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Blame the signaling: Role of cAMP for the resolution of inflammation

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ABSTRACT

A complex intracellular signaling governs different cellular responses in inflammation. Extracellular stimuli are sensed, amplified, and transduced through a dynamic cellular network of messengers converting the first signal into a proper response: production of specific mediators, cell activation, survival, or death. Several overlapping pathways are coordinated to ensure specific and timely induction of inflammation to neutralize potential harms to the tissue. Ideally, the inflammatory response must be controlled and self-limited. Resolution of inflammation is an active process that culminates with termination of inflammation and restoration of tissue homeostasis. Comparably to the onset of inflammation, resolution responses are triggered by coordinated intracellular signaling pathways that transduce the message to the nucleus. However, the key messengers and pathways involved in signaling transduction for resolution are still poorly understood in comparison to the inflammatory network. cAMP has long been recognized as an inducer of anti-inflammatory responses and cAMP-dependent pathways have been extensively exploited pharmacologically to treat inflammatory diseases. Recently, cAMP has been pointed out as coordinator of key steps of resolution of inflammation. Here, we summarize the evidence for the role of cAMP at inducing important features of resolution of inflammation.

1. Introduction

Inflammation is a protective pathophysiological response of vascularized tissues to infections or injury [1]. Complex inflammatory responses begin with the detection of an inciting stimulus leading to the production of pro-inflammatory mediators, recruitment and activation of leukocytes and terminates with reparative processes to restore tissue homeostasis and promote adaptive immunity [2]. Specific intracellular signaling events coordinate the three phases of inflammation: the onset, resolution and post-resolution [3–6].

The signaling pathways related to the onset of inflammation have been extensively studied [3]. Cellular sensors and receptors recognize harmful stimuli, activate signaling cascades and transmit the message to the nucleus through the activation of transcription factors, such as nuclear factor-kappa B (NF-κB), the prototypical activator of inflammatory genes [7,8]. This triggers the production of pro-inflammatory mediators, proteases, reactive oxygen species (ROS) and increases the expression of adhesion molecules on cell surface [7]. Consequently, neutralization of potential pathogens and clearance of debris are achieved while repair responses are promoted.

Every step of the inflammatory process must be finely tuned to ensure an effective defense against harmful stimuli and later induction of resolution, with minimal collateral damage [9]. Uncontrolled inflammation is an unifying feature of diseases such as cancer, vascular and other chronic inflammatory diseases [10–12]. Excessive or altered inflammatory responses may result from a failure in pro-resolution

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pathways [11,13,14]. Resolution of inflammation, as inflammation itself, is a complex active process that comprises (1) clearance of pathogens by phagocytosis, (2) apoptosis and efferocytosis of granulocytes and debris, (3) restoration of chemokine levels to baseline, (4) regeneration of damaged tissues, (5) regain of vascular integrity and (6) relief of inflammatory pain [15]. Pro-resolving mediators are lipids (specialized pro-resolving mediators – SPMs and short chain fatty acids), peptide or proteins (e.g. Annexin A1 - AnxA1), and gaseous molecules that coordinate resolution being produced during the initial stages of acute inflammation to program its termination [16,17]. This process (specially for SPMs) is evidenced by the called "mediator class switching" that is observed in self-limiting inflammatory conditions: pro-inflammatory mediators, such as leukotrienes, are replaced by SPMs, such as lipoxins and resolvins [15]. Understanding the signaling pathways of resolution of inflammation will pave the way for the development of new therapeutic strategies for inflammatory diseases.

Cyclic adenosine 3, 5′-monophosphate (cAMP) is one of the most versatile cellular second messenger and regulates important biological processes, including cell migration, activation, proliferation and survival [18,19]. The production of cAMP is triggered by the activation of membrane receptors (mainly G protein-coupled receptors - GPCRs) that activate cellular adenylyl cyclases (AC), which convert ATP to cAMP [20]. Downstream events include the activation of cyclic nucleotide-gated ion channels [21], exchange proteins directly activated by cAMP (Epac) [22] or protein kinase A (PKA), a heterotetramer formed by two regulatory subunits and two catalytic subunits [23]. cAMP activation of PKA is triggered by the binding of cAMP to the regulatory subunits of PKA followed by alterations of protein conformation that release the catalytic subunits to phosphorylate and activate the transcription factor CREB (cAMP response element binding protein). Moreover, PKA also phosphorylates and controls the activity of cellular motor proteins, ion channels and enzymes such as protein kinase C (PKC), phosphoinositide 3-kinase (PI3K) and phospholipase C [24]. Depending on the cell type and pathway activated, cAMP controls different processes during inflammation and resolution. Fig. 1 summarizes the described cAMP-dependent signaling pathways for resolution of inflammation that will be presented and discussed in the following sections of this review.

Elevations in the intracellular levels of cAMP modulate the activation of innate immune cells, including monocytes, macrophages, and neutrophils, through the modulation of key cellular effector functions: generation of inflammatory mediators (e.g., cytokines, chemokines, and lipids), chemotaxis, production of ROS and neutrophil extracellular traps (NETs), phagocytosis and killing of ingested pathogens, and cell survival [25–27]. The cellular levels of cAMP are regulated by cyclic nucleotide phosphodiesterases (PDEs) that catalyze the hydrolysis of cAMP to AMP. There are 11 families of mammalian PDEs (PDEs 1–11) of which PDEs 1, 2, 3, 4, 7, 8, 10 and 11 act on the inactivation of cAMP [28]. PDE4 is mainly expressed in leukocytes (neutrophils, eosinophils, cytotoxic T-lymphocytes and macrophages) and, therefore, is particularly important in inflammation [19,29]. During the onset of the inflammatory response, levels of PDE4 are increased and consequently those of cAMP are low [30]. As such, AC-mediated increase in cAMP is counter-regulated by PDE4 during inflammation.

This review summarizes the growing amount of evidence that supports the role of cAMP in triggering key features of resolution of inflammation: induction of pro-resolving mediators, apoptosis, efferocytosis and phagocytosis, nonphlogistic recruitment of macrophage, macrophage polarization, and return to tissue homeostasis.

2. The crosstalk between cAMP and pro-resolving mediators

Several, if not most, pro-resolving mediators that orchestrate the steps of resolution of inflammation act through specific GPCRs triggering a cascade of intracellular signaling events that synergize to induce pro-resolving cellular responses [14]. Of note, the production of a given pro-resolving molecule can induce biosynthetic pathways of other pro-resolving molecules potentiating the overall response [31]. Here, we discuss the role of cAMP as one central messenger in pro-resolving signaling pathways, being induced by or inducing the production of pro-resolving mediators (Fig. 1).

Annexin A1 (AnxA1) is an endogenous pro-resolving mediator, first characterized as a glucocorticoid (GC) induced protein active on phospholipase-A2 (PLA2) inhibition and prevention of eicosanoid synthesis [32–34]. AnxA1 is involved in several biological effects during inflammation and resolution [35]. AnxA1 is an agonist of the N-formyl peptide receptor 2/lipoxin A_4 receptor (FPR2/ALX) that has broad expression in leukocytes and non-immune cells [35]. In fact, AnxA1 was shown to be protective in several models of inflammatory diseases, including gout [36], tuberculosis [37], pleurisy [38], silicosis [39] and pneumonia [40] by regulating the amount of inflammation, triggering apoptosis and efferocytosis and decreasing the production of pro-inflammatory cytokines. Recently, we have shown that cAMP elevating agents increase the levels of AnxA1 that on its turn contributes to resolution of pleurisy and pneumonia in mice [41,42]. Indeed, activation of CREB, triggers the transactivation of the AnxA1 promoter [43] and the consequent transcription of the AnxA1 gene. Of interest, cAMP can regulate AnxA1 phosphorylation [44], an event that is important for protein mobilization and function [41,45]. Therefore, inducing AnxA1 may be an important mechanism of protection for cAMP-elevating agents during inflammatory diseases. In addition, cAMP-induced PKA activation promotes the phosphorylation of 5-lipoxygenase at serine 523 culminating with production of 15-epi-lipoxin A_4, a potent SPM [46]. Altogether, there is a growing amount of evidence for the role of cAMP signaling in mediating the production of mediators of resolution (Fig. 2).

Interestingly, pro-resolving mediators can also increase intracellular levels of cAMP (Fig. 1). The ability of AnxA1 to control the cellular pool of cAMP was suggested in culture of adipose explants from AnxA1 deficient mice (AnxA1 KO) that showed decreased cAMP at basal levels and after isoprenaline stimulation, as compared to WT mice [47]. The authors suggested that such effect of AnxA1 might be attributed to its inhibitory effect on phospholipase A_2, which depresses β-adrenoceptor-stimulated adenylyl cyclase [48]. Therefore, the direct effect of AnxA1 on the induction of intracellular levels of cAMP remains to be uncovered. Regulation of levels of cAMP is also observed as a mechanism of action of SPMs endogenously produced during resolution of inflammation, including Aspirin-triggered Resolvin D1 (AT-RvD1), Resolvin D1 (RvD1), Resolvin D2 (RvD2) and N-3 docosapentaenoic acid–derived Resolvin D5 (RvD5n-3 DPA) [49–51]. AT-RvD1 is an isoform of RvD1 that is produced by cyclooxygenase 2 (COX-2), after being covalently modified by aspirin [52]. AT-RvD1 can also be produced endogenously in the absence of aspirin by alternative pathways involving cytochrome P450 enzymes [53]. RvD1 and AT-RvD1 are agonists of resolution and, therefore, control several inflammatory diseases such as ischemia/reperfusion injury [54], infectious pneumonia [55], asthma [56], among others. The intracellular pathways triggered by AT-RvD1 are not completely understood; however, it was recently shown that it can increase cAMP levels with consequent activation of PKA in a FPR2/ALX dependent-manner [57]. Keeping with that, RvD1 (that also binds to FPR2/ALX) and RvD5 were shown to downregulate PDE4B in human macrophages stimulated with *Escherichia coli* [58]. Therefore, one can hypothesize that resolution of *E. coli* peritonitis induced by RvD1 and RvD5 may be associated with increased levels of cAMP. Keeping with that, RvD1 was shown to increase alveolar fluid clearance, an important feature of resolution of lung injury, in a cAMP-dependent manner [51].

In contrast, other lipid mediators such as Resolvin E1 (RvE1) can block adenylyl cyclase through the activation of ERV-1/ChemR23 or BLT1 receptors leading to a reduction in intracellular cAMP levels [59,60]. Therefore, the induction of cAMP by some SPMs might be receptor or cell specific. In agreement with this hypothesis, increased levels of cAMP were observed in airway epithelial cells after LXA_4

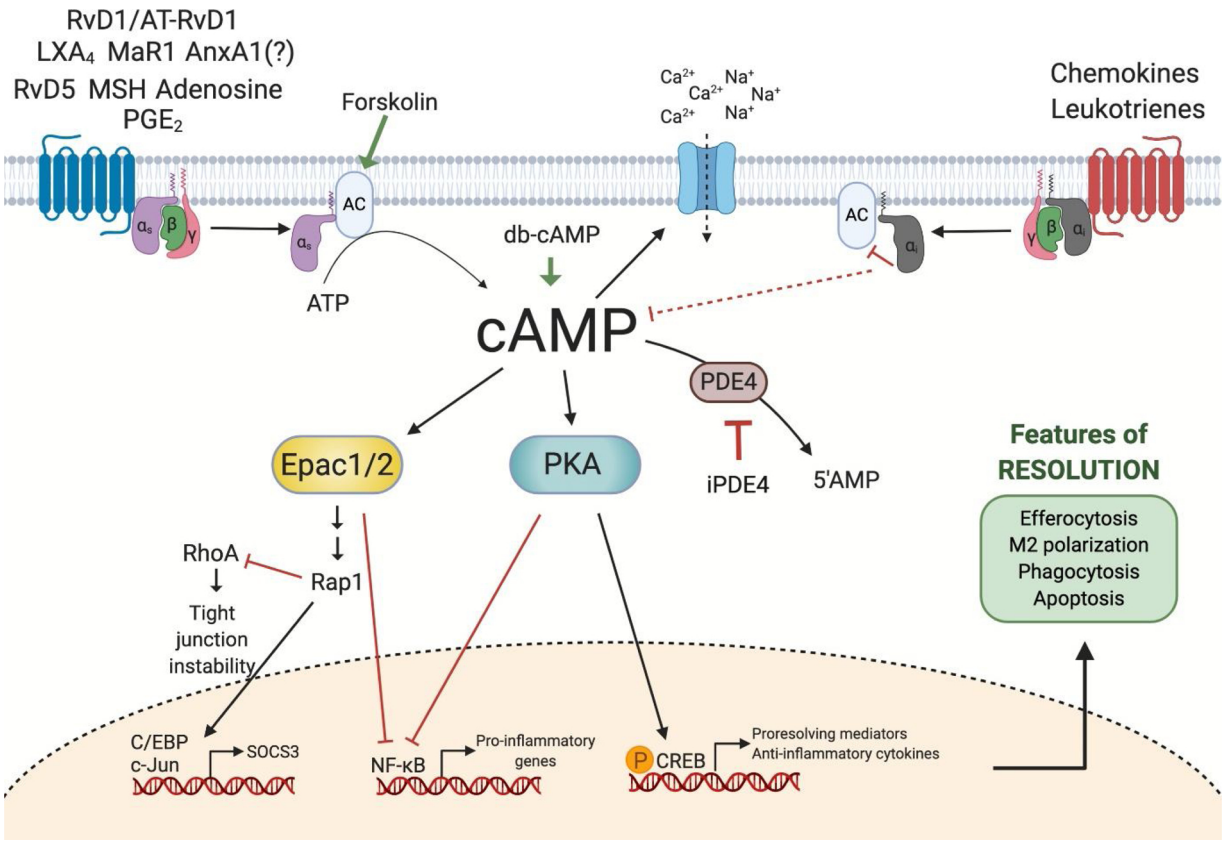

Fig. 1. Schematic representation of cAMP pathways in the context of the resolution of inflammation. cAMP levels are controlled by the activity of adenylyl cyclase (AC) and phosphodiesterase 4 (PDE4). AC increases cAMP levels by the conversion of ATP to cAMP, while PDE4 catalyzes the degradation of cAMP to AMP. Activation of GPCRs and release of the subunit Gα stimulates (Gαs) or inhibits (Gαi) AC activity. Chemokines and other inflammatory mediators, including leukotrienes, reduce the levels of cAMP by engaging GPCRs with subunits Gαi. Conversely, endogenous pro-resolving mediators are produced to induce and coordinate resolution of inflammation. Resolvins (RvD1, RvD5 and AT-RvD1), lipoxins (LXA_4), melanocortins (MSH), maresin 1 (MaR1), adenosine and potentially Annexin A1 (AnxA1), increase AC activity through binding to GPCRs with subunits Gαs. Mediators such prostaglandin E_2 (PGE_2) are also inducers of cAMP. Pharmacological inhibition of PDE4 inhibition (iPDE4), membrane-permeable cAMP analog (db-cAMP), and activator of AC (Forskolin) promote accumulation of intracellular cAMP. cAMP can activate protein kinase A (PKA), the exchange protein 1/2 activated by cAMP (Epac1/2), and cAMP gated ion channels. Activation of PKA leads to the phosphorylation of the cAMP-responsive element binding protein (CREB) that translocates to the nucleus promoting the production of pro-resolving mediators, anti-inflammatory cytokines, and stimulation of macrophage polarization, efferocytosis and granulocyte apoptosis – features of resolution of inflammation. In addition, PKA can reduce the transcriptional activity of NF-κB preventing the expression of inflammatory genes and by inhibiting PI3K/Akt – both signaling for cell survival. PKA-dependent activation of ERK1/2 mediates the secretion of CCL2, consequent nonphlogistic recruitment of macrophages and contributes to induction of macrophage polarization to pro-resolving phenotypes. cAMP also induces activation of STAT-3, STAT-6 and CRTC3→IL-10 that signaling M2 polarization. Activation of Epac1/2 inhibits the production of pro-inflammatory cytokines. Activation of cAMP signaling pathways represents a promising pharmacological strategy to treat inflammatory diseases by promoting resolution of inflammation. For simplicity, some of the pathways mediated by PKA, Epac, and Rap1 have been omitted. (Created with Biorender ®).

treatment, a pro-resolving lipid agonist of FPR2/ALX [61]. Curiously, it seems that treatment with LXA_4has an indirect effect on cAMP levels. By inducing increased secretion of ATP by epithelial cells, LXA_4 leads to the activation of purine receptors such as P2RY11 and consequent increase of cAMP levels [61]. Adding to the complexity of the role of cAMP in LXA_4 signaling, other studies have not reported increases in cAMP levels after LXA_4 treatment [62,63], which could be due to a cell-specific effect of this pro-resolving mediator. Maresin 1 (MaR1) is a SPM produced by macrophages that promotes resolution of inflammation and tissue repair responses [64]. Recently, cAMP production and signaling via CREB were observed as features of activation of LRG6, the newly recognized receptor for MaR1 [64]. Because cAMP is an important regulator of macrophage function and phenotype [65,66], cAMP might be one of the mechanisms of action of MaR1 agonists. Of interest, MaR1 belongs to a cluster of SPMs (including RvD1, RvD5 and LXB_4) that are upregulated during coagulation and activate ERK1/2 and CREB signaling pathways, promoting leukocyte antimicrobial responses [67]. Recently, the agonist of the GPR101 receptor, n-3 docosapentaenoic acid–derived resolvin D5 (RvD5n-3 DPA), was shown to induce cellular cAMP increases with increases in efferocytosis and phagocytosis [49].

More recently, the melanocortin system emerged as an important inducer of resolution of inflammation [68]. Melanocortins are peptides produced by proteolytic cleavage of pro-opiomelanocortin (POMC), a hormone expressed within the pituitary and peripheral cells and tissues. The melanocortin peptides adrenocorticotrophin (ACTH), α-, β-, γ-melanocyte stimulating hormone (MSH) bind to five melanocortin receptors (MC_{1-5}) that are small stimulatory GPCRs expressed in several cells and tissues such as leukocytes, endothelial cells, melanocytes and the hypothalamus [69]. Engagement of all the five MC receptors by the melanocortin peptides induce increased levels of cAMP and down-regulation of NF-κB [70]. Because of their expression in leukocytes, MC_1, MC_3 and MC_5 are the main receptors involved in regulation of the inflammatory responses [71]. Of note, MC_1 and MC_3 can be pharmacologically activated to induce resolution of inflammation. Agonists of melanocortin receptors can decrease the levels of pro-inflammatory cytokines and induce efferocytosis and IL-10 production, important features of resolution [71]. The direct role of cAMP as a major trigger of

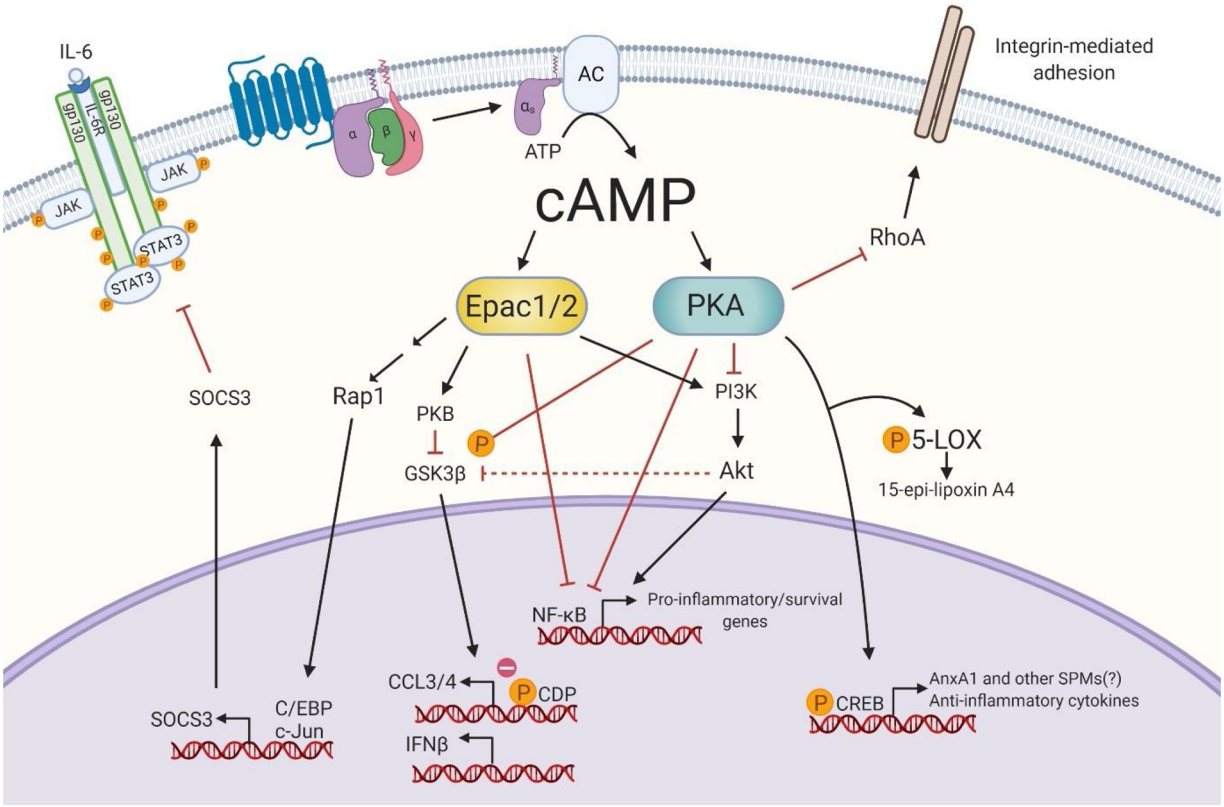

Fig. 2. Schematic representation of cAMP dependent regulation of pro-inflammatory cytokines and pro-resolving mediators. Elevations in cAMP intracellular levels activate protein kinase A (PKA) and the exchange protein directly activated by cAMP (Epac)-dependent pathways. PKA phosphorylates the transcription factor CREB, leading to the transcription of anti-inflammatory cytokines and production of pro-resolving mediators such as Annexin A1 (AnxA1), and the 5-lipoxygenase (5-LOX) enhancing the production of 15-epi-lipoxin A_4. In addition, PKA inhibits (glycogen synthase kinase 3) GSK3, the PI3K/Akt pathway and NF-κB decreasing secretion of pro-inflammatory cytokines and pro-survival signals. Moreover, PKA inhibits the Ras Homolog Family Member A (RhoA)-dependent expression of integrins in granulocytes. Among the cAMP/Epac-induced pathways, activation of the protein kinase B and consequent inhibition of GSK3β decreases production of pro-inflammatory cytokines, in part through the activation of the repressor transcription factor CCAAT displacement protein (CDP). Epac activates the Ras-proximate-1 or Ras-related protein 1 (Rap1) signaling leading to activation of c-Jun and C/EBP that promote the expression of SOCS-3 with consequent inhibition of IL-6 induced signaling. (Created with Biorender ®).

resolution induced by melanocortin peptides or synthetic agonists is still to be determined. Recent studies have shown that activation of non-canonical pathways, such as phosphorylation of ERK1/2, and not cAMP signaling pathway, might be major inducers of efferocytosis and modulators of cytokine production by inflammatory cells [72,73]. Therefore, more studies are necessary to clarify this controversy and to determine the exact role of cAMP as a mechanism for melanocortin-induced resolution of inflammation.

As mentioned before, there is still much to be understood in the signaling mechanisms of pro-resolving mediators. cAMP intracellular pathways are often triggered during self or drug-induced resolution of inflammation and therefore, must be considered as an important component of the complex network of resolution.

3. cAMP regulates pro-inflammatory cytokines and granulocyte recruitment

Among the features of resolution of inflammation, reduction of granulocyte recruitment and production of phlogistic cytokines are one the earliest steps of the effective termination of the inflammatory process [4]. Activation of cAMP pathways are long described to control pro-inflammatory cytokine production and leukocyte recruitment [19] (Fig. 2).

Pro-inflammatory cytokines such as chemokines, interleukins, and others, are relatively small proteins (< 40 kDa) produced by immune and non-immune cells during injury/infection [74]. Chemokines and cytokines act through different GPCRs leading to activation of leukocytes, endothelial cells and stromal cells culminating with the recruitment and/or activation of immune cells [75]. Differently from pro-resolving GPCRs, the chemokine receptors are Gαi/o-coupled and upon activation reduce adenylate cyclase activity limiting the intracellular levels of cAMP and PKA activation [76]. On the other hand, accumulation of intracellular cAMP with consequent activation of PKA and Epac1/2 is widely known to decrease the expression of pro-inflammatory cytokines and chemokines with consequent decreased activation and recruitment of leukocytes [25] (Fig. 2). Among the pro-inflammatory mediators, cAMP elevating agents were shown to diminish the levels of TNF-α [77], IL-12 [78], leukotriene B_4 (LTB_4) [79], IL-1β [80] and chemokines such as CCL3 [81], CXCL1 [42], CCL2, CCL4 [82] and CCL11 [83]. Therefore, it is not surprising that evidence from pre-clinical and human studies have shown that increased cAMP levels can decrease T cell activation [84], neutrophil oxidative responses [85], migration of eosinophils [86], and counter-regulate the expression of adhesion molecules in leukocytes and endothelial cells [87–91]. As illustrated in Fig. 2, cAMP activation of PKA inhibits RhoA-induced expression of integrins in the granulocyte surface [92]. The reduced expression of chemokines and adhesion molecules culminate with diminished recruitment of inflammatory leukocytes in tissues, which represents an interesting therapeutic strategy for several inflammatory diseases. Indeed, we and others have shown that PDE4 inhibitors reduce granulocyte recruitment and activation in the lungs and pleura [93], being protective in models of COPD, asthma [94], pleurisy [41,95,96]

and even pneumonia [42]. This modulation of inflammation was also shown to improve the outcome from diseases not related to the lungs, such as psoriasis, inflammatory bowel diseases, rheumatic arthritis, lupus, and neuroinflammation [97].

The effects of cAMP on the regulation of pro-inflammatory mediators were first described to be mediated by PKA, an effect that can lead to inhibition of NF-κB-induced gene transcription [98–100]. NF-κB is a family of transcription factors that, upon activation, promote the expression of genes related to inflammatory and anti-apoptotic responses [101,102]. Proteins from the IκB (inhibitor of κB) family sequester NF-κB in the cytoplasm. Pro-inflammatory signals (cytokines, pathogen related molecules, etc) activates a complex of cytoplasmic kinases (IκB kinase - IKK) that phosphorylates IκB molecules. After being phosphorylated IκB undergoes degradation by proteasome and release the NF-κB dimer (mainly composed by p50-p65) that can translocate to the nucleus inducing its target genes [103]. cAMP-activated PKA can inhibit NF-κB through different mechanisms depending on the cell type and inflammatory stimuli. cAMP/PKA activation can prevent IκB degradation, hence preventing NF-κB translocation [104,105], and inhibits signaling pathways that lead to NF-κB activation, such as PI3K/Akt [96]. In addition, there is evidence for a role of the cAMP/PKA pathway in repressing NF-κB-induced gene expression through the formation of the repressive dimer (i.e. p50-p50) instead of activating NF-κB complexes (i.e. p50-p65) that have transcriptional activity [106,107].

Activation of Epac by cAMP was also shown to reduce NF-κB activation [108]. In addition, cAMP/Epac pathway modulates pro-inflammatory cytokine responses through the induction of suppressor of cytokine signalling-3 (SOCS-3) that inhibits signaling from gp130-linked class I cytokine receptors such as the IL-6 receptor [109,110], preventing inflammation-induced dysfuction in endothelial cells [111]. Epac induction of SOCS3 prevents JAK-dependent phosphorylation and activation of STAT1/3, preventing further signaling from this class of cytokine receptors [112]. Moreover, Epac was shown to decrease the production of interferon-β and CCL3/4 through the activation of the protein kinase B (PKB) and PI3K/Akt pathway with consequent inhibition of glycogen synthase kinase 3 beta (GSK-3β) in murine macrophages [113] and dendritic cells [114]. Of note, inhibition of GSK-3 prevents the phosphorylation and activation of the transcriptional repressor CCAAT displacement protein (CDP), the downstream molecular mechanism for cAMP/Epac-mediated reduction of CCL3/4 levels [114].

The role of these different cAMP dependent pathways in reducing inflammatory cytokines may vary depending on the cell type and the mediator under investigation. Moreover, the balance of cAMP levels might also be important to determine the inhibitory action of this second messenger since overaccumulation of cAMP may induce, rather than block, chemokine release from macrophages *in vitro* [115]. Whether this effect also occurs *in vivo* and which type of cell would be recruited still need clarification.

4. cAMP modulates granulocyte apoptosis and phagocytosis

Induction of granulocyte apoptosis and subsequent efferocytosis ensures nonphlogistic cell death and clearance of cells in the tissue, preventing chronic inflammation [116]. The signaling pathways leading to apoptosis have been widely studied and explored therapeutically. Apoptosis of granulocytes can be triggered by two signaling cascades – intrinsic and extrinsic pathways [117]. The intrinsic pathway is triggered by extracellular or intracellular signals that increase mitochondrial membrane permeabilization to internal cytochrome C that is released to the cytosol [117]. Then, cytochrome C can associate with the adaptor protein Apaf-1 to form the apoptosome leading to downstream activation of caspase-9 that can further activate caspases-3 and 7 [117]. The extrinsic pathway is activated by the binding of the plasma membrane receptor Fas to Fas ligand (Fas-L) or other similar receptors, such as TNFR1 or TRAIL. Fas-L combines with Fas to form a death complex that recruits the death domain-containing protein (FADD) and pro-caspase-8, forming the death-inducing signaling complex (DISC). This protein complex cleaves and activates pro-caspase-8 that will further activate caspase-3. When activated by either the intrinsic or extrinsic pathways, caspases-3 and -7 can cleave different cell substrates, ultimately leading to phosphatidylserine exposure, nuclear condensation, membrane blebbing, and genomic DNA fragmentation - the classical features of apoptosis [117]. Of interest, neutrophil apoptosis through the extrinsic death receptor pathway is regulated by intrinsic pathway proteins, highlighting the pivotal role of the intrinsic pathway mediating cell death in this leukocyte [118,119].

cAMP modulates the apoptotic pathways in different cell types, increasing or delaying death [120]. We have previously determined the important role of cAMP in inducing resolution of acute inflammation by promoting granulocyte apoptosis *in vivo* [41,95,96]. We have demonstrated that activation of PKA triggered the intrinsic pathway leading to the ultimate neutrophil or eosinophil apoptosis [41,95,96]. Noteworthy, cAMP induced neutrophil apoptosis was shown to be dependent on AnxA1 and its receptor FPR2/ALX [41]. During inflammation, increased cAMP levels bends the balance between pro and anti-apoptotic proteins favoring the induction of cell death. Indeed, anti-apoptotic proteins, such as Mcl-1 and pro-survival proteins including PI3K/Akt and NF-κB were shown to be reduced by analogs of cAMP and PDE4 inhibitors [95]. In addition, increased cAMP enhanced the levels of the pro-apoptotic protein BAX [96], promoted caspase-3 activation [95] and phosphatidylserine eversion to outer cell membrane (as evaluated by Annexin V staining) [41,95,96] (Fig. 3A). The pro-apoptotic effect of cAMP elevating agents was also observed in cultured human neutrophils stimulated with bacterial lipopolysaccharides (LPS) [41]. Granulocyte fate (i.e. whether it undergoes apoptosis or remains viable) depends on the balance of pro-survival stimuli, such as LPS and GM-CSF, oxygen availability, and the presence of pro-apoptotic stimuli, including Fas ligand and TNF [121]. In contrast with our results, some *in vitro* studies have shown that increases of cAMP levels by cAMP analogs or PDE4 inhibitors delayed neutrophil apoptosis in the absence of pro-survival stimuli [122–125]. Interestingly, these studies also consider PKA as the mediator of the pro-survival responses. These contrasting results highlight the important role of the inflammatory milieu directing and dictating the cellular responses to cAMP. In pro-inflammatory conditions, the pro-survival stimuli to granulocytes found inside the inflammatory milieu may be counter-regulated by cAMP elevating agents [41]. Indeed, this environmental effect can also be noted in neutrophils treated with GCs. While GCs were shown to prolong neutrophil survival in culture [126], during inflammatory [38] or under hypoxia [126] conditions, the pro-survival stimuli for neutrophils are inhibited by GCs, leading to apoptosis.

In addition to inducing leukocyte apoptosis, several studies have also shown that increased levels of cAMP induce death of tumor cells [127–129]. cAMP was implicated as a regulator of cell growth inducing arrest of proliferation and apoptosis of different types of tumor cells while contributing to decreased inflammation, a key determinant of cancer progression [127–129]. In this regard, roflumilast, a selective PDE4 inhibitor, was shown to effectively inhibit proliferation, and induce apoptosis of two ovarian cancer cell lines *in vitro* and *in vivo*, acting through the activation of cAMP/PKA/CREB pathway [130].

Apoptotic cells must be cleared from the tissue to limit further inflammation. This task is achieved particularly by macrophages – but also other phagocytes – through a special type of phagocytosis, called efferocytosis. Efferocytosis has profound consequences on innate and adaptive immune responses in inflamed tissues [131] and is essential for normal tissue homeostasis and resolution of inflammation [132]. Soluble mediators such as cytokines (e.g. TGF-β and IL-10), serum proteins (e.g. complement factors, collectins), prostaglandins (e.g. PGE_2) and pro-resolving such AnxA1 and SPMs (e.g. lipoxins, maresins, resolvins) play a crucial role in the resolution of inflammation by inducing efferocytosis [132]. Indeed, the ability of macrophages to clear

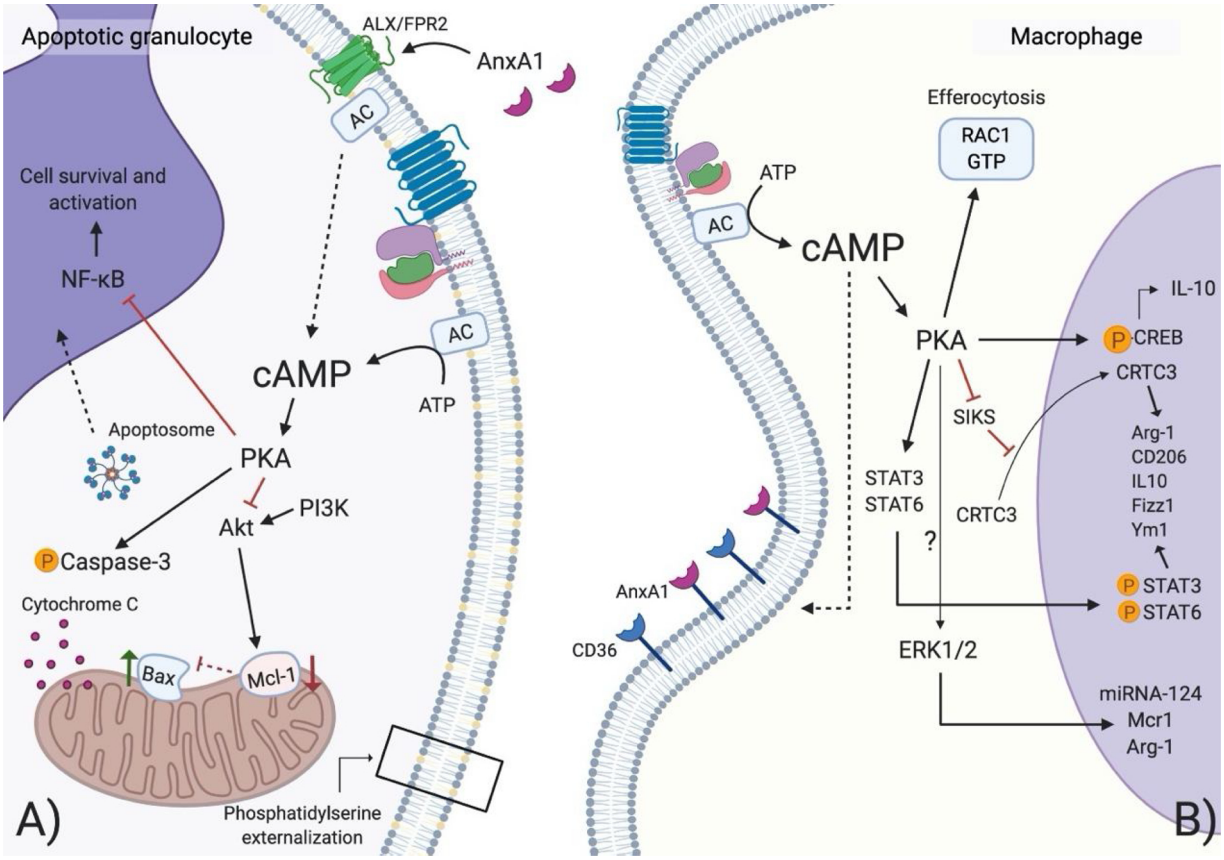

Fig. 3. Schematic representation of the cAMP dependent regulation of granulocyte apoptosis and efferocytosis. (A) Increased levels of cAMP and consequent activation of PKA in granulocytes leads to inhibition of pro-survival pathways (PI3K/Akt and NF-κB) and decrease the amount of Mcl-1, an anti-apoptotic member of the Bcl-2 family. Activation of PKA also leads to increased expression of the pro-apoptotic Bcl-2 family member Bax. By shifting the balance between pro-and anti-apoptotic members of Bcl-2 family, cytochrome C is released with further caspase-3 activation, promoting apoptosis. In addition, there is cAMP-dependent phosphatidylserine externalization, another marker of apoptosis. (B) cAMP enhances efferocytosis by promoting Annexin A1 (AnxA1) and CD36 expression in the phagocyte surface. In addition, cAMP/PKA activates Rac1 GTPAse that promotes cytoskeleton changes to favor efferocytosis. cAMP also induces macrophage polarization to the M2 phenotype. M2 macrophages are more prompt to engulf apoptotic cells and debris, enhancing resolution processes. PKA mediates the phosphorylation of STAT-3/6 and activates ERK1/2 leading to transcription of M2-associated genes. In addition, PKA inhibits SIK-inhibition of CRTC3 leading to its translocation to the nucleus and association with PKA-phosphorylated CREB (p-CREB) promoting the expression of M2 genes. p-CREB promotes the expression of anti-inflammatory cytokines such as IL-10. (Created with Biorender ®).

apoptotic cells critically determines the rate at which inflammation resolves [132,133]. Of note, there is abundant evidence that efferocytosis of apoptotic cells suppresses macrophage production of pro-inflammatory mediators such as TNF-α, IL-1, CXCL-1, IL-8, and leukotriene C4 (LTC_4) and promotes release of anti-inflammatory molecules, including TGF-β, IL-10, nitric oxide, and prostaglandin E2 (PGE_2) [134] and pro-resolving mediators such as resolvins and lipoxins [135]. In addition, failure to clear dead cells exacerbates inflammation emphasizing efferocytosis as a pivotal promoter of resolution [136–138].

Previously, we discussed the role of cAMP-triggered pathways in inducing apoptosis and shifting the production of pro-inflammatory to pro-resolving mediators. Therefore, one can hypothesize that cAMP could also be involved in induction of efferocytosis by different macrophages (Fig. 3B). Increased levels of cAMP in macrophages are associated with increased engulfment of apoptotic granulocytes through the activation of the Rac Family Small GTPase 1 (Rac1) in a PKA-dependent pathway [139]. In addition, enhanced levels of cAMP in macrophages, induced by binding to lysophosphatidylserine (lysoPS) expressed on apoptotic neutrophils, promotes eff ;erocytosis [140]. Recently, our group showed that db-cAMP, a cell-permeable cAMP mimetic, increases the expression of the brigding/engulfment molecules CD36 and AnxA1 in a murine model of LPS-induced pleurisy [65]. We have shown that the long term treatment with db-cAMP increased apoptotic neutrophil efferocytosis *in vivo* (7 h of treatment) and *in vitro* (24 h of treatment) in a PKA-dependent manner [65]. On the other hand, Rossi and colleagues have shown that short exposure of human monocyte-derived macrophages to PGE_2, PGD_2, db-cAMP or 8-Br-cAMP (a cAMP analog) decreased efferocytosis of neutrophils [141]. Given the divergence of these two studies, we hypothesize that longer treatment seems to be essential to alter macrophage responses through increasing expression of engulfment/bridging molecules involved in the recognition and engulfment processes [65].

Pro-resolving mediators that are known to increase the levels of cAMP, such as melanocortins, MaR1 and RvD5n-3 DPA, are also inducers of efferocytosis [49,68,142,143]. The agonism of melanocortin receptors, by LGR6 (the receptor for Mar1) or GPR101 (RvD5n-3 DPA receptor), profoundly enhanced the phagocytosis of human and mouse apoptotic neutrophils while increasing intracellular cAMP levels [49,64,142]. However, whether the induction of efferocytosis by SPMs is a direct action of cAMP remains to be described. In addition, RvD1 increases efferocytosis by preventing the oxidative apoptosis of macrophages. RvD1 is an agonist for FPR2/ALX in macrophages leading to increased intracellular cAMP levels and activation of PKA [144]. The triggered signaling pathway leads to reduction of pro-apoptotic proteins promoting macrophage survival and ensuring effective efferocytosis [144].

Not surprisingly, the role of cAMP during phagocytosis, as it is for efferocytosis, is controversial. Classically, increased intracellular levels

of cAMP are correlated with impaired phagocytosis and killing of bacteria or other particles [25,100]. Mechanistically, increased cAMP levels reduced expression of phagocytosis-related receptors such as complement receptors, scavenger receptors and Fc gamma receptors via PKA/Epac pathways [145,146]. In addition, cAMP was shown to inhibit the production of ROS impairing bacterial killing [145,147]. In contrast with these findings, there is evidence to suggest that the clearance of pathogens, a feature of resolution of inflammation, may be enhanced by cAMP [42,58,148,149]. Recently, treatment of *Mycobacterium tuberculosis* (Mtb)-infected macrophages with lysophosphatidylcholine (LPC) was shown to increase cAMP levels and activate PKA to promote phagosome maturation [148]. This process was dependent on the PKA phosphorylation of GSK3β that also reduced NF-κB, leading to decreased secretion of pro-inflammatory cytokines and increased production of anti-inflammatory cytokines. Therefore, LPC could effectively control Mtb growth by promoting phagosome maturation via cAMP-induced activation of the PKA–PI3K–p38 MAPK pathway [148]. Similarly, we have shown that PDE4 inhibition increases phagocytosis of *Streptococcus pneumoniae* during the later stages of lung infection [42]. In addition, increased cAMP levels was shown to increase the phagocytic uptake and ROS-mediated killing of bacteria in human and mouse neutrophils [149]. Moreover, treatment with RvD5 decreased expression of PDE4 (potentially increasing cAMP levels) leading to enhanced *E. coli* phagocytosis in human macrophages [58]. Furthermore, RvE1, RvD1, RvD5, LXB_4, and MaR1, a cluster of SPMs upregulated during normal coagulation, were shown to signal resolution through the CREB pathway increasing phagocytosis and killing of bacteria by human leukocytes [67]. The discrepancy among studies may be related to the timing and magnitude of cAMP intracellular levels. Moreover, depending on the environment (e.g. cytokines and other stimuli in the milieu), cAMP can trigger different pathways explaining these contrasting effects.

5. cAMP induces macrophage polarization

Macrophages coordinate both the onset and resolution of inflammation: promoting inflammation and clearance of pathogens; but also performing efferocytosis and production of pro-resolving molecules [135]. As such, different phenotypes of macrophages have been described to play segregated functions [6,138,150,151].

Recently, macrophage subtypes and nomenclature have been discussed. During inflammation, a continuum of macrophage phenotypes coexists orchestrating the different phases of the response. The categorization of macrophage phenotypes, although artificial, is helpful for research and teaching purposes. The most commonly accepted classification or nomenclature for the different macrophages phenotypes are M1 and M2 (M2a, M2b, M2c and M2d) or classically activated and alternatively activated, respectively [152,153]. *In vitro*, the M1 pro-inflammatory phenotype is induced by exposure to IFN-γ combined with LPS or TNF-α, whereas the M2 anti-inflammatory phenotype is induced by IL-4/IL-13 (M2a), immune complexes and Toll-like receptor agonists such as LPS (M2b), IL-10, TGF-β or glucocorticoid hormones (M2c) and, IL-6 and agonists for the adenosine receptor A2A (M2d) [152–156]. A variety of markers are used to determine these phenotypes, including proteins, cytokines, chemokines, receptors, and others. The most commonly used markers to characterize the M1 phenotype are the expression of inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-6 [152,153,157]. The traditional M2 markers are Arginase 1 (Arg-1), CD206 (mannose receptor) and anti-inflammatory cytokines, such as IL-10 and TGF-β [152,153,157].

Macrophage polarization is a feature of the resolution of inflammation [17] and might be induced before or after efferocytosis of apoptotic bodies [158–160]. Bystrom and colleagues firstly identified the involvement of cAMP pathways in macrophage polarization, showing increased production of cAMP by resolution phase macrophages (rMs) [66]. Later, the direct involvement of cAMP in polarization of murine macrophages (RAW 264.7) was determined through the cAMP dependent induction of Arg-1 expression [161]. In agreement with that, treatment with forskolin (activator of AC) in a model of autoimmune encephalomyelitis (EAE) increased M2 (miR-124, Arg-1, Mrc-1, Fizz-1 and Ym-1) and decreased M1 markers (NOS2 and CD86), a process dependent on ERK signaling, with an additional reduction of proinflammatory cytokines produced by pathogenic T CD4 cells [162]. Similarly, we have recently shown that db-cAMP increases the expression of Arg-1, CD206, Ym-1 and IL-10 levels (M2 markers) in bone marrow-derived macrophages (BMDMs) and RAW 264.7 macrophages, in a PKA-dependent manner [65]. Of note, db-cAMP promotes non-typical polarization to the M2 profile, as it induces iNOS expression, albeit at significantly lower levels than that induced by LPS/IFN-γ [65]. The expression of iNOS is indeed an important determinant for the clearance of bacteria, through the production of reactive nitrogen species (RNS) [163], and might explain the protective effect of rolipram during the late stages of bacterial infection [42]. Noteworthy, cAMP elevating agents (forskolin, IBMX, 8-Br-cAMP and db-cAMP) may synergize with IL-4 to induce M2 polarization of BMDMs, RAW 264.7 cells or human alveolar macrophages [65,161,162,164]. Mechanistically, cAMP induces M2 polarization through phosphorylation of STAT3 [65,165] and STAT6 signaling [161] and also re-educates M1 macrophages towards an M2-like phenotype by decreasing STAT1 phosphorylation [65]. In keeping with that, we have shown that db-cAMP decreases the number of M1 macrophages in LPS-induce pleurisy, while inhibition of the cAMP-pathway by using a PKA inhibitor prevents natural resolution of inflammation [65]. These results evidenced the critical role of cAMP not only in macrophage polarization, but also for the efficient resolution of inflammation. Fig. 3B illustrates the main signaling pathways triggered by cAMP to induce macrophage polarization.

Pro-resolving mediators known to induce cAMP-dependent cellular responses, such as RvD2 [50,166,167], RvD1 [144,167], melanocortin [168,169], MaR1 [64,170] and AnxA1 [41,171], promote the polarization of macrophages to a regulatory phenotype. As such, the potential antifungal and anti-inflammatory mechanism of the synthetic melanocortin peptides $(Ac\text{-}Cys\text{-}Lys\text{-}Pro\text{-}Val\text{-}NH_2)_2$ and $(CKPV)_2$ is the polarization of M1 macrophages to the M2 phenotype, what was associated with increased cAMP, Arg-1 and IL-10 levels in macrophages [169]. Similarly, PGE_2 induces the expression of several M2 markers in BMDMs via cAMP-induced CREB/CRTC2/3/KLF4 pathway [172] and in gilthead seabream macrophages through the cAMP/PKA/CREB pathway [173]. Of interest, PGE_2 signaling through CREB activation was shown to be protective in models of periodontal disease promoting tissue repair [174], a feature of M2 macrophages [151].

Additional signaling pathways related to macrophage polarization are beginning to be unraveled. The activation of salt-induced kinase 2 (SIK2) seems to be an important step to change macrophage phenotype. cAMP activated-PKA phosphorylates SIK2 that consequently prevents the phosphorylation of CREB-regulated transcription coactivator 3 (CRTC3). Unphosphorylated CRTC3 translocates to the nucleus to interact with CREB, which in turn can induce the transcription of M2 genes such as IL-10 in macrophages (Fig. 1) [175,176]. The mechanism of action of the clinically approved cancer drugs bosutinib and dasatinib is by the induction of CRTC3 dephosphorylation, interaction with CREB, transcription of IL-10 and polarization of macrophages to regulatory phenotypes. This effect occurs in a SIK2-dependent manner [177]. Indeed, pharmacological or genetic inhibition of SIKs also promotes the dephosphorylation of CRTC3 in BMDMs, resulting in the translation of CRTC3 into the nucleus, where it acts as a cofactor for the transcription of CREB-dependent genes, including a transcription of IL-10 and other macrophage markers (M2b), such as SPHK1, LIGHT and Arg-1 [178]. These studies demonstrate the importance of the cAMP→PKA→SIK→CTCR3→CREB→IL-10 axis in the polarization of macrophages to anti-inflammatory profiles, suggesting that drugs already

approved for different clinical uses might be assigned to treat inflammatory diseases. It is important to note that since PKA can directly activate CREB, the axis described above may not occur in a linear fashion. As such, different intracellular pathways can be triggered and converge in many points to induce macrophage polarization.

M2 macrophages produce IL-10 and favor further efferocytosis and resolution [179]. Part of the pro-resolving mechanism of some SPMs, such as RvD1, include macrophage polarization to M2 phenotype and increased expression of IL-10 [180]. We have shown that the pro-resolving proteins plasminogen and plasmin [181] also induce macrophage polarization associated with increased production of IL-10 [182]. Interestingly, LPS has been shown to have a synergistic effect with isoproterenol, a cAMP-inductor, on IL-10 expression through CREB activation, demonstrating that cAMP induces IL-10 not only in a non-inflammatory context, but also during inflammation, what contribute to modulation of the response and induction of resolution [183]. In agreement with that, we have shown that db-cAMP was able to induce IL-10 production during inflammation both *in vitro* and *in vivo*, reinforcing its resolving properties [65].

Polarization of macrophages is a coordinated process that can be initiated by the very same stimuli that induce their activation, such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [184]. Activated macrophages undergo metabolic alterations that lead to ATP production and release. Macrophage ectoenzymes in the cell surface, such as CD39 and CD73, can convert ATP to adenosine [184]. Adenosine is a purine nucleoside and mediator of metabolic stress that can act through binding to A2A and A2B receptors and activate AC to increase intracellular cAMP levels [185], leading to downregulation of inflammatory cytokines and induction of a regulatory state in macrophages [184]. Adenosine has been shown to have a synergistic effect with IL-4 and IL-13 on the induction of Arg1 and TIMP-1 (M2 markers) in murine macrophages via C/EBPβ and p38 pathways [186]. *In vitro* adenosine has been shown to have synergistic effects with IL-10 on the repolarization of macrophages to an M2c profile in RAW 264.7 macrophages by inducing Arg-1, BCL-3 and TIMP-1, an event dependent on the A2A, A2B receptors and STAT3 [187]. Interestingly, the induction of M2c phenotype in murine peritoneal macrophages increases the expression of CXCR4 leading to macrophage egression to the lymph nodes, what might contribute to resolution of inflammation [188]. Whether cAMP is directly involved with this import step remains to be determined.

The observation that macrophage functional phenotypes can be manipulated has drawn attention to macrophages as a potential therapeutic target [189,190]. Thus, elucidation of the signaling pathways that regulate macrophage functional polarization will aid in the design of strategies for modification of macrophage responses.

6. cAMP promotes nonphlogistic recruitment of monocytes/macrophages

Another key step for resolution of inflammation is the nonphlogistic recruitment of monocytes to the inflammatory site and further differentiation in macrophages with pro-resolving phenotypes [14,191]. This additional recruitment of cells provides a further opportunity to promote the clearance of debris and apoptotic neutrophils in the site of inflammation, preventing secondary necrosis and exposure to harmful neutrophil intracellular contents in the tissue [14]. Indeed, pro-resolving molecules including LXA_4 [192,193], AnxA1 [194,195] and plasminogen/plasmin [182,196] regulate the spatiotemporal nonphlogistic recruitment of monocytes/macrophages, events that contribute to efferocytosis and consequent resolution of inflammation. Of interest, we recently showed that db-cAMP promotes specific and timely regulated monocyte recruitment *in vivo*, which was associated with increased production of IL-10 and CCL2, but not other pro-inflammatory cytokines/chemokines such as IL-6, TNF-α and CXCL1. Mechanistically, db-cAMP-induced macrophage recruitment was shown to be dependent on the CCL2:CCR2 axis and activation of PKA [65]. Noteworthy, the MEK/ERK1/2 pathway, which triggers CCL2 production and controls cell migration [196–198], was shown to mediate the db-cAMP-induced mononuclear cell migration (unpublished data from our group). Therefore, a growing amount of evidence supports the role of cAMP in the recruitment and polarization of macrophages to the M2 phenotype contributing to resolution of inflammation.

7. Concluding remarks

The complexity of inflammation is well-appreciated by the myriad of intracellular signaling pathways that have been identified and studied in detail [7]. Different pro-inflammatory signals trigger a multifaceted net of synergic pathways that culminate with cell activation, migration, production of cytokines and other molecules, that ultimately ensure an effective immune response against an invading pathogen [3]. In the past years, the molecular mechanisms related to inflammatory responses have been vastly explored therapeutically [199]. On the other hand, the signaling pathways triggered and regulated during the resolution of inflammation are still less understood. Different pro-resolving mediators activate interconnected intracellular pathways leading to important features of resolution: apoptosis, efferocytosis/phagocytosis, reduction of inflammatory mediator production and recruitment of granulocytes, macrophage recruitment and polarization [200]. Of note, signaling pathways elicited during inflammation may induce the switch to the cell pro-resolutive status leading to the production of pro-resolving mediators [15,184]. Here we suggest cAMP as an important intracellular inducer of resolution during both sterile and infectious inflammatory responses. Evidence gathered by our group and others have shown that increased cAMP levels are associated with the induction and mechanism of action of different pro-resolving mediators (Fig. 4).

Increased intracellular levels of cAMP are often associated with anti-inflammatory effects such as production of IL-10 and blockage of leukocyte infiltration and pro-inflammatory cytokine production [25]. In the context of autoimmune or chronic inflammatory diseases, anti-inflammatory drugs are very useful to prevent overwhelming responses and tissue damage [11,13,25]. However, sustained anti-inflammation may increase host susceptibility to infections. Therefore, inflammation plays a dual role in the context of infectious diseases; ie. it is crucial for pathogen clearance but detrimental if uncontrolled/exaggerated, when it can cause tissue damage and contribute to morbidity and mortality [201]. In this regard, pro-resolving mediators and drugs that induce resolution, rather than block inflammation, represent a better pharmacological strategy. The growing field of resolution pharmacology is based on innovative approaches that utilize patients' endogenous resolution pathways for tissue protection and improvement of inflammatory diseases [202]. In this regard, cAMP elevating agents might represent new resolution therapeutics for inflammatory diseases. Noteworthy, PDE4 inhibitors have been recently suggested as a treatment option for the hyperinflammation seen in patients with pneumonia caused by the pandemic SARS-CoV-2 virus [203]. As for influenza infections, PDE4 inhibition might decrease inflammation in the lungs without causing immunosuppression and increased virus proliferation [204]. Indeed, the prolonged use of apremilast (a selective PDE4 inhibitor approved for psoriasis treatment) was associated to protection effect against COVID-19 severity, an effect considered secondary to drug-induced modulation of inflammation [205]. Further clinical studies with bigger cohort are needed to prove this hypothesis.

Here, we present evidence for a central role of cAMP as a messenger of resolution of inflammation promoting apoptosis of granulocytes, efferocytosis/phagocytosis, macrophage recruitment and polarization and production of pro-resolving molecules (Fig. 4). The cAMP effectors PKA and Epac have been shown to coordinate specific features of resolution triggering cell-dependent pathways. Importantly, while PKA is often suggested as the main effector of cAMP-responses in the induction

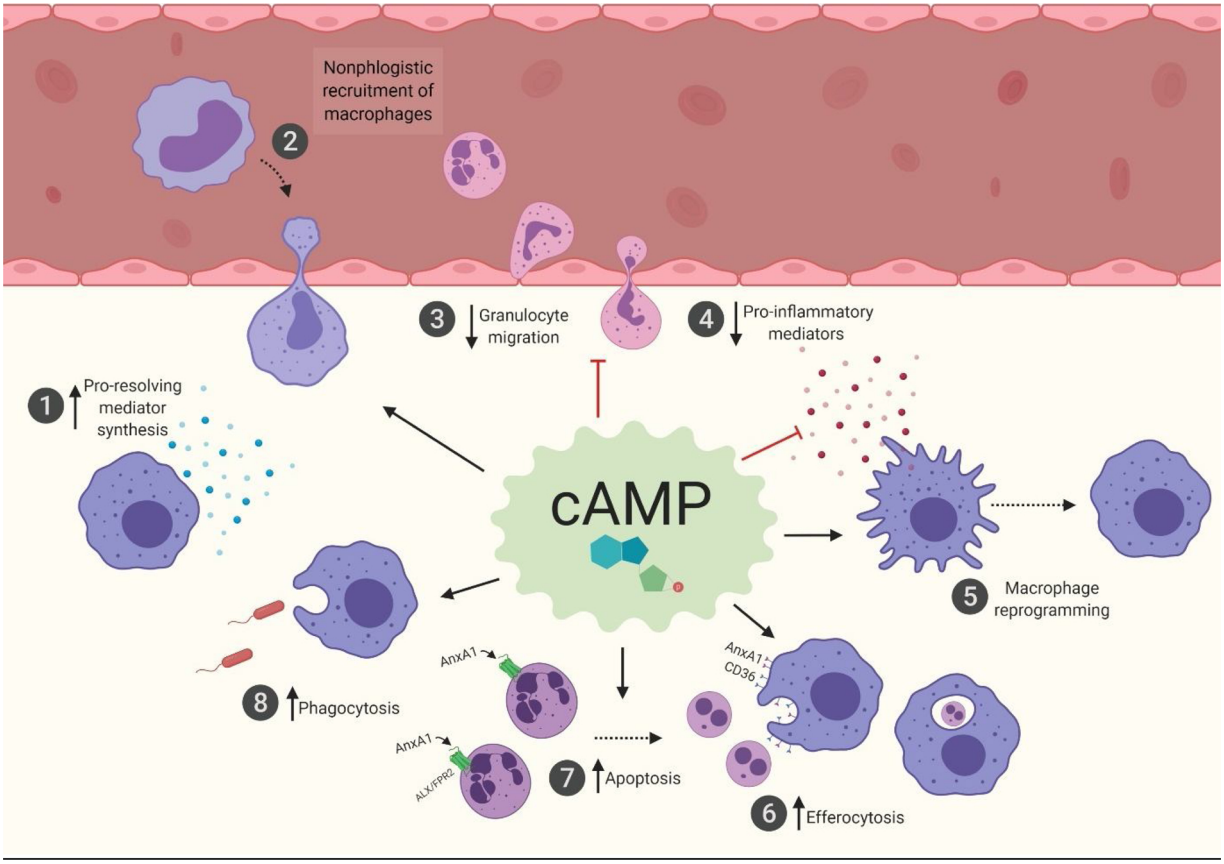

Fig. 4. cAMP regulates key steps for resolution of inflammation. Increased levels of cAMP were shown to (1) promote biosynthesis of pro-resolving molecules such as AnxA1 and (2) induce the non-phlogistic recruitment of monocytes into the tissue, while (3) restricting further recruitment of granulocytes. cAMP-triggered pathways (4) reduce the production of pro-inflammatory mediators in the sites of inflammation, (5) induce macrophage polarization to pro-resolving phenotypes and (6) increase efferocytosis, (7) apoptosis of granulocytes (dependent on AnxA1/FPR2) and (8) phagocytosis of microorganisms by macrophages. Overall, the dynamic intracellular circuit of cAMP signaling during inflammation coordinates key steps of resolution. (Created with Biorender ®).

of resolution, some of the evidence in the reported studies is supported solely by pharmacological inhibition of PKA by drugs such as H89. This compound is a competitive antagonist of the adenosine triphosphate (ATP) site on the PKA catalytic subunit, preventing its actions. Noteworthy, H89 has off-target actions as it also inhibits other kinases, including the mitogen- and stress-activated kinase-1 (MSK-1), which is a regulator of NF-κB, a promoter of inflammation and cell survival [206]. Trying to overcome the pharmacological problems of PKA inhibition, some of the studies utilized the compound Rp-adenosine-3′,5′-cyclic monophosphorothioate (Rp-cAMPS) [41]. Although often considered selective inhibitors of PKA, Rp-cAMPS can also bind to other molecules, including Epac, contain cyclic nucleotide–binding sites similarly to PKA [207]. Therefore, while there is robust amount of evidence for cAMP actions in the induction of resolution of inflammation, the use of relevant genetic strategies such as RNAi or gene knockout must be pursued to clarify the triggered effectors that signal the biological effect of cAMP. Understanding the molecular aspects and the dynamic of signaling pathways of resolution offers a new perspective on the development of novel therapeutic strategies for inflammatory diseases.

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Declarations of Competing Interest

The authors declare no conflict of interests regarding the publication of this paper.

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