perretti, M., and D'Acquisto, F. (2010) Design and characterization of a cleavage-resistant Annexin A1 mutant to control inflammation in the microvasculature. *Blood* **116**, 4288-4296
45. Yazid, S., Leoni, G., Getting, S. J., Cooper, D., Solito, E., Perretti, M., and Flower, R. J. (2010) Antiallergic cromones inhibit neutrophil recruitment onto vascular endothelium via annexin-A1 mobilization. *Arterioscler Thromb Vasc Biol* **30**, 1718-1724
46. Tabe, Y., Jin, L., Contractor, R., Gold, D., Ruvolo, P., Radke, S., Xu, Y., Tsutusmi-Ishii, Y., Miyake, K., Miyake, N., Kondo, S., Ohsaka, A., Nagaoka, I., Andreeff, M., and Konopleva, M. (2007) Novel role of HDAC inhibitors in AML1/ETO AML cells: activation of apoptosis and phagocytosis through induction of annexin A1. *Cell death and differentiation* **14**, 1443-1456
47. Rich, T. C., Xin, W., Mehats, C., Hassell, K. A., Piggott, L. A., Le, X., Karpen, J. W., and Conti, M. (2007) Cellular mechanisms underlying prostaglandin-induced transient cAMP signals near the plasma membrane of HEK-293 cells. *American journal of physiology. Cell physiology* **292**, C319-331
48. Ding, L., Zhang, F., Zhao, M. X., Ren, X. S., Chen, Q., Li, Y. H., Kang, Y. M., and Zhu, G. Q. (2016) Reduced lipolysis response to adipose afferent reflex involved in impaired activation of adrenoceptor-cAMP-PKA-hormone sensitive lipase pathway in obesity. *Scientific reports* **6**, 34374
49. Yang, J. H., Lee, E. O., Kim, S. E., Suh, Y. H., and Chong, Y. H. (2012) Norepinephrine differentially modulates the innate inflammatory response provoked by amyloid-beta peptide via action at beta-adrenoceptors and activation of cAMP/PKA pathway in human THP-1 macrophages. *Experimental neurology* **236**, 199-206
50. Takahashi, H. K., Liu, K., Wake, H., Mori, S., Zhang, J., Liu, R., Yoshino, T., and Nishibori, M. (2009) Prostaglandin E2 inhibits advanced glycation end product-induced adhesion molecule expression, cytokine production, and lymphocyte proliferation in human peripheral blood mononuclear cells. *The Journal of pharmacology and experimental therapeutics* **331**, 656-670
51. Minguet, S., Huber, M., Rosenkranz, L., Schamel, W. W., Reth, M., and Brummer, T. (2005) Adenosine and cAMP are potent inhibitors of the NF-kappa B pathway downstream of immunoreceptors. *European journal of immunology* **35**, 31-41
52. Antonicelli, F., De Coupade, C., Russo-Marie, F., and Le Garrec, Y. (2001) CREB is involved in mouse annexin A1 regulation by cAMP and glucocorticoids. *European journal of biochemistry* **268**, 62-69

53. Murphy, M. P., and Caraher, E. (2015) Mcl-1 is vital for neutrophil survival. *Immunologic research* **62**, 225-233
54. Milot, E., and Filep, J. G. (2011) Regulation of neutrophil survival/apoptosis by Mcl-1. *TheScientificWorldJournal* **11**, 1948-1962
55. Jones, H. R., Robb, C. T., Perretti, M., and Rossi, A. G. (2016) The role of neutrophils in inflammation resolution. *Seminars in immunology* **28**, 137-145
56. Page, C. P., and Spina, D. (2011) Phosphodiesterase inhibitors in the treatment of inflammatory diseases. *Handbook of experimental pharmacology*, 391-414
57. Hannon, R., Croxtall, J. D., Getting, S. J., Roviezzo, F., Yona, S., Paul-Clark, M. J., Gavins, F. N., Perretti, M., Morris, J. F., Buckingham, J. C., and Flower, R. J. (2003) Aberrant inflammation and resistance to glucocorticoids in annexin 1-/- mouse. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **17**, 253-255
58. Brancaleone, V., Dalli, J., Bena, S., Flower, R. J., Cirino, G., and Perretti, M. (2011) Evidence for an anti-inflammatory loop centered on polymorphonuclear leukocyte formyl peptide receptor 2/lipoxin A4 receptor and operative in the inflamed microvasculature. *J Immunol* **186**, 4905-4914
59. Nadkarni, S., Cooper, D., Brancaleone, V., Bena, S., and Perretti, M. (2011) Activation of the annexin A1 pathway underlies the protective effects exerted by estrogen in polymorphonuclear leukocytes. *Arterioscler Thromb Vasc Biol* **31**, 2749-2759
60. Parvathenani, L. K., Buescher, E. S., Chacon-Cruz, E., and Beebe, S. J. (1998) Type I cAMP-dependent protein kinase delays apoptosis in human neutrophils at a site upstream of caspase-3. *The Journal of biological chemistry* **273**, 6736-6743
61. Rossi, A. G., Cousin, J. M., Dransfield, I., Lawson, M. F., Chilvers, E. R., and Haslett, C. (1995) Agents that elevate cAMP inhibit human neutrophil apoptosis. *Biochemical and biophysical research communications* **217**, 892-899
62. Martin, M. C., Dransfield, I., Haslett, C., and Rossi, A. G. (2001) Cyclic AMP regulation of neutrophil apoptosis occurs via a novel protein kinase A-independent signaling pathway. *The Journal of biological chemistry* **276**, 45041-45050
63. Krakstad, C., Christensen, A. E., and Doskeland, S. O. (2004) cAMP protects neutrophils against TNF-alpha-induced apoptosis by activation of cAMP-dependent protein kinase, independently of exchange protein directly activated by cAMP (Epac). *Journal of leukocyte biology* **76**, 641-647
64. Parkkonen, J., Hasala, H., Moilanen, E., Giembycz, M. A., and Kankaanranta, H. (2008) Phosphodiesterase 4 inhibitors delay human eosinophil and neutrophil apoptosis in the absence and presence of salbutamol. *Pulmonary pharmacology & therapeutics* **21**, 499-506
65. Vong, L., D'Acquisto, F., Pederzoli-Ribeil, M., Lavagno, L., Flower, R. J., Witko-Sarsat, V., and Perretti, M. (2007) Annexin 1 cleavage in activated neutrophils: a pivotal role for proteinase 3. *The Journal of biological chemistry* **282**, 29998-30004
66. Tavares, L. P., Garcia, C. C., Vago, J. P., Queiroz-Junior, C. M., Galvao, I., David, B. A., Rachid, M. A., Silva, P. M., Russo, R. C., Teixeira, M. M., and Sousa, L. P. (2016) Inhibition of Phosphodiesterase-4 during Pneumococcal Pneumonia Reduces Inflammation and Lung Injury in Mice. *American journal of respiratory cell and molecular biology* **55**, 24-34
67. Lefkimmiatis, K., and Zaccolo, M. (2014) cAMP signaling in subcellular compartments. *Pharmacology & therapeutics* **143**, 295-304
68. Hsiao, H. M., Sapinoro, R. E., Thatcher, T. H., Croasdell, A., Levy, E. P., Fulton, R. A., Olsen, K. C., Pollock, S. J., Serhan, C. N., Phipps, R. P., and Sime, P. J. (2013) A novel anti-inflammatory and pro-resolving role for resolvin D1 in acute cigarette smoke-induced lung inflammation. *PLoS One* **8**, e58258
69. Titos, E., Rius, B., González-Périz, A., López-Vicario, C., Morán-Salvador, E., Martínez-Clemente, M., Arroyo, V., and Clària, J. (2011) Resolvin D1 and its precursor docosahexaenoic acid promote resolution of adipose tissue inflammation by eliciting macrophage polarization toward an M2-like phenotype. *J Immunol* **187**, 5408-5418

70. Shishikura, K., Horiuchi, T., Sakata, N., Trinh, D. A., Shirakawa, R., Kimura, T., Asada, Y., and Horiuchi, H. (2016) Prostaglandin E2 inhibits neutrophil extracellular trap formation through production of cyclic AMP. *British journal of pharmacology* **173**, 319-331
71. Aronoff, D. M., Carstens, J. K., Chen, G. H., Toews, G. B., and Peters-Golden, M. (2006) Short communication: differences between macrophages and dendritic cells in the cyclic AMP-dependent regulation of lipopolysaccharide-induced cytokine and chemokine synthesis. *J Interferon Cytokine Res* **26**, 827-833
72. Tiwari, S., Felekkis, K., Moon, E. Y., Flies, A., Sherr, D. H., and Lerner, A. (2004) Among circulating hematopoietic cells, B-CLL uniquely expresses functional EPAC1, but EPAC1-mediated Rap1 activation does not account for PDE4 inhibitor-induced apoptosis. *Blood* **103**, 2661-2667
73. Maurice, D. H., Ke, H., Ahmad, F., Wang, Y., Chung, J., and Manganiello, V. C. (2014) Advances in targeting cyclic nucleotide phosphodiesterases. *Nature reviews. Drug discovery* **13**, 290-314
74. Ahmad, F., Murata, T., Shimizu, K., Degerman, E., Maurice, D., and Manganiello, V. (2015) Cyclic nucleotide phosphodiesterases: important signaling modulators and therapeutic targets. *Oral diseases* **21**, e25-50
75. Solito, E., Mulla, A., Morris, J. F., Christian, H. C., Flower, R. J., and Buckingham, J. C. (2003) Dexamethasone induces rapid serine-phosphorylation and membrane translocation of annexin 1 in a human folliculostellate cell line via a novel nongenomic mechanism involving the glucocorticoid receptor, protein kinase C, phosphatidylinositol 3-kinase, and mitogen-activated protein kinase. *Endocrinology* **144**, 1164-1174
76. Englen, M. D., Valdez, Y. E., Lehnert, N. M., and Lehnert, B. E. (1995) Granulocyte/macrophage colony-stimulating factor is expressed and secreted in cultures of murine L929 cells. *J Immunol Methods* **184**, 281-283
77. Lucas, C. D., Allen, K. C., Dorward, D. A., Hoodless, L. J., Melrose, L. A., Marwick, J. A., Tucker, C. S., Haslett, C., Duffin, R., and Rossi, A. G. (2013) Flavones induce neutrophil apoptosis by down-regulation of Mcl-1 via a proteasomal-dependent pathway. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **27**, 1084-1094
78. Sousa, L. P., Silva, B. M., Brasil, B. S., Nogueira, S. V., Ferreira, P. C., Kroon, E. G., Kato, K., and Bonjardim, C. A. (2005) Plasminogen/plasmin regulates alpha-enolase expression through the MEK/ERK pathway. *Biochem Biophys Res Commun* **337**, 1065-1071
79. Souza, D. G., Vieira, A. T., Pinho, V., Sousa, L. P., Andrade, A. A., Bonjardim, C. A., McMillan, M., Kahn, M., and Teixeira, M. M. (2005) NF-kappaB plays a major role during the systemic and local acute inflammatory response following intestinal reperfusion injury. *Br J Pharmacol* **145**, 246-254

Footnotes

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[2] KML and JPV are equally contributed authors.

[3] The Abbreviations used are: AnxA1,annexin A1; αAnxA1, anti-AnxA1 antiserum; BOC-1, N-t-Boc-Met-Leu-Phe; cAMP, cyclic adenosine monophosphate, CRE, cAMP-responsive element; CREB, cAMP-responsive element binding protein; db-cAMP, dibutyryl cyclic AMP; Dexa, dexamethasone; DMSO, dimethyl sulfoxide; FPR2/ALX, formyl peptide receptor 2/ lipoxin A_4 receptor; GC, glucocorticoid; i.pl., intrapleural; i.p., intraperitonial; PKA, protein kinase A; PMA, 4α-phorbol 12-myristate 13-acetate; ROL, rolipram; WRW4, Trp-Arg-Trp-Trp-Trp-NH_2.

Figure legends

Figure 1. Effect of the treatment with rolipram and db-cAMP on resolution of acute inflammation. Evaluation of resolution indices: mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and 8 h later received an injection of ROL (6 mg/kg, i.p.) or db-cAMP (4 mg/kg, i.pl.). Pleural wash were performed at various time points after LPS injection and neutrophils were count from cytospin preparations (*A*) to calculate resolution indices (*B*). In C-F, mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and 4 h later received an injection of ROL or db-cAMP at the same dose of A, or Dexa (2 mg/kg, i.p.) as a control. The cells from pleural cavity were harvested and processed for neutrophils count (*C, D*) and western blot analysis (*E, F*) for detection of AnxA1 4 h later drug treatment, i.e., 8 h later LPS-challenge. Two different exposures time of the cleaved band of AnxA1 immunoblot are presented. Results are expressed as number of neutrophils/cavity and are shown as the mean ± SEM of at least five mice in each group. ***P < 0.001 when compared with PBS-injected mice; $^{\#\#}P < 0.01$ or $^{\#\#\#}P < 0.001$ when compared with LPS-challenged mice. For loading control, membranes were reprobed with anti-β-actin. Blots are representative of three independent experiments using pooled cells from at least five animals in each experiment.

Figure 2. Effect of rolipram on AnxA1 mRNA expression, protein levels and phosphorylation in THP-1 differentiated macrophages. Cells were differentiated using PMA (20 ng/mL) and serum deprived for 24 h. Later, the cells were untreated or treated with ROL at increasing concentrations for 6 h (*A, B, E*), or different time intervals (*C, D, E*) as indicated in the Figure. Whole cell extracts were obtained and subjected to Western blot analysis (*A, C, E*) to assess for AnxA1, *Ser*^{27}-phospho-AnxA1, and Phospho-CREB levels (as a marker of PKA activation) or for quantitative RT-PCR (*B, D*). For loading control, membranes were reprobed with anti-β-actin. Blots are representative of three independent experiments. qRT-PCR data were performed in biological triplicates performed with two technical replicates. The results are presented as fold increase of mRNA expression relative to the amount present in control samples. Data are mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$, when compared to untreated cells; $^{\#\#\#}P < 0.001$ when compared to ROL treatment at 10 µM or 6 h.

Figure 3. Effect of db-cAMP and Forskolin on AnxA1 expression and phosphorylation in THP-1 differentiated macrophages. Cells were differentiated using PMA (20 ng/mL) and serum deprived for 24 h. After starvation, the cells were untreated or treated with db-cAMP (*A, B*) or Forskolin (*D*) at different concentrations (6 h) and times as indicated in the Figures. Total cell extracts were obtained and subjected to Western blot analysis to assess for AnxA1 (*A, B, D*) or *Ser*^{27}-phospho-AnxA1 (*A*). Densitometry data are presented graphically in the panel *C*. For loading control, membranes were reprobed with anti-β-actin. Blots are representative of three independent experiments. Data are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, when compared to untreated cells.

Figure 4. Rolipram and db-cAMP resolve neutrophilic inflammation in a PKA-dependent manner. Mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and 4 h later received an injection of ROL (6 mg/kg, i.p.) or db-cAMP (4 mg/kg, i.pl.). Two groups of mice were pre-treated 30 min with H89 (4 mg/kg, i.pl.) before the drugs. The cells from pleural cavity were harvested and processed to neutrophils count (*A, B*) and western blot (*C, D*) for detection of AnxA1 4 h after drug treatment, i.e., 8 h after LPS challenge. Two different exposures time of the cleaved band of AnxA1 immunoblot are presented. Results are expressed as the number of neutrophils/cavity and are shown as the mean ± SEM of at least five mice in each group. $^{***}P < 0.001$ when compared to PBS-injected mice; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ when compared to LPS-challenged mice. Comparison between the groups H89 and H89+drugs are highlighted in the graphics. For loading control, membranes were reprobed with anti-β-actin. Blots are representative of three independent experiments in pools of cells from at least five animals in each experiment.

Figure 5. Effect of treatment with BOC-1, a FPR/ALX antagonist, on ROL and db-cAMP-induced resolution of acute inflammation. Mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and 4 h later received an injection of ROL (6 mg/kg, i.p.) or db-cAMP (4 mg/kg, i.pl.). An injection of BOC-1 (5 mg/kg, i.p.) was given 30 min before the drugs. The cells from pleural cavity

were harvested and processed to neutrophils count (*A, B*) and western blot analysis (*C, D*) for detection of AnxA1 and Mcl-1, 4 h later drug treatment, i.e., 8 h later LPS challenge. Two different exposures time of the cleaved band of AnxA1 immunoblot are presented. Number of apoptotic neutrophils was determined morphologically (*E*) and by flow cytometry of Annexin V^+ neutrophils (*F*) 24 h after LPS injection. Results are expressed as the number of neutrophils/cavity (*A, B*), percentage of neutrophils with apoptotic morphology (*E*) and number of apoptotic neutrophils ($Ly6G^+/F4/80^-/AnxAV^+/7AAD^-$) (*F*), and are shown as the mean ± SEM of at least five mice in each group. $^*P < 0.05$ or $^{***}P < 0.001$ when compared with PBS-injected mice; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ or $^{\#\#\#}P < 0.001$, when compared with LPS-challenged mice. Comparison between the groups BOC and BOC+drugs are highlighted in the graphics. For loading control, membranes were reprobed with anti-β-actin. Blots are representative of three independent experiments using pooled cells from at least five animals in each experiment.

Figure 6. Effect of treatment with anti-AnxA1 antiserum on ROL-induced resolution of acute inflammation. Mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and 4 h later received an injection of ROL (6 mg/kg, i.p.). Injections of anti-AnxA1 antiserum (αAnxA1, 200 μL, i.p.) was given 1 h before the challenge with LPS and again 1 h before ROL. Numbers of neutrophils (*A*), cells with distinctive apoptotic morphology (*B*), and western blot for Mcl-1 (*C*) were evaluated 4 h later drug treatment, i.e., 8 h later LPS challenge. The number of Annexin V^+ neutrophils (*D*) was evaluated by flow cytometry 24 h after LPS injection. Representative figures of nonapoptotic (asterisk) and apoptotic (arrows) and apoptotic cells inside macrophages (arrowheads) are showed in (*E*) - Original magnifications ×20. Results are expressed as the number of neutrophils/cavity (A), percentage of neutrophils with apoptotic morphology (C) and apoptotic neutrophils ($Ly6G^+/F4/80^-/AnxAV^+/7AAD^-$ (*D*) and are shown as the mean ± SEM of at least five mice in each group. $^*P < 0.05$ or $^{***}P < 0.001$ when compared with PBS-injected mice; $^{\#\#\#}P < 0.001$ when compared to LPS-challenged mice. Comparison between the groups ROL and ROL+αAnxA1 are highlighted in the graphics. For loading control, membranes were reprobed with anti-β-actin. Blots are representative of three independent experiments using pooled cells from at least five animals in each experiment.

Figure 7. Effect of treatment with anti-AnxA1 antiserum on db-cAMP-induced resolution of acute inflammation. Mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and 4 h later received an injection of db-cAMP (6 mg/kg, i.p.). Injections of anti-AnxA1 antiserum (αAnxA1, 200 μL, i.p.) was given 1 h before the challenge with LPS and again 1 h before db-cAMP. Numbers of neutrophils (*A*) and cells with distinctive apoptotic morphology (*B*) were evaluated 4 h later drug treatment, i.e., 8 h later LPS challenge. Representative figures of nonapoptotic (asterisk) and apoptotic (arrows) and apoptotic cells inside macrophages (arrowheads) are showed in (*C*) - Original magnifications ×20. The number of Annexin V^+ neutrophils (*D*) with representative dot plots (E) was evaluated by flow cytometry 24 h after LPS injection. Results are expressed as the number of neutrophils/cavity (*A*), percentage of neutrophils with apoptotic morphology (*B*) and apoptotic neutrophils ($Ly6G^+/F4/80^-/AnxAV^+/7AAD^-$ (*D*) and are shown as the mean ± SEM of at least five mice in each group. $^*P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$, when compared with PBS-injected mice; $^{\#}P < 0.05$ when compared to LPS-challenged mice. Comparison between the groups db-cAMP and db-cAMP+αAnxA1 are highlighted in the graphics.

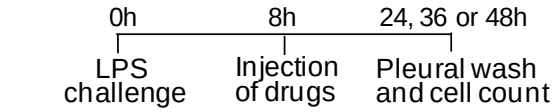
Figure 8. Effect of treatment with rolipram and db-cAMP on neutrophil apoptosis on wild-type and AnxA1-knockout mice. WT or AnxA-KO mice were injected with LPS (250 ng/cavity, i.pl.) and 4 h later received an injection of ROL (6 mg/kg, i.p.) or db-cAMP (4 mg/kg, i.pl.). The cells from pleural cavity were harvested and numbers of cells with distinctive apoptotic morphology were evaluated 4 h later drug treatment, i.e., 8 h later LPS challenge. Results are expressed as percentage of neutrophils with apoptotic morphology and are shown as the mean ± SEM of at least five mice in each group. $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ when compared to LPS-challenged mice.

Figure 9. Effect of treatment with rolipram and db-cAMP on human neutrophil apoptosis. Neutrophils isolated from human peripheral blood (1 x 10^6 cell per well) were cultured with LPS (500 ng/ml) for 1 h, and after with ROL (100 μM), db-cAMP (100 μM) (*A, D-F*) or different

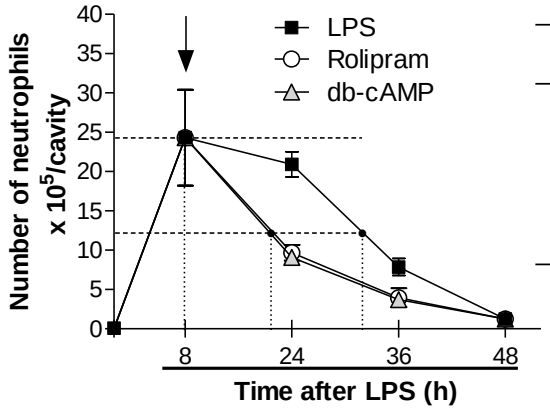
concentrations (*B, C*). The cells were also pre-treated with anti-AnxA1 antiserum (100 μg/mL) or WRW4 (10 μM), a specific FPR2/ALXR antagonist 1 hour before LPS (*D, E, F*). Sivelestat (100 μg/mL) was used as a positive control for neutrophil apoptosis (*A*). Neutrophils were processed for cytospin preparations for apoptosis count. Representative figures of nonapoptotic (asterisk) and apoptotic (arrows) neutrophils are show. Original magnifications ×100. $^{**}P < 0.01$ or $^{***}P < 0.001$ when compared LPS treated group with untreated (UT) neutrophils. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ when compared LPS treated group with drugs-treated neutrophils. The experiments were performed in biological quadruplicates.

Figure 10. Proposed mechanism by which rolipram and db-cAMP modulate AnxA1 and resolution of acute inflammation. The generation of cAMP is initiated when an extracellular first messenger binds to G protein coupled receptor (GPCR) at the plasma membrane, which is coupled to a stimulatory G protein α subunit (Gαs). The free Gαs subunit activates the enzyme adenylyl cyclase (AC) to convert ATP into cAMP. Forskolin directly activates adenylyl cyclase. Phosphodiesterases (PDEs), which degrade cAMP to 5'-AMP, are another regulator of intracellular cAMP levels. PDE inhibitors such as rolipram prevent cAMP degradation, resulting in accumulation of intracellular cAMP. Cyclic AMP can then to bind and to activate the protein kinase A (PKA), in which in turn phosphorylates CREB. P-CREB binds to the cAMP-response element (CRE) on the promoter region of AnxA1 gene and promotes transactivation. Dibutyryl cAMP (db-cAMP) is a cell-permeable cAMP analog that activates PKA. H89 and cAMPS-Rp are PKA inhibitors. Both rolipram and db-cAMP induce AnxA1 expression and its phosphorylation. Phosphorylated AnxA accumulates on the cell membrane and is externalized. Once in the extracellular medium, this protein exerts autocrine, paracrine and justacrine effects, which are mediated by the FPR2/ALXR. The peptide BOC-1 is a nonselective AnxA1 receptor antagonist. Our results shown that AnxA1 is at least one of the endogenous determinants mediating the proresolving properties of rolipram and db-cAMP.

Figure 1

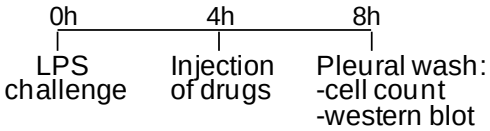


A



B

	ψ_{max}	T_{max}	T_{50}	Ri
LPS	24.3×10^5	~ 8h	~ 32h	~24h
LPS + Rolipram	-	~ 8h	~ 20h	~12h
LPS + db-cAMP	-	~ 8h	~ 20h	~12h



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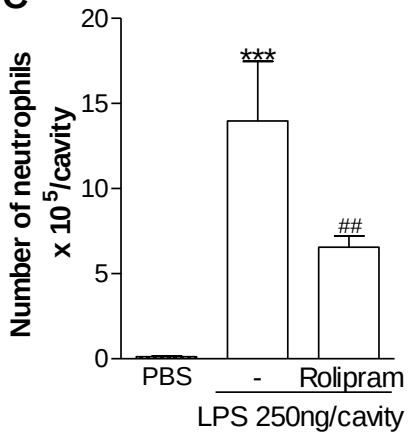

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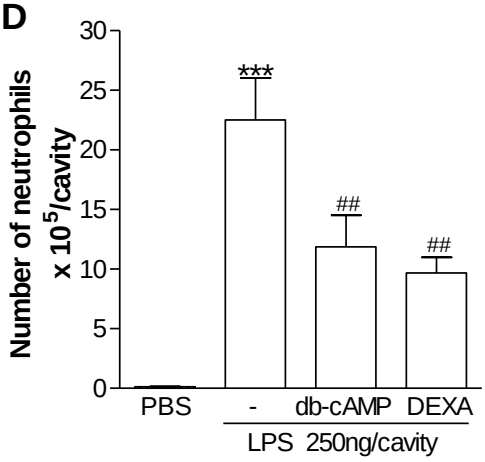

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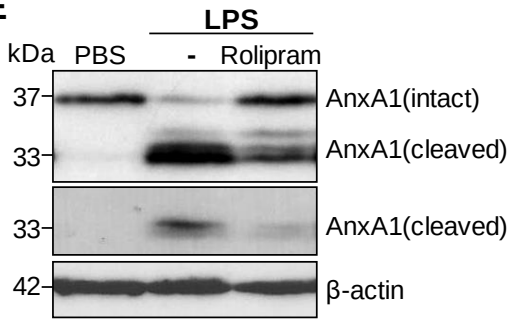

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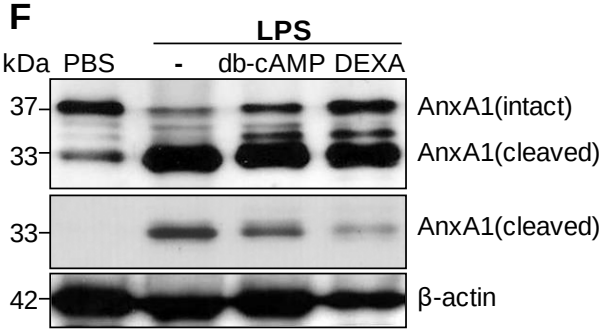

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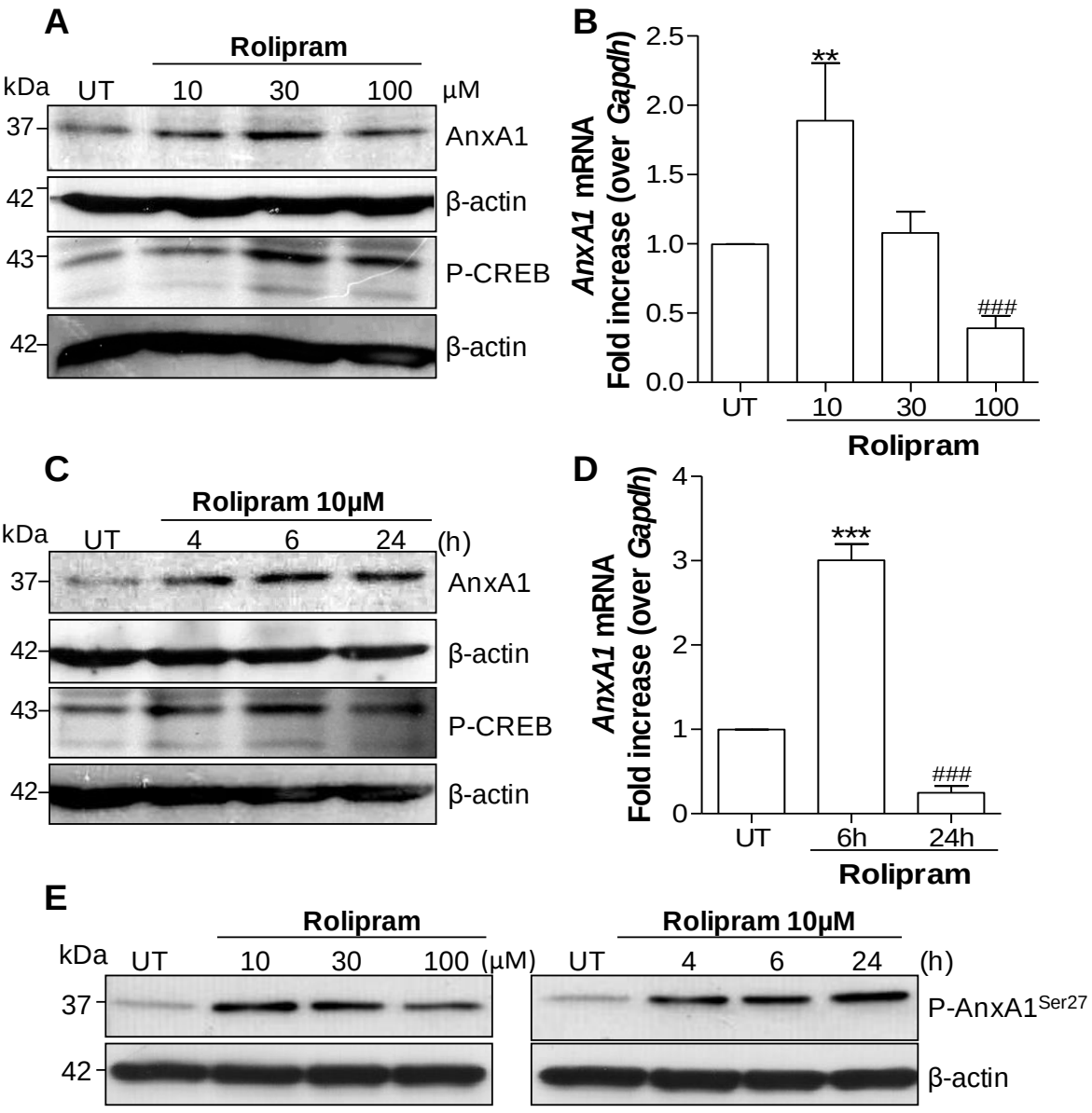

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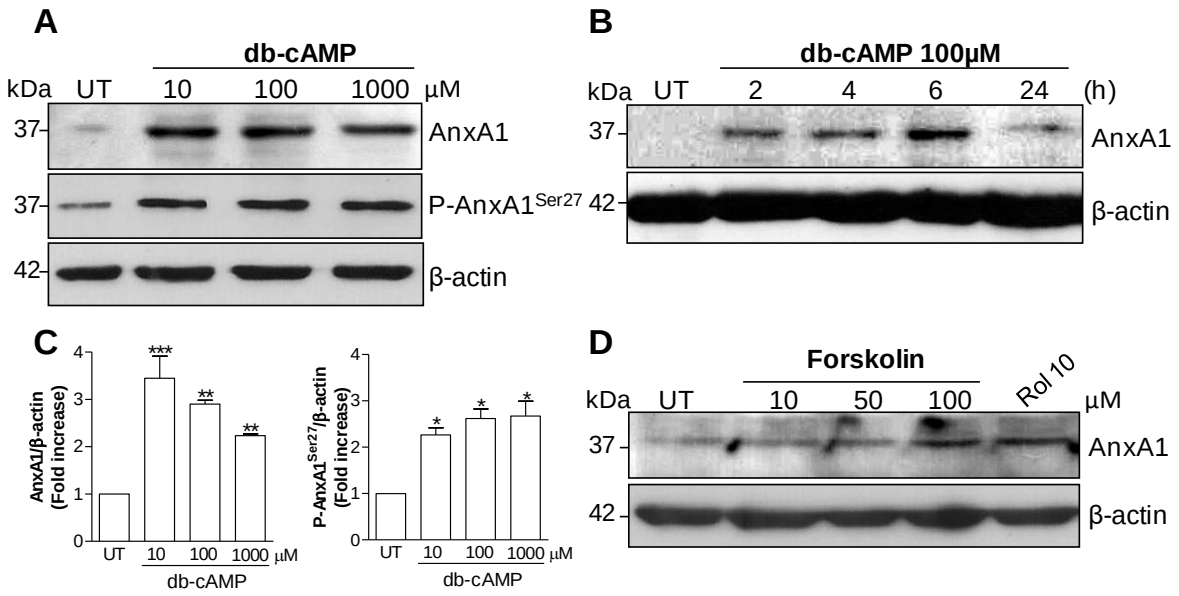

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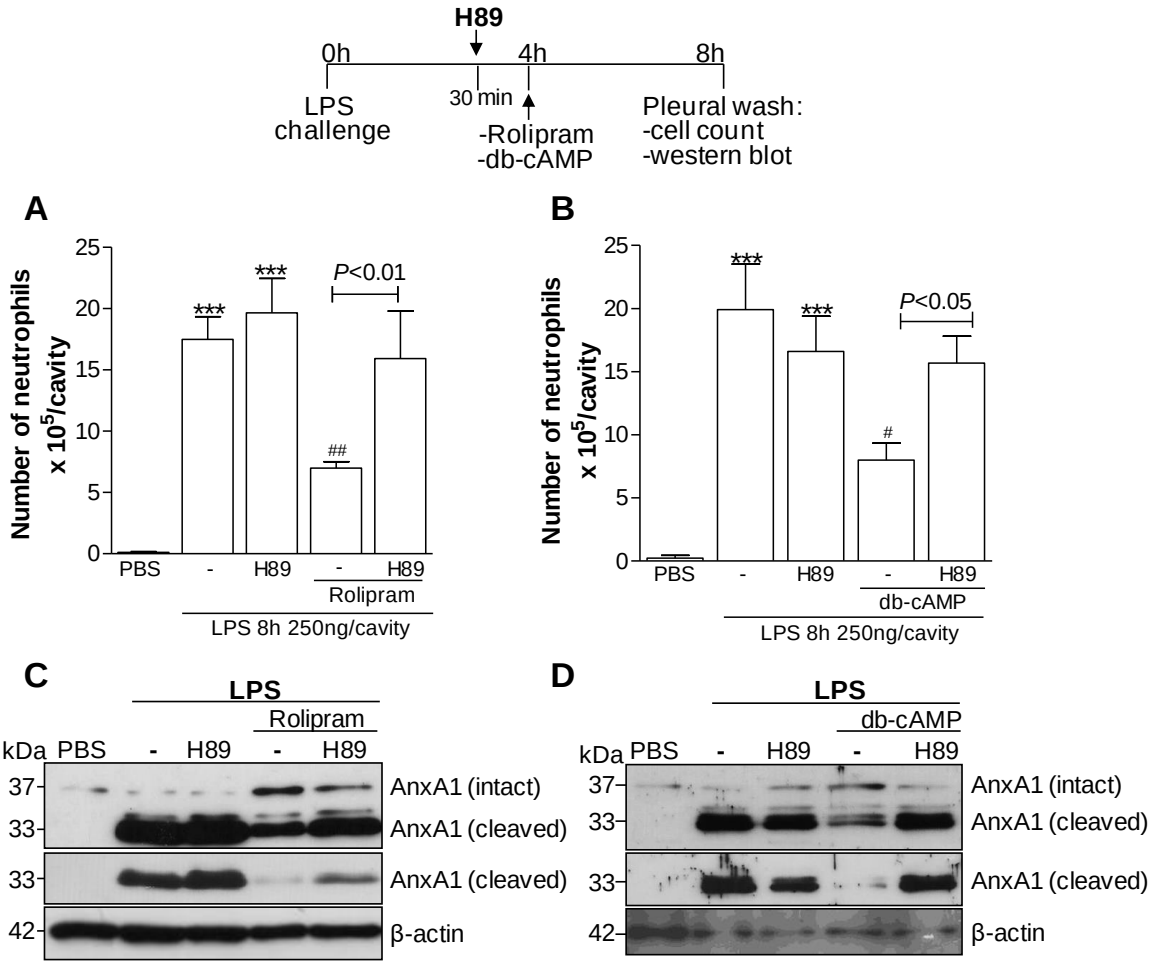


Figure 5

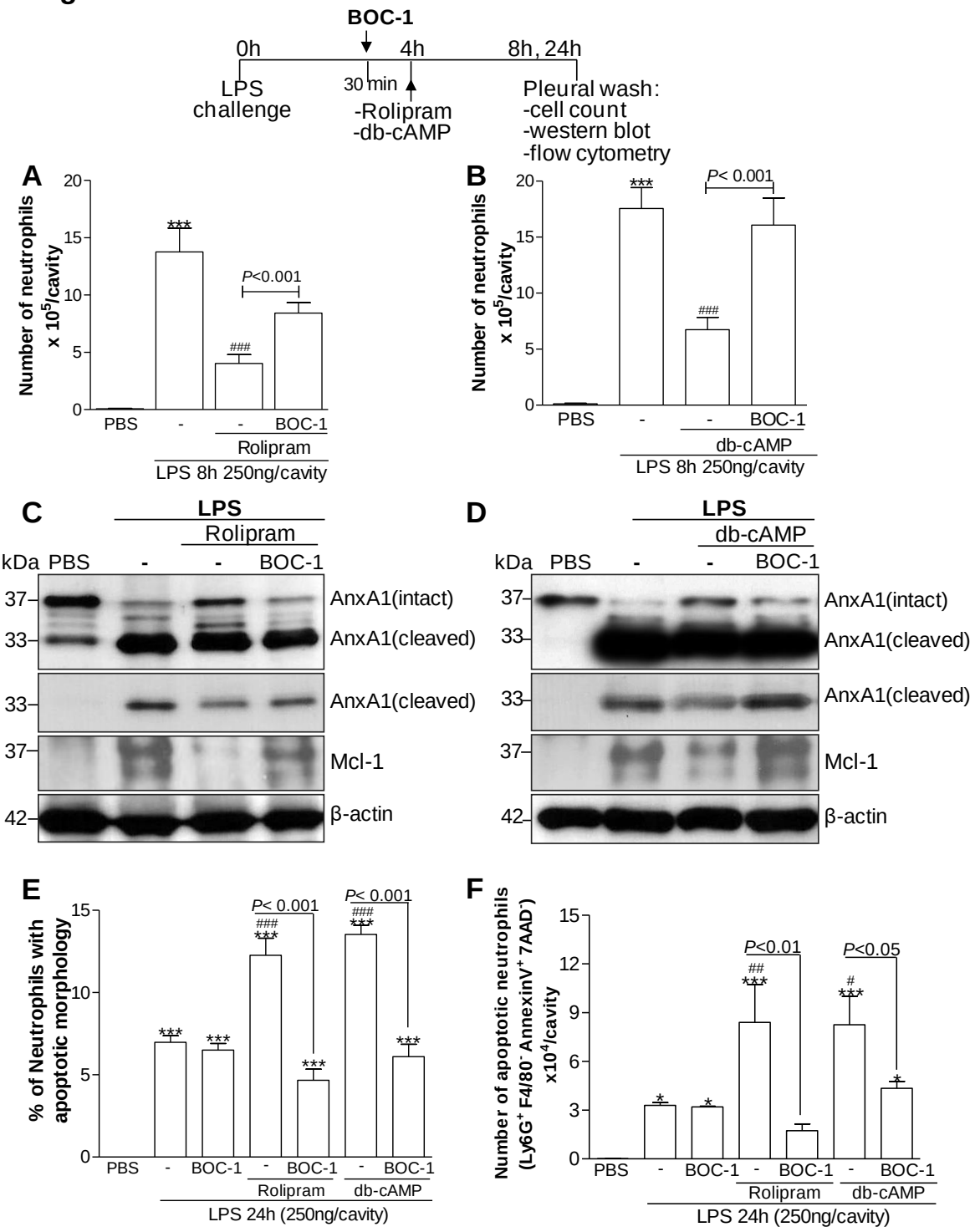


Figure 6

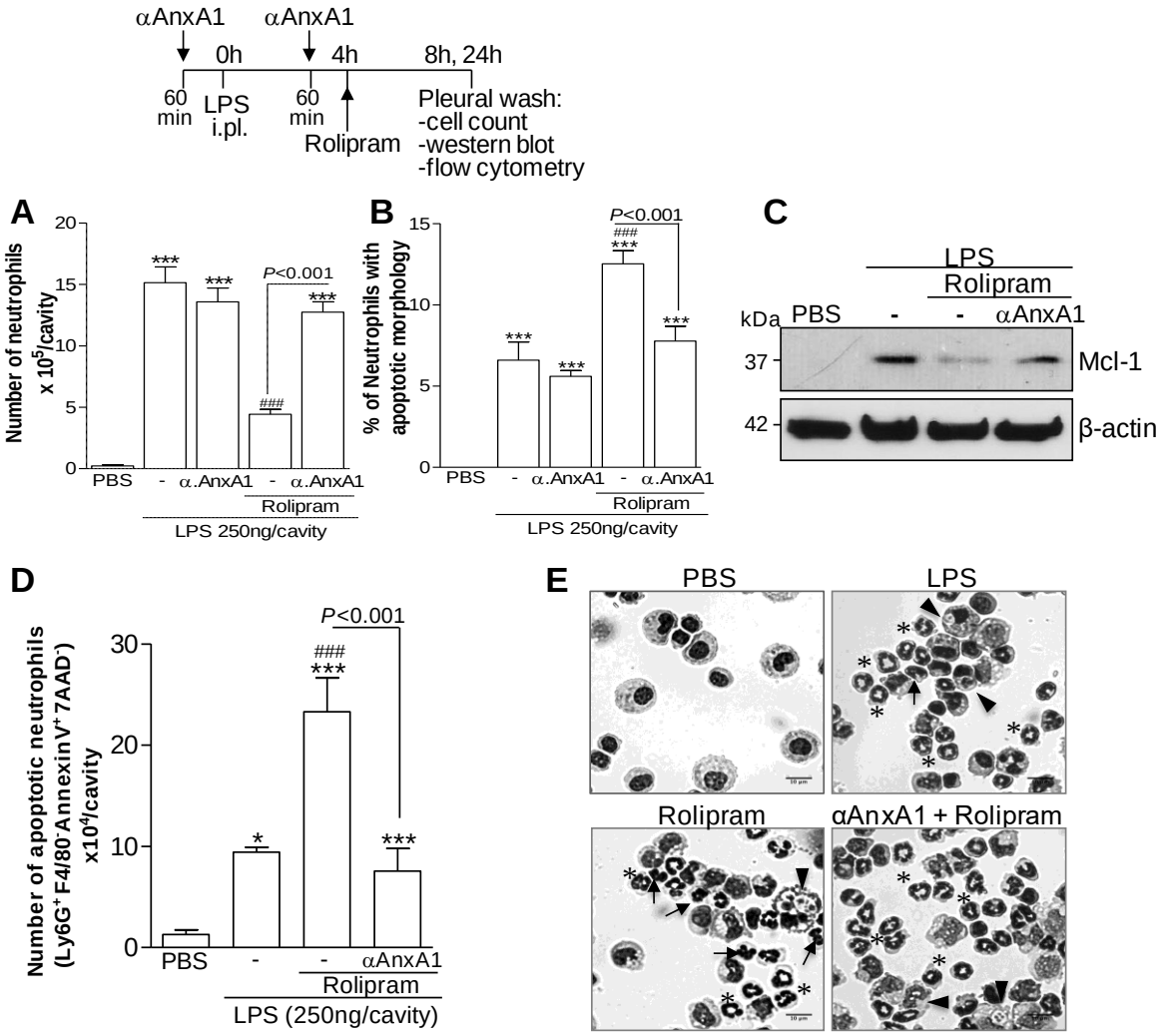


Figure 7

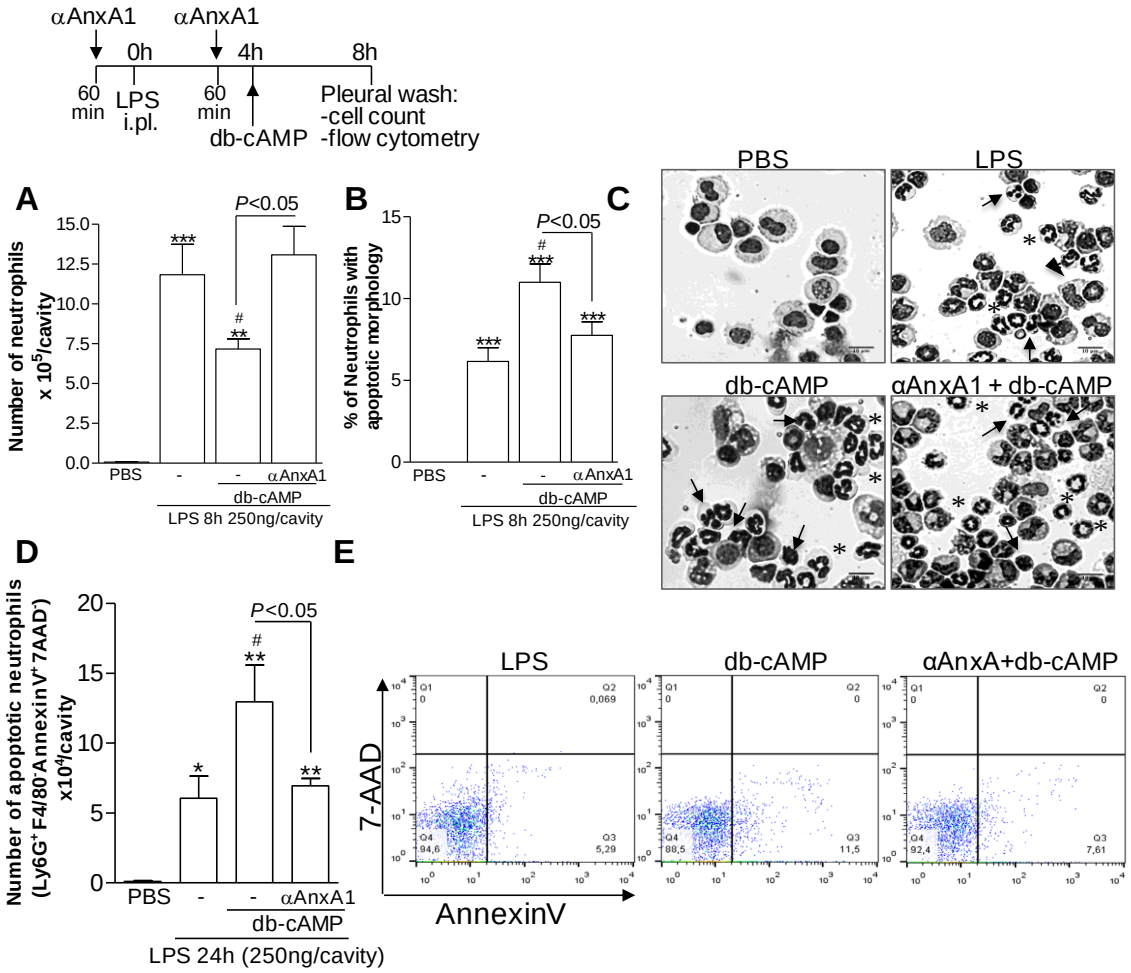


Figure 8

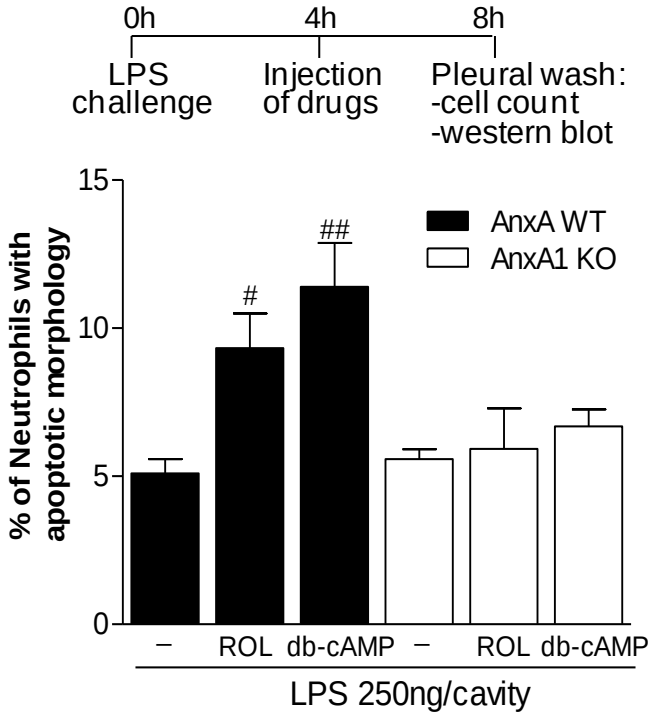


Figure 9

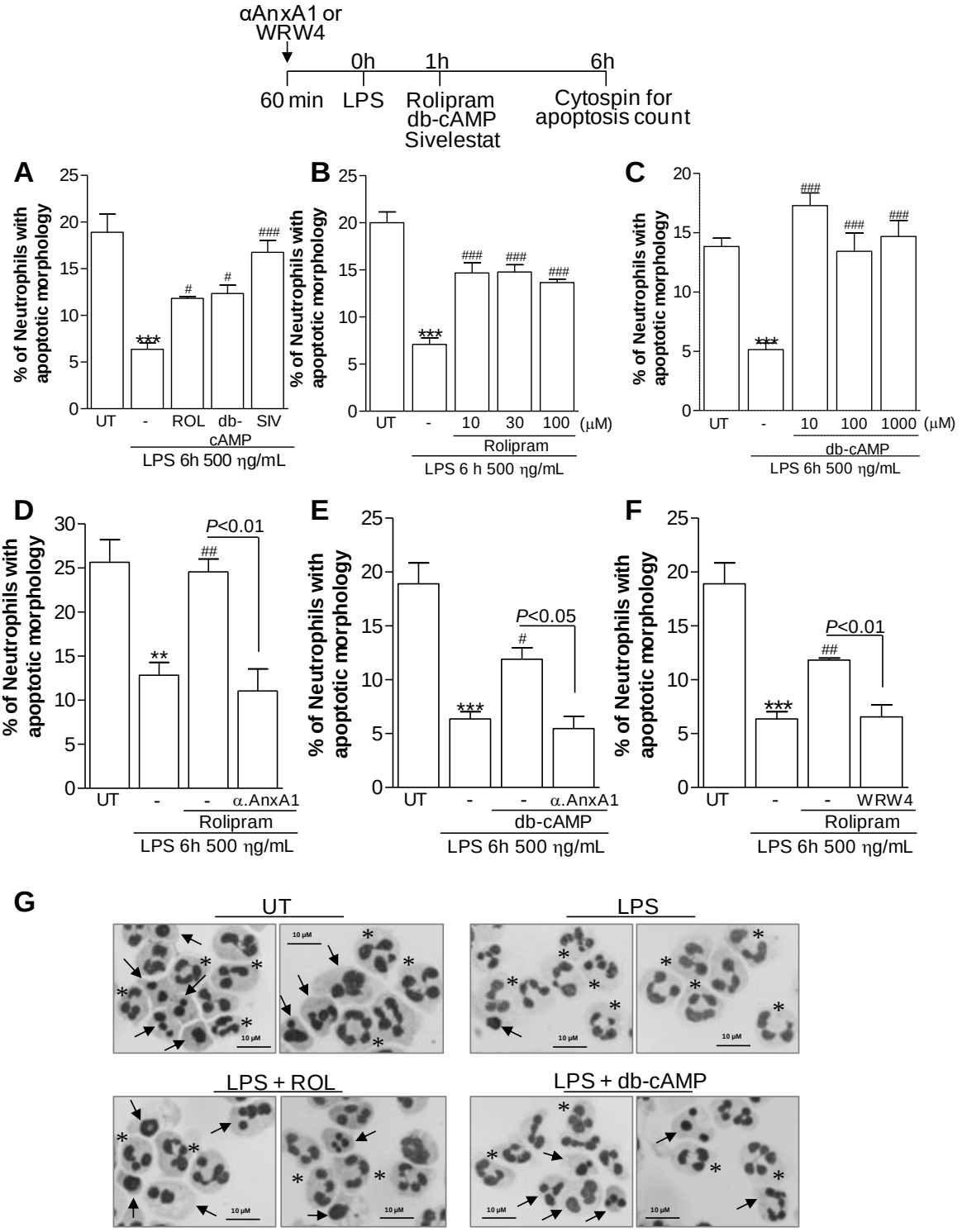
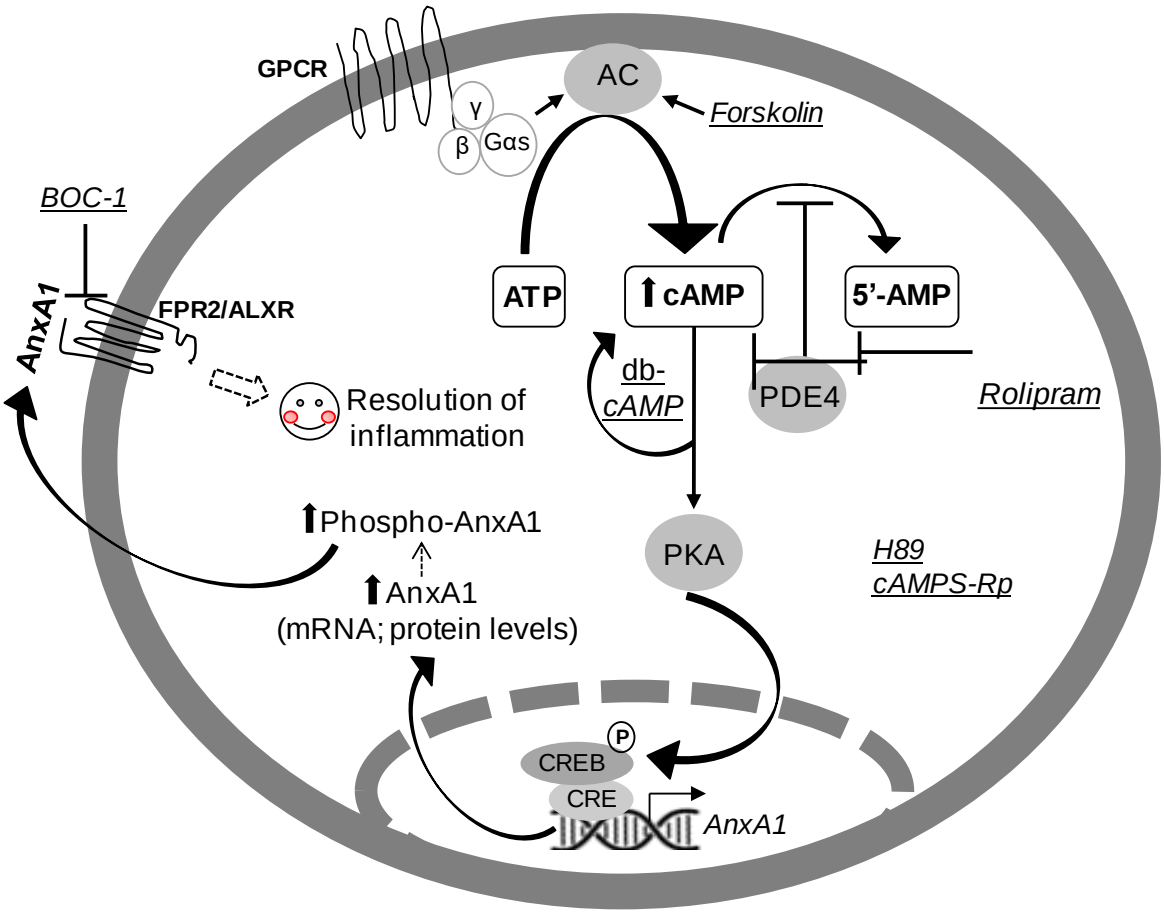


Figure 10



The resolution of acute Inflammation induced by cyclic AMP is dependent on annexin A1

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