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Pcyox1L Controls Immune Dynamics through Neutrophil Differentiation and Functionality

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A Thesis in the Field of Biology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

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Abstract

Autophagy is a cellular function that is essential in maintaining homeostasis at the molecular level. Over the years, autophagy has defined its role in neutrophil biology and innate immunity. Understanding the role of autophagy at the cellular level will prove very useful for future therapies in immunology. In this study, the functional significance of Pcyox1L and its influence on autophagy is explored to assess the Pcyox1L regulated neutrophil functions. While Pcyox1L is a known protein-coding gene, it currently has no know function. To examine the role of Pcyox1L on neutrophil function and differentiation, in vivo and in vitro experiments were demonstrated with mouse neutrophil cell lines and using a corneal mouse infection model. This study indicates that Pcyox1L alters the process of innate immunity by affecting neutrophil autophagy, which in turn affects the bactericidal capabilities, maturation, and viability of the neutrophil. Data from my studies highlight intracellular pathways influenced by commensal colonization induced an increase in Pcyox1L levels in PMNs. Conversely, Pcyox1L KO mice demonstrate an altered commensal microbiome in the gut and distant sites, such as the eye, and elevated susceptibility to *P. aeruginosa*.

Dedication

To my parents, who have been my guiding light and unconditional love and support.

Acknowledgments

My deepest gratitude to Dr. Mihaela Gadjeva for her kindness and for being a constant source of knowledge and inspiration.

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Chapter I.

Introduction

Little is known about how commensal colonization controls neutrophil differentiation and functions. Using system immunology approaches, previous studies discovered commensal and infection-induced changes in neutrophil proteomes. The identified differentially expressed genes were CRISPRed out in immortalized neutrophil progenitor cell lines transduced with lenti-guides. Analysis of maturation and bactericidal capacity of the generated CRISPRed clones ascribed a function to a currently unknown protein - termed prenylcysteine oxidase 1 like (Pcyox1L). Pcyox1L deficient neutrophils showed dramatic defects in maturation, autophagy, and bactericidal functions associated with significant reductions in SerpinB1a transcript and protein levels. CRISPR-ing out of SerpinB1a in neutrophils phenocopied the Pcyox1L KO phenotype signifying linked mechanisms. Commensal colonization induced an increase in Pcyox1L and SerpinB1a levels in PMNs and, conversely, Pcyox1L KO mice demonstrated altered commensal microbiome in the gut and at distant sites, such as the eye. Pcyox1L KO mice also showed elevated susceptibility to infection with a gram-negative pathogen, P. aeruginosa, exemplified with elevated bacterial burden at the infected site and diminished bactericidal functions. Cumulatively, our data highlight key intracellular pathways controlled by Pcyox1L and commensals that govern neutrophil functionality, longevity, and bactericidal properties.

Definition of Terms

"Autophagy"- An essential physiological process that involves the degradation of extracellular and intracellular bacterial pathogens that invade the cell

"Cytokines"- small proteins essential in cell signaling

"Defensins"-small proteins that act as host defense peptides

"Endotoxin"- a bacterial toxin that gets released in the host cell

"LC3"- Microtubule-associated proteins 1A/1B light chains

"mTORC"- Mammalian target of rapamycin

"PMN's"- white blood cells that composed of neutrophils, eosinophils, and basophils

"*Pseudomonas aeruginosa*"- a rod-shaped gram-negative bacteria

"p62"- Protein that is degraded upon the activation of autophagy

"ROS"- Reactive oxygen species.

"Pcyox1L" – prenylcysteine oxidase-1-like protein

Neutrophil Development

Neutrophils undergo differentiation in the bone marrow until reaching maturity, when they are released into the bloodstream.

Neutrophils are differentiated from lymphoid-primed multipotent progenitors (LMPPs), which are derived from hematopoietic stem cells (HSCs). LMPPs then go on to differentiate into granulocyte-monocyte progenitor cells (GMPs), which commit the lineage to myeloid development. From this phase, GMPs undergo subsequent differentiation to myeloblast, promyelocytes, myelocytes, metamyelocytes, and ultimately immature (band neutrophils) and mature neutrophils (Coffelt et al., 2016).

In granulopoiesis, the production of granulocytes is mediated mostly by G-CSF and, to a lesser extent, GM-CSF. In the granulocyte series, myeloblasts differentiate into promyelocytes and begin to process granules. Cell proliferation continues within this stage, and fine granules containing inflammatory-related proteins appear during myelocyte maturation. The nucleus of neutrophil progenitors becomes increasingly more condensed as the cell matures through its different stages, namely, myelocyte, metamyelocyte, band, and eventually the terminally differentiated neutrophil. The G-CSF receptor is primarily expressed on neutrophils and also bone marrow precursor cells and is the primary receptor responsible for the signaling pathway that causes the differentiation of mature neutrophils (Mehta et al., 2015).

Microbiota Drives Neutrophil Differentiation

Maintaining a healthy gut microenvironment is highly important to the homeostasis of the body. The gut is host to a complex community of gut commensal bacteria that aids in important physiological processes like digestion, metabolism, the protection against harmful pathogens, and regulation of the immune system. Gut microbiota maintains a beneficial symbiosis with the immune system; the production and function of neutrophils are influenced by metabolites and microbial components derived from microbiota (Zhang D et al., 2018). Apart from neutrophils, the bone marrow produces hematopoietic stem cells that give rise to progenitor cells that take place in the neutrophilic lineage (Zhu et al., 2018). Gut microbiota also plays a pivotal role in the regulation of neutrophil production and priming. Many studies have used germ-free mice to demonstrate that signals from the gut are critical for the development and regulation of neutrophils. Interestingly, neutrophil levels were significantly reduced in germ-free mice, the microbiota of these mice circulating neutrophil levels was restored with a heat-killed E.coli strain (Deshmukh et al.,2014). This is indicative that microbial components from commensals can drive neutrophil production. Furthermore, another study exploring the capabilities of gut microbiota to produce hematopoietic cells by Khosravi et al., 2014, concluded that signals derived from commensal bacteria regulate myelopoiesis. They found that microbial-associated molecular patterns and microbial metabolites (MAMPs) modulated immune responses. Specifically, MyD88, an adaptor for recognition of MAMPS, demonstrated GMP expansion and differentiation (Khosravi et al.,2014). Microbiota can regulate neutrophil production by recognition receptors like Toll-like

receptors (TLRs) and nucleotide oligomerization domain-containing proteins (NODs) that induce the production of IL-17 via the TLR4/MyD88 pathway, which drives the production of the master regulator of neutrophil differentiation, granulocyte colony-stimulating factor (GCSF) (Deshmukh et al., 2014). The source for IL-17 was identified, and ILC3-dependent mechanisms were implicated. Given that GCSF-deficient mice are not completely devoid of mature neutrophils, additional pathways are involved. Gram-positive commensals in the gut and nasopharyngeal mucosa trigger GM-CSF–driven pathways to control myeloid responses. Based on these findings, it appears that GCSF and GM-CSF pathways are fundamental to control neutrophil development and longevity at the infected site. While the GCSF and GMCSF-induced pathways in the context of granulopoiesis have been studied, they are not fully understood.

The laboratory I joined at HMS discovered that gut commensals control myeloid development, defining the frequency and characteristics of myeloid cells and specifically neutrophils (Kugadas et al., 2016). Lack of microbiota converted the outbred Swiss Webster (SW) strain of mice from normally resistant to P. aeruginosa keratitis phenotype to a susceptible one. Interestingly, reduction of gut commensals with nonabsorbable oral antibiotics in SW mice sensitized to infection pointing to the systemic effect of gut commensals on myeloid differentiation in the bone marrow.

Using system immunology approaches, the lab conducted a quantitative proteomic analysis of neutrophils generated in different environments: in the presence or absence of commensals and in the presence or absence of infection and identified novel proteins with previously unknown function. In total, 3,633 proteins were identified for

analysis from the quadruplicate samples of PMNs derived from different environments, including non-infected and infected SPF and GF SW mice, making our neutrophil proteome data the largest reported thus far. The results illustrated active remodeling of the proteomes in the mature neutrophils derived from the infected mice depending on the commensals present. Among the top 10 proteins with differential protein expression were proteins with an unidentified function such as Pcyox1L and proteins with a known function such as SerpinB1a.

Research Aims, Goals, and Hypothesis

The primary research goal of this thesis is to assess the different intracellular pathways that affect the Pcyox1L expression in mouse models and in vitro experiments. I hypothesize that Pcyox1L regulates neutrophil maturation and bactericidal capabilities through autophagy, affecting host responses to commensals and pathogens.

Specific Aim 1. Characterizing the phenotype of Pcyox1L deficiency in mouse neutrophil cell lines.

Rationale: Autophagy is an essential physiological process. Recently there has been a greater focus on how autophagy can influence the innate immune system (Xu et al., 2010). In mammals, autophagy is regulated by the mammalian target of rapamycin (mTOR) and directed by various autophagy-related proteins. Two protein complexes termed mTORC1 and mTORC2 control autophagosome assembly (Mizushima et al., 2011). Autophagy consists of 5 steps: initiation, elongation, formation of autophagosome, fusion with the lysosome, and degradation. The process of elongation results in a

completed autophagosome; two protein conjugation systems are essential in the step of elongation for the completion of a double membrane organelle. One system involves linked autophagy-related protein (ATG)12 and ATG5, and the other system consists of microtubule-associated proteins 1A/1B light chains (LC3) that are cleaved by ATG4 when autophagy is induced. This cleavage gives rise to cytoplasmic LC3I and the LC3II conjugation complex. LC3II is a common and important marker used to detect autophagic organelles because it is fused within the inner and outer membranes of the autophagosome. Before the fusion of the autophagosome with the lysosome, the autophagosome has to grow and close; the protein p62 binds to LC3II through polyubiquitinated protein aggregates. The binding of these two proteins will allow the autophagosome to grow and close. Generally, p62 is a protein that is degraded upon the activation of autophagy; contrastingly, the accumulation of p62 is brought on by the inhibition of autophagy (Yang et al., 2010; Yorimitsu et al., 2005; Bitto et al., 2014) (Supplemental figure 1).

Autophagy can be blocked at different steps by inhibitors. Chloroquine (CQ) is an inhibitor that can prevent lysosomal acidification that essentially blocks autophagic degradation. Weak bases like CQ can raise the pH in the lysosomal environment that leading to a cascade of events that results in disabling the degradation of the vesical cargo. It is important to mention that CQ is not the only inhibitor that can be used to block autophagy; bafilomycin A, ammonium chloride, and 3-methyladenine are all suitable inhibitors of autophagy; however, previous studies indicate that CQ is the most effective inhibitor (Myeku et al., 2011).

Various mouse models have suggested that autophagy is important for neutrophil functions, including differentiation, cytokine production, cell death, and phagocytosis.

Recent studies showed that autophagy has a role in assigning the identity and function of myeloid cells and regulates the differentiation of neutrophils (Huang et al., 2018). An increased level of autophagy is demonstrated in the myeloblast, promyelocyte, and myelocyte stages. However, the downregulation of autophagy is seen in the cells that have been terminally differentiated. Impairment of the autophagic pathways in myeloid cells can result in blood diseases like acute lymphoid leukemia (Koschade et al., 2019). The results of these studies delineate the crucial role autophagy has during the early stages of neutrophil development and seems to reciprocally regulate the differentiation of neutrophils at all stages of maturation (Shrestha et al., 2019). Previous studies conducted by Rožman et al., 2015, show that Atg5f/fLyz2Cre/Cre mice that have a deletion of the Atg5 gene in the myeloid cell lineage have an accelerated proliferation of neutrophil precursors in the bone marrow, as well as increased process of neutrophil differentiation. Increased production of neutrophils causes an accumulation of neutrophils in the spleen, blood, and bone marrow.

Hypothesis: Pcyox1L controls neutrophil differentiation and functionality by regulating autophagy and the expression of SerpinB1a.

Methods – Since autophagy controls neutrophil proliferation and maturation, we examined spontaneous and cytokine-induced autophagy in Pcyox1L and Serpin B1a KO myeloid cell lines.

Specific Aim 2. Characterize the phenotype of Pcyox1L KO mice upon challenge with keratitis clinical isolates of *P. aeruginosa.*

Rationale: Pseudomonas aeruginosa is an aerobic, gram-negative, non-spore-forming rod-shaped bacteria. It has a variety of virulence factors- a characteristic that accounts for its broad spectrum of infections. The versatility and potent defenses possessed by P. aeruginosa create an impressive resistance to antibiotics; consequently, selecting treatment options has become challenging to medical professionals (Wilson et al., 2020). The role of autophagy in the context of P. aeruginosa-induced infections has been considered; however, data are complex and contradictory. It has been suggested that P. aeruginosa inhibits autophagy through various virulence strategies to escape clearance. However, these data have been challenged by reports which propose that P. aeruginosa promotes autophagy to escape phagocyte killing (Deng et al.,2015) and that activation of autophagy by rapamycin treatment sensitizes to infection.

Hypothesis: Pcyox1L KO mice will be more susceptible to P. aeruginosa isolates due to decreased bactericidal capabilities of Pcyox1L KO neutrophils.

Methods: To examine the impact of Pcyox1L deficiency on resistance to keratitis in the context of autophagy, cohorts of Pcyox1L KO mice and WT littermates were infected with *P. aeruginosa* clinical isolates, laboratory strain MPAO1, and isogenic deletion mutants for proteins previously identified to be abundantly expressed in the infected corneas (Yeung, J.Gadjeva, M.Geddes-McAlister, J., 2020). To identify virulence factors that control autophagy, we screened for autophagy activation in primary

neutrophils. To examine whether alteration of autophagy induced by different *P.aeruginosa* strains affects the phenotype of Pcyox1L KO mice, we planned to examine bacterial load and pathology in the infected Pcyox1L KO mice exposed to virulent strains that alter autophagy and non-virulent strains which do not affect autophagy.

Specific Aim 3- Characterize commensal communities at the ocular and gut mucosa impacted by Pcyox1L deficiency.

Rationale: Although there is evidence showing that neutrophil differentiation is driven by gut microbiota, the mechanisms behind this interesting phenomenon are still not understood. The previous studies conducted by Kugadas et al., 2016 demonstrated that gut commensals have a role in the activation and development of neutrophils in the bone marrow, therefore, regulating neutrophil activation and recruitment. Indicative that the diverse community of gut commensal bacteria is not just confined to the regulation and development of the local intestinal immune system but also has a profound influence on systemic responses. Examining the link between communities of gut commensals and immune responses at distal sites like the ocular surface will bring a further understanding of the role Pcyox1L levels have on the innate immune system and its contributing factors.

Hypothesis: If the regulation of Pcyox1L by gut microbiota controls neutrophil functionality, then the absence of Pcyox1L functionality will diminish myeloid functionality and result in commensal disruption and or dysregulation and thereby influencing innate immune responses.

Methods-Using 16 S sequencing, we defined commensal presence in the presence and absence of Pcyox1L in mice.

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Chapter II.

Methods

All animal experiments were performed following National Institutes of Health guidelines for housing and care of laboratory animals and performed in accordance with institutional regulations after protocol review and approval by the BWH IACUC committee and were consistent with the Association for Research in Vision and Ophthalmology guidelines for studies in animals

Animals

C57BL/6 mice were used. Original breeders were purchased from Taconic Farms (Germany, NY). For infections, in vitro experiments, 7-10-week-old adult male and female mice were used. Animals were maintained in a pathogen-free animal facility at Brigham and Women's Hospital

Genotyping

Was conducted using the following sequences:

49020mutF2:5'AACACTTCCCTCACTCTTGGCTAAGC 3' (mutant forward primer)

49020-comR2: 5' CCTGCCACCCTCTTACTCGTCG 3' (common reverse primer)

49020-wtF: 5' ACAGTTCAATGCAGGCTCAG 3' (WT forward primer) from Integrated DNA Technologies (IDT) (Coralville, IA,USA) Taq DNA Polymerase

Invitrogen: #18038-042, 5U/ul, includes PCR reaction buffer, -MgCl2 and 50 mM MgCl2 (Invitrogen Waltham, MA USA) 2.5 mM dNTPs 100mM dNTP set, PCR grade, (cat # 10297-018 Invitrogen Waltham, MA USA).Mouse-ear biopsies were taken and digested in 1x of a 10x digestion buffer containing 50uL of 2-Mercaptoethanol (Sigma Aldrich cat#M3148, St Louis MO, USA) 1.5M Tris base(Sigma Aldrich Cat#TRIS RO, St Louis MO, USA) 2M NH4)2SO3(Sigma Aldrich Cat#A4418, St Louis MO, USA) 1M MgCl2 (Sigma Aldrich cat# 1374248, St Louis MO, USA) and 100% Triton X-100(Sigma Aldrich Cat#T8787, St Louis MO, USA) heated in a 95°C water bath for 3 minutes. Proteinase kinase 20mg/m was added immediately after (Qiagen cat no 158918, Hilden Germany) samples were left in a 55°C water bath for enzymatic digestion. The following day samples were reheated at 95°C to arrest the digestion. The polymerase chain reaction was run for a total of 4 hours and followed the cycle for genomic DNA, Initiation at 94°C for 2 minutes 1x, denaturation at 94°C for 10 seconds, annealing starting at 65°C and decreasing 1°C each cycle for 30 seconds 10x, elongation at 68□C for 2 minutes, denaturation at 94°C for 15 seconds, annealing for 55°C for 30 seconds, repeated 25 times. After PCR, gel electrophoresis to successfully visualize the genotyping. The gel was made with Omnipur agarose (Sigma Aldrich Cat #2120 St Louis MO USA) and supplemented with Ethidium bromide 10 mg/ml (Invitrogen Cat #15585-011 Waltham MA, USA) 1X TAE buffer (10X TAE buffer): (Invitrogen, Ultra-pure, Cat # 15558-026 Waltham MA, USA) 1 Kb plus DNA ladder (Invitrogen Cat# 10787018 Waltham MA, USA). The gel was run for 35 minutes at 90volts and imaged.

Bacterial strains and Inoculum

Invasive *Pseudomonas aeruginosa* clinical isolates 6294, MPAO1, and C02 were used throughout the experiments. Those bacterial strains were grown overnight at 37°C for 15 hours on trypticase soy agar plates (cat no BD 221261, BD, Franklin Lakes, NJ, USA) supplemented with 5% sheep blood. All suspensions were prepared in Hank's balanced salt solution (HBSS) (cat. no. SH3058802, HyClone Laboratories Inc, Logan, UT, USA) and used for bactericidal assays.

Infection Model

Infections were carried out as described previously [24]. Briefly, mice were anesthetized with intraperitoneal ketamine HCL (cat. no. EA2489-564, Zoetis, Parsippany, NJ, USA) and xylazine injections (cat. no. Sc-362950Rx, Lloyd Inc, Shenandoah, IA, USA). Three 5 mm scratches were made on the cornea with a 25G needle tip, and an inoculum of 1 x 10^6 CFU of *P. aeruginosa* was delivered in 5 µl onto the eye. Mice remained sedated for approximately 30 min. For evaluation of corneal pathology, daily scores were recorded by an observer unaware of the experimental conditions based on the following scoring system using a graded scale of 0 to 4 as follows: 0, eye macroscopically identical to the uninfected contra-lateral control eye; 1, faint opacity partially covering the pupil; 2, dense opacity covering the pupil; 3, dense opacity covering the entire anterior segment; and 4, perforation of the cornea, phthisis bulbi (shrinkage of the globe after inflammatory disease), or both. To determine bacterial corneal counts at 24h after infection, mice were sacrificed, the eyes were enucleated, and

the corneas were dissected from the ocular surface. To quantify *P. aeruginosa* levels, corneas were suspended in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4), 0.05% Triton X100 (cat. no.,), serially diluted and plated on *P. aeruginosa* selective MacConkey agar plates (cat. no. R01552, Thermo Fisher Scientific, Waltham, MA, USA).

Purification of PMNs and Bactericidal assays

Murine bone marrow was flushed from both hind limbs with PBS supplemented with 2% fetal bovine serum (BSA, cat no 00448-00100, American Bioanalytical, Inc., Natick, MA, USA) and 1 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, Saint Louis, MO, USA). The cells were washed 2x at 1200 rpm. After the washes, neutrophils were isolated using Mojo Sort Mouse neutrophil isolation kit (cat no 480058, BioLegend, San Diego, CA, USA) was used according to the manufacturer's instruction. Neutrophils were incubated at a density of 500,00 with *P. aeruginosa* strain MPAO1 and C02 at an MOI of 0.01, in a supplemented RPMI solution containing 1x Insulin transferrin (for 60 min at 37oC on a rotator. Aliquots were taken at time 0, and 60 min were serially diluted and plated on MacConkey agar (cat no R01552, Thermo Fisher Scientific, Waltham, MA, USA) to determine numbers of live *P. aeruginosa*. Percentage of killing ability of neutrophils was calculated

Neutrophil cell lines and treatments

SW and C57BL6/N neutrophil progenitor cell lines were established and maintained in RPMI-1640 medium (cat no 10-040-CV, Corning, Corning, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS, cat no 26140, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin/100 µg/ml streptomycin (cat no 15140-122, Thermo Fisher Scientific, Waltham, MA, USA), 2 mM L-Glutamine (cat no 25030081, Thermo Fisher Scientific, Waltham, MA, USA), 2% stem cell factor (SCF), and β-estradiol. The SCF was supplied as conditioned media from a CHO cell line engineered to produce SCF. 2% conditioned media was used, equating to an SCF concentration of ~100 ng/ml. The beta-estradiol (cat no E2758, Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in ethanol at 10 mM stock and used at a final concentration of 0.5µM. To differentiate into granulocytes, beta-estradiol was omitted from the growth media, and cells were cultured in RPMI-1640 media supplemented with 10% FBS in the presence or absence of 10 ng/ml GCSF (cat no 574602, BioLegend, San Diego, CA, USA) or 10ng/ml GCSF and GMCSF (cat no 576302, BioLegend, San Diego, CA, USA) and maintained for either 3 or 5 days before performing the killing experiments. Cells were seeded into 6-well plates at 2x106 cells per well. Autophagy was measured with Chloroquine stimulations (CaymanChem cat#14194 Ann Arbor MI, USA) at 50uM for 4 hours at 37□. Cells were made into protein lysates for western blot analysis.

Western blot analysis

Cell lysates of primary bone marrow-derived neutrophils, as well as SW- and C57BL6/N-derived progenitors were prepared at 4°C with RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 5 mM EDTA (cat. no. BP-115D, Boston BioProducts, Ashland, MA, USA). Right before using the RIPA buffer, the protease inhibitor cocktail (cat. no. 11836170001, Roche, Basel, Switzerland) was added. Cells were lysed for 5 min on ice with frequent vortexing. Supernatants were collected following high-speed centrifugation (13,200 rpm.) for 10 minutes at 4°C. In general, 20-25µg of protein was loaded onto a well of a pre-cast NuPAGE 4–12% w/v Bis-Tris 1.5-mm mini gel (cat. no. NP0335BOX, Invitrogen, Carlsbad, CA, USA). Electrophoresis was carried out at 200 V in NuPAGE MES SDS running buffer [50 mM Tris base, 50 mM MES, 0.1% w/v SDS, 1 mM EDTA, pH 7.3] (cat. no. NP0002, Thermo Fisher Scientific, Waltham, MA, USA) for 35 minutes for Rheb, Serpinb1a gene target and for 90 minutes for Pcyox1L gene target. The lysates were separated by electrophoresis under reducing conditions and transferred onto low fluorescence polyvinylidene difluoride (LF PVDF) membranes (cat. no. 162-0261, Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline solution (TBST) [20 mM Tris base, 137 mM NaCl, containing 0.1% Tween 20, pH 7.6] for 1 h and then incubated with primary antibodies in TBST and 5% non-fat dry milk at 4 °C overnight. Membranes were washed using TBST and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies for 1 h and visualized by chemiluminescence (ChemiDoc MP Imaging

System, Bio-Rad, Hercules, CA, USA). The protein in the cell lysates were analyzed by blotting with the following primary antibodies (Table 3): anti–Pcyox1L (cat. no. orb35904, Biorbyt, St Louis, MO, USA; 1:500 dilution), anti–Pcyox1L (cat. no. NBP2-31970, Novus Biologicals, Centennial, CO, USA; 1:500 dilution), anti-Rheb (cat. no. 13879, Cell Signaling Technologies, Danvers, MA, USA; 1:1,000 dilution), anti-Glyceraldehyde-3-Phosphate Dehydrogenase (cat. no. MAB374, Millipore, Burlington, MA, USA; 1:500 dilution). Goat anti-rabbit IgG– horseradish peroxidase 1:2,500 dilution and donkey anti-mouse IgG–horseradish peroxidase 1:5,000 dilution (Santa Cruz Biotechnology, Dallas, TX, USA) were used as secondary antibodies.

16s Metagenomic sequencing

Fecal tissue from Pcyox1L WT, HET, and KO mice was collected. A total of 25mg as needed for adequate analysis. DNA was purified using QiAmp DNA Stool Mini Kit (Qiagen cat#51504, Hilden Germany) according to manufacturer instructions. DNA purity was measured on a nanodrop machine at a 260/280 ratio from 1.8-2.0 and 40ug/uL. 30uL were sent out for 16s metagenomic sequencing at SeqMatic Fremont CA, USA.

Analysis

Statistical analysis of corneal pathology scores, bacterial burden, and cytokine levels were either by Unpaired Student's t-test upon normal distribution, Mann-Whitney

U test for pair-wise comparisons, or One-way ANOVA with Dunn's correction for Multigroup comparisons (Prism 4.0 for Macintosh. The analysis of the data from the bactericidal activity assays was based on the use of Unpaired Student's t-test or One-way ANOVA as appropriate. Differences were considered significant if the p-value was <0.05.

Chapter III.

Results

Pcyox1L KO PMNs show alterations in autophagy, a phenotype similar to SerpinB1a KO PMNs

Prenylcysteine oxidase 1 Like (Pcyox1L) is a protein whose function is unknown. The predicted function of this protein is one of an enzyme involved in the catabolism of prenylated proteins, controlling the last step of the process- the degradation of prenylcysteine to hydrogen peroxide, cysteine, and farnesol or geranylgeranyl SerpinB1a is a protein that is abundant in the neutrophil cytoplasm, it has the main function of inhibiting neutrophil serine proteases, elastase and cathepsin G. Mice who have the SerpinB1 germline ablated will show increased levels of tissue injury, prolonged inflammatory responses and cell death (Farley et al., 2012).

Comprehending the pathways that interconnect Pcyox1L and SerpinB1a and its effects on neutrophil maturation, cells in culture at the GMP stage were stimulated with maturation driving cytokines GCSF and GCSF/GMCSF. Western blots were used to analyze the protein levels of Rheb- the mTORC1 activator. Serpin B1a KO PMNs demonstrated decreased levels of Rheb protein when stimulated with maturation-inducing cytokines. These results suggest that the mTORC1 pathway was inhibited in the absence of SerpinB1a (Fig1a). Rheb levels in Pcyox1L KO PMNs were evaluated when matured with GCSF/GMCSF (Fig 1b). Reduced levels of Rheb protein were observed in two

different Pcyox1L KO clones (F4 and F6). Cumulatively, our data show that mTORC1 pathways were impaired in the absence of SerpinB1a and Pcyox1L.

Autophagy is a physiological process that controls neutrophil differentiation. Studies have shown that there is increased autophagic activity is increased in myeloblasts, promyelocytes, and myelocytes, which decrease at the metamyelocyte or band cell level (Rožman et al., 2014). Pcyox1L and SerpinB1a KO PMNs exhibit low levels of Rheb protein, which is correlated with low levels of mTORC activation and likely elevated autophagy. Autophagy was examined in WT, Pcyox1L KO, and SerpinB1a KO PMNs by monitoring LC3II fragment generation and p62/SQSTM1 degradation in the presence or absence of chloroquine treatments to distinguish between flux and autophagosomal activation.

At 1-day post differentiation, as cells transitioned from GMPs to myeloblasts and promyelocytes, the SerpinB1a KO cells exhibited heightened flux upon GCSF stimulation, measured by elevated levels of LC3II fragment appearance. The autophagosomal activation, measured by p62 degradation, was also significantly elevated in the absence of SerpinB1a in either vehicle or GCSF-matured cells (Fig. 2). The maturing Pcyox1L KO GMPs phenocopies the SerpinB1a KO clones demonstrating increased autophagosomal activation. This was in blunt contrast to the WT, where p62 degradation was mild in vehicle-treated cells and reduced upon GCSF-stimulation (Fig. 2). These data reveal dramatic deficiency to control autophagosomal activation at baseline or upon GCSF-stimulation during the early stages of neutrophil development. Expectedly, the SerpinB1a KO cells phenocopied the Pcyox1L KO cells, which also

demonstrated increased LC3II fragmentation upon GCSF stimulation and elevated p62 degradation (Fig. 2), suggesting that SerpinB1a controls autophagosomal activation.

When PMNs were primed with GMCSF in addition to GCSF, the elevated autophagosomal activation was still maintained in the Pcyox1L KO PMNs (Fig. 3). Similarly, SerpinB1a KO PMNs treated with either GCSF or GCSF/GMCSF showed increased spontaneous and induced autophagosomal degradation (Fig. 4).

Cumulatively, data show two different defects in Pcyox1L KO myeloid cells: 1) impaired flux and 2) increased autophagosomal degradation demonstrating the requirement for these proteins to control autophagy.

The role of Pcyox1L in controlling neutrophil functionality through autophagy in primary cells

Observing levels of autophagy in the mature neutrophils derived from bone marrow gives significant insights into Pcyox1L function. We tested the immune response with two *P. aeruginosa* strains: MPAO1 and C02 (PA 4221) and autophagic activity. Our preliminary data indicated that MPAO1 causes inhibition of autophagy flux, consistent with previously published data. In contrast, we found that the isogenic mutant CO2, lacking the fptA gene encoding the Fe(III)-pyochelin outer membrane receptor precursor failed to inhibit autophagy. For these experiments, the autophagic flux was characterized by the cleavage of LC3, whereas a measure of the autophagolysosome degradation efficacy was the monitoring of the p62 abundance. The results showed that there was little flux or autophagosomal degradation at baseline in non-activated

neutrophils. When neutrophils were exposed to *P.aeruginosa* wild-type strain MPAO1, there was less flux in the Pcyox1L KO PMNs when compared to WT PMNs. In between the HET and KO, there was no difference in LC3II, proposing that both have less autophagic flux. The overall degradation of P62 was not different and minimal, highlighting the reduced autophagolysosomal degradation.

When the isolated WT neutrophils were exposed to the C02 isogenic mutant strain lacking the fptA gene encoding the Fe(III)-pyochelin outer membrane receptor precursor, the accumulation of LC3 in the CQ stimulation indicated an elevation of the autophagy flux. The HET and KO isolated BM neutrophils showed less pronounced autophagic flux. Cumulatively, these data indicated impaired autophagy flux in the absence of Pcyox1L in primary BM PMNs.

Pcyox1L KO mice are susceptible to *P. aeruginosa*-induced keratitis

To examine the impact of Pcyox1L deficiency on resistance to infection, we tested the sensitivity to *P. aeruginosa*-induced ocular infection. Pcyox1L KO mice showed elevated susceptibility to infection with the lab strain MPAO1 and the clinical isolate 6294. This was exemplified with elevated bacterial burden detected at 24h post-infectious challenge and pathology

Pcyox1L deficiency shifts the balance of *Firmicutes* and *Bacteroidetes* in the gut

Delving into the 16s metagenomic sequencing data between Pcyox1L WT and KO mice, an overall difference in diversity between genus and species populations was

not seen. However, a dramatic shift with an expansion of Firmicutes and a reduction in Bacteroidetes was observed in Pcyox1L KO mice when compared to WT littermates. The main genus' investigated were *Prevotella, Faecalibacterium, Mucispirillium, Blautia, Ruminococcus,* and *Bacteroides* (Fig.7). Consistent with the shift at the phyla level, at the genus level, Pcyox1L showed an abundance of *Ruminococcus spp*, whereas WT littermates showed an abundance of *Prevotells spp*. Within each of these genus', there was a separate diversity index for the species. The species that showed to have the most notable differences were *Prevotella copri*, *Ruminococcus gnavus,* respectively (Fig.7).

Chapter IV.

Discussion

It is essential to understand the perplexing and reciprocal interaction between the gut microbiome and host immune system at distant sites. It has been now well recognized that the gut microbiome affects local mucosal immune responses through a wealth of mutually non-exclusive variety of mechanisms. It is also appreciated that gut commensals affect immune functions at distant sites such as BM, thereby defining the immune status of the organism. Our interest was to gain a greater understanding of the molecular mechanisms governing commensal-induced myeloid BM development. Our initial hypothesis was that we could uncover commensal-induced molecular signatures in myeloid cells by comparing neutrophil proteomes from cells derived from mice raised either under SPF or GF conditions. Through the experiments described in this thesis, we define a function for a newly discovered molecular termed, Pcyox1L that we show controls neutrophil development and is regulated by microbiota. Our data highlight a connection between Pcyox1L and SerpinB1a in determining neutrophil development. Further, we show that both factors inhibit autophagy during neutrophil development, thereby defining the size of the myeloid progenitor niche in the BM.

Autophagy has made its mark on the innate immune system and has established its importance in maintaining homeostasis inside and outside of the cellular environment. The experiments that were conducted have brought insight and knowledge to the role of Pcyox1L in regulating autophagy, impacting neutrophil differentiation and function.

In a study conducted by Riffelmacher et al., 2017autophagy was found to have different effects on neutrophil differentiation based on the maturation stage. They reported different levels of autophagy from the promyelocytic stage until the terminal differentiation stage. The highest levels of autophagy were observed during the GMP to myelocyte stage of differentiation. When reaching the metamyelocyte stage, autophagic activity was decreased as a consequence of the upregulation of the mTORC1 pathway. These data suggest that autophagy plays distinct roles during neutrophil differentiation and in mature PMNs.

Previous studies conducted by Rožman et al., 2015; used mice that were Atg5 deficient, an essential protein for the elongation step in autophagy and for the final completion of the autophagosome structure. The *Atg5* KO myeloid progenitors had elevated proliferative capacity and reduced autophagic flux, illustrating a dramatic impact of autophagy on neutrophil differentiation. When we examined the consequences of Pcyox1L and SerpinB1a deletion upon maturing the GMP cell lines with different maturation driving cytokines, we observed elevated autophagosomal degradation in the absence of Pcyox1L and SerpinB1a. Based on these data we predict, reduced proliferative capacity in the absence of these factors in the KO GMPs (which should be investigated in the future).

The increased autophagosomal activity was not significantly affected by GCSF or GCSF/GMSCF stimulations of Pcyox1L KO and SerpinB1a KO neutrophil progenitors, in contrast to the WT progenitors, where each treatment reduced autophagosomal activity. These observations highlight that Pcyox1L and SerpinB1a pathways were

downstream of GCSF and GCSF/GMCSF stimulations. Interestingly, protein levels of Pcyox1L were not elevated upon GCSF stimulation (data not shown) of GMPs, ruling out a direct transcriptional and translational effect on GCSF on the expression level of Pcyox1L. In contrast, SerpinB1a levels were elevated by GCSF (data not shown). These data bring the question of which signals control Pcyox1L expression in the context of GMP maturation. This question was only partially answered as dual treatment with GCSF and GMCSF elevated Pcyox1L protein levels and SerpinB1a transcription. Cumulatively our data show that Pcyox1lL and SerpinB1a control autophagosomal activity during neutrophil differentiation. These pathways are controlled at least partially via GCSF and GMCSF. The Pcyox1L and SerpinB1a pathways appear interconnected.

SerpinB1a KO mice appear to have elevated susceptibility to *P. aeruginosa* lung infection (Benerafa et al., 2007), characterized by elevated inflammatory responses and bacterial presence in the lungs, indicating dysregulated control over neutrophil functions in vivo. We examined susceptibility to infection in Pcyox1L KO mice, with the expectation that their phenotype will be similar to that of the infected SerpinB1a KO mice. Indeed, infected with the clinical isolate 6294 or laboratory strain, MPAO1 Pcyox1L KO mice showed elevated bacterial presence in the corneas and increased pathology when compared to the WT littermates. These findings indicate impaired bacterial clearance likely due to defects in neutrophil development. Our cell line data would support this conjecture. Experiments are in progress to examine neutrophil differentiation in vivo during infection.

P. aeruginosa inhibits autophagy in mature PMNs through a variety of virulence mechanisms (Deng et al.,2015). This is thought to promote initial infection. Since we identified a series of bacterial proteins which were overexpressed during infection, we obtained isogenic mutants lacking those factors from the Transposon Mutant Collection (University of Washington) and examined their impact on autophagy of mature C57BL6/J-derived PMNs. We found that exposure to MPAO1 reduced autophagic flux, an expected finding. In contrast, we discovered that exposure of C57BL6/J-derived PMNs to Fe(III)-pyochelin outer membrane receptor precursor isogenic deletion *P. aeruginosa* C02 mutant promoted autophagy. These data suggest that impairment of iron import into *P. aeruginosa* controls downstream virulence characteristics, thereby inhibiting the ability of the pathogen to control autophagy.

Because of the differences in autophagosomal degradation seen during neutrophil maturation in the absence of PcyoxlL, we also compared responses of mature neutrophils derived from C57BL6/J and Pcyox1L to both MPAO1 and C02 strains. MPAO1 inhibited autophagy flux while C02 activated it in C57BL/J PMNs but not in Pcyox1L KO PMNs. These data suggested that in mature neutrophils, the Pcyox1Lfunction is different from that of neutrophil progenitors and that in mature neutrophils, Pcyox1L is a positive regulator of autophagic flux. Experiments testing the clearance of C02 in vivo are in progress but have not been completed yet

Since we validated that commensal-induced signals upregulated Pcyox1L, we expected that the Pcyox1L KO mice might present reciprocal differences in the composition of the gut commensals. Metagenomic 16s sequencing results presented a

dramatic shift towards the expansion of the Firmicutes and reduction of Bacteroidetes genuses and species in the absence of Pcyox1L. At the genus level, Pcyox1L WT mice had a higher representation of *Prevotella spp*, specifically the species *Prevotella copri.* Conversely, Pcyox1L KO mice showed specific loss of *B. fragilis, B. plebeius,* and elevated *Faecalibacterium prausnitzii* and *Ruminococcus gnavus*. This phenotype is somewhat reminiscent of the IgA KO phenotype showing more *Firmicutes* and expansion of SFB (REF). Indeed, while we haven't yet analyzed the IgA producing B cell characteristics and frequencies or IgA levels at mucosal sites or in circulation, this similarity suggests a potential connection. Given that our mice were derived in the absence of SFB, it would also be of interest to colonize separate Pcyox1L KO and WT cohorts of mice to determine if colonization preference is maintained.

Previous studies into the impact of the gut microbiome on granulopoiesis highlighted the ILC3 release of IL-17, stimulating GCSF production as a driving force of granulopoiesis (Ruiz de Morales et al., 2020). As described previously, ILC3-derived IL-22 stimulates Th17 cell differentiation by driving IECs to produce SAA and other factors required for the induction of TH17 cells (Ivanov et al., 2007). Since Clostridium spp or SFB are potent IL-17 inducing commensals (Wang et al., 2019), it is possible that their relative expansion in the Pcyox1L KO mice is compensatory to defects in myeloid lineage development. This remains to be examined.

Interestingly, Pcyox1L KO mice showed relative expansion of Ruminococcus gnavus, a Gram-positive, obligate anaerobe with proinflammatory functions that has often been associated with Crohn's disease in humans (Hal et al., 2017). Another genus

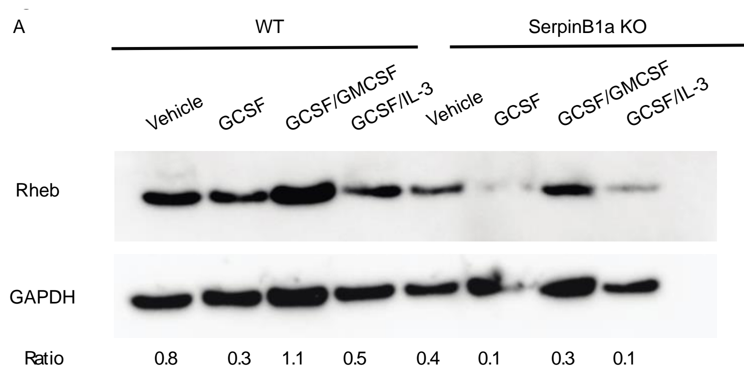
that was significantly enriched in Pcyox1L KO mice was that of Faecalibacterium prausnitzii. F. prausnitzii is a major butyrate producer (Lopez-Siles et al., 2016). The reasons for the relative abundance of F. prausnitzii in Pcyox1L KO mice are unclear and prompt to question if the response to Faecalibacterium prausnitzii and Ruminococcus gnavus is deficient in the absence of Pcyox1L? While experiments to address this issue are in progress, exciting insights come from the recent analysis of large patient cohorts undergoing chemotherapy followed by stem cell engraftment. This analysis included daily changes in circulating neutrophil, lymphocyte, and monocyte counts and more than 10,000 longitudinal microbiota samples revealing consistent associations between gut bacteria genuses and immune cell dynamics in the blood (Schluter et al., 2020). The data supported a strong, positive correlation between higher relative abundances of *Faecalibacterium spp* and *Ruminococcus spp* and increased neutrophil rates in blood. Further, when total bacterial abundances as predictors instead of relative abundances were used, this confirmed *Faecalibacterium* or be strongly associated with neutrophil dynamics in blood. Cumulatively, these data support a connection between neutrophil generation, specific commensal species, and Pcyox1L functionality.

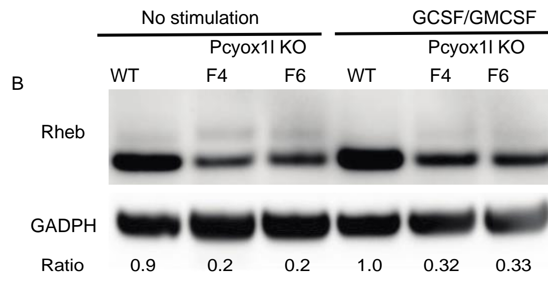
Conclusion

My data demonstrated a function for Pcyox1L in the regulation of autophagy during neutrophil development. Correspondingly, I showed alterations in bacterial clearance in Pcyox1L KO mice which correlate with alterations in commensal.

Cumulatively, data highlight previously unknown but important factor that is controlled by commensals and defines innate immunity.

Figures





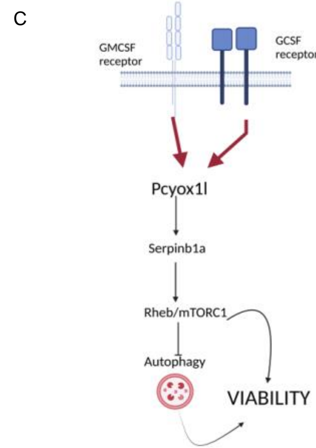


Figure 1. . Pcyox1L KO GMPs and SerpinB1a GMPs show decreased proliferative capacity.

(A)To understand the mechanisms connecting Pcyox1L and SerpinB1a-dependent pathways on neutrophil maturation, GMPs were stimulated with maturation-driving cytokines GCSF, GCSF/GMCSF, GCSF/IL-3, and analyzed protein levels of mTORC1 activator, Rheb when cells were matured with GCSF/GMCSF there was a decrease in Rheb, suggesting that in the absence of SerpinB1a, the activation of the mTORC1-driven pathways is impaired. (B) Rheb levels Pcyox1L KO PMNs matured with GCSF/GMCSF, mTORC1 pathways are impaired in the absence of SerpinB1a and Pcyox1L. (C) Representative image of GMCSF and GCSF mediated downstream signaling of Pcyox1L that leads to autophagy

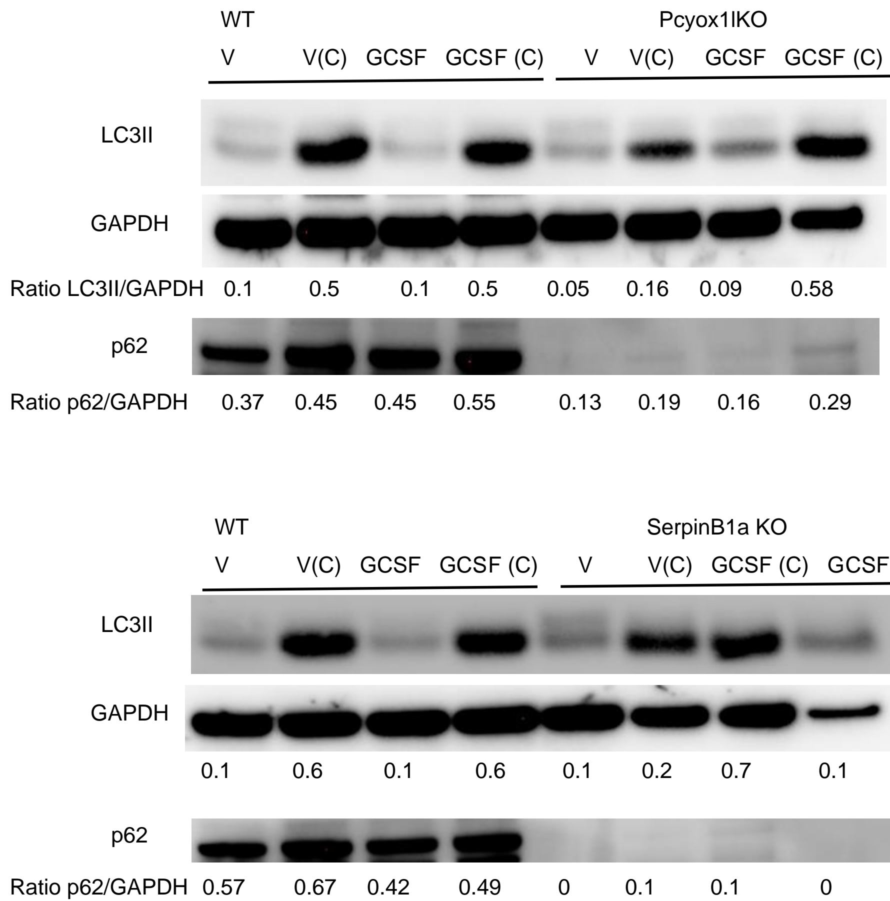


Figure 2. Pcyox1L KO and Serpin B1a GMPs have significantly altered autophagy.

At 1-day post differentiation, as cells transitioned from GMPs to myeloblasts and promyelocytes, the PcyoxLl KO and SerpinB1a KO cells exhibited heightened flux upon GCSF stimulation and challenged with chloroquine, measured by elevated levels of LC3II and p62. The autophagosomal activation, measured by p62 degradation, was also heightened in the absence of Pcyox1L in either vehicle or GCSF-matured cells

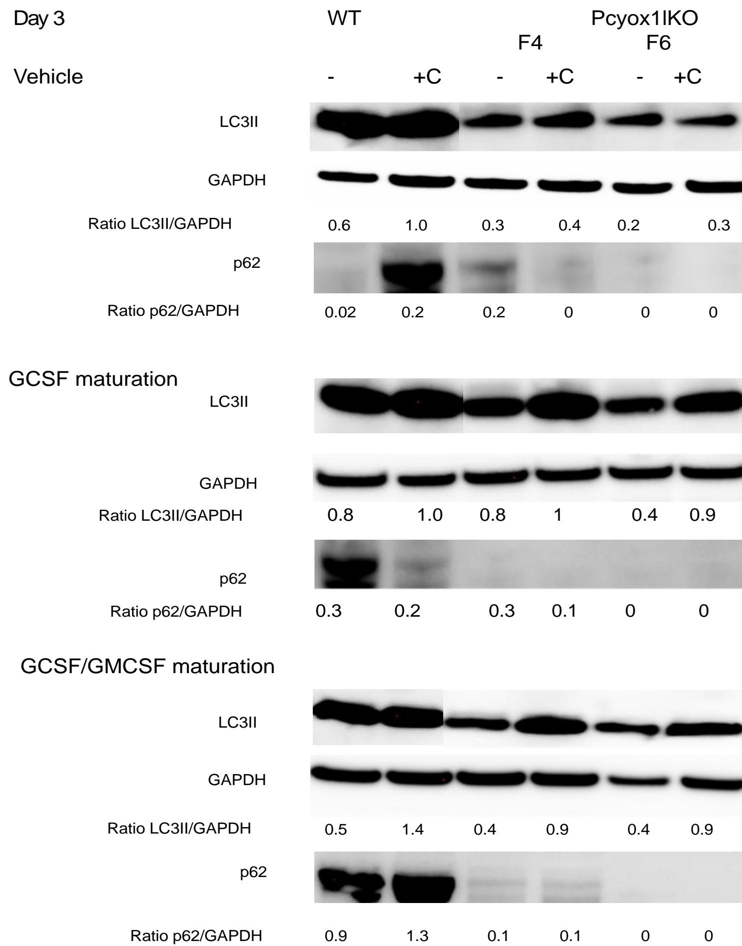


Figure 3. Pcyox1L KO mice show alterations of mucosal commensals.

At 3-day post differentiation, as cells transitioned to immature neutrophils, the Pcyox1L KO exhibited heightened flux upon GCSF stimulation and was challenged with chloroquine, measured by elevated levels of LC3II and p62 fragment appearance. PMNs were primed with GMCSF in addition to GCSF; the elevated autophagosomal activation was still maintained in the Pcyox1L KO PMNs.

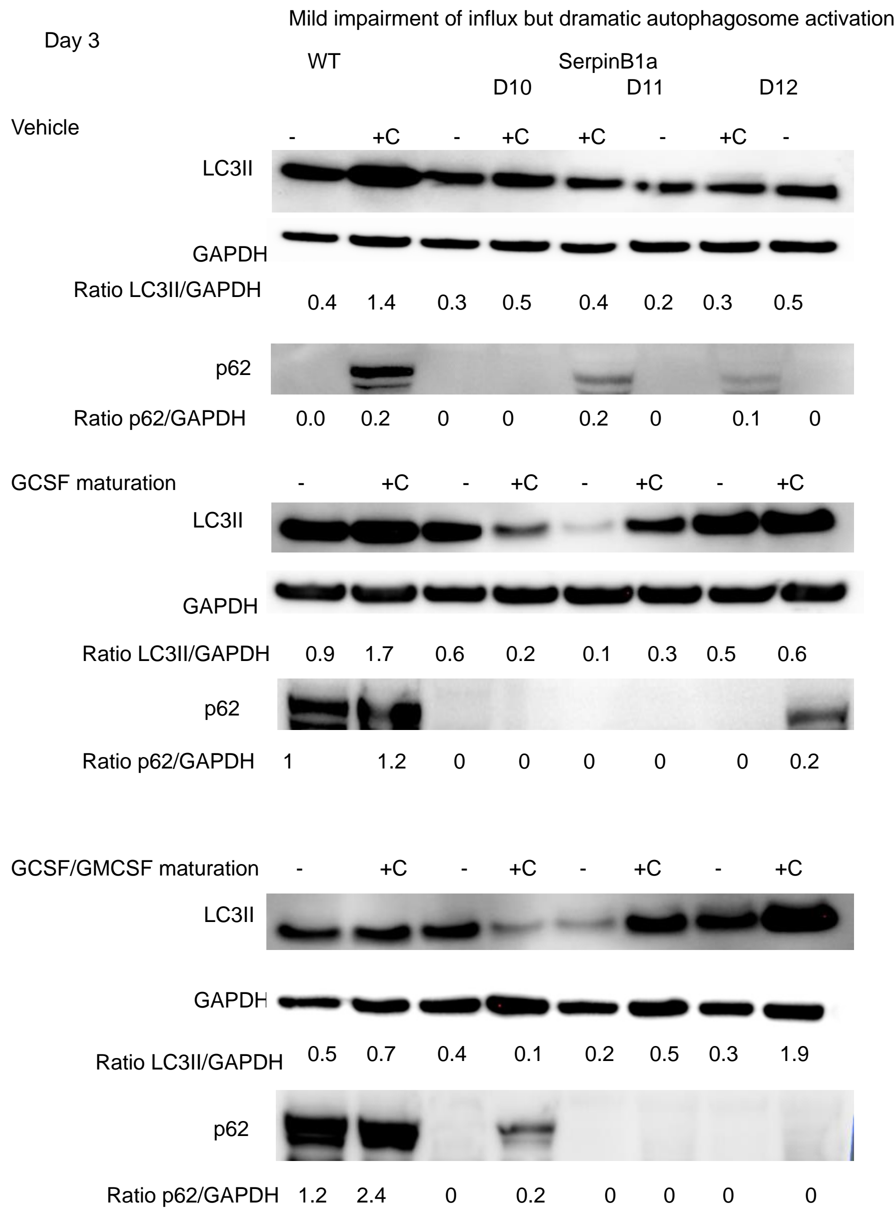


Figure 4. Pcyox1L KO mice show alterations of mucosal commensals

At 3-day post differentiation while cells are immature neutrophils, specifically in SerpinB1a KO cells exhibited heightened flux upon GCSF stimulation and challenged with chloroquine, SerpinB1a KO PMNs treated with either GCSF or GCSF/GMCSF

showed increased spontaneous and induced autophagosomal degradation

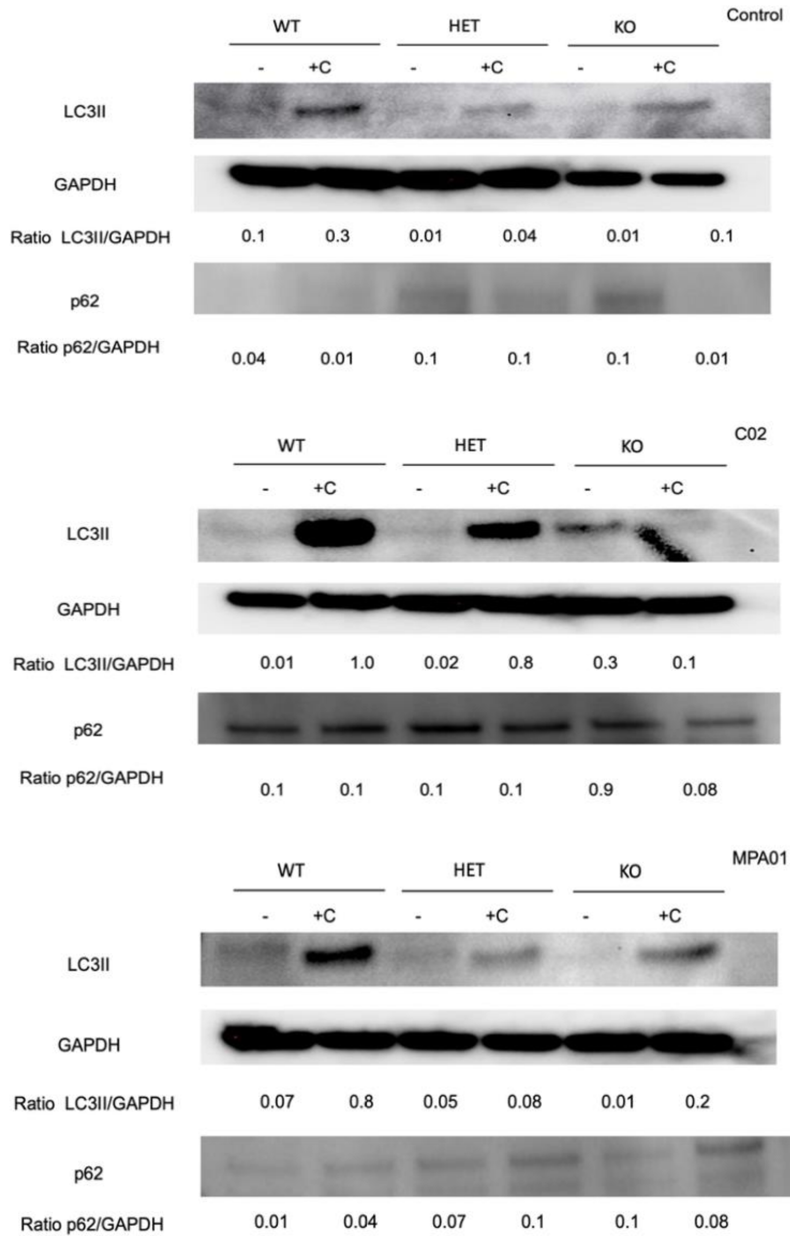


Figure 5. Autophagy in Pcyox1L PMNs upon infection.

The role of Pcyox1L in controlling infection by regulating neutrophil functionality through autophagy in vivo. Mouse PMNs were isolated and challenged with MPAO1 and C02 to assess the bactericidal capabilities of neutrophils during autophagy in the different Pcyox1L genotypes.

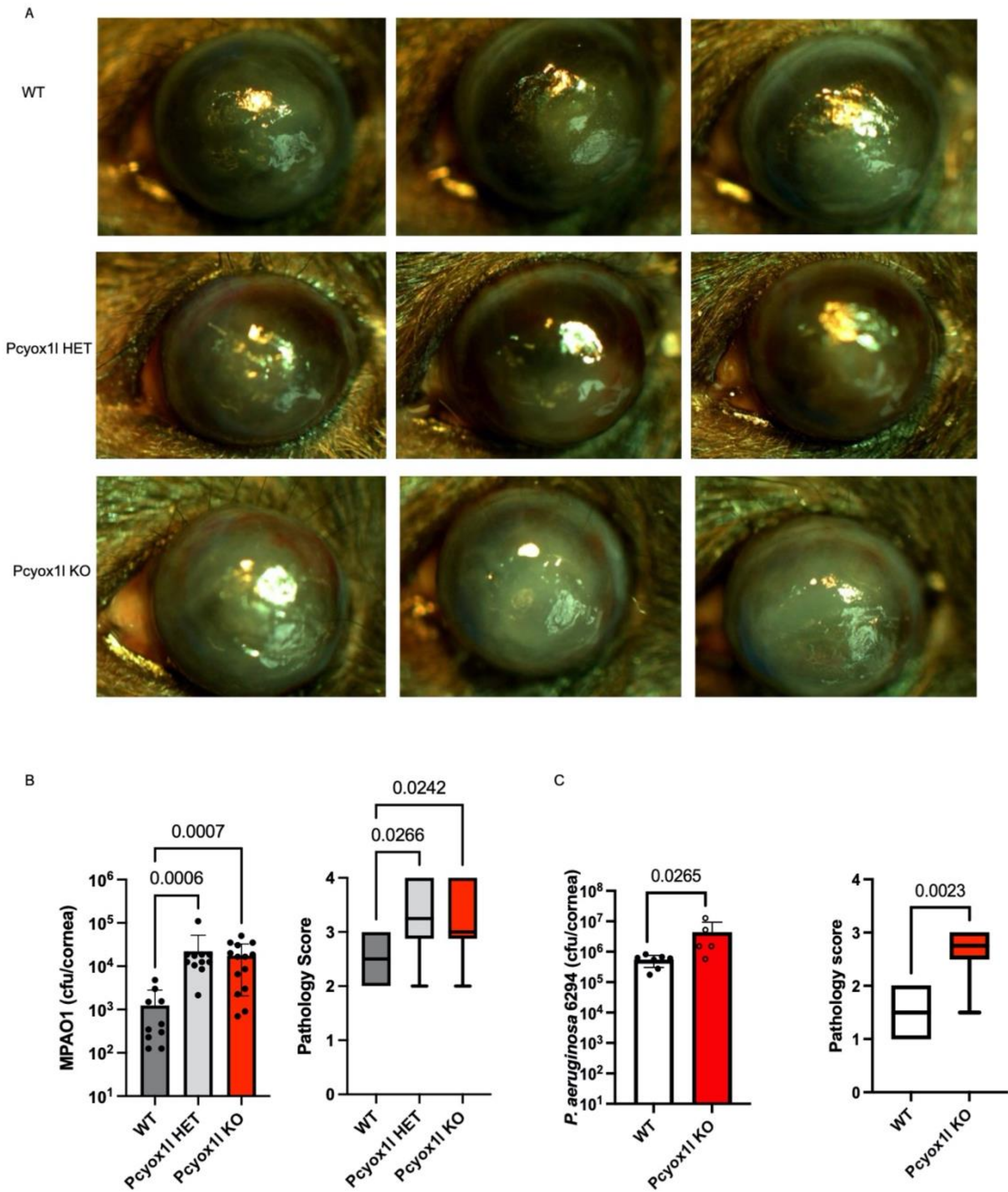


Figure 6. Ocular bacterial burden in Pcyox1L WT and KO mice.

To examine the impact of Pcyox1L deficiency on resistance to infection, we tested the sensitivity to P. aeruginosa-induced ocular infection. Pcyox1L KO mice showed elevated susceptibility to infection with the lab strain MPAO1 and the clinical isolate 6294.

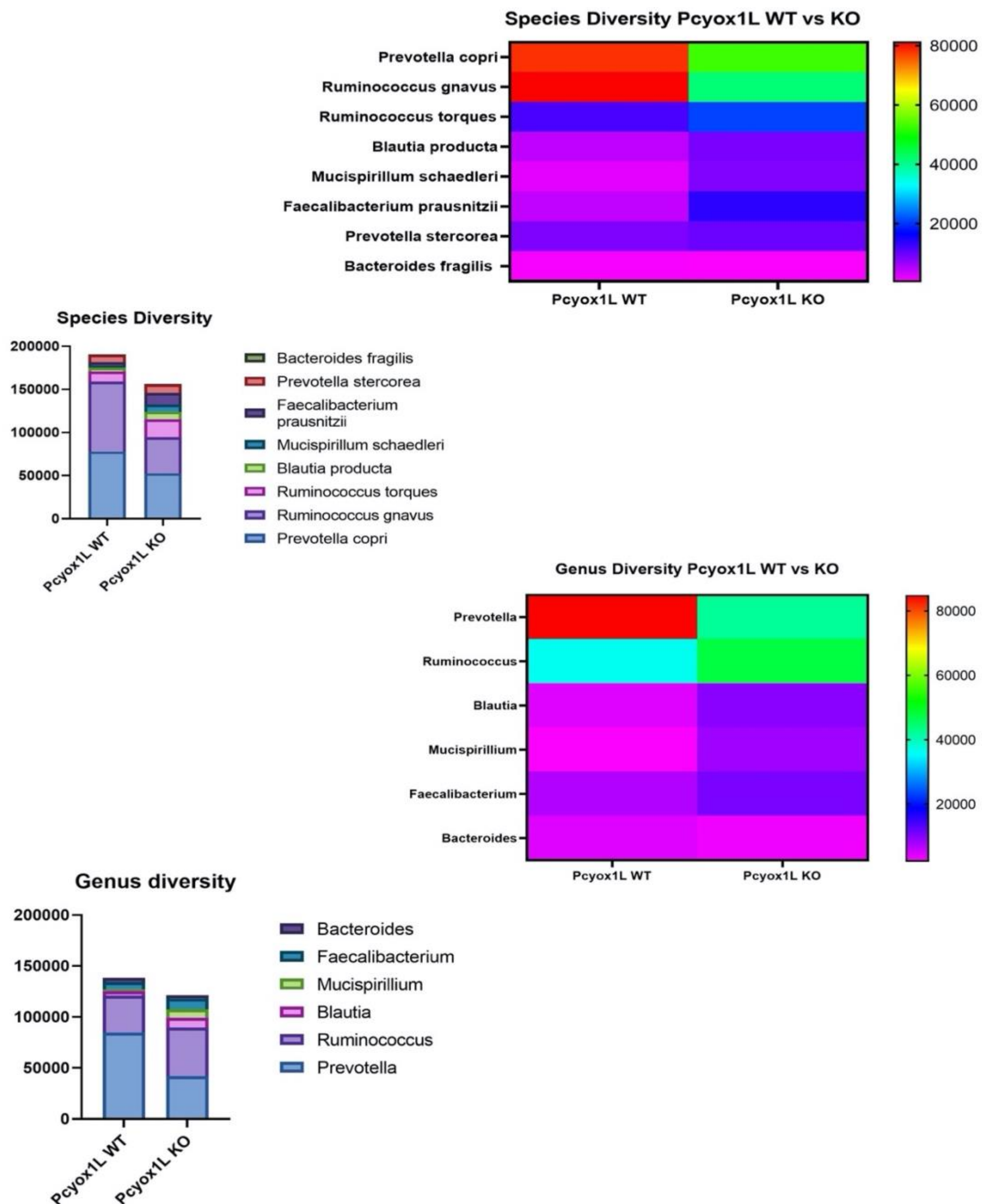


Figure 7. Pcyox1L KO mice show alterations of mucosal commensals.

16s metagenomic sequencing results. Heat Maps detailing the abundance of gut microbiota. Pcyox1L WT and KO mice overall differences in diversity between genus and species populations. The exhibition of slight differences d at the species level provided a

better understanding of the microbiome communities that inhabit these two different genotypes

Appendix 1.

Supplementary Figures

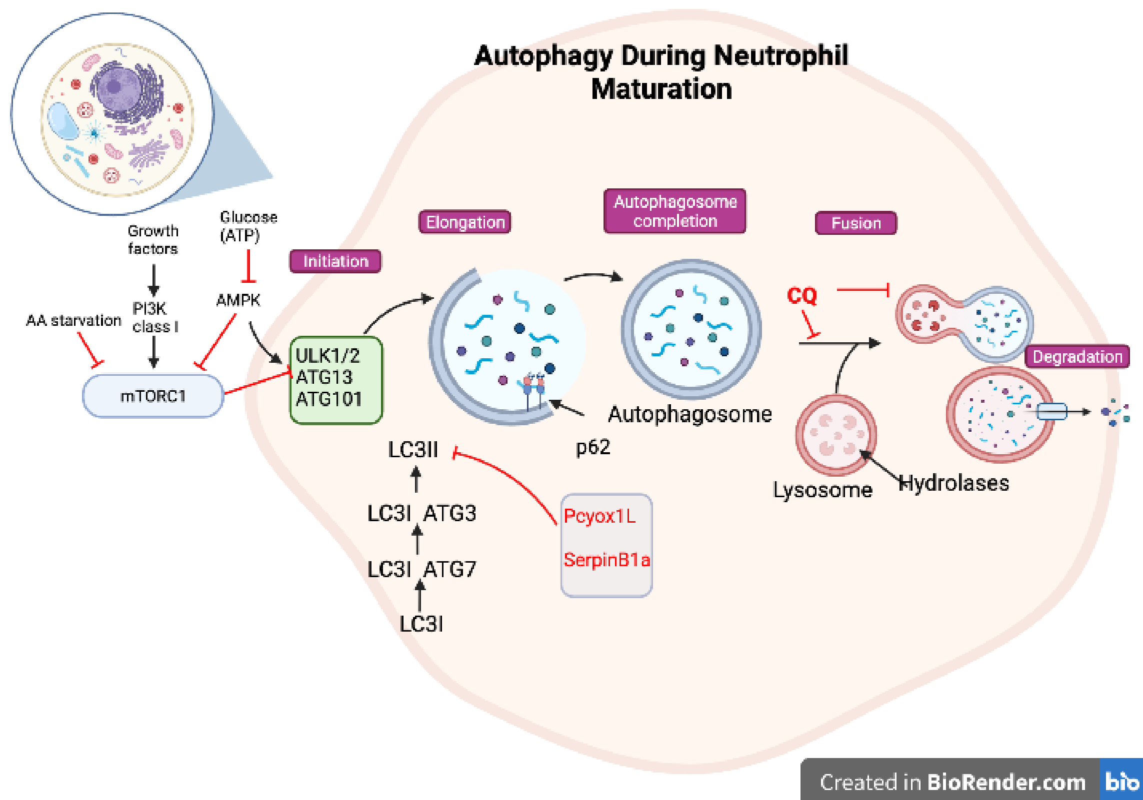


Figure 1. The 5 stages of autophagy during neutrophil maturation and how Pcyox1L and SerpinB1a contribute to the inhibition of autophagy.

Autophagy consists of 5 steps: initiation, elongation, formation of autophagosome, fusion with the lysosome, and degradation. The process of elongation results in a completed autophagosome. Two protein conjugation systems are essential in the step of elongation for the completion of a double membrane organelle.

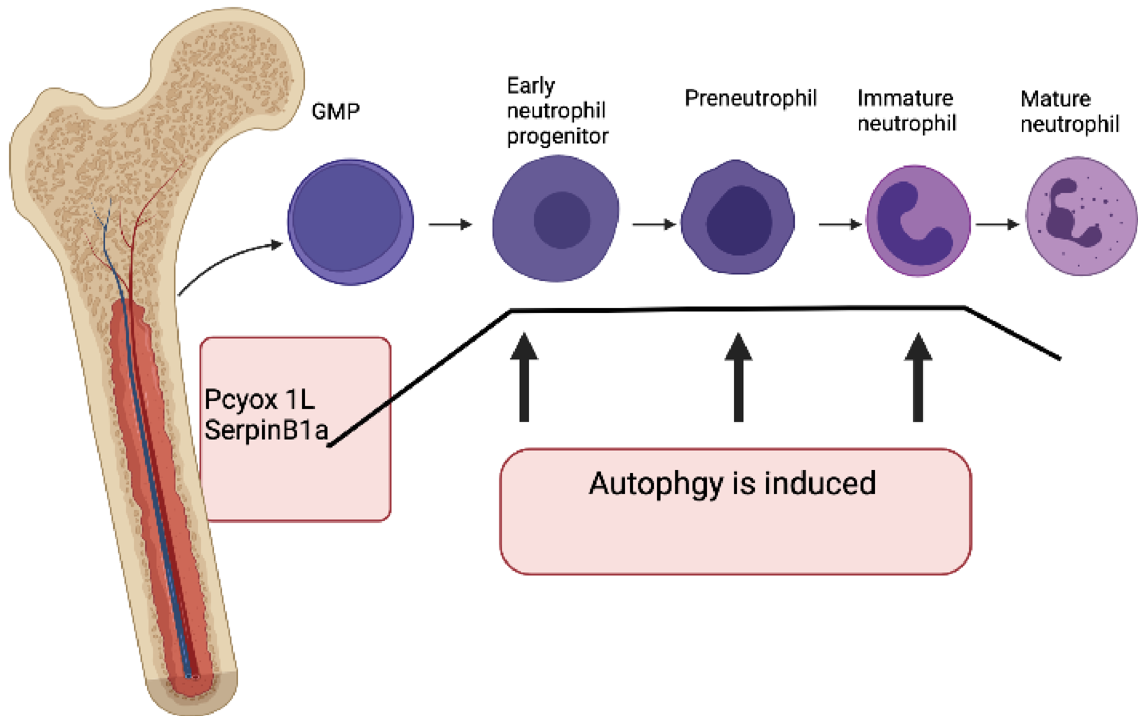


Figure 2. Pcyox1L and SerpinB1a induce autophagy during neutrophil maturation.

The induction of autophagy at the different stages of neutrophil development and where Pcyox1L and SerpinB1a initiate autophagy.

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