Functional Analysis of *Neutrophil Cytosolic Factor 4* (*NCF4*) in the pathogenesis of Pediatric Inflammatory Bowel Disease

by

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Department of Biochemistry

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Abstract

Genetic variants in NADPH oxidase are known to increase susceptibility of Very Early Onset Inflammatory Bowel Disease (VEOIBD). NADPH oxidase generates superoxide anions for bactericidal activity in phagocytes. Defects in NADPH oxidase lead to Crohn's disease (CD) and Chronic Granulomatous Disease (CGD) like phenotypes, characterized by defective respiratory burst and inefficient pathogen clearance. This study evaluates the functional consequences of an autosomal recessive nonsense variant in *NCF4* in the onset of pediatric IBD. It truncates p40phox (p40phox (W239X)), a subunit of the NADPH oxidase. It is reported that p40phox (W239X) loses its interaction with its known binding partner p67phox in NADPH oxidase and mislocalizes to early endosomes; instead of the cytoplasm. Impaired intracellular oxidative burst and reduced bactericidal activity were observed in macrophages stably expressing p40phox (W239X). It is proposed that p40phox (W239X) does not interact with NADPH oxidase causing its functional impairment and prompting the onset of pediatric IBD.

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Life is experienced in small moments, from the moment we learn how to walk to becoming an adult and when life brings you down on your knees, learning to get up and taking that first step that seems to be the hardest. The past 3 years have been an ongoing cycle of the latter, but the realization with each of that first step taken has been a lesson in itself. Graduate school tests you in every possible way: your mental strength, physical well being and mindset to succeed. The support system you establish in this time makes all the difference in pushing you to the end of this journey.

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I may have entered as a kid but I leave as a more knowledgeable, well informed and educated woman with all the energy to take on "real life" as people describe it in the "outside" world.

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List of Abbreviations

5-ASA: 5-aminosalicylic acid

APC: Antigen presenting cell

AR: Autosomal recessive

CADD: Combined annotation-dependent depletion

CD: Crohn's disease

CGD: Chronic granulomatous disease

CH: Compound heterozygote

CID: Combined immunodeficiency

Co-IP: Co-immunoprecipitation

CVID: Common variable immunodeficiency

DHR: Dihydrorhodamine

DPI: Diphenyleneiodonium

EEA-1: Early endosomal marker-1

FBS: Fetal bovine serum

fMLP: N-Formylmethionine-leucyl-phenylalanine

FMT: Faecal microbiota transplant

GI: Gastrointestinal

GWAS: Genome wide association studies

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP: Horseradish peroxidase

HSCT: Hematopoietic stem cell transplant

IBD: Inflammatory bowel disease

ICAM1: Intercellular cell adhesion molecule 1

IFN: Interferon

IL: Interleukin

IPEX: Immunodysregulation polyendocrinopathy enteropathy X-linked

LAD: Leukocyte adhesion deficiency

MDP: Muramyl dipeptide

MKD: Mevalonate kinase deficiency

MOI: Multiplicity of infection

MSMD: Mendelian susceptibility to mycobacterial disease

NADPH: Nicotinamide adenine dinucleotide phosphate

NBT: Nitro blue tetrazolium

NCF: Neutrophil cytosolic factor

NEMO: NFκB essential modulator

NET: Neutrophil extracellular trap

NFκB: Nuclear factor kappa light chain enhancer of activated B cells

NGS: Next generation sequencing

NMD: Non-sense mediated decay

NO_2: Nitrogen dioxide

NOBI: Neutrophil oxidative burst index

NOX: NADPH oxidase

PAGE: Polyacrylamide gel electrophoresis

PB1: Phagocyte oxidase and bem1

PBS: Phospho-buffered saline

PCR: Polymerase chain reaction

PFA: Paraformaldehyde

PI3K: Phosphoinositide 3 kinase

PI3P: Phosphatidylinositol 3-phosphate

PID: Primary immunodeficiency

PMA: Phorbol myristate acetate

PMSF: Phenylmethylsulfonyl fluoride

PX: PHOX homology

Rac: Ras-related C3 botulinum toxin substrate

RLU: Relative light units

ROS: Reactive oxygen species

TGFβ: Transforming growth factor β

T_H: T helper

TNFα: Tumor necrosis factor α

TLR: Toll like receptors

SCID: Severe combined immunodeficiency

SDS: Sodium dodecyl sulfate

SOD: Superoxide dismutase

UC: Ulcerative colitis

VEOIBD: Very early onset inflammatory bowel disease

WES: Whole exome sequencing

WT: Wild type

XIAP: X-linked inhibitor of apoptosis

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Chapter 1
Introduction

1.1 Inflammatory Bowel Disease

1.1.1 Background

Inflammatory bowel disease (IBD) is a form of chronic inflammatory disorder that affects the gastrointestinal (GI) tract [1]. It is a multifactorial condition known to arise due to various genetic, environmental, immunological and microbial factors (Fig. 1.1) [2]. Two major entities of IBD are Crohn's disease (CD) and Ulcerative colitis (UC) that result in chronic and relapsing inflammation [1]. The diagnosis of the disease is based on medical history (indications of genetic predispositions) as well as clinical and lab-based evaluation of endoscopy and histology [3]. Although UC and CD are distinguishable, both diseases share several clinical and pathological features. Patients with IBD frequently present with abdominal pain, bloody diarrhea and weight loss [2] and exhibit chronic and acute mucosal inflammation [4]. CD is defined by discontinuous lesions across the GI tract, granulomas, fistulas and deep penetrating ulcers and involves the ileum and perianal regions in severe cases [5]. UC is confined to the colon, with manifestation within the rectum that has the potential for proximal extension [3]. Ulceration is continuous in nature and inflammation is limited to the mucosa and submucosa [3]. A third category of IBD, IBD unspecified is applied in cases where a clear distinction cannot be made between UC and CD [6, 7]. These patients evolve symptoms of UC or CD over time. There is no cure that is currently known to treat IBD, but disease can be managed, and clinical remission is possible with the use of current medical therapy [4].

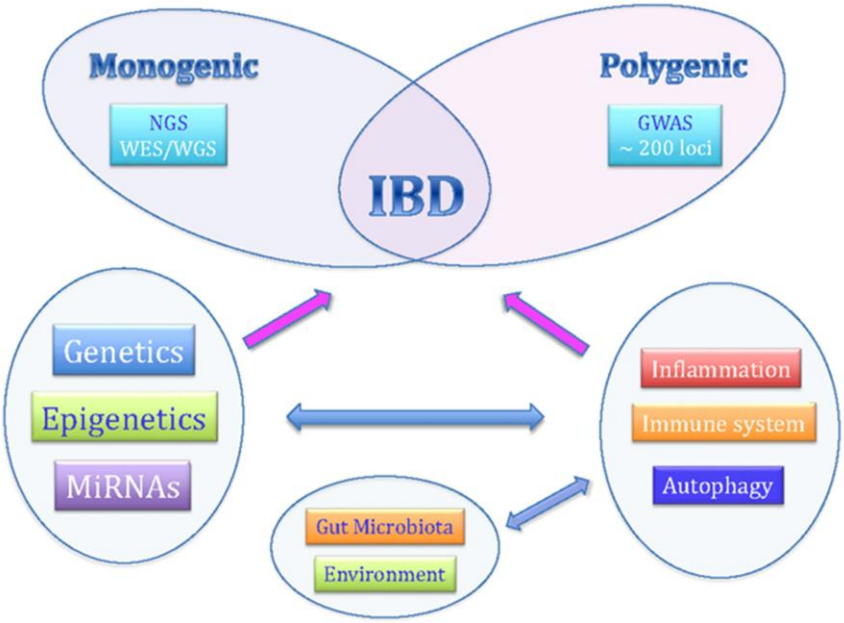

Monogenic
NGS
WES/WGS
Polygenic
GWAS
~ 200 loci
IBD
Genetics
Epigenetics
MiRNAs
Inflammation
Immune system
Autophagy
Gut Microbiota
Environment

Figure 1.1: Multifactorial nature of IBD. IBD is a complex disorder whose etiology remains largely unknown. However, various genetic, environmental, microbial and immunological factors are all known to contribute to its pathogenesis. Reproduced from Frontiers in Immunology: Mucosal Immunity. Loddo and Romano. Inflammatory bowel disease: genetics, epigenetics and pathogenesis [8]. 6: 55, 2015.

1.1.2 Disease Management and therapy

The overall goal of treatment in IBD is to induce clinical remission by treating symptoms (preferably steroid free), avoid hospitalization and surgery, heal the inflammation and ulcerations within the affected regions, maximize intestinal function and improve the quality of life [9]. Therapeutic pyramid is often used for treatment by physicians (Fig. 1.2) [10]. For optimal success, personalized therapy is recommended. Factors, such as the age of onset and diagnosis as well as extent, severity and duration of the disease are all considered when prescribing the treatment associated to the patient's disease [11].

A commonly used therapeutic strategy includes corticosteroids, such as 5-aminosalicylic acids (5-ASA), for the acute phase. This is followed by immunomodulatory drugs, like azathiopurine or mercaptopurine, to maintain remission. Severe cases are prescribed biological agents, such as anti-Tumor Necrosis Factor α (TNFα) agents like infliximab or adalimumab for inducing and maintaining remission [11].

Surgery is required for about 50% of CD patients and 20% of UC patients [12]. Meta-analysis conducted by Frolkis *et al.* (2013) showed that the risk of surgery has decreased for both UC and CD patients over time as a result of earlier detection methods and customized therapy [12]. For a small percentage of cases, with defects associated with the immune system, hematopoietic stem cell transplant (HSCT) is curative [13]. A successful case of allogenic HSCT was described by Glocker et al. (2009) in which a defect was recognized in genes *IL10RB* that encodes one part of the interleukin receptor-10 (IL-10) heterotetramer [14]. The patient was found to have a homozygous mutation in *IL10RB* causing a loss of IL-10 signaling. Upon HSCT, the patient entered remission for CD and was able to sustain it. However, several studies have been published that also describe cases where patients undergoing allogenic HSCT for IBD developed secondary conditions leading to more complications [15-17]. For this reason, HSCT is

only recommended in select cases. The precise pathological mechanisms leading to IBD remains elusive [18]. A better understanding of what causes IBD in different patients will help tailor therapeutic approach (i.e. personalized medicine) and hopefully improve their prognosis and quality of life.

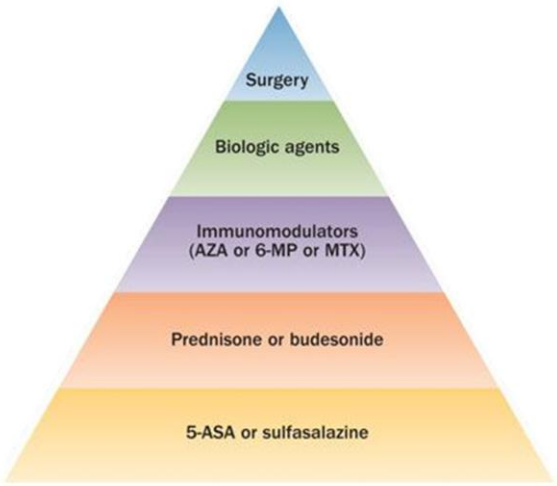

Surgery
Biologic agents
Immunomodulators
(AZA or 6-MP or MTX)
Prednisone or budesonide
5-ASA or sulfasalazine

Figure 1.2: Therapeutic pyramid used in the treatment of IBD patients. The pyramid describes the therapeutic approach opted by physicians in order treat patients with IBD. The treatment is designed to start with milder options which then moves into a stronger approach based on the patient's prognosis. Reprinted with permission from Nature Pub. Group: Nature Reviews Gastroenterology and Hepatology. Aloi et al. Advances in the medical management of pediatric IBD. 11 (99-108), 2014.

1.1.3 Classification

Due to the clinical variability observed in patients, the need for accurate classification of IBD became apparent [19]. These classifications considered the differences in location, behavior, severity of disease as well as the response to therapy in patient [19]. Clinically, it improves understanding of the disease prognosis in patients, and aid in patient counseling as well as prescribing therapy [19]. Additionally, it takes into account the variability in pathogenesis of the different categories of IBD [20]. In 1991, a classification system was proposed that distinguished IBD based on patient history, anatomical distribution and behavior of the disease [20]. However, this system was subsequently considered insufficient in providing an accurate classification of IBD [20]. In 1998, during the World Congress of Gastroenterology in Vienna, reclassification of this system was considered that came to be known as the Vienna classification [20]. Vienna classification considered the age of onset, location of the disease and its behavior as major determinants of the variable phenotypes as seen in IBD patients [20]. An improvement over the Vienna classification was proposed in 2005 at the Montreal World Congress of Gastroenterology with modifications in each category [20]. At the time, various studies suggested that the location of the disease, progression and response to therapy are age-dependent [20]. The Montreal classification system categorized patients with IBD according to age and defined patients with age of onset < 17 years as early onset IBD [20]. However, several weaknesses were found in the Montreal classification system [19]. Levine *et al.* (2011) highlighted that the Montreal classification failed to identify the variability in phenotypes in pediatric patients. The age of onset is not well classified, and the linear growth impairment was not considered at all [19]. Upon review, the Paris classification was proposed in which additional categories were added to the age of onset [19]. It was subdivided to include children who are diagnosed before the age of 6 as very early onset IBD (VEOIBD) and before the age of 2 as infantile IBD [19]. Clinical

manifestation is more severe for patients with VEOIBD compared to adult patients with IBD [19]. Moreover, disease location and behavior were also redefined, and growth monitoring was added as a category for CD patients [19]. For UC patients, the extent and severity of the disease were redefined [19].

1.1.4 Epidemiology

Previously considered rare, IBD is now classified as a global disease as its incidence and prevalence are increasing worldwide [21, 22]. Although the etiology remains elusive, "westernization" of society is believed to contribute to the observed increase in incidence [23]. The proof of principle lies in the highest incidence reported in North America and Europe [23]. Molodecky *et al.* (2012) conducted a systematic review of the literature reporting the epidemiology of IBD worldwide. The incidence rates differed across geographic regions [22]. Incidences for UC and CD in Europe, reported from 1930 to 2008, went from 0.6 to 24.3 and 0.3 to 12.7 per 100,000 respectively [22]. In North America, the rates reported from studies between 1920 and 2004 showed an increase from 0-19.2 and 0-20.2 per 100,000 for UC and CD [22]. Asian and Middle Eastern nations reported lower incidence rates compared to Europe and North America [22]. However, the increase in trend was evident with rates reported to be 0.1-6.3 per 100,000 for UC and 0.04-5 per 100,000 for CD between the years 1950 to 2008 [22]. Within these studies, the prevalence for UC was reported to be 505 per 100,000 in Europe, 248.6 per 100,000 in North America and 168.3 per 100,000 in Asia and the Middle East [22]. Estimates for CD were 322 per 100,000 in Europe, 318.5 per 100,000 in North America and 67.9 per 100,000 in Asia and the Middle East [22]. There was no obvious trend observed between sexes in these studies [22]. However, stratification of population based on age was possible as higher rates of incidences were reported in individuals between the age of 20 to 29 and 40 to 49 [22]. Another interesting trend derived from these studies was based on the ethnicity of the individuals who participated [22]. White and Jewish populations were reported to have the highest incidence, while a gradual increase was observed in Hispanic and Asian populations [22]. Additionally, individuals immigrating from developing countries to the Western world were reported to be at a higher risk of developing IBD, particularly the first-generation children belonging to these

families [24]. These findings point towards an environmental trigger in genetically susceptible individuals [25].

Twenty-five percent of patients worldwide develop IBD in childhood or adolescence [25]. A review conducted by Benchimol *et al.* (2011) summarized epidemiological trends seen in pediatric IBD worldwide. Overall, an increase in incidence for CD was reported, while early onset UC showed fairly stable numbers [25]. The authors indicated that the increase in incidence was quite significant in patients within Europe and North America [25]. However, they also reported the lack of information and surveys available from Asian, African, South American and Middle Eastern countries that made it difficult to derive conclusions [25]. Additionally, twin studies have been conducted that point towards the role of genetics in causing early onset IBD [26].

Epidemiological trends are an important asset to biomedical research in IBD, which is currently focused on elucidating mechanisms that result in its onset [27]. In 2017, Benchimol *et al.* reported that Canada has the highest incidence of early onset IBD. Researchers are using the current trends to identify risk factors (environmental, microbial, immunological or genetic) that are causing the increase in incidence of IBD in children [27].

1.1.5 Association of IBD with Immunodeficiency

IBD has been associated with primary immunodeficiencies (PID) as some PIDs show gastrointestinal inflammation as a leading symptom upon the onset of the disease [28]. Fifty monogenic disorders have been characterized by Uhlig *et al.* (2014) that depict IBD like phenotypes. These disorders can lead to epithelial defects and immune dysfunction. X-linked nuclear factor kappa light chain enhancer of activated B cells (NFκB) essential modulator (NEMO) deficient patients show epithelial barrier defects and commonly acquire IBD-related intestinal complications [28]. About 21% of patients show this trend and thus contribute to the association of this disease with IBD [28]. On the other hand, dysregulation of the innate immune response, especially those involving neutrophils, can cause IBD. One of the most common diseases of this type is Chronic Granulomatous Disease (CGD) [28]. Mutations within the phagocyte Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase are seen in patients with CGD [29]. This enzyme generates reactive oxygen species (ROS) that are directly involved in killing phagocytosed pathogens [29]. Defects within the enzyme lead to inefficiency in pathogen clearance [29]. A notable histopathological feature of a subset of CD patients is the formation of granulomas in the GI tract which is also seen in CGD patients [30]. The cause of the formation of granulomas is unknown but is thought to be a result of an attempt by the innate immune cells to clear infectious agents [30]. Additionally, defects in maturation and activation of T and B cells, as those seen in Common Variable Immunodeficiency (CVID), Combined Immunodeficiency (CID) and Severe Combined Immunodeficiency (SCID), lead to severe invasive infections causing inflammatory responses that are also seen in patients with IBD [28]. Auto inflammatory responses, related to X-linked inhibitor of apoptosis (*XIAP*) dysregulation and Mevalonate Kinase Deficiency (MKD), and defects in regulatory T cells as seen in patients

with Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome also affect patients with IBD [28].

The association of IBD with PID makes the disease even more complicated [28]. Methods to diagnose the disease at an earlier stage, such as genetic analysis via next generation sequencing (NGS), are required as this will determine the consequential prognosis and the therapy for the patient [28].

1.1.6 Mechanisms of IBD pathogenesis

1.1.6.1 Environment

Westernization of society has been largely implicated in causing the increase in incidence of IBD [31]. Risk factors, such as, changes in diet, exposure to pollution and various microbes, use of antibiotics and maintenance of hygiene are all associated to the urbanization of society [31]. At the center of these risk factors lies the dynamically changing gut microbiota, whose dysbiosis (changes in normal microbial ecology) can cause the development of IBD [31].

Antibiotics are needed to treat bacterial infections but can also act as a double-edged sword as they are known to cause extensive dysbiosis, associated with CD [32]. Studies have shown that a 5-day course of antibiotic ciprofloxacin can alter the microbial composition of the gut resulting in loss of diversity and evenness of the indigenous species [33, 34]. These changes were observed at the initiation of the treatment and were maintained at the 6 months follow up [33, 34]. Ungaro *et al.* (2014) performed a meta-analysis to establish an association of the use of several antibiotics with the onset of IBD. Apart from penicillin, all antibiotics showed a strong association with the onset of IBD, especially in children [35]. This signifies that the exposure to antibiotics leads to a predisposition to the disease [35]. Shaw *et al.* (2010) showed that children who are exposed to antibiotics in the early years of life are three times more prone to getting IBD [36].

Breast milk has been shown to play a significant role in the composition of gut microbiota [31]. It enhances the immune system by increasing tolerance to pathogenic antigens [31]. In fact, studies have shown that breast milk is able to reform microbiome dysbiosis, to a certain degree, in infants exposed to antibiotics intrapartum [37]. The association between breast feeding and UC had been established in 1961 [38]. Meta-analyses and systematic reviews have further established an inverse correlation between individuals who have been breast fed and the

incidence of early onset IBD within this cohort [39, 40]. This decreased risk of acquiring IBD has been attributed to a possible protective influence mediated by breast milk that leads to reduced production of pro-inflammatory cytokines and less severe inflammation [41, 42].

Diet is another major environmental factor that affects microbial composition of the gut [31]. Incorporation of increased dietary fiber from fruits and vegetables was shown to reduce the risk of developing CD [43]. Intake of n-3 polyunsaturated fatty acids was shown to reduce the risk of developing UC while n-6 polyunsaturated fatty acids had the opposite effect [31]. Consuming animal proteins, on the other hand, have been reported to increase susceptibility to IBD [44]. Moreover, micronutrients, like Vitamin D, have also shown to be associated with the pathogenesis of IBD [31]. Women with high plasma Vitamin D have been reported to be at a reduced risk of acquiring IBD [45]. Also, increased levels of Vitamin D have been shown to be associated with reduced risk of CD-related surgery in individuals with the disease [46].

Other environmental factors such as an increase in air pollutants like nitrogen dioxide (NO_2) [47], ulcerations due to the use of nonsteroidal anti-inflammatory drugs (NSAIDs) [48], inflammation as a result of hypoxia [31] have all been shown to have a positive association to the risk of acquiring IBD.

1.1.6.2 Microbial factors

Gut microbiota and its dysbiosis play an integral role in maintaining health and disease manifestation [31]. Composition of the gut microbiota varies between individuals within a population due to the influence of their genetics as well as environmental exposures [49, 50]. The importance of maintaining gut microbiome homeostasis lies in the fact that IBD patients have reduced diversity and complexity compared to healthy individuals [51]. Additionally, faecal microbiota transplant (FMT) has been used as a successful treatment strategy for selective cases of UC [52, 53].

Possible mechanism of pathogenesis, via commensal microbial stimuli, is the activation of the immune system where they should be acting as an adjuvant but can react as an antigen in disease [54]. It has been shown that occurrence of CD is associated with decreased diversity in Firmicutes and an increase in Enterobacteriaceae [55]. *Clostridium* species within Firmicutes, such as *F.prausnitzii*, are able to induce T regulatory (Treg) cells and a reduction of this species in the gut microbiota has been associated with the onset and severity of CD [56]. Within Enterobacteriaceae, *E. coli* is able to acquire virulent and invasive capabilities in CD [13, 57-59]. This species induces a chronic inflammatory response and when engulfed by macrophages, is highly resistant to the killing mechanism [60, 61]. Fungal and viral species are also known to affect the gut microbiome [51]. An increase in fungal profile was observed in patients with CD and UC compared to healthy controls, especially of Basidiomycota in a pediatric cohort [62, 63]. On the other hand, in a study by Cadwell *et al.* (2010), it was shown that viral infection was necessary in order to induce colitis in genetically susceptible mice with mutations in *ATG16L1*, in addition to disturbance in gut bacterial taxa [64]. CD- and UC-specific viruses have been derived from fecal samples of patients showing an increase in Caudovirales bacteriophage which

led to a decrease in diversity of gut bacterial taxa, indicating that viral particles can induces dysbiosis within the gut [65].

1.1.6.3 Immunological factors

Intestinal homeostasis is maintained because the immune system is able to distinguish between the commensal bacteria and pathogenic antigens [66]. IBD manifests when this tolerance is perturbed [66]. Various factors contribute to this perturbation, including disruption of the epithelial barrier, innate immune cells, lymphocyte defects as well as the dysregulated release of chemokines and cytokines [66].

An increased number of macrophages and dendritic cells are observed within the lamina propria in both UC and CD [54]. Furthermore, it has been observed that these patients also show an increase in expression of adhesion molecules such as intercellular cell adhesion molecule 1 (ICAM1) [54]. Adhesion proteins allow circulating cells to stick down and be able to penetrate the lumen through the endothelium [67]. This process is known as extravasation [67]. Resident innate immune cells have acquired the tolerance to commensal bacteria and thus do not respond to them [54]. It is these extravasated cells that recognize commensal bacteria as pathogens [54]. Binding of the bacterial components to receptors on the innate immune cells results in intracellular signaling that activates NFκB, a transcription factor that upregulates proinflammatory cytokine production [54]. Upregulation of TNFα, interleukin 1β (IL-1β), IL-6, IL-8 and other cytokines, that are direct targets of NFκB signaling, cause inflammation within the gut and are observed in active IBD [54]. Biological agents that specifically block some of these cytokines, such as anti-TNFα antibodies, are currently used to treat patients with CD and UC [68, 69].

Dendritic cells are antigen presenting cells (APCs) to naïve T cells within the lymph node which activates the adaptive immune system [54]. Active T cells secrete cytokines, whose profile differs between patients with CD and those with UC [54]. CD is characterized by an increase in production of cytokines by type 1 T helper cells (T_H1), with the predominance of interferon γ

(IFNγ) and IL-12 p40 [54]. Additionally, increased levels of IL-23 and IL-17, via the T_H17 axis, are also seen in CD [70, 71]. UC has been associated with T_H2 cytokine profile, characterized by elevated levels of IL-4 and IL-5, but with inconsistent results [72]. An atypical mode of activation of T_H2 signaling in experimentally induced colitis (oxazalone colitis model) in mice resulted in an increase in IL-13 levels via natural killer T cells [73]. APCs that interact with these natural killer T cells have unique receptors that bind lipids and present those to T cells instead of proteins [73]. The colitis within these mice resembled UC in humans [73]. Authors suggested that selective blocking of IL-13 can be a new mode of treatment for UC but this requires further validation [73].

1.1.6.4 Genetics in IBD

Traditionally, IBD has been regarded as a polygenic disorder [28]. Indeed, genome wide association studies (GWAS) and meta-analyses have identified over 230 loci that confer IBD risk [74]. GWAS have provided important insights into the genetic makeup of IBD patients compared to general population [74]. Out of the 230 non-overlapping genetic IBD risk loci, several loci are shared between CD and UC, suggesting similar mechanism of disease [74]. Significant differences are also found between CD and UC risk loci, which indicates pathological differences between the two diseases [1]. *NOD2* was the first risk gene to be implicated in IBD [1]. Thereafter, several genes associated with *NOD2* signaling were also identified in IBD risk loci [75]. NOD2 recognizes muramyl dipeptide (MDP) that leads to the activation of downstream signaling which regulates innate and adaptive immune system [1]. Of note, the activation of this pathway leads to induction of autophagy, T cell differentiation and immune tolerance [1]. These mechanisms are known to be perturbed in IBD patients [1]. *CARD9*, coding an adapter protein involved in activating various innate immune functions, is another gene that has been identified within the IBD risk loci in addition to *STAT3, IL23R* and autophagy related genes *ATG16L1* and *IRGM* [75]. More recently, de Lange *et al.* (2017) identified four new integrin genes, namely, *ITGA4, ITGAV, ITGB8* and *ITGAL*, present within IBD risk loci in their GWAS. These genes code for cell adhesion proteins that are crucial for regulating inflammation [76]. GWA studies have led to the identification of several high-risk variants that have been subjected to experimental validation, giving insights into the biological pathogenesis of IBD [75]. However, GWA studies are unable to capture the rarer genetic variants and must be complimented with more targeted approaches such as whole exome sequencing (WES) [76].

Recent advances in genomics have identified rare genetic variants that are known to exhibit IBD-like intestinal inflammation [28]. Over 50 genes have been identified to cause

monogenic VEOIBD [28]. The various monogenic forms of IBD are phenotypically heterogeneous and can exhibit disruption of epithelial barrier and hyper - and auto-inflammatory responses leading to dysregulation of T and B cells. [28]

The first known cause of monogenic disorder was reported in the case of severe pediatric IBD, wherein association analysis identified mutations in genes encoding the IL-10 receptor (*IL10RA* and *IL10RB*) [14]. This began an era of genomic technology that led to the identification of genetic disorders that present with intestinal inflammation via WES [4]. *NOD2* signaling was first reported in 2001 as a potential pathway whose disruption can increase the susceptibility to IBD [77, 78]. More recently, WES has identified rare missense variants in *PRDM1*, *NDP52*, *IL17REL* and *CSF2RB* genes that are also part of the *NOD2* pathway and confer risk to IBD [74]. Studies have shown that patients with defects in *SLC9A3*, encoding sodium hydrogen exchanger 3 expressed in the luminal epithelium, present with intestinal dysbiosis that causes inflammation [74]. Similar disease is seen in patients exhibiting gain of function mutations in *GUCY2C* gene, which leads to an increase in levels of cGMP, an inhibitor of the sodium hydrogen exchanger 3 [79]. Patients with *ARPC1B* mutations depict a disease that is similar to Wiskott-Aldrich syndrome where defects in actin filament formation leads to microthrombocytopenia, platelet malfunction, IBD, infections, vasculitis, etc. [80]. *TRIM22* and *NPC1* encode proteins involved in *NOD2* signaling, and mutations within these genes have been associated with CD-like disease [81, 82].

The power of genomic medicine lies in its immediate use for therapy [74]. Allogenic HSCT is curative in selective cases with significant risks of morbidity and mortality [74]. However, successful cases have been reported in patients with mutations affecting the immune system [74]. Mutations in *XIAP* cause granulomatous colitis and perianal disease [74]. Defects are recognized in the innate and adaptive immune response which makes patients with mutations

in *XIAP*, candidates for HSCT [74]. A case report has been published on a patient with defects in *XIAP* who failed all other modes of treatment for IBD but was cured using HSCT [83]. Additionally, genomic medicine has allowed clinicians to identify mutations that affect the epithelium, such as those in *EPCAM* and *TTC7A*, where HSCT is not a helpful treatment [74].

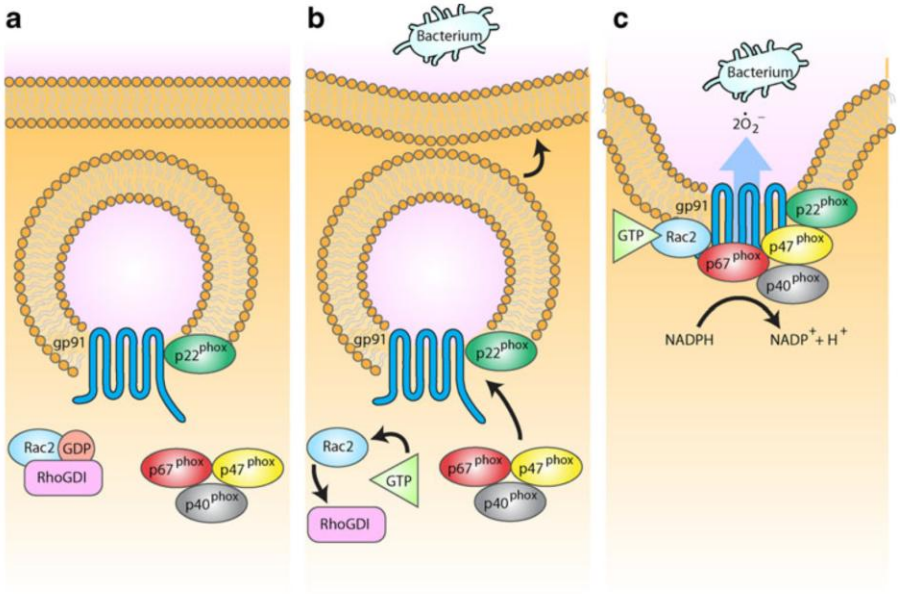
Current therapeutics are directed towards inducing and maintaining disease remission to reduce permanent damage [84]. Studies are now focused on elucidating the pathways by which monogenic IBD manifests itself as well as developing therapeutics targeting those defects for advancement in personalized medicine.

1.2 Role of NADPH oxidase (NOX) in IBD

1.2.1 Background

NADPH oxidase is a multiprotein enzyme that leads to the production of ROS, such as superoxide anion (O_2^-), by catalyzing the electron transfer between NADPH and oxygen (O_2) during phagocytosis (Fig. 1.3) [29]. It is composed of membrane bound gp91phox (gene: *CYBB*) and p22phox (gene: *CYBA*) proteins (collectively known as cytochrome b558), as well as cytosolic p47phox (gene: *NCF1)*, p67phox (gene: *NCF2*) and p40phox (gene: *NCF4)* proteins in a trimerized state and a small rho GTPase Ras-related C3 botulinum toxin substrate (Rac2) (gene: *RAC2*) [85].

In an inactivated state, the proteins within the NADPH oxidase remain in their respective locales, i.e. either membrane bound or within the cytoplasm [85]. Highly regulated signaling events take place within the cell that ensures proper assembly and activation of the enzyme [85]. NADPH oxidase assembles when the membrane bound receptors recognize molecules on the surface of pathogens [85]. Opsonic receptors, like Fcγ, recognize antibodies bound to pathogens where-as pattern recognition receptors, such as Toll like receptors (TLRs), recognize molecules exclusively present on the surface of pathogen [86]. Cytosolic components of the NADPH oxidase are regulated by kinases, such as protein kinase A [85], different isoforms of protein kinase C [85] and Phosphoinositide 3 Kinase (PI3K) [87]. These kinases phosphorylate residues within the cytosolic proteins that influence their conformation and state of activation [85]. Upon cellular activation, the cytosolic components are recruited to the membrane, forming an activated complex [85]. The electron transfer occurs through the gp91phox subunit, resulting in the release of superoxide anions [85]. These anions can then react to form hydrogen peroxide and hypochlorous acid for bactericidal activity [85].


a
b
c
Bacterium
Bacterium
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gp91
p22phox
GTP
Rac2
p67phox
p47phox
p40phox
NADPH
NADP+ + H+
Rac2
GDP
RhoGDI
Rac2
GTP
RhoGDI

Figure 1.3: NADPH oxidase assembly. A) NADPH oxidase is a membrane bound enzyme that facilitates the formation of reactive oxygen species in order to kill invading pathogens. In phagocytes, NADPH oxidase is composed of two membranes bound elements gp91phox and p22phox, three cytosolic proteins: p40phox, p47phox, p67phox and a small Rac G-protein. B) Gp91phox and p22phox proteins form a heterodimer linked permanently with the plasma membrane. Upon detection of a pathogen via cell surface receptors, p47phox gets phosphorylated and as a result, all three subunits translocate to the membrane. Rac GDP gets converted to Rac GTP and gets recruited to the membrane independently. C) This completes the complex that is able to generate a respiratory burst. Superoxide, produced as a result of this complex formation, can react to form hydrogen peroxide and hypochlorous acid, which together participate in bacterial killing. Reprinted with permission from SPRINGER-VERLAG BERLIN/ HEIDELBERG: Seminars in immunopathology. Lam et al. The many roles of NOX2 NADPH oxidase-derived ROS in immunity. 32:4 (415-430), 2010.

1.2.2 NADPH oxidase mediated immune response

The most well studied role of the leukocyte NADPH oxidase complex is its participation in innate immune defense. Superoxide anions that are generated as a result of assembly and activation of this enzyme can react to form strong oxidizing agents that become toxic to pathogens [85]. Generation of the strong oxidizing agents occurs via various mechanisms, such as the activity of superoxide dismutase (SOD) to form hydrogen peroxide [88], combining superoxide anions with nitric oxide to produce peroxynitrites [89], reaction of hydrogen peroxide with chloride ions to form hypochlorous acid [90] and the formation of hydroxyl radicals through the activity of ferric ions (Fig. 1.4) [85]. The anti-microbial activity of superoxide anions is exerted through these oxidizing agents that are able to oxidize pathogenic protein, lipids and DNA, thereby damaging their integrity [85].

ROS also participates in innate immunity by indirectly being involved in the activation of other mechanisms. One such method is by the regulation of ion buildup within phagosomes [91]. The electron transport within phagosomes, which occurs through the NADPH oxidase complex, builds up a negative charge within the organelle, causing an influx of positive potassium ions [91]. Buildup of charge, which results from the ion transport, leads to the activation of proteases that are further able to destroy the pathogen [91]. Moreover, NADPH oxidase is also believed to have a role in Neutrophil Extracellular Trap (NET) formation [92]. NETs are networks of degraded proteins and DNA that are formed by neutrophils as part of their microbial killing activity [92]. The exact mechanism is unknown but the stimulators for the assembly and activation of NADPH oxidase, such as Phorbol myristate acetate (PMA) and IL-8 have been shown to induce NET formation [92]. ROS produced by the NADPH oxidase has also been shown to influence NET formation as patients with CGD, who are unable to produce ROS effectively, were also unable to form NETs [92].

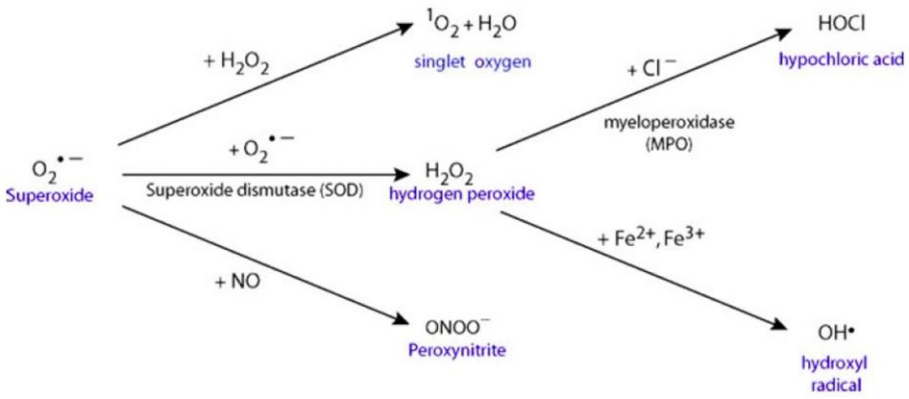

$^1O_2 + H_2O$
singlet oxygen
$+ H_2O_2$
HOCl
hypochloric acid
$+ Cl^-$
myeloperoxidase
(MPO)
$+ O_2^{\bullet -}$
$O_2^{\bullet -}$
Superoxide
Superoxide dismutase (SOD)
H_2O_2
hydrogen peroxide
$+ Fe^{2+}, Fe^{3+}$
$+ NO$
$ONOO^-$
Peroxynitrite
$OH^\bullet$
hydroxyl
radical

Figure 1.4: Various ROS products derived from the respiratory burst through the NADPH oxidase. Superoxide anion that is generated through the NADPH oxidase activity, is highly reactive and produces various ROS species that are anti-microbial. Reprinted with permission from SPRINGER-VERLAG BERLIN/ HEIDELBERG: Seminars in immunopathology. Lam et al. The many roles of NOX2 NADPH oxidase-derived ROS in immunity. 32:4 (415-430), 2010.

1.2.3 Homologues of NOX

NADPH oxidase in hematopoietic cells is also known as NOX2 [93]. There are 6 other homologues of NOX2 that are known to produce ROS [93]. The term NOX was used to classify proteins encoded by *CYBB* (gp91phox or NOX2). NOX2 is primarily expressed in hematopoietic cells and produces the majority of NADPH oxidase-derived ROS [93]. The NOX1 NADPH oxidase is expressed in colonic epithelium, where studies using NOX1-deficient mouse models have shown that NOX1 is involved in the maturation of colonic epithelium progenitor cells into colonocytes through Notch1 signaling [94]. Additionally, its expression in vascular smooth muscle cells is also noted and implications of its role in foam cell development and IL-8 secretion have also been found [95-97]. Schmausser *et al.* (2004) showed that activation of TLR-mediated signaling in guinea pig intestinal cells lead to increased production of ROS. The implication of this conclusion was that there seems to be a link between TLR-mediated signaling and ROS produced through NOX1 [98]. However, in another study, it was shown that ROS produced by NOX1 was not sufficient to control the growth of *Salmonella*, indicating that NOX1 mediated ROS production might be more significant in signaling than anti-microbial activity [99]. NOX3 is known to be specifically expressed in the inner ear and regulates balancing activity [100]. NOX4 was initially known to be expressed primarily in the kidney; however, more recent evidence has indicated that it is also expressed in other cell types [101, 102]. The protein is known to be expressed constitutively in these cell types and also produces ROS in the absence of a stimulus [101, 102]. Additionally, the expression can be upregulated via factors such as hypoxia in the lungs or transforming growth factor β (TGFβ) in cardiomyocytes [103, 104]. NOX5 NADPH oxidase produces ROS in a calcium-dependent manner and does not require other components of the complex to perform its function [93]. The expression of this homologue has been seen in the spleen, vascular muscle cells and testes [105]. On the other

hand, overexpression of NOX5 has been reported in cancerous B cells as well as Barrett's esophageal adenocarcinoma [105, 106]. The last two homologues of NOX2 are called DUOX1 and DUOX2 [93]. They are expressed in the thyroid and generate hydrogen peroxide which aids in the production of hormones within the thyroid gland [107-110]. DUOX2 is also expressed in intestinal enterocytes where it has anti-microbial roles [108, 111].

1.2.4 Role of NOX2 in inflammation

The role of NADPH oxidase in driving and sustaining inflammation has been extensively studied. Although a direct connection hasn't been established, studies have shown evidence that point towards a vital role of the enzyme in inflammation. In a study by Rutault *et al.* (1999), hydrogen peroxide, a derivative of ROS, was shown to play a role in dendritic cell activation and T cell proliferation [112]. Another important aspect of inflammation is the movement of immune cells to the site of infection, a process termed chemotaxis [113]. In a study by Hattori *et al.* (2010) it was shown that mice and human neutrophils that had a mutation in *CYBB* leading to reduced expression of gp91phox, had impaired chemotaxis compared to the controls. Hattori *et al.* (2010) also showed that when neutrophils are stimulated with chemoattractants, such as IL-8 or N-Formylmethionine-leucyl-phenylalanine (fMLP), cells that are treated with diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase, had reduced chemotaxis compared to untreated cells. This indicated the importance of this enzyme in the efficient movement of the cells to the site of infection [113]. Additionally, the fact that CGD patients have increased proinflammatory cytokine expression and are unable to resolve inflammation efficiently indicates the important role of NADPH oxidase in regulating inflammation [114, 115].

1.2.5 NADPH oxidase in Chronic Granulomatous Disease

CGD is a primary immunodeficiency that is caused by functional defects within the subunits of the NADPH oxidase in neutrophils and monocytes [116]. Like IBD, the onset of CGD can occur at any stage of life but most patients are diagnosed at less than 5 years of age [116]. CGD is characterized by the manifestation of granulomas in various tissues across the body, including the intestinal epithelium [117]. Clinical symptoms include repeated infections, malnutrition, granulomas, inflammation and colitis [117]. Approximately two thirds of CGD cases are caused by mutations in the X-linked *CYBB* gene that encodes for the gp91phox subunit [116]. Other mutations have been identified in *NCF1* (p47phox) that accounts for 20% of the cases, *CYBA* (p22phox) and *NCF2* (p67phox) which make up 5% of the cases and one case of *NCF4* (p40phox) [116]. An interesting study published in 2000 reported a patient that had a mutation within *RAC2* that led to expression of RAC2 protein that could not bind GTP, rendering it inactive at all times [118]. His main symptoms were severe and persistent bacterial infections and defects in wound healing [118]. His neutrophils had reduced ROS production and had defects in chemotaxis and polarization [118]. The disease within this patient was characterized as a combination of Leukocyte Adhesion Deficiency (LAD) and CGD [118]. Another case reported mutations within X-linked *CYBB* in two male patients that resulted in a variant of gp91phox that only affected macrophages [119]. Due to the cell specific effect, only macrophages had defective ROS production leading to the diagnosis of Mendelian Susceptibility to Mycobacterial Disease (MSMD), rather than CGD [119].

The western world, reportedly, has the highest incidence of CGD with 1/250,000 live births affected within Europe and North America [116]. Due to a lack of proper treatment and awareness, CGD was previously known as “fatal granulomatous disease” since patients did not survive beyond the age of 10 [116]. However, recent advances in therapy and modes of diagnosis

has allowed for an increase in life expectancy where a study has reported 90% survival beyond the age of 10 for CGD patients [120]. Current therapeutic strategy includes the use of antibiotic prophylaxis, interferon γ (IFNγ) and HSCT in more severe cases [116].

In a number of CGD cases, infections are caused by catalase-positive bacteria [116]. These infections are seen in the lungs, skin, lymph nodes and liver [116]. Bacterial species that are most commonly seen in patients with CGD are *Staphylococcus aureus, Burkholderia cepacia, Serratia marcescens, Nocardia spp.* and *Salmonella [116]*. 20-40% of CGD patients also exhibit fungal infections [116]. *Aspergillus spp., Rhizopus spp.* and *Trichosporon spp.* are fungal pathogens that are known to affect CGD patients [116]. These infections are severe and significantly contribute to CGD-related morbidity and mortality [116].

Inflammatory dysregulation is frequently seen in patients with CGD. A single cohort study from France reported that out of 98 patients, 69.4% had inflammatory complications and 88.2% of these patients had GI manifestations [121]. These complications are more frequently seen in X-linked CGD than autosomal recessive (AR) CGD [116]. It is believed that 30-60% of all patients with CGD develop inflammation within the GI tract [116]. The disease seems to affect the colon and patients with CGD are reported to develop perianal fistulae and perirectal abscesses [116].

1.2.6 NADPH oxidase genes in IBD

The implication of NADPH oxidase in the pathogenesis of IBD lies in the fact that a subset of CD patients show impaired ROS production [122]. Additionally, the use of antioxidant enzymes and drugs has been shown to have a protective effect against colitis in mouse models [123].

GWA studies have not shown a very strong association between genes encoding the NADPH oxidase complex and IBD. However, *NCF4* has been shown to be associated with ileal CD and *RAC2* with CD in adults [124, 125]. In a study by Muise *et al.* (2011), two variants of *RAC1* were shown to be associated with CD. One of the variants (rs10951982) led to an increased expression of RAC1 protein, which suggested that this increase may contribute to the onset of CD [126]. However, this association was not replicated in other cohorts with adult patients, similar to what has been seen in the case of *NCF4* [126, 127]. Therefore, in 2012 Muise *et al.* performed WES in 10 VEOIBD patients to see whether NADPH oxidase genes associate with the disease in younger patients, owing to the similarities in manifestation with CGD [124]. A rare coding variant in *NCF2* (p67phox R38Q) was found that led to reduced binding of p67phox to RAC2 causing an impaired oxidative burst [124].

In functional studies, Dhillon *et al.* (2014) reported 11 rare variants within the NADPH oxidase complex that are associated with increased VEOIBD susceptibility. It was suggested that the presence of these variants might affect the protein-protein interaction required to form a functional enzyme complex and therefore disrupts ROS production [128]. In a study by Hayes *et al.* (2015), targeted exome sequencing identified missense variants in *NOX1* and *DUOX2* genes in VEOIBD patients. Upon functional analysis, it was shown that model cell lines expressing these mutations had defective ROS production and were susceptible to *Campylobacter jejuni* infections [129]. In another study, a patient exhibiting a compound heterozygote (CH) mutation

in the *NCF4,* the gene encoding the p40phox subunit of NADPH oxidase, was reported by Matute *et al.* (2009). The patient had chronic granulomatous inflammation of the GI tract [130]. The two variants were a truncating allele (K52RfsX79) and a point mutation (R105Q) in the PHOX homology (PX) domain [130]. The p40phox subunit interacts with p67phox through its Phagocyte oxidase and Bem1 (PB1) domain and in turn is recruited to the phagosomal membrane during the formation of a functional enzyme complex [131]. However, for its stability at the membrane, its interaction with phosphatidylinositol 3-phosphate (PI3P) is essential via its PX domain [131]. It was shown that p40phox (R105Q) did not bind to PI3P, causing its early disappearance from the phagosome membrane and was unable to rescue the oxidase activity of p40phox-deficient neutrophils [130]. This study highlighted the importance of the p40phox subunit in the functionality of NADPH oxidase enzyme as well as the implications of its mutations in a disease context [130].

These findings suggest that the NADPH oxidase enzyme plays an essential role with regards to the pathogenesis of IBD. Further investigation is required to fully understand the consequences of impaired activity of this enzyme caused by mutations within the genes encoding the complex.

Chapter 2
Research Hypothesis and Aims

In the current study, NGS was performed on two sisters (from a consanguineous marriage) who were diagnosed with CD at ages 8 and 14, along with their unaffected family members. They exhibited symptoms similar to the patient reported by Matute *et al.* (2009). NGS can reveal hundreds of rare and potentially deleterious variants that may contribute to the development of IBD. Using inheritance modeling, a likely genetic candidate identified in these patients was a homozygous nonsense variant in the *NCF4* gene causing a premature stop codon at Trp239 in p40phox (c. 716 G>A NCF4 p.W239X).

2.1 Objective

To understand the pathobiological mechanism of a novel *NCF4* mutation that may be a significant contributor in the development of pediatric IBD.

2.2 Hypothesis

The truncation in p40phox (W239X) leads to impaired phagocyte oxidase activity which contributes to the onset of pediatric IBD.

2.3 Aims

Three **aims** have been established in order to test this hypothesis.

1. Define the disease phenotypes associated with the identified *NCF4* mutation.
2. Investigate the consequence of the reported mutation in p40phox on the known interaction with p67phox.
3. Establish a physiologically relevant model in order to understand the pathobiological mechanism of this mutation in contributing to the development of IBD.
4. Characterize the effect of this mutation on the innate immune defense.

Chapter 3
Materials and Methods

3.1 Mutation analysis: PCR and Sanger Sequencing

To confirm the mutation identified with NGS, the regions containing the reported mutation was amplified using Polymerase chain reaction (PCR) within the genomic DNA of the patients. The primers were designed around exon 8 of *NCF4*, including part of introns flanking the exon. Sequence for forward primer was: 5'-TCCTCCCTCTACAGAAGAAGAC-3' and reverse primer was: 5'-CCCATGAGAAGGTGATGTGAG-3'. Same primers were used for Sanger sequencing. The amplified product was sent to TCAG and results were retrieved.

3.2 Binding study

HEK293T cells were maintained in DMEM containing 10% heat-inactivated fetal bovine serum (FBS) and antibiotic-antimycotics at 37 °C in 5% CO_2. Monoclonal anti-Myc antibody and Polyclonal anti-GFP antibody were purchased from Millipore (05-724) and Invitrogen (A11122), respectively. Myc-tagged human p67phox and TTC7A (negative control) cDNA was cloned into the pCDNA3 vector (Invitrogen). GFP-tagged human p40phox cDNA vector was obtained from the lab of Dr. John Brumell (Sickkids, Toronto, Canada); GFP-tagged EGFP-C1 construct was obtained from the lab of Dr. Daniela Rotin (Sickkids, Toronto, Canada). Mutation p40phox (c. 716 G>A NCF4 p.W239X) was generated by site directed mutagenesis using PCR with QuikChange II Site-Directed Mutagenesis Kit, according to the manufacturer's instructions. All constructs were verified by sequencing. 293T cells were plated in 10-cm dishes and grown overnight to reach to 70% confluence. Cells were transiently co-transfected with constructs using

PolyJet transfection reagent. 24 hours after transfections, the cells were collected for cell lysis for protein analysis.

3.2.1 Co-immunoprecipitaion

For co-immunoprecipitation, 293T cells were washed with 1X PBS twice and extracted with standard cell lysis buffer (see below). Protein G-agarose beads and anti-GFP beads were washed twice with 1X PBS to make a 50% 1X PBS-agarose bead solution. 50 µl of the Protein G-agarose/anti-GFP bead solution was added to each 1mL solution of protein, followed by addition of 2µg of the appropriate primary antibody. The solution was rotated for 2 hours at 4°C. Afterwards, the solution was spun down and washed twice with lysis buffer and then resuspended in 30µl of 1X SDS protein sample buffer (40% glycerol, 240mM Tris/HCl, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol). The entire resulting solution was used for analysis by western blot.

3.3 Western blotting

All cells were lysed in cell lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1% Triton X-100), supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin, and 1 mM vanadate. Samples were resuspended in 1X SDS protein sample buffer (40% glycerol, 240mM Tris/HCl, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol) and loaded onto a gradient gel (BIO-RAD: 4568094). The gel was transferred onto PVDF membrane and blocked using 5% skim milk for 1 hour at room temperature (RT). The immunoblot was then incubated with appropriate primary and secondary antibodies for 1 hour at RT, respectively.

3.4 Densitometry

All densitometry measurements to indicate protein expression were measured using Chemidoc™ MP Image System (Bio-Rad). Electronic images of western blots were analyzed; band intensity levels of binding partners were measured and normalized to that of the immunoprecipitated protein.

3.5 Generation of RAW264.7 stable cell lines

Murine macrophages RAW264.7 were transfected with GFP-tagged human p40phox (WT and W239X) using FuGENE® 6 (Promega: E2691). 72 hours post transfection, geneticin (G418)-resistant cells were selected with 1.5 mg/mL G418 for one week. G418-selected macrophages were sorted using flow cytometry facility at SickKids. Cells were cultured in RPMI with 10% serum and allowed to recover to be used in functional assays.

3.6 Immunofluorescence

Stably transfected RAW264.7 cell lines expressing GFP-tagged WT or W239X p40phox were plated onto sterilized glass coverslips and were rested in RPMI with 10% FBS and antibiotic-antimycotics at 37 °C in 5% CO_2 overnight. Cells were fixed in 4% paraformaldehyde (PFA) for 20 mins and washed with PBS. Cells were then permeabilized in 0.1% Triton X-100 in PBS for 10 mins and blocked in BSA and 10% normal goat serum for 1 hour at RT. Antibodies for Rab5, Rab7 and Rab11 were obtained from cell signaling (C8B1), Novus Biologicals (NBP1-05048) and Invitrogen (71-5300) respectively. Cells were incubated with primary antibodies for Rab5, Rab7 or Rab11 overnight at 4°C and primary antibody for GFP for 1 hour at RT. This was followed by incubation with Alexa568 (Thermo Fisher) conjugated goat anti-rabbit and Alexa488 (Thermo Fisher) conjugated goat anti-mouse secondary antibodies for 1 hour at RT. DAPI was used for nuclear staining. Coverslips were then washed, mounted onto glass slides

using mounting medium (Dako Fluorescent Mounting Medium: S3023) and sealed using nail paint. Slides were visualized using Quorum Spinning Disk Confocal (Leica DMi8) set at 63X (oil imaging medium) for GFP, RFP and DAPI. Data was acquired at RT and images were analyzed and deconvolved using Velocity 6.3.0 (Perkin Elmer).

3.7 Live cell imaging

Quorum Spinning Disk Confocal (Leica DMi8) microscope was used at 63X objective (oil immersion lens) to film phagocytosis in RAW264.7 stable cell lines. Cells were plated on coverslip-bottom dishes and rested in RPMI with 10% FBS and antibiotic-antimycotics at 37 °C in 5% CO_2 overnight. To initiate phagocytosis, 20 µL of zymosan (Sigma, Cat. # Z4250) particles that had been freshly opsonized (original concentration = 2 mg/mL) with human IgG and labeled with Alexa555 succinimidyl ester (Invitrogen) (final concentration of 3 µg/mL) were fed to the cells. The particles were spun down at 1000 RPM for 1 minute for sedimentation onto the cells. The dish was mounted onto the microscope stage which was maintained at 37°C using a stage incubator. Fields were randomly selected to capture cells that were beginning to ingest a particle. Sequential images were collected with 488 nm and 568 nm excitation every 30 secs for 30-45 mins.

For opsonization of zymosan, particles at a concentration of 2 mg/mL were washed in PBS and then incubated with human IgG and Alexa555 succinimidyl ester for 60 mins at RT with end over end rotation.

3.8 Assay for NADPH oxidase activity

Generation of ROS was measured using chemiluminescence enhanced luminol or isoluminol. RAW264.7 stable cell lines, expressing WT and W239X p40phox, were counted using Cell Counter and plated ($1x10^5$ cells/well) and were rested in RPMI with 10% FBS and antibiotic-

antimycotics at 37 °C in 5% CO_2 overnight. Cells were washed and preincubated at 37°C for 10 minutes in PBSG (PBS plus 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM dextrose) in the presence of 50 µM isoluminol (Sigma: A8264) or 50 µM luminol (Sigma: 09253), without or with superoxide dismutase (SOD; final concentration: 75 µg/mL) (Sigma: S7571) with Horseradish peroxidase (HRP) (final concentration: 20 U/mL) (Sigma: 10814407001). Zymosan (1 mg/mL) or phorbol myristate acetate (PMA, 200 ng/mL) (Sigma: P8139) was added to activate cells (final volume: 200 µL), and the relative light units (RLU) were monitored at 60 second intervals for up to 1 hour by the Long Kinetic module in a Lmax microplate luminometer from Molecular Devices (Sunnyvale, CA). Integrated RLU values and area under curve (AUC) were calculated by SOFTmax software (Molecular Devices).

3.9 Gentamicin Protection Assay

RAW264.7 stable cell lines expressing WT and W239X p40phox were plated ($2x10^6$ cells/well) and were rested in RPMI with 10% FBS and antibiotic-antimycotics at 37 °C in 5% CO_2 overnight. Overnight culture for *E. coli* DH10β strain was started in polypropylene tubes and was incubated at 37°C shaking at 250 RPM. The next day, overnight culture was subcultured (1:1000) to a mid-log phase for 3 hours at 37 °C shaking at 250 RPM to give a reading of 0.5-0.6 at OD_{600} (used to determine bacterial count). Cells were washed with PBS and infected with multiplicity of infection (MOI) at 10:1 in serum free and antibiotic free RPMI. Internalization of bacteria was allowed to occur for 2 hours at 37 °C in 5% CO_2. Time point 0 was assumed post internalization period. Cells were washed and incubated in RPMI + 10% serum + gentamicin (100 µg/mL) for 2 hours. The media was replaced with RPMI + 10% serum + gentamicin (10 µg/mL) for the time points that followed. Cells were lysed in 0.1% Triton X-100 in PBS. The

lysate was diluted in LB medium and plated on LB plates that were incubated at 37 °C. Colonies were counted and plotted as colony forming units/well.

3.10 Statistical Analysis

All data represent three independent replicates, unless otherwise stated. Data is represented as means ± standard deviation from three independent biological replicates. Statistical analysis was performed using GraphPad Prism 5. P values were obtained using two-tailed Student's t-test, unless otherwise stated. A p value of < 0.05 was considered statistically significant.

Chapter 4
Results

4.1 p40phox (W239X) causes an atypical and less severe form of CGD-IBD.

Approximately 70% of patients with CGD have mutations in X-linked *CYBB* gene while the rest acquire autosomal recessive mutations in *CYBA, NCF1* and *NCF2* [116]. Only one case has been reported to date with a patient acquiring a mutation in *NCF4* gene [116]. Reported mutations in *CYBB, CYBA* and *NCF2* are heterogenous (missense, nonsense, deletion and frameshift mutations) and more severe than mutations within *NCF1* [132]. These mutations can lead to a total loss of the protein, downregulation of expression or loss of function [133, 134]. A GT deletion at the beginning of exon 2 is most frequently identified in *NCF1* which leads to a premature stop codon [134]. The frequency of this mutation is high which has been attributed to the presence of a structurally similar pseudogene that recombines with wildtype *NCF1* [134]. It is generally accepted that X-linked patients have a more severe phenotype than those with autosomal recessive mutations characterized by an early age of onset, high occurrence of inflammatory manifestations and increased risk of mortality in comparison to autosomal recessive patients [135]. However, significant clinical heterogeneity has been observed in CGD patients [133]. It is believed that residual ROS production determines the disease prognosis, where better clinical outcomes have been reported in certain patients with frameshift, nonsense, splice and deletion mutations in X-linked *CYBB* gene due to the presence of a partly functional protein [120]. Additionally, IBD is a common manifestation in patients with CGD with colon being the most affected site within the GI tract [136]. The treatment for patients with CGD-IBD is not without complications as it brings about a dilemma of treating an immunodeficiency with

anti-inflammatory drugs that would further impair the immune response [137]. Consequently, genetic and biochemical analysis are required to better characterize the phenotypic heterogeneity observed in CGD-IBD patients for personalized therapy.

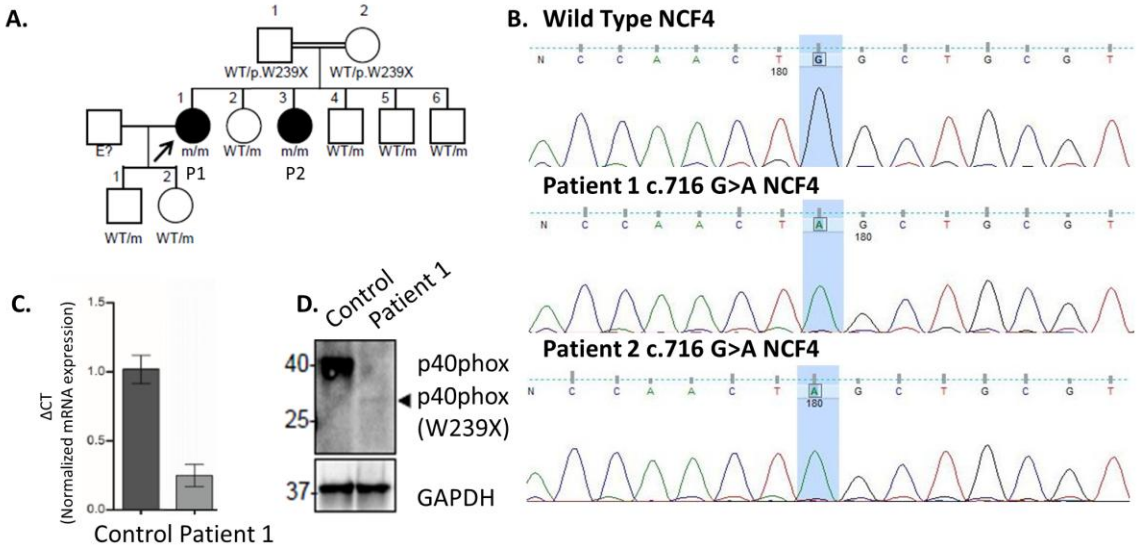
4.1.1 Results

In collaboration with Dr. Jean-Laurent Casanova and his group, we became part of an elaborate study, wherein, an international multicenter cohort of 20 patients with *NCF4* mutations in 12 families was studied [138]. The genotype and phenotype features of patients with various *NCF4* mutations were characterized. WES and NGS with a panel of PID genes identified eight novel variants within the *NCF4* gene.

This thesis is focused on patients bearing p40phox (W239X) mutations (a nonsense mutation within *NCF4*). The two sisters come from a consanguineous marriage with no reported disease in any other family member (Figure 4.1 A). A summary of phenotypes from these patients is provided in Table 4.1. The clinical phenotypes included Crohn's like-IBD. The mutation was validated with Sanger sequencing (Figure 4.1 B). The mode of inheritance was autosomal recessive as parents were heterozygous for the mutation. Furthermore, the combined annotation-dependent depletion (CADD) score was 40, classifying the mutation as deleterious. The mRNA expression within patient cells was lower when compared to control population but not completely abolished (Figure 4.1 C). A western blot from patient neutrophils showed that p40phox is being expressed but is downregulated and truncated, indicating the presence of the W239X mutation (Figure 4.1 D).

Table 4.1 Clinical details of sisters with p40phox (W239X) (adapted from van de Geer *et al.* (2018)).

Patient	Year of Birth	Mutation	Sex	Status	Age of onset	Clinical phenotypes	Treatment	Prophylaxis
1	1984	W239X	F	Alive	8	Gastritis, severe complex perianal fistula with recurrent abscesses and anal stenosis. Adenocarcinoma of anal canal.	Steroids, azathioprine, infliximab, golimumab. Chemo- and radiotherapy for adenocarcinoma.	none, post-HSCT
2	1979	W239X	F	Alive	14	Esophageal ulcerations and severe perianal fistula diagnosed as Crohn's disease. Recurrent skin abscesses. Episcleritis. Severe periodontitis.	Steroids, adalimumab, high-dose proton pump inhibitors	none, post-HSCT


A.
1
2
WT/p.W239X
WT/p.W239X
1
2
3
4
5
6
E?
m/m
WT/m
m/m
WT/m
WT/m
WT/m
P1
P2
1
2
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WT/m
B. Wild Type NCF4
Patient 1 c.716 G>A NCF4
Patient 2 c.716 G>A NCF4
C.
ΔCT
(Normalized mRNA expression)
1.5
1.0
0.5
0.0
Control
Patient 1
D.
Control
Patient 1
40
25
37
p40phox
p40phox
(W239X)
GAPDH

Figure 4.1: Identification and characterization of *NCF4* mutation in patients with CD. A) Pedigree chart of the patient family. Squares represent male family members and circles represent female family members. The affected sisters are shaded in black. 'E?' indicates an unknown genotype and the arrow indicates the index patient. Retrieved from van de Geer *et al.* (2018). B) DNA chromatograms corresponding to the wild type and patient alleles. C) mRNA expression of *NCF4* from control and patient neutrophils. ΔCt is the relative concentration of PCR product of *NCF4.* Retrieved and recreated from van de Geer *et al.* (2018). D) Western blot of total protein lysate showing the expression of p40phox (WT and W239X) in control and patient neutrophils. GAPDH was used as a loading control. Retrieved from van de Geer *et al.* (2018).

4.2 p40phox (W239X) does not interact with p67phox within the NADPH oxidase complex.

Previous studies have found that p40phox (WT) interacts with p67phox via its C-terminus [139-141]. This interaction has been well established by the use of several systems, such as yeast two-hybrid, co-immunoprecipitation and chromatography [139-141]. As mentioned before, p40phox, p47phox and p67phox exist as a heterotrimeric complex within the cytosol in a resting cell [142-144]. Upon activation, this complex is recruited to the membrane to complete the NADPH oxidase which is then able to generate an efficient respiratory burst as part of the innate immune defense mechanism [85]. The importance of the interaction between p67phox and p40phox lies in the recruitment of p67phox (directly involved in regulating electron flow within the NADPH oxidase) to the membrane [131]. It is hypothesized that the nonsense mutation within the patients that is causing the expression of a truncated p40phox without its C-terminus (p40phox (W239X)) is leading to the abrogation of interaction between p40phox and p67phox.

4.2.1 Results

To understand the biochemical significance of p40phox (W239X), plasmids encoding either the wild type or mutated versions of p40phox tagged with GFP (at the N-terminus) were expressed in HEK293T cells (Fig. 4.2 A). The cells were lysed 24 hours post transfection. Western blot analysis of the lysate revealed that p40phox expressed equally well in its wild type as well as its mutant configuration, when overexpressed in HEK293T cells with no significant difference in densitometry between the protein expression of the two constructs (Fig. 4.2 B). However, p40phox (W239X) expressed at a lower molecular weight than p40phox (WT) (around 10 kDa difference), indicating the presence of the truncation as a result of the premature stop codon. This truncation leads to the loss of the PB1 domain, a known protein-protein interaction domain,

which is important for the interaction between p40phox and p67phox. To determine whether this loss of the PB1 domain abrogates the interaction between p40phox and p67phox, plasmids encoding either the wild type or mutated versions of p40phox tagged with GFP (at the N-terminus) were co-expressed with a plasmid encoding wild type p67phox tagged with myc (at the C-terminus) in HEK293T cells and co-immunoprecipitation was performed (Fig. 4.3 A). The experiment was performed in a reciprocal manner using a GFP antibody to pull down GFP-p40phox (WT and W239X) as well as a myc antibody to pull down myc-p67phox (Fig. 4.3 B). Cells were lysed 24 hours post transfection, followed by 2 hours of immunoprecipitation. Immunoblots showed that whereas GFP-p40phox (WT) co-immunoprecipitated myc-p67phox, GFP-p40phox (W239X) did not. Similarly, whereas myc-p67phox co-immunoprecipitated GFP-p40phox (WT), it did not co-immunoprecipitate GFP-p40phox (W239X). The difference in binding between p40phox (WT) and p40phox (W239X) with p67phox was statistically significant (Fig. 4.3 C). This confirmed the loss in interaction between p67phox and p40phox (W239X).

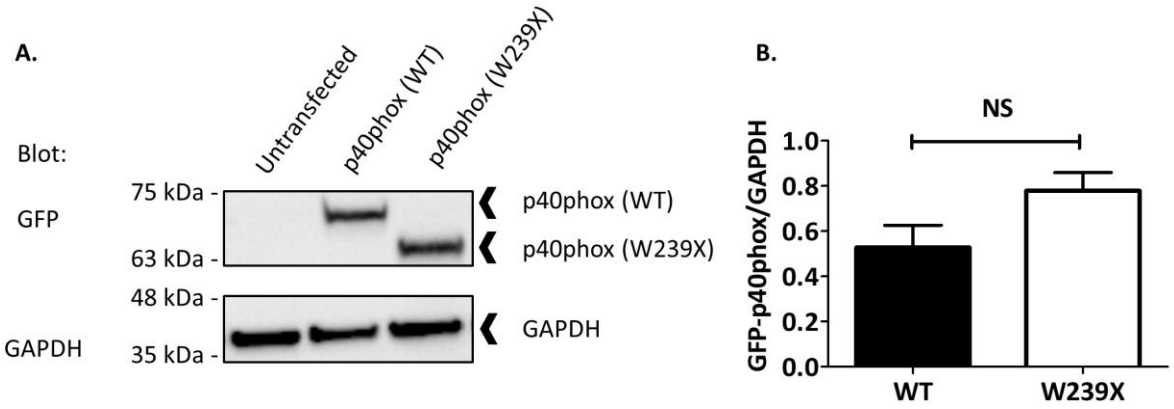

A.
Untransfected
p40phox (WT)
p40phox (W239X)
Blot:
GFP
75 kDa -
63 kDa -
p40phox (WT)
p40phox (W239X)
48 kDa -
35 kDa -
GAPDH
GAPDH
B.
NS
GFP-p40phox/GAPDH
1.0
0.8
0.6
0.4
0.2
0.0
WT
W239X

Figure 4.2: Protein quantification of HEK293T cells overexpressing GFP tagged-p40phox (WT and W239X). A) Lysates from HEK293T cells overexpressing GFP-p40phox (WT and W239X) were collected and analyzed for expression of p40phox using anti-GFP antibody. We probed with an anti-GAPDH as a loading control. B) Quantification of GFP-p40phox (WT and W239X) in HEK239T cells. Signal for GFP-p40phox (WT and W239X) was measured relative to GAPDH. Graph represents mean ± SD for n=3. NS: not significant (Student's *t* test).

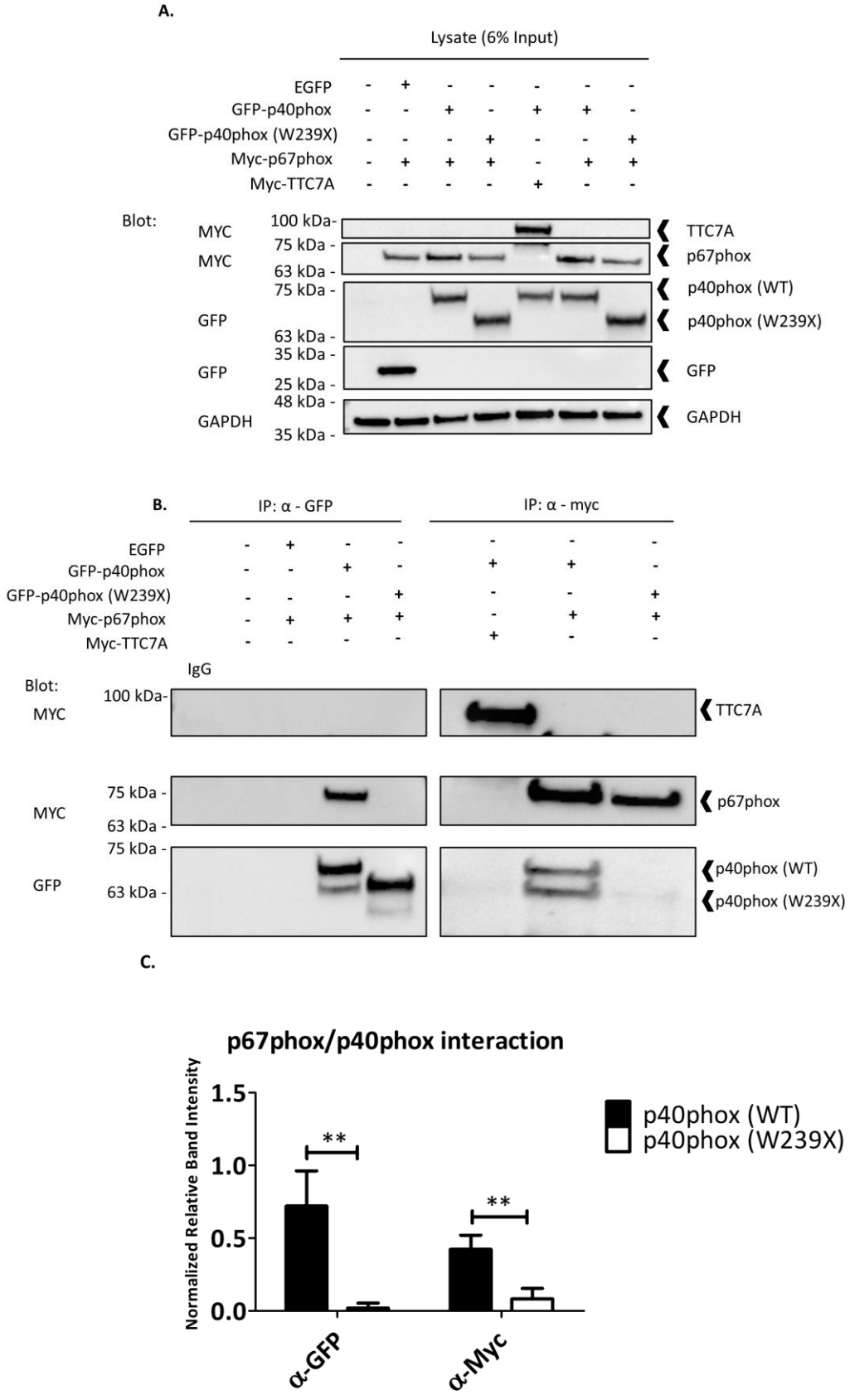

A.
Lysate (6% Input)
EGFP
GFP-p40phox
GFP-p40phox (W239X)
Myc-p67phox
Myc-TTC7A
Blot:
MYC
MYC
GFP
GFP
GAPDH
100 kDa-
75 kDa -
63 kDa -
75 kDa -
63 kDa -
35 kDa -
25 kDa -
48 kDa -
35 kDa -
TTC7A
p67phox
p40phox (WT)
p40phox (W239X)
GFP
GAPDH
B.
IP: α - GFP
IP: α - myc
EGFP
GFP-p40phox
GFP-p40phox (W239X)
Myc-p67phox
Myc-TTC7A
IgG
Blot:
MYC
100 kDa-
TTC7A
MYC
75 kDa -
63 kDa -
p67phox
GFP
75 kDa -
63 kDa -
p40phox (WT)
p40phox (W239X)
C.
p67phox/p40phox interaction
Normalized Relative Band Intensity
1.5
1.0
0.5
0.0
**
**
p40phox (WT)
p40phox (W239X)
α-GFP
α-Myc

Figure 4.3: Truncation of p40phox leads to loss of interaction with p67phox. HEK239T cells were co-transfected with myc-p67phox and GFP-p40phox (WT or W239X). A) Lysate blot for reciprocal co-immunoprecipitation of GFP-p40phox (WT and W239X) and myc-p67phox. EGFP and myc-TTC7A were used as negative controls. B) Immunoprecipitation was performed using anti-GFP beads to pull down GFP-p40phox (WT or W239X) and anti-myc-coupled Protein G beads to pull down myc-p67phox. C) Densitometry of immunoprecipitated myc-p67phox was normalized to total levels of immunoprecipitated GFP-p40phox (WT or W239X) and vice versa. n=3 experimental replicates; **p<.05 as calculated by Student's *t* test.

4.3 p40phox (W239X) is mislocalized to early endosomes within macrophages.

p40phox was the last subunit within the NADPH oxidase to have its role characterized. The expression of p40phox is restricted to bone marrow cells, such as, neutrophils, monocytes, basophils, eosinophils, mast cells, megakaryocytes, B and T cells [145]. As part of the NADPH oxidase, the integral role of p40phox in innate immune defense is deduced from studies in phagocytes [145]. p40phox is a 40kDa protein comprising of 339 amino acids and consists of 3 domains (Fig. 4.4) [145]. At the N-terminus is a PX domain that specifically binds PI3P, an SH3 domain that can interact with proline rich regions (though no direct interaction has been established) and a PB1 domain at the C-terminus through which it associates with p67phox [145]. In a resting cell, p40phox remains cytosolic in a complex with p67phox and p47phox [145]. However, upon activation via detection of pathogens by the cell surface receptors, the protein is recruited to the membranes of phagosomes that are known to be rich in PI3P [145]. Thus, the localization of p40phox within a resting and an activated cell and its domain interactions are important in modulating the function of the NADPH oxidase at different stages of phagocytosis.

4.3.1 Results

A physiologically relevant model was established by generating stable cell lines overexpressing GFP-p40phox (WT and W239X) in RAW264.7 murine macrophages, which were validated using western blot (Fig. 4.5 A). Immunofluorescence imaging showed that the expression pattern in the stables for p40phox (WT) was diffuse across the cytoplasm, while that of p40phox (W239X) was aggregated in punctae throughout the cell (Fig. 4.5 B). Previously, Ueyama *et al.* (2007, 2011) had shown that mutants missing the PB1 domain in p40phox localized to dot like structures in both HEK293T and RAW264.7 cells [131, 146]. This is in corroboration to our

findings as the predicted structure of p40phox (W239X) is also missing the PB1 domain. To determine the identity of these aggregates and the localization of p40phox (WT and W239X), RAW264.7 macrophages expressing p40phox (WT and W239X) were co-stained with Rab5, a GTPase and an early endosomal marker [147]. Co-localization of Rab5 and p40phox (W239X) was observed, indicating that p40phox (W239X) was being mislocalized to early endosomes in comparison to p40phox (WT) which remained cytosolic (Fig. 4.6 A). Furthermore, cells were also stained with Rab7, a late endosome marker [148] as well as Rab11, recycling endosome marker [149] to confirm the localization of the truncated protein. p40phox (W239X) showed minimal co-localization with Rab7 and no co-localization with Rab11, suggesting that the protein is localizing primarily to early endosomes (Fig. 4.6 B and C).

Additionally, live microscopy showed that GFP-p40phox (WT) transiently associates to phagosomes upon induction of phagocytosis via IgG-opsonized zymosan (as a stimulus to induce phagocytosis). This association was seen to be even more apparent in cells expressing GFP-p40phox (W239X) due to the proteins consistent presence on early endosomes (Fig. 4.7).

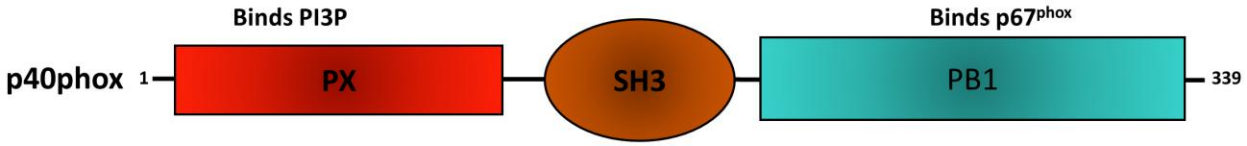
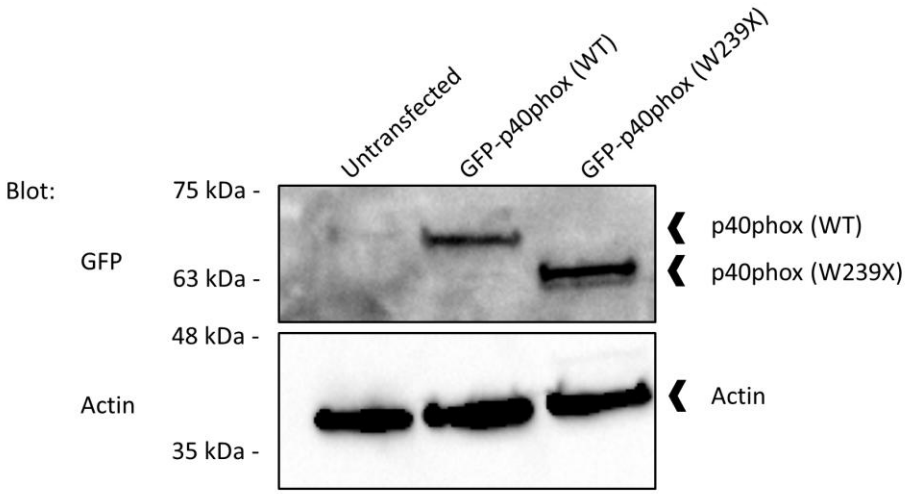


Figure 4.4: Domain structure of p40phox. p40phox comprises of 3 domains. PX domain binds PI3P with high affinity. Interaction of SH3 domain is not currently known, but presumably binds proline rich regions. Interaction of PB1 domain with p67phox has been well documented.

A.


Untransfected
GFP-p40phox (WT)
GFP-p40phox (W239X)
Blot:
75 kDa -
GFP
63 kDa -
48 kDa -
Actin
35 kDa -
p40phox (WT)
p40phox (W239X)
Actin

B.

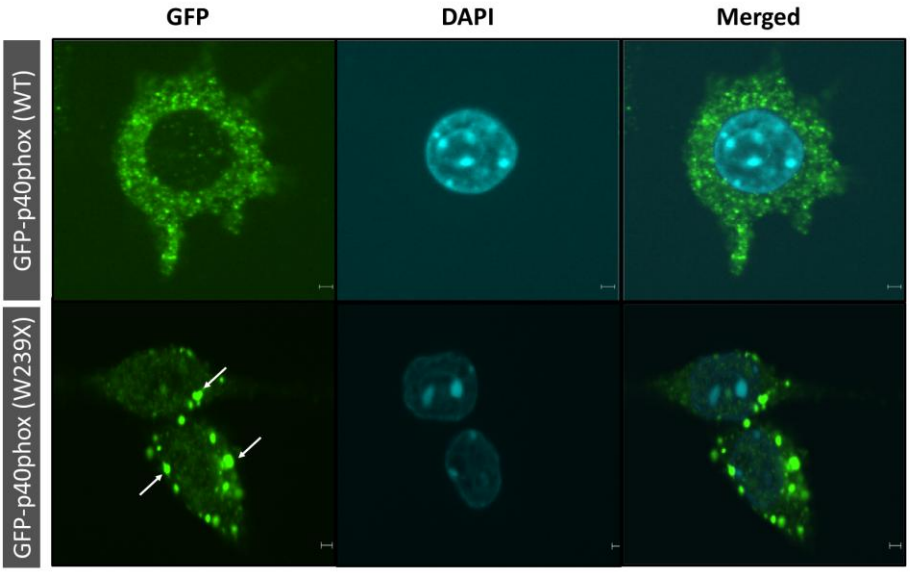
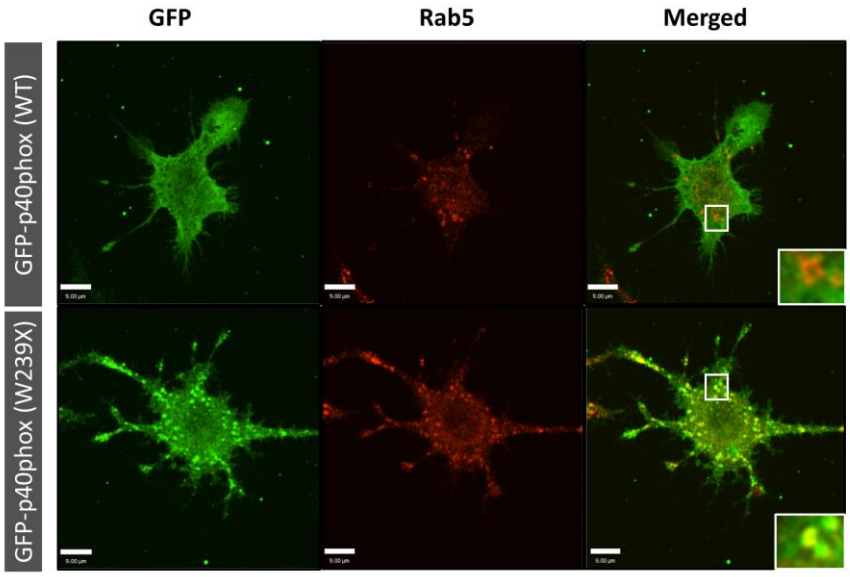
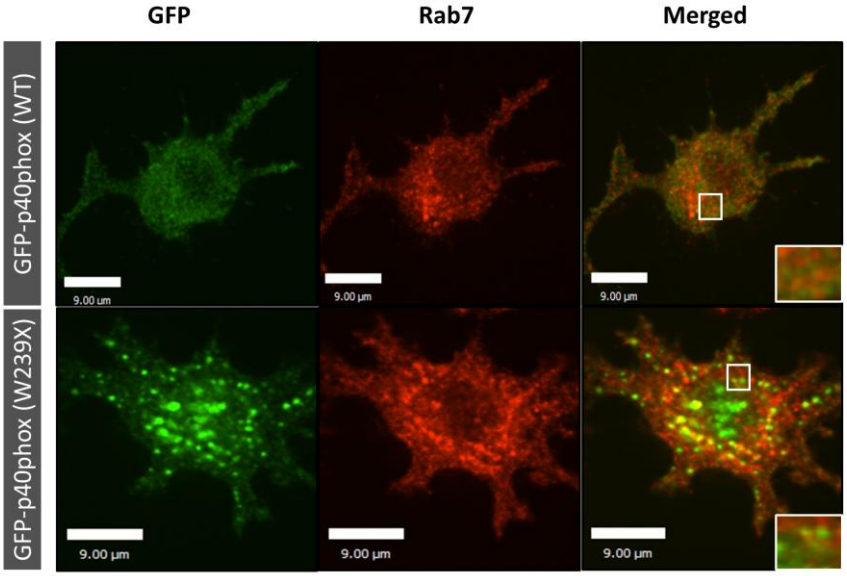

GFP
DAPI
Merged
GFP-p40phox (WT)
GFP-p40phox (W239X)

Figure 4.5: p40phox (W239X) mislocalizes and forms aggregates when stably expressed in RAW264.7 murine macrophages. A) Western blot validation of RAW264.7 murine macrophages stably expressing p40phox (WT and W239X). Actin was used as a loading control. B) Immunofluorescence images showing GFP tagged p40phox (WT) (top panel) and p40phox (W239X) (bottom panel) in RAW264.7 murine macrophages. p40phox(W239X) was seen to be mislocalized and formed aggregates (indicated by white arrows).

A.


GFP
Rab5
Merged
GFP-p40phox (WT)
GFP-p40phox (W239X)

B.


GFP
Rab7
Merged
GFP-p40phox (WT)
GFP-p40phox (W239X)
9.00 µm

C.

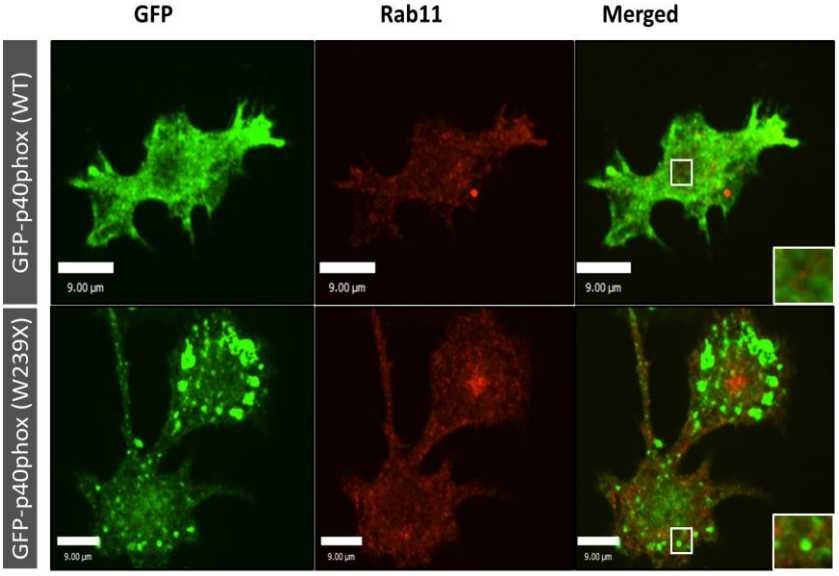

GFP
Rab11
Merged
GFP-p40phox (WT)
GFP-p40phox (W239X)
9.00 µm

Figure 4.6: p40phox (W239X) localizes to early endosomes. RAW264.7 cells stably transfected with either GFP-40phox (WT) or GFP-p40phox (W239X) were fixed and stained with GFP and endosome markers to detect the localization of p40phox (WT and W239X). A) Confocal images of p40phox (WT) (top panel) and p40phox (W239X) (bottom panel) showing that p40phox (W239X) co localizes with Rab 5 positive puntae. Scale bar: 9µm B) Confocal images showing that p40phox (WT) (top panel) does not co-localize with Rab7 but some degree of co-localization of p40phox (W239X) (bottom panel) with Rab7 positive punctae is observed. Scale bar: 9µm C) Confocal images showing neither p40phox (WT) (top panel) nor p40phox (W239X) (bottom panel) co-localize with Rab 11 positive puntae. Scale bar: 9µm

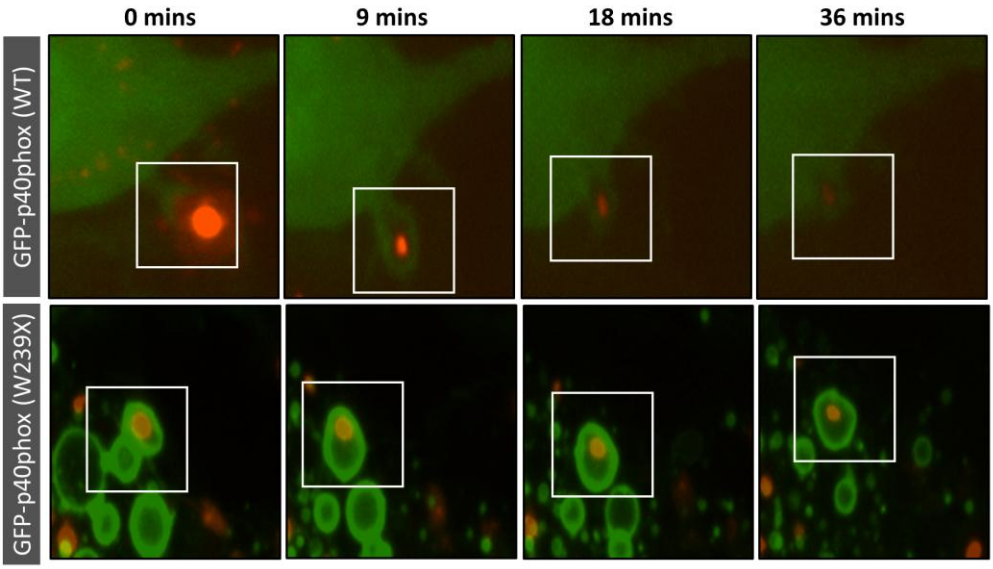

0 mins
9 mins
18 mins
36 mins
GFP-p40phox (WT)
GFP-p40phox (W239X)

Figure 4.7: p40phox (WT) transiently associates with phagosomes upon induction of phagocytosis while p40phox (W239X) persistently stays at the phagosome membrane. RAW264.7 macrophages were stably transfected with GFP-p40phox (WT) and GFP-p40phox (W239X). Cells were fed IgG opsonized-zymosan particles, labeled with Alexa555 succinimidyl ester, to induce phagocytosis. Live imaging was performed for 40 mins to capture the induction and diffusion of phagosome.

4.4 Macrophages expressing p40phox (W239X) have impaired oxidative burst in response to different stimuli.

Respiratory burst results in the formation of ROS that has antimicrobial effects on foreign pathogens [29]. NADPH oxidase is a key enzyme that is known to produce ROS as a defence mechanism elicited in phagocytes [29]. Interestingly, mutations within *NCF4*, the gene encoding p40phox, causing CGD-IBD has only been reported once by Matute *et al.* (2009) who showed that patient neutrophils had defective intracellular ROS production but not extracellular ROS production in response to various stimuli. It has also been previously shown that reduced binding between p40phox and p67phox reduces the cells ability to mount an effective respiratory burst to kill invading pathogens [128]. Since the mutation reported in this case causes a complete abrogation of interaction, it was hypothesized that phagocytes expressing p40phox (W239X) would exhibit impaired ROS production.

4.4.1 Results

A luminol-based chemiluminescence assay was performed to measure ROS production in the stable cell lines. This assay allows measuring extra- and intracellular ROS in real time. In the presence of horse radish peroxidase (HRP), isoluminol, which is a cell impermeable compound, reacts with ROS to produce a light reaction which is a quantitative measure of the presence of ROS [150]. Similarly, luminol (5-amino-2, 3-dihydro- 1, 4-phthalazinedione) measures extra- and intracellular ROS as it is a cell permeable compound (Fig. 4.8) [150, 151]. When the stable cell lines were stimulated with PMA, which activates PKC, thereby stimulating the accumulation of NADPH oxidase [85], no difference in extracellular oxidative burst was observed as seen in the dynamic curve (measuring relative light units (RLU) over time) (Fig. 4.9 A). Quantification of the area under curve of the isoluminol activity upon PMA stimulation also showed no

significant difference between p40phox (WT) and p40phox (W239X) (Fig 4.9 B). However, intracellular burst appeared to be impaired in p40phox (W239X) expressing cells as these cells were unable to sustain the burst as efficiently as the wild type (Fig. 4.9 C). This also affected the area under curve significantly between p40phox (WT) and p40phox (W239X) for the luminol activity (Fig. 4.9 D). Another stimulus was used to confirm these findings. Zymosan is known to be engulfed by phagocytes and primarily induces intracellular ROS [152]. Cells expressing p40phox (WT) and p40phox (W239X) showed minimal production of extracellular ROS, as seen by the dynamic curve for isoluminal based chemiluminescence (Fig. 4.10 A). The area under curve was quantified and was significantly different between p40phox (WT) and p40phox (W239X) (Fig. 4.10 B). But since the ROS production was negligible, this difference may not be of importance. The intracellular oxidative burst upon zymosan stimulation was impaired in cells expressing p40phox (W239X), similar to what was seen with PMA stimulation (Fig. 4.10 C and D).

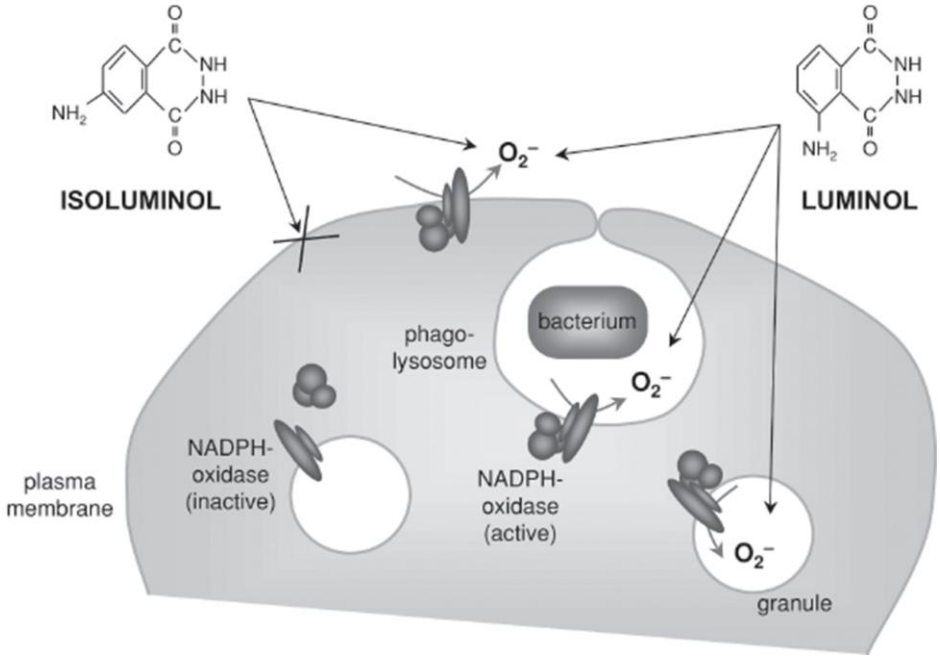

ISOLUMINOL
LUMINOL
O_2^-
bacterium
phago-
lysosome
O_2^-
NADPH-
oxidase
(inactive)
plasma
membrane
NADPH-
oxidase
(active)
O_2^-
granule

Figure 4.8: Schematic representation of chemiluminescence assay. Reprinted with permission from Methods in Molecular Biology. Neutrophil methods and protocol. Dahlgren *et al.* Measurement of respiratory burst products generated by professional phagocytes. 412: (349-363), 2007.

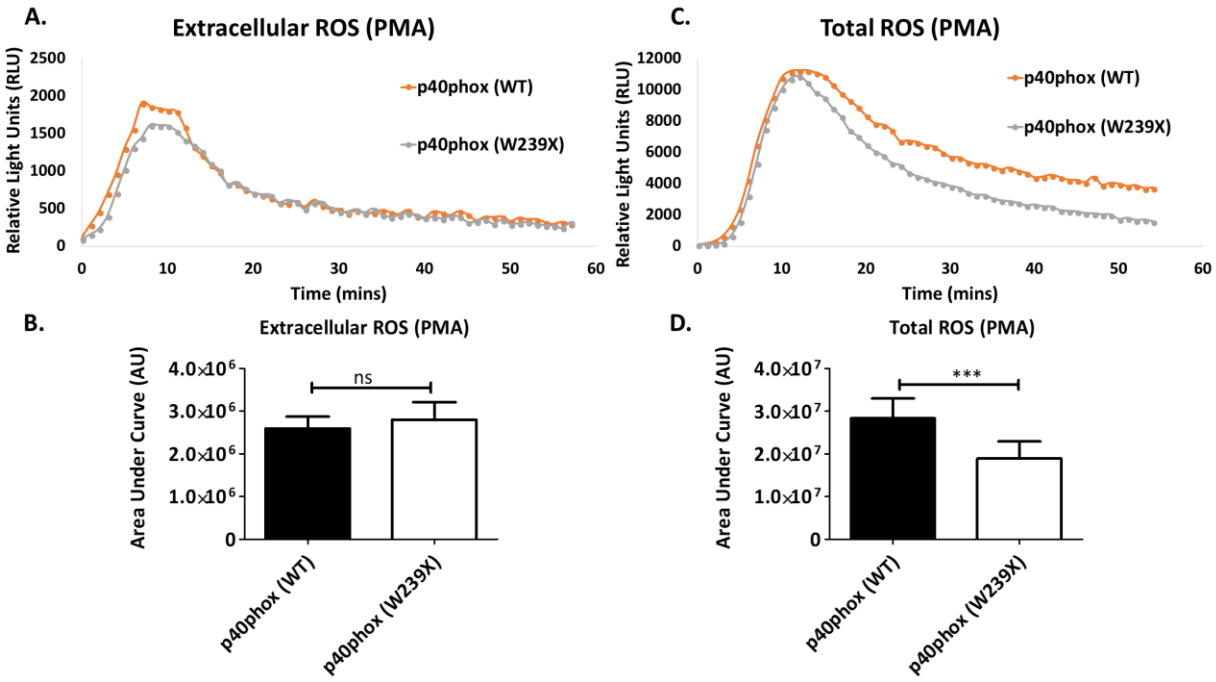

A.
Extracellular ROS (PMA)
Relative Light Units (RLU)
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
Time (mins)
p40phox (WT)
p40phox (W239X)
C.
Total ROS (PMA)
Relative Light Units (RLU)
12000
10000
8000
6000
4000
2000
0
0
10
20
30
40
50
60
Time (mins)
p40phox (WT)
p40phox (W239X)
B.
Extracellular ROS (PMA)
Area Under Curve (AU)
4.0x10^6
3.0x10^6
2.0x10^6
1.0x10^6
0
ns
p40phox (WT)
p40phox (W239X)
D.
Total ROS (PMA)
Area Under Curve (AU)
4.0x10^7
3.0x10^7
2.0x10^7
1.0x10^7
0
***
p40phox (WT)
p40phox (W239X)

Figure 4.9: RAW264.7 murine macrophages that stably express p40phox (W239X) show impaired intracellular oxidative burst when stimulated with PMA. A) Time course of isoluminol based chemiluminescence upon addition of 200 ng/mL PMA. B) Quantification of area under curve (AUC) for isoluminol based chemiluminescence. C) Time course of luminol based chemiluminescence upon addition of 200 ng/mL PMA. D) Quantification of area under curve (AUC) of luminol based chemiluminescence. n=3 experimental replicates. Results are expressed as mean ± SD for AUC. ***p< 0.001 with Student's *t* test. ns: not significant.

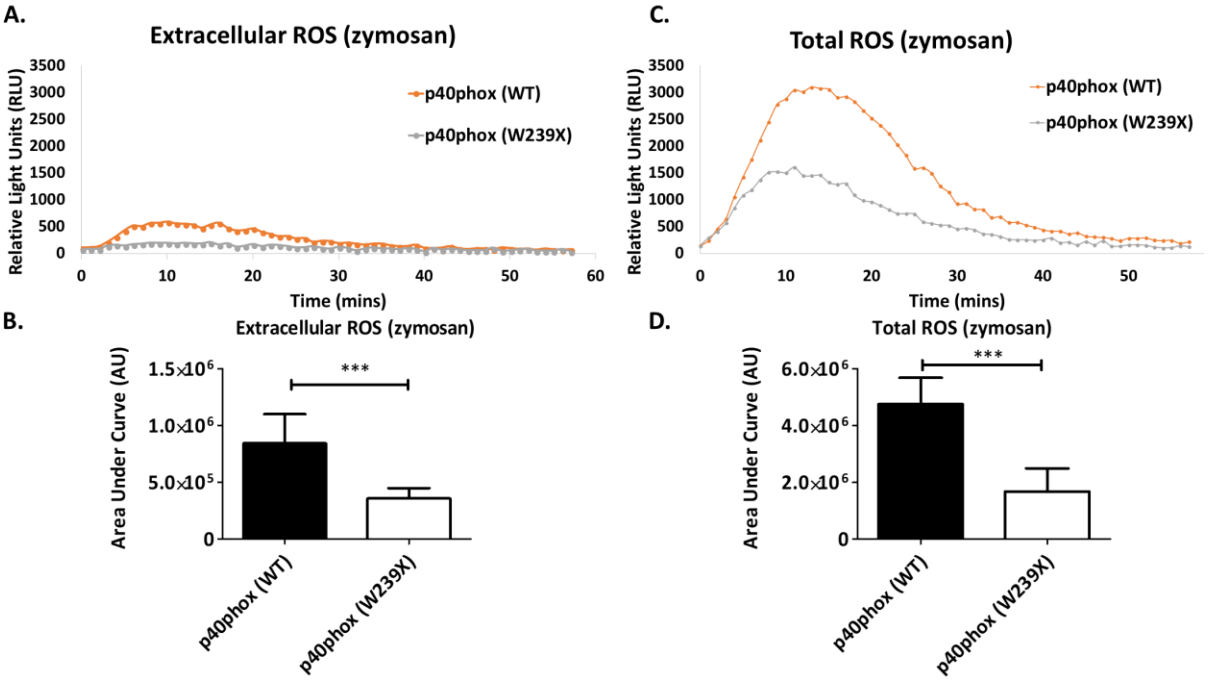

A.
Extracellular ROS (zymosan)
Relative Light Units (RLU)
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
Time (mins)
p40phox (WT)
p40phox (W239X)
C.
Total ROS (zymosan)
Relative Light Units (RLU)
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
Time (mins)
p40phox (WT)
p40phox (W239X)
B.
Extracellular ROS (zymosan)
Area Under Curve (AU)
1.5x10^6
1.0x10^6
5.0x10^5
0
***
p40phox (WT)
p40phox (W239X)
D.
Total ROS (zymosan)
Area Under Curve (AU)
6.0x10^6
4.0x10^6
2.0x10^6
0
***
p40phox (WT)
p40phox (W239X)

Figure 4.10: RAW264.7 murine macrophages that stably express p40phox (W239X) show impaired intracellular oxidative burst when stimulated with zymosan. A) Time course of isoluminol based chemiluminescence upon addition of 1 mg/mL zymosan. B) Quantification of area under curve (AUC) for isoluminol based chemiluminescence. C) Time course of luminol based chemiluminescence upon addition of 1 mg/mL zymosan. D) Quantification of area under curve (AUC) of luminol based chemiluminescence. n=3 experimental replicates. Results are expressed as mean ± SD for AUC. ***$p < 0.001$ with Student's *t* test.

4.5 Macrophages expressing p40phox (W239X) have reduced bactericidal activity in vitro.

ROS production is one of the central mechanisms whereby phagocytes defend the host against bacterial infections [30]. Patients with defects in the components of NADPH oxidase complex develop CGD and are known to have increased susceptibility to bacterial infections [30]. Previous studies have shown that neutrophils from p40phox$^{-/-}$ mice exhibited impaired bactericidal activity to *Staphylococcus aureus* both in vitro and in vivo [153]. A recent study has also shown that although; the recruitment of neutrophils and macrophages to the site of infection was not impaired in *Salmonella* infected p40phox$^{-/-}$ mice, the resolution of the inflammatory response, respiratory burst and the efficiency of bactericidal activity by the phagocytes was impaired [154]. This suggests that p40phox has an essential role in the killing of pathogens via NADPH oxidase complex. Based on this evidence, it was hypothesized that RAW264.7 macrophages expressing p40phox (W239X) will also have reduced bactericidal activity as a result of its loss of association with the NADPH oxidase.

4.5.1 Results

To directly test bacterial killing, a gentamicin protection assay was performed on the macrophages stably expressing GFP-p40phox (WT and W239X) (Fig. 4.11). Briefly, macrophages were incubated with a non-pathogenic strain of *E. coli* for various time points. Following internalization of bacteria, gentamicin containing media was used to incubate the cells as gentamicin is a non-cell permeable antibiotic and kills all extracellular bacteria [155]. The principle of this assay is centered on the ability of macrophages to engulf and kill bacteria [155]. The cells are lysed at various time points to release intracellular bacteria, followed by dilution in LB media and plating on LB agar plates to enumerate colony forming units (CFU) from the bacteria that survived. The results showed that more viable bacteria were recovered from

macrophages expressing p40phox (W239X) at the various time points tested (Fig. 4.12). The difference was significant. This suggests that p40phox (W239X) macrophages have reduced capacity to kill pathogens. This also suggests that patients expressing this mutation may have similar biological consequences, resulting in increased susceptibility to infections.

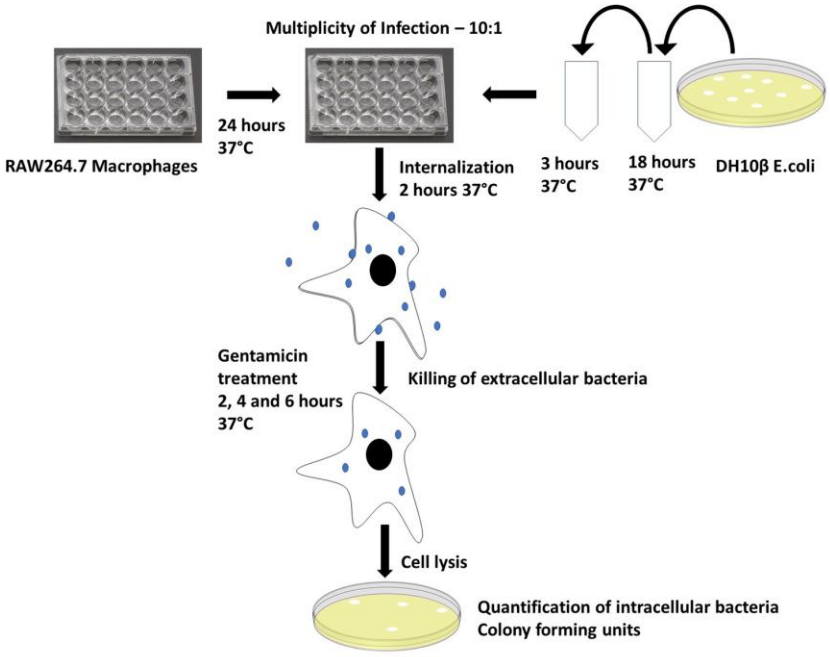


Figure 4.11: Schematic representation of gentamicin protection assay.

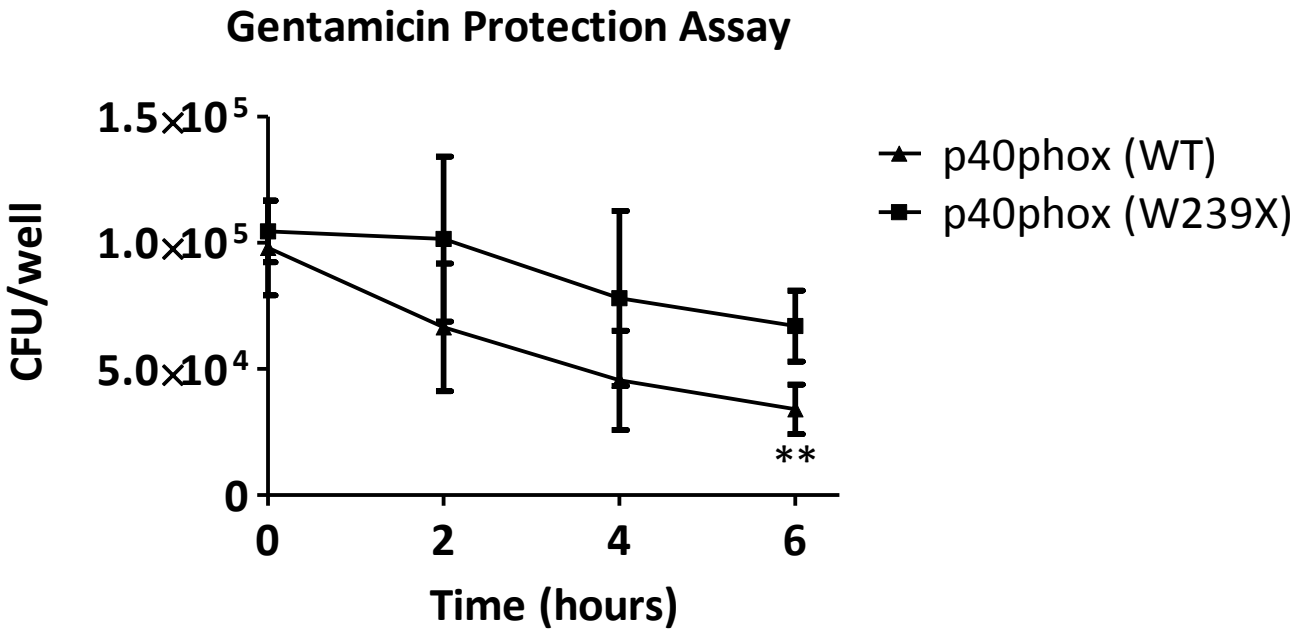

Gentamicin Protection Assay
1.5×10^5
1.0×10^5
5.0×10^4
0
CFU/well
0
2
4
6
Time (hours)
**
p40phox (WT)
p40phox (W239X)

Figure 4.12: p40phox (W239X) macrophages have reduced bactericidal activity. Stable cell lines expressing p40phox (WT and W239X) were infected with a DH10β strain of *Escherichia coli* at the rate of 1:10 (cell/bacteria). Intracellular bacteria were determined by counting colonies from lysate plated on LB agar plates at various time points. n=3 experimental replicates. The results are expressed as mean ± SD. **$p < 0.05$ with Student's *t* test.

Chapter 5
Discussion

NADPH oxidase is a membrane bound enzyme that generates ROS as part of the innate immune defense to kill invading pathogens [29]. Mutations within the components that make up this enzyme reportedly cause CGD [133]. X-linked mutations in the *CYBB* gene, encoding gp91phox, and autosomal recessive mutations in *NCF2* and *CYBA*, encoding p67phox and p22phox respectively, are known to cause severe disease while autosomal recessive mutations in *NCF1*, encoding p47phox, shows a less severe form of disease [132]. Only one case has been reported about a mutation within *NCF4*, encoding p40phox, that resulted in granulomatous colitis within the patient [130]. CGD patients suffer from recurrent bacterial and fungal infections and present with chronic inflammation, notably in the gastrointestinal tract [133]. For this reason, IBD is a common manifestation for CGD patients [133]. Previously, our lab has shown that variants within genes encoding NADPH oxidase increase susceptibility to VEOIBD [128]. However, the exact mechanism of how these genes contribute to the onset of IBD is largely unknown. In this study, a mechanistic approach was taken in order to characterize the effect of a nonsense mutation within *NCF4* in causing the onset of pediatric IBD.

5.1 p40phox (W239X) causes an atypical and less severe form of CGD-IBD.

Previously, only one patient has been reported to have a mutation within *NCF4* resulting in granulomatous colitis [130]. The patient had two variants in *NCF4*, a truncating allele (K52RfsX79) and a point mutation that encoded R105Q in the PHOX homology (PX) domain [130]. K52RfsX79 could not be detected at the protein level due to the lack of an appropriate antibody [130]. R105Q p40phox was expressed and analyzed for functional tests [130]. The patient phenotypes could not be distinguished from other CGD patients due to n of 1 [130]. However, a significant reduction in only intracellular ROS production was observed, which was different from other CGD patients showing defects in the production of both intra- and extracellular ROS [130].

To define distinguishable characteristics of the genetic subgroup that contains disease causing mutations in *NCF4,* a larger cohort had to be established. In collaboration with Dr. Jean Laurent Casanova's group, the genotypic and phenotypic characteristics of 24 patients from 12 different families were defined who carried a mutation within the *NCF4* gene [138]. Patients with defects within p40phox, the protein encoded by *NCF4*, exhibited different phenotypes than those of patients with CGD [138]. These patients primarily had peripheral infections, hyper-inflammation, in addition to Crohn's like-IBD (commonly seen in CGD patients) [138]. The clinical outcome for these patients was better than patients with CGD with no mortality reported within the cohort [138]. Treatment strategy for these patients was similar to that given to CGD patients with regimens of antibiotics and antifungal drugs, IFNγ and HSCT [138].

The patients described in this study came from a consanguineous marriage and inherited the autosomal recessive nonsense mutation in *NCF4.* This mutation was confirmed using Sanger sequencing. We predicted that the protein structure will be missing a part of the SH3 domain and

will have a complete loss of the PB1 domain resulting in a predicted molecular weight of 29 kDa instead of 40 kDa. This was confirmed by western blot of proteins from patient neutrophils. The mRNA and protein levels of p40phox in patient neutrophils were downregulated, as shown by data from quantitative PCR and western blot. This downregulation can be explained by a phenomenon called non-sense mediated decay (NMD). NMD is a surveillance mechanism that prevents the translation of truncated proteins that can have the potential to act in a dominant negative manner, thereby disturbing the physiological homeostasis within a cell [156]. However, since some degree of expression was seen at the protein level within patient samples, there may be a physiological relevance of this protein in the onset of pediatric IBD within these patients. To determine this relevance, functional analysis of this mutation was performed.

Future Directions: Patients with mutations in p40phox seem to exhibit phenotypes less severe than patients with mutations in other components of the NADPH oxidase [138]. In the study by van de Geer *et al.* (2018), eight novel disease causing variants were identified in p40phox, each depicting unique phenotypes with a few that overlapped between patients. This evidence brings about the need to establish a larger cohort and identify more patients with mutations in p40phox to better define the disease that is caused as a result of defective p40phox function. Furthermore, to perform biochemical analysis on patient mutations, *in vitro* and *in vivo* models might be useful in defining the pathobiological mechanism of the disease.

5.2 p40phox (W239X) does not interact with p67phox within the NADPH oxidase complex.

Protein-protein interactions are an important aspect in the functionality of the NAPDH oxidase [157]. Activation of the enzyme is a tightly regulated process that involves conformational changes in the protein structure, due to post-translational modifications, that expose domains and facilitate interactions between the different subunits, in order to form a functional enzyme [157]. Several genetic variants, that are disease causing, have been reported within the genes encoding NADPH oxidase complex components [158, 159]. Whether X-linked or AR, these mutations are heterogeneous and can lead to a total loss of the protein, downregulation of expression or loss of function [132]. Mutations affecting protein-protein interactions, reportedly, cause destabilization of the complex which impedes with its proper functionality. Diebold and Bokoch (2001) showed this by creating mutations that disrupted the binding between p67phox and Rac GTPase. This resulted in reduced binding of these proteins to gp91phox, having a direct consequence on the electron transport ability of the enzyme [160]. Additionally, in a study from our lab, Dhillon *et al.* (2014) showed that mutations in p67phox that result in reduced binding to p40phox increase susceptibility to VEOIBD. Reduction in binding caused impaired ROS production, as indicated by clinical tests, which may be the cause of increased susceptibility [128]. This suggests that interactions within this complex are integral for proper functionality. In this study, we report that the nonsense mutation in *NCF4* causes a truncation in p40phox that leads to the loss of PB1 domain of p40phox. Ueyama *et al.* (2007) proposed a model suggesting that p47phox initially recruits p67phox to the NADPH oxidase complex while p40phox stabilizes it at the membrane in later stages of phagocytosis [131]. In unstimulated conditions, reciprocal immunoprecipitation revealed that the absence of the C-terminal PB1 domain in p40phox (W239X) abrogates its interaction with p67phox. It was hypothesized that this result has direct consequences on the

destabilization of the enzyme at the phagosome membrane, since p40phox is known to be part of the enzyme as a result of its interaction with p67phox. Since p40phox (W239X) was shown to not bind to p67phox, it may not be interacting with the enzyme at all.

Future directions: To further understand what effect the loss in interaction between p40phox and p67phox have on enzyme activity, it is important to know whether p40phox (W239X) even interacts with the enzyme at all. Previous studies have suggested that the interaction of p40phox with NADPH oxidase is mediated by p67phox via the PB1 domains of both proteins [139-141]. Thus, it is hypothesized that the loss in interaction of p40phox with p67phox will also result in loss in interaction of p40phox with NADPH oxidase. This can be tested by using native blue polyacrylamide gel electrophoresis (PAGE) [161]. This technique separates proteins in a non-denaturing gel, which allows the protein to stay in its native configuration and keep its association to other proteins intact [161]. RAW264.7 macrophages that are stably expressing p40phox (WT and W239X) can be stimulated using PMA and the accumulation of the enzyme can be compared to unstimulated conditions for each cell line. Presumably, p40phox (W239X) will run separately from the entire complex upon stimulation, while p40phox (WT) will be seen as part of the complex, indicated by a band at high molecular weight.

5.3 p40phox (W239X) is mislocalized to early endosomes within macrophages.

Hematopoietic stem cells produce monocytes which can be differentiated into macrophages upon activation [162]. Monocytes are usually found circulating in the blood [162]. Upon detection of a pathogen, these cells are recruited to the site of infection, where they can differentiate into macrophages that proliferate and are able to phagocytose and kill [162]. A macrophage-mediated immune response results in the clearance of pathogen via secretion of extracellular bactericidal compounds or intracellular degradation [163]. NOX2 NADPH oxidase participates in the early stages of microbial killing by assembling on the membrane of the phagosome and producing ROS [163]. Moreover, a notable histopathological feature of CGD patients and a subset of CD patients is the formation of granulomas, which is a collection of immune cells at the site of infection [30]. The persistence of granulomas is attributed to the inefficiency of the innate immune cells in mounting a respiratory burst and their inability to resolve infection [133]. Based on this and the fact that p40phox is known to be primarily expressed in hematopoietic cells [145], RAW264.7 murine macrophages were used to understand the physiological consequences of p40phox (W239X).

When GFP tagged-p40phox (WT) and p40phox (W239X) were stably expressed in RAW264.7 macrophages, a distinct expression pattern was observed between the two cell lines. Whereas, p40phox (WT) was seen to be cytoplasmic, p40phox (W239X) was being mislocalized and forming aggregates. This was interesting since it is known that in resting cells, PX and PB1 domains of p40phox interact with each other to maintain a closed configuration that allows it to remain cytosolic [164]. In $p40^{phox}$ (W239X), the PX domain is not inhibited, which may be resulting in the protein binding to and sequestering PI3P (Fig. 5.1) [164]. Ueyama *et al.* (2007 and 2011) reported similar findings when they expressed the PX domain of p40phox (p40phox-

PX) in RAW264.7 and HEK293T cells. They co-labeled the cells with an early endosomal marker (EEA-1) and showed co-localization of EEA-1 with p40phox-PX [131, 146]. The early endosome is known to be enriched in PI3P causing p40phox-PX to be localized and reveal an aggregated pattern across the cell [131, 146, 165]. Similarly, when stable RAW264.7 cells were stained with Rab5, colocalization was seen in cells expressing p40phox (W239X) but not p40phox (WT). Conversely, when cells were stained with Rab7, some co-localization was seen with p40phox (W239X) but not with p40phox (WT). This can be attributed to the fact that Rab7 is present on early endosomes maturing into late endosomes. Rab11 showed no colocalization of with either p40phox (W239X) or p40phox (WT). This is due to the fact that recycling endosome membranes are not enriched in PI3P [165].

Live microscopy showed that when RAW264.7 cells are stimulated with IgG-opsonized zymosan particles, p40phox (WT) transiently associated with the phagosome that engulfed the particle. This is consistent with previous findings that have shown that p40phox (WT) undergoes conformational changes upon activation and binds PI3P, that form on the phagosomal membrane upon induction of phagocytosis [131, 166]. The first step in phagosome maturation is its fusion with early endosome [166]. Thus, the association of p40phox (W239X) was even more apparent, as the protein was already mislocalized to early endosomes.

Future directions: Based on the model proposed by Ueyama *et al.* (2007), it is predicted that p47phox recruits the cytoplasmic heterotrimeric complex of p47phox, p40phox and p67phox to the phagosomal membrane. The complex is recruited to the phagosomal membrane, where p40phox opens up and interacts with PI3P via the PX domain, while remaining in contact with p67phox via the PB1 domain [131]. This stabilizes the complex for an efficient respiratory burst,

where p67phox is able to regulate electron flow within the enzyme [131]. Since p40phox (W239X) is missing the PB1 domain and is not interacting with p67phox, it is possible that the enzyme doesn't remain intact for as long as it should. This can be tested by performing live imaging using RAW264.7 macrophages stably expressing GFP-tagged- p40phox (WT and W239X) that are transfected with a construct encoding p67phox tagged to a red fluorescent protein. Phagocytosis can be induced using labeled IgG-opsonized zymosan. Quantification of time from recruitment of p67phox to the disappearance of the phagosome, between RAW264.7 (WT and W239X) will show how long p67phox lasts at the phagosomal membrane between the two cell lines. Furthermore, this experiment will add evidence to the role of p40phox in stabilizing the enzyme in later stages of phagocytosis.

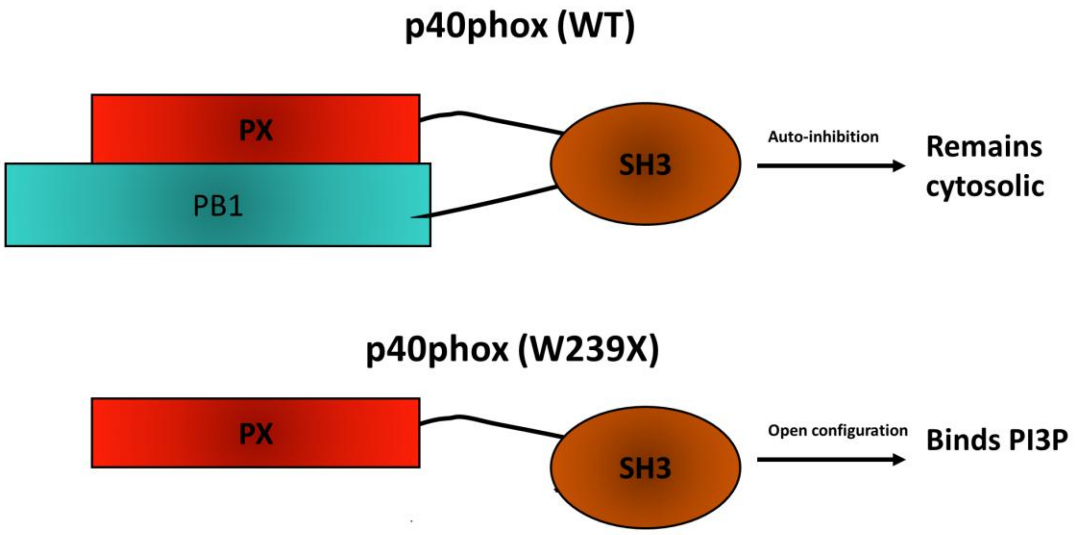


Figure 5.1: Schematic representation of autoinhibited p40phox (WT) and predicted open configuration of p40phox (W239X).

5.4 Macrophages expressing p40phox (W239X) have impaired oxidative burst in response to different stimuli.

RAW264.7 cells expressing the p40phox (W239X) mutation showed normal extracellular oxidative burst from its initiation to its resolution in response to PMA (measured by isoluminol). However, total oxidative burst (extracellular and intracellular ROS, measured by luminol) was impaired in these cells. The impairment was seen in the resolution phase indicating that although the peak of the oxidative burst was equivalent to p40phox (WT), the reaction was not sustained as efficiently as its wild type counterpart. To confirm these findings, stable RAW264.7 cells were stimulated with zymosan. Zymosan particles are derived from *Saccharomyces cerevisiae* and are engulfed by phagocytes [152]. Therefore, these particles are known to primarily produce intracellular ROS by inducing phagocytosis [152]. In accordance to this, isoluminol based chemiluminescence showed minimal ROS production. On the other hand, luminol based chemiluminescence showed reduced oxidase activity in RAW264.7 cells expressing p40phox (W239X).

Phagocytic leukocytes, i.e. macrophages and neutrophils, primarily express NOX2 NADPH oxidase [93]. As part of the oxidative burst within the phagosome, this enzyme is responsible for the electron transport that occurs between NADPH and oxygen to generate superoxide anions that kills invading bacteria and fungi [85]. ROS produced as a result of this action is also involved in further signaling to activate other parts of the immune system in order to effectively clear the infection [85]. Therefore, intracellular production of ROS via NADPH oxidase plays an essential role in innate immunity [85]. The importance of NADPH oxidase activity can be seen in patients with CGD that have defects in the components that make up the enzyme [133]. The clinical prognosis of the disease is directly correlated with the amount of residual ROS that the

mutant enzyme can produce, where severe cases are reported in patients with no ROS production [133].

ROS quantification can be of great importance in getting further insights on the mechanism of the disease onset. Several forms of ROS exist in cellular physiology due to its dynamic nature [167]. Superoxide's are highly reactive species [167]. In a reaction with nitric oxide, peroxynitrites are produced [167]. In a reaction catalyzed by superoxide dismutase, two superoxide's anions can react to form hydrogen peroxide (H_2O_2) [167]. H_2O_2 can further react with chloride ions (Cl^-) to produce hypochlorous acid (HOCl) or with metal ions, such as ferrous and ferric ions, to produce hydroxyl radicals (OH·) [167]. All these compounds are oxidizing agents and have roles in microbial killing [167]. However, it is challenging to capture the diversity of ROS species. To address this issue, spectroscopic, electrophysiologic, calorimetric, chemiluminescence and imaging-based techniques have been developed, with only a select few that can measure intracellular ROS [168].

IBD patients that are suspected to have mutations in the genes encoding the NADPH oxidase enzyme are subjected to clinical tests, such as dihydrorhodamine (DHR) oxidation tests, also known as Neutrophil Oxidative Burst Index (NOBI), to measure the oxidative ability of neutrophils in comparison to the control. This test is based on a cell permeable probe that fluoresces when oxidized. Essentially, DHR is a non-fluorescent probe but when oxidized, it becomes rhodamine 123, a red fluorescent product [169]. The fluorescence is measured by flow cytometry. The advantage of this probe is that it can detect various forms of ROS species, such as superoxides, H2O2, peroxynitrites as well as HOCl [169]. However, since it is a cell permeable molecule, it is hard to distinguish extracellular ROS from intracellular ROS [168]. Additionally, the reaction is terminal and can only detect the peak of the oxidative burst but

cannot tell how long the reaction lasted. Thus, it can distinguish between ROS+ cells and ROS- cells but does not give information on the dynamic state of the oxidative burst. This test was also performed on the neutrophils from patients with p40phox (W239X) mutation. The test results were normal for these patients, indicating that their neutrophils are able to generate an oxidative burst effectively.

In this study, luminol based chemiluminescence assay was used to measure ROS production in real time in stable RAW264.7 cells. In a reaction catalyzed by HRP, luminol reacts with ROS to produce luminol dianion [170, 171]. Luminal dianion further reacts with O2 to produce an unstable aminophthalate [172-174]. The electrons in aminophthalate undergo decay from an excited state to a ground state that results in the emission of a photon of light [172-174]. The light from this reaction can be quantified using a luminometer, which gives the indication of the presence of ROS within the cells [172-174]. The strength of this technique is that it can detect the different forms of ROS species like the DHR test [168]. It is also a cell permeable compound that can detect both extracellular and intracellular ROS [168]. Additionally, a derivate to luminol, called isoluminol, is a cell impermeable compound that only measures extracellular ROS [175]. Comparing results from luminol and isoluminol chemiluminescence assays gives information on the difference between extracellular and intracellular ROS production. Extracting this difference is important since Matute *et al.* (2009) showed that the patient with mutation in p40phox showed impaired intracellular ROS production but not extracellular ROS production.

Many other groups have also evaluated mutations within each domain of p40phox as well as deficiency of p40phox in neutrophils and macrophages and subsequent effect of these mutations on NADPH oxidase activity. Ellson *et al.* (2006) showed that neutrophils from p40phox$^{-/-}$ mice showed impaired ROS production in response to various stimuli, i.e. PMA, zymosan, IgG-coated

latex beads, *Staphylococcus aureus*, fMLP and TNFα. Although the reduction in response was variable between different stimuli, the impairment was consistent. In a parallel study, Ellson *et al.* (2006) generated mice that had a mutation within the PX domain of p40phox (R58A) that impairs its binding to PI3P. In this study, they showed that the binding of p40phox to PI3P is essential for the activity of NADPH oxidase [176]. Neutrophils from p40phox$^{R58A/-}$ mice showed reduced oxidase activity in response to *Staphylococcus aureus* when compared to p40phox$^{+/-}$ littermates [176]. Tian *et al.* (2008) were able to further validate the importance of p40phox and PI3P binding in regulating NADPH oxidase activity by showing similar results with another mutation p40phox (R105A). This mutation also abrogated the interaction between p40phox and PI3P and showed impaired oxidase activity in neutrophil-differentiated stable PLB-985 granulocytes [177]. Suh *et al.* (2006) generated mutations in different domains of p40phox and checked the activity of NADPH oxidase using IgG-opsonized red blood cells and nitro blue tetrazolium (NBT) quantification assay in COS cells (monkey kidney fibroblasts) [178]. NBT is a cell permeable compound that forms blue-black precipitate, called formazan, upon interaction with ROS [179]. This precipitate is solubilized in KOH and DMSO buffer which is followed by spectrophotometric measurement for the quantification of total superoxide present within the system [180]. Cells expressing point mutation in PB1 domain (D289A) that resulted in loss of interaction with p67phox, showed reduced number of NBT positive phagosomes [178]. However, the reduction was more apparent in mutations within PX domain (R58Q and R105A) that do not bind PI3P [178]. More recently, Bagaitkar *et al.* (2017) showed that macrophages from p40phox$^{R58A/R58A}$ mice also have impaired intracellular oxidative burst in response to various stimuli [181]. Taken together, these studies, in corroboration with the findings from the

current study, show that the interactions of p40phox are essential for proper functionality of NADPH oxidase, especially for intracellular ROS production.

Future directions: A common phenotype in patients expressing defective p40phox was granulomatous inflammation seen in the skin and GI tract. Enhanced inflammation has also been reported in mice that are deficient in p40phox. Li *et al.* (2017) showed that an increased expression of pro-inflammatory cytokines from $p40phox^{-/-}$ mice tissues. Therefore, it would be interesting to see the expression profile of inflammatory cytokines in macrophages that express p40phox (W239X) to further comment on the inflammation seen in these patients.

Previous studies have shown that ROS produced by phagocytes may have roles in the clearance of apoptotic bodies and inefficiency in doing this may result in inflammation [181]. The process of clearing cell debris and apoptotic corpses is termed efferocytosis, which is important in resolving inflammation [181]. Bagaitkar *et al.* (2017) have tested this phenomenon in $p40phox^{R58A/R58A}$ mice and have shown that the interaction of p40phox with PI3P is essential for resolving inflammation. Thus, it is predicted that p40phox (W239X) may also have a similar effect, due to defective ROS production. Further studies can explore this aspect to better understand excessive inflammation in patients with defects in p40phox.

5.5 Macrophages expressing p40phox (W239X) have reduced bactericidal activity in vitro.

The gentamicin protection assay evaluates intracellular microbicidal activity of cells by the use of gentamicin, a cell impermeable antibiotic [155]. It kills microbes present in the extracellular space without affecting the microbes that have entered the cell [155]. The purpose of this experiment was to evaluate the NADPH oxidase mediated microbicidal activity of RAW264.7 macrophages stably expressing p40phox (WT and W239X). The results showed that the number of internalized bacteria was similar in both p40phox (WT) and p40phox (W239X) macrophages as indicated by the recovered bacteria at time point 0 (2 hours after infection). However, more number of viable bacteria were recovered from p40phox (W239X) expressing macrophages at various time points, with a significant difference seen at 6 hours. This indicated that the macrophages expressing p40phox (W239X) had impaired bacterial killing capacity than macrophages expressing p40phox (WT).

ROS production by NOX2 NADPH oxidase can have context dependent biological function based on where it assembles in the cell. Within the phagosome, ROS generates a highly toxic environment for the microbes being engulfed in order to kill them [85]. On the contrary, the extracellular ROS is known to participate in signaling that leads to the activation of other parts of the innate immune response [182]. Experimental evidence has suggested that defects in p40phox show impaired intracellular ROS production, indicating that the protein is essential for mediating the microbicidal activity for NADPH oxidase [130, 153, 154, 181]. Therefore, the activity of RAW264.7 macrophages stably expressing p40phox (WT and W239X) was examined for bactericidal function.

A non-pathogenic strain of *E. coli* was used to infect cells. Although it has been previously shown that the mechanism of killing for *E. coli* by phagocytic leukocytes is NADPH oxidase

independent [183], these results are valid for pathogenic strains of *E. coli* that have developed unknown protective mechanism against the microbicidal activity of NADPH oxidase. The strain used in this study is DH10β, a common strain used in laboratories for transformation experiments. This strain in non-pathogenic and thus lacks the ability to colonize and infect cells.

The findings from this study are in corroboration with findings from studies that evaluated microbicidal activity of p40phox$^{-/-}$ neutrophils and macrophages. Ellson *et al.* (2006) recovered significantly higher number of *S. aureus* colonies from neutrophils of p40phox$^{-/-}$ mice, in comparison to p40phox$^{+/+}$ in vitro [153]. They also confirmed these findings in vivo by infecting mice and recovering a higher number of surviving bacteria from p40phox$^{-/-}$ mice, 24 hours post infection. More recently, Li *et al.* (2017) showed similar results in macrophages from p40phox$^{-/-}$ mice. They reasoned that although neutrophils are the first line of defense for infections, macrophages are present in higher numbers in tissues and contribute significantly in bacterial clearance as well as inflammatory response [154]. They utilized *Salmonella typhimurium* to infect cells as well as mice [154]. Of note, they showed that although similar numbers of bacteria were taken by cells, macrophages from p40phox$^{-/-}$ mice had significantly higher number of viable bacteria at 6 hours post infection compared to macrophages from p40phox$^{+/+}$ mice [154].

Future directions: This study was primarily done in macrophages. Neutrophils are another model that can be used to understand the consequences of p40phox (W239X) on innate immune defense to further validate the findings from this study. PLB-985 granulocytes are a human cell line that can be differentiated in neutrophils. These cells, like the macrophages, are also hard to transfect and thus, stable cell lines must be generated to effectively quantify ROS production and bacterial killing efficiency.

Chapter 6
Conclusion

This thesis analyzed the functional consequences of a nonsense mutation in *NCF4* in the pathogenesis of pediatric IBD. Patients with this mutation showed a truncation in p40phox, protein encoded by *NCF4*, leading to a loss in PB1 domain at its C-terminus. As part of the NADPH oxidase enzyme, p40phox has been previously shown to regulate an efficient intracellular oxidative burst to kill invading pathogens engulfed by phagocytes. Through experimental analysis, it was shown that loss of PB1 domain in p40phox abrogated its interaction with p67phox within the NADPH oxidase enzyme. Additionally, the mutant protein (p40phox (W239X)) was mislocalized to early endosomes. Cells expressing the mutant protein showed impaired intracellular oxidative burst as well as reduced bactericidal activity. Based on this experimental evidence, it is proposed that p40phox (W239X) fails to associate with NADPH oxidase enzyme and thus leads to the impairment in its activity (Fig. 6.1). This may have contributed in the onset of the patient's disease. Additionally, this study also supports the findings from previous studies in indicating that p40phox is essential for intracellular ROS production. Despite these advances, more questions remain to be answered. Further studies should look at the level of cytokines to comment on the extent of inflammation, as seen in the patients with this mutation. As well, the concept of efferocytosis must be explored to better understand the formation of granulomas in these patients.

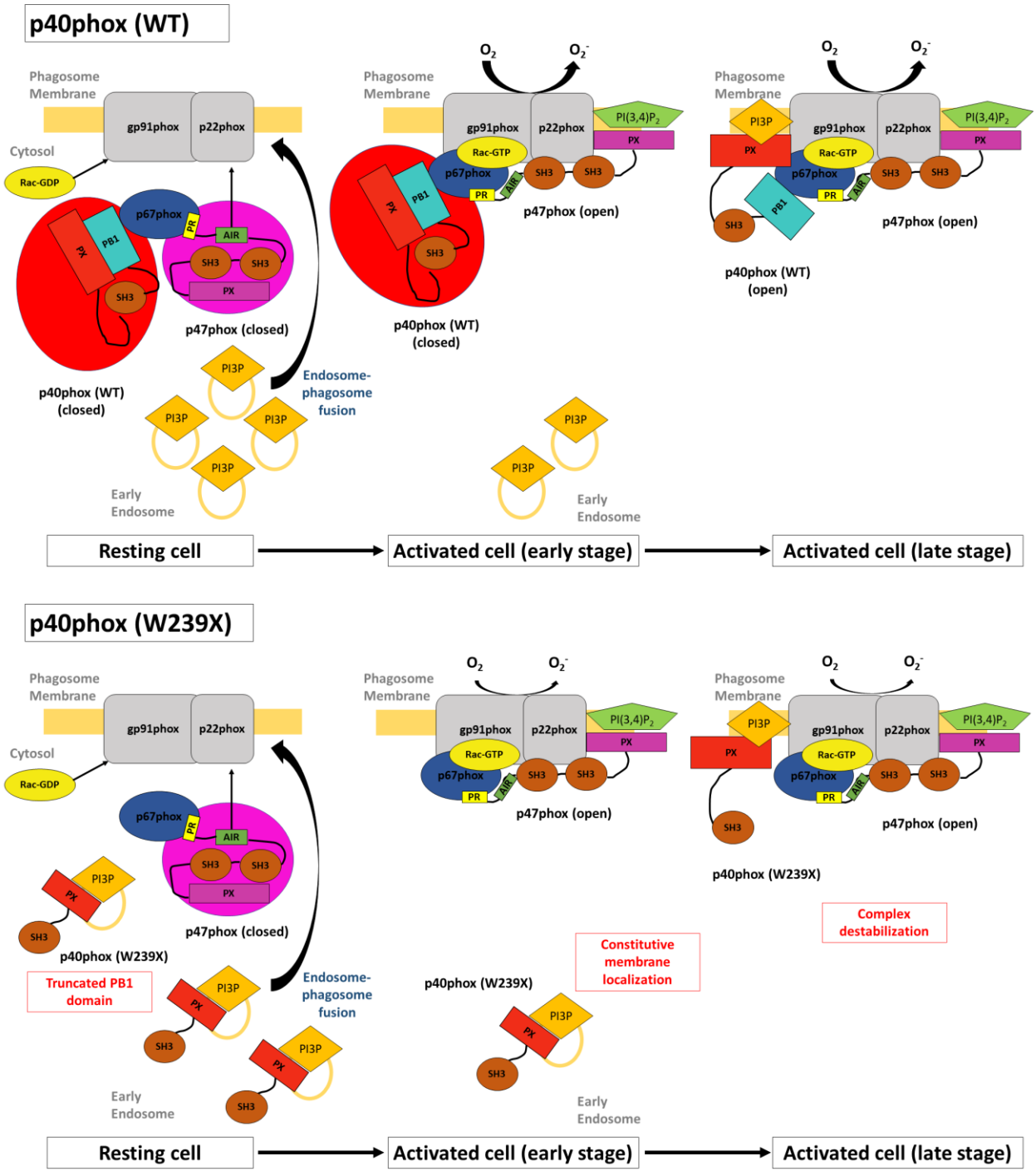

p40phox (WT)
Phagosome Membrane
gp91phox
p22phox
Cytosol
Rac-GDP
p67phox
PR
AIR
SH3
PX
PB1
p47phox (closed)
p40phox (WT) (closed)
PI3P
Endosome-phagosome fusion
Early Endosome
O2
O2-
PI(3,4)P2
Rac-GTP
p47phox (open)
p40phox (WT) (closed)
p40phox (WT) (open)
Resting cell
Activated cell (early stage)
Activated cell (late stage)
p40phox (W239X)
Truncated PB1 domain
p40phox (W239X)
Constitutive membrane localization
Complex destabilization

Figure 6.1: Proposed model of p40phox(W239X) and its role in the activity of NADPH oxidase in comparison to p40phox (WT). p40phox (W239X) does not interact with p67phox, because of the loss of PB1 domain. This leads to the loss of autoinhibition, causing the PX domain to be exposed. As a result, p40phox (W239X) localizes to early endosomes which are enriched in PI3P (due to the high affinity of its PX domain to PI3P). p40phox (W239X) fails to associate with NADPH oxidase enzyme and thus leads defective oxidative burst and impaired pathogen clearance. Adapted from: Molecular Biology of the cell. Ueyama et al. A regular adapted function of p40phox: distinct p67phox membrane targeting by p40phox and by p47phox. 18: 441-454, 2007.

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