Regulation of eosinophil functions by autophagy

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Abstract
Eosinophils are granule-containing leukocytes which develop in the bone marrow. For many years, eosinophils have been recognized as cytotoxic effector cells, but recent studies suggest that they perform additional immunomodulatory and homeostatic functions. Autophagy is a conserved intracellular process which preserves cellular homeostasis. Autophagy defects have been linked to the pathogenesis of many human disorders. Evidence for abnormal regulation of autophagy, including decreased or increased expression of autophagy-related (ATG) proteins, has been reported in several eosinophilic inflammatory disorders, such as Crohn’s disease, bronchial asthma, eosinophilic esophagitis, and chronic rhinosinusitis. Despite the increasing extent of research using preclinical models of immune cell-specific autophagy deficiency, the physiological relevance of autophagic pathway in eosinophils has remained unknown until recently. Owing to the increasing evidence that eosinophils play a role in keeping organismal homeostasis, the regulation of eosinophil functions is of considerable interest. Here, we discuss the most recent advances on the role of autophagy in eosinophils, placing particular emphasis on insights obtained in mouse models of infections and malignant diseases in which autophagy has genetically dismantled in the eosinophil lineage. These studies pointed to the possibility that autophagy-deficient eosinophils exaggerate inflammation. Therefore, the pharmacological modulation of the autophagic pathway in these cells could be used for therapeutic interventions.

Keywords Autophagy · Differentiation · Degranulation · Eosinophil · Eosinophilic disease · Eosinophilic leukemia

Introduction
Eosinophils are granulocytes which are characterized by their avidity for the acidic dye eosin [1]. Relatively few mature eosinophils are found in the peripheral blood of healthy humans (less than 400 per mm³) [2]. Moreover, under homeostatic conditions, eosinophils reside primarily in all regions of the digestive system except the esophagus [3]. Eosinophils are also present in lymphoid organs such as thymus, lymph nodes, and spleen. A physiological infiltration of eosinophils is also seen in non-lymphoid organs such as adipose and mammary gland tissues as well as in the uterus [4]. Moreover, the so-called regulatory eosinophils have been observed in normal human and mouse lungs [5]. In response to inflammatory stimuli, eosinophil differentiation in the bone marrow is increased and eosinophils migrate towards inflammatory tissues where their lifespan is prolonged [6, 7]. When blood eosinophil numbers exceed 400 per mm³, the term eosinophilia applies. A threshold of 1500 eosinophils per mm³ is usually employed to define blood hypereosinophilia [8]. A cytokine-independent eosinophilia is caused by genetic changes within the eosinophil lineage, and the resulting diseases are classified as primary or intrinsic eosinophilic disorders [9].

The most typical eosinophil characteristic which discriminates them from other granulocytes (neutrophils and basophils) is the presence of large specific granules in the cytoplasm [10, 11]. A significant amount of mediators, including cytotoxic cationic proteins, cytokines, chemokines, and growth factors, are preformed and stored within eosinophil specific granules, where they are available for a rapid, stimulus-dependent release [12, 13]. Other characteristic organelles in eosinophils are primary granules, known to contain...
the Charcot-Leyden crystal protein (also known as galectin-10), and lipid bodies, which are production sites of inflammatory lipid mediators such as cysteinyl leukotrienes, thromboxanes, and prostaglandins [14, 15].

Contrary to neutrophils, which are essential for host defense, pharmacological or genetic depletion of eosinophils does not cause evident functional consequences [16]. Nevertheless, the fact that eosinophil lineage in all vertebrates survived the evolution pressure over several thousands of years demonstrates the importance of eosinophils in health and disease [17]. While eosinophils have been traditionally perceived as cytotoxic effector cells, recent studies have revealed their additional immunomodulatory and homeostatic activities. The role of eosinophils in immunity is still a matter of dispute and remains to be ambiguously defined. Preclinical mouse models mimicking human eosinopenia [18, 19] and hypereosinophilia [20, 21] as well as the ability to specifically knockout genes in the eosinophil lineage [22] allow to investigate the potential roles of eosinophils in health and disease. Moreover, targeted anti-eosinophil therapies allow to draw conclusions regarding the contribution of eosinophils for physiological and pathophysiological processes in humans [23].

**Autophagy**

Macroautophagy (hereafter referred to as autophagy) is a highly dynamic intracellular degradation system by which cytoplasmic constituents are delivered to lysosomes for degradation [24, 25]. The main morphological feature of autophagy is the biogenesis of autophagosome, a unique double-membrane vesicle which encloses parts of the cytoplasm. The fusion between autophagosomes and lysosomes results in the formation of auto(phago)lysosomes, in which cargo is degraded by a number of hydrolytic enzymes. Breakdown products are then returned to the cytosol and reused [24, 25] (Fig. 1a).

The autophagic pathway is a central component of cellular stress response, and it assures a proper biological adaptation to the continually changing environment [26]. In general, autophagy has two principal functions: (1) provision of essential building blocks under the conditions of nutrient or energy deprivation and (2) maintenance of cellular homeostasis by elimination of damaged macromolecules and organelles [27]. Therefore, autophagy is a process which keeps individual cells in a healthy state. Any perturbation is expected to cause, or at least to contribute, to the pathogenesis of diseases. For instance, autophagy has been implicated in several pathologies, particularly neurodegeneration, cancer, inflammation, and infectious diseases [28–30]. Moreover, work in the last two decades has also demonstrated that autophagy is critical for multiple functions of the immune system such as removal of pathogens, differentiation of immune cells, antigen presentation, and regulation of inflammatory responses [31, 32].

The autophagic pathways have been characterized in excellent reviews, and general roles of autophagy in the regulation of immunity have been covered elsewhere [31–37]. Therefore, we only introduce the canonical autophagy pathway that requires autophagy-related (ATG) proteins. In the following, we discuss recently published work on the involvement of autophagy in eosinophil-associated diseases and review experimental work on the role of ATG proteins in eosinophil development and functions.

**The molecular mechanism of autophagy**

Understanding of the molecular mechanism of autophagy has progressively advanced since the identification of ATG genes. In mammals, the protein machinery is organized into functional units which are required for autophagosome biogenesis. Upon autophagy induction, the ULK complex (consisting of the protein kinase ULK1/2, ATG13, ATG101 and FIP200) is generated, arising from regions where ATG9 vesicles line up with the endoplasmic reticulum (ER) [38, 39]. ULK complex is required for the recruitment and activation of the class III phosphatidylinositol 3-kinase (PI3K) complex (consisting of the lipid kinase VPS34, VPS15, ATG14, and Beclin 1), locally producing phosphatidylinositol 3-kinase 3-phosphate (PI3P) [39, 40]. The function of PI3P could be the formation of a phagophore membrane by modifying the composition of the ER membrane and recruitment of PI3P effectors [41]. Two ubiquitin-like conjugation systems are essential for proper elongation and closure of the phagophore membrane. The ATG12-ATG5-ATG16L1 complex is located mainly on the outer side of the phagophore membrane, followed by the disassembly upon autophagosome completion [41]. In the next step, the ATG12-ATG5-ATG16L1 complex promotes microtubule-associated protein light chain 3 (LC3) conjugation with phosphatidylethanolamine (PE) on the expanding phagophore membrane. Lipidated LC3 (LC3-II) serves as a commonly used marker for detection of double-membrane organelles owing to its integration into both the inner and outer autophagosomal membrane [42, 43] (Fig. 1b).

Autophagic pathway is strongly regulated by the activity of mechanistic target of rapamycin complex 1 (mTORC1), which senses the levels of nutrients and growth factors [44]. A strong trigger for autophagy induction is the lack of amino acids, leading to inhibition of mTORC1 activity and activation of suppressed ULK complex [45, 46]. Another central autophagy regulator is an AMP-activated protein kinase (AMPK), a sensor of metabolic, oxidative, and genomic stress [47]. Under the conditions of inadequate glucose levels, hypoxia or DNA damage, the AMPK activity is induced and promotes autophagy either by mTORC1 inhibition or direct phosphorylation of ULK complex [48, 49]. In addition, class I PI3K responds to...
growth factor signaling and suppresses autophagy by positively regulating the mTORC1 activity \[50\](Fig. 1c). In contrast, class III PI3K (VPS34) forms a protein complex and produces the phospholipid PI3P, contributing to the initiation and progression of autophagy \[50\].

The protein p62 (sequestosome 1/SQSTM1) is a specific substrate for autophagy, and reduced autophagy is often related to excessive accumulation of p62 \[41\]. Besides, p62 selectively delivers ubiquitinated proteins to the phagophore membrane following the interaction with LC3-II \[51, 52\]. Finally, fusion between autophagosomes and lysosomes results in degradation of the auto(phago)lysosomal contents by lytic enzymes \[53\]. Despite the extensive involvement of ATG proteins in autophagy machinery, it has been shown that many ATGs also exhibit additional non-autophagic functions that are discussed elsewhere \[54, 55\].

**Regulation of the function of immune cells by autophagy**

The discovery of the ATG proteins was followed by in-depth investigation of the autophagy machinery and its critical involvement in the functioning of the immune system became evident \[32\]. The most direct approach of autophagy-dependent microbial removal is through xenophagy, a selective form of autophagy in which intracellular pathogens are targeted for autophagosomal sequestration \[56–60\]. Two possible mechanisms for the recognition of intracellular microbes by the autophagy machinery have been suggested. During xenophagy, the cytosolic bacteria are tagged by ubiquitin molecules and recognized by p62, which recruits the LC3-positive phagophores to capture the bacteria \[61, 62\]. Alternatively, ubiquitin is conjugated to host proteins on *Salmonella*-
containing endosomes and binds with ATG16L1 independently of LC3-ubiquitin interaction through adaptor proteins [63]. In addition, p62 is able to deliver the ribosomal protein precursor Fau to autolysosomes where it is metabolized into bactericidal peptides. As a consequence, autophagic organelles are endowed with unique antimicrobial properties [64].

Autophagy is involved in the regulation of inflammatory responses. For example, it can suppress the immune response through the inhibition of inflammasomes, leading to reduced activation of caspase-1 and secretion of the pro-inflammatory cytokines IL-1β in IL-18 [65–69]. Moreover, autophagy has an influence on the homeostasis, survival, activation, proliferation, and differentiation of multiple cells of the immune system such as natural killer (NK) cells, macrophages, dendritic cells (DCs), as well as T and B cells. For instance, autophagy contributes to the maturation and antiviral activities of NK cells [70, 71]. Autophagy also participates in antigen presentation by DCs, forming a link between the innate and adaptive immune systems. The autophagic sequestration promotes the delivery and presentation of endogenous antigens on major histocompatibility complex (MHC) class II molecules, resulting in enhanced CD4+ T helper cell responses [72, 73]. On the contrary, autophagy enhances internalization and degradation of MHC class I molecules, leading to compromised MHC class I antigen presentation and attenuated response of cytotoxic CD8+ T cells [74]. Previous reports have also demonstrated the significance of autophagy for the development, survival and effector functions of T [75–79] and B cells [80–84]. Furthermore, it has been suggested that autophagy supports the release of β-hexosaminidase and histamine from mast cells [85]. The involvement of autophagy has also been demonstrated in neutrophil differentiation [86, 87] and effector functions [88].

In contrast to other cells of the immune system, the relevance of autophagy and ATG proteins for the biology of eosinophils has long remained elusive. However, insightful evidence was recently generated from experimental mouse models in which Arg5 was specifically depleted in the eosinophil lineage, resulting in eosinophil-specific autophagy deficiency [89]. As discussed below, these models allowed testing the function of eosinophils under in vitro conditions in the presence and absence of ATG5. Moreover, these mouse lines were used in preclinical models of bacterial infection and primary hypereosinophilic disease.

Role of autophagy in eosinophilic diseases
Interest in the role of autophagy in immunity was partially driven by the association of ATG genes with inflammatory disorders. Genetic alterations in autophagy may be hereditary, predisposing individuals to autoimmune, autoinflammatory, or infectious diseases. The involvement of autophagy has been observed in several eosinophilic inflammatory diseases, such as Crohn’s disease (CD), bronchial asthma, eosinophilic esophagitis (EoE), and chronic rhinosinusitis (CRS). Study models of eosinophilic inflammatory diseases and observed results are summarized in Table 1.

Crohn’s disease (CD)
Inflammatory bowel disease (IBD) encompasses two types of chronic inflammatory disorders of the gastrointestinal tract, CD, and ulcerative colitis (UC) [90]. Accumulating evidence suggests that inflammatory conditions in the intestine result from the abnormal immune response to enteric microbes in genetically predisposed individuals [91]. Genetic studies of IBD have made great progress since 242 risk loci have been identified through genome-wide association studies (GWAS) associated with the presence of IBD, highlighting some major disease-associated pathways [92]. Moreover, NOD2 has been identified as a major susceptibility gene [93, 94].

Two genes involved in autophagy, ATG16L1 and immunity-related GTPase M (IRGM), have been strongly associated with CD but not with UC, suggesting that autophagy is involved in the pathogenesis of CD [91]. A GWAS reported a single-nucleotide polymorphism (SNP) encoding a susceptibility variant of ATG16L1 gene (rs2241880, Thr300Ala) which is associated with a significant risk for CD [95, 96]. Since its discovery, SNP rs2241880 remained one of the most clinically important variants in CD and a large number of subsequent association studies have replicated the strong association of this genetic variation with CD [97–100]. The ATG16L1 Thr300Ala variant is associated with an excessive production of the pro-inflammatory cytokines IL-1β and IL-6 that drive the chronic inflammation observed in CD [101]. Moreover, this variant is more susceptible to cleavage by caspase-3, resulting in compromised clearance of Yersinia (Y.) enterocolitica and Salmonella (S.) typhimurium, as well as elevated cytokine production [102, 103].

IRGM has been demonstrated to induce autophagy and generate large autolysosomal organelles as a mechanism to inhibit the survival of intracellular Mycobacterium (M.) tuberculosis [104]. Recently, it has been reported that IRGM physically interacts with ULK1 and Beclin 1, promoting their assembly and thus controlling the arrangement of autophagy initiation complexes. In addition, IRGM forms a molecular complex with NOD2 and ATG16L1, modulating autophagic responses to pathogens [105]. A significant association has been reported between CD in various ethnic cohorts and sequence variants in the IRGM gene (SNPs rs13361189, rs4958847, and rs10065172) [100, 106–109]. The SNP rs13361189 was found to increase the risk of CD clinical sub-phenotypes such as ileal disease, perianal disease, and intestinal resection [110]. Interestingly, a gene-gene interaction analysis showed a significant two-way interaction
between SNP rs2241880 (ATG16L1) and rs10065172 (IRGM), suggesting that ATG16L1 and IRGM work jointly toward CD pathogenesis [111].

Further insight into the role of ATG16L1 was obtained using genetically modified mice. Mice lacking Atg16L1 in hematopoietic cells revealed a strong susceptibility to acute colitis induced by dextran sulfate sodium, implying that Atg16L1 protects mice from intestinal inflammation [65]. Atg16L1 is also important in the biology of epithelial Paneth cells as Atg16L1-knockout Paneth cells demonstrated a defective granule exocytosis which might alter the intestinal microbiota [112]. However, despite the intense investigations of the IBD pathogenesis, the role of autophagy in eosinophils has not been addressed yet.

**Bronchial asthma**

Asthma is a heterogeneous disease characterized by chronic airway inflammation. Patients develop a variety of respiratory symptoms such as wheeze, breath shortness, chest tightness, cough, and expiratory airflow limitation [113]. Polymorphisms in ATG genes have suggested that a genetic predisposition may increase the chance to develop asthma. Since autophagy has been shown to regulate immune responses and inflammation, a possible association of genetic variants of ATG5 and ATG7 genes with childhood asthma was investigated. Two ATG5 SNPs, rs12201458 and rs510432, were significantly associated with asthma, the latter being functionally relevant by enhancing promoter activity [114].

Moreover, ATG5 gene expression was upregulated in nasal epithelial cells isolated from asthmatics with acute symptoms [114]. Similarly, the expression of ATG proteins (LC3-II, ATG4, ATG5-ATG12, ATG7) as well as the number of autophagic vacuoles was also increased in lung tissue from
patients with chronic obstructive pulmonary disease (COPD) [115]. Another study investigated the potential association of SNPs in ATG genes (ULK1, SQSTM1, MAP1LC3B, BECN1, and ATG5) with asthma. SNP rs12212740 of ATG5 exhibited a positive association with asthma [116]. Examination of bronchial tissue from asthmatic patients demonstrated an increased number of autophagosomes in fibroblasts and epithelial cells compared with healthy individuals [116].

Similarly to structural cells, autophagy levels in sputum granulocytes, blood leukocytes, and blood eosinophils from patients with severe asthma were significantly increased as compared with subjects with non-severe asthma and healthy controls [117]. Interestingly, autophagy was induced in isolated blood eosinophils and human eosinophil-like (HL-60) cells in response to IL-5 treatment. To confirm that IL-5 induced autophagy rather than inhibited autophagosome degradation, inhibitors were used which blocked autolysosome degradation or fusion of autophagosome with lysosome [117]. These findings stimulated investigations in ovalbumin (OVA)-specific mouse model of allergic asthma [118]. OVA-challenged mice exhibited an increased expression of LC3-II in lung homogenates and a higher abundance of autophagosomes in cells of the bronchoalveolar lavage fluid (BALF), particularly in eosinophils. The eosinophil count in BALF also positively correlated with the LC3-II expression in lung homogenates, suggesting that autophagy is closely correlated with the severity of asthma as well as the eosinophilic inflammation. Inhibition of autophagy by intraperitoneal injection of 3-methyladenine (3-MA) and intranasal treatment with Atg5 shRNA led to a reduced number of eosinophils and IL-5 levels in BALF, as well as improved histological inflammatory features [118]. However, it cannot be excluded that 3-MA also blocked cytokine signaling events in this model [119, 120]. Finally, intranasal administration of anti-IL-5 monoclonal antibody resulted in reduced LC3-II expression in lung homogenates, together with improved AHR and decreased eosinophil numbers in BALF [118]. Taken together, there is evidence that autophagy is induced in structural and inflammatory cells of the lungs in asthma, but it remains unclear how this phenomenon contributes to the pathogenesis of asthma. Therefore, it seems too early to propose novel therapeutic approaches for the treatment of asthma based on autophagy inhibition.

**Chronic rhinosinusitis (CRS)**

CRS is characterized by chronic inflammation of the sinonasal mucosa and clinically associated with sinus pressure, nasal congestion, and a decreased sense of smell persisting for more than 12 weeks [121]. CRS is often associated with pronounced eosinophil-dominant infiltration and inflammation and then classified as eosinophilic chronic rhinosinusitis (ECRS) [122].

The effect of autophagy on the development of ECRS was investigated in mice with a conditional knockout of Atg7 in myeloid cells (mainly neutrophils and macrophages), which was mediated using the LyzM-Cre (Lyz2-Cre) recombinase activity. An established mouse model of ECRS resulted in significantly increased eosinophil infiltration, epithelial hyperplasia, and mucosal thickening in Atg7flox/floxLyzM-Cre mice as compared with Atg7flox/− mice, possibly owing to increased prostaglandin (PG) D2 production [123]. Interestingly, eosinophil infiltration and histological abnormalities were significantly improved following macrophage depletion in Atg7flox/floxLyzM-Cre mice with ECERS. The autophagy-deficient macrophages exacerbate the eosinophilic inflammation in ECRS, at least partially, through the release of elevated IL-1β levels. Therefore, results of this study suggest a protective role of macrophage autophagy on eosinophilic inflammation [123].

**Eosinophilic esophagitis (EoE)**

EoE is defined as a chronic, immune-mediated disorder resulting in esophageal dysfunction and eosinophil-predominant inflammation leading to tissue remodeling and fibrotic stricture [124]. A study performed on a pediatric patient cohort revealed upregulated ATG7 gene expression in esophageal biopsies from active EoE patients as compared with esophagus-healthy control individuals. EoE patients in remission and patients with gastroesophageal reflux disease (GERD) [125]. Therefore, ATG7 might be used as a valuable tissue biomarker of active EoE, and other ATG genes may be explored to potentially identify novel biomarkers for EoE diagnosis, monitoring, and prognosis. A recent study revealed a possible cytoprotective mechanism of autophagy which supports cellular redox balance and homeostasis following exposure to the inflammatory EoE environment, providing mechanistic insights into the role of autophagy in EoE pathogenesis [126]. Specifically, TNF-α and IL-13 have been identified as triggers of autophagy within the epithelium of the esophagus under in vitro conditions, including an esophageal organoid model. Inhibition of autophagic flux via chloroquine treatment augmented basal cell hyperplasia in these model systems. Moreover, this study has demonstrated increased autophagy in epithelial cells of the esophagus in EoE patients in vivo [126]. However, it should be noted that also this study did not investigated the role of autophagy in eosinophils.

**Role of autophagy in eosinophil differentiation**

Understanding the differentiation of eosinophils is crucial since many eosinophilic diseases are associated with increased production of eosinophils in the bone marrow. Eosinophils are
Eosinophils are no longer mitotically active, and they are re-
progenitors (GMPs) in mice [128]. Terminally differentiated
genitors (CMPs) in human [127] and granulocyte-monocyte
progenitors (EoPs) which derive from common myeloid pro-
continuously produced from eosinophil lineage-committed
progenitors (EoPs) showing downregulation of
Gata-1, which might reflect the reduced and delayed eosino-
phil differentiation [89]. Moreover, the differentiation poten-
tial of Atg5-deficient eosinophil precursors was tested under
pathologic conditions, employing an established mouse model
of chronic eosinophilic leukemia (CEL). CEL has been initi-
at in mice by the combination of fusion protein FIP1L1-
PDGFRα (F/P) expression and IL-5 overexpression [139],
resulting in a less severe eosinophilia development in the
absence of Atg5. Similar results were obtained in EoL-1 cells,
a model of an established human CEL. Upon induced differen-
tiation of EoL-1 cells, significantly lower levels of surface
markers CD11b, Siglec-8 and CCR3 were observed in
ATG5-deficient EoL-1 cells, indicating decreased maturation
of eosinophil precursors [89] (Fig. 2). These observations sug-
gest that targeting ATG5 within the eosinophil lineage might
represent a possible future treatment of eosinophilic leukemia.
These findings were in contrast with the effect of Atg5-defi-
cency on neutrophil differentiation in Atg5flox/floxLyzM-Cre mice [136]. These mice exhibited an augmented production of eosinophil pro-
genitors together with deteriorated allergic airway inflamma-
tion after OVA exposure [138]. Collectively, these data dem-
strated the differential effects of mTOR in the regulation of
eosinophil development, likely due to the distinct functions of
mTORC1 and mTORC2 [138].

With the purpose of studying the developmental and func-
tional consequences of autophagy deficiency in eosinophils, a
novel mouse model with an eosinophil-specific knockout of
Atg5 was generated (Atg5flox/floxCre mice) [120]. Cre recombinase is expressed only after commitment to the eosin-
lineage and is absolutely specific for eosinophils (eoCre mice) [22]. Results obtained from Atg5flox/flexoCre mice showed elevated numbers of immature eosinophils in the bone
marrow and a significant drop of mature eosinophils in the
field [89] (Fig. 2). Knockout of Atg5 within eosinophils resulted in delayed and reduced eosinophil precursor prolif-
eration and maturation under in vitro conditions. No abnormal-
ities in cell death were observed in Atg5-knockout eosino-
phils. During in vitro eosinophil differentiation, a reduced
phosphorylation of p38 and p44/42 mitogen-activated protein
kinases (MAPKs) was detected in eosinophil precursors lack-
ing Atg5, which might explain, at least partially, the observed
phenotype. Eosinophil populations were further purified from
the bone marrow of hypereosinophilic H5 (IL-5) transgenic
mice (NJ.1638), showing downregulation of Gata-1, C/ebpe, Pu.1, and Tribl transcription factors in the absence of
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cency on neutrophil differentiation in Atg5flox/floxLyzM-
Cre mice [86]. These mice exhibited an increased proliferation
and differentiation of Atg5-knockout neutrophils, culminating
in neutrophil accumulation in the circulation and lymphoid
organs. Accelerated neutrophil differentiation was also ob-
served upon shRNA-mediated Atg5 knockdown in Hoxb8
neutrophils [86]. Furthermore, autophagy was studied in early granulopoiesis in mice with a conditional deletion of \( \text{Atg7} \) at the hematopoietic stem and progenitor level (\( \text{Atg7}^{\text{flox/floxVav-Cre}} \)), which caused accumulation of immature neutrophils in the spleen, blood, and peritoneum [87]. An expanded population of immature myeloblasts and myelocyte precursors was also observed in \( \text{Atg7}^{\text{flox/floxCebpa-Cre}} \) mice, expressing \( \text{C/ebp} \alpha \) promotor predominantly at the GMP stage. \( \text{Atg7} \)-knockout neutrophil precursors were unable to shift from glycolytic activity toward mitochondrial respiration, demonstrating accumulation of lipid bodies and decreased ATP production. Inhibition of autophagy-mediated lipid degradation failed to provide free fatty acids to support mitochondrial respiration and ATP production, resulting in a defective neutrophil differentiation [87].

Interestingly, it has been reported that the p38 MAPK activity differentially regulates eosinophil and neutrophil differentiation, potentially through the modulation of C/EBP\( \alpha \) transcriptional activity [140]. A recent study showed that Trib1 expression favors eosinophil development by restraining neutrophil lineage commitment by modulating C/EBP\( \alpha \), partly clarifying the regulation of granulocytic lineage selection and identity [141]. In addition, single-cell transcriptome analysis has determined two different myeloid progenitor subsets which separate early in the hematopoietic development. Subsets can be differentiated according to the presence (eosinophils, mast cells, megakaryocytes, erythrocytes) or absence (neutrophils, lymphocytes, monocytes) of \( \text{Gata1} \) expression [142]. The potential involvement of autophagy in the segregation and regulation of these two distinct myeloid progenitor differentiation pathways has not been established yet and could be the subject for future studies.

**Role of autophagy in eosinophil effector functions**

Activated eosinophils exert their effector functions mainly through degranulation and formation of eosinophil extracellular traps (EETs). While both degranulation and EETs are...
important innate immune effector functions against pathogens, they can also cause significant immunopathologies [14].

**Degranulation**

Eosinophil specific granules are rich in four major cationic proteins: major basic protein (MBP), eosinophil peroxidase (EPX), and the ribonucleases eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) [143]. Eosinophils release granule contents into the extracellular space through three commonly observed pathways, namely, piecemeal degranulation (PMD), exocytosis, or cytolysis [17]. During PMD, the specific granules are progressively emptied and eosinophil sombrero vesicles (EoSVs) transfer the selected secretory cargo to plasma membrane [144, 145]. Granules can release their entire contents following granule fusion with the plasma membrane in a process called exocytosis, which can be preceded by intracellular granule-granule fusion [146]. Eosinophil cytolysis is characterized by the disintegration of cytoplasmic membrane and release of nuclear DNA cloud upon cell lysis [147]. It has been demonstrated that eosinophil cytolysis is dependent on receptor-interacting protein kinase 3 (RIPK3)-mixed lineage kinase-like (MLKL) signaling pathway and can be counterregulated by autophagy induction, perhaps opening up new ways for therapeutic interventions [148]. PMD and cytolysis have been frequently reported to be associated with eosinophilic diseases such as asthma, nasal polyps, IBD, allergic rhinitis, and EoE [149–151]. On the contrary, exocytosis is rarely documented during inflammatory responses, but has been observed during the interaction of eosinophils with helminths [152] and certain environmental fungi [153].

The effect of autophagy on eosinophil degranulation has been studied in Atg5-knockout eosinophils, which intriguingly exhibited an enhanced capacity to degranulate in vitro as measured by increased CD63 surface expression following GM-CSF priming and C5a stimulation [89] (Fig. 2). Moreover, an experimental in vivo mouse model of bacterial infection with *Citrobacter (C.) rodentium* was used to test the infiltrating colonic eosinophils for their activation and degranulation status. Eosinophils lacking Atg5 exhibited higher Siglec-F and CD63 surface expression levels, supporting the in vitro observations [89]. In addition, the expression of ATG5 in human eosinophils was analyzed together with their degranulation status in human eosinophilic tissues. Patients with angiolymphoid hyperplasia, EoE, and sebaceous gland carcinoma demonstrated a positive correlation between ATG5 expression and intracellular EPX levels in tissue eosinophils, suggesting increased degranulation in ATG5low-expressing eosinophils. These data were supported by observations in hypereosinophilic syndrome (HES) patients, in which a significant negative correlation between ATG5 (and ATG7) mRNA expression in blood eosinophils and secreted EDN levels in plasma was found [89] (Fig. 2). These data supported the concept that Atg5-knockout eosinophils in mice and ATG5low-expressing human eosinophils are more susceptible to degranulation.

In contrast to eosinophils, autophagy was reported to be crucial for the degranulation of mouse neutrophils [88] and mast cells [85]. Mice with autophagy deficiency in myeloid cell lineage (*Atg7*–/–/LyzM-Cre) showed reduced severity of several neutrophil-mediated inflammatory and autoimmune disease models, including PMA-induced ear inflammation, LPS-induced breakdown of blood-brain barrier, and experimental autoimmune encephalomyelitis. The most likely mechanism was suggested to be a reduced NADPH oxidase-mediated production of reactive oxygen species (ROS) in *Atg7*-knockout neutrophils [88]. These findings suggest that autophagy has differential effects on eosinophil and neutrophil degranulation. It has also been reported that in contrast to neutrophils, which are absolutely required for antibacterial defense, pharmacological or genetic ablation of eosinophils does not result in obvious functional consequences [16].

**Extracellular trap (ET) formation**

Eosinophils are able to form ETs which consist of mitochondrial DNA and cationic granule proteins released from activated cells [154]. EETs perform antibacterial functions, and they enable the accumulation of toxic granule proteins directly onto pathogens captured in the DNA scaffold, limiting the damage of surrounding host tissues [155]. The formation of EETs has been demonstrated in various infectious, allergic, and autoimmune eosinophilic disorders [154, 156–159].

In addition to eosinophils, activated neutrophils are also able to form similar extracellular DNA structures, known as neutrophil extracellular traps (NETs) [160, 161]. Interestingly, the release of mitochondrial DNA does not require cell death neither does it limit the viability of the granulocytes [161]. On the other hand, neutrophil death-dependent mechanisms have also been described, and the scientific dispute regarding the requirement of cell death for NET formation is ongoing [155]. It has been demonstrated that NET formation by viable neutrophils depends on the activity of NADPH oxidase, cytoskeletal rearrangements, and glycolytic ATP production [162, 163].

A recent study reported the requirement for autophagy in the formation of EETs in the airway of asthmatic mice. Treatment with the autophagy inhibitor 3-MA attenuated EET formation and improved the lung inflammation, mitochondrial metabolism, and oxidative stress in OVA-challenged mice [164]. Moreover, treatment of neutrophils with autophagy inhibitor wortmannin reduced NET formation by activated neutrophils [165, 166]. 3-MA and wortmannin have been widely used as autophagy inhibitors.
based on their inhibitory effect on class III PI3K activity, which is known to be essential for autophagy induction [50]. The same inhibitors, however, are also reported to block class I PI3Ks which contribute to the activation of NADPH oxidase [167–169]. Therefore, decreased formation of EETs following 3-MA and wortmannin treatment can also be explained by inhibition of ROS production. A recent study has investigated this issue in details, and the reported results suggest that PI3K inhibitors, such as 3-MA and wortmannin, block both EET and NET formation in an autophagy-independent manner [120]. In addition, both Atg5-knockout eosinophils and Atg5-knockout neutrophils were fully capable to release extracellular DNA after stimulation, demonstrating that autophagy is not required for both EET and NET formation [120]. A careful quantitative analysis revealed that Atg5-knockout eosinophils exhibit an even increased ability of EET formation compared with control eosinophils in vitro, supporting the surprising findings of augmented degranulation in eosinophils lacking Atg5 [89]. Eosinophils lacking Atg5 also demonstrated elevated in vitro bacterial killing of Escherichia (E.) coli, suggesting increased effector function of eosinophils in the absence of Atg5. Moreover, the antibacterial defense of Atg5-knockout eosinophils was tested under in vivo conditions in the C. rodentium model. An improved local and systemic clearance of C. rodentium was observed in Atg5<sup>lox/lox</sup>eo<sup>Cre</sup> mice, together with an enhanced ability to form EETs [89]. Atg5-knockout eosinophils demonstrated an increased activity of Stat3, p38, and p44/42 signaling pathways following cytokine stimulation, providing a possible explanation for enhanced eosinophil effector functions in the absence of Atg5 [89].

Tumor-associated tissue eosinophilia is often observed in cancer patients and studies suggest their involvement in tumoricidal activities. Upon interaction with a colorectal carcinoma cell line Colo-205, eosinophils released their granule contents such as ECP, EDN, TNF-α, and granzyme A, which exerted cytotoxic responses against tumor cells [170]. Moreover, ablation of eosinophils severely compromised antitumor immunity in a colorectal cancer (CRC) mouse model, most likely owing to impaired Th1 and CD8<sup>+</sup> T cell responses. On the other hand, CRC patients with enhanced eosinophil tumor infiltration demonstrated robust CD8<sup>+</sup> T cell infiltrates, resulting in a better prognosis compared with patients with low-eosinophil infiltrating tumors [171]. Interestingly, the blockade of autophagy enhanced T helper 9 (Th9) cell anticancer functions in vivo, and mice with T cell-specific deletion of Atg5 exhibited reduced tumor growth in an IL-9-dependent manner [172]. Finally, it would be interesting to investigate the functional consequences of Atg5-knockout eosinophils in different disease models and explore their potential for cancer immunotherapy.

Concluding remarks

The findings summarized in this review article highlight the autophagic pathway as a protective mechanism of cells which contributes to the limitation of disease severity in eosinophilic diseases. Autophagy seems to secure the function of parenchymal cells under inflammatory conditions. This process appears to be particularly important in epithelial cells to maintain their barrier function. The generation of conditional/promoter-specific knockout mice has enabled researcher to investigate the role of autophagy in a cell-type specific manner. Recently, such experimental models were also developed to study the cell-type inherent function of autophagy in eosinophils. Surprisingly, and in contrast to other immune cells, eosinophils enhance effector functions when autophagy is impaired. Therefore, drug-induced inhibition of autophagy in chronic inflammatory eosinophilic diseases does not seem to be indicated because of two possible unwanted effects: (1) reduced epithelial barrier function and (2) increased eosinophil-mediated immunopathology. In contrast, however, blocking the autophagic pathway in eosinophils might be beneficial in eosinophilic tumors. Therefore, pharmacological impairment of autophagy might also be an option for the therapy of eosinophilic leukemias. Clearly, additional experimental work, including the analyses of human cells and tissues, is required to identify drug targets suitable for the modulation of the autophagic pathway as preventive or therapeutic intervention in eosinophilic diseases and other human pathologies.

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Declaration

Conflicts of interest H.U.S. is a consultant for GlaxoSmithKline. The other authors declare that they have no conflict of interest.

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