Phenotypical and Functional Characterization of Prenylcysteine Oxidase 1 Like (Pcyox1l) Knockout Murine Neutrophils.

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Abstract

Prenylcysteine oxidase 1 like (Pcyox1l) is a known protein coding gene where the protein has no described function. To examine the role of Pcyox1l on murine neutrophil function and metabolism, *in vitro* and *in vivo* experiments were performed on immortalized murine neutrophil cell lines and mice primary cells. This study demonstrated that the loss of Pcyox1l negatively impacts the viability of neutrophils *in vitro*. We further demonstrated Prenylcysteine oxidase 1 (Pcyox1) levels are increased with the absence of Pcyox1l in neutrophil cultures, suggesting a possible interaction between the two. We showed that the loss of Pcyox1l decreases prenylation, impacting functionality of prenylated proteins, and offering an explanation to the lowered effector function of Pcyox1l KO neutrophils against *P. aeruginosa*. Neutrophils are the most abundant leukocytes in mammals and one of the most important members of innate immunity. Understanding the effect of Pcyox1l can shed light on mechanisms that have potential therapeutic functions.

Dedication

To my mother To Wai Lai, who has sacrificed so much for me. To my love Zhang Jin, who provided me with unconditional love and support, a beacon of light during my difficult times.

Acknowledgments

My gratitude to Dr. Mihaela Gadjeva for her guidance and her sharp insights into the subject. I could not have done this without her expertise.

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Chapter I.

Introduction

Keratitis is the inflammation of the cornea. One cause of the illness is the infection by the bacteria *Pseudomonas aeruginosa*. Keratitis is a severe medical condition accounting for 5% of all vision loss cases and 30% of keratitis patients develop long-term moderate-to-severe vision loss (Ung et al. 2016). Current treatment methods include the use of antibiotics and the use of corticosteroids, yet all have their drawbacks that can lead to other complications.

The intense interest in commensal bacteria and microbiota in recent years have connected keratitis to commensal immunology. It has been demonstrated in Swiss Webster mice that SPF mice, which have a normal microbiota, are largely immune to *P. aeruginosa* while GF mice are heavily affected (Kugadas et al., 2016). Subsequent analysis pointed to a protein prenylcysteine oxidase 1 like (Pcyox1l) with no previously described function. In preliminary studies performed on Pcyox1l knockout (KO) and wildtype (WT) C57BL/6 mice, it was shown that the KO mice are easily infected with *P. aeruginosa* while the WT are not. Therefore, the study of Pcyox1l can better inform us on the immunity against diseases like keratitis.

Definition of Terms

Antimicrobial protein/peptide: Proteins produced by a cell that can prevent infection by pathogens.

Chemokine: Signaling protein that induce chemotaxis.

Chemotaxis: The movement of cells in response to chemokine.

Cytokine: Collective term of chemokines, interferons, interleukins, lymphokines, and tumor necrosis factors. Proteins for cell signaling.

Dysbiosis: An imbalance in the composition or function of the microbial species that are normally found in mammalian hosts.

Germ-free (GF) mice: A line of mice that does not have any microorganism living in them.

Specific-pathogen-free (SPF) mice: A line of mice that does not carry a specific pathogen.

Keratitis: Inflammation of cornea.

Metagenomic: The study of genetic material recovered directly from environmental samples.

Microbiota: An ecosystem of microorganisms, include those that are commensal, symbiotic and pathogenic, that exist in the body.

Mucosal immunology: Study of microbiota on the surface of mucosa and their effect on the immune system.

Neutropenia: Very low neutrophil count in the blood.

Pcyox1: PCYOX1 gene encodes the enzyme prenylcysteine oxidase 1.

Pcyox1l: PCYOX1L gene encodes the enzyme prenylcysteine oxidase 1 like, which has no described function.

Proteomic analysis: The study of all proteins that are produced by a cell.

Metabolomic analysis: The study of metabolite produced by a cell

Neutrophils

Neutrophils are the most abundant leukocytes in mammals. It is known as a member of granulocytes or polymorphonuclear (PMN) leukocytes along with basophils and eosinophils. Development into mature neutrophil starts with pluripotent hematopoietic stem cell in the bone marrow, then into multipotent common myeloid progenitor cells, to granulocyte–monocyte progenitor which will terminally differentiate into granulocytes or monocytes. The neutrophil subset contains pre neutrophil, which differentiates into non-proliferating immature neutrophils, and later to mature neutrophils. Mature neutrophils then enter circulation. In this study the mature neutrophils will be marked as CD45R-/NK1.1-/CD90-/TER119-/SiglecF-/CD11b+/cKIT-/CXCR4-/Ly6G+/CXCR2+ (Evrard et al., 2018).

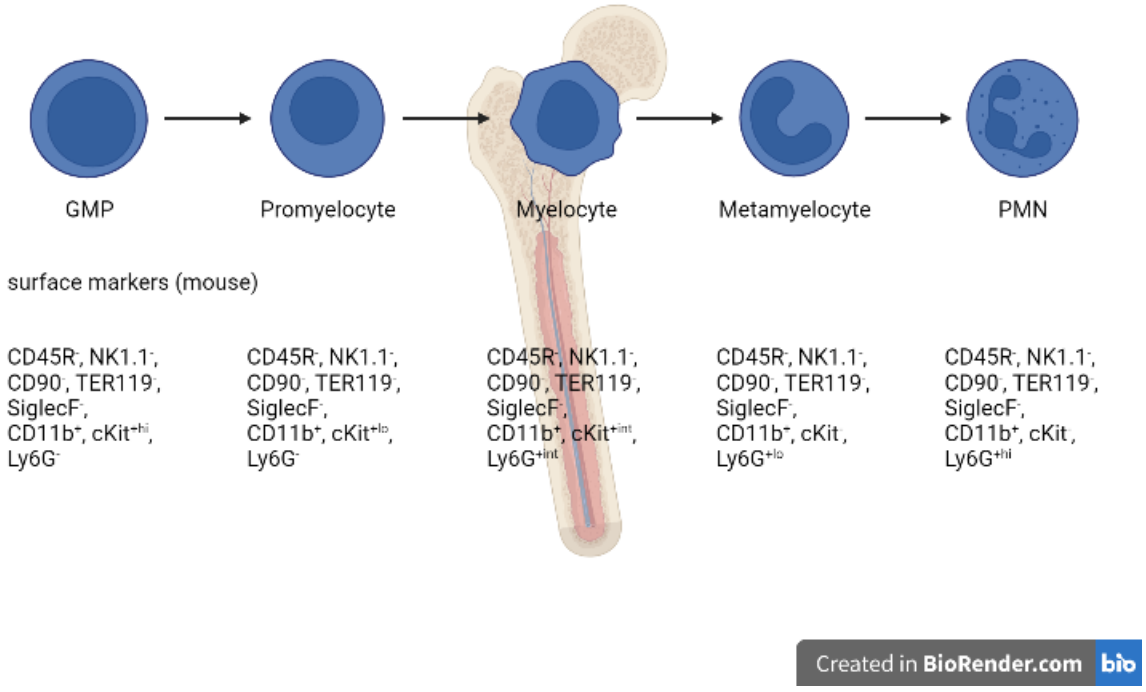

Figure 1. Stages of murine cell development from GMP to PMN.

Murine GMP cells undergo a number of stages of development, with cell surface markers shown.

Mature neutrophils in circulation are short lived and mobile. They await signals from site of infection or inflammation which include interleukin-8 (IL-8) and interferon gamma (IFN-γ). Upon receiving the signal, they will chemotaxis towards the target following the concentration of chemokines. Their function includes phagocytosis, the direct engulfment and digestion of pathogens. They can release antimicrobial proteins that destroys pathogen by various mechanisms. They can produce NETs, where they release DNA in the form of chromatin, forming a web that limits the movement of pathogens and also creates an environment with high concentration of antimicrobial proteins. They can also send signals to recruit more leukocytes to the site.

Microbiota

All multicellular organisms are covered with microorganisms inside and out. Different organs are colonized by different microbiota. Among many systems found in human, the gut microbiota receives the most attention. The colon has the highest density of microorganisms, comprising more than 1013 individual microbial cells (Rook & Garret, 2016). Large scale projects such as the Human Microbiome Project and the Metagenomics of the Human Intestinal Tract (MetaHIT) have defined the species of microorganisms in the gut microbiota (Qin et al., 2010). The composition varies greatly between individuals, and varies even in the same individual over time. Therefore, it is difficult to define an ideal microbiota.

It has long been established that the gut microbiota plays a pivotal role in the development and differentiation of hematopoietic progenitors. Evidence of the connection between microbiota and hematopoiesis was first derived from murine studies of germ-free (GF) mice, which lack microbiota entirely. These mice have well-known abnormalities in BM cell populations, smaller hematopoietic stem, and progenitor cell (HSPC) populations, and abnormal systemic peripheral and splenic myeloid cell function compared to their SPF counterparts (Yan et al., 2018). Although a reduction of granulocyte-macrophage progenitors (GMPs) is reported in GF mice, it has been shown that the introduction of human commensal bacteria *Clostridium scindens* in microbiota depleted mice increases GMPs populations in the BM (Burgess et al., 2020).

A more direct link between microbial dysbiosis and hematopoietic alterations is observed in patients receiving antibiotics. Antibiotic treatment is widely associated with different types of cytopenia, including neutropenia. Several recent studies indicate that

antibiotics impair normal hematopoiesis by depleting intestinal bacteria. For example, 5% to 15% of patients treated with B-lactam antibiotics for ten or more days developed neutropenia. Of these patients, 94% recovered neutrophil counts after stopping treatment. Importantly, hematologic abnormalities due to antibiotics are not limited to a specific antibiotic class (Neftel et al., 1985). This finding was corroborated in studies published in clinical cases reports that neutropenia is one of the most common adverse drug-related effects of outpatient parenteral antimicrobial therapy (OPAT) in patients regardless of the antibiotic agent used. Furthermore, TMP-SMZ is widely known to cause neutropenia, but the rate of TMP-SMZ mediated neutropenia is lower than that reported for other antibiotics, such as B-lactams (Khosravi et al., 2014). All in all, the prolonged use of oral antibiotics develops BM suppression. These effects are mostly independent of treatment duration, absorption, and type of antibiotic used.

Evidence indicates that the reduction or absence of microbiota, as induced by long term antibiotic treatment or germ-free state, is profoundly associated with reducing neutrophil numbers and their progenitors. Suppression of granulocytes and monocyte numbers in GF mice or antibiotics-treated mice was shown to be rescued by the oral provision of microbe-associated molecular pattern (MAMPs) from the gut microbiota or by recolonization with healthy microbiota. Consistent with these findings, a heat-resistant component of *E. coli* in serum was found to restore BM myeloid cell populations in GF mice (Iwamura et al., 2017). In their study, Iwamura et al., reported that nucleotide-binding oligomerization domain-containing protein 1 ligand (NOD1L), a heat-stable component of the peptidoglycan structure of *E. coli*, increased systemic levels of HSPC proliferation-stimulating cytokines in the serum of GF mice. These growth cytokines

included stem cells factor (SCF), IL-7, Flt3L, and ThPO and are produced in large part by MSCs in the BM niche. In summary, the intestinal microbiota products, such as NOD1L, enter the bloodstream and travel to the BM, where they promote the production of growth cytokines that support normal hematopoiesis.

Although progress has been made in determining the signals produced by intestinal microbes that trigger normal hematopoiesis, the host cells, and receptors by which those signals are detected have not yet been identified. However, studies have established that sensing of commensal microbiota through the TLR-MyD88 signaling pathway triggers several critical responses for maintaining host-microbial homeostasis, including the generation of hematopoietic progenitors. Stat 1-/- mice had BM HSPC and granulocyte counts as low as antibiotic-treated wild-type mice, and treating Stat1 -/- mice with antibiotics did not further suppress cell counts, suggesting that STAT1 signaling stimulated by the microbiota is required for normal hematopoiesis (Khosravi et al., 2014). These data indicate that basal inflammatory signaling, such as MyD88/TICAM, NOD1, and STAT1, is required to maintain normal hematopoiesis. All in all, murine studies now show that antibiotic-induced microbiota depletion in BM suppression is due to the absence of heat-stable microbial products that can circulate in the bloodstream and promote hematopoiesis through basal inflammatory signaling.

Keratitis

Keratitis is the inflammation of the cornea. One cause of the illness is the infection by the bacteria *Pseudomonas aeruginosa*. Keratitis is a severe medical condition accounting for 5% of all vision loss cases and 30% of keratitis patients develop long-term moderate-to-severe vision loss (Ung et al. 2016). Currently, there are three major methods of treatments, each with their own shortfall. The first and most traditional method is the simple application of antibiotic ointments. Antibiotics will kill all *P. aeruginosa* along with the rest of microbiota. With the modern understanding of the importance of microbiota this condition itself can lead to further complications. And even after pathogens are killed, the existing inflammation, caused by the influx of neutrophils that aimed to clear infection, can cause tissue damage in cornea tissue. The emergence of antimicrobial resistance may soon render antibiotic treatments unless all together (Ung et al. 2016). The second method focus on the inflammation caused by neutrophil influx. If neutrophil is responsible for inflammatory tissue damage, preventing neutrophil migration can prevent such damage. However, without an abundance of neutrophil at the infection site, *P. aeruginosa* cannot be eradicated effectively. The third method combines the use of antibiotics and corticosteroids. Antibiotics are first applied to kill *P. aeruginosa*, once that is achieved, corticosteroids are applied to decrease the inflammation (Ung et al. 2021). This method has been shown to be the most sophisticated and successful treatment of keratitis. However, with the usage of antibiotics the disruption of microbiota will still occur, leading to other long-term issues. More importantly, corticosteroids have been associated with glaucoma, an equally serious

condition that can result in blindness (Kersey and Broadway, 2006). As such, all of the commonly used strategies produce significant drawback.

With the intense interest in microbiota in recent years, focus of Keratitis has been shifted toward the relationship between the disease and microbiota. Swiss Webster mice are typically immune to the infection by *P. aeruginosa*. However, the removal of microbiota by antibiotics renders the mice susceptible. Curiously, removal of microbiota in the eye alone, in the gut alone, or in both eye and gut produced the same susceptibility (Kugadas et al., 2016). It appears microbiota has a strong immunity effect against *P. aeruginosa*. It is believed that microbiota prime innate immunity to produce both a bactericidal effect and anti-inflammatory effect.

Pcyox1l

As an extension to the above study, Gadjeva et al. studied the proteome of neutrophils depending on microbiota presence and infection state. Neutrophils generate unique proteome signatures for each state. This suggests that neutrophil response is not rigid. In the study Gedjeva et al. focused on murine Pcyox1l (prenylcysteine oxidase 1 like) gene that produce an enzyme with no described function. In preliminary study, Pcyox1l shares 88% homology with two predicted human transcripts and 94% amino acid homology. Preliminary study performed on C57BL/6 mice by Gadjeva et al. have shown that SPF mice with a Pcyox1l knockout produce a severe infection when infected with *P. aeruginosa* while WT mice is mostly immune, suggesting that Pcyox1l have a strong relationship to the immunity against *P. aeruginosa*.

Research Aims

Specific Aim 1: Characterization of Pcyox1l KO murine PMN cells *in vitro*. Anecdotal evidence has shown that in GMP cell cultures Pcyox1l KO cells produce less viable cells than the WT counterpart. Cell numbers, both live and dead, will be counted during cell maturation, which will provide definitive data on the mortality of Pcyox1l KO cells *in vitro*. To better understand the biochemistry of Pcyox1l, a metabolomic study will be performed. Structural analysis revealed that Pcyox1l contains a lyase domain similar to Pcyox1, thereby likely participating in the mevalonate/prenylation pathway. Farnesylation and geranylgeranylation are the main mechanisms for protein prenylation. A measurement of mass of farnesyl cysteine and geranylgeranyl cysteine between Pcyox1l KO and WT neutrophils can shed light on Pcyox1l activity and the status of the prenylation pathway. I hypothesized that if Pcyox1l controls the prenylation pathway, then the relative levels of either the precursors for protein prenylation or the metabolites arising from their degradation will be altered. The previously described Pcyox1 is similar to Pcyox1l structurally and may be redundant. To illustrate the possibility that Pcyox1 and Pcyox1l activities are interdependent, Pcyox1 level will be measured in Pcyox1l KO and WT cell lines.

Specific Aim 2: Characterization of Pcyox1l KO mice *in vivo*. Pcyox1l KO mice colony will be maintained and litters will be genotyped by PCR. Given that Pcyox1l KO mice are more susceptible to *P. aeruginosa* bacterial infection, it is reasonable to believe that Pcyox1l KO neutrophils could have certain defects. Mice will be sacrificed, blood collected for complete blood count (CBC). Their bone marrow (BM), spleen, and peripheral blood will be collected for neutrophil study by flow cytometry. Similar to the

rationale behind the metabolomic study in specific aim 1, farnesyl cysteine and geranylgeranyl cysteine will be measured. Lastly, to provide support that human Pcyox1l and murine Pcyox1l are similar, and the study of murine Pcyox1l is a good precursor to future human Pcyxo1l studies, human neutrophil Pcyox1l will be blotted using the same methodology as the murine counterpart.

With this study, we can better understand the previously undescribed Pcyox1l. It will provide a better understanding in the immunological pathway to the resistance against *P. aeruginosa* keratitis, potentially pave the way to the development of novel treatment method involving the manipulation of neutrophil development at cornea.

Chapter II.

Material and 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 later.

Neutrophil Cell Lines and Treatments

Existing murine GMP Pcyox1l KO (named F4, F5, F6 cell lines) and WT (H8) cell cultures were maintained in RPMI-1640 medium (cat no 61870127, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum (FBS) (cat no 10438026 Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (cat no 15140122, Thermo Fisher Scientific, Waltham, MA, USA), 2 mM L-Glutamine (cat no 25030081, Thermo Fisher Scientific, Waltham, MA, USA) 2% stem cell factor (SCF), with 0.5 μM β-estradiol (collectively named E+ medium) to prevent maturation and promote cell expansion. To mature GMP into neutrophils, all estradiol were removed. GMP were washed with PBS for at least 3 times, then suspend in the same medium described above without β-estradiol (collectively named E- medium). Maturation was stimulated in 3 different conditions for 72 hours. 1. In E- medium. 2. In E- medium with 80ng/ml GCSF (cat no 574602, BioLegend, San

Diego, CA, USA). 3. In E-medium and 80ng/ml GCSF, with 5ng/ml GM-CSF (cat no 576302, BioLegend, San Diego, CA, USA) added 48 hours after initiation. Cytospin was used to demonstrate that cells have developed a multilobed nucleus which indicates that they are maturing into neutrophils but not other cells types like monocytes.

Western Blot Analysis

To ensure no contamination of the cultured cell lines, western blot was used to validate the KO cells. Pcyox1l is a 55kDa protein that should present in the WT cells but absent in the KO cells. To prepare cell lysate from the KO and WT cell lines, RIPA buffer and Protease Inhibitor Cocktail (cat no 11836170001, Roche, Basel, Switzerland) were added to palleted cells in 4°C and on ice. The mixtures were well mixed with pipette and then centrifuged. Pallets were discarded and the supernatants transferred to new Eppendorf tubes for long term storage. To perform electrolysis, a NuPAGE 4–12% Bis-Tris gel (cat no NP0335BOX, Invitrogen, Carlsbad, CA, USA) was used. Lysate proteins were separated in MOPS buffer (cat no NP0001, Invitrogen, Carlsbad, CA, USA) under 200V for 95 minutes. Proteins from the gel were be transferred to a PVDF membrane (cat no LC2002, Invitrogen, Carlsbad, CA, USA) under 30V for 60 minutes. Membrane was blocked with 5% milk in TBST for 1 hour. Anti-Pcyox1l antibody (cat no orb35904, Biorbyt, St Louis, MO, USA) was diluted in 5% milk in TBST at a concentration of 1:500. 5ml of this primary antibody was added to the membrane and incubate in 4°C overnight. Membrane was then be washed in TBST. Secondary antibody Goat anti-rabbit IgG (cat no 4030-05, SouthernBiotech, Birmingham, AL, USA) was diluted in 5% milk in TBST at a concentration of 1:2500 and added to the membrane at room temperature for 1 hour. Chemiluminescence image was taken using West Femto

Maximum Sensitivity Substrate (cat no 34096, Thermo Fisher Scientific, Waltham, MA, USA) in ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA) to confirm the absence of 55kDa Pcyox1l band. Antibodies were then stripped and GAPDH will be used to normalize protein volume. Anti-GAPDH antibody (cat no MAB374, Sigma-Aldrich, St Louis MO, USA) will be diluted in 5% milk in TBST at a concentration of 1:2000. 5ml of this primary antibody was added to the membrane and incubate in 4°C overnight. Membrane was then washed in TBST. Secondary antibody anti-mouse (cat no 1031-05, SouthernBiotech, Birmingham, AL, USA) was diluted in 5% milk in TBST at a concentration of 1:5000 and added to the membrane at room temperature for 1 hour. Chemiluminescence images were taken as above.

Metabolomic Analysis of Cell Lines

To perform metabolomic study, a large number of cells is needed. KO and WT cells were matured for 3 days in E- medium, E- medium plus G-CSF, and E- medium plus G-CSF plus GM-CSG as described in methods. Refer to table 1 below.

12 million WT cells in 24ml E-medium	12 million KO cells in 24ml E-medium
12 million WT cells in 24ml E-medium G-CSF (80ng/ml)	12 million KO cells in 24ml E-medium G-CSF (80ng/ml)
12 million WT cells in 24ml E-medium G-CSF (80ng/ml) GM-CSF (5ng/ml) added 48 hours later	12 million KO cells in 24ml E-medium G-CSF (80ng/ml) GM-CSF (5ng/ml) added 48 hours later

Table 1. Cell preparation for metabolomic study.

After 3 days of maturation there were more than 30 million cells for each condition. Triplicates of 10 million cells were made, and cells were placed in cryotubes. Cells were

centrifuged and palleted to remove supernatant. Cryotubes were placed in liquid nitrogen for 2 minutes to lyse cells. Mass spectrometer analysis was outsourced to a third-party Creative Proteomics Inc. (Shirley, NY, USA) to measure geranylgeranylpyrophosphate (GGPP), farnesyl pyrophosphate (FPP), geranylgeranyl-cysteine, and farnesyl cysteine.

Animals and Genotyping

Pcyox1l KO mice colony were maintained in a pathogen-free facility. Litters were genotyped by PCR. Biopsy of ear were taken from mice. A digestion buffer containing Mercaptoethanol, Tris base, NH4SO3, MgCl2, Triton X-100 (cat no T8787, Sigma-Aldrich, St Louis MO, USA) was added to collected ear samples. The mixture was heated at 95°C for 3 minutes and Proteinase kinase (cat no 158918, Qiagen, Hilden, Germany) added immediately after. Mixture was placed in a 55°C water bath overnight. Samples were reheated at 95°C for 5 minutes to arrest the digestion. To perform PCR, the following primers (Integrated DNA Technologies Coralville, IA,USA) were used.

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

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

49020-wtF: 5' ACAGTTCAATGCAGGCTCAG 3' (WT primer)

Taq DNA Polymerase, PCR reaction buffer, 50 mM $MgCl_2$ (cat no 18038042, Invitrogen, Carlsbad, CA, USA), 2.5 mM dNTPs (cat no 10297018, Invitrogen, Carlsbad, CA, USA) were added to 0.3ul of ear extract. Samples were placed in a thermal cycler. Initiation at 94°C for 2 minutes, denaturation at 94°C for 10 seconds, annealing starts at 65°C and decreasing 1°C each cycle for 30 seconds repeated 10 times, elongation at 68 for 2 minutes, denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, repeated 25 times. After PCR, samples were added to a gel made with 2% agarose (cat no

A9539250G, Sigma-Aldrich, St Louis MO, USA) in TAE and 10mg/ml Ethidium bromide (cat no 15585011, Invitrogen, Carlsbad, CA, USA). DNA were separated with gel electrophoresis at 90V for 35 minutes. Ethidium bromide image showed the WT allele band at 227bp, Pcyox1l KO allele band at 280bp.

Complete Blood Count and Flow Cytometry

Mice 7-9 weeks of age were sacrificed. To perform complete blood counts, peripheral blood (50-100 ul) was sampled (retro-orbital) in anesthetized mice (isoflurane) and collected in heparinized capillary tubes. The blood was transferred to EDTA-containing tubes and the complete blood counts (CBCs) performed on an Heska Element HT5 analyzer.

To perform flow cytometric characterization of wild-type and Pcyox1l knock-out mice under homeostatic conditions, peripheral blood was sampled (retro-orbital) in anesthetized mice (isoflurane) and collected in heparinized capillary tubes. Hypotonic red blood cell lysis (ACK) was used to deplete red blood cells and the white blood cells were resuspended in FACS buffer (PBS, 2%FBS, 1 mM EDTA) prior to antibody staining for flow cytometry.

The spleen was dissected and macerated through a 40 micron filter using the rubber end of a 5 cc syringe plunger. Hypotonic red blood cell lysis (ACK) was used to deplete red blood cells and the white blood cells were resuspended in FACS buffer (PBS, 2%FBS, 1 mM EDTA) prior to antibody staining for flow cytometry.

The femurs were dissected and one end of the femur cut off with scissors. The inverted femur was transferred to a PCR tube in which a hole had been punched through the bottom with a 16G needle. The femur and PCR tube were placed inside a 1.5 mL tube

into which 200 ul of FACS buffer was aliquoted. A quick centrifugation (10-20 seconds, 10,000g) was used to “spin-flush” the bone marrow cells out of the femur and into the FACS buffer. The cells were resuspended quickly to prevent clotting. Hypotonic red blood cell lysis (ACK) was used to deplete red blood cells and the white blood cells were resuspended in FACS buffer (PBS, 2%FBS, 1 mM EDTA) prior to antibody staining for flow cytometry. Cells were stained with 3 different sets of florescence antibody for 3 different analyses.

Metabolomic Analysis of Primary Cells

To perform metabolomic study, mice aged 7 - 9 weeks were sacrificed and BM harvested. Comparison was made between 3 Pcyox1l KO mice and 3 WT mice. BM was collected from femur. The femurs were dissected and one end of the femur cut off with scissors. A syringe filled with PBS and 25G needle was used to flush BM our of femur into a conical tube. A 16G needle with syringe was used to homogenize the extracted BM with PBS. Neutrophils were purified using EasySep™ Mouse Neutrophil Enrichment Kit (Catalog # 19762, Stemcell technologies, Vancouver, Canada) following the instructions provided by manufacturer. 4 million of purified neutrophils were placed in cryotubes, palleted and discarded supernatant. Cryotubes were placed in liquid nitrogen for 2 minutes to lyse cells. Mass spectrometer analysis was outsourced to a third-party company Creative Proteomics Inc. (Shirley, NY, USA) to measure geranylgeranyl-cysteine.

Pcyox1l in Human Blood Neutrophils and HL-60 Cell Line

Human blood samples were provided by an adult male. Blood was drawn in to EDTA filled blood collection tube. Neutrophils will be purified using EasySep™ Direct Human Neutrophil Isolation Kit (Catalog # 19666, Stemcell technologies, Vancouver, Canada) following instructions provided by manufacturer. Purified neutrophils were lysed and proceed to Pcyox1l western blot analysis as described above.

Chapter III.

Results

Studying *in vitro* Pcyox1l KO Cell Lines

Immortalized cell lines, both WT (H8) and Pcyox1l KO (F4, F5, F6) are validated regularly by western blot (Figure 2). Pcyox1l is a 55kDa protein that is missing in the Pcyox1l KO cell lines. The cell lines were maintained as immature GMP cells.

To ensure that the maturation condition can indeed turn GMP into mature and immature neutrophils, Cytospin was used to visualize mature cells (Figure 3 and 4). GMP cells were stimulated with G-CSF and G-CSF+GM-CSF as described in Methods. The resulting mature cells showed the distinctive multilobed nucleus and donut shaped nucleus, indicating that they have differentiated into immature and matured neutrophils. Counting both dead and alive cells following maturation showed that Pcyox1l KO cells consistently produce less live cells and more dead cells than WT cell lines (Figure 5), implying that Pcyox1l KO neutrophils have increased mortality rates.

In mevalonate pathway Geranylgeranylpyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) are added onto proteins to generate their prenylated form, while geranylgeranylcysteine and farnesylcysteine metabolites arise from the degradation of the prenylated proteins (Figure 6A). Under GCSF maturation condition, GGPP, FPP, geranylgeranylcysteine, and farnesylcysteine levels were significantly lower in Pcyox1l KO cells than WT cells (Figure 6B and 6C). Additionally, GGPP and farnesylcysteine under vehicle condition were lower in Pcyox1l KO cells; farnesylcysteine under

GCSF/GM-CSF condition was lower in Pcyox1l KO cells (Figure 7). Data shows that there was decreased isoprene metabolites and *de novo* prenylated proteomes in the absence of Pcyox1l functionality, and by extension also shows a significant reduction of the mevalonate pathway. Given that this decrease is observed despite the presence of established prenylcystinelyase Pcyox1 in the Pcyox1l KO cells (data not shown), we conclude that not only is Pcyox1l a key enzyme in the mevalonate pathway, but it also has a larger role than the previously characterized enzyme Pcyox1.

To show a possible relationship between Pcyox1l and Pcyox1, western blot on Pcyox1 was performed on WT cell line and Pcyox1l KO cell lines (Figure 9). Pcyox1 level is higher in Pcyox1l KO cells than WT cells. It is possible the absence of Pcyox1l causes the upregulation of Pcyox1 in a compensatory mechanism.

Studying Neutrophils *in vivo* in Pcyox1l KO Mice

The same metabolomic study as described above was performed using mice primary cells. Mice were sacrificed and their BM collected. 4 million purified neutrophils were collected and frozen in liquid nitrogen. Samples were sent to Creative Proteomics Inc., where mass spectrometer analysis was performed. Figure 6D shows significantly less free geranylgeranyl cysteine were detected in primary BM-derived PMNs from Pcyox1l KO mice when compared to WT littermates. This result is consistent with the similar experiment with cell lines (Figure 6B and 6C).

Uninfected (homeostatic) WT and Pcyox1l KO mice are characterized by complete blood counts and flow cytometry to study myeloid cells and neutrophils in the peripheral blood, spleen, and bone marrow. Pcyox1l KO mice did not show differences in white blood cell counts, absolute neutrophil counts, or hematocrit (Figure 8A-C). The

frequency of mature neutrophils (CD11b+, GR1+) by flow cytometry did not differ between wild-type and KO mice in the peripheral blood, spleen or bone marrow (Figure 8 D-F). There was a small but significant reduction in the number of myeloid cells in the bone marrow in the Pcyox1l KO mice (Figure 8F).

To show that the mouse model used to study Pcyox1l KO protein can be applicable to human model and therefore capable of shedding light on human model, western blot was performed using human blood neutrophils and HL-60 cell line using the same experiment procedure and the same Pcyox1l antibody used in mouse model. The presence of pcyox1l band (Figure 10) shows that the mouse protein and human protein are similar, therefore it is reasonable to believe that the knowledge collected from mouse model is transferable to human model.

Chapter IV.

Discussion

This study shed light on the function of Pcyox1l protein. Pcyox1l has a predicted prenylcysteine lyase domain, suggesting that Pcyox1l may be involved in protein prenylation. Prenylation typically promotes peripheral association of the prenylated proteins with plasma and endomembranes and/or mediates interactions with other protein partners (Zhang & Casey, 1996). The process of adding a prenyl moiety to a protein is well described, however, the catabolic pathways responsible for the degradation of the prenylated proteins are poorly understood. The last step of the catabolic processing of a prenylated protein is the degradation of prenylcysteine metabolites to prenal, hydrogen peroxide and cysteine. This step is performed by the enzyme Pcyox1 alone (Herrera-Marcos et al., 2018; Huang et al., 2010). The pathway Pcyox1 KO cells use to perform prenylation and degradation is poorly understood, likely because the Pcyox1 KO mice had no major *in vivo* phenotypes (Beigneux et al., 2002). There is a possibility that other than Pcyox1, some other enzymes may exist to regulate the prenylation pathway. In this study we provide evidence that Pcyox1l, a predicted orthologue for Pcyox1, fundamentally controls the prenylation dynamics. We discovered that the Pcyox1l deficiency is clearly associated with reductions in *de novo* prenylation, reductions in prenylpyrophosphate, and prenylcysteine metabolites, pointing to the overall inhibition of the mevalonate pathway. Our data supports the conclusion that Pcyox1l has a regulatory role over prenylation.

The *de novo* prenylation of series of small GTPases including Rab1, Rab7, Rab5, Rab11, Rab14, Rab21, Rab24, Rab32 was reduced in the absence of Pcyox1l as reported in a parallel study by our group. Rab1 (Harper et al., 2010; Mochizuki et al., 2013; Ravikumar et al., 2010), Rab5 (Bridges et al., 2012; Ravikumar et al., 2008), Rab11 (Longatti et al., 2012; Longatti & Tooze 2012; Puri et al., 2018) and Rab 32 (Hirota & Tanaka, 2009) promote autophagosome initiation, while Rab7 (Jager et al., 2004; Liang et al., 2008), Rab 14 (Mauvezin et al., 2016), and Rab 21 (Jean et al., 2015) control autophagosome maturation and autophagosomelysosome formation. Rab24 localizes in spots decorated with LC3, mediating the clearance of late autophagic compartments after their acquisition of degradative capacity (Munafó & Colombo, 2012; Ylä-Anttila et al., 2015). Consistent with the decreases in the *de novo* prenylation of Rabs, our parallel study observed alterations in autophagy in matured neutrophil cell lines. These metabolic changes were associated with reduced cellular viability.

Complete blood count and flow cytometry in this study showed that Pcyox1l KO does not have an effect on the number of neutrophils or its maturation (except for the lowered number of myeloid cells in the BM). However, our parallel study shows that Pcyox1l KO mice have elevated susceptibility to *P. aeruginosa* infection. Therefore, Pcyox1l is related to the effector function of neutrophils. *In vitro* data shows that Pcyox1l KO cell line exhibit increased mortality (Figure 5). Future experiments utilizing cell fate tracing should address if similar processes take place *in vivo*.

A major method for neutrophils to destroy pathogens is to use reactive oxygen species (ROS). Our parallel study shows that prenylated Rac1 and Rac2 GTPases were 2 and 3-fold respectively decreased in the absence of Pcyox1l. Pcyox1l KO neutrophils

also produce less ROS than WT neutrophils, consistent with their lower ability to fight *P. aeruginosa* infections. ROS is produced by the membrane associated enzyme NADPH oxidase. The active form of this enzyme consists of the membrane bound cytochrome b558, and several associated proteins including Rac2 (Miyano & Sumimoto, 2012). When Rac2 is prenylated, its affinity to plasma membrane increases, and by coupling with cytochrome b558 the full NADPH oxidase is formed. It follows that in the situation of Pcyox1l KO, there is a lowered level of prenylation, therefore Rac2 is not bound to cytochrome b558, so it remains inactive and does not produce ROS. In effect, lack of Pcyox1l renders Rac2 useless, so the appearance should akin to Rac2 KO phenotype. Indeed, Rac2 deficiency in zebrafish resulted in elevated *P. aeruginosa* susceptibility due to reduced ROS production by neutrophils (Rosowski et al., 2016), and mouse Rac2 deficient neutrophils had impaired bactericidal functions and reduced superoxide production (Carstanjen et al., 2005; Nguyen et al., 2017). The variation in species also implied the evolutionary conservative nature of Rac2 and by extension Pcyox1l. Given that ROS is a major weapon that neutrophils use against pathogens, some pathogen developed a counter towards it. For instance, *adherent-invasive Escherichia coli (AIEC)* (Chargui et al., 2012), *P. aeruginosa* (Junkins et al., 2013; Zou et al., 2014), and *Streptococcus pneumoniae* (Ullah et al., 2017), can all dysregulate autophagolysosome formation. Promoting their survival and increasing bacterial burden of the host. Our results showing the role of Pcyox1l in prenylation connects with ROS production and *P. aeruginosa* keratitis, paving way for future studies to further illustrate the entwined relationship, and possibly therapeutics against keratitis.

Conclusion

This study provides insight into the function of Pcyox1l on prenylation metabolism. I demonstrated that the number neutrophils remain the same in Pcyox1l KO mice, the lowered immunity response of Pcyox1l KO mice is due to the lowered effector function of neutrophils. Pcyox1l is an enzyme that promotes protein prenylation, the lack of Pcyox1l will decrease *de novo* prenylation, and subsequently affect enzymes that require prenylation to function properly, for example the activity of NADPH oxidase and the decrease of ROS production in neutrophils. Consequently, the susceptibility towards diseases like *P. aeruginosa* keratitis increases.

Figures

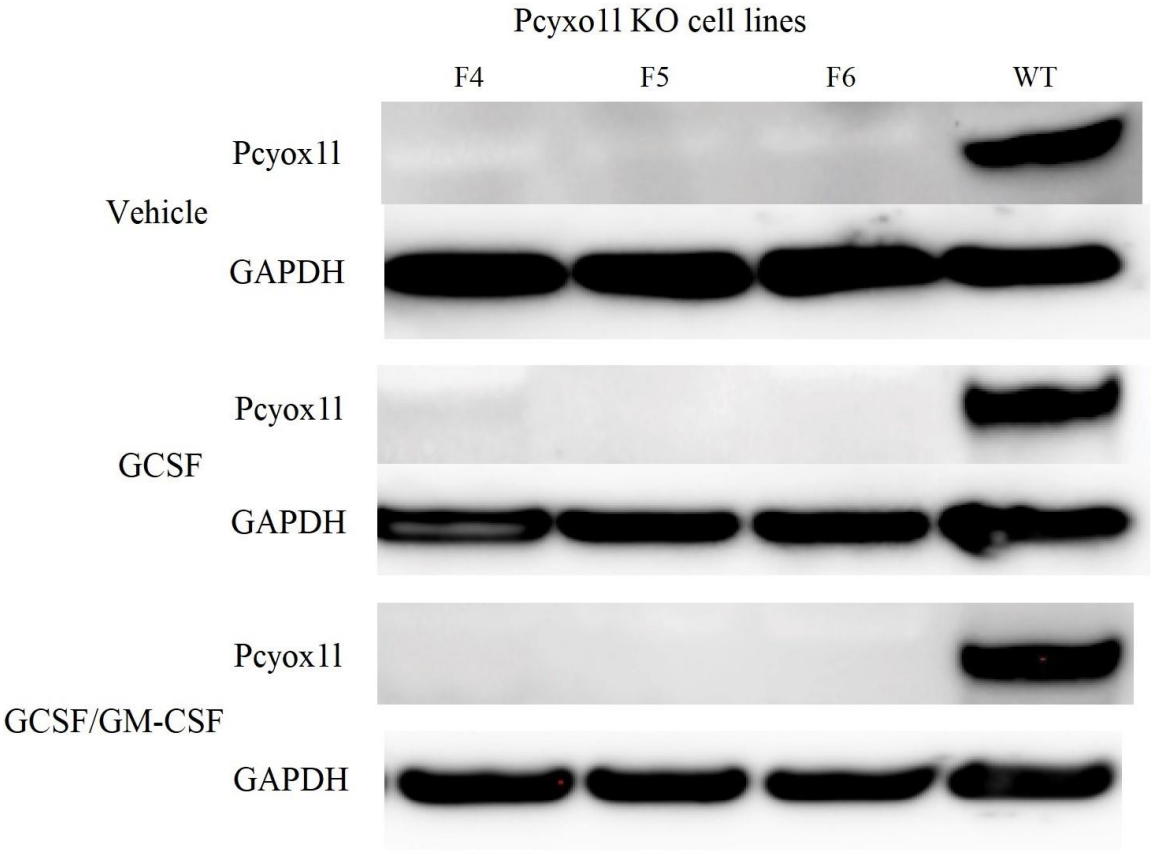

Figure 2. Cell line validation.

Validation of Pcyox1l KO cell lines by western blot. All the KO cells lack the 55kDa band that is present in WT cells.

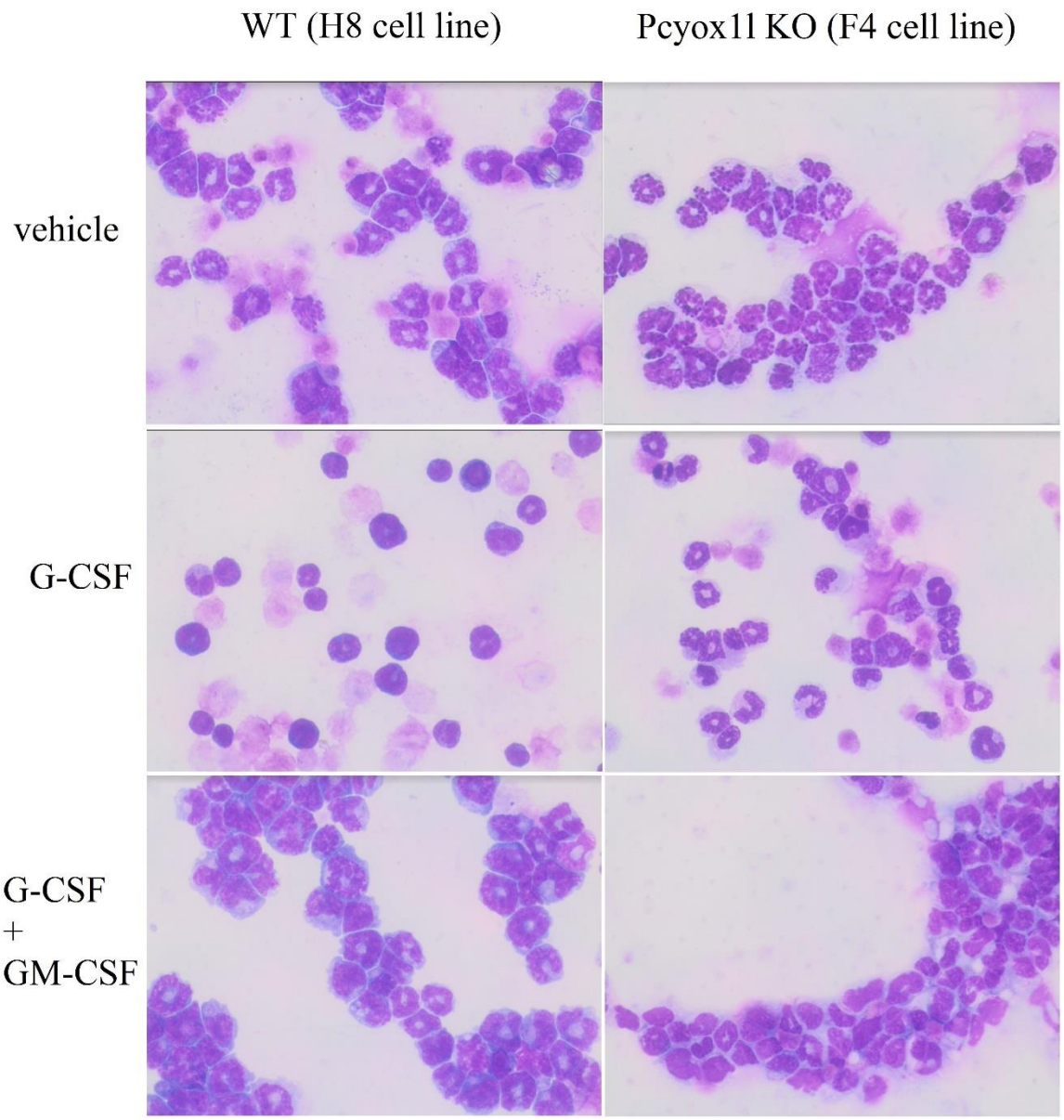

Figure 3. Cytospin of matured PMN cells.

Cytospin of *in vitro* cell line matured under vehicle, G-CSF, GM-CSF conditions. GMPs successfully developed into neutrophils, showing the multilobed and donut shaped nucleus.

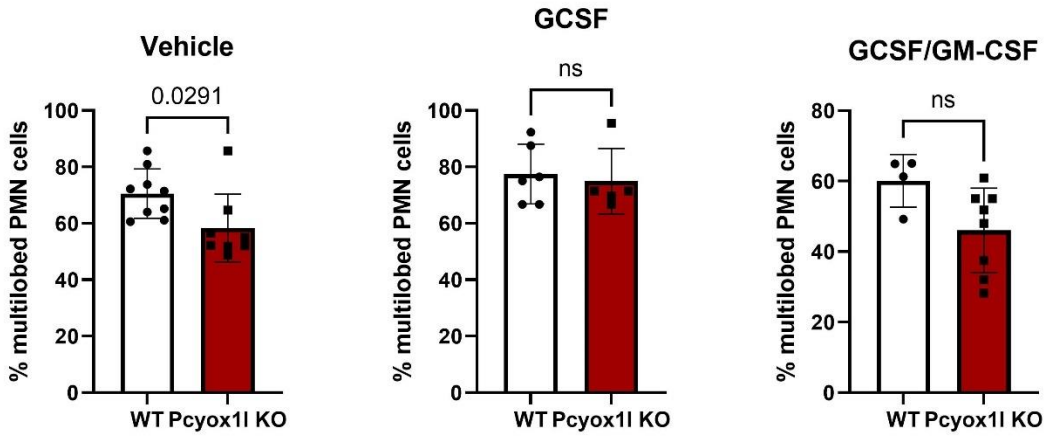

Figure 4. Percentage of cells displaying multilobed nucleus after Cytospin.

Cytospin of *in vitro* cell line matured under vehicle, GCSF, GCSF/GM-CSF conditions and subsequently have cells counted. Multiple pictures were taken with microscope from each Cytospin slide. Each picture is considered one field of view. Presented percentage is the number of cells with multilobed nucleus divided by total number of cells. Data are plotted as mean and SD. Each symbol represents one field of view. Student's t-test.

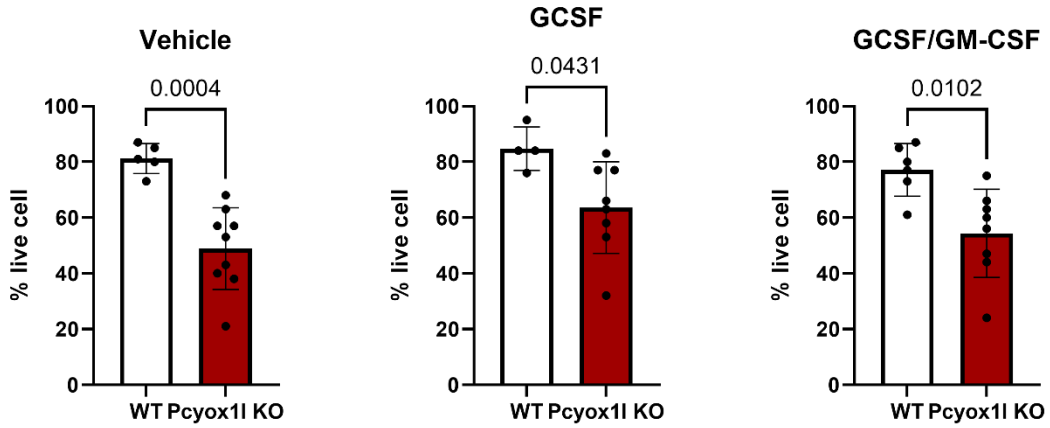

Figure 5. Percentage of live cells after maturation.

WT and Pcyox1l KO *in vitro* cell lines were matured under vehicle, GCSF, GCSF/GM-CSF conditions as described in methods. Number of cells, both live and dead cells, was counted. Percentage of live cells is the number of live cells divided by the total number of cells. Under all three maturation conditions there were significantly less live cells in the Pcyox1l KO cells compared to WT cells. Data are plotted as mean and SD. Each symbol represents one maturation event. Student's t-test.

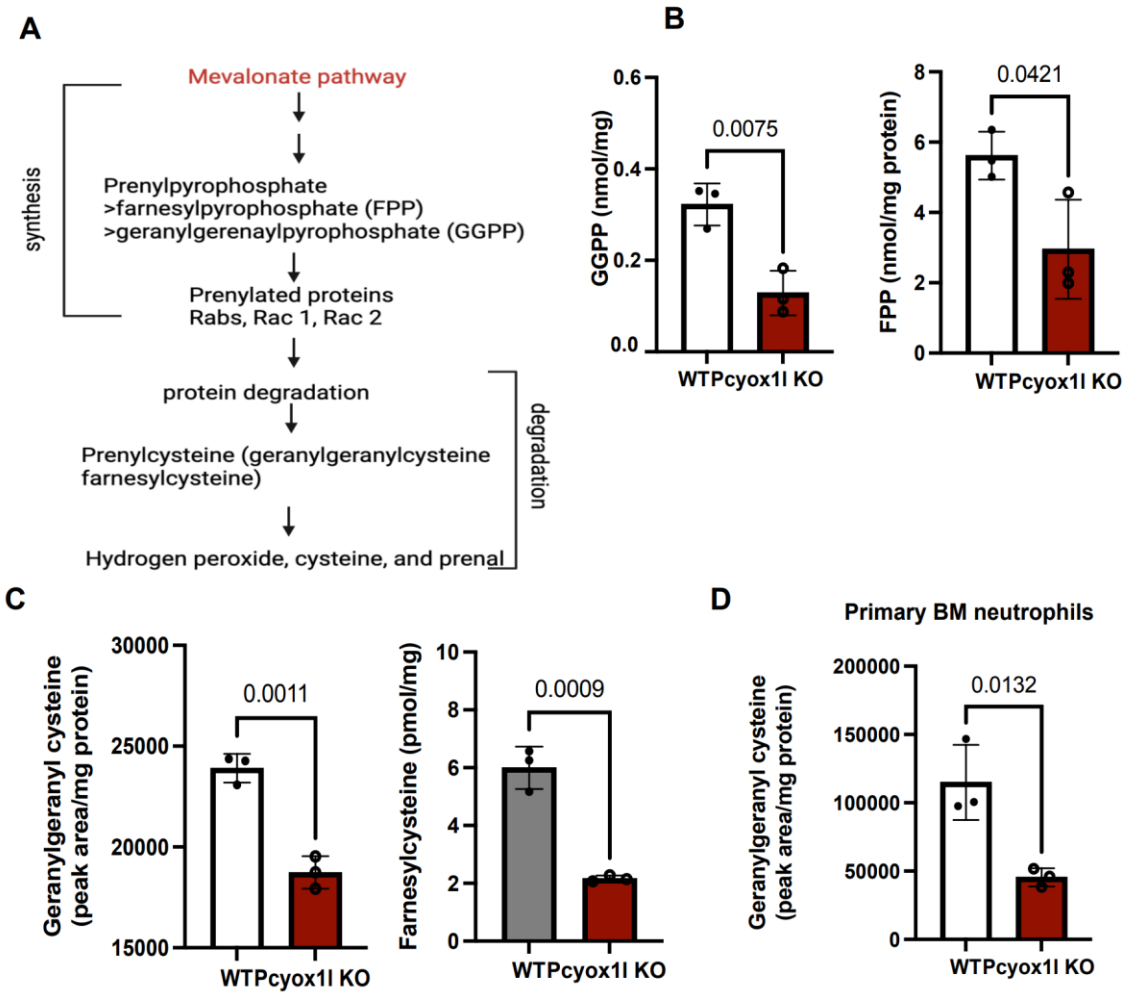

Figure 6. Metabolomic study results.

Pcyox1l deficient neutrophils show significant decreases in metabolites under GCSF condition.

A. Diagram showing the mevalonate pathway, from geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) to the synthesis of prenylated proteins and their free metabolites.

B. Metabolic quantification of GGPP and FPP in lysates from WT and Pcyox1l KO neutrophils matured *in vitro*. 12 million neutrophil progenitors were matured under GCSF condition as described in methods (E- medium, 80 ng/ml GCSF for 72 hours). Lysates

processed for relative metabolite presence. Data are plotted as mean and SD. Each symbol represents a biological replica. Student's t-test.

C. Metabolic quantification of free geranylgeranylcysteine and farnesylcysteine in lysates from WT and Pcyox1l KO neutrophils matured *in vitro*. 12 million neutrophil progenitors were matured under GCSF condition as described in methods (E- medium, 80 ng/ml GCSF for 72 hours). Lysates processed for relative metabolite presence. Data are plotted as mean and SD. Student's t-test.

D. Metabolic quantification of free geranylgeranylcysteine metabolite in lysates from WT and Pcyox1l KO BM primary neutrophils. Each data point represents individual animal. Data are representative of two independent experiments. Data are plotted as mean and SD. Student's t-test.

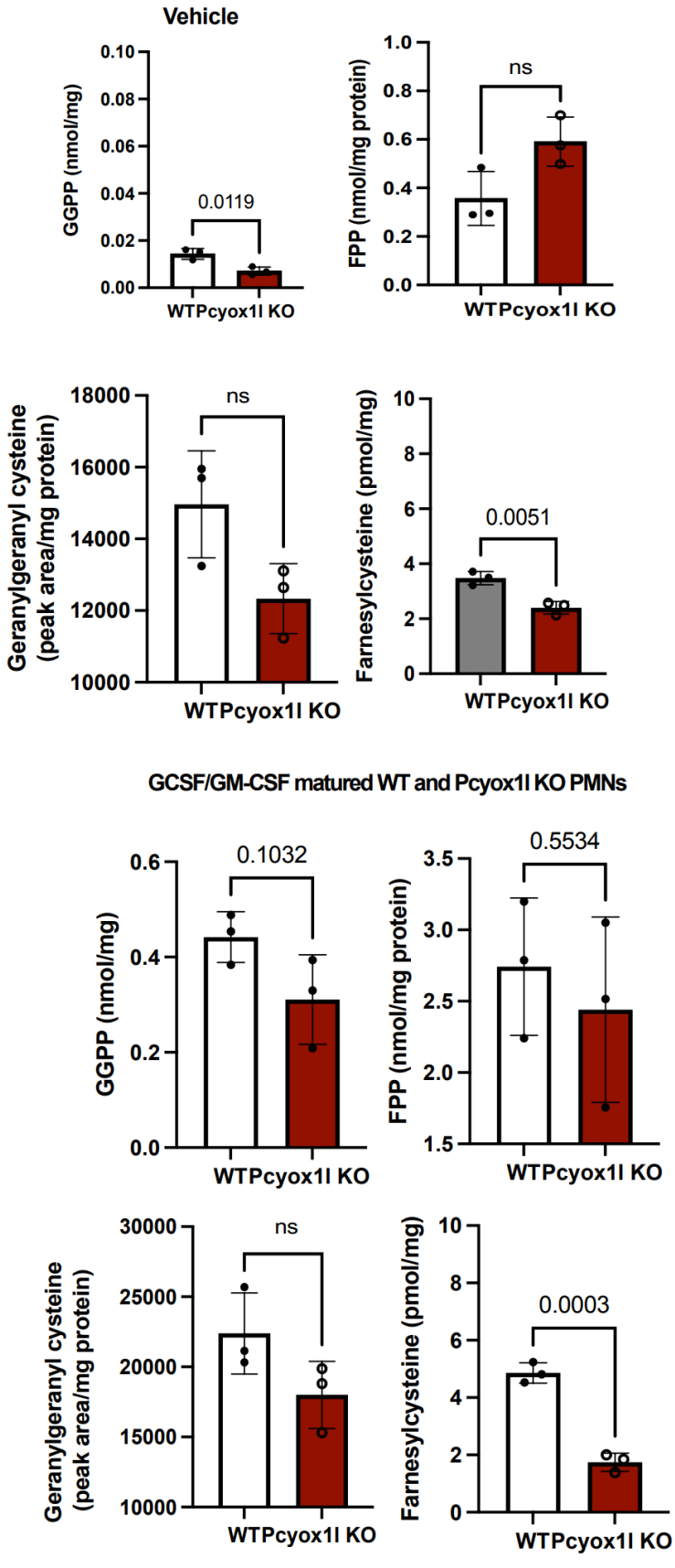
Vehicle
GGPP (nmol/mg)
0.10
0.08
0.06
0.04
0.02
0.00
0.0119
WTPcyox1l KO
FPP (nmol/mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
ns
WTPcyox1l KO
Geranylgeranyl cysteine (peak area/mg protein)
18000
16000
14000
12000
10000
ns
WTPcyox1l KO
Farnesylcysteine (pmol/mg)
10
8
6
4
2
0
0.0051
WTPcyox1l KO
GCSF/GM-CSF matured WT and Pcyox1l KO PMNs
GGPP (nmol/mg)
0.6
0.4
0.2
0.0
0.1032
WTPcyox1l KO
FPP (nmol/mg protein)
3.5
3.0
2.5
2.0
1.5
0.5534
WTPcyox1l KO
Geranylgeranyl cysteine (peak area/mg protein)
30000
25000
20000
15000
10000
ns
WTPcyox1l KO
Farnesylcysteine (pmol/mg)
10
8
6
4
2
0
0.0003
WTPcyox1l KO

Figure 7. Metabolomic study results under vehicle and GM-CSF condition. Decrease in metabolites in Pcyox1l deficient neutrophils under vehicle and GM-CSF condition is not as significant as in GCSF condition. Vehicle: 12 million neutrophil progenitors were matured in E- medium for 72 hours as described in methods. GCSF/GM-CSF: 12 million neutrophil progenitors matured in E- medium and 80ng/ml GCSF for 72 hours, addition of GM-CSF 5ng/ml 48 hours after initiation. Lysates processed for relative metabolite presence. Data are plotted as mean and SD. Student's t-test. Three measured metabolites are significantly decreased in Pcyox1l deficient neutrophils: GGPP and farnesylcysteine under vehicle condition, farnesylcysteine under GCSF/GM-CSF condition.

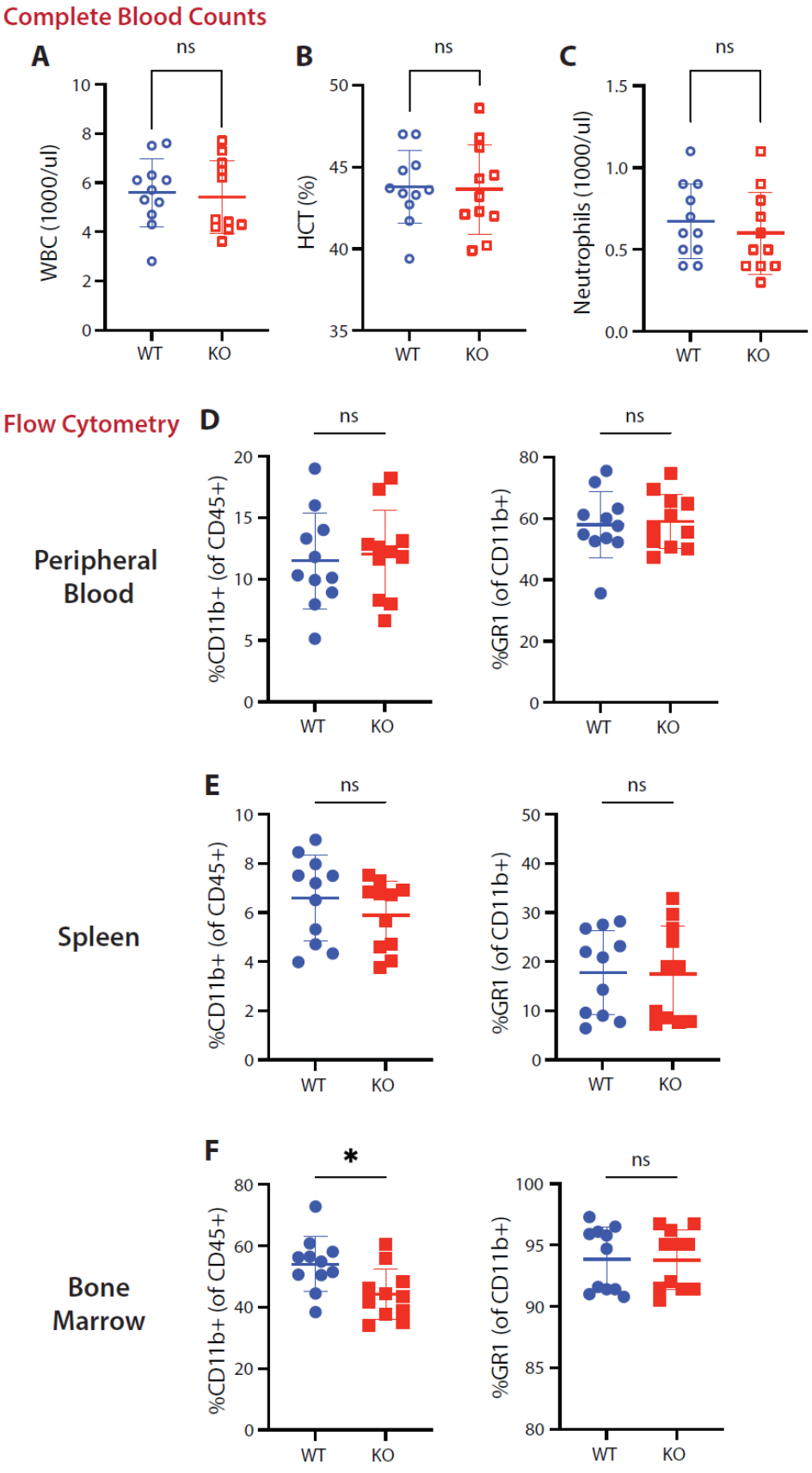
Complete Blood Counts
A
ns
WBC (1000/ul)
10
8
6
4
2
0
WT
KO
B
ns
HCT (%)
50
45
40
35
WT
KO
C
ns
Neutrophils (1000/ul)
1.5
1.0
0.5
0.0
WT
KO
Flow Cytometry
D
Peripheral Blood
ns
%CD11b+ (of CD45+)
20
15
10
5
0
WT
KO
ns
%GR1 (of CD11b+)
80
60
40
20
0
WT
KO
E
Spleen
ns
%CD11b+ (of CD45+)
10
8
6
4
2
0
WT
KO
ns
%GR1 (of CD11b+)
50
40
30
20
10
0
WT
KO
F
Bone Marrow
*
%CD11b+ (of CD45+)
80
60
40
20
0
WT
KO
ns
%GR1 (of CD11b+)
100
95
90
85
80
WT
KO

Figure 8. Hematopoietic characterization of Pcyox1l KO mice.

Complete peripheral blood counts of homeostatic (uninfected) wild-type and Pcyox1l KO mice showed no differences in (A) white blood cell count, (B) hematocrit, or (C) absolute neutrophil count. Flow cytometry was used to enumerate myeloid cells (CD45+, CD11b+) and neutrophils (CD45+, GR1+) in the (D) peripheral blood, (E) spleen, and (F) bone marrow. There was a small but significant reduction in the number of myeloid cells in the bone marrow in the Pcyox1l KO mice.

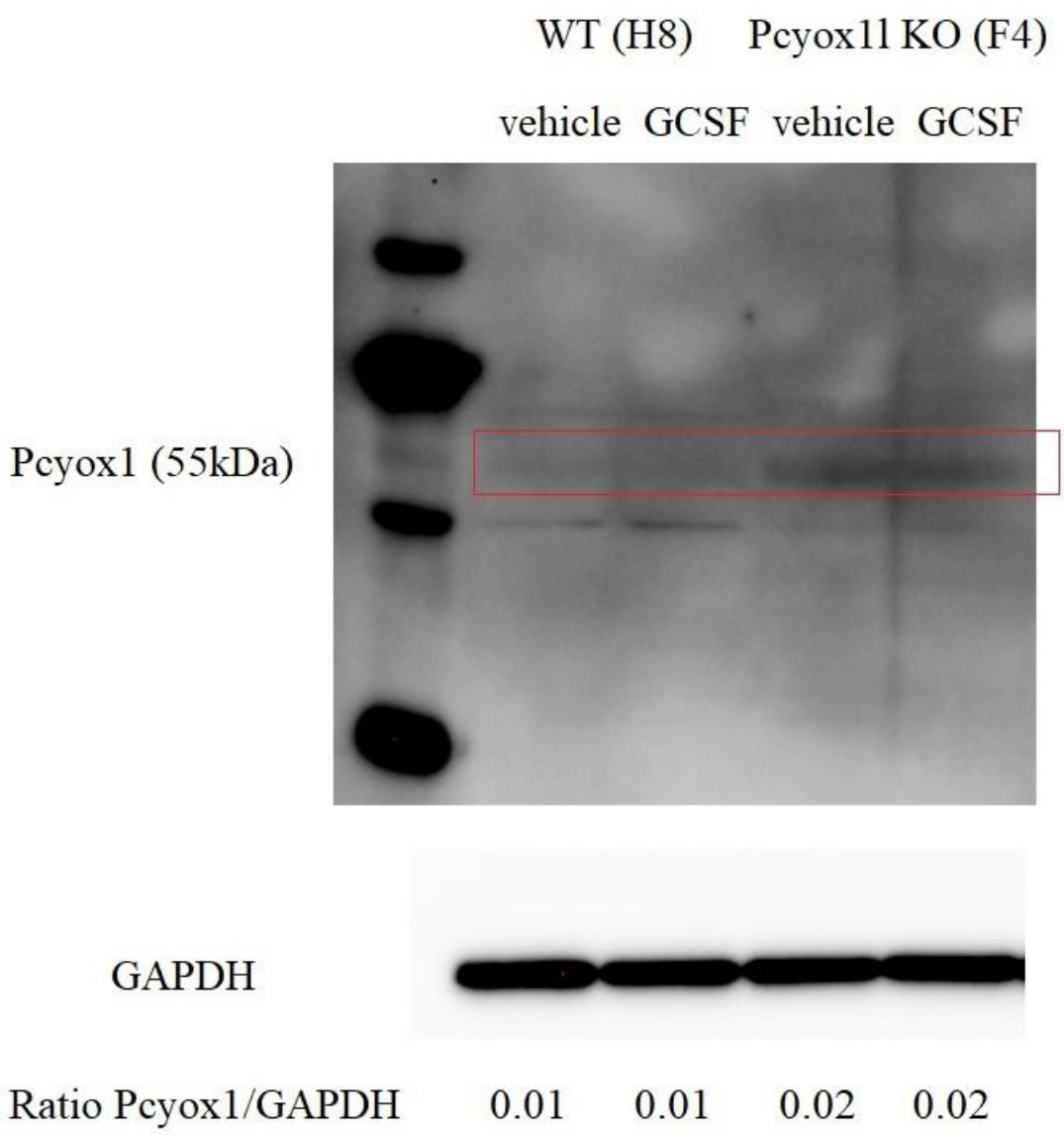

Figure 9. Pcyox1 level in Pcyxo1l cell lines.

Pcyox1l KO cell lines showed an increase in pcyox1 level. It is possible that the absence of Pcyox1l caused the upregulation of pcyox1.

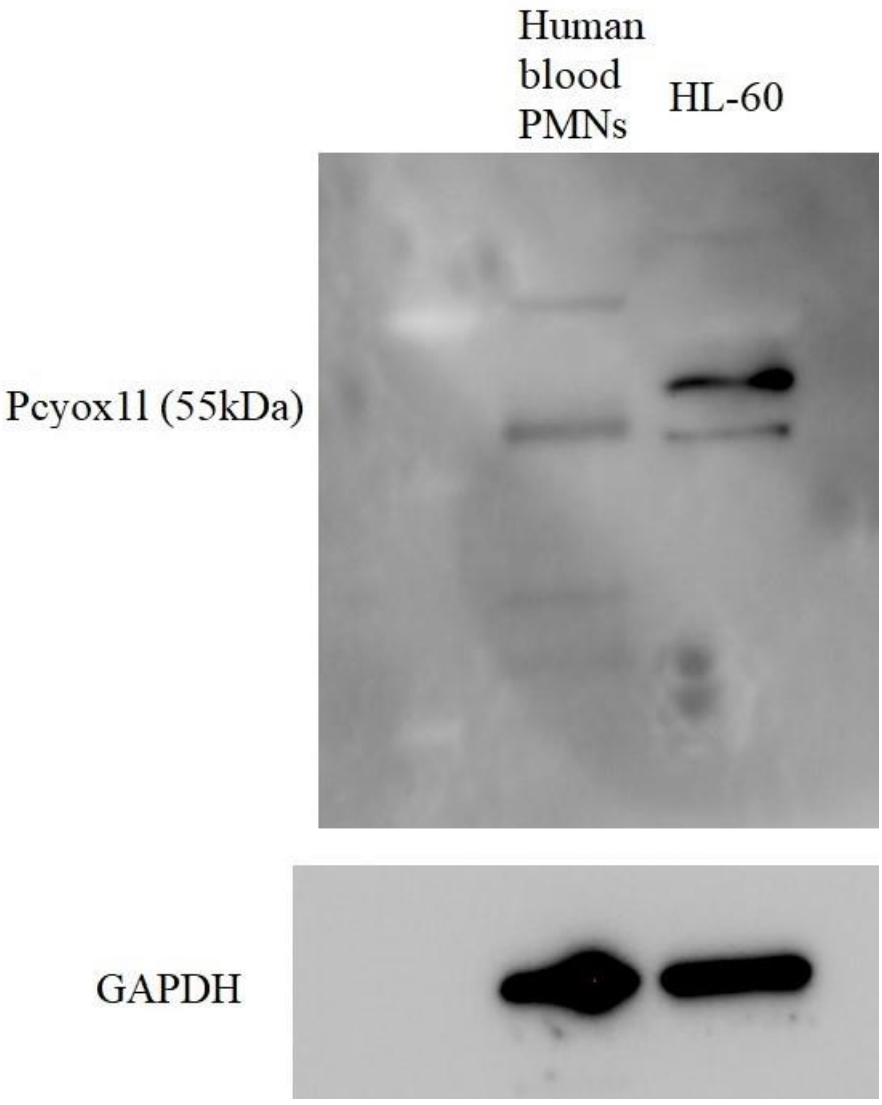

Figure 10. Pcyox1l in human PMN.

PMN purified from human blood and HL-60 cell line shows the Pcyox1l protein in western blot.

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