HHS Public Access

Author manuscript

Apoptosis. Author manuscript; available in PMC 2018 February 05.

Published in final edited form as:

Apoptosis. 2015 February ; 20(2): 224–234. doi:10.1007/s10495-014-1072-2.

Determinants of eosinophil survival and apoptotic cell death

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Abstract

Eosinophils (Eos) are potent inflammatory cells and abundantly present in the sputum and lung of patients with allergic asthma. During both transit to and residence in the lung, Eos contact prosurvival cytokines, particularly IL3, IL5 and GM-CSF, that attenuate cell death. Cytokine signaling modulates the expression and function of a number of intracellular pro- and anti-apoptotic molecules. Both intrinsic mitochondrial and extrinsic receptor-mediated pathways are affected. This article discusses the fundamental role of the extracellular and intracellular molecules that initiate and control survival decisions by human Eos and highlights the role of the *cis-trans* isomerase, Pin1 in controlling these processes.

Introduction

Proinflammatory cell infiltration of the bronchial airway, mucosa and wall in response to allergen is pathogneumonic of all forms of asthma (1). While all lineages are present, Eos typically comprise >50%, or the vast majority of the airway inflammatory infiltrate despite being a minor (1–3%), and rapidly turning over constituent of circulating white cell populations. After stimulation by IL-3, IL-5 and/or GM-CSF in the bone marrow, Eos differentiate from more primitive precursors and enter the circulation. Of these, IL-5 shows the greatest specificity for the Eos lineage that, not surprisingly, has led to monoclonal antibody (Mepolizumab) mediated therapeutic approaches targeting this cytokine. After maturation, Eos exit the bone marrow and enter the peripheral blood circulation in response to signals from chemokines (eotaxin-1 and eotixain-2). In the absence of allergic stimuli, Eos numbers in the peripheral blood are stable with balanced production and apoptotic cell death. Under nonpathologic conditions, approximately 33% of Eos turnover daily leading to a functional survival of ~3 days. Therefore, under basal conditions, Eos are programmed to die with replacement via production, making these cells a truly physiologic model to study the mechanisms and control of apoptosis. However, during infection, particularly with helminthes or allergen exposure in the allergic or cancer evolution Eos enter affected tissues such as lung, gastrointestinal tract, thymus, uterus, mammary gland, and some tumors (2, 3). Tissue entry is unidirectional with eosinophilic inflammation lasting for days to months as the cells resist apoptosis through the encounter with prosurvival signals induced by IL-3, IL-5, GM-CSF and other agonists. These signals can be maintained for weeks with attendant tissue accumulation of Eos. While there, they can present antigen, enhance inflammation and fibrosis by the release of cytokines and chemokines, remodel extracellular matrix (ECM) via metalloproteinases as well as other critical functions. Eventually, with the attenuation of exogenous stimuli and reductions in prosurvival agonists, default and

inducible proapoptotic signals predominate, leading to the death of tissue-based eosinophils (4).

In asthmatics, Eos are prominent in the sputum, airways, airway mucosa and wall. They are potent secretors of a wide array of cytotoxic granule proteins, cytokines and lipid mediators that contribute to the initiation and maintenance of the allergic inflammatory response. The persistent local inflammation arises from continued cell infiltration and/or failure/attenuation of the mechanisms normally responsible for resolution and restoration of tissue homeostasis. Apoptosis and subsequent clearance of tissue Eos by autologous macrophage and non-professional resident cells (e.g. dendritic cells, smooth muscle cells, small airway epithelial cells and lung fibroblasts) are an important mechanism to resolve local inflammation (5) and are clearly attenuated in asthma. IL-3, IL-5 and GM-CSF are key pro-survival cytokines that are derived from paracrine (inflammatory (e.g. T cells and macrophages) and epithelial cells in the lung) and autocrine (Eos) sources. In vitro, these cytokines prolong Eos survival for several weeks and do so at very low (pg) concentrations. Although the prosurvival effects of these cytokines on human Eos have been known for more than 30 years, their mechanisms of action and role in tissue eosinophilia has been unclear until recently.

Countering the action of GM-CSF and IL-3 are both exogenous (anti-inflammatory glucocorticoids, cytokine receptor blockers, anti-cytokine antibodies and bronchodilator theophylline) and endogenous (TNF-α and FasL) prodeath agonists. Glucocorticoids (GC; e.g. dexamethasone) can cause a striking reduction in eosinophil numbers in vivo (6) and remains a mainstay of anti-inflammatory therapy in asthma. Although the precise mechanism of its action remains to be determined, GCs are likely to exert their effects on Eos by both accelerating apoptosis and engulfment by lung macrophages (7), and by inhibiting the production of survival-enhancing cytokines. Alternative approaches targeting Eos have included anti-IL-5 antibodies (Mepoluzimab) or the GM-CSF receptor analogue E21R (8). Both manipulations induced the in vitro death of Eos obtained from normal or asthmatic patients (8), consistent with the notion that either GM-CSF or IL-5 can enhance Eos survival. Conversely, ligation of the death receptor Fas with monoclonal antibodies or with Fas ligand induces eosinophil apoptosis (9), which was enhanced in the presence of the Th1 cytokines IFN-γ and TNF-α, whereas the Th2 cytokines IL-3, IL-5 or GM-CSF inhibit this effect.

The intracellular pathways important in eosinophil apoptotic decisions include Lyn, Jak2, FADD, PI-3-K/Akt, Raf1 and MAPKs (9, 10). The role for the tyrosine kinase Lyn was demonstrated in Fas receptor-mediated apoptosis (11), and was sensitive to antioxidants (12), suggesting the levels of intracellular ROS species may also play a role. As in many cells, the Bcl-2 family of proteins are involved as regulators and downstream effectors of Eos apoptosis. These include Bcl-xL, Bax, Bid and Bcl-Xs. The relative levels of these proteins, location and molecular interactions are crucial in determining whether a cell will survive or become apoptotic.

The current review focuses on the proteins and signaling that have been experimentally associated with modulation of survival and/or apoptosis of terminally differentiated Eos.

Pro- and anti-survival cytokines

In active asthma, Eos accumulate and persist in the parenchyma, airways and airway wall. The accumulation reflects both pulmonary emigration as well as enhanced intrapulmonary survival. Eos persistence in the lung is determined by the presence or absence of survival factors. IL-3, IL-5, IL-13, and GM-CSF significanly prolong Eos survival beyond the 3-day life-span seen under non-pathologic conditions (13,14). Eos express these cytokines after cell activation and while the amounts are low (fg-pg), they are more than adequate to induce survival. Presumably the continuous autocrine release and signaling overcome the low concentrations. Other cytokine sources include T cells and neutrophils. Resident parenchyma including mast cells (15) and IL-1β-primed airway smooth muscle cells also elaborate GM-CSF (16), which in turn enhances eosinophil survival. GM-CSF or IL-5 signaling up-regulate Bcl-xL expression and inhibit caspase activation by suppressing the function of proapoptotic Bax.

Evignola et al (17) demonstrated that asthma severity correlated with GM-CSF production and pulmonary eosinophilia. These data provide evidence that prosurvival cytokine levels and the ensuing pulmonary eosinophilia are an important component driving asthma. Similarly, transgenic mice that overexpress IL-5 developed pulmonary eosinophilia with reduced Eos apoptosis (18). IL-13 overexpression led to pulmonary eosinophilia, airway hyper-responsiveness (AHR), increased mucus production as well as subepithelial, airway fibrosis (20a). The fibrotic response reflects excessive TGF-β production by activated Eos and other inflammatory cells and the initiation of signaling in fibroblasts, myofibroblasts and airway smooth muscle cells with the generation of ECM. Conversely, withdrawal or antagonism of these cytokines leads to the induction of Eos apoptosis and lessening of pulmonary eosinophilia. Administration of mepoluzimab, an anti-IL-5 antibody that has been employed clinically, led to reductions in airway eosinophils, TGF-β levels and the expression of the ECM components (20). Similarly, IL-13 blockade via the administration of a soluble form of the IL-13R chain, reversed airway hyper-reactivity (AHR) and mucus production (20a). Therefore, pharmacologic intervention aimed at modulating Eos apoptosis can have therapeutic effects on both acute and chronic features of asthma.

Other cytokines that support Eos survival include Leptin (21), TNF-α (22), IL-33 (23), and IFN-γ (24). Leptin regulates the function of the PI-3-K/AKT, PI-3-K/PKC and Erk MAPK pathways, all of which (25) induce the expression of pro-survival genes through activation of NF-κB. TNF-α along with fibronectin supports Eos survival likely through activation of integrins, NF-κB and Erk, that collectively increase the expression and stability of GM-CSF mRNA (22, 27). Recent studies (23, 28) showed that IL-33, which is produced by mucosal cells, is a potent survival cytokine for Eos. The IL-33 receptor (ST2) is expressed by Eos but not neutrophils. ST2-mediated enhancement of Eos survival is regulated by activation of the NF-κB, p38 MAPK and ERK MAPK pathways (30). Experimental mice challenged with allergen and injected with IL-33 display profound mucosal eosinophilia with associated pathologic changes while a single intranasal administration of soluble ST2 (Ad-sST2-Fc) before OVA challenge prevented eosinophil accumulation and reduced BAL cytokine (IL-4, IL-5 and IL-13) levels. Targeting IL-33/ST2 signaling may have therapeutic potential for the treatment of allergic pulmonary diseases. The role of IFN-γ in supporting Eos survival has

been demonstrated by several independent studies (31-34). IFN-γ prolongs Eos survival through both autocrine and paracrine induction of IL-3. IL-3 signaling through Jak2, maintains the level of Mcl-1 and prevents caspase 3 and 8 activation.

TGF-β, an anti-inflammatory mediator, can antagonize IL-5 and GM-CSF signaling and accelerate Eos cell death (35, 36). TGF-β levels are increased in the BAL of patients with allergic asthma and correlate well with airway and parenchymal remodeling (37). Pulmonary Eos are exposed to both autocrine and paracrine sources of TGF-β, that triggers canonical Smad dependent pathway and induce apoptotic cell death (35, 36, 38, 40). However, it remains unclear whether Smad or non-Smad signaling contributes to cell death and how TGF-β antagonizes Eos survival in the context of IL-5 or GM-CSF. We demonstrated that TGF- β antagonized IL-5 pro-survival signaling by inhibiting Akt phosphorylation leading to calpain activation (42). Blockade of calpain activation delayed spontaneous as well as TGF-β facilitated Eos apoptosis. Calpain activation was preceded by loss of calpastatin, its endogenous inhibitor. Calpains are highly conserved, non-lysosomal cysteine proteases that are selectively activated in response to calcium flux. Calpain inhibitors reduced spontaneous and Fas receptor-mediated death of neutrophil (43), while calpastatin-knock-down accelerated neutrophil apoptosis (44). Therefore, the calpastatin-calpain system is one of the control points in apoptotic decisions by Eos during the resolution of inflammation when TGF- β expression often increases.

Death-related receptors and ligands

Eos apoptosis is also regulated by extracellular ligands in addition to survival cytokines. So-called death factors induce apoptosis by engagement of cognate "death receptors" of the TNF-α receptor superfamily. Resting Eos express both the Fas receptor (CD95, APO-1) and Fas ligand (45). Eos obtained by bronchoalveolar lavage of sensitized mice after aerosol allergen challenge expressed Fas antigen and were sensitive to Fas-induced apoptosis (46). Moreover, inhalation of anti-Fas monoclonal antibody after pulmonary antigen challenge resulted in a marked reduction in the number of airway Eos (46). While corticosteroids have direct apoptotic effects, they also induce the expression of Fas and Fas ligand on Eos in the lung (48). Interestingly, airway epithelia showed increased anti-apoptotic Bcl-2 expression. Thus, steroid treatment directly and indirectly induces eosinophil apoptosis as well as enhanced, anti-apoptotic Bcl-2 expression by epithelium during recovery. Combined administration with steroid and Fas may thus be another option for the treatment steroid resistant, severe asthma.

Recently, a mechanism for the induction of apoptosis by Siglec-8 (sialic acid-binding immunoglobulin-like lectin 8) activation has been identified (49, 50). Siglec-8 is a single pass transmembrane inhibitory receptor found at high level on the surface of human and mouse Eos, basophils and mast cells. Siglec-8 binds to sialic acids in glycoproteins and glycolipids. The ligands are expressed by lung epithelium and enhanced during allergic inflammation and by cytokines such as IL-13. Antibody engagement or crosslinking of Siglec-8 on blood Eos resulted in mitochondria-dependent apoptosis and activation of caspase 8 and 3 despite in the presence of IL-5 and GM-CSF. The molecular mechanism underlying such process remains to be further elucidated.

Intracellular pro- and anti-apoptotic molecules

Members of the Bcl-2 family are important regulators of apoptosis in many cellular systems. Freshly isolated eosinophils express anti-apoptotic members of the Bcl-2 family such as Mcl-1, A1 and Bcl-xL, and pro-apoptotic Bax and Bid (10, 11). Among these, only Bcl-xL protein level is enhanced by IL-5 and GM-CSF whereas Mcl-1 protein was consistently detected in both normal and allergic subjects and unchanged after cytokine signaling (53). IL-5 treatment can induce modest expression of Bcl-2 mRNA; however, its protein level is variable and is likely to be dependent upon the state of maturation and/or activation of the cells (54-56). Thus, Bcl-2 likely plays a minor role in delaying apoptosis in Eos. The pro-apoptotic Bax molecule is expressed at high levels in Eos (10, 11) irrespective of activation status. Apoptotic stimulation triggers translocation of cytosolic Bax into the outer membrane of mitochondria where it forms pores, allowing the release of pro-apoptotic factors such as cytochrome c and the extramitochondrial assembly of the apoptosome. However, cytokine-mediated prosurvival signaling does not appear to involve reduced levels of Bax protein in Eos. As Bcl-xL opposes the activity of Bax, an increased Bcl-xL/Bax ratio and reduced activation status of Bax likely contribute to delayed eosinophil apoptosis in allergic and other eosinophilic diseases.

A number of kinases have been reported to regulate Eos migration, activation, cytokine production and survival signaling. Jak-STAT, Erk MAPK and PI3K/Akt are well known molecules that are activated by IL-5 and GM-CSF and all have been implicated in Eos development, activation and survival (10,11). Activation of Jak-STAT by IL5 or GM-CSF induces transcription of the gene encoding prosurvival protein Bcl-2, Bcl-xL, Mcl-1 and Pim-1 (10, 57). Activation of Erk is required for GM-CSF mRNA stability and cytokine production as well as GM-CSF and IL-5 receptor signaling to support Eos survival. For example, Eos transduced with TAT-MEK1(E), which constitutively activates Erk, showed increased GM-CSF mRNA stability, cytokine production and cell survival. This was not seen after transduction with TAT-MKK3b(E), which activates p38 (59). Further analysis identified that Erk was critical for the association of ARE binding proteins (YB-1, hnRNPC, AUF1 and HuR) with GM-CSF mRNA as well as the inactivation of Bax (60, 61). The role of PI3K/Akt in Eos migration and survival has been documented in several studies both in vivo and in vitro. Systemic treatment with PI3K inhibitors (Wortmannin and LY294002) before antigen challenge prevented Eos recruitment to the lung that was associated with decreased IL-5 production, Akt phosphorylation, and Eos release from bone marrow (62). Similarly, intratracheal administration of LY294002 significantly inhibited allergen-induced increases in Eos counts, and IL-5, IL-13, and eotaxin levels in BAL fluid (63). Western blot analysis of whole lung lysates revealed that LY294002 markedly attenuated Akt serine phosphorylation, a direct downstream substrate of PI-3-K. Using human peripheral blood Eos, we showed that IL-5 induces serine phosphorylation of Akt and Erk without affecting p38 and JNK. Antagonism of PI-3-K and Akt with LY294002 or Aktl attenuated IL-5-induced Eos survival (42), demonstrating the importance of this signaling cascade. Further studies are required to confirm these results using alternative approaches to avoid potential off-target effects of chemical inhibitors (64). The responsible PI-3-K isoform remains unclear but based on western blotting, PI3K-γ is moderately expressed in freshly purified

human blood Eos while others (PI-3-Kα and α are nearly undetectable (unpublished data). In sum, PI-3-K/Akt activation appears to be an essential pathway responsible for Eos migration and survival.

Other mediators

Bacterial and viral infections often precede asthma exacerbations in both children and adults. Direct activation of pulmonary Eos by microbes or microbe-derived molecules may represent an underlying mechanism. In support, recent studies (65, 66) showed that Eos constitutively express a panel of pathogen-associated molecular pattern (PAMPs) receptors that includes TLR1-10, NOD1, NOD2, Dectin1, RIG-1 and RAGE. These results suggest that Eos participate in innate immunity and may be activated by these receptors. Both mRNA or proteins for all TLRs except TLR8 have been detected in Eos, although at varying levels. Among these, TLR-7 is the only receptor that has been found to be fully functional in all studies (66a–66j). The expression of TLR-8 was up-regulated by IFN-γ but not by IL-4 or IL-5. Functional studies showed that R-848 and R-837 (structurally related to R-848), the ligands for TLR-7/8 and TLR-7, respectively, could activate the adhesion system (ICAM-1, CD18, ICAM-3 and L-selectin), enhance CCL5-induced migration, induce the release of IL-1β, IL-6, IL-8, GRO-α and superoxides, and prolong Eos survival by activating the NF-κB, PI-3-K, p38 MAPK and ERK MAPK signaling pathways (66b; 66k, 65). Moreover, several groups have demonstrated that CpG (ligand for TLR-9) can prolong Eos survival, facilitate migration, and increase the release of IL-8 and EDN (66; 66f; 66i; 66b). This response was enhanced in vitro after IL-5 priming of Eos, and the in vivo response was also higher in patients with allergic rhinitis compared with non-allergic normal controls. In support of this, a study showed that bacterial DNA delays Eos apoptosis in a TLR-9-dependent fashion by activating PI-3-K and NF-κB signaling cascades (66i). Taken together, these observations suggest that TLR7/8/9 system represents a unique mechanism of eosinophil activation and survival during viral and bacterial infection, linking microbial infection with the exacerbation of allergic inflammation.

Pin1 interacts with key pro- and anti-apoptotic proteins

Bcl-2, Bcl-x, and Mcl-1 are well characterized anti-apoptotic homologues which block Bax and Bid. Caspase 8 and Caspase 3 are effectors of apoptotic cascade activation. Caspase 8 acts upstream and can be directly activated during Fas ligation through physical association with the cytoplasmic portion of the Fas receptor via the FADD adapter molecule. Caspase 3 acts downstream and cleaves substrates critical to cell viability, as well as other caspases leading to their activation (amplification). IL-5 blocks spontaneous caspase activation and interferes with directed caspase activation after Fas ligation.

Increasing evidence suggests that Pin1 is an important regulator of apoptosis in neurons, tumors and immune cells. Pin1 **is** a peptidyl-prolyl isomerase (PPIase), and related to the cyclophilin and FKBP families ( ). Unlike these enzymes whose isomerase targets are X-Pro (where X = any amino acid), Pin1 has unique specificity for pSer-Pro or pThr-Pro dipeptide motifs (66m). Isomerization is bidirectional with cis to trans or trans to cis conversions but is approximately 1000× slower towards unphosphorylated Ser-Pro or Thr-Pro targets. As such,

if dephosphorylation occurs post-isomerization, the ‘new’ conformation (either cis or trans) is effectively stabilized. Pin1 sites are often targeted by the so-called proline directed kinases (MAPK, PKC, CDK, GSK, Akt etc). Thus, Pin1 can be seen as a critical molecule whose actions are required for secondary (and functional) signaling during phosphorylation driven cascades.

Not surprisingly, Pin1 is highly conserved from yeast to man and plays a role in plants ( ). Structurally, Pin1 is bipartite with a 40 amino acid N-terminal, WW domain and a C-terminal isomerase domain. The WW domain binds to pSer/pThr-Pro motifs while the catalytic domain is responsible for substrate isomerization. Pin1 mediated isomerization has profound effects on target protein folding, phosphorylation, protein-protein/RNA/DNA interactions, protein stability, and subcellular localization (66n–66p), thereby participating in a variety of cellular processes including cell cycle progression, apoptosis, innate and acquired immunity and cell senescence. Pin1 accelerates neuronal apoptosis by enhancing the expression and function of the proapoptotic Bcl-2 family member BimEL. However, in Alzheimer’s disease, depletion of nuclear Pin1 accelerates neuronal cell death through excessive phosphorylation of cis tau (66l) and the production of β-amyloid ( ). In tumor cells, Pin1 is commonly overexpressed and its levels correlate with tumor stage and grade in a variety of epithelial neoplasms of the breast, lung and GI track ( ). At a molecular level, Pin1 interacted with and regulated the apoptotic function of p73 in tumor cells lacking p53. These results suggest Pin1 coordinates the p53 family members’ activation pathway and is involved in controlling the accumulation and apoptotic function of p53 in cells exposed to genotoxic stress. Indeed, after DNA damage, Pin1 interacted with and altered the conformation of p53 in a phosphorylation (Ser33, Thr81 and Ser315) dependent manner, leading to its enhanced DNA-binding and trans-activation activity. Recently, we demonstrated that Pin1 interacts with Bax, FADD, Pim1, caspase-8 and cIAP2 in activated Eos (61, 92). Blockade of Pin1 antagonized IL-5 and GM-CSF-induced survival signaling and induced Eos death predominantly via Bax activation. Thus, Pin1 has complex and multiple functions in the apoptosis of dividing as well as quiescent, terminally differentiated cells such as Eos by interacting with and modulating the function of key pro- and anti-apoptotic molecules.

Bcl-2 belongings to a family of anti-apoptotic proteins that includes Bcl-xL, Bcl-w, Mcl-1 and A-1. These proteins function primarily at or immediately upstream or downstream of mitochondria to prevent apoptosis. They are highly expressed, along with proapoptotic Bax in neurons, tumors and neutrophils. Peripheral blood human Eos selectively express high level of Bax, Mcl-1 and A-1, while Bcl-2 and Bcl-xL are barely detectable even after IL-5 or GM-CSF stimulation. Thus, Eos employ less complex apoptotic signaling pathways and fewer effectors/regulators than many other cell types. Pin1 modulates apoptotic signaling through interactions with phosphorylated Bcl-2 in tumor cells arrested at G2-M phase by microtuble-targeting drugs (68) and control of Bcl-2 phosphorylation (67–69). Mutation studies suggested that Pin1 binds to Bcl-2 at Cdc2-phosphorylated Ser70-Pro71 and Ser87-Pro88, which are associated with inactivation of Bcl-2. Interestingly, phosphorylation did not alter the ability of Bcl-2 to homodimerize, or hterodimerize with Bax, BAG1 or other Bcl-2-binding proteins, but promoted translocation of the phospho-Bcl-2 to the nucleus where it binds Pin1. The Ser70-Pro71 and Ser87-Pro88 sites are part of a proline-rich loop

and involved in Bcl-2 auto-repression. As potential Pin1 sites also exist in Bcl-xL, Pin1 may play a role in determining the conformation and fate of proapoptotic, phosphorylated forms of both Bcl-2 and Bcl-xL, modulating intrinsic death signaling in activated Eos and in drug-treated tumors.

Pin1 is required for GM-CSF and IL-5 receptor signaling and prevents Bax activation

GM-CSF, IL-3 and IL-5 receptors (CD116, CD123 and CD125, respectively) consist of unique alpha and common beta subunits (70). IN Eos, engagement of the beta subunit triggers survival through the Syk-Lyn and Jak2-STAT1/5 pathways. The role of the alpha subunit in signal propagation remains murky but IL-5R-alpha is known to associate with Lyn irrespective of ligand engagement. The Syk-Lyn pathway predominantly activates Ras-Raf1-ERK-1/2 signaling, while Jak2-STAT1/5 induces the expression of Bcl-xL, which interacts with and inhibits members of the proapoptotic Bcl-2 homology 3 domain-only (BH3) family (Bad, Bid and Bim) and prevent downstream release of cytochrome c from mitochondria (10, 11). However, Eos express undetectable amounts of Bcl-2 and very low levels of Bcl-xL, even after treatment with prosurvival cytokines. Other anti-apoptotic genes that can be induced by both cascades include CIS1, SLP-76, Pim-1, DSP-5 and CD24 (71). Activation of the Ras-Raf1-Erk-1/2 cascade inhibits caspase activation by preventing the mitochondrial translocation and activation of Bax. Bax and Mcl-1 are highly expressed in resting Eos and largely unaffected by IL-5 or GM-CSF (10, 11, 75). These data suggest that the proapoptotic activity of BH3 family members must be attenuated by prosurvival cytokines such as IL-5 or GM-CSF. Possible options include conformational modification, blockade of activation or cleavage and/or prevention of mitochondrial translocation. Normally, Bax mainly resides in the cytosol as an inactive, monomeric protein. In response to apoptotic cues, Bax undergoes conformational change at both its N- and C-termini, cleavage, translocation to the mitochondrial outer membrane, and multimerization. Oligomerized Bax porates the mitochondrial outer membrane, leading to the release of cytochrome c from the mitochondrial inter-membrane space to the cytosol, where it binds to Apaf-1 and coordinates the formation of the Apaf-1/Caspase-9 apoptosome. In Eos, Bax is strongly expressed and its expression is unaffected by pro-survival IL-5 and GM-CSF (10, 11). However, pro-survival signaling triggers Bax phosphorylation at Thr167 by the threonine-serine kinase Erk1/2 MAPK (61). Bax is phosphorylated at three sites (Ser163-Tyr164, Thr167-Pro168 and Ser184-Lys185), which confers distinct cellular phenotypes. Whereas phosphorylation of Ser163 induces cell death in neurons by promoting the translocation of Bax to mitochondria, phosphorylation of Ser184 reverses this phenotype (77). Ser184-phosphorylated Bax can form heterodimers with the antiapoptotic molecules Mcl-1, Bcl-xL and A1 and thereby prevent neutrophil death (77). JNK- and p38-mediated phosphorylation of Thr167 accelerated apoptosis of human tumor cells (78), whereas Erk1/2 protected melanoma cells from apoptosis by inhibiting the release of Smac from mitochondria (79). Phosphorylation at Thr167-Pro168 in Eos enabled Bax-Pin1 interactions at this site. Once bound, Pin1 isomerized Bax, and prevented exposure of the proapoptotic N-terminus as assessed by 6A7 antibody staining. Conversely, blockade or knockdown of Pin1 accelerated N-terminal exposure, mitochondiral translocalization of Bax, and subsequent activation of

caspase-9 and caspase-3 with cell death. Thus, depending on the cell type and signaling, different MAPKs can modify Bax generally and Thr167 specifically with disparate apoptotic endpoints. Erk1/2 mediated Thr167-Pro168 phosphorylation is required for Pin1 to prevent Bax activation in long-lived Eos.

In addition to Pin1, several Bax partners have been previously identified and include Ku70, humanin, and clusterin. Ku70 interacted with the amino terminus of Bax (80) whereas humanin bound to the carboxyl terminus (81). Both molecules antagonized the activation of Bax. However, loss of Ku70 or humanin failed to induce translocation of Bax to mitochondria or apoptosis in the absence of appropriate stimuli. Clusterin interacted with mitochondria-associated Bax, which prevented the formation of oligomers and release of cytochrome c. Whether these anti-Bax molecules are expressed in or alter the activity of Bax in Eos is unknown. Several Pin1 sites are, however, present in these molecules suggesting Pin1 may interact with and/or modulate their potential interactions with Bax.

Pin1 mediates extrinsic death pathways through FADD

Like other cells, Eos apoptosis can be initiated by the ligation of CD95L (Fas ligand) to the membrane-bound APO-1/CD95 (Fas) receptor. This triggers the formation of the death induced signaling complex (DISC) that consists of Fas, FasL, Fas-associated death domain protein (FADD) and procaspase 8. FADD activates caspase 8, leading to either mitochondria-independent (extrinsic) or mitochondrial-dependent (intrinsic) death of Eos. In intrinsic pathway, activated caspase 8 cleaves Bcl-2 interacting protein (Bid) which then translocates to the mitochondria causing the release of cytochrome c into the cytoplasm. In the extrinsic pathway, activated caspase 8 directly cleaves procaspases 3 and 7, activating the effector caspases that culminate in DNA fragmentation and cell death.

FasL is produced and secreted by mast cells, activated T and B cells and macrophages in the inflamed lung. Fas ligand expression has the potential to contribute to the resolution of eosinophilic inflammation. Indeed, inhalation of anti-Fas monoclonal antibody after the induction of asthma resulted in a marked reduction in the number of airways Eos (89). Consistent with these results, airway Eos obtained from allergen sensitized and challenged mice expressed Fas and were sensitive to FasL-induced apoptosis. These data suggest that there are adequate levels of FasL expressed during acute episodes of asthma to overcome the prosurvival actions of GM-CSF and IL-5 and eventually cause Eos apoptosis. This is a potential mechanism for the eventual eradication of eosinophilic inflammation. Mechanistically, Fas induces the phosphorylation of FADD at Ser194 (in human) or Ser191 (in mouse cells) (90, 91). However, it remains unclear how this modification facilitates FADD mediated activation of caspase 8 or leads to FADD's accumulation in the nucleus. Recently, we demonstrated that Pin1 participates in Fas-induced Eos apoptosis (92). In Eos activated by IL-5/GM-CSF, Pin1 bound to FADD at P-Ser194-Pro195 which blocked nuclear translocation, and proapoptotic signaling. These data indicate that Pin1 can attenuate Eos death initiated through both the intrinsic as well as extrinsic pathways in the presence of prosurvival signaling. In animal models of asthma, blockade of Pin1 in reduced the numbers of BAL Eos, induced caspase 3 activation and apoptosis. These data collectively show that

Pin1 could be a novel target for anti-Eos therapeutics geared at reducing allergic airway inflammation.

Pin1 is required for GM-CSF expression

Clearly, Eos accumulation in the lung is determined not only by the rate of cell recruitment from the marrow but also by the longevity of inflammatory cells. The latter is driven by IL-3, IL-5, IL-13 and GM-CSF and the absence of mitigators such as FasL that leads to apoptosis. For example, normally long-lived Eos found in bronchoalveolar lavage (BAL) fluid after allergen challenge rapidly die in the presence of anti-GM-CSF. Asthma severity correlates well with GM-CSF production, and was associated with the presence of activated eosinophils (95). This evidence suggests that GM-CSF signaling leading to the inhibition of apoptosis is an important component to maintain eosinophilic inflammation in asthma. GM-CSF is produced by activated cells such T cells, neutrophils as well as Eos that constitute the majority of the inflammatory cells in the lung during exacerbations of asthma. Other resident cells including mast cells (96) and IL-1β-primed airway smooth muscle cells also elaborate GM-CSF (97). Sub-picogram amounts of GM-CSF are adequate to prolong the in vitro survival of Eos, suggesting similar requirements in vivo.

The expression of GM-CSF is regulated at the post-transcriptional level through 3′ untranslated region AU-rich elements (AREs). AREs are found in many cytokine and proto-oncogene mRNAs and are responsible for the rapid decay seen for these mRNAs in resting cells (98). The effects of AREs are modified by various ARE-binding proteins (ARE-BP) that can either promote or prevent the decay of these mRNAs. Eos and T cells express a number of ARE-binding proteins including AUF1, hnRNP C, YB-1 and HuR (60). AUF1 has four isoforms of 37, 40, 42 and 44 kD (p37, p40, p42 and p44, respectively), all of which are expressed in Eos. Interestingly, different isoforms have been implicated as stabilizers or destabilizers of ARE-containing mRNAs. This may reflect the cell types used for the analysis as well as the common paradigm of expressing them individually rather than collectively. However, it remains unclear how these ARE-binding proteins/isoforms are regulated, ultimately affecting the accumulation of the target mRNA and cytokine secretion.

Recently, we showed that Pin1 was an essential component of the ribonucleoprotein complex responsible for stabilization of GM-CSF mRNA in activated Eos and T cells (60, 99). Pin1 was highly expressed in Eos and its isomerase activity was significantly increased by cell activation. Under these conditions, Pin1 was bound to and modulated the mRNA binding capacity of all four AUF1 isoforms, preventing interactions with GM-CSF mRNA. The functional outcome of these events was an increase in the stability of GM-CSF mRNA and enhanced GM-CSF secretion. Conversely, Pin1 inhibition prevented GM-CSF mRNA stabilization or cytokine secretion. Knock-down of p37 AUF1 also stabilized GM-CSF mRNA whereas overexpression of all four isoforms induced GM-CSF mRNA-AUF1 interactions, and more rapid decay. Again, cytokine secretion was inversely related to mRNA half-life. These observations indicate that Pin1 is a key mediator of GM-CSF production by Eos.

Because AUF1 controls the decay of many ARE containing mRNAs, Pin1 likely plays a broader role in the expression of additional cytokines. Dysregulation of Pin1 expression or its isomerase activity is thus likely relevant for many pathologies where aberrant cytokine production contributes to the disease. For example, macrophage accumulation with severe tissue damage and inflammatory mediator release, autoimmune gastritis, myeloproliferative syndromes, or eosinophilia all occur in the context of GM-CSF overexpression (100-103). A variety of epithelial tumors (breast, lung, colon) overexpress Pin1 and its expression is a independent negative predictor of patient survival. Pin1 likely plays a role in bone disease through its regulation of parathyroid hormone (PTH) mRNA stability (104, 105).

From a therapeutic perspective, it seems unlikely Pin1 blockade could be specific enough to selectively treat disease. While Pin1 clearly has functions in all cells of the body, the unique physiology and anatomy of the lung and gut provides a means of organ specific drug delivery. For example, aerosols have been used for decades to deliver corticosteroids to asthmatic lungs with minimal systemic effects. Secondly, Eos seem to be particularly sensitive to Pin1 blockade as compared to other inflammatory cells. For example, sensitized and allergen challenged rats who received the Pin1 inhibitor juglone, showed 25% the airway Eos counts of untreated controls, without alteration in the counts of other inflammatory cells (lymphocytes, neutrophils and macrophages) (106). BAL cell expression of GM-CSF and IL-5 mRNAs were significantly decreased after Pin1 inhibition, whereas IL-4 and eotaxin were unchanged. Moreover, bone marrow differential counts showed no significant change in Eos numbers after Pin1 blockade. These data suggest that the attenuation in pulmonary Eos after Pin1 blockade is likely due to increased cell death, not because of reduced Eos recruitment to the lung and impaired Eos generation in the bone. These results further suggest that anti-Pin1 therapeutics could be effective in allergic diseases of the lung.

Conclusion and future perspectives

The body of knowledge regarding the mechanisms controlling eosinophil inflammation in allergic and parasitic diseases of the gut and lung has increased steadily. The process is highly dependent on the balance between cell recruitment and programmed cell death. Dysregulation of either limb can cause substantial pathology. Under normal circumstances, the eventual loss of allergic drivers reduces the expression of prosurvival cytokines, making Eos susceptible to default apoptosis. Cell death can also be initiated, admittedly with less intensity, after Fas activation, despite the ongoing presence of GM-CSF or IL-5. It is likely Fas signaling is one of the principle means by which eosinophilia is attenuated and normal lung physiology restored. Pin1 participates in the regulation of both pro-survival as well as pro-death signaling, making it an attractive therapeutic target. Critical issues to be resolved are how to deliver specific anti-Pin1 therapeutics into selective locations and avoid deleterious side effects. At present, there are no Pin1 inhibitors with sufficiently low IC50 to be used clinically. Thus a concerted drug discovery process is both warranted and needed and could lead to novel approaches for the treatment of allergic inflammation and cancer.

Acknowledgments

This study was supported by P01 HL088594 to JSM.

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