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Intestinal Exposure to PCB 153 Induces Inflammation via the ATM/NEMO Pathway

Matthew C. Phillips
University of Miami, mcphillips@med.miami.edu

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UNIVERSITY OF MIAMI

INTESTINAL EXPOSURE TO PCB 153 INDUCES INFLAMMATION VIA THE ATM/NEMO PATHWAY

By

Matthew C. Phillips

A DISSERTATION

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of the University of Miami
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INTESTINAL EXPOSURE TO PCB 153 INDUCES
INFLAMMATION VIA THE ATM/NEMO PATHWAY

Matthew C. Phillips

Approved:

Maria T. Abreu, M.D.
Professor of Medicine

Michal J. Toborek, M.D., Ph.D.
Professor of Biochemistry and
Molecular Biology

Emmanuel Thomas, M.D., Ph.D.
Assistant Professor of Cell Biology

Rebecca Adkins, Ph.D.
Professor of Microbiology and
Immunology

Noula Shembade, Ph.D.
Assistant Professor of Microbiology and
Immunology

Guillermo Prado, Ph.D.
Dean of the Graduate School

Margaret O. James, Ph.D.
Professor of Medicinal Chemistry
University of Florida
Polychlorinated biphenyls (PCBs) are persistent organic pollutants that adversely affect human health. Although production of PCBs has stopped, they remain a pressing environmental problem due to their slow biodegradation and high lipophilicity. These properties enable PCBs to bio-accumulate in food chains leading to high levels of PCBs in the tissues of foodstuffs, especially fish. Despite dietary exposure being one of the main routes of exposure to PCBs, the gut has been widely ignored when studying the effects of PCBs. We set out to investigate the effects of PCB 153, the most environmentally prevalent PCB, on the gut, looking specifically for proinflammatory effects and a breakdown of the intestinal barrier. We further wanted to address the mechanism by which PCB 153 affected intestinal permeability or inflammation. Mice were orally exposed to PCB 153 and gut permeability was assessed. Intestinal epithelial cells (IECs) were collected and evaluated for evidence of genotoxicity and inflammation. A human IEC line (SW480) was used to examine the direct effects of PCB 153 on epithelial function. NF-κB activation was measured using a reporter assay, DNA damage was assessed, and cytokine expression was ascertained with real-time qPCR. Mice orally exposed to PCB 153 had an increase in intestinal permeability and inflammatory cytokine expression in their IECs; inhibition of NF-κB ameliorated both these effects. This inflammation was associated with genotoxic damage and NF-κB activation. Exposure of SW480 cells to PCB 153 led to similar effects...
as seen in vivo. We found that activation of the ATM/NEMO pathway by genotoxic stress was upstream of NF-kB activation. These results demonstrate that oral exposure to PCB 153 is genotoxic to IECs and induces downstream inflammation and barrier dysfunction via NF-kB in the intestinal epithelium.
For my family.
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Chapter 1. Enter Exposome

Humans evolved 200,000 years ago. Since then, we have invented farming and subsequently formed cities, ancient Romans used bellow furnaces to smelt lead, and around 250 years ago humans entered the industrial revolution. On the morning of June 26, 2000, President Bill Clinton and British Prime Minister Tony Blair announced that we had sequenced the human genome, with the much hyped promise that this new found understanding would herald a new understanding of human disease. While there have certainly been disease causing changes in the human genome on the large time scale described above, over the course of a single, modern human’s lifetime, there have been significant increases in multiple types of non-infectious diseases such as autoimmune disease, cancer, metabolic syndrome, and even psychological diseases. At the currently level of sequencing resolution, it has been estimated that disease causing variants in genes can only spread through human populations on the time scale of approximately 2000 years\(^1\). This understanding explains why people from different genetic backgrounds have different susceptibilities to disease, but does little to explain the rapid increases in multiple types of diseases over the past century.

Epidemiologic data collected from multiple populations has consistently shown staggering increases in multiple allergic and autoimmune diseases in the past few decades. A study of Swedish schoolchildren found that the prevalence of asthma and allergies had nearly doubled between 1979 and 1991\(^2\). Even more strikingly, between 2001 and 2009, the incidence of type 1 diabetes in adolescent rose 23% in the United States\(^3\). In fact, over the past 30 years, the incidence of all autoimmune diseases has risen at a rate of 19.1% per year worldwide\(^4\).
Likewise, cancer rates have increased dramatically in recent years as well. A study estimating cancer risks in Great Britain found that the lifetime risk for cancer has increased from 38.5% for men born in 1930 up to 53.5% for men born in 1960. The lifetime risk for women during this time period also increased, from 36.7% in 1930 to 47.5% in 1960. For people born after 1960, the authors conclude that the lifetime risk for cancer will be greater than 50 percent\(^5\).

Metabolic syndrome, a condition characterized by central obesity, hypertension, dyslipidemia, and hyperglycemia, has also seen recent, dramatic increases, especially in the developed world. Between 2003 and 2012 in the United States, there was an 18.3% increase in the prevalence of metabolic syndrome in those between the ages of 20 and 29. Even more dramatic, there was a 46.7% increase in the population 60 years of age or higher\(^6\).

Even psychological diseases have also shown this upward trend. Five times more young adults scored above common cutoffs for psychological pathologies in 2007 as compared to seventy years prior\(^7\). This trend has only amplified in the past decade\(^8\).

These rapid increases in disease incidence, risks, and prevalence are occurring at rates that far exceed those that could be produced by genomic changes. If most of us still have similar genomes to our ancestors who lived alongside the ancient Romans, why are modern humans getting sick at an ever growing rate? What is the human genome incapable of telling us?

There is a paradigm in the field of environmental health that can be summed up in the pseudo-mathematical equation: G x E = P. Our genotype, crossed with our
environment, gives us our phenotype. In other words, who we are, our phenotype, is dependent not only on our genome but also on our exposome. Broadly defined, our exposome is the sum total of all our environmental exposures. The exposome consists of the food we eat, the air we breathe, the microbes we are exposed to, the stresses we feel, and all the chemicals we have come into contact with over the course of our biological existence. Since the gametes that fuse to give rise to our bodies existed within our parents, our exposome extends back to even before the time of our conception. Going back to the equation, if there has been a large increase in a given phenotype, for our purposes this will be disease, and there has been relatively little change in the genes, then algebraically, there must be a large change in our exposures.

Certainly over the past several thousand years, the human exposome has changed in dramatic ways. Farming has concentrated and filled our environment with fertilizers. Cities have moved humans, and their waste, into much greater and closer contact with one another. Ancient Roman smelters released such large quantities of lead into the environment that some have hypothesize contributed to the fall of their empire. Possibly the most dramatic changes to the human exposome have occurred since the industrial revolution. The burst of manufacturing and factories, driven by a massive increase in energy from fossil fuels, filled the environment with novel and exogenous compounds. Coal burning plants introduced mercury, nitrogen oxide, sulfur dioxide, arsenic, and volatile organic carbon compounds into the atmosphere; while textile factories released chemical into streams and waterways. While many of the exposures from the industrial revolution have been reduced or remedied in the past century, the legacy of many of these pollutants is still with us in modern times. The human exposome continues to grow and
evolve every year and these changes continue to have very real effects on human health. According to the World Health Organization, an estimated 12.6 million people died from an unhealthy environment in the year 2012; nearly 1 out of every 4 deaths worldwide\textsuperscript{12}.

While the causes of the recent, dramatic increase in non-infectious diseases are certainly multifactorial, most likely involving the totality of changes in the human exposome, more accurate reporting of diseases, and changing demographics; one stark correlation with the rise in disease incidence has been the increase in synthetic chemical production. The best estimates for the number of chemicals in circulation come from the U.S. Environmental Protection Agency (EPA) which was tasked in 1976 by the Toxic Substance Control Act (TSCA) with creating an inventory of chemicals produced in the United States\textsuperscript{13}. The EPA’s initial report, released in 1982, contained approximately 62,000 chemicals. Over the next 20 years, the EPA added approximated 22,000 chemicals to this inventory, suggesting that there are potentially over 84,000 chemicals in circulation today\textsuperscript{14}. This number vastly understates the totality of chemical exposures in the human exposome however, as the TSCA does not cover food, food additives, drugs, or cosmetics, which are covered by the Food and Drug Administration (FDA). Further evidence for this rapid increase in synthetic chemicals comes from the growth of the total amount of them in commerce. Between 1970 and 1995, the total volume of synthetic chemicals being produced went from 50 million tons to over 150 million tons\textsuperscript{15}. This growth is projected to only accelerate in the near future. Global pesticide usage is growing at over 3 times the rate it was in 1970, and the diversity of pharmaceuticals are increasing at over twice the rate they were 40 years ago\textsuperscript{16}. Global sales of chemicals have increased, based on one estimate,
by a factor of 25 since 1975 and global production is projected to nearly triple in the next 30 years\textsuperscript{14}.

While the coinciding increases in synthetic chemicals and incidence of non-infectious diseases are purely correlative, a growing body of data suggest that many of these chemicals can be epidemiologically linked or even experimentally cause many of these diseases. Phthalates, found in common household plastics, have been linked to asthma in children\textsuperscript{17}. Propylene glycol and glycol ethers, solvents used in paints and cleaners, have also been shown to exacerbate and asthma, rhinitis and eczema in preschool age children\textsuperscript{18}. Type 1 diabetes has been linked both epidemiologically and experimentally to nitrates, nitrites, and N-nitroso-compounds; all substances found in fertilizers\textsuperscript{19}. The International Agency for Research on Cancer has listed over 50 synthetic chemicals as either “definite” or “probable” carcinogens and over 100 as “possible” human carcinogens\textsuperscript{20}. Metabolic syndrome has been epidemiologically linked to multiple persistent organic pollutants, and chemicals such as the plasticizer bisphenol A have been shown to increase adiposity in animal models\textsuperscript{21}. Depression and psychological pathologies have been linked to environmental mercury exposure as well\textsuperscript{22,23}. While clearly not an exhaustive review of all possible chemicals that could be causing the observed increases in disease, a wealth of literature strongly suggest that chemical exposure is a major contributing factor to modern society’s increased burden of disease.

One large class of these synthetic chemicals that has been shown to cause adverse health effects, Polychlorinated Biphenyls (PCBs), will be the focus of the remainder of this dissertation.
Chapter 2. 110 Years of Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) are ubiquitous and persistent organic pollutants that adversely affect human health. They consist of a central biphenyl moiety, surrounded by up to 10 chlorine atoms (example in figure 2.1). This allows for up to 209 different PCB congeners to exist. In practice however, only approximately 130 of these individual congeners are found in PCB products. The position and number of the chlorine atoms in the different congeners of PCBs allow them to take on multiple structural forms. Coplanar PCBs lack chlorine atoms in the ortho position around the central bond. This allows them to take on a flat, coplanar shape. These are sometimes referred to as dioxin-like PCBs because their shape and toxicities can resemble that of polychlorinated dibenzodioxins (known simply as dioxins). When chlorine atoms occupy one or more of the ortho positions of a PCB, it is referred to as a non-coplanar PCB or non-dioxin-like PCB. The chlorine atom in the ortho position sterically inhibits the PCB from taking a coplanar shape. These distinctions are important because they give PCBs from these two groups different chemical and biological properties. In general though, PCBs are extremely chemically and thermally stable. They are nonflammable, have a high dielectric constant, and are extremely heat resistant as well. These properties made PCBs an attractive substance for commercial and industrial use.

PCBs were first discovered in 1865 as a byproduct of coal tar, but were not able to be commercially produced until 1881, when the German chemists, Schmidt and Schultz, developed a laboratory method for their chemical synthesis. While widespread production would not start until the beginning of the 20th century, enough PCBs were released into the environment in the first few decades to be detected in museum specimens...
of bird feather from that time\textsuperscript{26}. Widespread industrial use began when PCBs started being commercially produced in 1929 by Swann Chemical Company. Production further increased when Swann was bought out by Monsanto Chemical Company in 1935 and mixtures of PCB congeners began being sold. They gained widespread popularity and were used as heat exchangers, dialectic fluids, hydraulic fluid, lubricating fluid, and plasticizers. PCBs also became ingredients in paints, inks, carbon-copying paper, caulking compounds, adhesives, flame retardants, and pesticides\textsuperscript{27}. Such extensive usage in such a wide variety of applications led to the production of over 1.3 million tons of PCBs in the 50 years they were commercially produced\textsuperscript{28}. Despite this prolific production, the scientific community was broadly unaware of the environmental accumulation of PCBs until almost 40 years after their widespread industrial usage.

![Figure 2.1. The structure of a PCB. Shown is the chemical structure of 2,2',4,4',5,5'-Hexachlorobiphenyl (PCB 153). This is an example of a non-coplanar PCB and is the congener primarily used in experiments presented in this dissertation.](image)
In the 1964, a Swedish chemist named Sören Jensen began noticing unknown peaks on gas chromatographs while investigating the accumulation of the pesticide dichlorodiphenyltrichloro-ethane (DDT) in the environment. These unknown peaks had retention times that did not match any known chlorinated pesticide described in the literature. After ensuring that the peaks did not come from a natural product, Jensen began collaborating with ecologist and was able to identify these unknown substances in the feathers of eagles dating back to 1942. This was important, because chlorinated pesticides, like DDT, were not used until 1945 meaning the unknown substance was not a metabolite of any chlorinated pesticide. After two years of analytical chemistry detective work, Jensen positively identified the substance as PCBs. In 1966, Jensen published his work, documenting the presence of PCBs in an eagle and numerous fish found all over Sweden. Jensen theorizes several reasons for the 40 year lag time between the beginning of widespread PCB usage and their discovery as an environmental contaminant. The first is a lack of a clear and immediate cause and effect. After DDT became wildly sprayed into the environment to control mosquito populations, the birds in those areas began to disappear. Likewise, when farmers used seeds that had been treated with mercury, the birds that ate those seeds died. Since PCBs were primarily used internally within manufacturing processes, as various fluids within machinery, they enter the environment though less obvious means, such as emissions and leaks. These surreptitious contamination events made it much harder for biologist to form hypotheses about PCBs effects that could then go on to be confirmed by analytical chemistry. The second, and possibly more important, reason for the delay in identifying PCBs as an environmental pollutant, was the lack of sufficiently sensitive analytic techniques to detect substances at the parts per billion level.
With the advent of gas chromatography in the 1950s, the technological requisites were in place for more thorough investigation into environmental contaminants. For these reasons, Jensen’s 1966 paper was the first report of the ability of PCBs to persist in the environment and bioaccumulate in food chains\(^\text{30}\).

PCBs, by their nature, are incredible stable molecules. Indeed this was a large part of their appeal for industrial usage. Unfortunately, this property also contributes to their persistence in the environment. The chemical structure of PCBs make them extremely lipophilic, giving them the ability to build up in lipid droplets and fatty tissues of organisms. As larger animals consume smaller, PCB laden food sources, these PCBs build up in their fatty tissues and accumulate to higher and higher tissue levels as they travel up the food chain. Apex predators such as whales, seals, wolves, and especially humans, can end up with the largest burdens of PCBs. Since Jensen’s first report of PCB bioaccumulation in nature, there have been numerous reports from all over the world of high tissue levels of PCBs in organisms ranging from wolves in Russia\(^\text{31}\), to penguins in Antarctica\(^\text{32}\), to humans across the United States and Europe\(^\text{33,34}\). Despite the recognition of PCBs as a persistent environmental contaminant, significant regulatory action was not undertaken until the widespread public health risk became readily apparent.

The first reports of adverse human health effects of PCBs came 4 years after they began being commercially produced, and were published in the journal *Archives of Dermatology and Syphilology* in 1936 by Jones and Alden\(^\text{35}\). The case study details a patient who had worked at a factory distilling PCBs from 1930 until 1933. In 1933, he developed an acne-like eruption of blackheads, cysts and pustules which was diagnosed as chloracne, a specific kind of acne known to be associated with the exposure to halogenated
compounds. They further went on to describe that out of the 24 men working at the PCB manufacturing plant, 23 had developed similar chloracne. Upon investigation, it was found that the distilling apparatus used in the factory for manufacturing PCBs was not enclosed. Upon installation of a ventilation fan and enclosure of the distilling apparatus, the factory workers experienced a gradual improvement of the chloracne\textsuperscript{36}. The same year as this initial publication, the U.S. Public Health Service put out a report detailing a similar case of chloracne in the wife and child of an employee at a PCB manufacturing plant. The wife and child had become exposed when they came into contact with the employees’ work clothes and subsequently developed comedones and pustules. In addition to the skin lesions, workers at this same factory also displayed symptoms of systemic poisoning such as digestive disturbances, burning of the eyes, impotence, and hematuria\textsuperscript{37}.

The first reported fatalities from occupational exposure to PCBs came the following year in 1937. Three workers manufacturing coating for electrical wires in New York City developed chloracne and subsequently became jaundiced. They were hospitalized with abdominal pain and distension. Upon autopsy, two of the patients were found to have liver cirrhosis and yellow atrophy\textsuperscript{38}. These deaths prompted the wire factory to commission Cecil K. Drinker, the researcher who published the initial report, to investigate PCBs toxicities in the laboratory with the hope of elucidating an underlying cause. Drinker exposed rats exposed to varying levels of PCBs (and other chemical vapors found in the factory) meant to represent conditions experienced by the workers in the plant. These chemical fumes caused morphological changes in the livers of rats exposed to these occupationally relevant concentrations\textsuperscript{39}. Interestingly, after these initial reports, very little
was published on the toxic effects of PCBs in humans until a mass exposure event in Japan once again raised the alarm about PCBs.

In early 1968, poultry farmers in the western area of Japan started reporting an unknown illness afflicting their birds. This illness, which caused difficulty breathing, resulted in the deaths of over 400 thousand birds over the next several months. By fall, effects on humans began being reported and in October of 1968, the Japanese government announced an epidemic of a mysterious skin disease. Patients primarily presented with swelling of their upper eyelids and acne-form eruptions on their skin. Other initial symptoms included edematous swelling of limbs, digestive disturbances, and odd skin discoloration. Soon after the epidemic was announced, the cause of the illness was found to be the ingestion of rice oil that had become contaminated with a large amount of PCBs. Starting earlier that year, a leak in the heating coils at the Kanemi Company had contaminated the rice oil they produced with PCBs, polychlorodibenzofuran, and polychloroquaterphenyl. Since the disease was caused by ingestion of this rice oil, it became known as “Yusho” disease, which literally translated means “oil symptoms”.

By the end of the incident, the total number of patients affected by Yusho disease would total over 1,800. This cohort, along with another rice oil-PCB exposure that produced similar symptoms in Taiwan several years later (called Yu-cheng disease), gave scientist and physicians their best look at the human health effects of PCBs. Besides the dermatological and ocular manifestations listed above, 60% of exposed women experienced irregular menstrual cycles and several whom were pregnant at the time of exposure had still births. Approximately 40% of patients developed chronic bronchitis and neurological symptoms like numbness, headaches, and hearing difficulties. There were
also hints of metabolic and liver dysfunction such as high serum triglycerides and abnormal liver function tests\textsuperscript{43}. In Yusho patients, the clinical severity of these symptoms was correlated with blood PCB concentrations\textsuperscript{42}. Many of these symptoms gradually decreased over the following years but long term follow up has shed light on the lasting effects of PCB exposure.

Measurable levels of PCBs have been detected in the serum and tissue of Yusho patients up to 40 years after the initial exposure. These levels are higher than the general population suggesting that they have remained elevated due to the Yusho incident\textsuperscript{44}. This is perhaps to be expected given the lipophilic nature of PCBs, causing them to be stored in adipose tissue, and their long half-life in humans (highly chlorinated PCBs have a half-life of over 15 years)\textsuperscript{43}. This retention of PCBs is accompanied by multiple long term health effects. In approximately 20\% of the chronic bronchitis cases from Yusho, the symptoms lasted for over 10 years. Decreased respiratory function has been reported 20 years after the incident\textsuperscript{45}. Men exposed in the Yusho incident had a twofold increase in their odds of mortality from malignant cancers, driven primarily from a 5-fold increase in liver cancer mortalities in these patients. Women also experienced increase mortality from liver cancer\textsuperscript{46}. Persistent metabolic and hormone related symptoms such as high thyroxin, decreased sperm motility, goiter, endometriosis, and an increased risk for diabetes were also observed in Yusho and other similarly exposed population. Immune dysregulation, involving abnormal immunoglobulin levels were also seen in Yusho patients\textsuperscript{44}. Children born after the Yusho incident, even those not exposed \textit{in utero}, also showed signs of PCB toxicity; possibly from exposure via the mother’s milk, changes in the maternal reproductive system, or lasting changes to the mother’s gametes. Children were born with
lower birth weights, experienced stunted growth, and had an increased incidence of learning deficits and lower IQ\textsuperscript{42,44}.

The Yusho incident quickly thrust PCBs into the spotlight and served as a catalyst for both an increase in scientific scrutiny of PCB toxicity, and increased governmental oversight of PCBs in food. In 1973, the FDA issued tolerances for the amount of PCBs that were allowable in food. While based on the best available data at the time, these guidelines were quickly challenged based on the influx of new scientific data on the toxicity of PCBs. In 1979, the FDA, equipped with this expanded body of knowledge, revised their tolerances for PCBs. That same year, the EPA, which had been granted regulatory authority over PCBs by the Toxic Substance Control Act 3 years prior, banned the further use of PCBs in food, drug, and cosmetic manufacturing plants. The EPA would further go on to ban PCBs entirely in 1984, two years after the international community agreed to do so at the Stockholm Convention in 1982\textsuperscript{47}.

While the industrial production of PCBs has been discontinued, over 1.5 million tons were produced worldwide and there remains an estimated 350,000 tons of PCBs in the environment of North America alone\textsuperscript{48}. Because of this, PCBs remain a pressing environmental concern. They continue to bioaccumulate in a variety of wildlife including in fish species important for human consumption\textsuperscript{49}. Indeed the people now at the highest risk of PCB toxicity are those from high fish-consuming populations\textsuperscript{50}. PCBs also accumulate within humans as they age making it more likely for the elderly to experience PCB-related toxicities. A large study out of Germany, sampling over 2,700 people, found that serum PCBs steadily increased with age reaching levels above which human health effects may occur \textsuperscript{34}. With the global population’s median age expected to increase over
70% within the next century, the burden of PCB-induced toxicities will only increase making the study of the human health effects of PCBs more essential than ever\textsuperscript{51}. 
Chapter 3. Immunotoxic Effects of PCBs

One key observation made after the Yusho incident in 1968 was that patients exposed to PCBs experienced immune dysregulation\textsuperscript{42}. This observation serendipitously occurred just as the field of immunotoxicology was emerging in the early 1970s\textsuperscript{52}. Subsequent investigation into the immunotoxicity of PCBs revealed that immune function is exquisitely effected by exposure to PCBs and that the immune system may be one of the most sensitive targets for their toxic effects\textsuperscript{53}.

The immune system is a complex network of interconnected tissues, cells, and molecules that work together to protect the body from infection and remove neoplastic or damaged tissue. Due to the breadth and importance of these task, the immune system evolved a great deal of functional and structural complexity involving multiple cell types, numerous chemical messengers, and a multitude of cell-cell interactions. These cells and messengers coordinate to recognize and mount an immune response in order to contain or remove threats to the organism. Importantly, the immune system is also self-regulating and will resolve immune responses to prevent self-inflicted damage to healthy cells. While immune cells circulate in the blood and are maintained systemically in peripheral lymphoid organs such as the spleen, mucosal tissue, and lymph nodes; they all originate in the primary lymphoid organs: the bone marrow and thymus. The immune system is broadly separated into two separate yet interconnected branches: the innate immune system and the adaptive immune system\textsuperscript{54}.

The innate immune system provides the first line of defense, forfeiting specificity for a swifter response time after an insult. The innate immune system is comprised of cells such as natural killer cells, eosinophils, basophils, mast cells, and phagocytes such as...
macrophages and neutrophils. It also is composed of molecular components, such as the complement system and antimicrobial peptides. Mechanical barriers, such as the intestinal epithelium, are important components of the innate immune system, involved in the initiation and coordination of acute inflammation via chemical factors such as chemokines and cytokines. Cytokines can be produced by a wide variety of tissues and serve as a chemical warning of immunological insults. Aside from serving as the first line of defense, the innate immune system also primes and initiates the adaptive immune response.

The adaptive immune system, while considerably slower in response time than the innate immune system, yields a much greater specificity for a given threat and has the capacity for long-term memory. This gives the adaptive immune system the ability to mount a quick and more vigorous response after repeat exposures to the initial agent. The adaptive immune system is composed of two types of lymphocytes: B cells and T cells. B cells, originating and maturing in the bone marrow, are responsible for the body’s humoral immune response. They release proteins called immunoglobulins which bind to and aid in the elimination of extracellular pathogens. T cells, while also originating in the bone marrow, mature in the thymus and are responsible for mediating the cellular immune response. Different classes of T cells (helper, cytotoxic, and regulatory) perform and tightly modulate the various aspects of this process. It is here in the adaptive immune system that scientists first found evidence for the immunotoxic effects of PCBs.

Initial clinical analysis of patients after both the Yusho incident, and the Yu-cheng exposure a decade later, revealed that those acutely exposed to PCBs had suppressed humoral and cellular immunity in the first few years after the exposure. Patients after both incidents had decreased levels of multiple types of immunoglobulins in their serum
including IgA and IgM. They did not have reduced total B cells however, suggesting this was not due to a direct cytocidal effect on the B lymphocytes. Patients in the Yu-cheng incident also had a decreased percentage of total T cells, driven primarily by a reduction in the T helper cell population. These observations in humans have been replicated in non-human primates exposed to similar levels of PCBs, providing considerable evidence that acute exposure to PCBs causes immunosuppression.

Epidemiologically, there was also evidence of immunosuppression in these acutely PCB-exposed populations found by looking at rates of infectious diseases. Following Yusho, patients experienced a high frequency of respiratory infections and persistent respiratory distress. Children were found to be particularly sensitive to the infections linked to immunosuppression by PCBs. Infants after the Yu-cheng exposure experienced higher frequencies of bronchitis and influenza attacks. Higher rates of ear infections and respiratory tract infections were reported in older children as well.

Similar immunosuppressive effects have been seen in human populations chronically exposed to PCBs in their diet. In Northern Canada, Inuit children who experienced high, prenatal exposure to PCBs had increased incidences of acute otitis media, recurrent otitis media, and lower respiratory tract infections. Similarly, PCB burden in Dutch preschool children was associated with a higher prevalence of recurrent otitis media and chicken pox. This increased incidence of recurrent otitis media persisted in this cohort of children into later childhood. PCB exposure in the Dutch cohort was also associated with lower antibody levels against measles and mumps following primary vaccination against these pathogens. Two birth cohorts of children from the Faroe Islands, exposed to PCBs via their diet, also experienced insufficient responses to vaccinations.
against tetanus and diphtheria. For each doubling of cumulative PCB exposure at 18 months of age, antibody response to diphtheria decreased by over 24%. At 7 years of age, the children’s antibody response to tetanus was reduced by over 16% for each doubling of PCB exposure. Studies in non-human primates fed diets high in PCBs have demonstrated similarly stunted humoral immune responses following immunizations\textsuperscript{53,57}.

Suppression of the cellular immune response has also been associated with exposure to PCBs. Multiple \textit{ex vivo} studies utilizing immune cells from PCB exposed populations have shown decreased functional capacity of T lymphocytes determined by measuring their proliferation, secretion, and their ability to mount a cellular immune response. To assess proliferation, cells extracted from umbilical cord blood were stimulated with mitogens, chemical substances meant to stimulate cell division. Lymphocytes from PCB-exposed populations constantly showed reduced proliferation and clonal expansion as compared to lymphocytes from unexposed populations\textsuperscript{61,62}. Clonal expansion is the process by which a lymphocyte, having been exposed to an antigen, rapidly divides to create replicate cells specific to the antigen from which it was originally exposed. This process, which is important for both the cellular and humoral immune response, is dependent upon presentation of this antigen by other cells. In highly PCB exposed populations, cell surface markers associated with this antigen presentation are significantly reduced\textsuperscript{63}. Cytokine secretion, the molecular messaging service of the immune system, was also suppressed in lymphocytes stimulated \textit{ex vivo}; further suggesting PCB exposure reduces the functional capacity of these cells\textsuperscript{62}.

This lack of ability to clonally expand, present antigens, and secrete cytokines suggest that PCBs could severely hamper cellular immune responses. To more globally test
this, a scientist investigated whether PCB exposure could affect delayed-type hypersensitivity reactions. Delayed type hypersensitivity, or type 4 hypersensitivity, is an immune response mediated specifically by T cells in response to a soluble antigen. It is unique because it does not involve immunoglobulins like other hypersensitivity reactions so specifically tests the function of the cellular immune response. Delayed type hypersensitivity was assessed in exposed populations by intracutaneously injecting various bacterial antigens and then assessing skin reactivity after several days. Yu-cheng patients have significantly less reactivity compared to controls in response to both tuberculin (PPD) antigen and to streptokinase/streptodornase (SK/SD) antigen mixtures. These results persisted up to 4 years following PCB exposure. Interestingly, the amount of immunosuppression measured in the skin reactivity tests was correlated with the severity of the skin manifestations of PCB toxicity, and with total PCB concentrations in the patient’s blood.35 Chronically PCB-exposed populations in Northern Europe have also been reported to have impaired hypersensitivity reactions which were correlated with PCB exposure.64

A considerable amount of supporting evidence for the immunosuppressive effects of PCBs on the adaptive immune system comes from wild mammal species not traditionally associated with the biomedical sciences. Due to the large amount of PCBs still in the environment, particularly in fish, marine mammals consuming these fish are consistently exposed to high levels of PCBs. This makes them excellent subjects for studying the immunotoxic effects of PCBs. A recent meta-analysis of over 20 published manuscripts on PCBs in marine mammals, compiled data from both in vivo and in vitro analysis of lymphocyte proliferation from grey seals, harbor seals, ringed seals, beluga,
bottle nose dolphins, and polar bears. The authors found a remarkably robust, dose-response to PCB exposure on lymphocyte proliferation. This reduction in lymphocyte proliferation closely mirrors results obtained from humans. In a study of harbor seals at a rehabilitation facility fed a diet high in PCBs for 2.5 years, it was found that PCB-exposed animals mounted a significantly lower humoral immune response when immunized with a foreign protein as compared to seals fed a low PCB control diet. These seals also mounted a significantly lower delayed type hypersensitivity reaction as compared with controls, thus causally linking PCB exposure with defects in the cellular immune response. Many of these immunosuppressive effects of PCBs have also been confirmed in controlled laboratory experiments using model organisms. While studies on these animals are well suited for providing mechanistic insights, recent evidence suggest that the relative sterility of the laboratory environment artificially under-develops the immune system of model organisms like laboratory mice. This could potentially limit the generalizability of immunology findings from laboratory animals and makes the study of the immunotoxic effects of PCBs on wild animals all the more relevant.

While PCBs exert a primarily suppressive effect on the adaptive immune system, evidence suggest that PCBs exert a predominantly stimulatory effect on the innate immune system. While in vivo data on the proinflammatory effects of PCBs in humans is limited, a study looking at Yusho patients, compared to age matched controls, found that Yusho patients had significantly higher serum levels of several proinflammatory cytokines including interleukin (IL)-17, IL-23, IL-1β, and tumor necrosis factor-α (TNFα). Most striking about this study was that it was conducted 40 years after the Yusho incident suggesting that PCBs may have a lasting proinflammatory effect in humans. Expression
of Cyclooxygenase-2 (COX-2), an enzyme important for the synthesis of acute inflammatory mediators such as prostaglandins, was also found to correlate with the amount of blood PCBs in a cohort of Japanese children chronically exposed to PCBs\textsuperscript{70}. Multiple studies looking at PCB exposed seals in the wild have also found increased circulating levels of IL-1\(\beta\), a potent proinflammatory cytokine which can activate COX-2\textsuperscript{71,72}.

These human and wildlife studies are augmented by toxicological data from animal models and \textit{in vitro} experiments which support the proinflammatory effects of PCBs. Mice exposed orally to PCBs rapidly accumulated measurable amounts of PCBs in their brains, lungs, and livers. Analysis of these organs revealed an upregulation of important proteins for initiating immune responses such as intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1)\textsuperscript{73}. ICAM-1 and VCAM-1 are expressed in endothelial cells and leukocytes, and serve as molecular tethers used for trafficking immune cells to the site of inflammation. Analysis of vascular endothelium also has shown upregulation of VCAM-1 following intraperitoneal injections of PCBs\textsuperscript{74}. After oral exposure to PCBs, mice upregulated monocyte chemoattractant protein 1 (MCP-1, also referred to as CCL2), in their brain, liver, and lungs\textsuperscript{73}. MCP-1 is an important chemokine for attracting other immune cells to the site of an inflammatory insult. MCP-1 has also been shown to be upregulated in adipocytes exposed \textit{in vitro} to PCBs\textsuperscript{75}. Matrix metalloproteinases (MMPs) are a family of excreted enzymes that are important for the degradation of the extracellular matrix and serve as important modulators of the innate immune response\textsuperscript{76}. MMPs, specifically MMP-3 and MMP-7, were also upregulated in the brain, lungs, and liver of mice following oral exposure to PCBs\textsuperscript{73}.
Innate immune activation, as measured by an increase in inflammatory cytokine production, has also been linked to PCB exposure \textit{in vitro}. Both porcine and human vascular endothelial cells, in response to PCB exposure, upregulate IL-6 which is an important mediator of fever and acute inflammation\textsuperscript{74,77}. Human vascular endothelial cells also upregulated TNFα, another potent proinflammatory cytokine responsible for both local and systemic inflammation\textsuperscript{77}. Stimulation of mature adipocytes with PCBs also caused significant increases in the production of these cytokines\textsuperscript{78}. Increased cytokine production from innate immune cells has also been reported in response to PCBs. Mast cells are innate immune cells located within other tissues and are responsible for allergic reactions and defense against parasitic worms. \textit{In vitro} stimulation of human mast cells with PCBs caused significant increases in the mRNA of COX-2 and the inflammatory cytokines TNFα, IL-6 and IL-1β\textsuperscript{79}. Primary cultures of kidney cells have also been shown to upregulate IL-1β following PCB exposure\textsuperscript{80}. Many of the inflammatory proteins that are upregulated \textit{in vitro}, namely COX-2, TNFα, and IL-1β, are also seen at increased levels in the blood of PCB exposed human and wildlife populations. Taken together, the upregulation of proinflammatory cytokines across multiple tissue types, in response to PCB exposure provides robust evidence of innate immune activation.

Neutrophils are another important component of the innate immune system which have been shown to be sensitive to the proinflammatory effects of PCBs. Neutrophils are the most abundant type of white blood cell in our blood and are capable of defending against microorganisms in multiple ways. As phagocytes, neutrophils can engulf pathogens and subsequently destroy them through a biochemical process known as the “respiratory burst” which involves the creation of reactive oxygen species (ROS). They can also release
a suite of antimicrobial molecules into their surroundings by a process called degranulation. These antimicrobial molecules can damage host tissues if produced in excess. Both of these processes have been shown to be effected by PCBs. Human and rat neutrophils, upon exposure to PCBs, produce excessive quantities of ROS\textsuperscript{81-83}. Excess ROS could go on to damage healthy tissue and cause further inflammation. These increases in free radicals have been linked to PCB-induced inhibition of superoxide dismutase, an important enzyme that converts ROS into less volatile molecules\textsuperscript{84}. Increased respiratory bursts have also been reported in multiple species of marine mammals exposed to PCBs\textsuperscript{65}. Neutrophils exposed to PCBs also undergo the process of degranulation. This increase in degranulation appears to be dependent on multiple signal transduction pathways and tends to be congener specific\textsuperscript{81,83}. PCB-induced increases in both ROS production and degranulation could potentially damage healthy tissues and contribute to the toxic effects of PCBs.

As seen with both the increase in cytokine production by mast cells and the activation of neutrophils, cells of the innate immune system, despite originating in the bone marrow with lymphocytes, experience stimulatory effect from PCBs as oppose to the immunosuppression. While these proinflammatory effect on the innate immune system may appear at odds with the immunosuppressive effects of PCBs on the adaptive immune system; it has been shown that chronic, low level inflammation can suppress the adaptive immune system and decrease responsiveness to vaccinations\textsuperscript{85}. This could imply that the chronic, innate immune activation caused by exposure to PCBs eventually goes on to suppress the adaptive immune response leading to an increase in infections and decreases in vaccine response. Further studies linking these two immunotoxic effects of PCBs would be necessary to confirm this hypothesis.
As detailed in this chapter, PCBs have been shown to cause inflammation in brain, liver, lungs, adipocytes, and endothelial cells. They have been found to exert immunosuppressive effects on lymphocytes and have even been shown to cause gross and histopathological damage to more traditional immune organs such as the thymus, spleen, and bone marrow\textsuperscript{86}. Despite this relative abundance of immunotoxicology literature, there remains a dearth of knowledge about how PCBs effect the largest immune organ, the gut. Over 70\% of the immune system resides in the gut and the gastrointestinal tract makes up the largest surface area exposed to the environment\textsuperscript{87}. Now that industrial production and thus occupational exposure to PCBs has stopped, dietary exposure is now the main route of PCB exposure. Despite this, the gastrointestinal tract has been widely ignored when studying the effects of PCBs.
Chapter 4 – PCB 153 Causes Inflammation in IECs

The gastrointestinal tract is ground zero for modern exposures to PCBs and constitutes the largest epithelial surface exposed to the outside world. This surface is predominantly composed of intestinal epithelial cells (IECs). These cells have a vital role in maintaining this physical barrier and are also imperative for the intestinal immune homeostasis. The gut is not only exposed to a wide variety of antigens and potential exposure from the environment, but is also home to trillions microbes important for a variety of homeostatic processes in organisms. The importance of the microbiota will be discussed in a later chapter but it is important to note that IECs integrate and regulate the microbial-host relationship through a variety of innate immune mechanisms that serve as sensors for the intestinal environment. Pattern recognition receptors (PRRs), such as toll-like receptors (TLR) and NOD-like receptors, sense the presence of microbial derived molecules and initiate complex intracellular signaling cascades. These intracellular signals converge and lead to the transcription and release of immunomodulatory molecules by the IECs\textsuperscript{88}. This integration of signals from PRRs is critical because unlike other tissues where the presence of microbes would signal an invasion by pathogens, the gut is permanently colonized by a wide range of microorganisms. This makes the IECs responsible for maintaining a delicate balance. Too much immune activation and there is a risk that rampant inflammation would damage healthy tissue, as is seen in inflammatory bowel disease. Too little immune activation and the commensal microbiota would be left unchecked and potentially escape the intestinal lumen. This would lead to systemic inflammation and a rapid deterioration of the organism’s health. Therefore the integration of these signals from PRRs, and the subsequent orchestration of the intestinal immune
response through the release of cytokines, makes IECs imperative for the health of the organism.

We now have a picture of three important facts that underpin the rationale for the work presented in this dissertation. First, PCB exposure primarily happens through the diet making IECs some of the first cells exposed to their toxic effects. Second, PCBs have been shown to have a variety of immunomodulatory effects on a variety of other cell types and tissues. And finally, dysregulation of the balance between inflammation and tolerance that is maintained by IECs can have harmful human health outcomes. For these reasons, it is important to investigate potentially immunotoxic effects of PCBs on the intestinal epithelium.

Given the primarily proinflammatory effects of PCBs on innate immune responses in other tissues in the body, we hypothesized they would also have proinflammatory effects on IECs. If this was the case we also wanted to interrogate the mechanism of this inflammation. In the environment, PCBs exist as mixtures of different congeners which together can activate a multitude of different cellular mechanisms. In order to provide sufficient biological clarity to provide a mechanistic understanding of potential inflammation, we chose to focus on a single, environmentally relevant PCB congener: 2,2',4,4',5,5'-Hexachlorobiphenyl (PCB 153). PCB 153 is a non-coplanar PCB that has been shown to be especially prevalent in the environment and in fish consumed by humans. Indeed, PCB 153 constitutes the bulk of PCB congeners found in humans—making it a vital PCB to study. Additionally, PCB 153 has been shown to cause upregulation of inflammatory cytokines in human mast cells exposed in vitro and to induce inflammation in the livers, lungs, and brain of mice orally exposed to PCB 153. Given its
environmental importance and demonstrated proinflammatory potential, we investigated whether oral exposure to PCB 153 would lead to inflammation in the intestinal epithelium.

To test this hypothesis, that PCB 153 causes intestinal inflammation, we developed a mouse model to replicate an acute, oral exposure to PCB 153. Mice were exposed to 300 μmol/kg of PCB 153 dissolved in stripped safflower oil and administered via oral gavage. This dosage was chosen based on typical amounts of PCBs found in fish meals in highly polluted areas and meant to represent the upper bounds of reported daily intake of PCBs \(^{90,91}\). Control animals received an equal volume safflower oil. Mice were exposed once per day, for two days. On the third day, mice were euthanized and IECs from the proximal small intestine, distal small intestine, and the colon were isolated.

To measure inflammation caused by PCB 153, we measured the upregulation of two important proinflammatory cytokines: IL-6 and TNFα. Both these cytokines are produced locally in the intestine and have been implicated in intestinal inflammatory diseases\(^{92}\). Prior work looking at the proinflammatory effects of PCBs, has demonstrated that these cytokines are upregulated in other cell types after PCB 153 exposure\(^{79}\). RNA was isolated from the IECs that had been isolated from the three sections of the intestines and reverse transcribed to make cDNA. Quantitative-PCR was then performed looking at the expression of the IL-6 and TNFα.

Following oral exposure to PCB 153, transcription of these inflammatory cytokines significantly increased as compared to unexposed controls (figure 4.1a and 4.1b). Expression of IL-6 was upregulated (15.5 fold, P<0.01) in the IECs of the proximal small intestine, as was TNFα (6.5 fold, P<0.001). There was no increase in inflammatory cytokine production in the distal small intestine and colon (figure 4.2a and 4.2b). This
Figure 4.1 PCB 153 causes NF-κB dependent inflammation in the proximal small intestine. Mice were orally exposed once per day to PCB 153 (300μmol/kg) or vehicle for 2 days, following IP injections of the NF-κB inhibitor PDTC or control (PBS). IECs from the proximal small intestine were isolated, RNA was extracted and RT-qPCR run for (A.) IL-6 and (B.) TNFα. N=5 mice per group. Two-way AVOVA with Tukey’s multiple comparison test. **P<0.01, ***P<0.001
Figure 4.2. PCB 153 does not cause inflammation in the distal small intestine or colon. Mice were orally exposed PCB 153 as described above. IECs from the (A.) distal small intestine and (B.) colon were isolated, RNA was extracted and RT-qPCR run for IL-6 and TNFα. N=5 mice per group.
suggested to us that PCB 153 was specifically upregulating these cytokines and causing inflammation in the proximal part of the small intestine.

The central regulator of the innate immune response in IECs is the transcription factor NF-κB\(^94\). It is also a major driver in the transcription of IL-6 and TNFα\(^92,93\). Additionally, PCB 153 has been shown in the literature to increase nuclear localization of NF-κB in the livers of mice and to induce the expression of NF-κB-controlled inflammatory cytokines in a mast cell line \(^79,94\). We hypothesized that NF-κB could potentially be responsible for the observed inflammation caused by PCB 153 in IECs. To test this, we wanted to specifically inhibit the activation of NF-κB in PCB 153 exposed mice by using the inhibitor, pyrrolidine dithiocarbamate (PDTC). PDTC prevents NF-κB activation by preventing the phosphorylation and subsequent dissociation of the iκB subunit from the rest of the NF-κB protein complex, thus preventing the activation of NF-κB\(^95\).

Mice were intraperitoneally injected with PDTC (120mg/kg dissolved in 100uL of PBS) or vehicle (PBS), 30 minutes prior to PCB 153 exposure. This dosage was based on previously reported effective and nontoxic dosages\(^95\). Following injection, mice were exposed to PCB 153 as described above. Once again, IECs were isolated and RT-qPCR was performed to measure the expression levels of IL-6 and TNFα. Strikingly, the blockade of NF-κB dramatically inhibited induction of inflammatory cytokines (figure 4.1a and 4.1b). Both IL-6 and TNFα were significantly reduced in the proximal small intestine of mice after administration of the NF-κB inhibitor, PDTC (IL-6: \(P<0.001\); TNFα: \(P<0.001\)). These data suggested an important role for the transcription factor NF-κB in the mechanism of PCB 153 induced intestinal inflammation.
In our *in vivo* model, the IECs are exposed not only to PCB 153, but also to the mouse’s microbiota and other luminal contents. While this adds to the biological relevance of this exposure model, it also adds potentially confounding variables. With the mechanistic aspirations of this project, we needed an *in vitro* model of IEC exposure to further interrogate how PCB 153 induces inflammation. For these experiments, we used the intestinal epithelial cell line SW480s. Cells were grown to confluence and then exposed to PCB 153. Dosages of PCB 153 were chosen based on the range of possible concentrations that intestinal epithelial cells would be exposed to after a meal high in PCBs\(^9\). Cells were also exposed to lipopolysaccharide (LPS) and etoposide, two proinflammatory substances known to act through the activation of NF-κB, although through different mechanisms. A relatively short exposure time of 3 hours was chosen for the *in vitro* exposure. This exposure time was chosen in part to simulate the speed of intestinal transit, but also to reduce possible feedback processes, such as TNFα signaling, that could obscure the initial mechanism leading to inflammation by PCB 153.

Similar to the *in vivo* model, statistically significant increases in gene expression of inflammatory cytokines were observed in response to PCB 153 exposure. Expression of TNFα experienced a 3.4 fold increase (P<0.05) and IL-6 expression increased 3.2 fold (P<0.05, figure 4.3). This suggested to us that PCB 153 acts, at least in part, directly on the IECs and that we could use this *in vitro* model to interrogate the mechanism of that inflammation.

First however, given importance of NF-κB in the induction of IEC inflammation *in vivo*, we wanted to investigate if NF-κB was also important for the only other reported effect of PCBs on the gut.
Figure 4.3 PCB 153 causes upregulation of inflammatory cytokines in vitro. SW480 cells were exposed to, LPS (50ng/mL), Etoposide (10uM), PCB 153 (50uM) or vehicle (DMSO) for 3 hrs. RNA was collected and RT-qPCR run for IL-6 and TNFα. (N>4) Kruskal-Wallis with Dunn's multiple comparisons test. *P<0.05, ****P<0.0001
Chapter 5 - PCB 153 Increases Intestinal Permeability via NF-κB

As previously stated, despite dietary exposure being the primary means by which humans are exposed to PCBs, the effects of PCBs on the gut remain conspicuously unstudied. One notable exception to this oversight is a reported increase in intestinal permeability after oral PCB exposure in mice. Mice orally gavaged with PCB 153 were shown to have disrupted intestinal morphology and displayed a “leaky gut” phenotype. This phenotype was defined in two ways. The first was via the leakage of LPS into the blood stream of the mice. LPS, a major component in the cell membranes of gram negative bacteria, is unsurprisingly found at very high levels in the intestinal lumen; accompanying the trillions of bacteria that make up the gut microbiota. In the blood however, LPS is potently inflammatory and at high concentrations can cause sepsis. The second way the “leaky gut” phenotype was defined was by measuring the leakage of FITC-dextran out of the intestinal lumen into systemic circulation. FITC-dextran consist of a carbohydrate (dextran) attached to a fluorophore (FITC). When the intestinal barrier is disrupted, FITC-dextran can paracellularly leak into systemic circulation. Since it is not readily excreted by the kidneys, it remains in circulation for extended periods of time and can easily be measured in the serum by detecting the fluorescence of the FITC molecule. An increase in fluorescence corresponds to an increase in the permeability of the intestinal barrier. Mice exposed to PCB 153 had significantly higher FITC-dextran levels in their serum.

Given the dramatic amelioration of inflammation seen upon inhibition of NF-κB, we wanted to investigate if the “leaky gut” phenotype seen after PCB 153 exposure was similarly dependent on NF-κB. To investigate this, we once again turned to our in vivo
model of PCB 153 exposure. Mice were exposed to PCB 153 or vehicle control for 2 days, either with or without NF-κB inhibition from PDTC. On the third day, 4 hours prior to euthanasia, mice were orally gavaged with FITC-dextran at a concentration of 60mg/100g body weight. Blood was collected via cardiac puncture and centrifuged to isolate serum. The concentration of FITC-dextran in the serum was measured via fluorescence.

Consistent with previous reports, oral administration of PCB 153 significantly increased (P<0.05) intestinal permeability compared to vehicle-treated controls (figure 5.1). Strikingly, the inhibition of NF-κB via PDTC significantly ameliorated (P<0.001) the observed increase in serum FITC-dextran. While these data convincingly point to the importance of NF-κB in the “leaky gut” phenotype, we wanted to further establish a more functional read out of this barrier dysfunction.

To do this, we quantified viable bacteria in the mesenteric lymph nodes (MLN) from mice after PCB 153 exposure. Lymphatic vessels from the intestines primarily drain to the MLNs thus making them a likely location to isolate bacteria that have translocated out of the intestinal lumen. Increases in the amount of bacteria isolated from the MLNs would correspond to a break down in the integrity of the intestinal barrier. At the time of sacrifice, MLNs were aseptically isolated and homogenized. They were then plated onto a general culture medium and incubated in both aerobic and anaerobic conditions.

Consistent with the increase in serum FITC-dextran, there was an increase in viable bacteria cultured from the MLNs of PCB 153-exposed mice compared to vehicle-treated controls. A greater quantity of both anaerobic and aerobic bacteria were found in the PCB-153 exposed MLNs. This increase in bacterial translocation was prevented in mice that had been pretreated with the NF-κB inhibitor, PDTC (figure 5.2). Taken together with the
Figure 5.1. PCB 153 causes NF-κB dependent increases in intestinal permeability.

Mice were orally exposed once per day to PCB 153 (300μmol/kg) or vehicle for 2 days, following IP injections of the NF-κB inhibitor PDTC or control (PBS). Four hours prior to sacrifice, mice were orally gavaged with 60mg/100g body weight of FITC-dextran. At the time of euthanasia, serum was collected and levels of FITC-dextran were quantified. N=5 mice per group. Two-way AVOVA with Tukey’s multiple comparison test. *P<0.05, ***P<0.001
Figure 5.2. PCB 153 exposure increased the number of bacteria translocated out of the intestine to the Mesenteric lymph Nodes (MLN). Mice were orally exposed to PCB 153 with or without NF-κB inhibition as described above. MLNs were isolated, homogenized and cultured for 24-48hrs to measure bacterial translocation out of the intestine. N=5 mice per group.
FITC-dextran experiments, these data suggest that the observed increase in NF-κB activation is likely responsible for the barrier dysfunction observed after oral exposure to PCB 153.

NF-κB could be altering permeability in a number of ways. Oral exposure to PCB 153 in mice has been shown to activate NF-κB in other organs and induce the expression of MMPs. Breaking down the extracellular matrix in the gut by these MMPs could reduce the barrier integrity and cause leakage. PCB 153 can also alter the expression of tight junctional proteins such as zonula occludens-1 and occludin, both of which have been shown to be under the control of NF-κB. Tight junctions (TJs) bind together adjacent IECs, preventing the luminal contents of the intestine from leaking out into circulation. Proper functioning of TJs is important for maintaining intestinal barrier integrity and the downregulation of TJ proteins has been seen after oral exposure to PCB 153 in vivo. Alternatively, the TNFα produced by IECs could be acting in an autocrine and paracrine fashion to increase intestinal permeability. In response to an inflammatory insult, TNFα facilitates the movement of other immune cells to the site of inflammation by increasing permeability and thus increasing access to the site of inflammation. PCB 153-induced increases in local TNFα levels could aberrantly increase intestinal permeability causing unintended health consequences on the organism. Further research is needed to fully elucidate the mechanism behind NF-κB dependent increases in intestinal permeability caused by PCB 153.

Regardless of the specific molecular players, the breakdown of the intestinal barrier, such as after exposure to PCB 153, can have dire consequences for the organism. Under homeostatic conditions, the intestinal barrier effectively compartmentalizes a
massive quantity of proinflammatory microbial molecules as well as the microbes themselves. Disruptions of the epithelium that allow for the systemic spread of these inflammatory substances undermines the mechanical barrier role of IECs as part of the innate immune system. Given the importance of NF-κB in PCB 153-induced disruption of the intestinal barrier, and in the observed inflammation in IECs, we wanted to further investigate this NF-κB activation.
Chapter 6 – PCB 153 Activates NF-κB in IECs

Having observed that NF-κB inhibition ameliorated both inflammation and increases in intestinal permeability, we wanted to investigate the nature of this activation.

NF-κB transcription factors are vital for cell survival and are crucial regulators of immunity, stress response, apoptosis, and cellular growth and differentiation. They are constitutively present in cells, existing in an inactive state in the cytoplasm, thus allowing NF-κB to be a molecular first responder to a wide variety of stimuli. Inflammatory cytokines, interaction with antigens, sensing of bacterial products, oxidative stress, and DNA damage are just some of the many stimuli that have been shown to activate NF-κB. Given the multiplicity of stimuli and processes that are controlled by NF-κB, and the speed at which it can be activated, NF-κB is tightly regulated by both positive and negative feedback mechanisms and coordinates with other cell signaling pathways. Perhaps unsurprisingly given its importance, dysregulation of NF-κB has been linked to many metabolic, neoplastic, and immune related diseases.

As previously mentioned, NF-κB is the central regulator of the innate immune response in IECs and a major driver of the inflammatory cytokines induced by PCB 153. In vivo, we saw that inhibition of NF-κB via PDTC was sufficient to ameliorate the upregulation of IL-6 and TNFα, and the increase in permeability. While these data provided significant evidence for the role of NF-κB, we wanted to further confirm that PCB 153 was indeed increasing the activity of NF-κB in IECs. To do this, we again returned to our in vivo PCB 153 exposure model. Following exposure to PCB 153 as described previously, we again isolated IECs from the proximal small intestine of the mice, where we had previously observed increases in inflammation. Following isolation, we isolated
nuclear extracts from these IECs. The activity of NF-κB was tested utilizing a colorimetric, ELISA based assay. Activated NF-κB protein from the nuclear extracts binds to NF-κB consensus sequences of DNA which are attached to the bottom of clear wells. Following an enzymatic reaction, relative NF-κB activity can be determined by measuring the absorbance in a spectrophotometer.

In the proximal small intestine of PCB 153 exposed mice, there was a subtle but statistically significant increase (P<0.05) in the DNA binding activity of NF-κB compared to the control mice, suggesting an increase in NF-κB activity in response to PCB 153 exposure (figure 6.1). Similar magnitudes of in vivo increases in the activity of NF-κB have been linked to increases in inflammation, increases in gene expression, and distinct phenotypic changes in other tissues101,102. To further investigate the mechanism behind this PCB 153-induced NF-κB activation, we then switched to the in vitro exposure of IECs to PCB 153.

Investigation into the mechanism of NF-κB activation in vitro relied on the human intestinal cell line, SW480, which, when exposed to PCB 153, had a similar inflammatory responses as to what was observed in vivo. To measure NF-κB activity, we utilized a luciferase reporter assay. SW480 cells were transfected with an NF-κB reporter plasmid, which expresses several copies of firefly luciferase under the control of promoters where NF-κB binds. Cells were also transfected with a control plasmid, which constitutively produce Renilla luciferase as a means of normalizing the transfection efficiency. Twenty-four hours after transfection, cells were exposed to PCB 153, LPS or DMSO for 3 hours. LPS is a potent activator of NF-κB and was used as the positive control. DMSO is the solvent used to dissolve the stock of PCB 153. As such, DMSO was used as a negative
control. After 3 hours, cells were harvested and luciferase activity was measured. Increases in firefly luciferase, normalized to the number of cells as measured by the *Renilla* luciferase, signify increases in NF-κB activity as a result of the exposures.

**Figure 6.1.** PCB 153 activated NF-κB in IECs of the proximal small intestine. Mice were orally exposed once per day to PCB 153 (300μmol/kg) or vehicle for 2 days. IECs from the proximal small intestine were isolated on the 3rd day and the nuclear fraction was extracted. An NF-κB binding assay was performed. (N=9-10) Unpaired, two-tailed T test.

*P<0.05
In agreement with the *in vivo* data, we observed a significant increase in luciferase production in cells exposed to PCB 153 at both the 100μM (2.8 fold, P<0.05) and 10μM (2.4 fold, P<0.01) doses as compared to the DMSO control (figure 6.2).

PCBs and PCB-like chemicals have been shown produce free radicals, which are known to activate NF-κB\(^{103}\). In order to see if the production of free radicals was involved in the activation of NF-κB in the intestinal epithelium, we pretreated the SW480 cells with N-acetyl cysteine (NAC), a free radical scavenger shown to be effective in preventing oxidative damage from PCB-like chemicals in other cell lines\(^{104}\). Following a 1 hour pretreatment with 5mM NAC, cells were exposed to PCB 153 in the same manner as described above. NAC pretreatment attenuated the PCB 153-induced NF-κB activation, significantly decreasing (P<0.05) the amount of luciferase produced by the cells (figure 6.2). This suggests that the production of free radicals may be an important factor in the inflammatory effects of PCB 153 on intestinal epithelial cells. Indeed, PCB-induced free radical production has been reported in the literature and PCB 153 has been linked to increases in free radical producing enzymes\(^{96,105,106}\).

Importantly, pretreatment of cells with NAC did not affect the activation of NF-kB by LPS. As previously stated, many different signaling pathways converge on NF-kB to affect gene transcription. LPS is sensed by TLR-4 and subsequently activates NF-kB through what is known as the canonical pathway. This pathway is also activated by most other physiologic NF-kB stimuli\(^{100}\). Had PCB 153 been activating NF-κB via the canonical pathway, we would have expected it to behave similarly to LPS. Since PCB 153 behaved differently, it most likely is activating NF-κB via a different pathway, potentially in response to genotoxic damage. Free radicals, such as reactive oxygen species, are known
to cause DNA damage when present in excess. Given this link between PCBs, free radicals, and damage to the DNA; and that NF-κB can be activated in response to genotoxic damage, we wanted to investigate this pathway as a possible mechanism for PCB 153-induced NF-κB activation.

To address if the observed NF-κB activation is driven by the genotoxic response pathway, we once again used the luciferase reporter assay. For these experiments, we used *Clostridium difficile* Toxin B as an inhibitor of the genotoxic activation of NF-κB. *Clostridium difficile* Toxin B has been shown to specifically inhibit the genotoxic activation of NF-κB, while not effecting the canonical activation of NF-κB \(^{107}\). The day after transfection with the NF-κB and control reporter plasmids, cells were pretreated for 2 hours with *Clostridium difficile* Toxin B (100ng/mL or 50ng/mL) and then subsequently exposed for 3 hours to DMSO, PCB 153, etoposide, or LPS. LPS was used as a positive control for the activation of NF-κB through the canonical pathway. Etoposide damages DNA through inhibiting topoisomerase II and is known to cause genotoxic activation of NF-κB \(^{108}\). As such we used etoposide as a positive control for NF-κB activation from the genotoxic response pathway. Pretreatment of the transfected cells with 100ng/mL of *Clostridium difficile* Toxin B significantly decreased NF-κB driven luciferase production in cells exposed to etoposide and PCB 153 as compared to controls (P<0.05), while not significantly affecting cells exposed to LPS (figure 6.3a). A similar trend could be seen in all exposure groups after pretreatment of cells with 50ng/mL of *Clostridium difficile* Toxin B (figure 6.3b).
Taken together, these data point towards PCB 153 activating NF-κB not through the canonical pathway, like LPS, but rather through a different pathway involving response to genotoxic insults.
Figure 6.2. *In vitro* activation of NF-κB by PCB 153 is ameliorated by free radical scavenger. SW480 cells were transfected with an NF-κB reporter plasmid. Cells were pretreated for 1 hr with 5mM N-Acetyl-Cysteine (NAC), a free radical scavenger, or control (PBS). Cells were subsequently exposed to LPS, PCB 153, or vehicle (DMSO) for 3 hrs. (N=5) Two-way AVOVA with Tukey’s multiple comparison test. *P<0.05, **P<0.01, ****P<0.0001
Figure 6.3. Inhibition of the genotoxic response pathway reduces PCB 153-induced NF-κB activation. SW480 cells were transfected with a NF-κB reporter plasmid and were pretreated for 2hrs with (A.) 100 ng/mL or (B.) 50ng/mL of C. difficile Toxin B (Tox) to specifically inhibit genotoxic activation of NF-κB while allowing LPS-induced activation. They were then exposed to vehicle (DMSO), PCB153 (10μM), etoposide (10μM), or LPS (50 ng/mL) for 3 hrs. (N>4) Two-way AVOVA with Tukey’s multiple comparison test. ns = not significant. *P<0.05
Chapter 7 – PCB 153 is Genotoxic to IECs

As touched upon in the previous chapter, PCBs are known to increase free radical production and cause oxidative stress, a consequence of which is DNA damage. This increase in oxidative stress appears to be persistent following PCB exposure as well. One study measuring urinary biomarkers of oxidative damage to lipids, or lipid peroxidation, in Yusho patients found evidence of high levels of oxidative stress 35 years after the initial PCB exposure as compared to age matched controls\(^\text{109}\). Genotoxicity of PCBs have also been observed in wild bottlenose dolphin populations\(^\text{110}\). Both these studies looked at populations exposed to mixtures of PCBs, not specifically non-coplanar PCB congeners like PCB 153. *In vitro* experiments looking at human lymphocytes have demonstrated that PCB 52, a non-coplanar PCB, has the potential to cause extensive DNA damage\(^\text{111}\). PCB 153 has been shown to interact with hepatic DNA in rats following oral exposure, but this study stopped short of showing any genotoxic damage\(^\text{112}\).

The best evidence suggesting that PCB 153 is genotoxic comes from *in vitro* experiments utilizing the RTG-2 cell line, an established fibroblast-like line from gonadal tissue of rainbow trout. Following PCB 153 exposures for both 2 and 24 hours, exposed cells shown increases in DNA damage as measured via the micronucleus test and the alkaline comet assay\(^\text{113}\). Micronuclei are produced when parts of chromosomes break off during mitosis and are subsequently not incorporated into the nuclei of the daughter cells. This breakage is a sign of DNA damage and thus micronuclei are signs of the genotoxicity by PCB 153. The alkaline comet assay is another method of directly measuring DNA damage and will be discussed in detail later in this chapter. Lipid peroxidation, like that seen in Yusho patients, was also reported in these RTG-2 cells after exposure to PCB.
153. While this study provides strong evidence of PCB 153’s potential for genotoxicity, no such effects have yet been reported in the gut. Given the evidence that the NF-κB activation by PCB 153 may be in response to genotoxic damage, we wanted to investigate if PCB 153 was indeed capable of causing DNA damage in IECs.

Genotoxicity of PCB 153 was assessed utilizing the alkaline comet assay. This simple assay, cleverly uses some of the basic physical properties of DNA to sensitively measure DNA damage. Following exposure to a genotoxic substance, cells are suspended in a semisolid gel on a microscope slide and their cellular and nuclear membranes are lysed. This allows the DNA from the cell’s nucleus to freely travel in the gel. Slides are then exposed to an electrical gradient, pulling the negatively charged DNA towards the positive charge. The alkaline conditions denature the double helix, making the DNA single stranded. The undamaged DNA strands are larger and do not migrate as far in the gel when exposed to the electrical gradient. If a genotoxic agent has broken the DNA into smaller fragments however, these small fragments travel freely through the gel and will migrate farther than the undamaged DNA. The DNA is then stained and visualized under a microscope. DNA that has been exposed to a genotoxic substance will form a comet shape due to the damaged, smaller DNA fragments (the tail) getting pulled away from the rest of the DNA (the head). The intensity of the comet’s tail, relative to the comet’s head, is used to calculate the “tail moment” and is a reflection of the severity of the genotoxic damage. Exposure conditions similar to previous in vitro experiments were used for the alkaline comet assay. SW480 cells were exposed for 3 hours to either the positive control etoposide, PCB 153, or the negative control DMSO. As previously mentioned, etoposide is a topoisomerase II inhibitor and causes DNA damage by preventing the re-legation of
DNA following strand breaks meant to relax torsional strain on the double helix during replication. Cells from 4 biological replicates for each exposure condition were pooled and 100 random cells (50 per slide) were assessed for DNA damage. There was a significant increase (P<0.0001) in the tail moment in cells exposed to PCB 153 as compared to cells exposed to DMSO (figure 7.1). Tail moments were comparable to the prior study that demonstrated PCB 153 can be genotoxic in RTG-2 cells\textsuperscript{113}.

We further investigated the genotoxicity of PCB 153 utilizing the protein $\gamma$-H2A.X, a phosphorylated variant of the H2A histone which serves as a sensitive biomarker of genotoxic damage. When the DNA is damaged, this histone variant is phosphorylated by kinases such as ATM or ATM-Rad3-related (ATR)\textsuperscript{115}. Once phosphorylated, $\gamma$-H2A.X allows for DNA damage repair proteins to be recruited to the site of the break. This protein serves as the first step in recruiting DNA repair proteins to the site of the DNA damage. As such it is one of the earliest marks of DNA damage and has been used in past studies investigating the genotoxicity of PCB metabolites\textsuperscript{104}. The ubiquity of $\gamma$-H2A.X in the presence of genotoxic damage makes it a sensitive and reliable marker of multiple types of DNA damage both \textit{in vitro} and \textit{in vivo}\textsuperscript{116}.

\textit{In vitro}, we once again exposed SW480 cells for 3 hours to etoposide, PCB 153, and DMSO. Following the exposure, cells were harvested, lysed, and protein was collected for western blotting. Expression levels of $\gamma$-H2A.X were quantified using densitometry. As seen with the comet assay, PCB 153 caused significant increases in $\gamma$-H2A.X (10$\mu$M: P<0.05, 100$\mu$M: P<0.0001). These increases in $\gamma$-H2A.X were also dose dependent for both PCB 153 and etoposide. Taken together with the results from the alkaline comet assay,
Figure 7.1. PCB 153 causes DNA damage in vitro. An alkaline comet assay was performed to determine genotoxicity. (A.) Representative comets from SW480 cells exposed to vehicle (DMSO), PCB 153 (50μM) and Etoposide (10μM). (B.) Tail Moment taken from comet assay. Four biological replicates for each exposure condition were pooled and 100 random cells (50 per slide) were assessed for DNA damage. Unpaired, two-tailed T test. ****P<0.0001
Figure 7.2. PCB 153 causes an increase in the marker of genotoxic damage, \(\gamma\)-H2A.X, \textit{in vitro}. (A.) Protein was extracted from SW480 cells after exposure to vehicle (DMSO), PCB 153, Etoposide, or LPS. Western blots were run for \(\gamma\)-H2A.X, (representative blot shown). (B.) Densitometry quantification of 3C. \(\text{N}=4\) experiments. One way ANOVA with Dunnett’s multiple comparison test. *\(P<0.05\), ***\(P<0.001\), ****\(P<0.0001\)
the increase in $\gamma$-H2A.X indicates that PCB 153 causes DNA damage in vitro to an intestinal epithelial cell line (figure 7.2).

Interestingly, while there was significant DNA damage in response to both etoposide and PCB 153, the extent of this damage diverged based on assay, with etoposide causing more damage as measured by the comet assay, and PCB 153 causing more dramatic increases in $\gamma$-H2A.X. These data potentially reflect PCB 153 possessing a wider repertoire of molecular mechanisms to damage DNA than etoposide. Etoposide primarily damages DNA through strand breaks during cell division\textsuperscript{117}. This type of DNA damage is specifically measured in the comet assay\textsuperscript{118}. Cells use $\gamma$-H2A.X in response to a wider range of DNA damage, including oxidative damage and the formation of DNA adducts\textsuperscript{119-121}. PCB 153 has been shown to form DNA adducts and increase the production of reactive oxygen species (ROS), which can lead to oxidative DNA damage\textsuperscript{122,123}. Additionally, PCB 153 has been shown to induce the activation of NADPH oxidase, a major source of ROS within cells\textsuperscript{96}. ROS can cause a wide array of different types of DNA damage, base modifications, single-strand breaks, protein-DNA adducts, and both intra- and inter-strand DNA crosslinks\textsuperscript{124}. All of these PCB 153-mediated genotoxic effects, in addition to its ability to directly break the DNA, could increase the levels of $\gamma$-H2A.X. While our results do demonstrate the genotoxicity of PCB 153 in IECs, the mechanism of this genotoxicity, and whether it is caused directly by PCB 153 or via its breakdown products, has yet to be determined.

While the data point to PCB 153 being directly genotoxic to IECs in vitro, we wanted to assess if PCB 153 was also genotoxic in our in vivo exposure model. This was important because the genotoxicity of PCB 153 after oral exposure could potentially be
modified by confounding factors such as the intestinal microbiota. To assess genotoxicity 
in vivo we once again used \( \gamma \)-H2A.X. The sensitivity of this biomarker makes it ideal for 
detecting genotoxicity \( \textit{in vivo} \), and to the point where it has even been suggested as a 
possible biomarker for humans exposed to radiation\textsuperscript{125}. Utilizing the same exposure 
scheme used to assess permeability and inflammatory cytokine expression, mice were 
exposed to PCB 153 or vehicle control for 2 days. On the third day, mice were euthanized 
and IECs were collected from the proximal small intestine, the distal small intestine, and 
the colon. Protein was then extracted from the IECs and \( \gamma \)-H2A.X was quantified via 
western blot. Similar to what was observed \( \textit{in vitro} \), mice exposed to PCB 153 had 
significantly higher (\( P<0.01 \)) expression of \( \gamma \)-H2A.X as compared to vehicle treated 
controls, thus indicating DNA damage (figure 7.3a and 7.3b). Interestingly, as seen with 
the expression of inflammatory cytokines, this effect was observed only in the proximal 
small intestine. There was no DNA damage in the distal small intestine and colon 
suggesting that PCB 153-induced genotoxicity is localized to the proximal small intestine 
(figure 7.3c and 7.3d).

Thus far, we have observed DNA damage, NF-\( \kappa \)B activation, and an increase in 
inflammatory cytokine expression, all localized to the proximal small intestine following 
\( \textit{in vivo} \) exposure to PCB 153. \( \textit{In vitro} \), PCB 153 also causes these same effects. We next 
set out to connect these dots to more fully elucidate a mechanism by which PCB 153 exerts 
these effects on IECs.
Figure 7.3. PCB 153 causes DNA damage in IECs of the proximal small intestine \textit{in vivo}. IECs from the proximal small intestine and were isolated from mice after 2 days of exposure to 300μmol/kg of PCB 153 or vehicle via oral gavage. \textbf{(A.)} Protein was extracted and westerns run for γ-H2A.X. \textbf{(B.)} Densitometry quantification of \textbf{(A.)}. \textbf{(C.)} PCB 153 exposure does not cause DNA damage in the distal small intestine or \textbf{(D.)} the colon. (Note: samples were pooled for colon western blot analysis due to small amount of colonic IECs isolated). N=4-5 mice per group. Unpaired, two-tailed T test. **P<0.01
Chapter 8 - PCB 153 Activates NF-κB and Causes Inflammation via ATM and NEMO

Genotoxic agents have long been known to cause inflammation. This response highlights the role of the immune system in protecting the organism from cancer. Damage to DNA could potentially cause oncogenic mutations in cells exposed to genotoxic substances. Inflammation mobilizes the immune system to surveil for and, if needed, destroy these potentially neoplastic cells. This inflammation can involve the upregulation of inflammatory cytokines, such as IL-6, and has been linked to the activation of the transcription factor NF-κB\textsuperscript{126,127}.

The genotoxic response pathway that links DNA damage to NF-κB activation occurs through the phosphorylation of several key intermediaries\textsuperscript{108}. First, the damage to the DNA causes the phosphorylation of ATM. ATM then phosphorylates and binds to NF-κB essential modulator (NEMO), also known as inhibitor of NF-κB kinase subunit gamma (IKK-γ). This ATM-NEMO complex is subsequently transported out of the nucleus. Once in the cytoplasm, NEMO is then responsible for the activation of NF-κB.

Phosphorylation of ATM and NEMO would provide strong evidence that PCB 153 was activating NF-κB through its genotoxic effects. To test this we once again used our \textit{in vitro} exposure model. SW480 cells were exposed for 3 hours to either the positive control etoposide, PCB 153, or the negative control DMSO. Following the exposure, protein was extracted from the cells. Western blots for phosphorylated ATM (Phospho S1981) showed an increase in phosphorylation after exposure to PCB 153 (figure 8.1). Next we measured the phosphorylated form of NEMO (Phospho-S85) and once again found an increase in the cells exposed to PCB 153 (figure 8.1). Taken together these data show that PCB 153 is activating ATM and NEMO, two key components of the genotoxic response pathway.
Figure 8.1. PCB 153 activates ATM and NEMO. Protein was extracted from SW480 cells after exposure to etoposide (10μM), PCB153 (100uM), or vehicle (DMSO). Westerns were run for phosphorylated-ATM (Phospho S1981), ATM, phosphorylated-NEMO (Phospho S85), NEMO and β-Actin. Blots shown are representative of at least 3 biological replicates.
Figure 8.2. Inhibition of ATM reduces PCB 153-induced NF-κB activation. SW480 cells were transfected with a NF-κB reporter plasmid and were pretreated for 1hr with 10μM of KU-55833, an inhibitor of ATM. They were then exposed to etoposide and PCB153 for 3 hrs. (N=5) One way ANOVA with Dunnett’s multiple comparison test. *P<0.05, ***P<0.001, ****P<0.0001
We next wanted to test if the activation of ATM/NEMO by PCB 153 was indeed responsible for the previously observed increases in NF-κB activity. To do this, we specifically inhibited ATM utilizing the competitive ATM inhibitor, KU-55933. KU-55933 has been shown to exhibit great specificity and a lack of known off target effects, even on ATM’s most closely related enzyme, ATR\textsuperscript{128}. Utilizing the NF-κB reporter assay, transfected cells were pretreated for 1 hour with 10μM of KU-55933 before being exposed utilizing the same exposure scheme used above. ATM inhibition significantly inhibited NF-κB activation in both the high (34% inhibition, P<0.001), and the low dosages (48% inhibition, P<0.0001) of PCB 153 (figure 8.2).

We finally wanted to link the increases in inflammatory cytokine expression induced by PCB 153 with the activation of the ATM/NEMO genotoxic response pathway. We did this through chemical inhibition, with KU-55933, and by silencing ATM. For the chemical inhibition experiments, we used the same exposure scheme used in prior \textit{in vitro} experiments. SW480 cells were pretreated for 1 hour with KU-55933 and subsequently exposed to etoposide, PCB 153, and DMSO for 3 hours. RT-qPCR was performed following extraction of RNA from the cells. Pretreatment with the ATM inhibitor significantly reduced the induction of both IL-6 (P<0.05) and TNFα (P<0.01) following exposure to PCB 153 (figure 8.3).

Despite the specificity of KU-55933, chemical inhibition can have unintended off target effects so to further strengthen the involvement of ATM in PCB 153-induced inflammation, siRNA was used to silence the expression of ATM in SW480 cells. Cells were transfected with either an ATM-specific siRNA or a scrambled control. They were then allowed to grow an additional 48 hours to allow for more complete silencing of ATM.
Figure 8.3. Chemical inhibition of ATM ameliorates upregulation of inflammatory cytokines. SW480 cells were pretreated for 1hr with 10μM of KU-55833. They were then exposed to etoposide (10μM) and PCB153 (50μM) or vehicle (DMSO) for 3 hrs. RNA was collected and RT-qPCR run for (A.) IL-6 and (B.) TNFα. (N=4) Two-way AVOVA with Tukey’s multiple comparison test. *P<0.05, **P<0.01
Efficacy of the knockdown was confirmed via western blot (figure 8.4a). After ATM was effectively silenced, cells were once again exposed for 3 hours to etoposide, PCB 153, or DMSO. Following exposure to PCB 153, the increase in expression of IL-6 was significantly down-regulated (P<0.05) in cells lacking ATM (figure 8.4b). Similar trends were seen in the expression of TNFα after exposure to PCB 153 (figure 8.4c). Taken together, these data support the role of the ATM/NEMO in the increases in inflammatory cytokines in IECs following exposure to PCB 153.

Our data suggest that the DNA damage caused by PCB 153 is associated with the activation of NF-κB through ATM and NEMO. PCB 153 directly leads to the phosphorylation of both ATM and NEMO, the major components of the NF-κB genotoxic response pathway. The chemical inhibition of ATM significantly prevented NF-κB activation by PCB 153. Additionally, when ATM was chemically inhibited or silenced, subsequent increases in inflammatory cytokines were significantly reduced. This suggests that activation of NF-κB by the ATM/NEMO pathway contributes to the inflammatory effects of PCB 153. A summary of the proposed mechanism can be found in figure 8.5.

It is important to note that PCB 153 could also be activating ATM through more than one mechanism. While the activation of NF-κB through ATM and NEMO has never been conclusively shown to occur in the absence of DNA damage, a variety of other cellular stresses have been shown to impact this pathway. When exposed to a genotoxic agent, DNA can be damaged in a variety of ways that can activate NF-κB. These include, but are not limited to, double strand breaks, single strand breaks, intra- and inter-strand crosslinking, and other base modifications. Any of these events can potentially initiate the ATM/NEMO genotoxic response pathway but other extra-nuclear events can also have
effects on this signal transduction. ROS and other free radicals, intracellular calcium concentration, and input from other signaling pathways all can affect NF-κB signaling after genotoxic damage. The pleiotropic nature of ATM can help explain some of these effects. In addition to being activated by DNA damage, ATM can be activated by variety of known and unknown mechanisms.

One of these mechanisms is oxidative stress. ATM normally resides as an inactive dimer, undergoing auto-phosphorylation and subsequent monomerization rapidly after sensing DNA damage. When cells are exposed to oxidative stress, ROS can directly oxidize ATM creating an active ATM dimer. This suggest that ATM may have a role in cellular redox sensing. This is potentially relevant to our understanding of the ATM-dependent activation of NF-κB by PCB 153. Compared to our positive control etoposide, PCB 153-induced activation of NF-κB was much more dramatically attenuated after chemical inhibition (figure 8.2). As discussed chapter 7, PCB 153 may have a wider array of genotoxic and cellular effects as etoposide, including the creation of ROS. The production of ROS, combined with the possibility that PCB 153 is damaging DNA in multiple ways, could induce multiple mechanism of ATM activation and lead to a much greater degree of ATM-dependent NF-κB activation. Indeed, western blots of activated ATM seem to visually suggest more ATM activation following PCB 153 exposure than etoposide exposure (figure 8.1). Unfortunately, we currently lack the basic knowledge to fully understand the extent and importance of this increase ATM activation. While only 10% of phosphorylation and dephosphorylation events following DNA damage occur at consensus ATM recognition sites, over 60% are ATM dependent, suggesting a multifaceted role for ATM in a variety of other signaling cascades. Because of the presence of both ATM-
Figure 8.4. Silencing of ATM reduces upregulation of inflammatory cytokines by PCB 153.

SW480 cells transfected with siRNA to silence ATM. (A.) Protein was extracted, and western blots for ATM were performed to determine the efficacy of the silencing procedure. Following the silencing of ATM, SW480 cells were exposed to etoposide (10μM) and PCB153 (50μM) or vehicle (DMSO) for 3 hrs. RNA was collected and RT-qPCR run for (B.) IL-6 and (C.) TNFα. (N=4). Two-way AVOVA with Tukey’s multiple comparison test. ns = not significant. *P<0.05, **P<0.01
dependent and other concurrent molecular events following exposure to genotoxic agents, there is likely a variety of interwoven mechanisms at play, each with differing kinetics and competing dominance over the genotoxic response pathway of NF-κB\textsuperscript{129}.

While there are still many interesting questions left to be answered about the necessary and sufficient events required for genotoxic activation of NF-κB, the observed activation of ATM and NEMO by PCB 153 provides us with sufficient resolution to conclude that this pathway is indeed activated in IECs. The activation of this pathway by PCB 153 subsequently leads to the activation of NF-κB, which in turn leads to inflammation in IECs (figure 8.5).
Figure 8.5 Proposed mechanism.
Chapter 9 – The Geography of PCB 153 Induced Inflammation in the Gut

An interesting result to emerge from the in vivo mouse exposures to PCB 153 was the specific localization of the genotoxic and inflammatory effects. The increases in γ-H2A.X, and subsequent NF-κB activation and inflammation, were only seen in the proximal small intestine while the distal small intestine and colon were spared from the effects of PCB 153. This localization most likely reflects the digestive specialization of this particular segment of the gastrointestinal (GI) tract.

The lower GI tract can be broadly separated into the small intestine and large intestine, or colon. The small intestine is then further subdivided (proximally to distally) into the duodenum, the jejunum, and the ileum. Each of these segments have unique roles in the digestion and absorption of nutrients. The contents of the stomach enter the small intestine in the duodenum, where they meet a large quantity of enzymes released by the pancreas. These enzymes are largely responsible for the breakdown of ingested macromolecules. Amino acids, iron, and monosaccharides are absorbed in this region of the intestine. Following the duodenum is the jejunum. A wide variety of molecules are absorbed in this segment of the intestine including calcium, monoglycerides, free fatty acids, and fat-soluble vitamins. The distal segment, the ileum, takes up over half the total length of the small intestine and is responsible for absorption of water, sodium, bile acids, and water-soluble vitamins. The colon also absorbs a small amount of water and is responsible for absorbing the metabolites produced by the large quantity of bacteria which liberate some of the remaining nutrients\textsuperscript{132}.

Given the anatomic specialization of the intestines, the location of PCB 153’s genotoxic and inflammatory effects make sense. PCBs are extremely lipophilic molecules.
As such, they are absorbed much like fat soluble vitamins, primarily in the second segment of the proximal small intestine, the jejunum. The IECs in the jejunum would thus be more exposed to PCB 153 than cells in other segments, and distal parts of the intestine may be further spared since they would potentially be exposed to less PCB 153 consequent of jejunal absorption.

The regional specialization in the GI tract naturally means that the IECs of the different segments of the intestines may have differing physiologic properties. Enzymatic profiles will change based on the types and quantities of molecules the IECs in that segment are required to absorb. One important type of enzymes for the metabolism of PCB 153, are the cytochrome P450 (CYPs) enzymes. CYPs are not uniformly distributed along the GI tract and can help further explain the localized phenotype following oral exposure to PCB 153.

CYPs are an important superfamily of proteins responsible for the metabolism of endogenous substances like bilirubin, hormone synthesis and breakdown, cholesterol synthesis, and vitamin D. Perhaps the most important function of CYPs however is their vital role in the metabolism of xenobiotics. In humans, CYP enzymes account for over 75% of all drug metabolism. They are also responsible for the initial metabolism of PCBs, including PCB 153. CYPs catalyze the oxidation of PCBs which converts the PCBs into a more metabolically active form. While CYPs and drug metabolism are typically associated with the liver, the gut wall is an often overlooked and incredibly important site of xenobiotic metabolism.

Before reaching the liver, all ingested xenobiotics must first enter the intestinal lumen and pass through the IECs to reach portal circulation and subsequently the liver.
Considering the absorptive function of the intestines, it stands to reason that IECs would have a substantial role in xenobiotic metabolism given the importance of this barrier to the systemic uptake of these substances. The CYP enzymes, performing the bulk of this metabolism, are thus found throughout the intestines. While the total CYP content of intestinal epithelium is considerably lower than that in the liver (depending on the segment, intestinal CYP content is 24-44% that of the liver), \textit{in vivo} data suggest that for certain xenobiotics, the clearance by the intestines matches or even exceeds that of the liver\textsuperscript{135}.

CYPs are not uniformly distributed in the intestines but rather are found in much higher quantities in the proximal small intestine, specifically the jejunum\textsuperscript{136}. Total CYPs expression increases from the duodenum into the jejunum then falls along the length of the ileum. CYP expression also differs topographically in the intestinal epithelium. Total CYPs in the intestine are found at the highest concentration in the mature enterocytes of the villus tip, where orally administered compounds such as PCBs would be absorbed\textsuperscript{135}. The CYP3A subfamily, is the most prevalent type of CYP in the small intestine, making up approximately 80% of the total immunoquantified intestinal CYPs\textsuperscript{135}. Similar to the total CYP content in the intestine, CYP3A has also been reported to be highest in the proximal small intestine\textsuperscript{136}. Multiple studies have linked the CYP3A enzymes to PCB 153\textsuperscript{137,138}. \textit{In vitro} experiments utilizing the human intestinal epithelial cell line, Caco-2, found that following exposure to PCB 153, cells dramatically upregulated the expression of CYP3A\textsuperscript{138}. This upregulation could dramatically alter the toxicity of PCB 153 in the proximal small intestine.

This increased presence of CYP enzymes where the majority of PCB 153 is being absorbed means that not only will IECs in the jejunum be exposed to more PCB 153, they
will also be exposed to more biologically active PCB 153. The initial oxidation by the CYP enzyme causes the formation of an arene oxide intermediate. Subsequent hydroxylation makes biphenylols (OH-PCBs), and hydroquinones which are then further transformed into PCB-quinones. The production of these quinones releases ROS\textsuperscript{139}. As discussed in chapter 7, ROS are known to cause DNA damage through oxidation and can cause a wide array of different types of DNA damage\textsuperscript{124}. OH-PCBs have also been shown to cause genotoxic damage to cells\textsuperscript{140}. One of the most common OH-PCB congeners found in human blood is 3-OH-PCB 153, a hydroxylated metabolite of PCB 153\textsuperscript{139}. The higher concentration of CYPs in the jejunum makes it more likely that the jejunal IECs are exposed to higher concentrations of these genotoxic metabolites of PCB 153.

Taken together, the observed geographic specificity of the DNA damage and subsequent inflammation induced by PCB 153 in our studies could be explained by the location of intestinal absorption of PCBs and the subsequent metabolism into metabolically active, and potentially genotoxic, metabolites.
Chapter 10 – PCBs and the Microbiome

Previous chapters have focused on the mechanism of action that PCB 153 has directly on the IECs themselves. *In vitro* experiments allowed us to isolate these effects, and similar results seen *in vivo* supported the conclusions these experiments produced. *In vivo* though, IECs are not only exposed to PCB 153, but are constantly exposed to the microbiota, the trillions of commensal microorganisms that reside in the intestinal tract.

The bulk of the human microbiota is made up of 10-100 trillion bacteria representing approximately 400-1000 different individual species. These bacteria interact with each other, the host, and a variety of other microorganisms such as viruses and fungi, in a dynamic ecosystem within the gut. It has become clear in recent years that the microbiota functions basically as an additional organ, performing a wide variety of essential host functions such as assisting with digestion, protection against pathogens, and the training and modulation of the immune system. Pathological alterations in the microbiota, known as dysbiosis, have been associated with a wide variety of diseases such as inflammatory bowel disease, colorectal cancer, obesity, diabetes, allergies, and even psychological conditions like depression, anxiety, and autism\textsuperscript{141-143}.

Given the importance of the microbiota, and the consequences should dysbiosis occur, it is imperative that the host control the abundance, location, and composition of the microbiota. As introduced in chapter 4, the IECs play a critical role in orchestrating these processes primarily through the surveillance activity of PRRs. A wide variety of cell types in the epithelium also participate in regulating the microbiota. Mucins, released by goblet cells, help create a protective mucus layer which keep the bacteria specially separated from the intestinal epithelium. This special separation prevents an extensive inflammatory
response from the host. Antimicrobial peptides (AMPs), released into the lumen primarily by paneth cells, exert a bactericidal effect by interfering with bacterial cell membranes. These AMPs act both to regulate the bacterial community and to prevent intrusion of the gut bacteria into the epithelium. Intestinal B cells release immunoglobulin A (IgA) into the intestinal lumen which also controls bacterial growth and location. Specificity of these IgAs allow the host to limit the growth of more proinflammatory bacteria thus maintaining a more benign community composition in the intestinal lumen. IECs can even release microRNAs into the intestinal lumen that modulate bacterial gene expression and control their growth.

This exquisite control over the microbiota by the host is accompanied by a surprisingly large amount of bacterial control over host processes as well. Beyond innate immune recognition of proinflammatory microbial molecules, bacteria in the gut also fine tune the host’s immune system. Segmented filamentous bacteria tightly adhere to IECs and drive the production of proinflammatory T cell subsets. This effectively heightens tissue immunity and has been demonstrated to promote a protective response to intestinal pathogens. Bacterial-produced molecules like short-chain fatty acids and polysaccharide A exert the opposite effect, increasing the regulatory and anti-inflammatory functions of the mucosal immune system. On a much grander scale, there is considerable evidence that the microbiota can even exert control over higher host functions such as regulation of host appetite and energy homeostasis.

This crosstalk between the host and microbiota produces an experimental conundrum for studying environmental exposures in the gut. Any in vivo phenotype of a given exposure could represent an effect of the exposure on the microbiota, the host, or
both. This includes both host phenotypes, like inflammation, and microbiota phenotypes, like changes in the community structure and relative abundances. Studies utilizing germ free mice, without a microbiota, and fecal microbial transplants can begin to dissect this complexity. The picture becomes all the more complex when studying environmental pollutants. The gut microbiota has an extensive capacity to metabolize and biotransform xenobiotics, including environmental pollutants, thus modulating their toxicities in a variety of ways. Biotransformation of xenobiotics by the microbiota can increase their primary absorption by the host, and even reverse the chemical modifications made by the liver to detoxify and excrete xenobiotics. The microbiota can also directly increase the toxicity of xenobiotics, by chemically modifying them into more damaging metabolites\textsuperscript{150}. Despite the importance of the gut microbiota, there is still much work needed to be done to understand the role of the microbiota after exposure to environmental pollutants, including PCBs.

Similar to the dearth of knowledge about PCBs effects on the gut, there is also very little is known about the effects of PCBs on the gut microbiota. To date, only two published manuscripts have looked into the effects on PCB exposure on the composition of the gut microbiota. The first study looked at the effects of dietary exposure to PCB 126, a coplanar PCB, on the development of the microbiome of northern leopard frogs. Larval frogs were fed either a diet high in PCBs or a control PCB-free diet. The PCB diet was discontinued after they reached adulthood and all frogs were fed the same, PCB-free diet. The gut microbiome of the tadpoles and adult frogs were analyzed by 16sRNA sequencing. A diet high in PCBs changed the community structure of the tadpoles’ gut microbiota. PCB fed tadpoles had increases in phylum Firmicutes and decreases in Proteobacteria. There was
also an increased presence of *Pseudomonas* in PCB exposed tadpoles as compared to controls. Interestingly, the changes in the microbiome of the frogs persisted and became more defined when the frogs reached adulthood, well after exposure to PCBs had ceased. Adult frogs showed increases in Bacteroidetes and a corresponding decrease in Firmicutes. The PCB-exposed frog microbiome was also enriched with Fusobacteria. Both PCB-exposed tadpoles and adult frogs had increases in the species richness and phylogenic diversity of their gut microbiota. There were no reported phenotypic outcomes on the host frogs reported but the persistence of the changes in the microbiota following PCB exposure suggest that any consequences to the host from the PCB-exposed microbiota could persist for long periods of time.

The second study looked at the effects of dietary exposure to PCBs on the gut microbiota of mice. The effect of exercise on the PCB-exposed gut microbiota was also assessed. Mice were exposed to a mixture of PCB 153, PCB 138, and PCB 180, meant to represent a typical environmental exposure. The gut microbiota of PCB exposed mice was altered, primarily by a decrease in Proteobacteria. This decrease was especially pronounced in multiple species of *Pseudomonas* which experienced up to a 5.6 fold decrease. The opposite was seen in PCB 126 exposed frogs suggesting that the effects of PCBs on the microbiota varies depending on the species studied and the specific PCB congeners used. While less pronounced, there were increases in abundance of a wide range of bacterial taxa, including a nearly 2 fold increase in the common skin microbe, *Staphylococcus epidermidis*. While there no significant changes described in the biodiversity of the microbiota, there did appear to be a tendency towards increased biodiversity in the PCB-exposed, exercised mice. Most striking about this study was that the changes in the
microbiota caused by PCBs were largely ameliorated in the exercised mice. This provides an attractive therapeutic avenue should the microbiota changes caused by PCB exposure prove to have negative human health consequences\textsuperscript{152}.

One possible negative health consequence comes from an as yet unpublished report on the effect of dietary PCB exposure on germ free mice. Dietary exposure to PCBs significantly changed the bile acid profile found in the intestines of mice. In germ free mice, the lack of a microbiota amplified these PCB-mediated effects on bile acids\textsuperscript{153}. Bile acids have wide spread roles in digestion, cholesterol metabolism, inflammation, and can also act as hormones. The microbiota is important in the modification of bile acids so, given the importance of bile acids in homeostasis, this may prove to be an important microbiota related effect following dietary exposure to PCBs. Studies utilizing germ free mice have also shown that the metabolism of PCBs by the microbiota is important for absorption and tissue retention of PCBs, although no toxic effects were reported\textsuperscript{154}.

As discussed above, the composition of the microbiota is influenced by the host. Our data demonstrated that PCB 153 causes inflammation in IECs. This inflammation may in turn increase host control mechanisms over the microbiota, such as the increasing the production of mucins, IgA, or AMPs. These would alter the composition of the microbiota. PCBs could also be directly effecting the composition of the microbiota without host input. To begin to tease apart the cause and effect of PCB-induced changes in the gut microbiota, we tested the effects of PCBs on gut microbes \textit{ex vivo}.

Utilizing the data from the prior study on the PCB-exposed mouse microbiota, we chose one species that increased in abundance, \textit{Staphylococcus epidermidis}, and one genus that decreased in abundance, \textit{Pseudomonas}, to investigate \textit{ex vivo} (table 10.1). We then
investigated the effects of PCBs on the growth rates of these bacteria. *S. epidermidis* and *Pseudomonas* (*P. aeruginosa*) were grown overnight in general culture media. They were then diluted to a set optical density (OD$_{600}$=0.01) and split into subcultures. Subcultures were exposed to multiple concentrations of PCB 153 (non-coplanar), PCB 126 (coplanar), PCB 105 (a PCB with mixed properties of both a coplanar and non-coplanar PCB), or DMSO as a negative control. The optical density was measured every 30 minutes to measure the growth rate of the bacteria. The growth rate was then plotted and used to calculate the doubling time (example shown in figure 10.1). Decreases in the doubling time signified a faster growth rate, and increases in the doubling time signified a lower growth rate.

Table 10.1. Bacterial taxa in mouse microbiota that changed *in vivo* following PCB exposure. Adapted from Choi et al. 2013$^{152}$. Bacteria from the bolded taxa were used for experiments.

<table>
<thead>
<tr>
<th>Advantageous Effect</th>
<th>Phylum</th>
<th>Family</th>
<th>Genus/Species</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteroidetes</td>
<td>Saprospiraceae</td>
<td><em>Candidatus aquirestis</em></td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>Actinobacteria</td>
<td>Corynebacteriaceae</td>
<td>-</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Verrucomicrobia</td>
<td>Verrucomicrobiaceae</td>
<td>-</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Firmicutes</td>
<td>Staphylococcaceae</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>1.7</td>
</tr>
<tr>
<td>Deleterious Effect</td>
<td>Phylum</td>
<td>Family</td>
<td>Genus/Species</td>
<td>Fold Change</td>
</tr>
<tr>
<td></td>
<td>Proteobacteria</td>
<td>Pseudomonadaceae</td>
<td><em>Pseudomonas plecoglossicida</em></td>
<td>-5.6</td>
</tr>
<tr>
<td></td>
<td>Proteobacteria</td>
<td>Pseudomonadaceae</td>
<td>-</td>
<td>-4.8</td>
</tr>
<tr>
<td></td>
<td>Proteobacteria</td>
<td>Comamonadaceae</td>
<td>-</td>
<td>-4.4</td>
</tr>
<tr>
<td></td>
<td>Proteobacteria</td>
<td>Pseudomonadaceae</td>
<td><em>Pseudomonas plecoglossicida</em></td>
<td>-4.3</td>
</tr>
</tbody>
</table>
Figure 10.1. An example plot tracking the growth rate of bacteria exposed to PCBs.

Shown is the growth of *S. epidermidis* upon being exposed to PCB 105. Growth was measured via optical density and converted into colony forming units (CFUs) utilizing a standard curve generated from plated cultures.
Following exposure to all three PCB congeners, there was a dose-dependent decrease in the doubling time, and thus increase in growth rate, of *S. epidermidis* as compared with controls (figure 10.2a). *P. aeruginosa* experienced the opposite effect, showing a dose-dependent increase in doubling time, signifying a slower rate of growth (figure 10.2b). These changes in growth rate mimicked the changes in gut microbiota seen in vivo after oral exposure to PCBs. *S. epidermidis* increased in abundance in the gut microbiota in vivo and also increased in growth rate following PCB exposure ex vivo. Similarly, *Pseudomonas* decreased in abundance in vivo and decreased in growth rate ex vivo following exposure to all three PCBs. Given the community dynamics of the microbiota, with a multitude of different species competing for a finite amount of resources, PCB’s effects on the growth rate of these bacteria could dramatically affect their fitness and ability to persist in the gut environment. The actual mechanism of these effects remains to be elucidated. The *S. epidermidis* could potentially be using the PCBs as an energy source thus increasing their growth rates. PCBs could also be exerting a toxic effect on *Pseudomonas*, thus hampering their growth rate. These direct effect of PCBs on the bacteria could be responsible for the altered abundances seen in vivo after oral exposure to PCBs.

Published data has shown changes in the relative abundances of bacterial taxa following PCB exposure and our data suggest that these changes could be partially mediated by the effects of the PCBs on the bacteria themselves. While these descriptive studies shed light on the community structure of the gut microbiome, the actual phenotypic effects on the organism has yet to be determined. Further experiments utilizing germ free animals would be able to determine functional effects of these changes in the microbiome.
They may also be able to assign cause and effect to the changes in gut bacterial communities, determining if the changes in the microbiota originate from the direct effects of PCBs on the bacteria or from their effects on the host immune system. Most likely, it is a combination of both these PCB driven effects.
Figure 10.2. *Ex vivo* PCB exposure changes the doubling time of intestinal bacteria.

Bacterial cultures of (A) *S. epidermidis* and (B) *P. aeruginosa* were grown overnight and split into subcultures. They were subsequently exposed to DMSO, PCB 105, PCB 126, or PCB 153 at concentrations of 10μM, 50μM, or 100μM. Optical density was measured every 30 minutes to calculate doubling time which was then statistically compared to DMSO controls. Axis on the figures are inverted to more easily conceptualize increases (up) and decreases (down) in growth rate. (N=4) Kruskal-Wallis with Dunn's multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001
Chapter 11 – Implications for Human Health and Disease

Our data explore some of the initial physiological responses following oral exposure to PCB 153. While no specific disease endpoint was studied, genotoxic damage in the IECs, the subsequent intestinal inflammation, and the potential for dysbiosis all could link PCB 153 exposure with a variety of human pathologies. Populations exposed to PCB 153, have been epidemiologically shown to be at risk for a variety of health concerns associated with inflammation; such as cancer, metabolic syndrome, and immune dysfunction\textsuperscript{155,156}.

The ATM/NEMO pathway activating NF-κB has not previously been reported in IECs and thus has not been associated with gut pathologies. It has however, been shown to be dysregulated in acute myeloid leukemia (AML) cell lines and primary tissue samples from AML and myelodysplastic syndrome (MDS) patients. In AML cell lines, ATM is constitutively active and NEMO is consistently found within the nucleus. ATM/NEMO complexes were also detected suggesting this pathway is indeed active. When the authors chemically inhibited ATM using KU-55933, or when the knocked down ATM, the constitutive NF-κB activity was lost. These findings, including the inhibition of NF-κB via chemical inhibition of ATM, were also demonstrated in primary patient samples of AML and MDS\textsuperscript{157}. While unknown whether this pathway could be contributing to pathologies in the gut, these data provide compelling evidence that ATM and NEMO, and thus the genotoxic pathway, are important in maintaining constitutive NF-κB signaling in blood based malignancies.

Despite our evidence demonstrating the genotoxic effects of PCB 153 on the small intestine, there is no evidence, epidemiological or otherwise, that PCB exposure increases
risk of small intestinal cancers. One possible reason for the lack of PCB-induced malignances is the relative resistance of the small intestine to cancer. While the exact reason for this resistance is not known, several hypotheses have been suggested including rapid turnover of IECs, the alkaline environment of the proximal small intestine, lower density of bacteria, and faster luminal transit times\textsuperscript{158}. After absorption by the small intestine however, PCBs are transported to the liver where they have been shown to cause DNA damage and have been linked both experimentally and epidemiologically to hepatocellular carcinoma (HCC)\textsuperscript{103}. \textit{In vitro} evidence has demonstrated that genotoxic activation of NF-\kappaB by other chemicals can impair the chemosensitivity of HCC cell lines, suggesting a role of this pathway in the pathology and treatment of HCC\textsuperscript{159}. Persistent NF-\kappaB activation in the liver, mediated by multiple pathways, has also been widely associated with HCC\textsuperscript{160}. Although not linked to neoplasms, experiments exposing rats to PCB 153 have demonstrated that exposure causes an increase in NF-\kappaB activity in the liver\textsuperscript{94}. While as-yet unlinked, the evidence in the literature does show that PCBs induce DNA damage in hepatocytes, cause NF-\kappaB activation in the liver, and that the genotoxic response pathway may be important in the pathology of HCC. Additionally, while the role of ATM is understudied in the gut, NEMO in IECs has been shown to be an important mediator linking innate immune activation, similar to that seen after exposure to PCB 153, with chronic intestinal inflammation such as that seen in inflammatory bowel disease\textsuperscript{161}. Further study is warranted looking for evidence of PCB-induced pathologies, in the gut, liver, and beyond, caused by ATM/NEMO mediated activation of NF-\kappaB in response to genotoxic damage.
In both our *in vitro* and *in vivo* models, exposure to PCB 153 led to an increase in expression of the inflammatory cytokines IL-6 and TNFα, driven by NF-κB. In humans, increased expression of these cytokines has been linked to the pathogenesis of a variety of inflammatory diseases and, in the intestine, NF-κB-mediated inflammation has been linked to inflammatory bowel disease. Studies performed on a variety of cell types, both within the gut and in other tissues, have also implicated NF-κB and NF-κB-induced cytokine production as a key event in the early pathogenesis of diabetes mellitus. Recent work on adipocytes have even linked oxidative stress caused by PCB 153 to NF-κB-mediated inflammation. This inflammation was responsible for glucose and lipid dysregulation in these adipocytes, an important indicator of metabolic syndrome. This is important because, as mentioned before, epidemiological data has linked exposure to PCB 153 to diabetes and the development of metabolic syndrome. These systemic, metabolic effects could also be originating in the gut. Data from *in vivo* models have demonstrated that intestinal inflammation, and increases in the IL-6 and TNFα, precedes and is predictive of development of obesity and insulin resistance in mice fed a high fat diet. The exact mechanism connecting inflammation in the IECs and obesity have yet to be fully elucidated but it could be related to energy absorption or a bystander of pathological changes in the microbiota. PCB 153 has also been shown to activate PXR and constitutive androstane receptor (CAR), both of which have been linked to lipid metabolism and glucose homeostasis. Activation of these two receptors could also contribute to the observed metabolic consequences seen in epidemiological studies. While our data clearly show increases in NF-κB-induced intestinal inflammation caused by PCB 153, further studies
should investigate if there is a causal link between PCB exposure, intestinal inflammation, and the onset of metabolic syndrome.

Preliminary work conducted in our lab, utilizing a chronic exposure model of PCB exposure, does seem to suggest that PCBs can act as an obesogen, an obesity causing substance. Mice were fed either a low fat diet (LFD) consisting of 10% fat, or a high fat diet (HFD) consisting of 60% fat for one month. Mice from both diets were then exposed to PCBs or a vehicle control, daily, for 30 days. For these experiments, mice were exposed to an environmentally relevant mixture of PCBs that had been used in the mouse model looking for changes in the microbiome: PCB 153, PCB 138, and PCB 180. Mice were continued on their respective diets throughout the experiment. At the conclusion of the chronic exposure, mice fed a HFD had gained more weight than their LFD counterparts. Interestingly, mice fed a LFD and exposed to PCBs gained a similar amount of weight as the HFD, non-exposed mice. More strikingly, mice fed a HFD and exposed to PCBs gained the most weight out of any of the experimental groups (figure 11.1). While more research on these effects needs to be conducted, including whether intestinal inflammation may be playing a role, these data are in agreement with epidemiological data linking PCB exposure to obesity and metabolic syndrome\textsuperscript{164}. 
Figure 11.1. Chronic PCB exposure increase weight gain in mice. Mice were fed either a low fat diet (LFD) or a high fat diet (HFD) for one month then exposed to a mixture of PCBs (PCB 153, PCB 138, and PCB 180) at a dosage of 150 μmol/kg or a vehicle control, daily, for 30 days. Weight was measured at the beginning and conclusion of the PCB exposure. (N=5 mice per group).
Metabolic syndrome has also been associated with a “leaky gut” phenotype. Disruption of the intestinal barrier is also seen in many other inflammatory diseases as well, such as diabetes mellitus, inflammatory bowel disease, celiac disease and multiple sclerosis. Previous work has shown that oral exposure to PCB 153 can cause an increase in intestinal permeability in mice, allowing for the leakage of bacterial products such as LPS. We build on this finding by demonstrating that not only is there an increase in permeability, but that it can lead to an increase in bacterial translocation to the MLNs. Additionally we link the activation of NF-kB to this breakdown of the intestinal barrier suggesting that inflammation may play a role in the increased permeability caused by PCB 153. This “leaky gut” phenotype can have serious human health consequences. Under normal conditions, the gut in very good at sequestering a large quantity of pro-inflammatory substances, from the microbiota, away from systemic circulation. When this barrier breaks down, much like it does after exposure to PCB 153, these pro-inflammatory substances can leak from the intestinal lumen into the portal circulation and end up in the liver. When these microbial molecules, such as LPS, reach the liver, they trigger multiple immune processes, including the activation of NF-kB, which can lead to liver fibrosis and eventually HCC. This breakdown of the intestinal barrier provides yet another possible link between exposure to PCBs and the epidemiological observation of increased HCC in PCB exposed populations.

This leakage of bacterial products from the intestinal lumen can also pass through the liver and reach systemic circulation, causing widespread inflammation. This chronic, low level, systemic inflammation has been shown to impair the adaptive immune system and decrease responsiveness to vaccinations. High serum levels of PCBs in humans have
also been shown to impair the humoral immune response\textsuperscript{168}. Whether the leakage of pro-inflammatory molecules from the intestinal lumen is to blame for this PCB-induced immunosuppression has yet to be investigated, but the breakdown in the intestinal barrier after exposure to PCBs could be responsible for far reaching health consequences such as these.

It is quite possible that, even staying within the lumen of the gut, microbes could be playing a role in the diseases associated with PCB exposure. Previously published work by Choi et al. (2013) showing PCB-induced changes in the microbiota, as well as our ex vivo data showing that PCBs can directly affect the microbiota, could potentially contribute to multiple PCB-associated pathologies\textsuperscript{152}. As discussed in the previous chapter, the gut microbiota extensively interacts the host and can control a wide variety of homeostatic processes. When the composition of the microbiota changes in a way that no longer supports these functions, or even actively causes disease, it is referred to as a dysbiosis. Dysbiosis has been linked to a variety of diseases, many of which have also been epidemiologically linked to PCBs. Most notable of these is metabolic syndrome and obesity, as dysbiosis has been shown to be causally linked to the disease though careful experimentation. Deep sequencing of human microbiota has shown that there is a core, obese microbiota that differs from the lean microbiota in its increased capacity to extract energy from food\textsuperscript{169}. This obese microbiota, when transplanted from an obese human donor into germ free mice, caused a significant increase in adiposity and body mass as compared to mice transplanted with an identical, lean twin’s microbiota\textsuperscript{170}. This establishes a link between obesity and the microbiota by showing that the disease is transmissible through the microbiota. While this link is relatively new to medicine, farmers have long taken
advantage of the connection between dysbiosis and obesity by feeding a potent inducer of dysbiosis, antibiotics, to livestock in order to increase their body mass. Taken together, this suggests a causal link between the obese, dysbiotic microbiota and the development of obesity and metabolic dysfunction. This pathological role of the microbiota could also potentially apply to dysbiosis following PCB exposure.

In vivo experiments conducted by Choi et al. (2013) showed that mice exposed to PCBs had a dramatic reduction in the phylum Proteobacteria. Our ex vivo data utilizing Pseudomonas, one genus of many in Proteobacteria, similarly found PCBs to have a deleterious effect on their growth. While the literature on this taxa’s relation to obesity is admittedly mixed, multiple studies have reported that this phylum is linked to better insulin sensitivity and is reduced in mice fed a high fat diet. It is possible that the reduction in Proteobacteria following PCB exposure could be contributing to PCB’s epidemiologic link to metabolic syndrome.

While yet to be studied, given the intimate link between the microbiota and the immune system, PCB-mediated changes in the microbiota could be significantly contributing to the immunotoxic effects of PCBs discussed in chapter 3. Even subtle, PCB-induced changes in individual bacterial taxa could have dramatic ramifications on immune parameters such as populations of T cell subsets and lymphocyte function. Both of these effects have been associated with exposure to PCBs.

It is important to note that the microbiota changes following PCB exposure have not yet been experimentally shown to be dysbiotic, however given the diseases linked to PCBs, it is not outlandish to hypothesize that the microbiota could potentially be contributing to the pathologies and definitely warrants further investigation.
It is striking, although not surprising, that many of the human health impacts seen today in PCB exposed population could originate with, or be exacerbated by, the effects of PCBs on the gut. Given how diet is the main route of PCB exposure, this is perhaps to be expected, although the potential for far reaching and systemic ramifications of these intestinal effects remain salient. Knowledge gained from basic research into the physiologic effects of PCBs on the gut opens potentially new therapeutic avenues for preventing this toxicity. For example: a dietary intervention such as increased intake of antioxidants, could potentially prevent the genotoxic damage and subsequent intestinal inflammation we have shown to be caused by PCB 153. Any subsequent systemic effects might be ameliorated if this initial domino was prevented from falling. This is clearly all speculative but highlights the need for increased scientific inquiry on the effects of PCBs on the gut.
Chapter 12 - Limitations and Conclusions

The results of this study present evidence that PCB 153 causes DNA damage, driving inflammation and barrier dysfunction in the intestinal epithelium. Our data suggest that the driver of this inflammation and increase in permeability is the transcription factor NF-κB, which can become activated through ATM and NEMO. A summary of the proposed mechanism can be found in figure 8.5. PCBs were also shown to have similar effects on intestinal bacteria ex vivo as seen in previous studies looking at the effects of PCBs on the microbiota.

One of the major limitations of our experiments is reconciling the differences between PCB exposure in the environment and PCB exposure in the laboratory. In the environment, PCBs exist as complex mixtures of multiple congeners, each with unique chemical properties that can exert different biological effects. Additionally, it is extremely challenging to accurately establish levels of dietary exposure to PCBs. Because of the mechanistic nature of this study, we chose to focus on only one congener, PCB 153, and levels of exposures that intestinal epithelial cells would encounter after a meal high in PCBs. While this dosage admittedly only represents a subset of the PCB exposed population, it was chosen in order to glean mechanistic insights into the initial physiologic response of IECs following oral exposure to PCB 153.

Though our experiments on the inflammatory effects of PCB 153 on IECs focused exclusively on PCB 153 induced activation of NF-κB, this represents only one of many ways exposure to PCB 153 can potentially cause inflammation and toxicity. PCB 153 has been shown in various cell types and conditions to activate CAR, PXR, Activator Protein-1 (AP-1), and NADPH oxidase. The full scope of PCB 153 induced toxicity on the
intestinal epithelial cells most likely involves multiple mechanisms and a diverse array of transcription factors.

While further studies need to be done, the data presented here suggest that effects of PCBs in the gut, and specifically inflammation in the intestinal epithelium, may be important drivers for pathologies that have been epidemiologically linked to PCB exposure in humans. The gut could represent an attractive and accessible therapeutic target to combat PCB-induced inflammation.
Chapter 13: Afterword: The Importance of the Gut Exposome

“Tell me what you eat, and I will tell you what you are.”
-Anthelme Brillat-Savarin, *Physiologie du Gout*, 1825

The human exposome represents the totality of experiences our biological selves have encountered; every microbe, metabolite, and molecule that we’ve ever come into contact with in our environment. Evolution has crafted life in such a way that clearly demonstrates we experience our environment through our gut. Over 600 million years of multicellular evolution has strategically placed over 70% of our body’s defenses, the immune system, in the gut. This bastion of cells is expressly fashioned to protect us from the constant onslaught of exposures experienced by the single largest surface in our body. Evolution’s shrewd focus on the importance of exposures in the gut underscores the importance of scientific inquiry into the intestinal effects of environmental exposures, including pollutants like PCBs. The dynamism of the gut microbiota, paired with its potent influence over host physiology, adds a layer of complexity while amplifying the potential for pervasive health consequences following intestinal exposures. Given the expanse of the exposure surface and the breadth of systemic influences, it is not unreasonable to suspect that systemic, pathophysiological consequences observed following environmental exposures originate with, or are influenced by, antecedent impacts on the gut. Understanding the nature and ramifications of these intestinal manifestations could lead to better prevention, prophylactic interventions, and novel therapeutics for combating the consequences of environmental exposures.
Appendix 1: Detailed Materials and Methods

Chemicals and reagents.

PCB 153 (2,2’,4,4´,5,5´-hexachlorobiphenyl) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO, EMD Millipore Corp., Billerica, MA) to make the stock solution (10mM). For in vivo use, PCB 153 stock solution was diluted in safflower oil. The same amount of DMSO diluted in safflower oil was used a negative control. Etoposide was purchased from Sigma-Aldrich (St. Louis, MO) and LPS was purchased from Invivogen (San Diego, CA). Etoposide was used as a positive control for genotoxic damage and genotoxic activation of NF-κB, and LPS was used as a positive control for NF-κB activation by the canonical, non-genotoxic pathway. The free radical scavenger, N-acetylcysteine (NAC), the NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC), and the inhibitor of genotoxic NF-κB activation, Clostridium difficile Toxin B, were all purchased from Sigma-Aldrich (St. Louis, MO). The ATM inhibitor, KU-55933 was purchased from Abcam (Cambridge, United Kingdom).

Cell culture and PCB 153 Treatment.

The human intestinal cell line SW480 (ATCC CCL-228) was used for all in vitro experiments. SW480s were chosen because of their high level of responsiveness to other activators of NF-κB such as LPS. Cells were grown at 37°C in Dulbecco's Modified Eagle Medium (DMEM, Corning, Corning, NY) supplemented with 10% fetal bovine serum (Gemini Bio Products, West Sacramento, CA) and 1% Penicillin-Streptomycin (Sigma-Aldrich, St. Louis, MO). For experiments, DMEM without FBS was used. For inhibition experiments, cells were pretreated for 1 hour with either 5mM NAC or 10μM of KU-55833; or for 2 hours with 100 ng/mL or 50 ng/mL of C. difficile Toxin B, before PCB 153
exposure. Cells were exposed to either 50ng/mL LPS, 10μM or 1μM Etoposide, 100μM, 50μM, or 10μM PCB 153, or DMSO. Dosages of PCB 153 were chosen based on the range of possible concentrations that intestinal epithelial cells would be exposed to after a meal high in PCBs. Cells were exposed for 3 hours and then collected for analysis. Treatments at these dosages did not affect cell growth or viability of the cells as determined by an MTT assay (ATCC, Manassas, VA) performed as per manufacturer’s instructions (figure A1.1).

Figure A1.1. PCB 153 exposure and treatments used in paper are not cytotoxic to SW480 cells in the exposure conditions used. Cell viability was measured via MTT Assay (N=3)
Transfection and NF-κB Reporter Assay.

Transfections were performed in Opti-MEM, Reduced-Serum Medium (Thermo-Fisher, Waltham, MA). Lipofectamine 2000 (Thermo-Fisher) was used as per manufactures protocol. For the NF-κB reporter assay, cells were transfected with two plasmids: pGL4.32[luc2P/NF-κB-RE/Hygro] vector containing five copies of an NF-κB response element and pRL Renilla Luciferase Control Reporter Vector to normalize for number of cells, both from Promega (Madison, WI). The Dual-Luciferase Reporter Assay System from Promega (Madison, WI) was used to measure luciferase activity and read on a luminometer (Promega GloMax). Data are presented as fold change in luminescence as compared to the DMSO exposed controls.

Animals and PCB 153 Exposure.

Male, C57BL/6 mice between 8-12 weeks of age were obtained from Jackson Laboratory (Bar Harbor, ME) and housed under 12:12 hour light/dark conditions and given access to food (Teklad Global 18% Protein Rodent Chow; Envigo, Madison, WI) and water ad libitum. Animals were exposed to 300 μmol/kg of PCB 153 stock solution dissolved in 100μL stripped safflower oil (Alfa Aesar, Ward Hill, MA) and administered via oral gavage. This dosage was chosen based on typical amounts of PCBs found in fish meals of a highly polluted area and meant to represent the high end of reported daily intake of PCBs in a single day. Control animals received an equal volume of DMSO as the PCB 153 stock solution, also dissolved in 100μL safflower oil (<1% DMSO by volume) For inhibition experiments, mice were intraperitoneally injected with PDTC (120mg/kg dissolved in 100uL of PBS) or vehicle (PBS), 30 minutes prior to PCB 153 exposure based on previously reported effective and nontoxic dosages. Mice were exposed once per day.
for two days and then euthanized on the third day. Upon euthanasia, samples of intestinal tissue were collected and processed as outlined below.

For the chronic exposure model, mice were fed either a low fat diet (10% fat) or high fat diet (60% fat) for 4 weeks prior to experimentation. The three environmentally relevant PCB congeners, PCB138, PCB153, and PCB180 (AccuStandard, New Haven, CT), were mixed at a molar ratio of 1.7:3.2:1 in stripped safflower oil. For 4 weeks, mice were orally gavaged with either the PCB mixture or vehicle control. Weight was measured weekly throughout the study.

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Miami Miller School of Medicine and followed National Institutes of Health guidelines (National Research Council 2011).

**Intestinal Permeability**

Intestinal epithelial integrity was determined using a FITC-dextran permeability assay as previously described 174. Briefly, mice were orally gavaged with 4 kDa FITC-dextran (Sigma-Aldrich, St Louis, MO) at a concentration of 60mg/100g body weight 4 hours prior to sacrifice. Blood was collected via cardiac puncture and centrifuged to isolate serum. The concentration of FITC-dextran in the serum was measured by Gemini EM fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) at 490/525 nm.

**Mesenteric lymph node isolation and bacterial culture**

Mesenteric lymph nodes (MLN) were aseptically isolated at the time of euthanasia. MLNs were homogenized in sterile PBS and serial dilutions were plated onto Tryptic Soy Agar (TSA) supplemented with 5% sheep’s blood (Hardy Diagnostics, Santa Maria, CA). Cultures were incubated at 37°C for 24-48 hrs under both aerobic and anaerobic conditions.
Quantity of translocated bacteria was expressed as colony-forming units (CFU) per mg of MLN tissue.

*Intestinal epithelial cell isolation.*

Sections of proximal and distal small intestine, and colon were removed following euthanasia. Small intestinal samples were cut longitudinally and transferred to 10mL of cold HBSS with gentle agitation to remove debris. The small intestinal samples were then cut into 1cm pieces and transferred to cold HBSS containing 3mM ethylenediaminetetraacetic acid (EDTA) for 30 minutes on ice with 200rpm agitation on an orbital shaker. Colons were processed similarly to small intestines, but were incubated in 60mM EDTA in HBSS for one hour. The pieces of intestine were then transferred to cold HBSS and shaken vigorously at 450rpm on an orbital shaker for 5 minutes. Released epithelial cells in the supernatant were filtered through 70μm strainer. IECs were washed twice with 1% FBS in HBSS to remove any residual EDTA and then pelleted for further analysis.

*Western Blotting and RT-qPCR.*

Total cell lysate from cultured cells or IECs were obtained by using M-PER protein extraction reagent (Thermo Scientific, Waltham, MA) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, MA). Protein lysates were loaded into precast, NuPAGE 4%-12% Bis-Tris Gels (Novex by Life Technologies, NY), electrophoresed (200V, 1h) and then transferred (30V, 1.25h for anti-γH2A.X; 30V, 16hr for ATM) onto polyvinylidene fluoride microporous membranes. Membranes were blocked for 1 hour in 5% bovine serum albumin (Sigma-Aldrich, St Louis, MO) and incubated with their respective antibody (1:1000 dilution) overnight at 4°C. Mouse anti-
γH2A.X, mouse anti-NEMO, mouse anti-phospho NEMO (s85), mouse anti-ATM, and mouse anti-phospho-ATM (s1981) antibodies were obtained from Abcam (Cambridge, United Kingdom). Membranes were washed and incubated with either goat anti-mouse (anti-γH2A.X and anti-phospho-ATM), or goat anti-rabbit antibody (anti-ATM, anti-NEMO, and anti-phospho NEMO), both conjugated to horseradish peroxidase (1:10,000 dilution; Invitrogen, MA) for 30 minutes. Anti-β-Actin conjugated to horseradish peroxidase (1:10,000 dilution; Sigma, St Louis, MO) was used as the loading control. Membranes were developed using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) as per manufacturer instructions and imaged on a myECL Imager (Thermo Scientific). Total RNA was extracted from IECs or cultured cells using RNA Bee (Tel-test, Friendswood, TX) and Direct-zol RNA MiniPreps (Zymo Research, Irvine, CA). RNA was reverse transcribed using transcriptor reverse transcriptase enzyme and random hexamers (Roche life science, Indianapolis, IN) and qPCR was performed using SYBR Premix Ex Taq (Clontech Laboratories, Mountain View, CA) on a Roche LightCycler 480 (Roche, Indianapolis, IN). See appendix table 1 for primer pairs. Relative expression levels were calculated using the ΔΔCt method normalizing to Gapdh or β-Actin as the housekeeping genes for mouse or human cell culture samples, respectively.
### Table A1.1 Primer pairs used in in vivo (mouse) and in vitro (human) experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reverse Primer</th>
<th>Forward Primer</th>
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<tbody>
<tr>
<td>Human TNFα</td>
<td>5’-CAGCCTCTTCTCTTTCTCTTGAT</td>
<td>5’-GCCAGAGGGCTGATTAGAGA</td>
</tr>
<tr>
<td>Human IL-1β</td>
<td>5’-TACCTGTCTTGGCTGGTGGAA</td>
<td>5’-TCTTTGGGTAATTTTTGGGATCT</td>
</tr>
<tr>
<td>Human IL-6</td>
<td>5’-GATGAGTACAAGATCCCTTGATCCA</td>
<td>5’-CTGCAAGCCACTGGTTCTGT</td>
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<tr>
<td>Human β-Actin</td>
<td>5’-CCAAGCGCCGAGAGATGA</td>
<td>5’-CCAGAGGCGTACAGGGATAC</td>
</tr>
<tr>
<td>Mouse TNFα</td>
<td>5’-TCTTCTATGCTTCTTTCTTGG</td>
<td>5’-GGTCTTGCGCCATAGAAGTA</td>
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<tr>
<td>Mouse IL-1β</td>
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<tr>
<td>Mouse IL-6</td>
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<td>Mouse GAPDH</td>
<td>5’-GTGGGTCCACACCCACATCAGA</td>
<td>5’-CCATCACCATCTTTCCAGAG</td>
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</table>

**Nuclear fractionation and NF-κB Transcription Factor Assay.**

IECs, isolated from mice, were pelleted after isolation. In order to extract the nuclear contents of the cells, pellets were resuspended in buffer containing 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.5mM MgCl₂, 10mM KCl, 0.5mM Dithiothreitol (DTT), 0.05% NP40, and brought to a pH of 7.9. After addition of the buffer, cells were incubated on ice for 10 min to allow for lysis then spun down at 3000rpm for 10 min. The supernatant was removed and the pellet was resuspended in buffer containing 20mM HEPES, 400mM NaCl, 1mM EDTA, 1mM DTT, and supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, MA). This was homogenized using a dounce homogenizer and left on ice for 30 minutes. The solution was then centrifuged for 30min at 12,000g and the supernatant containing the nuclear fraction of proteins was collected for analysis. NFκB p65 Transcription Factor Assay Kit (Abcam, Cambridge, United Kingdom) was used as per
manufacturer’s instructions to assess DNA binding activity of NF-κB in the nuclear fraction.

*Alkaline comet assay.*

Alkaline comet assay was performed utilizing the CometAssay Kit from Trevigen (Gaithersburg, MD) as per manufacturer’s instructions. Briefly, following exposure, SW480 cells from four biological replicates were collected and suspended in L-Magarose and plated onto slides in duplicate. Slides were immersed in lysis solution and then placed in the CometAssay ES Tank for alkaline electrophoresis at 21V for 30 minutes. Slides were then fixed and left to dry overnight. Slides were then stained with SYBR Gold (ThermoFisher) and photographed utilizing the FITC filter on a Keyence BZ-X710 All-in-one Fluorescence Microscope (Okasaka, Japan). Fifty, randomly selected cells were analyzed on for each slide (total of 100 cells per condition) and data are presented as “tail moment”. Images were analyzed using ImageJ with the OpenComet software.

*Gene silencing.*

SW480 cells were grown to approximately 60-70% confluence and were transfected using Lipofectamine RNAiMAX reagent (Invitrogen, MA) with either scrambled (Silencer Negative Control No. 2, Thermo-Fisher Scientific) or ATM-specific siRNA (s1708, Thermo-Fisher). Cells were incubated with the transfection mixtures for 12 hours, allowed to recover in complete medium for 48hr, and subsequently exposed to PCB 153, etoposide, or DMSO.

*Bacterial Growth Assay.*

*S. epidermidis* and *P. aeruginosa* were grown overnight in general culture media (Luria broth). Primary cultures were then diluted to set optical density (OD$_{600}$=0.01) and
split into subcultures. Subcultures were exposed to 10μM, 50μM, and 100μM of PCB 153 (non-coplanar), PCB 126 (coplanar), PCB 105 (a PCB with mixed properties of both a coplanar and non-coplanar PCB), or DMSO as a negative control. The optical density was measured every 30 minutes at 600nm to measure the growth rate of the bacteria. A standard curve correlating optical density with CFUs/mL was made for the analysis of the data. The growth rate was then plotted vs time and log transformed to calculate the doubling time. Decreases in the doubling time signified a faster growth rate, and increases in the doubling time signified a lower growth rate.

**Statistics.**

Data are expressed as mean ± standard error of the mean. Experiments were powered to show significance based on prior published data both from our laboratory and others utilizing the method outlined in Eng 2003. Multiple fully powered *in vivo* experiments were conducted to collect necessary amounts of tissue for all assays conducted with markers of inflammation collected from each trial. Further details on power calculations can be found in the supplement. Normality was assessed using the Shapiro-Wilk test. Statistical significance for data determined to be parametric was assessed using student T test, for comparison of two means and one-way analysis of variance (ANOVA) for comparison of more than two means with Dunnett’s test for multiple comparisons. For non-parametric data involving more than two groups we used the Kruskal-Wallis H test with Dunn’s test for multiple comparisons. For experiments examining the influence of two different independent variables, two-way ANOVAs were used with Tukey’s multiple comparison test. All comparisons of means were made *a priori*. All analyses were
performed in GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). Differences were considered statistically significant when P<0.05.
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Matthew C. Phillips was born in Augusta, Georgia and then proceeded to move to Kansas, Alaska, Kansas again, New York, and Maryland before moving down to Miami for college in 2006. He graduated University of Miami in 2010 with a B.S.M.A.S. in Marine Science and Biology with minors in Chemistry, Psychology, and Sociology. During college, Matthew worked at the U.S. Army Center for Environmental Health Research in Frederick, MD, doing analytical chemistry and aquaculture. He also worked as an epidemiological field worker with Professor Lora Fleming at the UM Oceans and Human Health Center, studying the health effects of swimming at recreational beaches. This introduced him to the field of oceans and human health (OHH), which soon became his passion. For the remainder of college, and for a year following, Matthew worked with Dr. Helena Solo-Gabriele doing environmental engineering research within the OHH center. Over the next two and a half years, they would go on to characterize multiple mechanisms of microbial transport out of beach sand and find correlations between the quantity of microbes in beach sands and the water quality at those beaches.

As Matthew was exploring the field of OHH, he became more interested in not only researching human health, but also the clinical practice of medicine. Because of this he applied and was later accepted into the M.D./Ph.D. program at University of Miami Miller School of Medicine. He started his graduate training with Dr. Lisa Plano, where he had the opportunity to study the zoonotic transmission of methicillin-resistant *S. aureus* between humans and whales at a rehabilitation facility in the Florida Keys.

For his dissertation research, Matthew was fortunate to be mentored by Dr. Maria Abreu, who allowed him to continue to pursue his passion for OHH, this time from a
biomedical perspective, looking at the effects of ocean pollutants on the gut. During graduate school, Matthew had the opportunity to be actively involved in the UM community. His first year of graduate school, he co-founded the Biomedical Graduate Student Government and helped start the Research Intersections Initiative to promote interdisciplinary research at UM. His extracurricular involvement culminated with serving as the student body president of the graduate students at UM on the Graduate Student Association.

Matthew was granted his Ph.D. in Microbiology and Immunology in December of 2017.