HHS Public Access

Author manuscript

*Trends Immunol*. Author manuscript; available in PMC 2023 January 17.

Published in final edited form as:

*Trends Immunol*. 2021 October ; 42(10): 904–919. doi:10.1016/j.it.2021.08.004.

# What's the deal with efferocytosis and asthma?

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## Abstract

Mucosal sites, such as the lung, serve as crucial, yet vulnerable barriers to environmental insults such as pathogens, allergens, and toxins. Often, these exposures induce massive infiltration and death of short-lived immune cells in the lung, and efficient clearance of these cells is important for preventing hyperinflammation and resolving immunopathology. Herein, we review recent advances in our understanding of efferocytosis – a process whereby phagocytes clear dead cells in a non-inflammatory manner. We further discuss how efferocytosis impacts the onset and severity of asthma in humans and mammalian animal models of disease. Finally, we explore how recently identified genetic perturbations or biological pathway modulations affect pathogenesis and shed light on novel therapies aimed at treating or preventing asthma.

## Efferocytosis

> 'According to most studies, people's number one fear is public speaking. Number two is death. Death is number two. Does that sound right? This means to the average person, if you go to a funeral, you're better off in the casket than doing the eulogy.' – Jerry Seinfeld

An integral component of maintaining homeostasis is the immunotolerant clearance of dead and dying cells, a process termed efferocytosis [1]. This occurs naturally on a daily basis, as billions of cells undergo genetically-programmed cell death, are sensed by tissue-resident and recruited phagocytic cells, and are engulfed and processed in a immunosilent manner [1]. Efferocytosis also promotes the return to homeostasis, once the imminent threat, such as exposure to a pathogen or allergen, is resolved [2]. Often, immune responses to such challenges cause as much tissue damage as the provoking agent itself, leaving large numbers of dying cells and cellular debris that must be removed [3]. In health, phagocytic cells sense the presence of these dying cells and facilitate their clearance without further inciting inflammation (Figure 1) [2, 4, 5]. However, it is becoming clear that failure to efficiently remove dying cells can lead to increased inflammatory responses that likely contribute to the severity of multiple diseases, including atherosclerosis, autoimmune disorders, and asthma. Improved mechanistic understanding of how efferocytosis occurs is already revealing

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exciting new molecular targets that might be therapeutically manipulated to effect changes in disease progression. Here, we review the molecular and cellular basis of efferocytosis, and discuss how failure to efficiently clear dead cells might contribute to the severity of asthma.

Efferocytosis (Latin: ‘to take to the grave’) requires active participation from both the dying cell and the phagocyte. Dying cells release molecules termed ‘find-me’ signals, to recruit phagocytic cells to sites of cellular death (Figure 2A, Key Figure). In mammals, these ‘find-me’ signals include nucleotides, such as ATP and UTP; chemokines, such as CX3CL1 (CX3C motif chemokine ligand 1; fractalkine); and lipid mediators, such as S1P (sphingosine-1-phosphate) and LPC (lysophosphatidylcholine) [6]. Sensing of these molecules through their cognate receptors on phagocytes results in their chemotaxis toward dying cells [7]. In addition, some molecules, such as S1P, have been shown to exert an additional priming effect on the phagocyte, wherein lipid sensing and engulfment receptors are upregulated for sustained efferocytosis [8].

Once phagocytes are recruited, they must distinguish between healthy cells and dying cells in need of removal (Figure 2B, Key Figure). To this end, dying cells display ‘eat-me’ signals that allow phagocytes to discriminate them from living cells [1, 3]. The best characterized ‘eat-me’ signal is phosphatidylserine (PS), a lipid located on the intracellular side of the plasma membrane of viable cells that is actively flipped extracellularly by caspase activity during apoptosis [9]. Phagocytes express a variety of receptors that specifically recognize PS, and hence discriminate between the living and the dead [6]. These receptors can be either membrane-bound, such as TIM1, TIM4, BAI1, RAGE, Stabilin1/2, and CD300f, or circulating, such as MFGE8 and GAS6/Protein S, which link PS-coated dying cells to phagocytes via interaction with αvβ5 integrin and TAM receptors (TYRO3, AXL, and MerTK), respectively [4].

Engagement of PS and other ‘eat me’ signals by specific receptors during efferocytosis triggers an immunosilent genetic program via ‘tolerate-me’ signaling pathways (Figure 2C, Key Figure) [3]. Dying cells are laden with lipids, and their uptake activates nuclear receptors, such as liver X receptors (LXRs) and peroxisome proliferator-activated receptors (PPARs), which control lipid metabolism, cholesterol efflux, and transport [10, 11]. Importantly, activation of these receptors, which heterodimerize with retinoid X receptors (RXRs), also upregulates efferocytosis receptors, such as *Mertk* and *Mfge8*, thus further promoting dead cell clearance [1]. The net result of this signaling is the efficient breakdown and shuttling of these surplus lipids [12, 13], the suppression of pro-inflammatory mediators such as IL-12 and IL-6, and the production of immunotolerant molecules such as IL-10, TGF β, and prostaglandin $PGE_2$ [5, 14].

Trafficking of engulfed cellular corpses to the lysosomal pathway plays a crucial role in preventing inflammation, as proper degradation and processing of cargo requires the activity of the lysosomal acidic environment [15]. In the absence of efficient degradation and processing, phagosomal contents can leak into the cytosol, activating cytosolic receptors such as cGAS/STING, AIM2, and ZBP1 [16]. Activation of the receptors by nucleic acids triggers a robust type I interferon (IFN) response commonly associated with autoimmunity [17]. Recent studies have identified a form of non-canonical autophagy termed LC3-

associated phagocytosis (LAP) that is pivotal for the efficient and anti-inflammatory removal of dead cells (Figure 2D) [18, 19]. In addition to engagement of toll-like receptors (TLRs) by pathogens, or Fc receptors by immune complexes, LAP can be triggered during efferocytosis via engagement of PS receptors, such as TIM4, on macrophages as well as non-professional phagocytes, such as epithelial cells [18, 20, 21]. This signaling during engulfment results in the recruitment of some, but not all, of the ATG family of proteins to the cargo-containing phagosome. This recruitment of LAP machinery, demonstrated in *Caenorhabditis elegans* and *Drosophila melanogaster* in addition to mammals, allows for efficient and proper processing of engulfed cargo [22]. Despite some molecular overlap between LAP and canonical, starvation-induced autophagy [23], LAP is molecularly distinct [24]. LAP proceeds independently of the most upstream complex of autophagy, the pre-initiation complex composed of ULK1, ATG13, and FIP200 [18, 19, 25]. LAP utilizes a Class III PI3K complex containing Beclin1, VPS34, and UVRAG and does not require ATG14 [25–27]. Proteomic analysis of the LAP-engaged phagosome revealed that Rubicon (RUBCN) forms a complex with the Class III PI3K complex and is uniquely and significantly associated with the cargo-containing phagosome [25]. During LAP, RUBCN supports the generation of the signaling molecule, PI(3)P, by VPS34, presumably via stabilization of the Class III PI3K complex, as opposed to RUBCN's role during canonical autophagy [25]. Importantly, RUBCN binds p22phox, a component of the NADPH complex NOX2, to stabilize and promote the production of reactive oxygen species (ROS) [25, 28, 29]. NOX2 activity is also positively regulated by RUBCN-mediated PI(3)P, which binds the NOX2 component p40phox and stabilizes its association with the cargo-containing phagosome [25, 29]. Both PI(3)P and ROS are required for the association and activation of downstream conjugation systems, such as the ATG5–12 conjugation system and the LC3-PE conjugation [25, 30, 31]. The net result is the decoration of the single-membrane, cargo-containing phagosome with lipidated LC3-II to form a structure termed the LAPosome. Fusion of LAPosome with the lysosome allows the intralysosomal acidic environment to properly process and degrade the engulfed cargo [25].

The absence of LAP has a dramatic effect on the immunological outcome of efferocytosis. The normally immunosilent process of dead cell clearance becomes hyperinflammatory in LAP-deficient conditions, such as in the *Rubcn*$^{-/-}$ mouse [32]. *Rubcn*-deficiency results in age-associated autoinflammation [32], increased onset and severity of Alzheimer's disease [30, 33], increased skin inflammation [34], and enhanced anti-tumor T cell immunity (LLC carcinoma mouse models) [31]. Despite these findings, it remains to be determined how LAP might impact type 2 immunopathologies, such as asthma.

## Asthma

> 'The sea was angry that day, my friends. Like an old man trying to return soup at a deli! I got about fifty feet out and then suddenly the great beast appeared before me. I tell you, he was ten stories high if he was a foot. As if sensing my presence, he let out a great bellow. I said, 'Easy, big fella!' And then, as I watched him struggling, I realized something was obstructing its breathing.' – George Costanza

Asthma is a common disease affecting approximately 26 million patients in the U.S. In addition to its profound effects on the quality of life of affected individuals, asthma exacts an enormous economic burden in the form of costs associated with physician visits, hospital stays, and lost work and school hours [35]. The prevalence of this disease has remained high, and new approaches are needed to better manage the symptoms of asthma, and presumably, to eventually prevent its onset.

It is well-established that asthma can stem from inappropriate, or maladaptive, immune responses to otherwise harmless agents in the environment. These responses in turn give rise to inflammation of the airway, excessive mucus production, and bronchial airway hyperresponsiveness (AHR). Consequently, individuals with asthma suffer from periodic episodes of dyspnea, or shortness of breath [36]. For many years, asthma was regarded as a single disease because virtually all affected individuals displayed airway inflammation and shortness of breath. However, it has long been known that not all asthmatics are responsive to glucocorticoid administration, the gold standard treatment for asthma. Indeed, it has recently become clear that despite these overlapping pathologies, or phenotypes, asthma is in fact heterogeneous in nature (Figure 3). The relatively new concept of asthma ‘endotypes’ postulates that different forms of asthma arise from perturbations of distinct molecular and cellular pathways [37]. The most common and best-characterized form of this disease is allergic asthma, characterized by eosinophilic airway inflammation, increased production of type 2 cytokines IL-4, IL-5, and IL-13, and elevated allergen-specific IgE titers [37, 38]. Fortunately, this so-called ‘type 2’ (T2) form of asthma generally responds well to treatment with inhaled or oral glucocorticoids. However, approximately half of asthmatic patients display non-eosinophilic forms of disease [39]. These patients often display neutrophilic inflammation of the airway [40] and are notoriously resistant to inhaled corticosteroids [41]. An improved understanding of the molecular and cellular basis of non-eosinophilic asthma endotypes should reveal novel pathways that might be selectively targeted to treat these forms of asthma.

While asthma is a debilitating disease, it is also associated with increased risk of other pulmonary diseases, including chronic bronchitis, emphysema, and chronic obstructive pulmonary disease (COPD) [42]. One might anticipate, therefore, that individuals with asthma might also be at increased risk of developing more severe Coronavirus disease 2019 (COVID-19), and some studies have indeed shown that. Indeed, studies have shown that severe asthmatics are at higher risk of developing more severe COVID-19 [43], or dying from it, than individuals who not presenting with severe asthma [44].

However, several other studies have failed to demonstrate such a relationship [45]. It is possible that these apparently discrepant findings arise from differences in patient groups. In support of this idea, a recent study found that the increased overall risk of asthmatics might be driven primarily by individuals with nonallergic asthma, whereas there has been no statistically significant association of more severe disease in individuals with allergic asthma [43]. This is an intriguing result, as it suggests that therapies targeting non-allergic asthma might be particularly effective in preventing COVID-19-related deaths. Additional, larger studies are needed to determine whether the risk for severe COVID-19 disease is dependent on asthma endotypes.

Given that non-eosinophilic forms of asthma are glucocorticoid-resistant and often associated with airway neutrophilia [41], it will be important to identify putative immune pathways whose perturbation might give rise to this form of inflammation. As in all forms of inflammation, the numbers of any particular leukocyte cell type are determined by the balance between recruitment of new cells to that tissue and the clearance of those cells. Chemokines such as IL-8, which promote neutrophil recruitment, are present at much higher concentrations in asthmatics than in individuals with healthy airways, and IL-8 concentrations correlate with neutrophil numbers [46]. Airway neutrophilia is also associated with the production of IL-17 by Th17 cells in both humans [47] and mouse models of asthma [48]. IL-17 indirectly recruits neutrophils by binding to its receptor IL17RA on epithelial cells, which in turn, produce neutrophil-recruiting chemokines [49]. A major source of IL-17 is Th17 cells, whose master transcription factor is RORγt. Inverse agonists of RORγt were recently shown to suppress IL-17 production, neutrophil recruitment, and AHR, in a mouse model of neutrophilic asthma in which lipopolysaccharide functioned as an adjuvant to promote immune responses to inhaled ovalbumin [50]. These experiments showed that inhibiting neutrophil recruitment could reduce asthma-like features in animals, further suggesting that augmented clearance of these cells through efferocytosis might also potentially be an effective therapeutic approach in asthma.

## Efferocytosis and Asthma

> ‘Poor little bubble boy. He’s sitting there waiting for you in his bubble, or igloo thing or whatever.’ – Elaine Benes

While defects at any point of the efferocytosis pathway can result in inflammatory pathology in any tissue, the lung is especially susceptible, as it contains an inordinate number and variety of phagocytes -- specifically alveolar macrophages (AM) and dendritic cells, with varying expression of PS receptors and other core efferocytosis machinery molecules such as *Rac1*, *Dock1*, and *Elmo1* (Figure 4A). In addition to the mere clean-up function of efferocytosis, $CD103^+$ dendritic cells play an essential role in engulfing dying cells and cross-presenting antigens to $CD8^+$ T cells in regional lymph nodes. Further, non-immune cells, such as airway epithelial cells and endothelial cells (Figure 4B), are crucial executioners of efferocytosis in the lung [51, 52]. Thus, in a murine model of asthma, efferocytotic defects in airway epithelial cells have resulted in increased disease severity [53].

The efficiency of pulmonary efferocytosis is underscored by the virtual absence of uncleared apoptotic cells in healthy lung tissue [54]. During acute inflammatory lung diseases such as pneumonia and acute respiratory distress syndrome, apoptotic cell presence remains relatively low, no greater than 2% [55]. Conversely, chronic inflammatory pulmonary pathologies such as COPD, cystic fibrosis, idiopathic pulmonary fibrosis, and asthma, often feature uncleared dead and dying cells, suggesting a crucial role for efferocytosis in the onset and severity of these diseases. The observations that apoptotic cells are increased in asthma and that patients with glucocorticoid-resistant disease have ‘M1’(inflammatory)-

skewing of monocytes and AM raises the possibility that defective efferocytosis might contribute to asthma pathogenesis in at least some forms of that disease [55, 56].

Because asthma represents a spectrum of pathologies that differ in terms of triggers and immune responses, the role that efferocytosis plays mirrors the heterogeneity of the diseases themselves. Compared with macrophages from patients with eosinophilic asthma, macrophages from non-eosinophilic asthmatics displayed less *ex vivo* efferocytosis of epithelial cells rendered apoptotic by exposure to UV light [57, 58]. These studies suggest that defective efferocytosis by AM might at least partly explain persistent airway neutrophilia in non-eosinophilic asthma. Furthermore, both bronchoalveolar lavage fluid (BALF)- and induced sputum-derived macrophages from non-eosinophilic asthmatics have exhibited defective efferocytotic capacity, suggesting that the latter procedure might represent a rapid method for testing efferocytotic activity of macrophages [57]. However, efferocytosis of neutrophils, which comprise the bulk of cellular corpses in the lungs of non-eosinophilic asthmatics, was not directly assessed in that study, nor was the extent to which efferocytosis occurred in bronchial tissue, as opposed to the airway lumen [59]. A subsequent study did not reveal a significant difference in efferocytosis in macrophages derived from monocytes of eosinophilic and non-eosinophilic donors [60], but it is possible that such differences would only be seen in lung macrophages, which might have received different cues in these two different groups of asthmatics.

Disease severity can correlate with levels of efferocytosis. Thus, AM from patients with severe asthma demonstrated significantly lower levels of dead cell clearance, both *in vivo* and *ex vivo*, than AMs isolated from lungs of healthy human controls or from patients with mild-to-moderate asthma [61]. Furthermore, when cultured *ex vivo*, AMs from severe asthmatics produced higher amounts of the proinflammatory molecule, TNF-α and lower amounts of the immunotolerant molecules $PGE_2$ and 15-hydroxyeicosataenoic acid [61]. Co-morbidities in asthmatic patients can also have a dramatic effect on efferocytotic capacity of airway cells in asthmatic patients. Obese patients with asthma, who typically have glucocorticoid-resistant disease, were also found to have reduced efferocytosis, as determined by counting sputum-derived macrophages that contained pyknotic nuclei [62]. Thus, a growing body of associative evidence now links efferocytosis to the severity of asthma.

## ‘Find-me’ signaling

‘If she can’t find me, she can’t break up with me.’ – George Costanza

Defects in ‘find-me’ signaling (see below) (Figure 2A) can contribute to asthma pathogenesis; asthmatic patients have shown upregulation of chemoattractants such as fractalkine in airway monocytes and S1P in BAL fluid [63, 64]. In line with these observations, recent studies have shown that the variants of the fractalkine receptor *CX3CR1* (rs938203, rs2669849, rs1050592, T280M, and V249I) have a positive association with the development of asthma, whereas minor alleles (rs2669849 and V249I) of *CX3CR1* confer protection [65].

Not all find-me signaling is created equally. Relative to wildtype mice, $P2Y_2^{-/-}$ mice (deficient in the purinergic receptor P2Y(2)) have shown reduced allergic airway inflammation and pathology in an OVA-alum model of asthma, due to defective chemotaxis of DCs and eosinophils in response to ATP -- a key 'find-me' signal [66]. Indeed, P2Y2R expression has been reported to be significantly increased in DCs and eosinophils from human asthmatics compared to healthy controls [66]. However, HSP70, a ubiquitous, stress-induced chaperone with ATPase activity, was found to be increased in the sputum and plasma of severe asthmatics, compared to non-asthmatics and mild asthmatics [67].

## 'Eat-me' signaling

> 'You dipped the chip. You took a bite. And you dipped again. That's like putting your whole mouth right in the dip! From now on, when you take a chip — just take one dip and end it.' — Timmy to George Costanza

Recognition and physical uptake of dying cells (Figure 2B) is also important for the regulation of airway inflammation. *AXL* is a member of the TAM (*TYRO3*, *AXL,* and *MERTK*) receptor tyrosine kinase family that recognizes PS-bound Gas6 or Protein S, and DNA methylation of AXL at birth is associated with increased risk for asthma in childhood [68]. Cleavage of AXL from the plasma membrane of airway macrophages to generate soluble AXL (sAXL) results in decreased efferocytosis, and sputum from patients with moderate-to-severe asthma contains increased sAXL relative to controls, suggesting diminished efferocytotic capacity [69]. Murine studies have been less clear. In a model of asthma in which mice were first sensitized with antigens from *Aspergillus fumigatus* and then challenged by oropharyngeal administration of live conidia from that organism, intraperitoneal delivery of monoclonal antibodies against AXL decreased plethysmograph-measured airway hyperresponsiveness, diminished concentrations of IL-4 and IL-13, and reduced numbers of mucus-secreting goblet cells, compared with controls [70]. Another group, however, demonstrated that *Axl* was exclusively expressed on murine AM, and that $Axl^{-/-}$ mice developed exacerbated lung inflammation during influenza virus infection (IAV), compared with controls [71]. Another member of the TAM family, MerTK, has also been implicated in allergic airway inflammation: *Mer*-deficient ($Mer^{KD}$) mice exhibited delayed resolution of allergic asthma and increased AHR levels and BALF protein amounts, compared to controls. Further, after intraperitoneal administration of the glucocorticoid dexamethasone, asthmatic *Mer*-deficient mice displayed defective efferocytosis of apoptotic eosinophils [72].

Milk Fat Globule-EGF factor 8 (MFGE8) is a bridging molecule that links PS-displaying dead cells to phagocytes via αvβ3–5 integrin, and studies have demonstrated that protein amounts of MFGE8 are decreased in endobronchial biopsies from human asthmatics compared to healthy patients [73]. Similarly, $Mfge8^{-/-}$ mice develop exacerbated immunopathology and AHR in experimental models of ovalbumin (OVA)-induced asthma relative to controls [73, 74].

Galectin-3 (encoded by *LGALS3*) is a β-galactoside-binding, endogenous lectin that mediates effects on immune cells intracellularly and extracellularly [75]. Upon stress or

stimulation, macrophages secrete Galectin-3, which can act as a bridging molecule between dying cells and phagocytes to promote efferocytosis [76, 77]. Studies have demonstrated that *LGALS3* expression in AM is lower in asthmatics compared with healthy controls, and that recombinant Galectin-3 improves efferocytosis *in vitro* by macrophages derived from patients with neutrophilic asthma [60]. This finding is consistent with previous studies showing that Galectin-3 amounts are particularly low in the sputum from individuals with neutrophilic asthma, and that the ratio of galectin-3 to galectin-3-binding protein is also reduced relative to controls [78]. Together, these data suggest that reduced galectin-3-mediated efferocytosis can contribute to the severity of neutrophilic asthma.

In terms of pulmonary disorders, the most well-characterized ‘eat-me’ receptor is RAGE (receptor for advanced glycation end products). Genome-wide association studies (GWAS) identified a variant (rs2070600) in *AGER* (encoding RAGE) that increases ligand binding affinity and is associated with increased pulmonary/asthmatic disease severity [79–81]. In addition, sputum samples of asthma patients contain higher expression of endogenous and soluble RAGE and correlate with disease severity [82, 83].

In addition to recognizing advanced glycation end products, RAGE binds numerous danger-associated molecular patterns (DAMPs), such as S100A8/A9 or HMGB1, which are associated with asthma [84]. Both of these ligands are elevated in the sputum of asthmatics, compared to healthy patients [83, 85]. Noteworthy, increased expression of HMGB1 correlates with increased asthma severity, immune activation, and inflammation in humans [86], while S100A8/A9 induces mast cell degranulation and IgE responses in the lung [85].

The generation of the $Ager^{-/-}$ mouse (RAGE-KO) has facilitated a better understanding of the molecular mechanisms by which RAGE mediates airway inflammation. RAGE-KO mice exhibited reduced asthma immunopathology in two models of experimental allergic airway inflammation. While IL-4 production was equivalent between RAGE-KO and controls, IL-5 and IL-13 (type 2 cytokines crucial for eosinophils and mucus production, respectively), were absent in RAGE-KO mice [87]. The alarmin IL-33 was also reduced in the lungs of RAGE-KO mice during allergic asthma, compared to controls; as a result, after induction of allergic asthma, RAGE-KO mice failed to accumulate another type 2 cell subset, group 2 innate lymphoid cells (ILC2s) in the lung, as evidenced by flow cytometry [88]. The cells in which RAGE primarily acts, however, are still being determined.

The engulfment machinery downstream of PS receptor engagement is also an important mediator of airway inflammation. Efferocytosis by bronchial epithelial cells is required to limit allergic airway inflammation and promote immunotolerant responses. Rac1 is a Rho-family GTPase that functions downstream of multiple signaling pathways to induce actin reorganization during phagocytosis [3]. Another member of Rho-family GTPases, RhoA, acts as an antagonist to Rac1 to inhibit uptake [89]. Mice harboring bronchial epithelial cells that were deficient in Rac1 (CCSP-$Cre/Rac1^{flox/flox}$ mice) failed to efficiently clear apoptotic cells in vivo, and when these mice were challenged with house dust mite (HDM), they exhibited significantly increased pulmonary inflammation, production of type 2 cytokines, and AHR, relative to controls. These CCSP-$Cre/Rac1^{flox/flox}$ mice

failed to produce IL-10 during challenge with apoptotic cells and airway inflammation, and recombinant IL-10, delivered intranasally, dampened hyperinflammation in knockout mice [53]. This study highlights the role that non-professional phagocytes such as epithelial cells, play in efferocytosis. It is now clear that both immune and non-immune cells play important roles in clearing the inevitable cellular graveyard during allergic airway inflammation, and defects in this process can exacerbate inflammation and pathology.

## ‘Tolerate-me’ signaling

> ‘I’m lactose intolerant. I have no tolerance for lactose and I won’t stand for it!’ – Jerry Seinfeld

At the heart of efferocytosis is the question of how a phagocyte handles the burden of dramatically increasing its load of lipids and cholesterol when it engulfs a dying cell. LXRα/β are isoforms of LXR nuclear receptors that mediate cellular lipid and cholesterol metabolism. Consistent with the inverse correlation between circulating cholesterol concentrations and asthma [90], *Lxrα*$^{-/-}$*β*$^{-/-}$ mice exhibit reduced AHR, type 2 cytokine production, and immunopathology in the HDM model of eosinophilic asthma, relative to controls [91]. Conversely, activation of the LXR pathway (with the LXR agonist GW3965, for example) has decreased the production of pro-inflammatory mediators such as IL-6 via LPS-stimulated human airway macrophages, and has reduced lung neutrophilia in LPS-challenged rodents [92].

The PPAR family of nuclear receptors are also crucial regulators of immunotolerance associated with efferocytosis. *Cc10-Cre/Pparg*$^{flox/flox}$ mice, which lack *Pparg* in airway epithelial cells, display heightened asthma-like responses, including increased AHR and elevated production of type 2 cytokines and alarmins [93]. Similarly, the PPARγ agonist rosiglitazone, improved lung function in mice in an OVA-induced asthma model [94] as well as in human asthmatic smokers [95]. In addition, mice with a ubiquitous *Pparα* deletion (*Pparα*$^{-/-}$ mice) phenocopy *Cc10-Cre/Pparg*$^{flox/flox}$ mice and exhibit increased allergic airway responses relative to controls [96].

Given the contrasting role of LXRs and PPARs in asthma pathogenesis, researchers have examined the role of downstream cholesterol transporter, ATP-binding cassette (ABC), subfamily A, member 1 (ABCA1) in asthma. Mice that overexpress human ABCA1 in their vascular endothelial cells (Tie2-human *Abca1* mice) are significantly protected against OVA-induced neutrophilic asthma, with reduced peribronchial inflammation, decreased titers of OVA-specific IgE, and reduced airway epithelial thickness, relative to controls [97].

How LAP and other mediators of dead cell processing affect allergic airway inflammation remains unknown. Collectively, these studies demonstrate a delicate balance in terms of efferocytosis, cholesterol metabolism, and airway inflammation, with more in-depth studies needed to explore the role that each of these molecules plays in differing types of asthma.

## Concluding Remarks (and therapeutic options)

> ‘Mother Nature’s a mad scientist, Jerry.’ – Cosmo Kramer

The discovery that the efficient clearance of dying cells plays an important role in asthma pathogenesis has opened many therapeutic avenues. Glucocorticoids, the gold standard for the treatment of asthma, have been shown to enhance efferocytosis by upregulating key efferocytotic machinery, such as *MERTK, LXRs, PPARs, and RXRs* [98]. Macrolide antibiotics, such as azithromycin, have anti-inflammatory effects and have been reported to promote efferocytosis in lung macrophages [99]. Similarly, studies have demonstrated that the macrolide antibiotic, telithromycin, can significantly reduce asthmatic symptoms and improve lung function in patients with moderate-to-severe asthma [100].

Drugs that modulate phagocytosis are also possible candidates. One such candidate is lipoxin A4, which is an endogenous lipoxygenase-derived eicosanoid mediator that promotes cytoskeletal rearrangement and phagocytosis, and has been shown to display anti-inflammatory properties [101]. Indeed, pediatric patients with severe asthma present decreased sputum concentrations of lipoxin A4, compared to those with mild-to-moderate asthma [102]. Recent studies have demonstrated that lipoxin A4 can promote efferocytosis and reduce inflammation and pathology in murine models of acute lung inflammation [103, 104]. However, targeting an axis with such wide-ranging roles in fundamental biological processes could result in unwanted side effects.

Given the connection between cholesterol and asthma pathogenesis, statins have been explored as a potential therapeutic for airway inflammation. Widely used as cholesterol-lowering agents, statins also have broad, anti-inflammatory effects and have been demonstrated to increase efferocytotic capacity *in vitro* and *ex vivo* in a mouse model of lung inflammation [105]. Statin use is also associated with reduced asthma-related hospitalization or severe events [106]. However, clinical trials exploring the efficacy of statins as a treatment for asthmatics has yielded mixed results [106], suggesting further studies are needed.

Manipulating cholesterol efflux via PPAR agonism is also a potential treatment option. Synthetic PPAR agonists such as thiazolidinediones have been shown to enhance dead cell clearance and improve resolution in a mouse model of lung inflammation [107, 108].

These agonists have also decreased immunopathology in mouse models of asthma [109, 110]. While different PPAR isoforms retain specific activity in response to pharmacological PPAR agonists, an alternative approach would be to promote the production of broadly-acting natural PPAR ligands, such as eicosanoids, polyunsaturated fatty acids (PUFAs), and endocannabinoids [109].

Recent studies have highlighted the association of autophagic processes and allergic airway inflammation. Epithelial cells and airway smooth muscle from human asthmatics express increased *BECN1* and *ATG5*, compared to non-asthmatic control tissues [111]. A SNP in *ATG5* (rs510432) was found to be associated with childhood asthma, with the minor variant allele associated with increased asthma risk. [112]. Given this association, pharmacological modulation of autophagy has been explored in murine models of asthma. Intranasal administration of chloroquine, which inhibits lysosomal fusion [113], during HDM exposure resulted in decreased inflammation, AHR, and airway remodeling relative to controls [111].

However, chloroquine acts broadly on the lysosomal network and does not distinguish between autophagy and LAP. By specifically targeting RUBCN -- which is required for LAP but not autophagy, one might preserve vital autophagy quality control mechanisms while modulating the LAP response. One such candidate is Tat-N8, a cell-penetrating, RUBCN inhibitory peptide, comprised of the HIV protein Tat, conjugated to an N-terminal 8-amino acid sequence derived from the NOX2 subunit, p22phox. Tat-N8 can robustly block the RUBCN-p22phox interaction, which is required for LAP [114]. While studies have demonstrated the ability of Tat-N8's to increase survival in a murine model of sepsis [114], this peptide (or its derivatives) have not yet been examined in the context of experimental asthma. It will be of great interest to determine whether selective targeting of LAP might represents an effective candidate therapy to treat asthma or other inflammatory diseases (see outstanding questions).

## Acknowledgments

This work was supported by NIH Intramural Research Program 1ZIAES10328601 (to JM) and ZIA ES102025-13 (to DNC).

## Glossary

**Absent in melanoma 2 (AIM2)**
Cytosolic DNA sensor that forms the AIM2 inflammasome with ASC and Caspase-1 to mediate pro-inflammatory cytokine production and pyroptosis

**Airway hyperresponsiveness**
Hallmark of asthma: defined as increased sensitivity and reactivity of the airways to normally innocuous stimuli; correlated to disease severity

**Autophagy**
Evolutionarily conserved process, classically triggered by nutrient deprivation; cellular components are sequestered into de novo autophagosomal structures and trafficked to lysosomes for recycling and degradation

**Autophagy-related genes (ATG)**
Evolutionarily conserved set of genes required for autophagy

**Cyclic GMP-AMP synthase (cGAS)**
Cytosolic DNA sensor that regulates the synthesis of cGAMP, which subsequently activates the type I IFN response via STING-mediated signaling

**Damage-associated molecular patterns (DAMPs)**
Small molecular motifs produced or released by damaged and dying cells that subsequently activate pathogen recognition receptors to stimulate innate immunity

**Efferocytosis**
clearance of dying cells; recruitment of phagocytes via 'find-me' signals, the recognition and engulfment of dying cells via 'eat-me' signaling, and an immunotolerant response via 'tolerate-me' signaling

**Fc receptors (FcRs)**
Family of surface receptors that recognize the Fc region of antibodies bound to opsonized particles, such infected cells or pathogens

**LAPosome**
Cargo-containing, LC3-decorated phagosome generated during LAP

**LC3-associated phagocytosis (LAP)**
Form of non-canonical autophagy triggered by receptor engagement during phagocytosis wherein RUBCN and components of the autophagy machinery are recruited to the cargo-containing phagosome to facilitate trafficking to the lysosomal network

**Liver X Receptor (LXR)**
Member of the nuclear receptor family of transcription factors; regulators of cholesterol, lipid, and general metabolic homeostasis

**Peroxisome proliferator-activated receptors (PPARs)**
Members (alpha, gamma, and delta (beta) of the nuclear receptor family of transcription factors; regulators of cellular differentiation, tumorigenesis, and metabolic homeostasis

**Pyknotic nuclei**
Nuclei of cells undergoing apoptosis or necrosis, wherein chromatin is characterized as dense, compact, and possibly fragmented

**Retinoid X receptor (RXR)**
Member of the steroid/thyroid hormone nuclear receptor superfamily of transcription factors; regulators of cellular differentiation, metabolism, and cell death

**Stimulator of interferon genes (STING)**
Cytosolic five-transmembrane protein activated by cGAMP to mediate the type I IFN response

**Toll-like receptors (TLRs)**
Family of membrane-bound pathogen recognition receptors that recognize and respond to PAMPs or DAMPs to mediate immune responses

**Z-DNA-binding protein 1 (ZBP1)**
cytosolic sensor of Z-DNA and Z-RNA, present in numerous viruses; promotes the production of antiviral mediators, such as IFN β

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## Highlights

- Efferocytosis is a carefully orchestrated process that requires dying cells to release and display signals to facilitate recruitment of and recognition by phagocytes.
- Efferocytosis is characterized by its immunotolerant response, and defects in the efferocytotic machinery are associated with autoimmune and inflammatory disorders in mice and humans.
- Asthma affects over 200 million people worldwide and results from inappropriate immune responses to inhaled allergens leading to airway inflammation, excessive mucus production, and bronchial airway hyperresponsiveness (AHR).
- Impaired efferocytosis has been observed in asthmatics, and defects in efferocytosis are associated with increased risk or severity of asthma.
- Therapeutics that promote efferocytosis are promising candidates for the treatment of asthma, in which certain endotypes present unmet medical needs.

### Outstanding Questions

- The lung contains an inordinate number of phagocytic cells - do professional phagocytes and non-phagocytes in the lung play distinct efferocytotic roles during asthma?
- Do different endotypes of asthma exhibit different requirements for efferocytosis? Do these differences, whether in expression or function, represent possible therapeutic targets to treat asthma?
- What role does LC3-associated phagocytosis (LAP) play in the onset and severity of asthma?
- Would PS-receptor agonists or LAP modulators be an effective therapeutic against asthma?

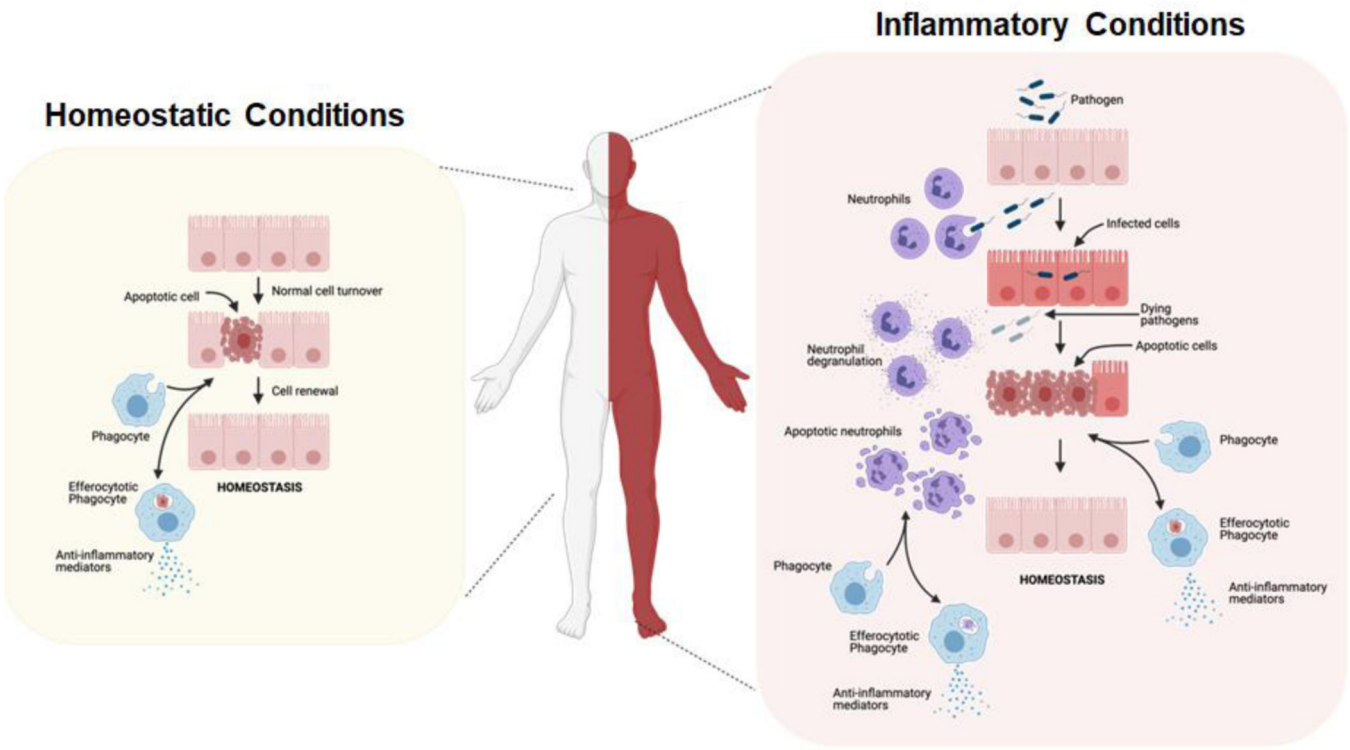



**Figure 1: Efferocytosis is required for immunotolerance during homeostasis and inflammation.**

Efferocytosis is required to maintain homeostasis during normal cellular turnover (left), as well as during inflammation in an evolutionarily conserved manner (right). During homeostasis, normal cell turnover via apoptosis is tolerated by the immunosilent clearance of cellular corpses by local and recruited phagocytes. During inflammation, innate immune cells, such as neutrophils, are recruited to sites of damage, including pathogen infection. Short-lived neutrophils, as well as infected host cells, undergo cell death, and cellular corpses are cleared by local and recruited phagocytes in a tolerant manner [1]. This figure was created using BioRender (https://biorender.com/).

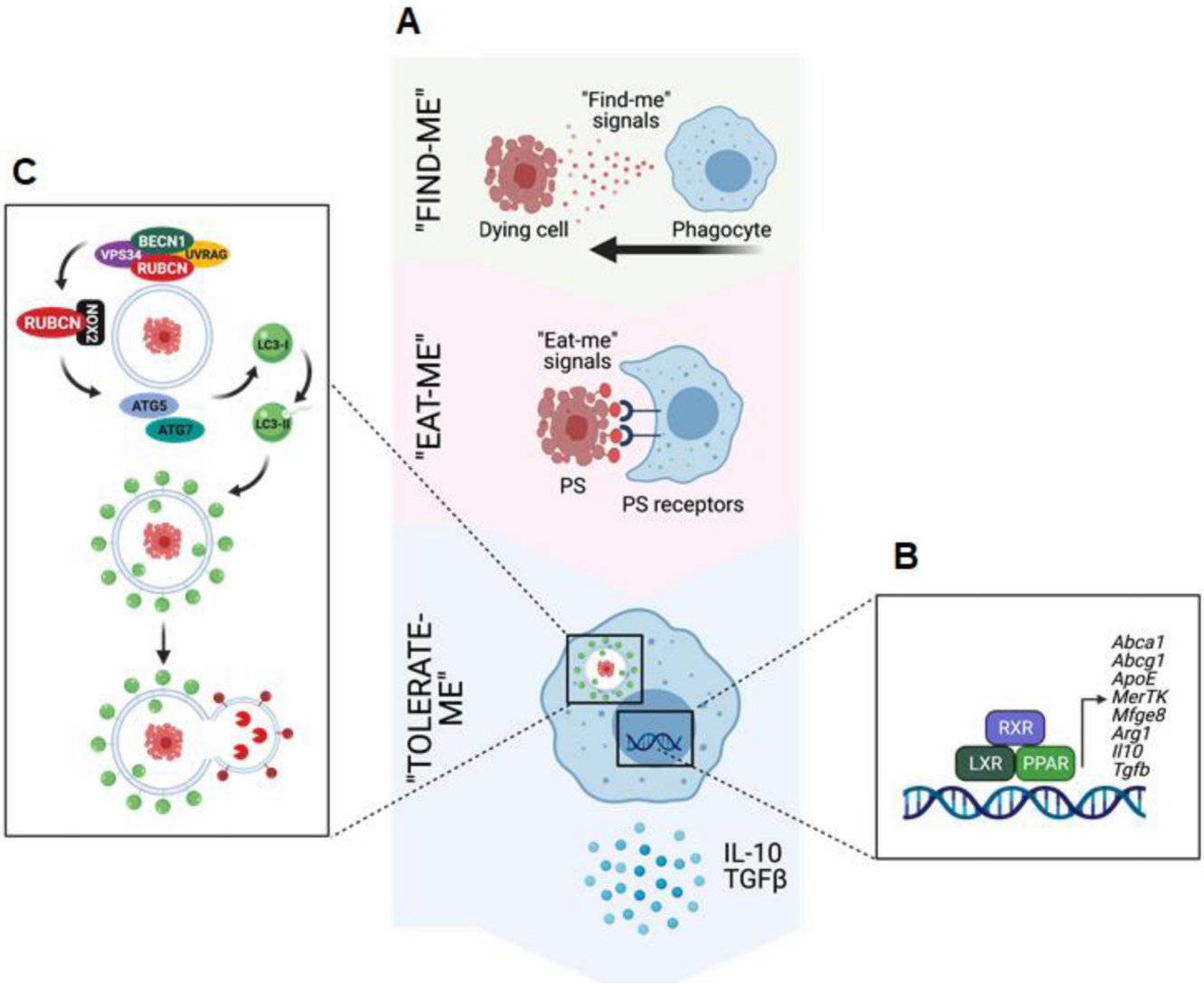



**Figure 2: Mechanisms of efferocytosis in vertebrates.**

**A**. Efferocytosis is mediated by the recruitment of phagocytes to sites of cell death via “Find-me” signals, such as ATP and CXCL3, released by dying cells. Dying cells are distinguished from viable cells via active display of “Eat-me” signals, such as phosphatidylserine (PS). Phagocytes employ receptors that recognize “Eat-me” signals, such as PS receptors, which facilitate recognition and engulfment of dying cells. Efferocytosis is ultimately an immunotolerant process, and proper trafficking and processing of engulfed dying cells initiates “Tolerate-me” signaling via activation of LXR, RXR, and PPAR pathways [4]. **B**. Activation of nuclear receptors, such as LXRs, PPARs, and RXRs, largely mediates the immunotolerant response by inducing the transcription of genes such as *Abca1, Il10,* and *Tgfb*. **C**. A form of non-canonical autophagy called LC3-associated phagocytosis (LAP) is required for immunotolerant efferocytosis. Uptake of dying cells via engagement of PS receptors triggers the recruitment of the Class III PI3K complex, containing BECN1, VPS34, UVRAG, and RUBCN, to the dead cell-containing phagosome (or LAPosome). RUBCN binds to and stabilizes the NOX2 complex to promote production of ROS, which is

required for recruitment of the downstream ATG5-ATG12 and LC3-PE conjugation systems. Decoration of the single-membraned LAPosome is required for fusion to the lysosomal network [7]. This figure was created using BioRender (https://biorender.com/).

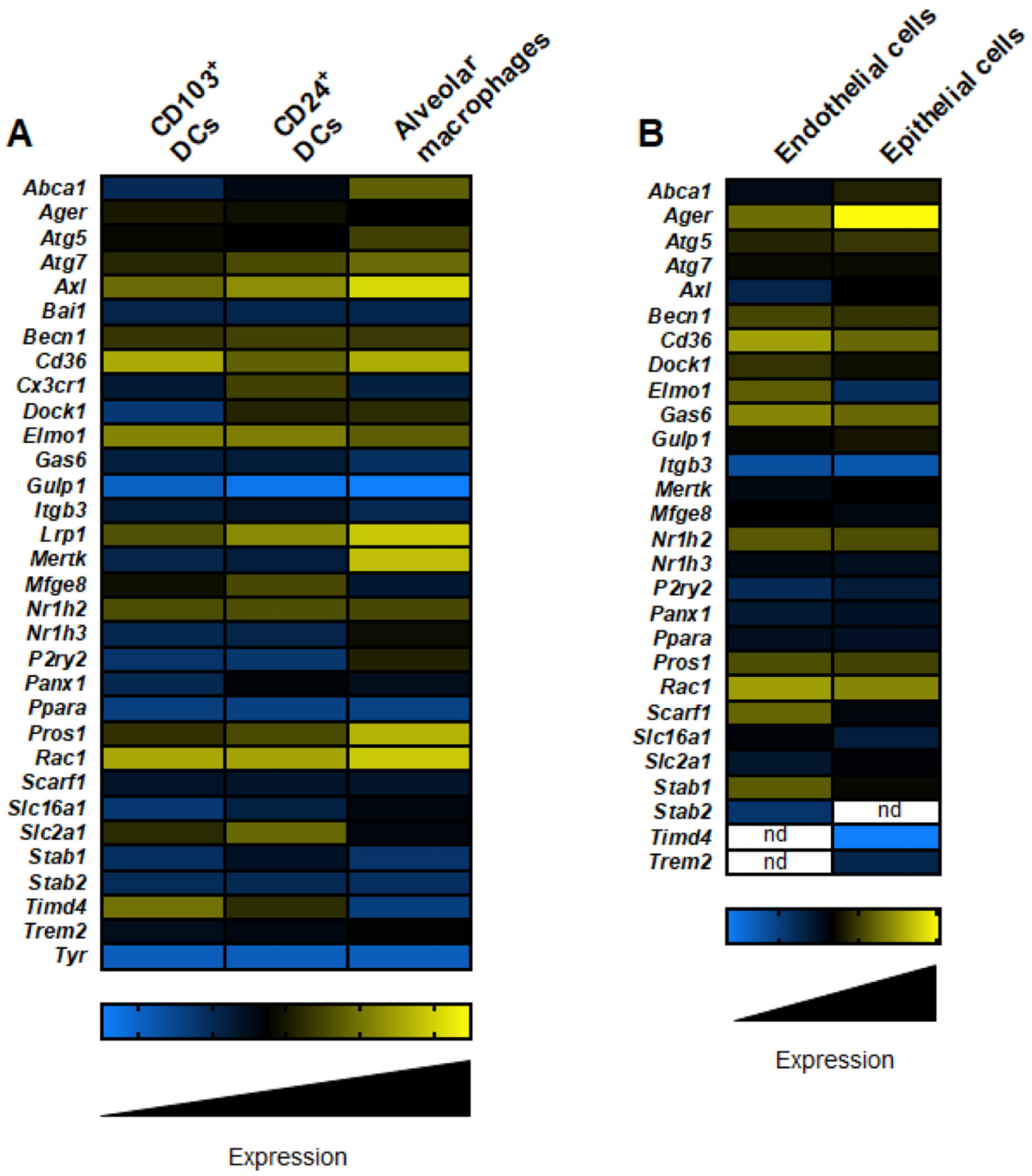



**Figure 3: Expression of efferocytotic machinery in human lung resident cells.**

The schematic depicts the relative RNA expression amounts of key efferocytotic machinery molecules in lung resident immune cells (**A**) and non-immune cells (**B**). Immune cell expression amounts were acquired from ImmGen (https://www.immgen.org/), and non-immune cell expression amounts were acquired from Human Lung Cell Atlas (https://hlca.ds.czbiohub.org/) [115]. n.d. denotes not determined. This figure was created using BioRender (https://biorender.com/).

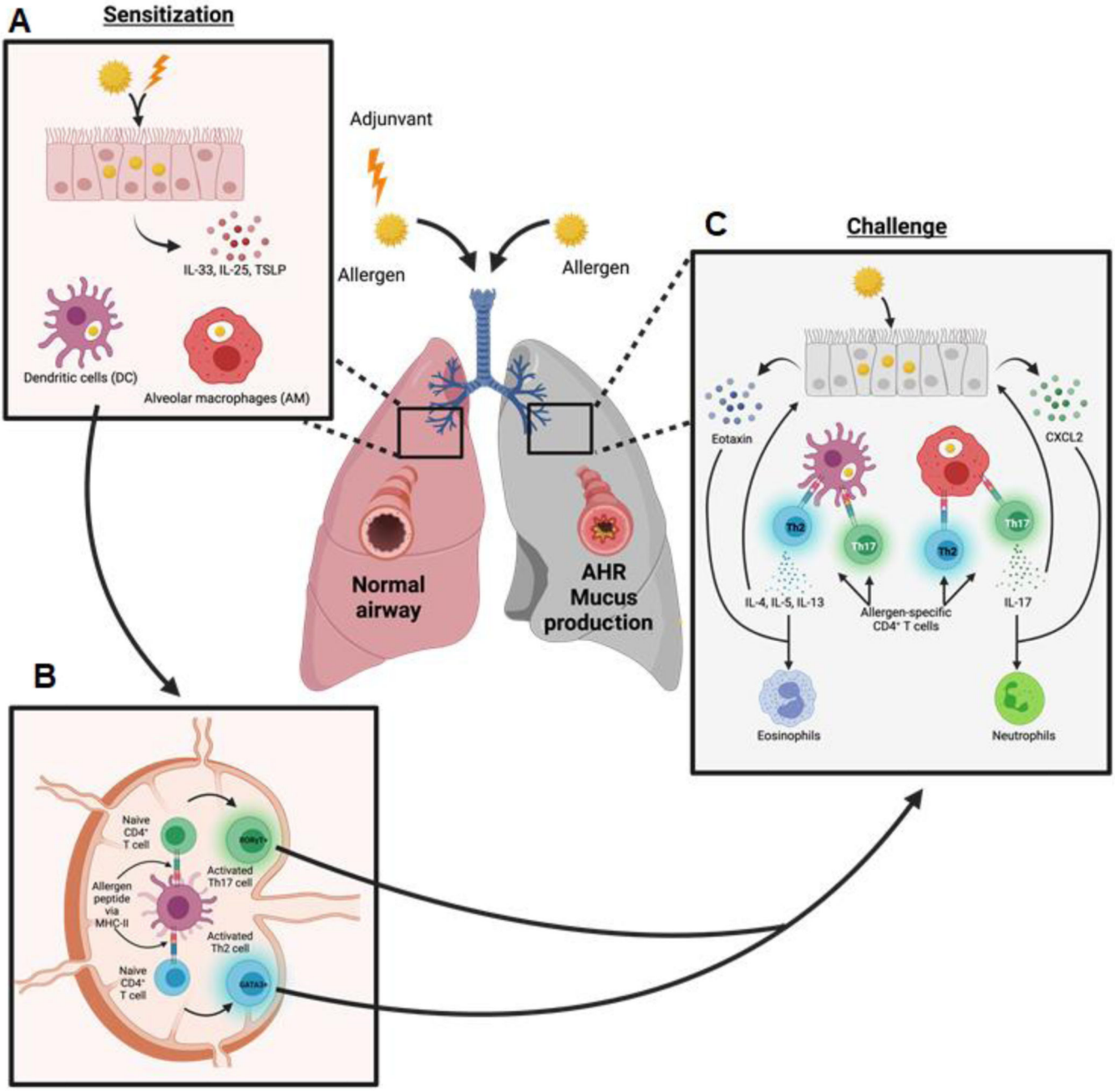


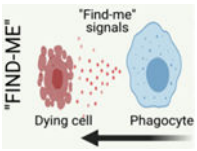
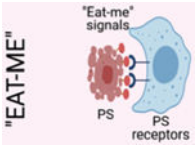
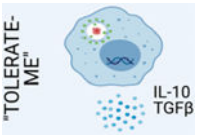

**Figure 4: General model of asthma in mammals.**

**A.** During allergic sensitization, inhaled allergens and adjuvants are taken up by lung resident cells, such as dendritic cells (DCs), alveolar macrophages (AMs), and epithelial cells. Stressed epithelial cells produce alarmins, such as IL-33, IL-25, and TSLP, which are important for a type 2 immune response [116]. Alveolar macrophages and dendritic cells (DCs) also take up allergen and become activated [117]. **B.** Activated DCs traffic to draining lymph nodes where they present antigen to naïve $CD4^+$ T cells and promote the differentiation of allergen-specific Th2 ($GATA3^+$) and Th17 (ROR $\gamma t^+$) cells [118]. **C.** During challenge, Th2 and Th17 cells become activated and produce their signature

cytokines. Th2 cell production of IL-5, and IL-13 promotes and synergizes with epithelial cell-derived eotaxin to drive eosinophil recruitment and activation [119]. Th17-derived IL-17 induces and synergizes with epithelial cell-derived CXCL2 to promote the recruitment and activation of neutrophils [106]. This figure was created using BioRender (https://biorender.com/).

## Table 1.

Efferocytotic machinery associated with asthma[a,b,c]

| | | | |
|---|---|---|---|
|   | **"Find-me" signaling** | | |
| | ***Molecule*** | ***Phenotype*** | ***Ref*** |
| | Fractalkine | Increased upon induction of asthma | 63 |
| | S1P | Increased upon induction of asthma | 64 |
| | CX3CR1 | Variant associated with increased risk of asthma | 65 |
| | | Possible protective effect of minor allele | 65 |
| | PY(2)R | *Py2r*-deficient mice exhibit protection against experimental asthma | 66 |
| | HSP70 | Circulating and sputum concentrations are increased in asthmatics | 67 |
|   | **"Eat-me" signaling** | | |
| | ***Molecule*** | ***Phenotype*** | ***Ref*** |
| | AXL | DNA methylation associated with increased risk of childhood asthma | 68 |
| | | Sputum concentrations of soluble AXL (sAXL) are increased in asthmatics | 69 |
| | | Antibodies against AXL mitigate asthma-like features in mice | 70 |
| | MerTK | *Mer*-deficient mice exhibit increased susceptibility to, and delayed resolution of, experimental asthma | 72 |
| | MFGE8 | *Mfge8*-deficient mice exhibit increased AHR and immunopathology in a model of experimental asthma | 73, 74 |
| | Galectin-3 | Galectin-3 expression decreased in alveolar macrophages from asthmatics | 60 |
| | | Sputum concentrations of Galectin-3 and the ratio of Galectin-3 to Galectin-3 binding protein are particularly low in individuals with neutrophilic asthma | 78 |
| | | Recombinant Galectin-3 improves efferocytosis in macrophages derived from asthmatics with neutrophilic disease | 60 |
| | RAGE | RAGE-KO mice are protected from asthma and exhibit reduced inflammation, AHR. And type 2 cytokine production | 87 |
| | Rac-1 | Mice with *Rac1*-deficient bronchial epithelial cells develop more severe allergic airway inflammation and fail to mount an immunotolerant response | 53 |
|   | **"Tolerate-me" signaling** | | |
| | ***Molecule*** | ***Phenotype*** | |
| | LXRα/β | LXRα/β-deficient mice exhibit protection from asthma and reduced AHR | 91 |
| | PPARγ | *Ppparg*-deficient mice exhibit more severe asthma and increased AHR | 93 |
| | PPARα | *Pppara*-deficient mice exhibit more severe asthma and increased AHR | 96 |
| | ABCA1 | Overexpression of human ABCA1 in murine endothelial cells improved AHR and lung function | 97 |

[a]The table includes molecules within the efferocytosis pathway that affect asthma pathogenesis.

[b]This table was created using BioRender (https://biorender.com/).

[c]Abbreviations: AHR, airway hyperresponsiveness; KO, knockout.