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Resolution of inflammation: Mechanisms and opportunity for drug development

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ABSTRACT

Inflammation is a beneficial host reaction to tissue damage and has the essential primary purpose of restoring tissue homeostasis. Inflammation plays a major role in containing and resolving infection and may also occur under sterile conditions. The cardinal signs of inflammation *dolor*, *calor*, *tumor* and *rubor* are intrinsically associated with events including vasodilatation, edema and leukocyte trafficking into the site of inflammation. If uncontrolled or unresolved, inflammation itself can lead to further tissue damage and give rise to chronic inflammatory diseases and autoimmunity with eventual loss of organ function. It is now evident that the resolution of inflammation is an active continuous process that occurs during an acute inflammatory episode. Successful resolution requires activation of endogenous programs with switch from production of pro-inflammatory towards pro-resolving molecules, such as specific lipid mediators and annexin A1, and the non-phlogistic elimination of granulocytes by apoptosis with subsequent removal by surrounding macrophages. These processes ensure rapid restoration of tissue homeostasis. Here, we review recent advances in the understanding of resolution of inflammation, highlighting the pharmacological strategies that may interfere with the molecular pathways which control leukocyte survival and clearance. Such strategies have proved beneficial in several pre-clinical models of inflammatory diseases, suggesting that pharmacological modulation of the resolution process may be useful for the treatment of chronic inflammatory diseases in humans.

Contents

Abbreviations: α-MSH, α-melanocyte-stimulating hormone; AA, arachidonic acid; AnxA1, annexin A1; AP-1, activating protein 1; ATL, aspirin-triggered lipoxin; Bcl-2, B-cell lymphoma 2; C5a, complement factor 5a; cAMP, cyclic adenosine monophosphate; CDKi, cyclin-dependent-kinase inhibitor; COX, cyclooxygenase; CyPG, cyclopentenone prostaglandin; DHA, docosahexaenoic acid; DNA, deoxyribonucleic acid; ERK, extracellular-signal-regulated kinase; fMLP, formyl-Met-Leu-Phe; FPR2/ALXR, formylpeptide receptor 2/lipoxin A_4 receptor; GC, glucocorticoid; GILZ, glucocorticoid-induced leucine zipper; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon gamma; JNK, c-Jun N-terminal kinase; H_2O_2, hydrogen peroxide; iNOS, inducible nitric oxide synthase; IL, interleukin; LX, lipoxin; LO, lipoxygenase; LPS, lipopolysaccharide; LT, leukotriene; MAPK, mitogen-activated protein kinase; Mcl-1, myeloid leukemia cell differentiation protein 1; MKP-1/DUSP-1, mitogen-activated protein kinase phosphatase/dual specificity phosphatase; NF-κB, nuclear factor kappa B; NADPH, nicotinamide adenine dinucleotide phosphate; PAF, platelet-activating factor; PDE, phosphodiesterase; PG, prostaglandin; PI3K, phosphoinositide 3 kinase; PPARγ, peroxisome proliferator-activated receptor gamma; PS, phosphatidylserine; RNA, ribonucleic acid; ROS, reactive oxygen species; Rv, resolvin; SOD, superoxide dismutase; SCFA, short chain fatty acid; TGF-β, transforming growth factor-β; TLR, toll-like receptor; TNF-α, tumor necrosis factor alpha.

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1. Introduction

Inflammation is a salutary host response against invading pathogens or following sterile tissue injury. The inflammatory response is a spatially and temporally orchestrated event in which cells and mediators collaborate to neutralize and eliminate the damaging stimuli to allow maintenance of homeostasis (Cotran et al., 1999; Medzhitov, 2010). Although inflammation is primarily a physiological and beneficial process, non-resolving inflammatory processes may be involved in the pathogenesis and progression of many inflammatory diseases, including asthma, atherosclerosis, rheumatoid arthritis, multiple sclerosis, rhinitis and ischemia–reperfusion injury (McFarland & Martin, 2007; Waldburger & Firestein, 2009; Nathan & Ding, 2010; Eltzschig & Eckle, 2011; Mandhane et al., 2011; Van-Assche et al., 2011; Chung, 2012).

Traditionally, anti-inflammatory therapies have focused on strategies to decrease or neutralize the level of pro-inflammatory mediators and/or inhibit the recruitment of leukocytes and their activation. These therapies include non-steroidal anti-inflammatory drugs (NSAID), glucocorticoid (GC) receptor agonists (synthetic GCs) and antibodies or inhibitors targeting specific pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1. However, in the past few years, it has been recognized that pro-resolution-based strategies have potential for the treatment of multiple inflammatory conditions (Gilroy et al., 2004; Rossi et al., 2007; Hallett et al., 2008; Serhan et al., 2008; Duffin et al., 2010). Indeed, resolution of inflammation is regarded as a targetable process that is distinct from targeting acute inflammatory processes. Resolution is an active process involving biochemical mediators and signaling pathways controlling 1) termination of the inflammatory response (mainly by diminishing granulocyte recruitment and reversing vasodilatation and vascular permeability); 2) switching from pro-inflammatory mediator generation to production of pro-resolution mediators; 3) turning off signaling pathways associated with cytokine production and leukocyte survival; 4) apoptosis of recruited inflammatory cells; 5) phagocyte clearance of apoptotic cells (especially by macrophages in a non-phlogistic process) and; 6) switching from pro-inflammatory cell phenotypes to pro-resolution phenotypes (especially relevant to macrophages).

There are defined molecular pathways that promote leukocyte survival (or prevent death) once non-resident inflammatory cells migrate into tissue. Thus, understanding mechanisms that regulate apoptosis is vital in providing clues as to which molecular pathways can be pharmacologically modulated. In this review, we will discuss important signaling pathways involved in the control of apoptosis, with a focus on key pharmacological agents that are able to manipulate those pathways in vivo and are therefore potentially relevant as therapies for the treatment of autoimmune and chronic inflammatory diseases.

1.1. General aspects of acute inflammation

Macroscopically the inflammatory reaction is recognized by the cardinal signs of *calor* (heat), *rubor* (redness), *tumor* (swelling), *dolor* (pain) and loss of function, the first four of which were described by Cornelius Celsus in the first century. The first step of the inflammatory cascade is primarily a vascular response with hyperemia and increase in permeability of the vessel wall. Initially, transient arteriolar vasoconstriction is observed and promoted by the contraction of vascular smooth muscles. Subsequently, arteriolar vasodilatation leads to increased blood flow into the damaged area resulting in local hyperemia. At this early stage, alterations in the vascular endothelium are readily detectable with consequent exudation of plasma proteins and fluid from the blood into the tissue. This is followed by migration of leukocytes from the circulation into tissue (Cotran et al., 1999; Lawrence et al., 2002).

In response to an appropriate stimulus (e.g. infection, mechanical trauma, ischemia, toxins, minerals, crystals, chemicals, and antigens), circulating leukocytes interact with post-capillary venule endothelial cells (or capillary endothelial cells in the pulmonary circulation). This process is mediated predominantly by selectins (glycoprotein adhesion molecules) present on leukocytes (e.g. L-selectin) and on endothelial cells (e.g. P and E-selectin) and their carbohydrate ligands (PSGL-1, ESL-1 and CD34) (Kubes, 2002; Petri et al., 2008). Leukocytes rolling on the endothelium are now capable of interacting with chemoattractant factors, including complement factor 5a (C5a), IL-8/CXCL8, platelet-activating factor (PAF), eotaxin/CCL11 and leukotriene (LT) B_4, which sit on the luminal surface of the endothelium. The activation of relevant chemoattractant receptors on the rolling leukocyte results in up-regulated expression and affinity of integrins (CD11/CD18 family; very late antigen-4, VLA-4) (Baggiolini, 1998; Baggiolini & Loetscher, 2000; Sallusto & Baggiolini, 2008). Integrins promote firm adhesion of activated leukocytes to endothelial cells by binding to their ligands, including intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). Subsequently, leukocytes crawl on endothelial cells until they find appropriate spots where they migrate into the interstitium (Phillipson et al., 2006). Migration into tissue relies on integrins and other cell adhesion molecules, including platelet endothelial cell adhesion molecule 1 (PECAM-1) present in intercellular junctions (Kubes, 2002; Petri et al., 2008). Once in the tissue, leukocytes can be further activated and become an important source of a range of substances which include colony stimulating factors, cytokines, chemokines, lipid mediators and reactive oxygen species (ROS). Besides promoting the elimination of injurious stimuli, the inflammatory process itself may contribute to damage of adjacent tissues and can therefore enhance the severity of symptoms (Cara et al., 2000).

Apoptosis of granulocytes followed by recognition and removal by surrounding phagocytes, such as macrophages, are important events in the resolution of acute inflammation. The latter events limit tissue injury caused by leukocytes at sites of inflammation and are important for switching macrophage phenotype towards pro-resolution (discussed later). Ideally, the initial inflammatory response to protect the host is self-limiting and progresses to complete resolution (Hallett et al., 2008). However, if dysregulated acute inflammation can progress to a more chronic situation that can eventually result in scarring and fibrosis. Thus, the induction of granulocyte apoptosis could be of potential benefit in the control of acute inflammatory diseases (Savill, 1997; Gilroy et al., 2004; Rossi et al., 2007; Hallett et al., 2008; Sousa et al., 2009, 2010; Alessandri et al., 2011; Vago et al., 2012). Fig. 1 highlights the different phases of acute inflammatory processes towards successful resolution.

1.2. Mechanisms of leukocyte death

The accumulation of leukocytes in tissue depends not only on the number of cells being recruited at any particular time, but also on the number of cells that are cleared or leave the tissue (Gilroy et al., 2004; Hallett et al., 2008; Duffin et al., 2010). Inflammatory cells can be eliminated by systemic re-circulation, lymphatic drainage or local cell death. Different modalities of cell death, based on morphological, molecular and functional criteria, have been described. Examples include apoptosis, excitotoxicity, pyroptosis, necrosis, necroptosis and NET-osis (Kroemer et al., 2009; Han et al., 2011).

Granulocytes in particular undergo apoptosis during both physiological and inflammatory conditions. However, leukocytes may also die by autophagy, NET-osis and necrosis, with the mode of cell death impacting on the process of inflammation (Mitroulis et al., 2010; Geering & Simon, 2011). Our groups and others have examined a number of key molecules and pathways involved in maintenance of leukocyte survival or apoptotic death, including nuclear factor kappa B (NF-κB), phosphoinositide-3-kinase (PI3K), myeloid leukemia cell differentiation protein 1 (Mcl-1) and cyclic adenosine monophosphate (cAMP). Studies with these molecules or pathways have highlighted the importance of the subsequent regulated clearance of apoptotic cells by inflammatory macrophages in vitro and in vivo (Rossi et al., 1998; Ward et al., 2002; Duffin et al., 2009; Sousa et al., 2010;

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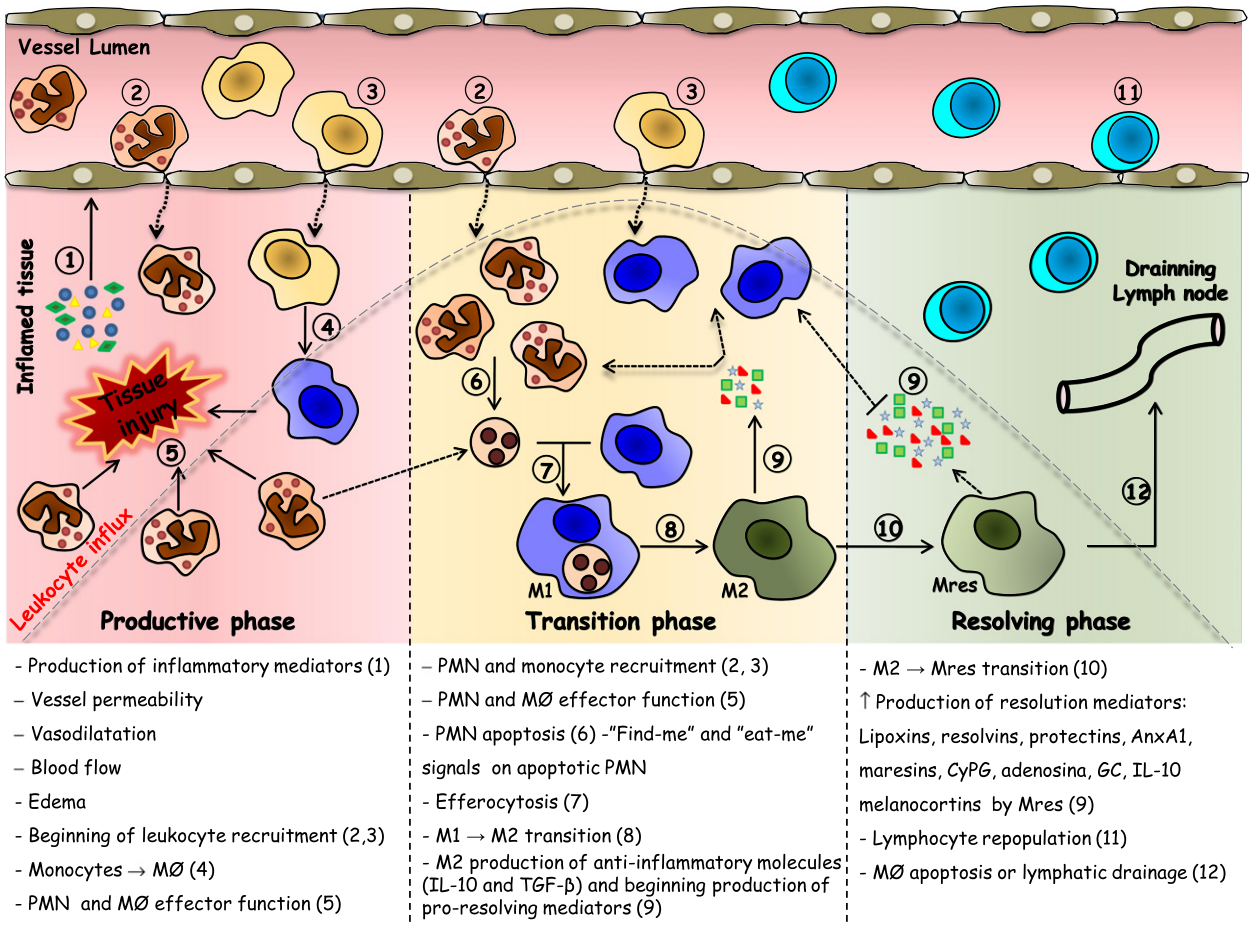

Fig. 1. Orchestrated series of events in a successful self-resolving acute inflammatory process. Tissue injury (sterile or following infection) leads to recognition of molecular pattern (DAMPs and PAMPs) by resident cells (tissue macrophage, dendritic cells and epithelial cells) that rapidly produce several pro-inflammatory mediators (1). In this *productive phase* of inflammation, mediators act by promoting vasodilatation and consequent increase of local blood flow, and modifying endothelial wall permeability. These events are accompanied by hyperemia and exudation of plasma proteins and fluid (edema). Endothelial cells are also activated to express cell adhesion molecules (selectins and integrins) and to present chemoattractant mediators which will allow the capture and extravasation of leukocytes (2–4). Polymorphonuclear leukocytes (mainly neutrophils) are the first cells that extravasate into inflamed tissues (2) followed by mononuclear cells (3). With progression of the inflammatory response, there is intense leukocyte influx into inflamed tissue. These cells can be activated and become an important source of a range of mediators, including growth factors, cytokines, chemokines, lipid mediators and ROS (5), which will allow infiltrating leukocytes to perform their effector functions in tissues. In the *transition phase* of inflammation, despite the intense leukocyte accumulation, PMNs under the action of pro-resolving signals (mediated by pro-resolving mediators and decreased levels of survival modifying agents) start undergoing apoptosis (6) followed by phagocytosis by tissue macrophages (efferocytosis) (7). This process involves several signals that lead to attraction, binding and removal of apoptotic cells by macrophages. During efferocytosis, macrophage phenotypes switch from M1 to M2-phenothype (8). M2 (or M2-like) macrophages are highly efferocytic and produce anti-inflammatory molecules (such as IL-10 and TGF-β) and pro-resolving mediators (9). Such mediators have the potential to inhibit additional PMN recruitment, intensify monocyte migration and amplify efferocytosis. M2 macrophages then switch to Mresolution (Mres) phenotype (10), which display reduced phagocytosis, but, instead, produce anti-fibrotic and anti-oxidant proteins that limit tissue damage and fibrosis. Such events, pave the way to the *resolution phase* of inflammation. The increased production of pro-resolving mediators, anti-inflammatory and anti-fibrotic agents by resolution macrophage (Mres), lymphocyte repopulations (11) and lymph node drainage or apoptosis of macrophage (12) close the inflammatory process and restore tissue homeostasis.

Alessandri et al., 2011). Regulated clearance of apoptotic cells leads to shutdown of cellular activity and inhibition of inflammatory responses. Therefore, it is considered that granulocyte apoptosis followed by phagocytosis is the most desirable form of cell death for successful resolution (Savill, 1997; Gilroy et al., 2004; Rossi et al., 2007; Hallett et al., 2008).

Morphologically, apoptosis proceeds with chromatin condensation, nuclear fragmentation with rounding up and apoptotic body formation, organelle involution and cytoplasm shrinkage (Kroemer et al., 2009). Along with morphological evaluation performed by light and scanning electron microscopy (Rossi et al., 1993), apoptosis can be assessed by a number of techniques, including deoxyribonucleic acid (DNA) gel electrophoresis (Ward et al., 1999), western blotting (Duffin et al., 2009), TUNEL (terminal deoxy-nucleotidyl-transferase mediated dUTP nick end labeling; which detects DNA fragmentation) (Gorczyca et al., 1992) and flow cytometry (van et al., 1998; Duffin et al., 2009). Flow cytometric assessment of apoptosis is generally performed using labeled annexin-V, which binds to phosphatidylserine (PS) exposed on the surface of apoptotic cells, and propidium iodide (PI), which marks necrotic cells.

There are much data supporting a critical role for caspases in the mechanism underlying leukocyte apoptosis (Weinmann et al., 1999; Daigle & Simon, 2001; Duffin et al., 2009; Sousa et al., 2009, 2010; Yazdi et al., 2010; Alessandri et al., 2011; Vago et al., 2012). Caspases (cysteinyl aspartate proteases) are involved in both apoptosis pathways, namely the extrinsic and intrinsic pathways. The extrinsic pathway is triggered when an extrinsic ligand stimulates a death receptor (e.g. Fas/Fas ligand and TNF-TNFR) and activates pro-caspase 8 (Krammer, 2000; Geering et al., 2011). The clustering of procaspase-8 molecules initiates an autocatalytic caspase activation cascade with subsequent cleavage and activation of effector caspases (in particular caspase-3) which drive apoptosis. Caspase-8 can either directly cleave procaspase-3 to caspase-3 which causes apoptosis without mitochondrial depolarization (Huang et al., 1999) or caspase-8 can cleave the

pro-apoptotic B-cell lymphoma 2 (Bcl-2) homolog Bid (the Bcl-2 family proteins will be discussed later) to its truncated form (tBid) with activation of the intrinsic pathway and release of pro-apoptotic factors from mitochondria. Conversely, the intrinsic pathway is largely dependent on the relative balance of pro-apoptotic versus anti-apoptotic proteins from the Bcl-2 family. The intrinsic pathway controls apoptosis in response to stimuli such as cytotoxic agents, UV radiation or oxidative stress (Siegel, 2006). A relative abundance of pro-apoptotic Bcl-2 proteins (or relative decrease in anti-apoptotic proteins) results in mitochondrial outer-membrane permeabilization with release of proteins from the mitochondrial inner membrane space into the cytosol. These proteins include cytochrome c which interacts with pro-caspase-9 and the cytosolic protein apoptotic-protease-activating factor-1 (APAF1) to form the apoptosome complex which activates the effector caspase-3 driving extensive cleavage of substrates and irreversible cell death (Acehan et al., 2002). Other proteins such as second mitochondria-derived activator of caspases (SMAC/DIABLO) are also released from mitochondria, with SMAC/DIABLO inhibiting the inhibitors of apoptosis (IAP) proteins which further augments activation of the caspase cascade.

In addition to apoptotic death, several studies have described non-apoptotic forms of granulocyte death, particularly in neutrophils (von et al., 2005; Huang et al., 2009; Mitroulis et al., 2010; Mihalache et al., 2011; Prince et al., 2012). For example, there is evidence that autophagy occurs in human and mouse neutrophils in both a phagocytosis-independent and dependent manner (Huang et al., 2009; Mitroulis et al., 2010). Autophagic cell death is morphologically defined as cell death that occurs in the absence of chromatin condensation and it is accompanied by massive autophagic vacuolization of cytoplasm (Kroemer et al., 2009). It has been reported that induction of autophagy is associated with an adaptive response to cellular stressors, such as nutrient deprivation or growth factor withdrawal. Moreover, inflammation-related agents including pathogens, ROS and hypoxia also induce autophagy and autophagy-like neutrophils have been observed in vivo in septic shock, rheumatoid arthritis, cystic fibrosis and skin disorders (Moreau et al., 2010; Mihalache et al., 2011). Similarly, phagocytosis of *Escherichia coli* or Ig-G coated beads induces recruitment of the autophagy protein LC3 to phagosomes suggesting induction of autophagy (Huang et al., 2009; Mitroulis et al., 2011). Mitroulis et al. (2010) have shown that neutrophil exposure to rapamycin, toll-like receptor (TLR) agonists or phorbol myristate acetate (PMA) resulted in cytoplasmic vacuole accumulation, previously characterized as autophagy-like vacuoles, suggesting phagocytosis-independent autophagy. High levels of intracellular ROS generated by neutrophil nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are suggested as essential for autophagy induction induced by PMA, TLR activation and phagocytosis (Huang et al., 2009). A role for ROS in CD44-induced neutrophil cell death in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) or formyl-Met-Leu-Phe (fMLP) has also been suggested (Mihalache et al., 2011).

The production of neutrophil extracellular traps (NETs) composed mainly of DNA and granule constituents leads to non-apoptotic neutrophil death termed NETosis (Brinkmann et al., 2004; Amulic & Hayes, 2011). The primary function of NETs seems to be the binding and killing of bacteria and fungi. Nevertheless, NETs may also enhance inflammation, as suggested by NET production in neutrophils isolated from synovial fluid or peripheral blood of patients with acute gout. Inhibition of phagolysosomal fusion or IL-1β blockade prevents NET formation suggesting that NETosis is associated with IL-1β and autophagy-related signaling in vivo. Interestingly it has also been reported that DNA can be released from neutrophils which do not undergo cell death (Yousefi et al., 2009) and as such the role of NETosis on neutrophil death is not yet fully understood. Moreover, it has recently been demonstrated that infection may induce NETosis in neutrophils that are crawling in the microcirculation following infection. In this case, neutrophils do not undergo lysis and retain their ability to multitask in the context of infection (Yipp et al., 2012).

Finally, many agents have been reported to induce cell necrosis, morphologically characterized by a gain in cell volume, swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents (Kroemer et al., 2009). Necrosis can proceed either as a primary event (primary necrosis) or can occur following apoptosis when phagocytosis is delayed (secondary necrosis). Until recently, necrosis was considered a non-specific mode of cell death, but a body of evidence has demonstrated that certain types of necrosis are finely regulated by signal transduction pathways leading to the term necroptosis (programmed necrosis) (Degterev et al., 2005; Han et al., 2011). It has been reported that necroptosis contributes to physiological and pathological processes including maintaining homeostasis and inflammatory diseases (Han et al., 2011). In this context the protein serine/threonine kinase receptor interacting protein 1 (RIP1) family member RIP3 has been referred as a key important mediator of caspase-independent cell death (Cho et al., 2009; Zhang et al., 2009).

It has been demonstrated that isolated human neutrophils undergo necroptosis after infection with virulent *Shigella flexneri* requiring type III secretion, IpaB and IpaC invasins, and actin polymerization (François et al., 2000). In agreement with this, Kobayashi et al. (2010) have shown that neutrophils containing ingested *Staphylococcus aureus* undergo morphological changes leading to rapid lysis. Importantly, phagosome membranes remained intact until the point of cell destruction suggesting that *S. aureus* was inducing programmed necrosis rather than non-specific lysis caused by escape of the pathogen from phagosomes or by the cytolytic action of secreted pore-forming toxins. In fact, it has been demonstrated in monocyte-macrophages, neutrophils and eosinophils that pathogens can disrupt death pathways as a survival strategy impairing antimicrobial functions of immune cells (Dockrell & Whyte, 2006; Feoktistova et al., 2011; Prince et al., 2012).

Although necrosis has been traditionally correlated with enhanced inflammation and tissue injury and is therefore considered unwanted cell death, few reports have conversely suggested that necrotic cells can inhibit inflammatory reactions (Hirt & Leist, 2003; Casares et al., 2005). Of note, it has been recently shown that annexin A1 (AnxA1) externalization during secondary necrosis provides an important fail-safe mechanism counteracting inflammatory responses when the timely clearance of apoptotic cells has failed (Blume et al., 2012). This change of paradigm faces many challenges and there is no consensus on mechanisms driving necrosis and how this may be beneficially controlled. Table 1 summarizes the key modalities of cell death and their role in inflammation resolution.

1.3. Recognition, response and removal of apoptotic cells by phagocytes

An important consequence of the apoptotic process is induction of alterations on the expression of molecules on the cell surface of the dying cell. Such alterations lead to rapid recognition and phagocytosis of apoptotic cells (Fadok et al., 2001a; Gardai et al., 2006; Gregory & Pound, 2010). Defective clearance of apoptotic bodies has been associated with autoimmunity and chronic inflammation (Munoz et al., 2005; Donnelly & Barnes, 2012). As such, efficient phagocytosis of apoptotic cells is crucial for the resolution of inflammation (Savill, 1997; Hallett et al., 2008). Gregory and colleagues have rationalized three main phases and dynamic stages that connect apoptotic cells to the engulfing phagocyte, namely *recognition*, *response and removal* (Gregory & Pound, 2010, 2011; Gregory et al., 2011).

The *recognition* phase involves migratory responses of mononuclear phagocytes in response to chemoattractants released from apoptotic cells signaling their location. Besides the classical chemokines CX3CL1 and CCL2 (Kobara et al., 2008; Truman et al., 2008), other chemoattractants include the lipid signals lysophosphatidylcholine and sphingosine-1-phosphate (S1P) and the nucleotides ATP and UTP (Lauber et al., 2003; Gude et al., 2008; Elliott et al., 2009; McDonald et

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Table 1
Types of cell death.

Type of cell death	Key molecules	Major characteristics	Role in the resolution of inflammation
Apoptosis	Caspases-3, 6, 7, 8, 9, Bcl-2 family of proteins	Chromatin condensation, nuclear fragmentation with rounding up and apoptotic body formation, organelle involution and cytoplasm shrinkage.	It is considered the most desirable form of cell death for successful resolution and is an important event in the resolution of acute inflammation which limits tissue injury caused by leukocytes at sites of inflammation. Furthermore, regulated clearance of apoptotic cells leads to shutdown of cellular activity and inhibition of inflammatory responses.
Necroptosis	RIP1 (also known as RIPK1) and RIP3 (also known as RIPK3)	It is considered programmed necrosis associated with caspase-independent cell death.	It has been reported that necroptosis contributes to inflammatory diseases.
Autophagy	Bcl-2, Bcl-XL, Beclin1, ATG5-ATG12, ATG8 (LC3)	Auto-digestive process that promotes delivery of intracellular components from the cytoplasm to lysosomal or vacuolar compartments for terminal degradation and recycling.	Not clearly determined.
Necrosis	No signal determined	It is characterized by a gain in cell volume, swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents	It has been traditionally correlated with enhanced inflammation and tissue injury.
NET-osis	Neutrophil Extracellular Traps (NETs)	It is a form of pathogen-induced cell death involving disintegration of the nuclear envelope, mixing of cytoplasmic and nuclear materials, and loss of internal membranes and cytoplasmic organelles	Not clearly understood
Pyroptosis	Caspase-1-dependent	Involves cellular lysis and release of the cytosolic contents to the extracellular space.	It is predicted to be inherently inflammatory and coincides with interleukin-1β (IL-1β) and IL-18 secretion.

al., 2010; Marques et al., 2012). In addition, it has been reported that molecules such as covalent dimer of ribosomal protein S19, endothelial monocyte-activating polypeptide II (EMAP II), AnxA1, transforming growth factor-β (TGF-β) and thrombospondin 1 direct mononuclear phagocytes towards apoptotic cells (Horino et al., 1998; Knies et al., 1998; Chen et al., 2001; Scannell et al., 2007; Peter et al., 2010).

Interestingly, it has been suggested that along with chemoattractant molecules, apoptotic cells also release agents such as lipoxins (LXs), lactoferrin and AnxA1 which inhibit granulocyte recruitment, contributing towards the predominant presence of mononuclear cells seen during the resolution phase (Schwab et al., 2007; Bournazou et al., 2009a; Vago et al., 2012). Bournazou et al. (2009a, 2009b) have shown in vitro and in vivo that lactoferrin, which is released by apoptotic cells, selectively inhibited migration of granulocytes but not mononuclear phagocytes. Lactoferrin concentration-dependently decreased the migration of neutrophils despite the presence of powerful neutrophil chemoattractants including fMLP, C5a, IL-8 and LTB_4. The inhibitory ability of lactoferrin on neutrophil migration has also been confirmed in vivo using a murine peritonitis model induced by thioglycollate, whereas the migration of other types of leukocytes was not reduced (Bournazou et al., 2009a). Moreover, we have recently shown that an AnxA1 peptidomimetic, Ac2-26, was able to decrease neutrophil influx into the pleural cavity without alterations in mononuclear cell numbers (Vago et al., 2012).

Apoptotic cells signal the requirement to be removed by loss of 'don't-eat-me' signals, such as CD31 (Brown et al., 2002) and CD47 (Gardai et al., 2005), as well as release and display of various 'eat-me' signals, including phospholipids, nucleotides and by externalization of the molecule PS (Fadok et al., 2001b). Macrophages differentiated from recruited monocytes as well as tissue resident macrophages recognize these "eat-me" signals via receptors which include CD36, CD14, integrins αvβ3 and αvβ5, lectin, vitronectin and PS receptors (e.g. BAI1, TIM-4 and stabilin-2) (Savill et al., 1992; Devitt et al., 1998; Park et al., 2007; Gregory & Pound, 2011). The interaction between "eat-me" signals on apoptotic cells with receptors on macrophages leads to firm tethering and subsequent *activation of engulfment pathways*. This ensures that apoptotic cells are efficiently removed from tissue prior to membrane rupture with release of cytotoxic mediators, minimizing tissue damage and perpetuation of the inflammatory response.

A body of in vitro and in vivo evidence shows that phagocytes that consume apoptotic granulocytes switch towards a pro-resolution phenotype that suppresses the inflammatory response. This helps to contribute to a non-phlogistic environment that paves the way towards successful resolution of inflammation. Macrophages are usually classified as either classically (M1) or alternatively (M2) activated. Classically activated (M1) are considered pro-inflammatory and are driven by interferon-gamma (IFN-γ) produced by Th1 cells or by recognition of pathogen-associated molecule patterns to promote pro-inflammatory responses. Alternatively activated (M2) are driven by IL-4 and/or IL-13 produced by Th2 cells and produce IL-10 and TGF-β, which have several anti-inflammatory actions. Early efferocytosis (non-phlogistic phagocytosis of apoptotic cells) leads to a mixed resolution-phase macrophage population with a more alternatively activated phenotype as well as enhanced phagocytosis/efferocytosis. Subsequently, macrophages convert to a resolution-promoting macrophage (Mres) profile which limits dysregulated tissue repair/fibrosis and migrate to the local draining lymph nodes promoting macrophage regulatory properties at remote sites (Bellingan et al., 1996; Bystrom et al., 2008; Schif-Zuck et al., 2011; Stables et al., 2011; Ariel & Serhan, 2012).

Interestingly, Ramachandran et al. (2012) have recently identified and characterize the macrophage phenotype responsible for tissue remodeling following hepatic injury (restorative macrophage). Using a CCl4-induced model of reversible hepatic fibrosis, it has been reported that a $CD11B^{hi}$ $F4/80^{intermediate}$ $Ly\text{-}6C^{lo}$ monocyte-derived macrophage subset was more abundant in livers observed during maximal fibrosis resolution and depletion of this population caused a failure of scar remodeling. Importantly these restorative macrophages derive from recruited $Ly\text{-}6C^{hi}$ monocytes (indicative of a phenotypic switch in vivo conferring pro-resolution properties) and presented features of being pos phagocytic. In vivo the administration of liposomes containing fluorescent microspheres, which enhance macrophage efferocytosis, caused increased restorative macrophage number and accelerated hepatic fibrosis resolution, suggesting a novel strategy for the treatment of tissue fibrosis.

Uptake of apoptotic cells by phagocytes leads to up-regulation of cell-surface expression of co-inhibitory molecules such as PD-L1 and ICOS-L; release of anti-inflammatory cytokines IL-10 and TGF-β, secretion of PAF, prostaglandin E_2 (PGE_2) and cAMP (Fadok et al., 1998a; McDonald et al., 1999; Huynh et al., 2005; Girkontaite et al., 2007; Bystrom et al., 2008; Gregory & Pound, 2010). It has been also reported that macrophages which ingested apoptotic cells release pro-resolving lipid mediators such as LXA_4, resolvin (Rv) E1, protectin D1 and maresins contributing to termination of the inflammatory

process (Schwab et al., 2007; Yazid et al., 2011). Conversely, engulfment of apoptotic cells inhibits the release of pro-inflammatory cytokines, including TNF-α, GM-CSF, IL-12, IL-1β and IL-18 (Fadok et al., 1998b; McDonald et al., 1999). The uptake of apoptotic cells also drives inhibition of inducible NO synthase (iNOS) expression and stimulates the expression of arginase-1 in murine macrophages resulting in suppression of nitric oxide (NO) synthesis (Freire-de-Lima et al., 2006). Moreover, it has been shown in vitro that IL-10 and dexamethasone significantly increase uptake of apoptotic cells by human macrophages (Liu et al., 1999; Giles et al., 2001; Michlewska et al., 2009), whereas macrophages from IL-10-deficient mice have decreased phagocytosis in comparison to wild type mouse macrophages (Michlewska et al., 2009).

There is ongoing interest in delineating further mechanisms underlying the interactions between apoptotic cells and phagocytes, especially in vivo. Better understanding in this area may be potentially relevant to the treatment of chronic diseases such as systemic lupus erythematous where defective removal of apoptotic cells represents an important aspect in the pathogenesis of the disease.

2. Evidence for natural and drug-induced resolution of inflammation

Studies in vitro provide essential background information and understanding as to how resolution may occur in vivo but do not describe the complexity of in vivo phenomena. There has been much recent interest in the understanding of the signal transduction pathways governing leukocyte survival and apoptosis in vivo. Although a number of pathways have been evaluated, studies are difficult as many pathways become interrelated or co-dependent in vivo. On top of this complexity, it is not possible to extrapolate pathways governing cell death from one leukocyte type to another. For instance, although neutrophils and eosinophils are closely related granulocytes, glucocorticoids induce opposing effects on these two cell types. It has been shown that while these powerful anti-inflammatory drugs induce apoptosis in lymphocytes as well as eosinophils, they delay constitutive apoptosis of neutrophils, at least in vitro (Meagher et al., 1996). Several studies have described a number of key molecules and pathways involved in the maintenance of leukocyte survival and apoptotic death in vitro and in vivo and have highlighted the importance of the subsequent regulated clearance of apoptotic cells by inflammatory macrophages (Rossi et al., 1998; Liu et al., 1999; Ward et al., 1999; Rossi et al., 2006; Sousa et al., 2009, 2010; Alessandri et al., 2011; Lopes et al., 2011; Vago et al., 2012). It is believed that defined molecular pathways promote leukocyte survival (or prevent death) once these cells migrate into inflammatory tissue. Therefore, the use of animal models of inflammation together with knowledge of mechanisms regulating human leukocyte apoptosis in vitro is important in the quest to understand fully the molecular pathways relevant to human inflammatory diseases. Delineating these key pathways will likely reveal potential opportunities for pharmacological interventions (apoptosis-based) which treat inflammatory disorders by blocking the undesired effect of inappropriate leukocyte accumulation, activation or removal.

2.1. Mediators of natural resolution with focus on pro-resolution mediators

2.1.1. Pro-resolving lipid mediators (lipoxins, resolvins, protectins, maresins)

Following investigations mainly by Serhan and colleagues, a number of lipid mediators derived from polyunsaturated fatty acids (PUFAs) have been discovered as anti-inflammatory and pro-resolution agents. These include lipoxins, E-series resolvins, D-series resolvins, protectins/neuroprotectins and maresins (please see reviews by Serhan, 2007; Serhan & Chiang, 2008; Serhan et al., 2008; Spite & Serhan, 2010 for definition and chemical structures of the lipids). It has been demonstrated that lipid mediators have a modulatory role in various acute and chronic animal models of inflammatory conditions, including arthritis, peritonitis, ischemia–reperfusion injury, inflammatory pain and asthma (Levy et al., 2007; Souza et al., 2007; Conte et al., 2010; Xu et al., 2010; Serhan et al., 2012).

Lipoxin A_4 (LXA_4), lipoxin B_4 (LXB_4) and aspirin-triggered lipoxins (ATLs) were the first mediators biosynthesized from arachidonic acid (AA) to be recognized as anti-inflammatory, pro-resolving lipid mediators (Chiang et al., 2005). LXs and ATLs stimulate the formyl-peptide receptor 2/lipoxin A_4 receptor (FPR2/ALXR) inhibiting cellular recruitment as well as enhancing non-phlogistic phagocytosis of apoptotic cells by macrophages (Godson et al., 2000; Fierro et al., 2003; Maderna & Godson, 2005). Moreover a number of investigations have provided strong evidence that LXs are relevant for resolution in animal models (Souza et al., 2007; Conte et al., 2010 and reviewed by Ryan & Godson, 2010). For instance, aspirin-triggered 15-epi-LXA_4 enhances resolution of acute lung inflammation by overriding the anti-apoptotic signal from myeloperoxidase (MPO) to induce neutrophil apoptosis (El Kebir et al., 2009). Conversely, Prieto et al. (2010) have shown that LXA_4 protects human and murine macrophages from apoptosis through the activation of PI3K/Akt and extracellular-signal-regulated kinase (ERK) pathway suggesting that pro-resolution molecules may act differently dependent on the cell type under investigation.

Additionally, the association between LXs and IL-10 production has been studied by Souza et al. (2007) who evaluated the role of LXA_4 and AnxA1 in germ free mice. These mice, which have no detectable bacteria in their intestines, generate little evidence of local or systemic injury following intestinal ischemia and reperfusion dependent upon high levels of LXA_4, AnxA1 and IL-10. The treatment of conventional mice with the 15-epi-LXA_4 analog or an AnxA1-derived peptide markedly inhibited the reperfusion-associated inflammatory response and improved survival. This was associated with reduced TNF-α production in the intestine, lung and serum whereas IL-10 production was increased. The ability of LXA_4 and AnxA1 to prevent reperfusion injury was lost in IL-10 deficient-mice. In order to further investigate the role of these mediators on the production of IL-10 in vivo, the effect of LXA_4 was inhibited using either an inhibitor of 5-LO (ZM230487) or a non-specific antagonist of ALXR receptor (BOC-1) following reperfusion injury. Treatment with ZM230487 or BOC-1 led to increased production of TNF-α and reduced survival. Similarly the treatment of germ-free mice with anti-AnxA1 antiserum enhanced tissue injury, TNF-α production and lethality similar to that found in conventional animals. Taken together these findings suggest that increased production of IL-10 may be an important action of LXA_4 and AnxA1 in vivo. Furthermore, the presence of microbiota alters endogenous production of anti-inflammatory substances and modulates inflammatory responsiveness. Future studies are necessary to demonstrate whether IL-10 is also necessary in mediating actions of lipoxins in the context of the resolution of inflammation. Moreover, the impact of the microbiota in the context of resolution of inflammation needs to be defined in greater detail.

Besides producing AA derived mediators, PUFAs generate omega-3 fatty acids, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These, in turn, are enzymatically transformed to generate E series (RvE), D series (RvD) resolvins, protectins and maresins (Serhan & Petasis, 2011). Rvs, also named specialized pro-resolving mediators (SPMs), share some of the actions of the LXs regarding resolution of the inflammatory process. Rvs decrease the recruitment of neutrophils in peritonitis and induce resolution in several animal models including peritonitis, ischemia/reperfusion injury, inflammatory pain, and allergic airway inflammation (Hasturk et al., 2005; Duffield et al., 2006; Schwab et al., 2007; Xu et al., 2010; El Kebir et al., 2012; Rogerio et al., 2012). Moreover RvE1 enhances phagocytosis of zymosan A by human macrophages (Ohira et al., 2010). Interestingly RvE1 and RvD1 bind to receptors that until recently were orphan receptors named ChemR23 and GPR32, respectively (Arita et al., 2007; Krishnamoorthy et al., 2010; Oh et al.,

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2011). Rvs have been shown to promote tear production, prevent loss of corneal epithelial cell barrier integrity, inhibit keratocyte transformation to myofibroblasts, decrease inflammation, modulate T-lymphocyte-cell responsiveness and reduce goblet cell apoptosis (Li et al., 2010; Zhang et al., 2010b; Dartt et al., 2011). These effects have encouraged and guided current phase I and phase II clinical trials for dry eye syndrome (keratoconjunctivitis sicca) where individuals suffer from insufficient tears and/or have tears with incorrect osmolarity and evaporate rapidly resulting in irritation, inflammation and pain (www.clinicaltrial.gov).

Protectins (and the isoforms biosynthesized in neural tissues termed neuroprotectins) have a key role in controlling inflammatory processes with effects seen in animal models of peritonitis, kidney ischemia/reperfusion, asthma and stroke (Marcheselli et al., 2003; Bannenberg et al., 2005; Duffield et al., 2006; Levy et al., 2007).

Maresins, originally isolated from exudates of murine macrophages, play pro-resolving actions during murine peritonitis in vivo with potency similar to RvE1 and protectin D1 (Serhan et al., 2009). Recently, the anti-inflammatory and pro-resolving effects of maresins have been confirmed with a synthetic compound (MaR1) based on maresin 1 produced by human macrophages (Serhan et al., 2012). MaR1 limited neutrophil infiltration in murine peritonitis and enhanced uptake of apoptotic neutrophils by human macrophages. Furthermore, MaR1and RvE1 enhanced the rate of tissue regeneration in planaria. MaR1 also dose-dependently blocks transient receptor potential cation channel subfamily V member 1 (TRPV1) currents in primary sensory neurons and reduced both inflammation- and chemotherapy-induced neuropathic pain in mice suggesting that maresins modulate different aspects of the inflammatory process including resolution, tissue regeneration, and pain resolution (Serhan et al., 2012). Taken together the aforementioned studies, including clinical trials, highlight how understanding the endogenous control of resolving inflammation by pro-resolving lipid mediators can help develop novel therapeutics for clinical use.

2.1.2. Glucocorticoid induced proteins (AnxA-1, GILZ and MKP-1)

Glucocorticoids (GCs) are potent anti-inflammatory and immunosuppressive drugs that are used therapeutically for the treatment of many inflammatory conditions (Clark, 2007; Perretti & D'Acquisto, 2009; Beaulieu & Morand, 2011; Clark & Belvisi, 2012). During inflammation, endogenous GCs are produced by the adrenal glands and play a critical role during the resolution of inflammation. The broad-spectrum anti-inflammatory and immunosuppressive effects of GCs depend on their effects on signaling pathways, such as NF-κB and activating protein 1 (AP-1) pathways, and their capacity to induce anti-inflammatory regulatory proteins. However, it has been thought that the metabolic side effects of GCs are also dependent on GC-induced gene expression (Clark, 2007; Perretti & D'Acquisto, 2009; Beaulieu & Morand, 2011). These aspects of the mechanism of action and side effects of glucocorticoid therapy have been recently discussed by Clark and Belvisi (2012).

There is growing interest in three GC-induced proteins, namely AnxA1, glucocorticoid-induced leucine zipper (GILZ) and mitogen-activated protein kinase phosphatase/dual specificity phosphatase (MKP-1/DUSP-1). In fact, GILZ and MKP-1/DUSP-1 have presented significant anti-inflammatory in vivo and AnxA1 has shown anti-inflammatory and pro-resolving properties in several experimental models of inflammation (Ayroldi & Riccardi, 2009; Li et al., 2009; Perretti & D'Acquisto, 2009; Beaulieu & Morand, 2011; Vago et al., 2012). Knowledge of such proteins may allow dissociation of anti-inflammatory effects of GC from their adverse metabolic effects and the main effects of these proteins during inflammatory conditions. AnxA1 (also known as lipocortin-1) is a 37-kDa calcium-dependent phospholipid-binding protein from the annexin superfamily that binds to and activates FPR2/ALXR, a receptor also shared with LXs (Perretti & D'Acquisto, 2009). AnxA1 is a known anti-inflammatory molecule, with its activities studied predominantly in the context of inhibition of pro-inflammatory PGs and leukocyte recruitment in various models of inflammation (Getting et al., 1997; Bandeira-Melo et al., 2005; Souza et al., 2007; Babbin et al., 2008). AnxA1 knockout mice show exacerbated inflammatory responses and are resistant to some of the anti-inflammatory effects of GCs (Hannon et al., 2002; Yang et al., 2004). Exogenous and endogenous AnxA1 exerts exquisite control on leukocyte recruitment to inflammatory sites by modulating both pro-inflammatory mediators (including those derived from activation of phospholipase A2, COX-2 and iNOS synthase) as well as the anti-inflammatory cytokine IL-10. In addition to the anti-inflammatory activities of AnxA1, some in vitro and in vivo proprieties of this protein show that it promotes resolution of inflammation mainly by inducing neutrophil apoptosis and increasing efferocytosis by macrophages (reviewed by Perretti, 2012). It is known that apoptotic neutrophils release AnxA1, which then act on macrophages to promote efferocytosis (Scannell et al., 2007). Macrophages treated with GCs also secrete AnxA1, which contributes to the augmented phagocytosis of apoptotic neutrophils (Maderna et al., 2005). Microglial-derived AnxA1 targets apoptotic neurons, serving as both an "eat me" signal and a bridge between phosphatidylserine on the dying cell and FPR2 on the phagocytosing microglia (McArthur et al., 2010). AnxA1 released from apoptotic cells acts through FPR to dampen inflammatory monocyte activation, contributing to immunomodulatory effect of apoptotic cells on inflammation (Pupjalis et al., 2011). In addition, AnxA1 exposure during secondary necrosis provides an important failsafe mechanism counteracting inflammatory responses, even when the timely clearance of apoptotic cells has failed (Blume et al., 2009). The follow-up study of these authors demonstrated that the AnxA1 released peptide and culture supernatants of secondary necrotic-AnxA1-externalizing cells induced chemoattraction of monocytes (Blume et al., 2012). Thus, AnxA1 is conveying the well-known pro-resolution proprieties of apoptotic cells and seems to be part of the apoptotic cell-associated molecular patterns (ACAMPs) that interact with PRRs to convey a switch towards an anti-inflammatory response (Pupjalis et al., 2011). A recent paper using AnxA1 knockout mice has demonstrated that expression of AnxA1 by resident bone marrow macrophages is required for the efficient phagocytosis of apoptotic neutrophils under homeostatic conditions in vivo (Dalli et al., 2012). In the context of neutrophil apoptosis, it has been shown that AnxA1 regulates leukocyte apoptosis not only in vitro (Solito et al., 2003; Parente & Solito, 2004) but also in vivo under inflammatory settings (Vago et al., 2012). Collectively, we can propose that during ongoing inflammation, endogenous and exogenous AnxA1 can exert exquisite modulation on the fate of recruited neutrophils by promoting their death by apoptosis, as well as their safe removal by phagocytes (efferocytosis), dampens inflammatory mediators and promotes monocyte subendothelial space locomotion. Both apoptosis and efferocytosis have been shown to be modulated by AnxA1 in vivo and are crucial for resolution of inflammation (Vago et al, 2012).

Our group has demonstrated the relevance and dynamics of AnxA1 in driving natural and dexamethasone induced resolution of inflammation by its ability to induce neutrophil apoptosis in vivo (Vago et al., 2012). AnxA1 is up-regulated during natural and drug-induced resolution of acute inflammation, and such increase is associated with increased Bax and caspase-3 cleavage and appearance of apoptotic neutrophils at the site of inflammation. Neutralization of AnxA1, by use of a specific antiserum or by blocking its receptor, prevented natural and GC-induced resolution. Importantly, use of the AnxA1 peptidomimetic Ac2-26 promoted caspase-dependent resolution of neutrophilic inflammation in the pleural cavity associated with the induction of neutrophil apoptosis. Therefore, this provides strong evidence that AnxA1 is a mediator of natural and GC-induced resolution of inflammation by promoting apoptosis of neutrophils in vivo (Vago et al., 2012). Importantly, AnxA1 cleavage was maximal at times of neutrophil recruitment and treatment of inflamed mice with anti-inflammatory drugs prevented AnxA1 cleavage. This is in accordance with recent data showing that AnxA1 cleavage resistant protein is effective in ameliorating several aspects of inflammation

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(Pederzoli-Ribeil et al., 2010; Patel et al., 2012), reinforcing the idea that AnxA1 cleavage resistant mutant or its peptido-mimetic may represent a powerful anti-inflammatory strategy for the treatment of diseases, in which neutrophil accumulation plays a relevant role.

GILZ was first identified in 1997 in a study which identified GC-induced genes involved with apoptosis (D'Adamio et al., 1997). GILZ has since been shown as a novel GC-induced protein in several cell types which mediates many anti-inflammatory effects of GCs in leukocytes (Ayroldi & Riccardi, 2009; Beaulieu & Morand, 2011). GILZ binds and inhibits several main pathways involved in inflammation, including the transcription factors NF-κB and AP-1 and the mitogen-activated protein kinase (MAPK) pathway. Inhibition of these pathways is believed to be central to the ability of GILZ to inhibit inflammation (Ayroldi & Riccardi, 2009). In addition, GILZ binds to Ras/Raf and reduces Akt phosphorylation, suggesting that GILZ may also affect the Akt survival pathway (Ayroldi et al., 2001, 2002, 2007).

GILZ has been mostly studied in T cells, where it has been shown to have both pro- and anti-apoptotic properties (D'Adamio et al., 1997; Asselin-Labat et al., 2004; Delfino et al., 2004). The involvement of GILZ in the context of apoptosis has been shown in GILZ transgenic mice, which overexpress GILZ in the T cell lineage. Thymocytes from these mice undergo apoptosis, activate caspase-8 and down-regulate Bcl-xL, suggesting that GILZ has a similar effect to GCs. In contrast, GILZ does not induce apoptosis in mature mouse T-cell lymphocytes (Delfino et al., 2004; Ayroldi & Riccardi, 2009). Inhibition of the PI3K/Akt pathway resulted in up-regulation of GILZ and increased apoptosis of multiple myeloma cells (Grugan et al., 2008). From our in vivo studies using a model of lipopolysaccharide (LPS) induced pleural inflammation, we have found that GILZ expression paralleled spontaneous resolution of inflammation and was associated with neutrophil apoptosis. In addition, there was increased GILZ expression in inflammatory exudates of mice after treatment with anti-inflammatory drugs (Sousa and Teixeira, unpublished observations).

Interestingly, GILZ is a target of the inhibitory effects of AnxA1 (Yang et al., 2009), supporting our findings that the kinetics of GILZ expression is remarkably similar to the kinetics of AnxA1 expression. Strategies that inhibit AnxA1, such as use of a specific antiserum or by blocking its receptor also cause GILZ inhibition (Sousa and Teixeira, unpublished observations), suggesting that GILZ could be an AnxA1 target in vivo. However, at present the mechanism governing AnxA1 regulation of GILZ expression remains unknown. Like AnxA1, GILZ has been shown to mediate several effects of GC, including modulation of T-lymphocyte activation, apoptosis and proliferation, and is up-regulated by IL-10 and TGF-β in several cell types (D'Adamio et al., 1997; Ayroldi & Riccardi, 2009). GILZ appears to have a physiological role in the regulation of inflammation, however there are few reports exploring the role of GILZ in inflammatory diseases (Cannarile et al., 2009; Beaulieu et al., 2010; Srinivasan & Janardhanam, 2011; Esposito et al., 2012). Cannarile et al (2009) have shown that mice that overexpress GILZ in T-cells are less prone to dinitrobenzene sulfonic acid-induced colitis with reductions in intestinal damage associated with inhibition of nuclear translocation of NF-κB and inhibition of TNF-α, IFN-α and IL-1 production in CD4+ T lymphocytes of the lamina propria. Another study showed that endogenous GILZ controls the inflammatory response in a mouse model of collagen induced arthritis, and was able to modulate the expression of pro-inflammatory cytokines. In this study the inhibition of endogenous GILZ by small interfering ribonucleic acid (siRNA) increased the severity of disease with enhanced production of TNF-α and IL-1β (Beaulieu et al., 2010). GILZ over-expression in T lymphocytes inhibited inflammation and tissue damage in spinal cord injury (Esposito et al., 2012), while treatment of mice with a GILZ peptide was beneficial in experimental autoimmune encephalomyelitis (Srinivasan & Janardhanam, 2011). Taken together, these recent studies suggest that a GILZ-based strategy could be an emerging therapeutic tool to treat inflammatory conditions without the metabolic effects of GCs. Future research with recently generated GILZ deficient mice (Bruscoli et al., 2012; Suarez et al., 2012) will help shed light on the role of GILZ on resolution of inflammation.

MKP-1 was the first described member of a large family of phosphatases that catalyze removal of phosphate from serine, threonine or tyrosine residues and has been shown as an important negative regulator of inflammatory responses (Clark, 2007). MKP-1 is also known as DUSP-1 since it dephosphorylates and inactivates members of the MAPK family, a key signaling pathway activated by pro-inflammatory agonists. It has been reported that certain anti-inflammatory effects of dexamethasone are partially mediated by MKP-1 (Abraham & Clark, 2006; Abraham et al., 2006). King et al. (2009) have observed that dexamethasone induces expression of MKP-1 in human bronchial epithelial and pulmonary cells that mirrored reduced TNFα-stimulated p38 MAPK phosphorylation, induction of IL-8 expression and NF-kB-dependent transcription. Several studies have appreciated that MKP-1 may interfere with the inflammatory process by targeting MAPK such as c-Jun N-terminal kinase (JNK), p38 and ERK1/2 (Franklin & Kraft, 1997; Chen et al., 2002; Abraham & Clark, 2006; Salojin et al., 2006). For instance, Salojin et al. (2006) have reported a pivotal role of MKP-1 in the negative control of innate immune responses in vivo. Specifically MKP-$1^{-/-}$ mice were given low-dose LPS challenge and exhibited significantly increased serum TNF-α, IL-6, IL-12, monocyte chemotactic protein-1 (MCP-1), IFN-γ and IL-10. Furthermore, the absence of MKP-1 increased systemic levels of pro-inflammatory cytokines and enhanced the incidence and severity of autoimmune arthritis. Bone marrow-derived MKP-$1^{-/-}$ macrophages enhanced constitutive and TLR-induced activation of p38 and ERK1/2 MAPK pathways. Similarly, the disease-modifying anti-rheumatic drug aurothiomalate, which drives anti-inflammatory and anti-erosive actions through increased expression of MKP-1, in turn reduces activation of p38 MAPK and suppresses expression of COX-2, matrix metalloproteinase-3 (MMP-3) and IL-6 (Nieminen et al., 2010). A very recent study showed that knockout of MKP-1 exacerbated colitis in IL-10-deficient mice and proposed a pivotal role of MKP-1 as a negative regulator of mucosal immune responses (Matta et al., 2012). Another role for MKP-1 has been proposed by Perdiguero et al. (2012). They found that the pro- to anti-inflammatory macrophage polarization switch is controlled by the balance of p38 MAPK and MKP-1 during the process of muscle healing. The studies mentioned above do show the correlation between expression of MKP-1, actions of steroids and decrease of inflammation. In agreement we also observed that MKP-1 expression is associated with natural resolution of acute inflammation and decreased phosphorylation of p38 e JNK MAPKs (unpublished). However, it remains to be determined whether MKP-1 does indeed contribute to resolution of inflammation in vivo and whether it is a useful target for the development of pro-resolving therapies.

2.1.3. Reactive oxygen species (ROS)

Reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH) are normally produced during cellular energy production in aerobic cells and are removed by antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and peroxiredoxins (Prxs). ROS can be generated either by the mitochondrial electron transport chain or by the NADPH oxidase (NOX) complex in the plasma membrane and cytosol (Forman & Torres, 2002). Stimulation of ROS was first reported in neutrophils and macrophages and named "the respiratory burst" due to the brief necessity of oxygen. ROS influence many physiological processes including hormone biosynthesis, intracellular signaling and host defense against different pathogens (Bogdan et al., 2000; Dröge, 2002).

Besides the relevance of ROS for an adequate anti-microbial response, ROS may also damage host DNA, lipids, and proteins and therefore lead to cellular injury and death (Prütz, 1996; Shacter, 2000; Niethammer et al., 2009). It has been suggested that ROS may have a role in the development of many inflammatory diseases, including cancer, atherosclerosis,

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arthritis, diabetes and degenerative diseases (Quinlan et al., 1994; Dröge, 2002; Riedl & Nel, 2008; Phillips et al., 2010).

Several transcription factors and signaling pathways important for pro-inflammatory processes, such as NF-κB, AP-1, MAPKs and PI3K, are activated by ROS (Li et al., 2002; Chen et al., 2004; Carreras & Poderoso, 2007; Groeger et al., 2009; Lopes et al., 2011). In fact, oxidative stress promotes changes in nuclear histones by acetylation, methylation, and phosphorylation leading to chromatin remodeling, recruitment of basal transcription factors and activation of RNA polymerase II. These, in turn, increase gene expression of pro-inflammatory mediators (Sanlioglu et al., 2001; Forman & Torres, 2002; Wang et al., 2002). Furthermore, the release of ROS from damaged tissue can form a concentration gradient that directs leukocyte recruitment towards the site of tissue injury, demonstrating that ROS can act as damage-associated molecular patterns (DAMPS) and thus modulate inflammatory responses in tissue (Niethammer et al., 2009). Based on these findings it has been suggested that anti-oxidative agents may be a useful strategy to decrease ROS levels and act as anti-inflammatory therapies (Yasui & Baba, 2006; Impellizzeri et al., 2011).

Conversely several studies suggest that ROS production may regulate negatively inflammation. For example, Lee et al. (2011) have reported that NOX2-deficient mice spontaneously develop severe arthritis with increased inflammatory cytokines such as TNF-α and IL-1β. It was observed that NOX2 deficiency or ROS depletion significantly affected the development of CD11b+ myeloid cells and Th17/Treg cells, promoting inflammatory cytokine production and inflammatory arthritis development. In agreement with the latter findings, Deng et al. (2012) have argued that generation of ROS regulated acute lung inflammation. Deficiency of the $p47^{phox}$ component of NADPH oxidase in macrophages was associated with increased inflammation in a model of systemic endotoxin. ROS deficiency was associated with decreased activation of the Akt/GSK3-β pathway that resulted in deficiency of IL-10 production. These findings highlight a complex role of ROS in the context of inflammation and suggest that temporal control of ROS production may determine whether ROS act as pro- or anti-inflammatory agents.

Recent studies have also demonstrated a role for ROS in the regulation of leukocyte survival in vitro and in vivo (Kankaanranta et al., 2002; Lee & Shin, 2009; Lopes et al., 2011; Honda et al., 2012). Neutrophils from patients with SAPHO syndrome (synovitis, acne, pustulosis, hyperostosis and osteitis) have a specific defect in ROS production, indicating that decreased production of these molecules is involved in the development and maintenance of this inflammatory disease (Ferguson et al., 2008). Moreover patients with chronic granulomatous disease, characterized by a genetic defect in superoxide anion production and consequently hydrogen peroxide, have decreased ability to resolve inflammation (Coxon et al., 1996). Interestingly, in patients with Down's syndrome in which the gene for SOD is over-expressed, spontaneous apoptosis as well as that induced by anti-Fas is accelerated making these patients more susceptible to infectious diseases (Yasui et al., 2005). Investigating the molecular mechanisms underlying the involvement of ROS induced leukocyte apoptosis, Honda et al. (2012) have implicated the kinase Btk. Btk-deficient neutrophils had enhanced ROS production after TNF-α or TLR stimulation. This was associated with accelerated human neutrophil apoptosis which was reversed by transduction of wild-type Btk.

Hydrogen peroxide has also been described as an important mediator of cell death, as shown by studies that H_2O_2 induced apoptosis in neutrophils (Lopes et al., 2011), eosinophils (Lee & Shin, 2009), epithelial cells (Hussain et al., 2010), endothelial cells (Fang et al., 2010), human hepatoma cells (Kim et al., 2010) and lymphoma cells (Klamt et al., 2009). Moreover, it has been shown that *gp91phox*−/− mice, which lack the ability to assemble NADPH oxidase and are therefore incapable of generating ROS, manifest an increased susceptibility to infection and prolonged inflammatory reactions that may result in granulomatous lesions (Pollock et al., 1995; Rajakariar et al., 2009). Similarly, experiments using transgenic mice that have high levels of superoxide dismutase demonstrate reduced number of neutrophils in bronchoalveolar lavage after intratracheal administration of particulate pollutants (Yasui et al., 2005). Although the mechanisms of cell apoptosis induced by H_2O_2 are not fully elucidated, Aikawa et al (2010) have demonstrated that H_2O_2-induced apoptotic death of Jurkat cells is inhibited by overexpression of human Bcl-2 or by ablation of Bax/Bak, suggesting that H_2O_2-induced apoptosis is mediated by the intrinsic apoptotic pathway.

Our group has also investigated the effects of H_2O_2 on the resolution of inflammation. Lopes et al. (2011) have shown by both genetic and pharmacological approaches a role for H_2O_2 in resolving neutrophilic inflammation in a murine model of antigen-induced arthritis. In this model, challenge with antigen induces neutrophil recruitment which is sustained for 12–24 h prior to subsequent resolution by 48 h. H_2O_2 production peaked at 24 h, prior to induction of resolution. In *gp91phox*−/− mice the neutrophil recruitment was similar to wild mice. However, there was delayed resolution in *gp91phox*−/− mice or after administration of catalase to wild-type animal, suggesting that H_2O_2 contributes to natural neutrophil clearance (Lopes et al., 2011). Animals which were treated with either low dose H_2O_2 or SOD at the peak of the inflammatory process (thereby increasing the local levels of H_2O_2) had enhanced resolution of inflammation concurrent with an increased number of apoptotic neutrophils and accumulation of the pro-apoptotic protein Bax and activated caspase-3. Importantly, macrophage counts were unaffected, indicating relative specificity for neutrophils. Inhibition of Akt phosphorylation and decreased NF-κB p65 translocation to the nucleus appeared to be the major mechanisms by which H_2O_2 affected neutrophil survival in murine antigen-induced arthritis (Lopes et al., 2011). Administration of intravenous immunoglobulin preparations, which are beneficial therapeutic agents in the treatment of autoimmune systemic inflammatory diseases, may also increase the production of intracellular H_2O_2 and induce apoptosis of LPS-stimulated neutrophils in vitro (Takeshita et al., 2005). As such pharmacological modulation of H_2O_2 production may represent a novel therapeutic target to modulate neutrophilic inflammation.

2.1.4. COX2-derived cyclopentenone prostaglandins

Evidence suggests that it may be possible to exploit the anti-inflammatory and pro-resolution effects of PGD_2 and its cyclopentenone PG (CyPG) metabolites (Ward et al., 2002; Gilroy et al., 2003; Rajakariar et al., 2007; Gilroy, 2010; Surh et al., 2011). Cells can metabolize AA into PGH_2 which is further metabolized to thromboxane (TX) A_2, PGE_2, PGD_2, $PGF_2\alpha$ and PGI_2 depending on the enzymes present in the cell. PGD_2, in turn, can be further metabolized to PGJ_2, Δ^{12-14} PGJ_2 and 15-deoxy-$\Delta^{12,14}$ (15d-PGJ_2) (Straus & Glass, 2001; Gilroy, 2010). CyPG metabolites may play an important role in both homeostatic and inflammatory processes including inhibition of cell cycle progression, platelet aggregation and cytokine production in immune cells (Straus & Glass, 2001; Cuzzocrea et al., 2002; Rajakariar et al., 2007). Furthermore, endogenous CyPGs, especially 15d-PGJ_2, are produced during inflammatory responses and exert potent immunomodulatory and anti-inflammatory effects (Lawrence et al., 2002; Gilroy et al., 2003; Scher & Pillinger, 2005; Surh et al., 2011). In addition to activating intracellular peroxisome proliferator-activated receptors γ (PPARγ) and inhibiting tumor growth (Ishihara et al., 2004), it has been reported that 15d-PGJ_2 regulates redox-sensitive transcription (Kim & Surh, 2006) and apoptosis of granulocyte, macrophage and myofibroblast cells (Li et al., 2001; Ward et al., 2002; Castrillo et al., 2003; Gilroy et al., 2003).

PPARγ is a member of a nuclear receptor family which regulates transcription of several adipocyte-specific genes involved in lipid synthesis and storage (Lehrke & Lazar, 2005). PPARγ also regulates inflammatory signaling pathways with stimulation leading to inhibition of pro-inflammatory cytokine expression, suppression of iNOS and regulation of ROS (Jiang et al., 1998; Ricote et al., 1998; Li et al., 2000; von et al., 2007; Schmidt et al., 2010; Bystrom et al., 2011). Several synthetic PPARγ ligands have been shown to effectively induce inflammatory

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cell apoptosis (Kim et al., 2007; Kang et al., 2008; Zaytseva et al., 2008), with 15d-PGJ_2 being a natural ligand of PPARγ (Forman et al., 1995). Despite the involvement of PPARγ in mediating various effects induced by PGD_2 metabolites, it has been observed that $\Delta^{12}PGJ_2$ and 15d-PGJ2 can inhibit the activation of NF-κB. Indeed LPS and TNF-α induce IκB-α degradation in granulocytes in a PPARγ independent manner (Rossi et al., 2000; Ward et al., 2002). In accordance with this finding, it has been recently described that 15d-PGJ2 induces apoptosis in human non-small cell lung carcinoma (A549 cells) via ROS production with subsequent Akt down-regulation and caspase activation in a PPARγ-independent fashion (Wang & Mak, 2011).

Cuzzocrea et al. (2003) have shown that 15d-PGJ_2 treatment is protective in experimental colitis in the rat. Moreover, treatment with 15d-PGJ2 is effective in other in vivo models including adjuvant-induced arthritis, autoimmune encephalomyelitis (Diab et al., 2002) and ischemia/reperfusion kidney injury (Chatterjee et al., 2004). Rajakariar et al. (2007) have also shown that endogenous 15d-PGJ_2 controls the levels of pro- and anti-inflammatory cytokines and subsequent leukocyte accumulation in peritonitis. Furthermore, it was observed that the resolution phase was characterized by macrophage and lymphocyte influx. This is in keeping with previous findings reporting a role for 15d-PGJ2 on induction of macrophage apoptosis, therefore limiting an acute inflammatory episode and preventing development of chronic inflammation (Gilroy et al., 2003). Although some reports describe a detrimental effect of CyPGs on the resolution of the inflammatory response (Matsuoka et al., 2000), most evidence suggests that CyPGs accelerate resolution of the inflammatory phase by preventing cell recruitment and inducing apoptosis (Gilroy et al., 2003; Rajakariar et al., 2007; Napimoga et al., 2008) via both PPARγ-dependent and independent mechanisms. Further studies to clarify the exact role of CyPGs metabolites on the inflammatory process are necessary prior to their application as potential pro-resolutive agents.

2.1.5. Interleukin 10 (IL-10)

IL-10 belongs to the class II family of α-helical cytokines and regulates innate and adaptive immunity (Moore et al., 2001). This cytokine is secreted by several cell types including macrophages, monocytes, dendritic cells, granulocytes, epithelial cells, keratinocytes, mast cells, B lymphocytes and regulatory T cells (Moore et al., 2001; Fontenot et al., 2003; O'Garra et al., 2004; Chung et al., 2007; Saraiva & O'Garra, 2010). Upon binding of IL-10 to the tetrameric IL-10 receptor (IL-10R), Jak1 and Tyk2 are stimulated which in turn stimulate the transcription factor STAT3 which is responsible for the majority of IL-10 mediated immune responses (Moore et al., 2001; Glocker et al., 2011). Other mechanisms have also been implicated in the biological effects of IL-10 such as inhibition of NFκB, up-regulated expression of the suppressor of cytokine signaling 3 (SOCS3) protein, down-regulation of TLR4 and activation of PI3K (Schottelius et al., 1999; Muzio et al., 2000; Saraiva & O'Garra, 2010). IL-10 modulates inflammatory mediator production by neutrophils, monocytes, and macrophages, limiting the secretion of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8 and IL-12. Additionally IL-10 reduces co-stimulatory and MHC class II molecule expression (Cassatella et al., 1993; Voll et al., 1997; Saraiva & O'Garra, 2010; Zhang et al., 2010a). Besides its actions on innate immunity, IL-10 plays an immune regulatory role on the processing of antigens and differentiation and proliferation of T cells, and B cells. Furthermore, it has been reported that IL-10 is a potent stimulator of natural killer (NK) cells and CD8+ cytotoxic T cells (Fiorentino et al., 1991; Saraiva & O'Garra, 2010; Zhang et al., 2010a).

In vivo studies have confirmed the ability of IL-10 to restrict the inflammatory process in diverse settings including respiratory virus infection, acute myocarditis, endotoxic shock, colitis and ischemia and reperfusion (Fuss et al., 2002; Souza et al., 2004, 2007; Loebbermann et al., 2012; Roffê et al., 2012). Specifically, Souza et al. (2004) have argued that the high levels of IL-10 found in germ-free mice are correlated to the hyporesponsiveness to inflammatory response since the inhibition of this cytokine is accompanied by an increase of TNF-α-production, neutrophil recruitment and reperfusion-induced tissue injury and lethality.

Mice deficient in IL-10 (IL-10−/−) or the IL-10R β-chain (IL-10rb−/−) develop severe spontaneous enterocolitis (Kühn et al., 1993). Interestingly it has been reported that tumor-associated macrophages (TAM) release high levels of IL-10 (Ley et al., 2012). Moreover, patients with diffuse large B-cell lymphoma have elevated IL-10 levels and JAK/STAT activation suggesting that IL-10 contributes to tumor proliferation and longevity by dampening the inflammatory reaction directed against the tumor (Gupta et al., 2012).

Owing to these anti-inflammatory and immunosuppressive abilities, we investigated whether IL-10 could induce neutrophil apoptosis or regulate efferocytosis. IL-10 did not directly induce neutrophil apoptosis, but inhibited neutrophil survival induced by LPS by modifying ERK signaling. IL-10 did not override the effect of other pro-survival agents, including GM-CSF, dexamethasone, cAMP and TNF-α (Ward et al., 2005). We have also shown that IL-10 markedly increased the ability of macrophages to phagocytose apoptotic cells (Michlewska et al., 2009), with phagocytosis in turn leading to greater release of IL-10.

Taken together, these findings suggest that the IL-10 (produced either by phagocytes from the engulfment of apoptotic cell or other sources) may contribute to the resolution of the inflammatory process by prevention of longevity induced by bacterial products, decreased pro-inflammatory and increased anti-inflammatory cytokine production and enhanced macrophage ability to phagocytose apoptotic cells. However, formal demonstration of the role of IL-10 in either natural or drug-induced resolution of inflammation is still lacking.

2.1.6. Melanocortin system

The melanocortin (MC) peptides, which include adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), β-MSH, and γ-MSH are derived from a larger precursor molecule called the pro-opiomelanocortin (POMC) gene (Catania, 2007). One of the main peptides of the MC system is α-MSH which exerts neuro-immunomodulatory effects via activation of the G-protein coupled MC receptors (MCRs). So far, 5 MCRs have been cloned (Wikberg et al., 2000). In vitro, it has been shown that binding of α-MSH to MCRs leads to down-regulation of various pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α, IL-2, IFN-γ, IL-4, and IL-13 (Delgado et al., 1998; Mandrika et al., 2001; Catania, 2007). At the molecular level, α-MSH activates cAMP-dependent pathways and may mediate its anti-inflammatory effects by modulation of NF-κB activation (Haycock et al., 1999; Catania et al., 2010).

MC peptides and their receptors have been appreciated as anti-inflammatory agents in many experimental models of acute and chronic inflammation including atopic dermatitis, arthritis, allergic inflammation, inflammatory bowel diseases and ischemia–reperfusion injury (Raap et al., 2003; Getting et al., 2008; Kannengiesser et al., 2008; Leoni et al., 2008; Catania et al., 2010; Patel et al., 2010; Montero-Melendez et al., 2011a; Etori et al., 2012). Evidence shows that ACTH, α-MSH, and other MC agonists can activate MC1 and MC3 on macrophages through cAMP and/or NF-κB-dependent mechanisms to abrogate pro-inflammatory cytokines, chemokines, and NO and enhance anti-inflammatory mediator levels such as IL-10 and HO-1 (revised in Patel et al., 2011).

Getting et al. (2008) investigated the potential of MC peptides in murine models of allergic and non-allergic inflammation. In an allergic model of inflammation, MC peptides inhibited leukocyte accumulation in wild type-mice. The pan-agonist α-MSH markedly decreased eosinophil accumulation in mutant MCR1 mice (which receptor is unable to couple to adenylate cyclase and activate cAMP synthesis) but not in MC3R-null mice, suggesting a preferential role for MC3R in mediating the anti-inflammatory effects of MC in this model. In vitro analysis identified the presence of two receptors (MC1R and MC3R) on alveolar macrophages. Alveolar macrophage incubation with the selective MC3R

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agonist [D-TRP8]-g-MSH and pan-agonist α-MSH increased levels of cAMP. Similarly in a model of LPS-induced lung inflammation, MC peptides displayed significant attenuation of neutrophil accumulation and inhibition of TNF-α release, highlighting the beneficial potential of MC peptides in inflammatory lung disorders (Getting et al., 2008). In agreement with these findings, Montero-Melendez et al. (2011b) have reported that a promising analog of α-MSH (AP214, which has high binding affinity for MCRs) inhibited cell infiltration and cytokine release in zymosan-induced peritonitis. Furthermore, AP214 augmented the uptake of zymosan particles and human apoptotic neutrophils by wild-type macrophages. In experimental arthritis, AP214 caused significant reductions in clinical score by acting on the endogenous MCR3 receptor. Importantly mice treated with AP214 present increased efferocytosis of human apoptotic neutrophil, showing that this peptide also modulates a key step of resolution response.

AP214 has been tested in other preclinical studies including sepsis-induced acute kidney injury (Doi et al., 2008), ischemia-induced acute kidney injury (Simmons et al., 2010) and endotoxin-induced systemic inflammatory response syndrome (Kristensen et al., 2011). Importantly, clinical evidence indicates that AP214 is effective in preventing post-surgical kidney injury being investigated with initial results suggesting good efficacy, safety and tolerability. (www.clinicaltrials.gov). Further development of α-MSH-peptide analogs for long term anti-inflammatory treatment may include the development of MCR3 specific agonists. It must be pointed out, however, that the studies described above mainly focus on the anti-inflammatory effects of MC peptides and analogs and further demonstration of their effects in the context of resolution of inflammation needs to be shown.

In Table 2, we summarize the effects of several mediators for their ability to induce resolution of inflammation in vivo or to induce biological effects in vitro thought to be relevant for the resolution process.

2.2. Receptors implicated in mediating resolution

2.2.1. GPR43 and GPR120

Free fatty acids (FFAs) which bind G-protein-coupled receptors (GPCRs) including GRP119, GPR84, GPR120, GPR40 (FFAR1), GPR43 (FFAR2) and GPR41 elicit several biological effects including adipogenesis, regulation of appetite, release of regulatory peptides and potential anti-inflammatory effects (Hong et al., 2005; Karaki et al., 2006; Gotoh et al., 2007; Maslowski et al., 2009; Sina et al., 2009; Oh et al., 2010; Vinolo et al., 2011a). GPR120 and GPR40 are activated by medium- and long-chain FFAs, and GPR119 is activated by long-chain fatty acids; GPR84 is activated by medium-chain FFAs; GPR43 and GPR41 are activated by short-chain fatty acids (Milligan et al., 2006). GPR43 and GPR120 (Brown et al., 2003; Le Poul et al., 2003) have received attention for their potential therapeutic application in the treatment of several inflammatory diseases, including obesity and type 2 diabetes mellitus (Hirasawa et al., 2005; Maslowski et al., 2009; Oh et al., 2010; Cornall et al., 2011; Talukdar et al., 2011). GPR43 is expressed in adipose tissue, gastrointestinal endocrine cells and leukocytes, with particularly high levels on monocytes and neutrophils (Le Poul et al., 2003; Nilsson et al., 2003; Karaki et al., 2006; Maslowski et al., 2009).

Table 2
Selected mediators of inflammation and their known effects on key events associated with inhibition of inflammation or induction of resolution of inflammation.

Mediator	Evidence for anti-inflammatory effects in vivo	Evidence for pro-resolution effects in vivo	Evidence for induction of granulocyte apoptosis in vitro	Evidence for induction of phagocytosis in vitro
LXs and ATLs	Yes	Yes	Yes	Yes
Rvs	Yes	Yes	No	Yes
Protectins	Yes	Yes	Yes	Yes
Maresin	Yes	Yes	No	Yes
AnxA1	Yes	Yes	Yes	Yes
GILZ	Yes	No	No	No
MKP-1	Yes	No	No	No
H_2O_2	No	Yes	Yes	Yes
PGD2 and 15d-PGJ_2	Yes	Yes	Yes	No
IL-10	Yes	No	No	Yes
α-MSH	Yes	Yes	No	Yes

Binding of short chain fatty acids (SCFAs), such as acetate, propionate and butyrate or synthetic agonists (e.g. phenylacetamide-1), to GPR43 induces neutrophil chemotaxis in vitro (Vinolo et al., 2011b). SCFAs and phenylacetamide-1 also promote GPR43-dependent activation of multiple pathways including PKB, protein kinase C, p38, ERK and the transcriptional factor activating transcriptional factor-2 (ATF-2) (Maslowski et al., 2009). In vivo, there have been conflicting reports on the effects of SCFAs and GPR43 on the inflammatory response (Maslowski et al., 2009; Sina et al., 2009). We have previously shown that GPR43$^{-/-}$ mice showed greater unresolved inflammation in models of colitis, arthritis and asthma suggesting that stimulation of GPR43 was necessary for normal resolution of inflammation (Maslowski et al., 2009). Indeed, GPR43$^{-/-}$ mice had increased bronchoalveolar lavage inflammatory cells as well as enhanced levels of eosinophil peroxide in the lung tissue in ovalbumin (OVA)-induced allergic airway inflammation. Moreover, it has been reported that germ-free mice, which are devoid of bacteria and express little or no SCFAs, showed a similar dysregulation of certain inflammatory responses. Specifically, dextran sulfate sodium (DSS)-induced intestinal inflammation in germ-free mice caused enhanced colonic inflammation and the increased intake of fermentable dietary fiber or SCFAs improved clinical parameters, corroborating a body of evidence which has suggested that intestinal microbiota modulates host immune responses and protects against intestinal inflammatory diseases (Mazmanian et al., 2008; Wen et al., 2008). In contrast, Sina et al. (2009) found that GPR43-deficient mice had attenuated cell infiltration and higher mortality in chronic colitis. Death was associated with dissemination of *Clostridium* spp. It is difficult to reconcile the different findings between the studies of Sina and Malowski, which could be related to the nature of the models used (acute versus chronic colitis). However, it is clear that under certain conditions, GPR43 could be playing a role in limiting the intensity and duration of the inflammatory response. However, formal demonstration of the ability of GPR43 in modulating resolution of inflammation still needs to be shown.

GPR120 is broadly distributed and highly expressed in adipose tissue (Gotoh et al., 2007; Oh et al., 2010), macrophages (Oh et al., 2010), gastrointestinal tract, lung (Hirasawa et al., 2005; Gotoh et al., 2007) and pituitary (Gotoh et al., 2007). GPR120 shares effects with GPR43 being reported as a potent anti-inflammatory and insulin-sensitization mediator (Hirasawa et al., 2005; Maslowski et al., 2009; Oh et al., 2010; Cornall et al., 2011; Talukdar et al., 2011). It has been reported that GPR120 couples to Gq and β-arrestin 2 elevating intracellular calcium levels and activating the ERK cascade (Hirasawa et al., 2005). Oh et al. (2010) have shown that the stimulation of GPR120 with ω-3 FAs (DHA or EPA) or a GPR120 agonist, GW9508, causes anti-inflammatory effects on RAW264.7 cells and monocytic peritoneal macrophages. For instance, DHA and GW9508 markedly inhibited LPS-induced phosphorylation of JNK and IKKb, IkB degradation, cytokine secretion and inflammatory gene expression. All effects were abolished by siRNA mediated knockdown of GPR120. Importantly GPR120−/− mice become insulin resistant, with hyperinsulinemia and decreased insulin stimulated glucose disposal. Taking the results together, it is suggested that GPR120 mediates potent insulin sensitizing and anti-diabetic effects in vivo by preventing macrophage-induced tissue inflammation. The aforementioned effects of GPR43 and GPR120 on the inflammatory process are encouraging and supportive for further studies to formally demonstrate their capacity to modify the resolution phase of inflammation.

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2.2.2. ChemR23

ChemR23 (also called chemokine-like receptor-1) is a GPCR expressed on monocytes/macrophages, immature dendritic cells (DCs), plasmacytoid DCs, NK cells and on chondrocytes (Samson et al., 1998; Parolini et al., 2007; Luangsay et al., 2009; Berg et al., 2010). Its natural agonist, chemerin, binds to ChemR23 at low concentration and acts as a chemoattractant for monocytes/macrophages and immature DCs (Wittamer et al., 2003; Vermi et al., 2005; Luangsay et al., 2009). In addition to chemerin, RvE1 also binds to ChemR23 to regulate inflammation (Arita et al., 2007; Oh et al., 2011). The role of ChemR23 in the context of inflammation has been studied recently in vitro and in vivo suggesting an important role for ChemR23 in mediating inflammation and, potentially, pro-resolution pathways (Cash et al., 2008, 2010; Bondue et al., 2011; Demoor et al., 2011; Iannone & Lapadula, 2011). For example, Bondue et al (2011) studied the role of chemerin/ChemR23 in cellular recruitment and resolution of viral-induced pulmonary inflammatory in wild type and ChemR23 knockout mice. ChemR23$^{-/-}$ mice displayed higher morbidity and mortality with enhanced respiratory dysfunction, delayed viral clearance and increased neutrophilic infiltration. Chemerin levels increased in bronchoalveolar lavage during viral infection in both groups but they were higher in ChemR23$^{-/-}$ mice. Furthermore these mice had impaired recruitment of plasmacytoid DCs and a reduction in type I interferon, key players of anti-viral immunity. However, plasmacytoid DC depletion and adoptive transfer experiments showed that increased morbidity/mortality in ChemR23$^{-/-}$ mice was not due to impaired plasmacytoid DC recruitment, but to a more severe inflammatory status involving ChemR23 expressed by non-leukocytic cells. Cash et al (2008) found that the administration of chemerin in zymosan-induced peritoneal inflammation promoted zymosan clearance and apoptotic neutrophil ingestion by macrophages in wild-type but not ChemR23$^{-/-}$ mice, suggesting an important role for chemerin/ChemR23 in promoting efferocytosis. Therefore, Chemerin/ChemR23 interaction seems to preferentially limit inflammation and has effects associated with greater resolution, albeit formal demonstration of the capacity of this ligand to induce resolution in vivo is lacking.

2.3. Signaling pathways of survival and death

The inflammatory response is characterized by coordinated activation of various signaling pathways that regulate expression of both pro- and anti-inflammatory mediators in resident tissue cells and leukocytes recruited from blood (Lawrence & Fong, 2010). Granulocytes survive in the circulation for only a few hours but mediators released at the site of inflammation can extend the lifespan of recruited granulocytes. For instance neutrophils may only survive in the circulation for 7–10 h but their longevity is extended by cytokines (e.g. IL-6, IL-8 and GM-CSF), bacterial products (e.g. LPS) and local factors such as oxygen tension (Rossi et al., 2007). Likewise, eosinophil survival is augmented at sites of inflammation by cytokines such as IL-3, IL-5 and GM-CSF (Cara et al., 2000). Several signaling pathways have been implicated in leukocyte survival, including NF-κB, PI3K/Akt and MAPKs. Generally these signaling pathways lead to enhanced levels of pro-survival molecules and diminished level of pro-apoptotic molecules. In Fig. 2, we show an example of the crosstalk between signaling pathways and pro-resolution mediators in vivo. The interaction described between cAMP, H_2O_2 and AnxA1 and certain intracellular pathways is derived from our own studies evaluating natural and drug (steroid)-induced resolution in models of acute neutrophilic inflammation in mice. It is unlikely that this figure represents the interaction among various mediators and pathways in more complex models of acute and chronic inflammation, but it serves to describe the interaction of certain effectors in the regulation of resolution of inflammation by apoptosis.

The manipulation of key intracellular pathways could be of potential benefit in the control of inflammatory disease. However, it is noteworthy that these pathways participate in numerous physiological functions and, therefore, potential therapeutic use of blocking survival pathways should be considered with great care in clinical development.

2.3.1. Nuclear factor kappa B (NF-κB)

NF-κB is a transcription factor recognized as a key regulator of innate and adaptive immune responses, cell survival and oncogenesis (Bonizzi

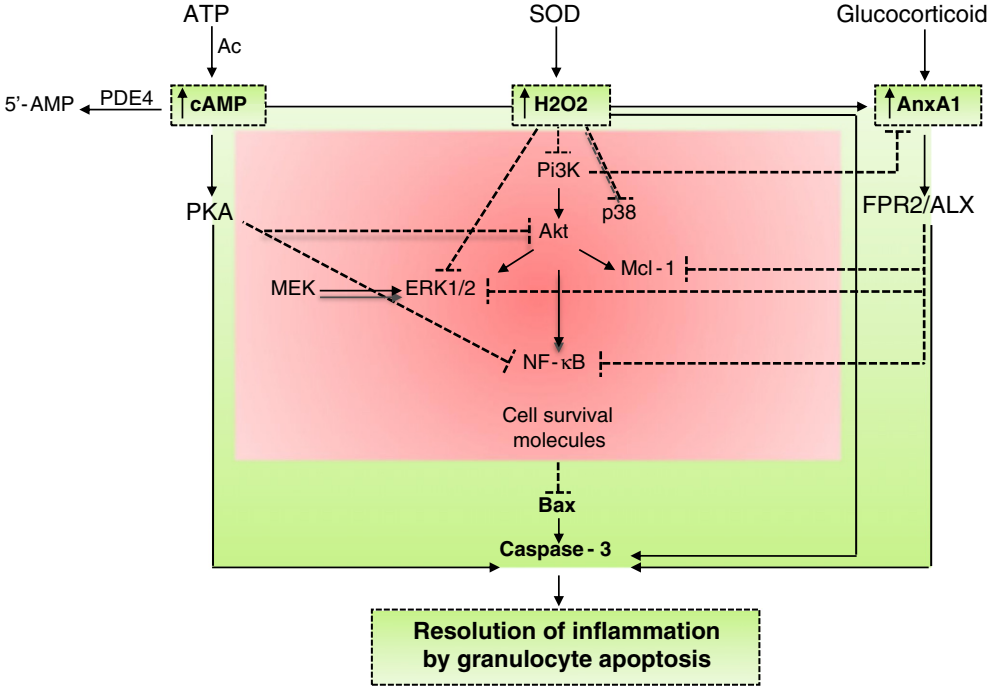

Fig. 2. Crosstalk between signaling pathways and pro-resolution mediators in the regulation of the resolution of inflammation by apoptosis.

& Karin, 2004; Lawrence & Fong, 2010). Although NF-κB had initially been thought of as only controlling pro-inflammatory signaling pathways, consistent evidence has now shown that NF-κB controls genes involved in both the onset as well as the resolution phase of the inflammatory process (Lawrence et al., 2001; Greten et al., 2007; Lawrence & Fong, 2010). NF-κB activation can drive at least two signaling pathways – canonical and alternative – which enable NF-κB to translocate towards the nucleus. There, it binds to consensus sites in the DNA of responsive genes, activating gene transcription (Bonizzi & Karin, 2004). The canonical pathway is triggered by microbial products and pro-inflammatory cytokines (e.g. TNF-α and IL-1) and regulated by IκB kinase (IKKβ) inducing phosphorylation of IκBs. Such phosphorylation targets IkB to proteosomal degradation allowing the release and nuclear translocation of p50/RelA heterodimers (Karin & Ben-Neriah, 2000). Conversely, the alternative pathway is stimulated by TNF-family cytokines, including lymphotoxin β (TNFSF3), CD40 ligand (CD40L and TNFSF5), B cell activating factor (BAFF and TNFSF13B) and receptor activator of NF-κB (RANKL and TNFSF11) (Senftleben et al., 2001; Novack et al., 2003; Bonizzi & Karin, 2004). The latter pathway is characterized by the requirement of IKKα for activation through phosphorylation followed by proteosomal processing of p100 (the p52 precursor) and nuclear translocation of p52/RelB heterodimers (Bonizzi & Karin, 2004). NF-κB activates the transcription of many pro-inflammatory mediators including cytokines (e.g. IL-6, TNF-α, IL1-β), chemokines (e.g. CXCL8, MCP-1), enzymes that produce secondary inflammatory mediators (e.g. iNOS, COX-2) and adhesion molecules (e.g. VCAM-1, ICAM-1, E-selectin) contributing to the induction and perpetuation of inflammation (Elewaut et al., 1999; Maeda et al., 2005). As such IKKβ has been suggested as relevant for the development of new anti-inflammatory therapies (Karin et al., 2004; Lawrence & Fong, 2010).

Activation of NF-κB and IKKα influences granulocyte survival and apoptosis, therefore influencing the resolution phase of the inflammatory response (Ward et al., 1999; Lawrence et al., 2001; Fujihara et al., 2002; Ward et al., 2004; Greten et al., 2007; Sousa et al., 2009). NF-κB controls the expression of many intracellular pro-survival proteins, such as Bcl-2 family members (e.g. Bcl-xL, XIAP), which appear relevant for their capacity to enhance survival of inflammatory cells (Ward et al., 2004). Several inhibitors of NF-κB have been developed and tested both in vitro and in vivo. For instance, the fungal metabolite gliotoxin inhibits NF-κB and increases the rate of constitutive apoptosis of human neutrophils and eosinophils (Ward et al., 1999) and increases the apoptotic effects of TNF-α (Ward et al., 1999; Fujihara et al., 2002).

Inhibition of NF-κB in vivo promotes resolution of established neutrophilic and eosinophilic inflammation (Sousa et al., 2009; Lopes et al., 2011). NF-κB inhibition in a mouse model of arthritis was associated with enhanced neutrophil apoptosis and resolution of inflammation (Lopes et al., 2011) while inhibition in a model of allergic pleurisy caused eosinophil apoptosis with consequent resolution of inflammation. However, the use of the NF-κB inhibitors PDTC and SN-50 in a model of LPS-induced pleurisy failed to promote resolution of neutrophil inflammation despite LPS inducing significant NF-κB activation. Furthermore, inhibition of NF-κB in a model of carrageenan-induced neutrophilic inflammation prolonged the inflammatory response and prevented apoptosis (Lawrence et al., 2001). These apparently conflicting results may be explained by the timing of NF-κB inhibition, as RelA/p50 heterodimers are prevalent at the beginning of inflammation to drive pro-inflammatory mediator production and leukocyte recruitment and survival, whereas during times of resolution p50/p50 heterodimers predominate and are responsible for pro-inflammatory mediator suppression and induction of apoptotic genes (Lawrence et al., 2001; Gilroy et al., 2004). Therefore, in vivo manipulation of the NF-κB system likely requires either a timely inhibition or inhibition of specific NF-κB subunits to ensure an anti-inflammatory and pro-resolution outcome.

2.3.2. PI3K/Akt

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that phosphorylate the 3′-hydroxyl group of phosphatidylinositols $(PtdIns)^3$ resulting in the formation of PtdIns(3)P, PtdIns(3,4)P2 and PtdIns(3,4,5)P3, collectively named 3′-PtdIns lipids. PI3Ks are subdivided into three different classes according to their substrate and sequence homology (Vanhaesebroeck & Waterfield, 1999). PI3K is activated by several stimuli including antigen receptors, cytokine and chemokine receptors leading to the activation of signaling pathways including Ser/Thr kinases, Akt (or PKB), PDK1 and BTk (Toker & Cantley, 1997; Fruman & Cantley, 2002; Engelman et al., 2006). PI3Ks and their downstream signaling pathways have a central role in numerous processes including cell metabolism, cell cycle and survival, protein synthesis, cell polarity and motility, vesicle trafficking and immune function (Berwick et al., 2002; Burgering & Medema, 2003; Nobukuni et al., 2005; Vanhaesebroeck et al., 2012). It has also been demonstrated that PI3K controls fundamental leukocyte functions such as growth, proliferation, recruitment, activation and survival (Sasaki et al., 2000; Yum et al., 2001; Yang et al., 2003; Puri et al., 2004; Pinho et al., 2005; Russo et al., 2011). For example, our group has demonstrated that there is a tissue- and stimulus-dependent role for different isoforms of PI3K in regulating neutrophil migration in vivo in response to different chemoattractants (Pinho et al., 2007). For instance, neutrophil recruitment into the alveolar space or in response to the exogenous administration of C5a or fMLP was dependent upon only PI3Kγ, whereas CXCL1-induced neutrophil recruitment to the pleural cavity induced similar neutrophil influx in PI3Kγ-deficient and wild-type animals.

With regard to the molecular mechanisms behind the ability of PI3K to influence cell survival, studies have indicated that the intracellular pro-apoptotic protein BAD (Bcl-2-associated death promoter) is an important downstream target of PI3K/Akt. Akt phosphorylates BAD, thereby suppressing apoptosis and promoting cell survival (Datta et al., 1997; Song et al., 2005). Moreover, the PI3K/Akt pathway is relevant in mediating the anti-apoptotic and pro-inflammatory signals activated by LPS, GM-CSF and TNF-α in granulocytes (Yang et al., 2003; Cowburn et al., 2004).

Our group has shown that PI3K contributes to the resolution phase of inflammation by influencing granulocyte apoptosis (Pinho et al., 2005; Sousa et al., 2009; Rodrigues et al., 2010). In vivo, using OVA challenge as a model of eosinophilic inflammation (Klein et al., 2002; Alessandri et al., 2003; Pinho et al., 2003), we found that treatment with the PI3K inhibitors wortmannin or LY294002 at the peak of inflammation after the cells had migrated markedly reduced eosinophil numbers, an effect associated with inhibition of Akt phosphorylation and increased eosinophil apoptosis (Pinho et al., 2005; Sousa et al., 2009). Likewise, treatment with dexamethasone 24 h after antigen challenge of sensitized mice rapidly cleared eosinophils from the pleural cavity (Sousa et al., 2009) a feature already observed in vitro (Liu et al., 1999). Morphologically, apoptotic eosinophils could be observed inside macrophages as early as 6 h after the administration of dexamethasone. A major caveat of the studies discussed above is that they made use of non-selective PI3K inhibitors and the role of the various family members is yet to be determined.

One study from our group showed that in MOG-induced experimental autoimmune encephalomyelitis, PI3Kγ-deficient animals developed milder clinical disease as well as decreased levels of the chemokines CCL2 and CCL5 in brain tissue (Rodrigues et al., 2010). However neither PI3Kγ-deficiency nor use of the PI3K inhibitor AS-605240 affected leukocyte rolling or adhesion as assessed by intravital microscopy. On the other hand, there was a marked increase in the percentage of leukocytes undergoing apoptosis in PI3Kγ-deficient mice suggesting that the role of PI3Kγ in this model appeared to be the mediation of leukocyte survival. Further demonstration of this pro-survival role of PI3Kγ in models in vivo is necessary.

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2.3.3. Cyclic AMP (cAMP)

cAMP is a ubiquitous second messenger produced after adenylate cyclase activation in response to several stimuli. The two main cAMP effectors are heterodimeric cyclic AMP-dependent protein kinase PKA and EPAC (exchange protein directly activated by cAMP) but there are additional, less well-characterized effector proteins. Once activated by cAMP, PKA acts by causing dissociation of the catalytic subunit C, which phosphorylates several enzymes regulating a variety of cellular process. Cyclic AMP plays an important role in the immune system, usually promoting suppressive effects on the functions of inflammatory cells (Serezani et al., 2008). Intracellular levels of cyclic AMP levels depend on efflux from the cells or are controlled by phosphodiesterases (PDEs) (Soderling & Beavo, 2000).

PDEs are enzymes that hydrolyze cyclic adenosine and guanosine 3′,5′-monophosphate enzymes (cAMP/cGMP) to their corresponding 5′-mononucleotides 5′-AMP and 5′-GMP, which do not activate cyclic nucleotide-dependent protein kinases. Inhibition of these enzymes enhances cAMP and cGMP levels in the cells (Schudt et al., 2011). There are different families of PDEs with important roles in different cells or tissues. For instance, PDE_4 isozyme plays a particularly important role in the immune system and is the predominant PDE in inflammatory cells such as mast cells, eosinophils, neutrophils, T cells and macrophages (Page & Spina, 2011). It has been well appreciated that selective inhibition of PDE_4 has anti-inflammatory properties (Teixeira et al., 1997; Souza et al., 2001; Bopp et al., 2009; Sousa et al., 2009, 2010).

Recently, we described in an allergic pleurisy model that cAMP elevation promoted by administration of a PDE_4 inhibitor or by cAMP mimetic drugs at the peak of inflammation induced a significant reduction of eosinophils, without a change in mononuclear cells (Sousa et al., 2009). This reduction of eosinophils was associated with an increase in the number of apoptotic cells in the pleural cavity and correlated to an increased expression of the pro-apoptotic protein Bax. Moreover, we showed that this resolution of eosinophilic inflammation by rolipram, a PDE_4 inhibitor, was PKA-dependent and due to inhibition of Akt phosphorylation and NF-κB activation. An important role for cAMP during the resolution process is supported by studies which described that drugs that elevate the intracellular levels of cAMP regulate granulocyte apoptosis in vitro (Rossi et al., 1995; Martin et al., 2001; Parkkonen et al., 2008) and their subsequent recognition and uptake by macrophages (Rossi et al., 1998). Another agent that can elevate cAMP, PGD_2, selectively induces caspase-dependent eosinophil apoptosis in a peroxisome-activated receptor-γ (PPAR-γ)-independent manner (Ward et al., 2002).

By using a model of LPS-induced pleurisy, Sousa et al. (2010) induced neutrophil accumulation by intrapleural administration of LPS and evaluated the effect and mechanisms of PDE_4 inhibition and other cAMP-elevating agents or mimetics on the resolution of the neutrophilic inflammation. Treatment with rolipram 4 h after LPS challenge (when the cell influx was already established but had not peaked yet) dose-dependently reduced the number of neutrophils in the pleural cavity via PKA, the best known cAMP effector. Furthermore, the administration of forskolin (an adenylate cyclase activator) or db-cAMP (a cell-permeable cAMP analog) into the pleural cavity prevented neutrophil accumulation mirrored by an increased number of apoptotic cells in the cavity. Rolipram-induced neutrophil apoptosis was associated with decreased expression of Mcl-1, a key anti-apoptotic protein regulating neutrophil survival, and increased caspase-3 cleavage. Rolipram-induced resolution was caspase-dependent since treatment with the pan-caspase inhibitor zVAD-fmk inhibited inflammatory cell apoptosis and prevented resolution of neutrophil numbers. Since rolipram-induced resolution of neutrophilic inflammation was associated with Akt and NF-κB inhibition, we evaluated these pathways in greater detail using the LPS-induced pleurisy model. The use of a PI3K inhibitor (LY294002) and an Akt inhibitor (Akt inhibitor V) also enhanced apoptosis and promoted neutrophil clearance. Conversely NF-κB inhibitors failed to resolve neutrophil accumulation in our LPS-induced pleurisy model, showing that although rolipram promotes inhibition of NF-κB activation, this pathway appears not to be critical for rolipram-induced resolution of neutrophilic inflammation (Sousa et al., 2010).

These in vivo findings using LPS-induced pleurisy are in contrast with in vitro studies using neutrophils, which have shown that PDE_4 inhibition or an increase of cAMP levels can delay or inhibit spontaneous neutrophil apoptosis (Rossi et al., 1995; Conran et al., 2007; Parkkonen et al., 2008). However, it is noteworthy that these studies used mainly human peripheral blood neutrophils and therefore the complex in vivo inflammatory environment was not replicated. In addition it has also been reported that elevating cAMP induces a switch of the pro-inflammatory macrophages into resolution-phase macrophages (Bystrom et al., 2008) emphasizing that cAMP manipulation in vivo can act on multiple interacting cell types. As such cAMP elevating drugs may be a useful therapeutic strategy to induce resolution of inflammation.

2.3.4. MAPK (mitogen-activated protein kinase) pathways

The MAPK family of serine threonine kinases integrates and processes multiple cell surface signals (Kumar et al., 2003; Kaminska, 2005). There are three main groups of distinctly regulated MAP kinase cascades known in humans that lead to differential gene expression: extracellular-signal-regulated kinase ERK1/2, c-Jun N-terminal kinases (JNKs) and p38 MAP kinase (Pearson et al., 2001; Kaminska, 2005). MAPKs phosphorylate and activate transcription factors present in the cytoplasm or nucleus, driving the expression of genes and consequently biological responses (Pearson et al., 2001; Kumar et al., 2003; Saba-El-Leil et al., 2003). Commonly, ERKs are activated by mitogenic and proliferative stimuli, whereas JNKs and p38 MAPKs respond to environmental stress, including UV radiation, heat, osmotic shock and inflammatory cytokines (Stevenson et al., 1994; Lee & Young, 1996; Pearson et al., 2001). However, in in vivo settings these patterns of activation are not straightforward and these proteins cooperate in response to various stimuli.

There is much evidence demonstrating that MAPKs contribute to the inflammatory process by regulating expression of cytokines, chemokine cell adhesion molecules and recruitment of leukocytes (Suzuki et al., 1999; Kumar et al., 2003; Sawatzky et al., 2006; Junttila et al., 2008). Moreover, it is clear that all MAPK subtypes play a role in modulating granulocyte apoptosis. The relevance of each family member varies according to cell type and experimental condition (Suzuki et al., 2001; Alvarado-Kristensson & Andersson, 2005; Derouet et al., 2006; reviewed by Junttila et al., 2008). The relevance of MAPKs for the resolution of inflammation in vivo is less well known, making it very difficult to understand the precise relevance of these mediators in vivo and the potential of inhibitors of these enzymes for resolution of inflammation.

In vivo data using carrageenan-induced pleurisy have demonstrated that the ERK1/2 inhibitor PD98059 enhanced resolution of inflammation with reduced numbers of neutrophils and macrophages in the pleural cavity and increased neutrophil apoptosis (Sawatzky et al., 2006). Interestingly, PD98059 does not have a direct effect on constitutive neutrophil apoptosis in non-inflammatory conditions (Ward et al., 2005; Sawatzky et al., 2006), suggesting that its pro-apoptotic effect is a result of inhibition of survival factor-induced anti-apoptotic effects. The pro-resolving effect of endogenous and exogenous anxA1 was associated with ERK inhibition (Vago et al., 2012). Indeed AnxA1 knockout mice showed exacerbated MAPK activation and production of pro-inflammatory cytokines (Yang et al., 2006).

While ERK is best known for its role in the regulation of activation and survival of a plethora of cell types, including granulocytes (Pagès et al., 1993; Geest et al., 2009; Chapman & Miner, 2011) the exact role of p38 in the control of granulocytes apoptosis/survival is controversial and both pro- and anti-apoptotic effects have been described (Junttila et al., 2008; Langereis et al., 2010; Lin et al., 2011). In the

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context of resolution of inflammation, there is a recent report showing that pro- to anti-inflammatory macrophage polarization switch is controlled by the balance of p38 MAPK and the MAPK phosphatase MKP-1 during muscle healing process (Perdiguero et al., 2012). Precise demonstration of pro-survival roles of p38 in vivo is necessary.

2.3.5. B-cell lymphoma 2 (Bcl-2) family

The Bcl-2 family comprises a series of intracellular proteins with a central regulatory role on mitochondrial integrity and caspase activation, thus controlling the intrinsic pathway of apoptosis (Cory & Adams, 2002). Bcl-2 family members can be either pro-apoptotic or anti-apoptotic and are divided into three distinct groups on the basis of their possession of the four conserved Bcl-2 homology domains (BH1–4). Pro-apoptotic Bcl-2 proteins share homology in either two or three BH domains. This group includes Bax and Bak which themselves induce mitochondrial outer membrane permeabilization (MOMP), causing the release of caspase-activating proteins and other cell death mediators. The pro-apoptotic BH3-only proteins (including Bad, Bid, Bim and Puma) possess homology only in the BH3 region and act to activate Bax and Bak, either directly or binding and down-regulating anti-apoptotic proteins. Finally, the anti-apoptotic group is composed of proteins, such as Bcl-2, Bcl-X_L, Mcl-1 and Bcl2A1, which show sequence homology through BH1 to BH4. These members inhibit apoptosis mainly by sequestering BH3-only proteins and thereby preventing activation of Bax or Bak (Cory & Adams, 2002).

Owing to their modulatory role in apoptosis, Bcl-2 family proteins are potential therapeutic targets for conditions where cellular apoptosis/survival is dysregulated such as inflammatory diseases (Adams & Cory, 2007). In fact it has been observed that in sputum of patients with severe asthma, there is increased expression of the anti-apoptotic protein Bcl-2 (Jang et al., 2000; El-Gamal et al., 2004). Moreover, a significant inverse correlation was found between eosinophils that expressed Bcl-2 and peak expiratory flow rate (El-Gamal et al., 2004).

There is a substantial body of evidence supporting the notion that neutrophils and eosinophils express Bcl-2 family proteins and the balance between the expression of the pro-apoptotic and anti-apoptotic members represents a relevant factor determining the lifespan of granulocytes (Simon, 2001; Bianchi et al., 2006). For instance some members of the Bcl-2 family are involved in eosinophil survival. Specifically, increased expression of Bcl-X_L (Dibbert et al., 1998), maintained Bid cleavage (Segal et al., 2007), inhibition of Bax translocation to the mitochondria (Dewson et al., 2001), and delayed Bax cleavage (Conus et al., 2005) result in maintained mitochondrial integrity and inhibition of caspase activation (Dewson et al., 2001; Conus et al., 2005). Likewise, neutrophils express anti-apoptotic members (Bcl-X_L, Bcl2A1 and Mcl-1) as well as pro-apoptotic proteins (Bax, Bak, Bid, Bim and Puma) and those proteins regulate constitutive as well as inducible apoptosis/survival pathways (Weinmann et al., 1999; Moulding et al., 2001; Guo et al., 2006; Cowburn et al., 2011).

Experiments in vivo using carrageenan induced pleurisy demonstrated that inflammatory cells from the onset of the inflammatory express Bcl-X_L and defective levels of Bax. However, during resolution the profile is inverted with increased expression of Bax and low levels of Bcl-X_L. Furthermore the Bax inhibitor (peptide V5) given at the peak of inflammation enhanced the number of macrophages/monocytes and neutrophils in the pleural cavity associated with a decrease in the amount of neutrophil apoptosis preventing resolution (Sawatzky et al., 2006). In agreement with the ability of Bax to accelerate inflammation resolution by shifting the balance towards a pro-apoptotic direction, our group has shown that strategies which enhance resolution of inflammation – i.e. treatment with rolipram, SOD, dexamethasone, wortmannin and AnxA1 peptidomimetics – were able to increase Bax expression which was associated with granulocyte apoptosis and resolution of inflammation (Sousa et al., 2009, 2010; Lopes et al., 2011; Vago et al., 2012).

Recently, Iwata et al. (2010) have reported that administration of the anti-apoptotic recombinant human (rh) Bcl-2 or rhBcl-2A1 protein reduced apoptosis and tissue damage in murine models of hind limb and myocardial ischemia–reperfusion injury. Moreover, the same group also evaluated the effect of rhBcl-2 or rhBcl-2A1 on a murine model of sepsis induced by cecal ligation and puncture (CLP) (Iwata et al., 2011). The administration of anti-apoptotic proteins markedly improved animal survival. Protection was accompanied by decreased number of apoptotic cells and reduced cleaved caspase-3 expression in the intestine and heart. Guo et al (2006) using the same CLP model also showed increased levels of Bcl-X_L and decreased expression of Bim in neutrophils isolated from blood of septic animals.

A few studies have highlighted an important role of Mcl-1 on eosinophil and neutrophil survival and apoptosis (Dzhagalov et al., 2007; Sivertson et al., 2007; Duffin et al., 2009; Leitch et al., 2010; Sousa et al., 2010; Milot & Filep, 2011; Lucas et al., 2012; Vago et al., 2012). In fact the anti-apoptotic protein Mcl-1 plays a major role in controlling the rate at which neutrophils undergo apoptosis. Mcl-1 transcripts are particularly unstable with rapid turnover and a half-life of ~3 h (Moulding et al., 2001; Edwards et al., 2004). Additionally it has been shown that either Mcl-1 expression or turnover is regulated by several agents such as GM-CSF, TNF-α (Moulding et al., 2001; Derouet et al., 2004), sodium salicylate (Derouet et al., 2006), cAMP and PI3K/Akt (Sousa et al., 2010), cyclin-dependent-kinase inhibitors (CDKi) (Rossi et al., 2006; Duffin et al., 2009; Leitch et al., 2010, 2012) and more recently AnxA1 peptide, Ac2-26 (Vago et al., 2012). Cross et al. (2008) have reported that in human neutrophils, TNF-α at high concentrations was able to increase the rate of Mcl-1 degradation via activation of caspases whereas low concentrations induced anti-apoptotic Bfl-1 expression. These concentration-dependent responses might help explain the discrepant effects of TNF on neutrophil survival/death (Salamone et al., 2001; van den Berg et al., 2001) and understand the complicated scenario in vivo. Another study reported that the opposing effects of dexamethasone on granulocytes relied on the ability of glucocorticoids to stabilize Mcl-1 in neutrophils but not in eosinophils (Sivertson et al., 2007). This might be one of the mechanisms underlying the induction of eosinophil apoptosis by glucocorticoids and its success as a therapy for eosinophil dominant inflammation such as asthma, while explaining the reduced efficacy of glucocorticoids on neutrophil dominant diseases such as chronic obstructive pulmonary disease (COPD). From our findings, we demonstrated that eosinophils and neutrophils underwent apoptosis in vitro following treatment with the CDK inhibitor R-roscovitine preceded by down-regulation of the protein Mcl-1 (discussed below). Additionally we have shown that the pro-apoptotic effects of rolipram, PI3K inhibitors and Ac2-26 in LPS-induced pleurisy (as discussed) were associated with decreased cellular levels of Mcl-1 suggesting that the down-regulation of this protein contributes to the resolution proprieties of these agents (Sousa et al., 2010; Vago et al., 2012). Importantly, Wardle et al. (2011) have recently shown in vitro that decreased levels of Mcl-1 may be the signal for apoptosis leading to caspase activity in neutrophils. Altogether, the findings mentioned above provide strong evidence for a role of Bcl-2 members in driving inflammatory persistence or resolution of inflammation in vivo, suggesting that these molecules could potentially be pharmacological targets for novel anti-inflammatory therapies.

2.3.6. Cyclin-dependent-kinases (CDK)

CDK inhibitor drugs (CDKi) are a class of potential therapeutic agents that have shown much potential as novel anti-inflammatory and pro-resolution drugs (Rossi et al., 2006; Duffin et al., 2009; Koedel et al., 2009; Leitch et al., 2010; Alessandri et al., 2011; Leitch et al., 2012). CDKs are protein kinases that bind to cyclin partners to mediate phosphorylation reactions within cells. CDKs have been associated with transcription, neural functions and apoptosis (Knockaert et al., 2002; Alarcón et al., 2009; Koedel et al., 2009; Lange et al., 2009; Leitch et al., 2009; Menn et al., 2010; Wang et al., 2012). CDKi

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insert into the ATP-binding pocket of CDKs resulting inhibition of CDK activity (Leitch et al., 2009). CDKi are under clinical trials for oesophageal, lung, prostate and non-small-cell lung cancers (Senderowicz, 2003).

Rossi et al. (2006) have established that the CDKi, R-roscovitine, can induce human neutrophil apoptosis in a time-, concentration- and caspase-dependent manner and override delayed neutrophil apoptosis induced by pro-survival factors such as GM-CSF, LPS and GCs (Leitch et al., 2010). Importantly, it was reported that R-roscovitine enhanced the resolution of established neutrophil-dependent inflammation in vivo using several models of inflammation, including carrageenan-induced pleurisy, arthritis and bleomycin-induced lung injury (Rossi et al., 2006). Further investigating the molecular mechanisms associated with the resolution-inducing effects of CDKi, we have demonstrated recently that the phosphorylation of RNA polymerase II (RNA pol II) by CDKs 7 and 9 is inhibited by R-roscovitine and that specific effects on neutrophil transcriptional capacity are responsible for neutrophil apoptosis (Leitch et al., 2012). Specifically, RNA pol II is present in neutrophils and is phosphorylated by CDKs 7 and 9 in order to enhance transcriptional capacity. When neutrophils were incubated with the R-roscovitine there was a significant time-dependent loss of RNApolII phosphorylation suggesting that CDKi down-regulates gene transcription. Moreover, LPS treatment increased neutrophil CDK7 gene transcription and this process was down-regulated by R-roscovitine. Importantly, R-roscovitine drove neutrophil apoptosis by Mcl-1 down-regulation whereas other pro-survival Bcl-2 homologs were unaffected at gene expression level. Confirming the in vitro findings, a CDK7/9 specific pharmacological agent (DRB) and R-roscovitine drove resolution of inflammation in a bleomycin-induced lung injury model. A similar important role for CDK9 has been demonstrated by Wang et al. (2012).

R-roscovitine also drives rapid human eosinophil apoptosis in a caspase-dependent fashion by loss of mitochondrial membrane potential as well as down-regulation of the survival protein Mcl-1 (Duffin et al., 2009; Alessandri et al., 2011; Farahi et al., 2011). AT7519 is another CDKi which has an attractive biological profile including water solubility (Squires et al., 2009; Santo et al., 2010; Squires et al., 2010), which is also currently in clinical trials as an anti-cancer therapy (Mahadevan et al., 2011). Recently, we have shown that AT7519 and R-roscovitine were able to induce apoptosis of human eosinophils in a concentration-dependent manner (Alessandri et al., 2011). However, AT7519 appeared to be roughly 50 times more potent than R-roscovitine. Having shown that AT7519 promoted direct eosinophil apoptosis in vitro we evaluated the ability of this agent to resolve eosinophil-dominant inflammation in vivo. By utilizing a murine model of allergic pleurisy induced by OVA, we have found that systemic administration of AT7519 at the peak of the inflammatory process (24 h) dramatically reduced the number of total leukocytes, eosinophils and mononuclear cells in the pleural cavity at 48 h after challenge, consistent with enhanced resolution of established eosinophilic inflammation. Moreover, the pro-resolution action of AT7519 was due to its ability to drive eosinophil apoptosis prior to subsequent clearance of apoptotic cells by macrophages, as administration of the caspase inhibitor zVAD-fmk prevented AT7519-induced resolution of inflammation. This highlights the importance of phagocytic clearance of inflammatory cells to the resolution process.

The effect of the CDKi, R-roscovitine, has also been investigated in a zebrafish model of sterile inflammation following tail transection (Loynes et al., 2010). This in vivo model enables the monitoring and tracking of all stages of the inflammatory process at the single organism level. Renshaw et al. (2006) have shown that the natural resolution of inflammation occurring between 6 and 24 h post tail transection was significantly reduced by treatment with zVD-fmk whereas treatment with R-roscovitine enhanced the resolution. Moreover, Koedel et al (2009) have shown that R-roscovitine, in combination with antibiotic therapy, enhanced resolution of experimental pneumococcal meningitis in mice by promoting neutrophil apoptosis.

Recently, we have reported that CDK inhibition overrides neutrophil survival mediated by a major gram positive bacterial cell wall component, lipoteichoic acid (Tait et al., 2012) and in agreement with our own data (unpublished) it has also been reported that R-Roscovitine-driven neutrophil apoptosis reduces lung inflammation in vivo induced by lipoteichoic acid and *Streptococcus pneumoniae* (Hoogendijk et al., 2012a) as well as lung damage induced by mechanical ventilation (Hoogendijk et al., 2012b).

Taken together these findings suggest CDK as a promising pharmacological target for treatment of inflammatory conditions. Further studies using novel inhibitors that are more selective will help clarify the mechanisms underlying the effects on transcription activity and apoptosis induction. This knowledge will be useful for the development of more apoptosis-oriented therapeutics and hopefully minimize any deleterious side effects.

3. Concluding remarks

Timely resolution of inflammation is essential to maintain tissue health after stimuli that cause tissue dysfunction or damage. Resolution of inflammation is an active process which involves key events including leukocyte apoptosis, recognition and phagocytosis of dying cells (efferocytosis). There are now several studies clearly showing that induction of resolution is possible in vivo and may be useful to control inflammatory responses. Therefore, it is possible that induction of resolution may be used to develop new therapies to treat chronic inflammatory diseases.

Herein, we have reviewed the role of several mediators, receptors and signaling pathways which have been shown to modulate the inflammation in vivo interfering in crucial events for the resolution of inflammation. For many of these mediators and pathways, however, formal demonstration of their role in the resolution of inflammation in vivo is lacking. It is crucial that we are able to differentiate mediators or pathways which are necessary for the resolution process from those necessary for the onset of inflammation. Knowledge of the exact role of these mediators will help in defining the therapeutic potential in blocking or mimicking their activity.

Another important point to be made is that the capacity to induce apoptosis and efferocytosis in vitro will not necessarily translate into capacity to induce resolution in vivo. The in vivo scenario is certainly much more complicated than an isolated cell exposed to high concentrations of mediators without relevant matrices. Conversely, there are several examples of molecules which induce resolution in vivo but not necessarily via direct effects on apoptosis or efferocytosis in vitro. Therefore, there is much need for in vivo experimentation in the field and the forthcoming years will define roles and potential mechanisms of action of molecular pathways in vivo.

Another challenge in this promising and exciting field is to translate the current findings to human clinical trials in order to determine the real usefulness of resolution-based strategies to treat patients over current therapies. Indeed, new avenues have been opened in this area with α-MSH analogs and resolvins. α-MSH analogs are being evaluated for post-surgical kidney injury after major thoracic surgery and resolvins are being studied in the context of dry eye syndrome (www.clinicaltrial.gov). These interesting studies open new avenues for tests in other pro-resolution scenarios.

Additionally to the aforementioned points and equally important is the belief that an ideal approach to treat chronic inflammatory disease aims to inhibit persistent inflammation and restore tissue function. Current pharmacological strategies are based upon inhibition of endogenous factors that participate in normal physiology and therefore, they may trigger imbalance of the homeostasis and undesirable side effects. For instance, glucocorticoids may induce osteoporosis and NSAIDs can trigger gastric ulceration. As regards to resolution-based therapies, it is potentially concerning whether side effects may include inappropriately early termination of inflammation thus preventing control and

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elimination of the inciting insult. However, as resolution-based strategies mimic the actions of mediators which are crucial for the natural response towards successful termination of inflammation this may help limit side effects. In addition resolution based strategies may also enhance innate immune responses to bacterial infections by enhancing the ability of macrophages to recognize and phagocytose those agents.

Finally, how will molecules which induce resolution of inflammation interact with molecules which prevent induction of inflammation in the clinical setting? Will they potentiate the activity of one another and will this not increase the risk of immunosuppression? Will drugs which induce resolution of inflammation be better used to induce remission of disease followed by drugs which then prevent flares (productive phase)? Is this a valid therapeutic promise? Before all of this is tested, we do need to take into the clinic molecules which are proven to be pro-resolving in vivo in pre-clinical models. The years to come will hopefully see exceptional developments in the field.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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