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Eosinophils, Pin1 and the Response to Respiratory Viral Infection and Allergic Stimuli

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ABSTRACT: Eosinophils (Eos) are prominent inflammatory cells found in the sputum, airways, and airway walls during and after exacerbations of allergic asthma. These cells are potent secretors of a wide array of cytotoxic granule proteins, cytokines, and lipid mediators involved in the initiation and maintenance of the Th2-type inflammatory reaction. Even though respiratory viral and bacterial infections are known risk factors contributing to severity and prognosis, the induction of anti-microbial Th1 type responses can offset allergic Th2 responses. Emerging evidence suggests that the prolyl isomerase Pin1 plays important roles in both Th1 and Th2 immunity in the lung and especially during allergic disease. In this review, we summarize recent findings related to the role of Pin1 in the regulation of Eos differentiation, survival, migration, cytokine expression, and antiviral immunity in asthma.

KEY WORDS: eosinophils, asthma, virus, allergy, Pin1, TLR7

ABBREVIATIONS: Pin1, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; **Eos**, eosinophils; **BAL**, bronchoalveolar lavage; **UPR**, unfolded protein response; **TLR**, toll-like receptor; PPIase, peptidyl-prolyl isomerase; **OVA**, ovalbumin; **KO**, knockout; **WT**, wild type; **ERK**, extracellular signal-regulated kinase; **MAPK**, mitogen-activated protein kinase; **PKC**, protein kinase C; **Akt/PKB**, protein kinase B; **GSK**, glycogen synthase kinase; **EBI2**, Epstein-Barr virus-induced gene 2; **MBP**, major basic protein; **EDN**, eosinophil derived endotoxin; **BM**, bone marrow; **ARE**, AU-rich element; **EPX**, eosinophil peroxidase

I. INTRODUCTION

The allergic variant is the most common type of asthma, found in approximately 60% of patients. It is characterized by high allergen-specific IgE, elevated Eos counts (sputum and lung), and Th2-predominant airway inflammation.[1] These patients show variably steroid-sensitive lung obstruction, undergo frequent exacerbations, and if these conditions are chronic, these patients are at greater risk for loss of lung function. Exacerbations, especially in children, are often associated with single-stranded (ss) RNA virus pulmonary infection.[2] Viral infection triggers innate immunity involving the toll-like receptor (TLR) family. ssRNA is sensed by TLR7, which activates Th1 antiviral responses including the local release of IFN-α/β from monocytes, epithelia, and Eos, promoting viral clearance.[3] In humans but not mice, TLR8 can also bind ssRNA and initiate antiviral immunity.[4] If infection includes double-stranded RNA viruses, TLR3 can become activated while TLR9 is triggered by unmethylated viral DNA.[5] However, whether dsRNA/DNA of ssRNA viruses generated during the viral life cycle are sensed by TLR3/9 *in vivo* remains unclear, although double-stranded nucleic acids can induce IFNs *in vitro*. Nevertheless, robust Th1 cytokine induction after viral infection is highly associated with protection from exacerbation, whereas Th2 cytokines are correlated with increased disease severity.[6] The balance between inflammation and infection, along with other risk factors (e.g., genetic predisposition, allergic sensitization and bronchial anatomy), likely determines the pathologic trajectory of asthma.

Systemic responses to allergen exposure include enhanced Eos differentiation in the bone marrow (BM). Eos differentiate from hematopoietic stem cells (HSC) under the control of multiple cytokines, particularly IL-5, which induces terminal differentiation of Eos progenitors (EoP) from common myeloid

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progenitors (CMP) and stimulates the exit of mature cells from the BM.[7] Eos typically reside within the BM and peripheral barrier tissues, including the gut and skin. In response to specific stimuli (inflammatory or parasitic), Eos are expanded and recruited to specific sites by the cooperative action of eotaxin and IL-5. Eos differentiation is also dependent on the coordinated expression of additional cytokines and regulatory proteins. These include Xbp1, which regulates the unfolded protein response (UPR) and is absolutely required for the differentiation and maturation of EoP into Eos.[8] Xbp1 KO mice lack detectable, circulating Eos.[8] Humans with loss-of-function mutations in the IL-33 gene have reduced peripheral blood Eos counts and show reduced incidence of asthma.[9] KO of Eos-specific granule proteins MBP1 and EPX reduced eosinophilopoiesis[10] and attenuated major hallmarks of asthma pathophysiology, including airway epithelial damage, hyperreactivity, and remodeling with smooth muscle hyperplasia and subepithelial fibrosis after allergen sensitization and challenge. Taken together, these and other data establish the centrality of Eos to asthma pathology. In addition, a variety of chemokines (RANTES, MIP-1α, and MCP-3),[11] cytokines (TNF-α, TGF-β and IFN-γ),[12,13] hormones (glucocorticoids and leptin),[14,15] and lipids[16] influence and regulate eosinophil migration, function, and survival in inflamed tissues.

Like other cell lineages, Eos must evoke a variety of intracellular changes to respond to changing environmental cues. These necessarily include outside-in, phosphorylation-dependent signaling, usually initiated by contact with cytokines, antibodies, or infectious agents. One critical downstream pathway involves protein phosphorylation by so-called proline-directed kinases, including MAPK, PKC, Akt, and GSK. Such posttranslational modifications often are targeted to Ser-Pro or Thr-Pro dipeptide motifs. A growing body of evidence suggests that these phosphosites are recognized by Pin1, a ubiquitously expressed *cis-trans* peptidyl-prolyl isomerase (PPIase). Unlike other prolyl isomerases, such as cyclophilin and FKBP, which target X-Pro (where X = any amino acid), Pin1 uniquely interacts with and isomerizes only phosphorylated Ser-Pro or Thr-Pro motifs (pS/pT-P), altering target protein stability, function, and/or catabolism. Many studies have independently established that Pin1 is an important intermediary in the signaling that drives Eos differentiation, survival, migration, and cytokine expression as well as the responses to viral infection in asthma.[16–24] These studies have revealed how Pin1 contributes to Eos biology and pathology including asthma, and Pin1 may provide a novel target for treating eosinophilic disorders.

II. THE ROLE OF PIN1 IN EOSINOPHIL ACCUMULATION IN THE LUNG

Mouse and rat models of the acute and chronic allergic response to inhaled allergens have been used to elucidate the mechanisms by which Pin1 regulates the immunologic and inflammatory responses of Eos in asthma. In a pioneering study, juglone, which covalently binds to and at low concentrations (0.1–1 μM) irreversibly inhibits Pin1 PPIase activity with reasonable specificity,[25] was delivered I.P. in brown Norway rats. In this well-established, acute allergic airway inflammation model,[26] eosinophils typically comprise 50%–60% of lung inflammatory cells. To characterize the role of Pin1 in responses to allergen, juglone was injected (1 mg/kg) between sensitization and aerosol challenge. BAL fluid and MBP-stained sections of lung tissue from rats who received juglone showed significantly reduced (by approximately 50–78%) pulmonary Eos accumulation without alterations in other cell populations,[23] suggesting selective effects of Pin1 inhibition on Eos accumulation in the lung. Later studies demonstrated that Pin1 KO mice show similar attenuation of acute airway eosinophilic inflammation after ovalbumin (OVA) sensitization and acute challenge[20] and reduced collagen deposition in chronic challenge models.[22] Some of these phenotypes likely reflect loss of Pin1- dependent IL-33 signaling.[20] Wild-type mice given IL-33 exhibited moderate-to-severe pulmonary inflammation and abundant airway Eos, whereas germ-line, Pin1 KO mice showed few inflammatory infiltrates and significantly lower BAL Eos counts. The levels of BAL Th2 cytokines, such

as GM-CSF, IL-4, IL-5, IL-6 and IL-13, were also all significantly lower while the number of apoptotic Eos in BM increased. In WT animals, Pin1 interacted with and isomerized IRAK-M, contributing to Th2 cytokine expression and pulmonary inflammation. Pin1 blockade in purified human Eos or PBMC prevented GM-CSF production and signaling and induced Eos apoptosis, consistent with these *in vivo* data. These results suggest a broad role of Pin1 in allergic asthma that impacts Eos, lymphocyte, and dendritic cell function and contributes to allergic airway inflammation and remodeling through multiple mechanisms (Fig. 1). Currently, both germ-line and conditional KO mice are available[19,22] with the later permitting novel, cell-specific analysis of Pin1 function in allergic and other diseases.[19]

III. EFFECT OF PIN1 ON CYTOKINE EXPRESSION BY IMMUNE CELLS AND LUNG FIBROBLASTS

Pin1 was originally identified as a cell-cycle regulator that interacted with CDK2.[27,28] However, Pin1 is expressed in all cells consistent with its broad function in facilitating intracellular signaling. The highest levels are found in brain, whereas Pin1 is overexpressed in many epithelial derived human cancers (e.g., prostate and breast cancers)[29,30] with

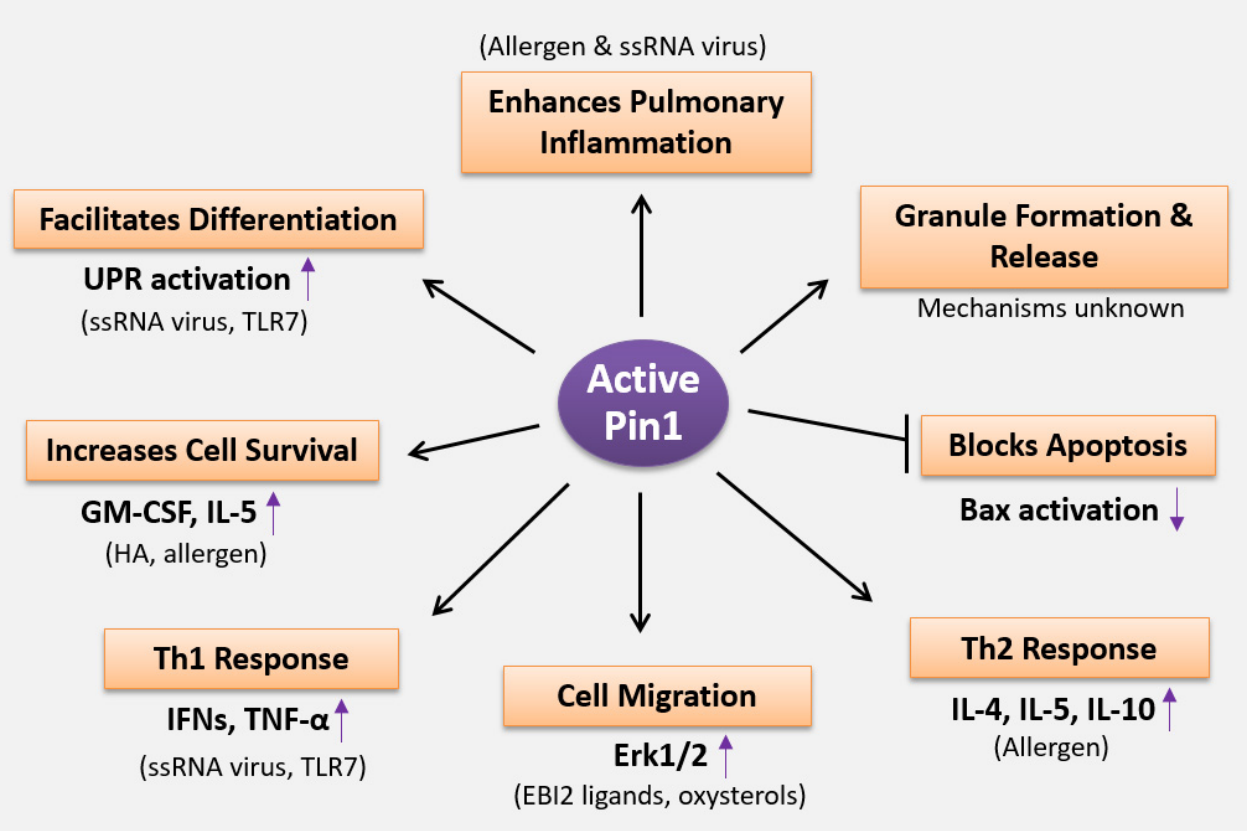

FIG. 1: Eosinophil functions affected by Pin1 in the presence and absence of viral infection. Pin1 is activated by IL-5 family cytokines or TLR-7 agonists as well as in the lung of allergic asthma. After activation, Pin1 binds to and isomerizes targeting molecules, such as AUF1, IRAK1/4 and Bax, to regulate downstream signaling (mRNA stabilization, transcription and caspase activation) and cellular functions (cytokine expression, differentiation and survival). Ultimately, Pin1 is required for eosinophilic airway inflammation as well as antiviral immune response of the cells in the context of respiratory virus infection.

prognosis inversely related to the degree of expression. The functions of Pin1 in immune cells were first identified in 2005,[24] when immunoblots showed high levels of Pin1 in Eos, T cells, and monocytes. Treatment with Pin1 inhibitors accelerated Eos apoptosis, antagonized the pro-survival effects of GM-CSF or IL-5, and reduced cytokine expression. Pin1-mediated regulation of the expression of Th1 (IFNs and TNF-α) and Th2 (IL-4, IL-5 and IL-10) cytokines (Fig. 1) was predominantly posttranscriptional, involving mRNA binding proteins (AUF1, HuR, and hnRNPC).[22,24,31] In dendritic cells, Pin1 regulated the expression of IL-6, CSF3, CXCL2, and CCL5[20] by modulating IRAK-M signaling and localization. These data suggest that Pin1 participates in asthma pathogenesis by influencing Th1 and Th2 pathways depending on cell type and initial signaling events that determine inflammatory cell migration and ultimately survival in tissue (Fig. 1).

In the lung, Pin1 regulates the expression of profibrotic cytokines including TGF-β, PAI-1, IL-1β, and CTGF by resident cells such as epithelia and fibroblasts. In doing so, collagens and other ECM proteins (MMPs and TIMPs) are up- or downregulated.[32] Primary lung fibroblasts derived from Pin1 KO mice exhibit defective Smad2/3 signaling, reducing collagen and ECM protein production in response to TGF-β compared to WT cells. Normally Pin1 interacts with receptor-regulated Smad3 and inhibitory Smad6, contributing to their subcellular localization and regulating their binding partners.[32–34] In the absence of Pin1, Smad6 showed excessive cytoplasmic localization, preventing Smad3 from binding Smad4 and nuclear translocation.[32] Again, these results are consistent with *in vivo* observations with Pin1 KO mice showing reduced lung collagens and MMPs during chronic airway inflammation.[32]

IV. PIN1 REGULATES EOS SURVIVAL THROUGH INCREASED GM-CSF PRODUCTION AND GM-CSF/IL-5 SIGNALING

Eos accumulation in the lung reflects the rates of cell entry versus egress and tissue-based cell survival. Pulmonary Eos is exposed to a rich soup of prosurvival cytokines such as IL-3, IL-5, IL-13, and GM-CSF. Cell death is accelerated both through the cessation of pro-survival and initiation of pro-apoptotic signaling induced by FasL.[35] Thus, viable Eos found in bronchoalveolar lavage (BAL) fluid after allergen challenge rapidly dies in the presence of anti-GM-CSF antibody. Asthma severity is well correlated with pulmonary GM-CSF levels, which in turn supports long-lived and activated Eos.[36] GM-CSF is produced locally by T cells, neutrophils, and Eos themselves, as well as resident parenchyma including fibroblasts and epithelial.[37, 38]

The expression of GM-CSF is regulated at multiple levels. In immune cells, posttranscriptional control is exercised through 3′ untranslated region AU-rich elements (AREs) and cognate binding proteins (ARE-BPs). Pin1 interacts with a subset of ARE-BPs,[39] including all four AUF1 isoforms that are highly expressed in Eos and T cells.[24,40,41] Different isoforms of AUF1 have been implicated in the stabilization or destabilization of ARE-containing mRNAs.[42] Once activated, Pin1 alters the binding affinity of the isoforms, effectively reducing destabilizing AUF1 isoform interactions with GM-CSF mRNA and preventing delivery of GM-CSF mRNA to the decay machinery.[24] Enhanced stability of GM-CSF mRNA was responsible for activation dependent, GM-CSF secretion by Eos. Conversely, Pin1 inhibition prevented GM-CSF mRNA stabilization, cytokine secretion, thereby accelerating Eos apoptosis.

Pin1 has also been implicated in the regulation of GM-CSF/IL5 prosurvival signaling. GM-CSF, IL-3, and IL-5 receptors (CD116, CD123, and CD125, respectively) consist of unique alpha and common beta subunits.[43] In Eos, engagement of the beta subunit triggers survival signaling through the Syk-Lyn and Jak2-STAT1/5 pathways.[44–46] The role of the alpha subunit in signal propagation remains unknown, 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, whereas Jak2-STAT1/5 induces the expression of antiapoptotic Bcl-xL, which interacts with and inhibits members of the proapoptotic Bcl-2 homology 3 domain-only (BH3)

family (Bad, Bid, and Bim) and prevents release of cytochrome c from mitochondria.[47,48] Activation of the Syk-Lyn-Ras-Raf1-Erk-1/2 cascade inhibits caspase activation by blocking Bax function. Conversely, in response to apoptotic cues, monomeric, cytosolic Bax undergoes spontaneous conformational change, cleavage, and translocation to and multimerization at the mitochondrial outer membrane, leading to cytochrome c release and cell death. Prosurvival signaling by GM-CSF/IL-5 simultaneously triggers Bax phosphorylation by Erk 1/2 at Thr167-Pro168 and Pin1 activation.[18] The phosphorylation of Thr167-Pro168 facilitates Pin1 binding to and isomerization of Bax, thus preventing spontaneous exposure of its proapoptotic N-terminus. Blockade or knockdown of Pin1 accelerated N-terminal exposure, mitochondrial translocation of Bax, and cell death.

V. PIN1 PARTICIPATES IN EOSINOPHIL MIGRATION INDUCED BY EBI2

Eos accumulation in the lung is partially a function of the rate of cell recruitment from the BM and blood. Much evidence implicates C-C chemokines in allergic inflammation. RANTES, eotaxins, MIP-1, and monocyte-chemotactic protein (MCP)-3 and -4 are significantly increased in rodent models and humans with asthma.[49,50] These chemoattractants are released by activated Th2 lymphocytes, macrophages, epithelial and mast cells, and they show variable specificity and potency on Eos. They bind to and signal through a common receptor, CCR3. Our RNA-Seq and proteomic data showed that both human[40,41] and murine Eos (unpublished data) express dozens of defined and orphan G-protein-coupled receptors (GPR), including Epstein-Barr virus-induced gene 2 (EBI2). EBI2, a seven-transmembrane GPR, is a guidance receptor to position B cells within lymph nodes and spleen,[51] and it has been associated with inflammatory autoimmune diseases.[52,53] EBI2 is constitutively expressed in blood Eos, and not surprisingly, these cells show direct migration towards EBI2 ligands (7β, 27-DHC or 25-DHC), which was enhanced by priming with physiologic doses of IL-5.[16] Migration was suppressed by inhibitors of Pin1 or extracellular signal-regulated kinases (ERK)-1 and -2. Movement necessitates cell polarization which in turn requires F-actin reorganization. Based on these and data from neurons,[54] we sought to determine whether Pin1 regulates cellular shape. Indeed, IL-5 induced actin polymerization and migration toward chemokines were dependent on Pin1, possibly through the regulation of GTPase function (Shen et al., manuscript in preparation). These data suggest that Pin1 regulates Eos function at multiple levels from vascular transmigration, cell survival, and gene expression in the inflamed lung.

EBI2 oxysterol ligands are upregulated in a variety of human inflammatory diseases, including atherosclerosis, autoimmunity, diabetes, and cancer.[55,56] The concentration of EBI2 ligands (7β, 27-DHC, 25-HC, and 27-HC) is significantly increased in the bronchoalveolar lavage fluid (BALF) 48 hours after segmental allergen challenge and strongly correlated with the increased numbers of Eos, lymphocytes, and neutrophils in the airways of human asthmatics.[16] The increased airway oxysterols are potentially a new class of Eos chemoattractants that contribute to allergic inflammation. Because the production of oxysterols can be pharmacologically manipulated, their targeting could provide additional approaches to reduce inflammation in asthma.

VI. PIN1 AND ANTIVIRAL IMMUNITY

Pin1 has been repeatedly connected to the regulation of antiviral immunity. Early studies by Saitoh et al.[57] showed that Pin1 regulates dsRNA-induced (sensed by TLR3), IRF3-dependent production of IFN-β by primary macrophages and cancer cell lines. IRF3, a ubiquitously expressed transcription factor, is responsible for the primary induction of IFN-β and critical in the establishment of innate antiviral or antibacterial immunity. Pin1 directly interacted with phosphorylated Ser339-Pro340 of IRF3 after TLR3 activation. Interestingly, overexpression of Pin1 suppressed IRF3 activation and IFN-β production suggesting the existence of a negative feedback loop to cap IFN-β production. However, this allowed the replication and persistence of vesicular stomatitis

virus (VSV) infection in host cells and may explain why Pin1-overexpressing cancer cells are more susceptible to lytic infection by VSV.[58]

Similar but more direct regulation by Pin1 was observed in the lifecycle of HIV-1. Pin1 facilitates three key steps of the HIV-1 lifecycle: uncoating, replication, and integration. First, Pin1 modulates the function of capsid protein. After binding to pSer16-Pro17, Pin1 facilitates HIV-1 uncoating.[59] Inhibition of Pin1 isomerase activity increases the number of capsid cores in the cytoplasm with impairment of viral replication and reverse transcription. Second, the conformation and stability of viral integrase is regulated by Pin1. Pin1 interacts with phosphorylated integrase at Ser57-Pro58[60] and inhibition of Pin1 impaired viral integration into the host cell genome, thus suppressing HIV-1 infection and propagation. Finally, Pin1 has been physically associated with and destabilized A3G,[61,62] a cellular protein known to restrict viral replication. HIV-1 infection increases Pin1 isomerase activity, attenuating A3G function and enhancing viral replication in infected cells.

In addition to its regulation of antiviral cytokine expression and HIV-1 replication, Pin1 has been implicated in tumorigenesis induced by hepatitis B virus (HBV),[63–65] hepatitis C virus (HCV),[66] Kaposi's sarcoma-associated herpes virus (KSHV),[67 68] human T-cell lymphotropic virus type 1 (HTLV-1),[69,70] Epstein-Barr virus (EBV),[71] and human cytomegalovirus (HCMV).[71] Typically, Pin1 enhances tumorigenesis, tumor growth, and metastasis through the regulation of the conformation, stability and/or activity of virus-encoded proteins that directly affect critical host cellular functions such as transcription, cell-cycle progression, or apoptosis. For example, Pin1 interacts with a pSer-Pro motif of hepatitis B virus X-protein (HBx) and the human T-cell leukemia virus type 1 tax oncoprotein to facilitate carcinogenesis. Pin1 overexpression increases HBx stability and downstream cellular proliferation, whereas Pin1 blockade attenuates HBx-enhanced tumor growth in nude mice. This Pin1-mediated regulation of HBx provides a previously unappreciated pathogenic mechanism for HBV-related HCC. Two independent studies[69,70] have demonstrated that Pin1 also interacts with HTLV-1 tax at pSer160-Pro161 during mitosis, leading to reduced ubiquitination, lysosomal degradation, and prolonged protein half-life. Excess tax contributed to malignant transformation by activating NF-κB. Not surprisingly, Pin1 is frequently overexpressed in hepatocellular carcinoma associated with viral infection, and Pin1 levels negatively correlate with prognosis.

VII. PIN1 REGULATION OF TLR7 SIGNALING IN EOSINOPHILS

Pin1 participates in TLR7 signaling[72] in dendritic cells and lymphocytes. Activation of TLR7 by ssRNA induces Pin1 to bind to IRAK1, leading to IRF7-dependent, IFN-α/β production. Conversely, Pin1 blockade prevented IFN production by DC in response to infection. Recently, we demonstrated that Pin1 plays an important role at multiple levels in the regulation of TLR7 signaling and antiviral immunity in Eos.[19] Pin1 was dispensable for Eos differentiation in *in vitro* BM cultures under basal, unstressed conditions. However, after ssRNA viral infection or TLR7 activation on day 8 of culture, Pin1 KO Eos showed reduced cell division and accelerated apoptotic cell death, which was evident from day 14. Similar effects were observed in TLR7-agonized WT BM cultures after treatment with Pin1 inhibitors. Accelerated cell death was exclusively triggered by TLR7 but not by agonists that engage other TLRs or by cytokines (IL-4, IL-8, IL-10, IL-12, IL-1β, TNF-α, eotaxin, or TGF-β). Cell death was entirely prevented in KO cells if reconstituted with exogenous Pin1 from day 12 onward. Interestingly, when TLR7 agonists were added to BM culture containing mature Eos (day 14 and onward), Pin1 KO had no effect on cell survival. However, the induction of IFN-α/β production after TLR7 activation remained significantly reduced in Pin1 KO compared with WT cells irrespective of cell maturity. Similar data were observed in mature human Eos, which showed reduced IFN expression after TLR7 agonists if Pin1 was inhibited.

The molecular mechanisms underlying the defect of TLR7 signaling in Pin1 KO Eos have been explored. After interacting with its ligands in endosomes, TLR7/9 is cleaved as a prelude to recruiting adaptors including MyD88, IRAK1/4, and TRAFs and

the initiation of downstream signaling.[73,74] Wild type BM Eos cultures treated on days 8–10 of culture with the synthetic TLR7 agonist R848 showed cleavage of full-length TLR7 (110 kDa) and the appearance of 60 kDa N-terminal and 53 kDa C-terminal products that were significantly reduced in Pin1 KO Eos. TLR7 processing has also been observed in human blood Eos after TLR7 activation with ligands. Immunostaining of day 12, unstimulated WT Eos cultures with an anti–C-terminal Ab showed a combination of full length and cleaved TLR7 that were predominantly endosomal.[73] R848-treated WT cells showed increased, punctate C-terminal fragment staining, consistent with cleavage and endosomal localization. Untreated KO cells exhibited similar postendosomal distribution as resting WT Eos but with reduced total TLR7. However, treatment with R848 resulted in receptor retention in the endosomal compartments.

As mentioned previously, TLR7 engagement induces myddosome formation composed of cleaved TLR7, MyD88, IRAK1, and IRAK4.[75] IRAK4 phosphorylation activates IRAK1 and causes dissociation of both IRAK1 and IRAK4 from MyD88. Phospho-IRAK1 then associates with TRAF6 before being ubiquitylated and degraded by the proteasome. Both Pin1 and IRAK4 were required for IFN production after R848.[19] Co-immunoprecipitation showed that Pin1 interacted with the cleaved C-terminal (32 kDa) but not with full-length IRAK4. Activation of TLR7 did not alter IRAK4 expression (protein or mRNA) nor the interaction of Pin1 with cleaved IRAK4. These results suggest that Pin1 regulates TLR7 processing and localization and the function of the myddosome complex. UNC93B, which has been implicated in the regulation of TLR3/7/9 transport,[76] was unaffected by Pin1 KO. Although it remains hypothetical, Pin1 might regulate the expression or activity of proteases involved in TLRs processing, particularly the acidic pH-dependent cathepsins B/K,[77] furin,[78] or AEP.[79]

VIII. PIN1 REGULATES UPR AND EOS DEATH DURING DIFFERENTIATION

Eos differentiate from hematopoietic stem cells (HSC) in bone marrow. Between days 8 and 12 of differentiation, Eos actively synthesize granule proteins, including EDN, EPX, and MBP.[80] In the lumen of the ER, newly synthesized proteins fold into their correct conformation with the assistance of the protein glycosylation machinery and dedicated chaperones. Under certain stress conditions, such as elevated synthesis of secretory proteins, the abundance of misfolded proteins exceeds the capacity of the protein folding machinery. In response to these perturbations, three sensors located in the ER membrane direct the induction of the UPR. These include inositol-requiring enzyme 1 (IRE1), double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6).[8,81] The IRE1-Xbp1 pathway is a central component of the three evolutionarily conserved UPR pathways.[82] In response to the stress, Xbp1 mRNA is spliced by IRE1, which, after translation, directs the transcription of genes that, in aggregate, suppress ER protein influx, catabolize misfolded proteins, and improve protein folding. Consistent with the importance of this response, Eos are completely eliminated in the BM of Xbp1 KO mice.[8] On the other hand, external stimuli such as viral and bacterial infections can exert ER stress and induce or modulate the UPR due to their impact on protein synthesis, replication, and host cell survival.[83] For example, hepatitis C virus, whose ssRNA genome activates TLR7, downregulates the IRE1-Xbp1 pathway to promote viral replication,[84,85] whereas rotavirus (a dsRNA virus that activates TLR3) modulates the UPR via sequestration of UPR components.[86,87] Although our understanding of the precise molecular mechanisms leading to activation of these complex ER sensors is incomplete, emerging evidence supports new inputs to this process.[88,89]

Pin1 participates in the UPR response to TLR7 signaling.[19] Analysis of immature, day-12, BM-derived Eos showed a significant reduction in UPR activation in Pin1 KO cells compared to WT after TLR7 agonists. Immunoblots revealed reductions in spliced Xbp1 (Xbp1s) amounts and ATF-6α cleavage in KO Eos, whereas PERK phosphorylation was unaffected. Pin1 inhibition at day 8–12 of differentiating WT BM Eos cultures also reduced Xbp1s expression and ATF-6α cleavage. Conversely, KO cells reconsti-

tuted with Pin1 showed restoration of Xbp1s mRNA even in the absence of IL-5, confirming that Pin1 is also required for TLR7-induced UPR induction and cell survival in immature Eos. Not surprisingly, terminally differentiated and mature Eos completely downregulated expression of Xbp1 and ATF-6α, coincident with completion of granule production. In support, RNASeq and proteomic data also showed only trace amounts of Xbp1s and ATF-6α in mature, human blood Eos.[40,90] In the absence of Xbp1, differentiating Eos displayed defective maturation and granule protein production, leading to reduced cell survival.[8] Similar to these phenotypes, Pin1 KO Eos contained reduced EDN and ECP. The transcripts of granule proteins and master Eos lineage-commitment factors GATA1 and GATA2 were all unchanged by Pin1 deficiency, suggesting that Pin1 is required for UPR-mediated, posttranslational granule protein processing and maturation in differentiating Eos and that failure of this program leads to cell death during ssRNA viral infection (Fig. 1).

IX. THE EFFECT OF PIN1 ON AIRWAY EOS IN THE CONTEXT OF TLR7 ACTIVATION

In 50%–80% of cases, exacerbations of asthma with eosinophilic inflammation are associated with viral infection.[91,92] Pulmonary viruses can induce inflammatory responses through protein kinase R[93,94] and multiple toll-like receptors.[95–98] Most respiratory viruses have single-stranded RNA genomes which predominantly activate TLR7 signaling.[2] Activation of TLR7 leads to the rapid generation of innate Th1-type immune responses, including the local release of IFN-α and IFN–β from mononuclear cells and epithelium.[3] The balance between Th2 and Th1 responses likely determines the disease trajectory. A growing body of animal data and ongoing clinical trials highlight the important role for and therapeutic potential of TLR7 in asthma.[95,99-101] The expression of TLR7 is high[102] in the lung, and interestingly, polymorphisms are associated with asthma.[103,104] However, it remains unclear how Eos themselves contribute to viral surveillance and eradication. Recently, Pin1 was implicated in the regulation of viral immunity (see above) and Eos responses to respiratory viral infection.

The roles of Pin1 and Eos in antirespiratory viral immunity were first studied in mice with the Eos-specific deletion of Pin1.[19] These mice were the offspring of conditional $Pin1^{f/f}$ mice bred to eoCre mice in which Cre recombinase was driven by the Eos-specific EPX promoter and expressed at the EoP stage.[105] To see Pin1's effect, adult Pin1 KO or WT mice were sensitized and challenged with OVA and injected with a single IT dose of R848 (a potent, cell-permeable TLR7 agonist[106]) followed by euthanasia 1 or 4 day(s) later. In the absence of R848, total BAL inflammatory cell counts were Eos predominant and similar between genotypes. R848-treated WT mice showed dose-dependent shifts in BAL inflammatory infiltrates highlighted by reductions in Eos and lymphocytes but increased neutrophils and monocytes.[19,107] However, BAL Eos counts were significantly reduced in Pin1 KO compared with WT controls 1 day after the completion of allergen challenge.[19] Eos were the only BAL inflammatory cells negatively affected by Pin1 deficiency after R848. The reduced Eos counts were also observed in the peripheral blood and, most notably, in the BM of KO mice. Similar data were obtained in mice harvested 4 days after the last OVA challenge or if R848 was administered i.v. rather than i.t. WT and KO BAL and blood Eos showed similar viability and similar expression of cell-surface IL5Rα and BAL chemokines (eotaxin, RANTES, and MCP-1). Unexpectedly, the B lymphocyte chemoattractant (CXCL13) and LPS-induced CXC chemokine, which are not predominantly produced by Eos, were decreased in KO BALF, implying an indirect effect of Pin1 KO on other cells. Similar lung histology was observed after TLR7 agonists in WT and KO mice. These data suggest that Eos reductions in KO mice are possibly due to reduced proliferation and enhanced Eos death in the BM and/or reduced blood Eos migration to the lung after TLR7 activation as described earlier. Reduced pulmonary accumulation of anti-viral Eos[108-111] and the lack of anti-viral Pin1[72] have been hypothesized to contribute to a higher viral burden in infected lung. Because KO Eos accumulate fewer granules and express reduced IFNs, these physiologic

defects were possibly responsible for exaggerated infection in Pin1 KO mice. This was tested by infecting WT and Pin1 KO mice with Sendai virus (SeV) after the last aerosol allergen challenge and measuring residual virus in BAL. As was expected, viral clearance was significantly attenuated (~8-fold) in KO mice compared with WT.[19] In the absence of R848, BAL granule contents (MBP and EDN) were equivalent in WT and KO mice. Although R848 treatment increased BAL release irrespective of genotype, EDN was significantly lower in KO mice compared to WT even after normalization by absolute Eos number. These results suggest that KO Eos contain lower granular contents and/or have reduced degranulation. The levels of BAL reactive oxygen species (ROS) post infection (normalized by absolute Eos counts or by BAL volume) were not significantly different between WT and KO. However, antiviral IFNs in the BAL were significantly reduced in KO mice treated with R848,[19] suggesting the change was due to reduced production by infiltrating Eos. This observation was supported by *in vitro* data in which pretreatment of peripheral blood Eos with Pin1 inhibitors suppressed TLR7 agonist-induced, upregulation of IFN mRNAs without affecting cell viability.[19] Similar results were observed with murine, BM-derived Pin1 KO Eos. In addition, Pin1 isomerase activity was increased by TLR7 agonists or IL-5/GM-CSF in purified Eos.[19,72] These results indicate that Pin1 is activated by and required for TLR7 signaling that culminates in a variety of antiviral response in both circulating as well as tissue-based Eos in allergic condition (Fig. 1).

X. CONCLUSIONS AND PERSPECTIVES

The *in vivo* roles of Eos in antiviral and allergic responses are emerging but the underlying mechanisms remain unclear. Although data reported from different experimental models and systems is often divergent, antiviral responses of Eos likely include the effects of granule contents, anti-viral cytokines and/or other mediators in a complex cellular environment. Nevertheless, TLR7 agonists remain promising as antiasthma therapeutics due to their effects on Eos function, infiltration and differentiation. Pin1-mediated phosphorylation signaling in concert with innate TLR7 sensing play important roles in Eos differentiation, tissue recruitment and the production of antiviral mediators in active asthma with viral infection. Several TLR7 agonists and Pin1 inhibitors are being explored as therapeutics; however, their specificity, toxicity, and modes of action *in vivo* need additional study. Given the multiple roles of Pin1 in antiviral immunity and asthma pathogenesis, the concurrent administration of TLR7 and Pin1 agonists, possibly as an aerosol directly to the lung could provide benefit for the treatment of eosinophilic allergic asthma with ongoing respiratory virus infection.

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